# The use of molecular markers in the study of the origin and evolution of Japanese Knotweed *sensu lato*

A thesis submitted to the University of Leicester for the degree of Doctor of Philosophy

By

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September 2003

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## Declaration

I hereby declare that no part of this thesis has been previously submitted to this or any other university as part of the requirements for a higher degree. The work described here, unless otherwise acknowledged in the text or by reference, was conducted by the undersigned who is fully responsible.

Signed ……..

## Dedication

In loving memory of my grandparents, Margaret Bratcher and Walter Pashley.
Also to my parents, Megan and David, thank you for your love and support.

## Acknowledgments

Firstly I would like to thank my supervisors John Bailey and Colin Ferris for their support, guidance and supervision. Dr Knotweed himself, John Bailey has been an excellent supervisor these last six years, and great company on the many collection trips around Britain, as well as to Holland and Japan. Thank you for your help in the lab and the encouragement you have given me when things have been stressful. I also thank John for producing the majority of chromosome counts reported in this work. Also to Colin Ferris, thank you for your assistance with a lot of the molecular data, in particular with sequence analysis, and your guidance with my writing.

Ann Conolly deserves a special mention for her support, encouragement, helpful conversations, and company on some field trips. Most notably Holland where she had to row John and I out of danger, then teach me how to row.

This work was primarily funded by the Midlands Asthma & Allergy Association (MAARA), to whom I am grateful. Additional funding to enable the first collection trip to Japan came from the British Ecological Society (BES), Botanical Society of the British Isles (BSBI), Botanical Research Fund, and Leicester University.

The production of this thesis has been made easier by the help of many people. Thanks go to Mike Wilkinson from the University of Reading for providing me with some double-anchored ISSR primers, Michelle Hollingsworth for helpful advice on working with Japanese Knotweed, Dave Stevenson for the aid in cloning chloroplast fragments for sequencing, Andy King for practical advice in the lab and help with data analysis, Clive Stace for explaining many taxonomy related queries I have had, and Trude Schwarzacher & Pat Heslop-Harrison, both for advice and access to their computers, scanner and printer.

I am also very grateful to the many people who aided in the plant collection. For plants from the introduced range I would like to thank Tony Leach (Hempstead), Jerry Heath (Colchester), Peter Jepson (Preston), R. Hemming (South Woodchester), Caroline Wilson (Dolgellau), K. Pyne (Leeds), James Partridge (Warwick), G. Hutchinson (Coed-y-felin-Woods), Peter Zika (Seattle), and James MacFarlene (Cornwall). For plants from the native range, Kazuhito Matsuo, Jun Suzuka, Hiroyuki Shibaike, and Tatuyoshi Morita, Shin Taketa and Takehiro Endo were of great assistance. I should like to also mention Hiroki Yamaji, Yoshihide Uyama, Fujita Mika, Ōki Yasuyoshi, Narumi Tomoko, Dr T. Sano, Dr T. Fujita, Dr Hasegawa, Midori Abe, and Aki Kobayashi who accompanied us in the field in Japan

during August 1999. In addition I thank Dick Shaw and Harry Evans who collected many more Japanese accessions with John Bailey in 2000.

Thanks are due to my friends, past and present, from the Biology department at Leicester University, and to the excellent technical and support staff that have made my time here much easier. In particular Martha Clokie, ex-lab mate and very good friend. Thank you for being there for me, and for opening my eyes (and taste buds) to such wonderful food. And to Helen Chambers, my buddy who has been going through a lot of the same writing stresses at a similar time to me, thank you – we did it eventually, now we just have to make it up the Eiffel tower! A special thanks goes to Trudie Allen, Tony Wardle, Dave Baines, and Bill Hawes, who have always been up for a beer or three. Also, Helen Pearson, Carol McAuley, Sally Adams, Amy Baldwin, Veryan Codd, Jodi Maple, my Brazilian friends Kiko, Camila, Samira, and Luciana – it's been great getting to know you all. Thanks also to the many I haven't mentioned but have socialised with at some stage or other.

Being in Leicester has been made a lot easier by having some very special friends 'back home' who I couldn't be without. My fellow "slamming sisters", Hannah Thorpe, Izzy Douglas-Smith, Catherine Smith, Hayley Pink, Dawn Jones and Sam Sorci. Thank you for always being there, for your visits to Leicester, and for your support when things have been tough.

To the friends I have made outside of the department in Leicester I owe much for what remains of my sanity. In particular Nadia Koucheksarai who has been a great friend to me this last year. But not forgetting Garry Smart, Dave Watson, Andy, Margaret, Min & Phil, and Steve & Sue. Here's to many more nights at the rock and goth clubs! Also to Toby Morrison, thank you for being a good friend these last three years.

Finally, and most importantly, I thank my family for all their love and support. Mum & Mike, Dad & Sue, Nikki, Simon, and Dorothy, I couldn't have done this without you.

# Abstract

Japanese Knotweed *s.l.* comprises taxa from the genus *Fallopia* section *Reynoutria,* and were introduced to Britain from Asia during the 19th century. The hybrids are believed to have arisen since the introduction of the parental species.

Inter-simple-sequence-repeat (ISSR) PCR was used to determine genotypes to examine the potential for sexual reproduction of *F*. x *bohemica* in Britain.

At one site in Wales the distribution of the two parents and the resultant hybrid offspring were mapped, their genotypes assessed, and the relationships between the genotypes discussed. Evidence was found that both clonal spread and sexual reproduction play a role in the distribution of these plants. Twenty-six genotypes of established hexaploid and two of octoploid *F*. x *bohemica* were detected, indicative of continuous new recruitment to the population.

Three locations in Britain where both male-fertile and male-sterile tetraploid hybrids are found were analysed for both chloroplast haplotype and genotype. Chloroplast haplotypes were determined by RFLP-PCR analysis of the chloroplast region *trn*C-*trn*D, and used to identify the direction of the cross that produced the *F*. x *bohemica*. Hybridisation was shown to occur in both directions. In total, ten different genotypes of established tetraploid *F*. x *bohemica* were detected, with no common genotype found between the different sites.

Genetic diversity among British *F. sachalinensis* was examined. Most were found to be one of two genotypes, either a widespread male-fertile, or a widespread male-sterile clone.

A molecular biogeographical study of *F. japonica* and *F. sachalinensis* in Japan was undertaken using PCR RFLPs of six chloroplast regions, *trn*K[1]-*trn*K[2], *trn*C-*trn*D, *trn*F-*trn*V, *trn*H-*trn*K, *trn*D-*trn*T, and *trn*M-*rbc*L. The probable region of the source of the introduced material was identified. Additionally a sub-set of the plants was sequenced to better understand the relationships between the native taxa. To complement the molecular analysis, morphological and cytological investigations were also conducted.

# Contents

# Chapter 1. Introduction to Biological Invasions.

## 1.1 Existence of alien invasive plants and associated problems

One of the earliest books, which was regarded by some as being the start for invasion ecology as a new discipline, was Charles Elton's 'The Ecology of Invasions by Animal and Plants' (1958) (Rejmanek, 2000; Richardson *et al.*, 2000). In this volume Elton states 'One of the primary reasons for the spread and establishment of species has been quite simply the movement around the world by man of plants, especially those intentionally bought for crops or garden ornament or forestry.' He also quotes Fairchild, who was head of the United States Office of Plant Introduction, who apparently mentions casually in a travel book about the tropics that the work of this organisation 'has resulted in the introduction of nearly 200,000 named species and varieties of plants from all over the world'.

Alien species are now a common component of the floras of most countries. In the British Isles, out of 3565 plants (including apomicts) recorded in the revised List of Vascular plants of the British Isles (Kent & Stace, 2000), 34.3% were recorded as being alien. The Swiss flora contains about 300 naturalized alien vascular plant species that make 11% of all vascular plants in Switzerland (Weber 1999, in (Weber, 2000)). The Polish flora consists of nearly 2,300 species of vascular plants, of which approximately 10% were introduced by man and are already well established (Kornas, 1990). These are just selected examples of floras to give an indication of the sorts of levels alien plants have attained. There are in fact very few areas left where man has not had an effect on the flora and fauna to some extent (Callaway & Aschehoug, 2000; Milne & Abbott, 2000; Sakai *et al.*, 2001; Willis *et al.*, 2000).

Due to the increasing number of papers published in this field, there has been considerable confusion regarding the terminology. As a direct result of this, Richardson *et al*. (2000) examined definitions from various dictionaries, encyclopaedias, books dealing with plant invasions, and scientific papers, and arrived at the recommended terminology for plant invasion ecology that will be adopted for this work. These definitions can be found in Table 1.1

Not all alien plants that are introduced to a new country become invasive. In fact only a subset of those that become naturalised will be invasive taxa (Rejmanek, 2000). Williamson (1993) estimated that approximately 1% of British invaders become what he classifies as pests, and quotes Groves (1986) as giving a figure of 2.5%, or less, of plant species

introduced into Australia that had become pests. The percentages of alien plants that become invasive weeds may be small, but the impact these plants have can be immense.

Invasive species can have severe economic impacts, as well as having critical negative consequences for biodiversity (Sakai *et al.,* 2001). The invasion of alien plants is considered to be one of the primary threats to rare and endangered species and to the integrity and function of ecosystems (Blossey *et al.*, 2001). Parker (1999) grouped the biological impact effects of an invader into five levels: (1) effects on individuals, (2) genetic effects (including hybridisation), (3) population dynamic effects (abundance, population growth etc.), (4) community effects (species richness, diversity, trophic structure), and (5) effects on ecosystem processes (nutrient availability, primary productivity etc.). Often invasive plants can have impacts in more than one of these areas.

In some instances the alien plant will enter a new environment and displace native plants. For example, the Chinese Tallow tree *Sapium sebiferum* (L.) Roxb., a native to Asia, was introduced to Georgia in the late 18$^{th}$ century and now aggressively displaces native plants and forms monospecific stands in many areas of the southeastern United States (Siemann & Rogers, 2001). Likewise *Pueraria lobata* Ohwi (kudzu) was repeatedly introduced into the southeastern United States throughout the late 19$^{th}$ century and the first half of the 20$^{th}$ century, firstly as an ornamental and subsequently as fodder and a means of erosion control. Blanketed fields of kudzu are now a familiar site throughout the region (Pappert *et al.,* 2000). *Rhododendron ponticum* L., introduced from the Iberian Peninsula, is extensively naturalized in the British Isles, and recognized as a threat to native communities and as a pest of forestry (Milne & Abbott, 2000). Purple loosestrife (*Lythrum salicaria* L.) introduced to North America in the early 1800s has since spread throughout the continent, now occurring in all lower 48 states (except Florida) of the US and in 9 Canadian provinces, where it out-competes native species causing local reductions in native plant species richness (Blossey *et al.*, 2001). Another alien species currently causing negative effects in its introduced region is the water hyacinth, *Eichhornia crassipes* (Mart) Solms. It was introduced into Africa from South America in the early 1900s (Masifwa *et al.*, 2001), and since 1989 has been spreading through all parts of Lake Victoria. Lake Victoria is shared between Kenya, Uganda and Tanzania and is a very important water resource in the region, supporting the livelihood of millions of people (Lung'ayia *et al.*, 2001). The weed forms a permanent floating fringe at the wetland/open water interface zone, alters the food web, and affects biological diversity (Masifwa *et al.*, 2001).

Some invasive plants can have impacts beyond physically displacing native species. For example, most of the formerly forested lowland ecosystems within the North Kona region of the island Hawaii are now dominated by invasive species such as fountain grass (*Pennisetum setaceum* (Forsskål) Chiov.), an alien perennial bunch grass which as well as out-competing and suppressing native vegetation, promotes fires that have proved devastating to the native flora (Cabin *et al.*, 2000).

All the examples given above are alien plants that have invaded a habitat, displacing native species and thereby altering the environment. There are additionally other effects that can be attributed to alien species. As pointed out by Parker *et al*. (1999), alien plants can also have an impact on native species by genetic means such as hybridisation.

**1.2 Hybridisation and its effect on alien invasive plants**

Hybridisation is a completely natural phenomenon that occurs within many different groups of organisms, including plants, birds, fish and mammals. It is an exceptionally frequent occurrence within the plant kingdom. The New Flora of the British Isles (Stace, 1997) lists 715 hybrids, and of those known to have been produced in the British Isles, 70 are deemed to be products of hybridisation between native and alien species, 21 between two alien species, and four between an introduced hybrid and a native species.

Hybridisation from a taxonomic perspective tends to refer to interspecific or intergeneric hybrids, although obviously the term hybrid can relate to hybrids between taxa of the same species (Stace, 1989). An interspecific hybrid backcrossing to one or both parents leads to the infiltration of genes from one species to another. Such interspecific gene flow is known as introgression. The genetic and ecological consequences of introgression can include: increased genetic diversity; origin or transfer of adaptations; origin of ecotypes or species; breakdown or reinforcement of isolating barriers, and promotion of dispersion and colonization (Hardig *et al.*, 2000).

Generally when a taxon is introduced only a small proportion of the native genetic diversity is brought in. In some cases it can even be a single clone. The introduction of plants to new environments often brings together species that may not have been sympatric in their native range. These factors often lead to interspecific hybridisation occurring between a native and an invading plant species, or two invading species. This hybridisation can have numerous effects, the extremes being the evolution of new plant taxa e.g. *Iris nelsonii* Randolph (Abbott, 1992), or the extinction of the native species (Sakai *et al.*, 2001). One of the classic

examples of the evolution of a new species is that of *Senecio cambrensis* Rosser. It arose as an allohexaploid hybrid between Groundsel, *S. vulgaris* L., and the introduced species, Oxford Ragwort, *S. squalidus* L. (Harris & Ingram, 1992). There have been at least two independent origins of *S. cambrensis* in Britain, and there is still the potential for new origins to occur elsewhere in Britain (Ashton & Abbott, 1992).

Hybridisation events within the genus *Spartina* Schreb. can be used to illustrate both of these scenarios. The introduced *Spartina alterniflora* Loisel. hybridised with the British native *S. maritima* (Curtis) Fernald to produce *S.* x *townsendii* H. & J. Groves, a sterile hybrid that underwent chromosome doubling to produce *S. anglica* C. E. Hubb. (Raybould *et al.*, 1991). Furthermore, in South San Francisco Bay, the alien invasive *S. alterniflora* has readily hybridised with the native *S. foliosa* Trin., and the hybrid has been shown to readily backcross with both parents to the extent that it is now almost impossible to find pure native *S. foliosa* (Ayres *et al.*, 1999).

**1.3 Investigation of biological invasions**

Biological invasions can be natural phenomenon caused by an environmental change, such as those that lead to the post-glacial migration of both plant and animal taxa, or can be a result of the actions of man. Invasions caused by the actions of man have occurred over the last 5 – 6 millenia. These invasions can be split into two kinds, those that are natural consequences of man-induced changes in the environment, and those that result from voluntary or involuntary introductions of organisms to a new environment. The discipline of paleoecology can be used to distinguish between the two (Pons *et al.*, 1990).

Paleoecology covers techniques such as pollen analysis, radiocarbon dating, and prehistoric charcoal analysis, and can be extremely useful for studying the pattern of spread of different trees, in particular to follow post glacial migrations. Pollen analysis mainly distinguishes at the generic level, as it is not usually possible to distinguish species of the same genus. On a continental scale, the cover of samples can be patchy, and there are fairly large areas for which there are, as yet, no pollen records (Williamson, 1996). A difficulty with radiocarbon dating is that there is no simple linear relationship between radioactivity and time. Many dates are quoted as uncalibrated year before present using the half-life of $^{14}C$ to estimate dates before AD 1950. In the worst case an uncalibrated year can correspond to several years (Williamson, 1996). Prehistoric charcoal analysis includes the study of wood directly collected and used by man. Combustion generally facilitates the conservation of wood and many taxa (up to 30) can be found in each stratified charcoal deposit. Charcoal deposit

studies span the last 20 millennia, the period of the development of modern man and the transition from hunting-gathering to agriculture, in particular it includes the last glacial maximum and the period of post-glacial warming (Vernet, 1990).

Unfortunately there is little paleoecological information available concerning more recent invasions, resulting from voluntary or involuntary introductions by man. Primarily because, ever since man began to clear land for cultivation, he has also been removing his own traces (Pons *et al.*, 1990).

Since the early interest in biological invasions in the 1950s, the number of studies, and number of techniques utilised has escalated. Pyšek (1995) reviewed available literature for the years 1974-1993, and found that more than a quarter of the studies available dealt with control and conservation, whilst historical studies were quite rare. A further area gaining interest during that time period focused upon the biology and ecology of invasive species, and their interactions at the community level.

### 1.3.1 *Classical research techniques*

Basic scientific research has been used to attempt to identify the mechanisms underlying the invasion process, the impact of alien species on the native flora, and also to search for the general properties of invasive species and invaded communities (Pyšek, 1995). Most of the studies available are concerned with a single taxon, or small group of taxa, although there are a few publications that deal with more widespread issues, for example Weber (2000) who discussed invasive plant species issues for Switzerland, and Andersen (1995) who compared dispersal strategies of alien and native species within the Danish flora.

Historical reconstructions of the dynamics of spread tend to be based on published floristic records, unpublished floristic data obtained through personal communication, and herbarium specimens (Pyšek & Prach, 1994). In Britain in the mid-19$^{th}$ century, H. C. Watson decided the sizes of the British counties were unsatisfactorily variable for recording the distribution of British plants. He devised the vice-county system of dividing the larger counties and merging small ones with their neighbours, leaving the middle sized ones untouched. This allowed him to record distributions using the maps of the day. The average size of a vice-county is about 2200 km$^2$, making it only slightly smaller than the 50 km grid squares, which are used to map European distributions. So even now vice-counties are a useful way to map distribution, and they have the additional advantage that records dating back to the middle of the 19$^{th}$ century

and earlier can be used. This tradition is continued to this day by the Botanical Society of the British Isles (Williamson, 1996).

The choice of traditional characteristics used to study a biological invasion is often influenced by the particular speciality of the investigator, however there are some broad generalisations that can be made. Traits chosen often include the area of origin, plant stature, life form, life strategy, pollination agents, dispersal agents, mode of spread, planting history, and ecological requirements (Pyšek *et al.*, 1995) This includes looking at characters such as mean height, maximum height, minimum juvenile period, mean longevity, mean seed mass, seed-wing loading index, average percentage of germination, and mean interval between large seed crops (Rejmanek, 1995). Basic genetic attributes that may affect the invasive potential of a species, include ploidy level, genome size, and mating system.

Single species studies can be grouped into a number of different areas. For example the general biology and ecology of an invasive taxa has been reported, amongst others, for *Tamarix* spp. (salt cedar) (Brock, 1994), the Giant Hogweed, *Heracleum mantegazzianum* Somm. *et* Levier (Pyšek, 1994) and *Plantago* spp. (Matsuo, 1997). Control and management studies have been published for several taxa including, *Crassula helmsii* (Kirk) Cockayne (Child & Spencer-Jones, 1995), *Rhododendron ponticum* (Gritten, 1995) and the Giant Hogweed (Dodd *et al.*, 1994; Lundström & Darby, 1994). Additionally topics such as the effect of climatic variables (Beerling, 1994), or the reproductive biology of a species (Bailey, 1994) have been covered.

With regards to the Japanese Knotweed *s.l.* invasion a number of these methods have been utilised. The historical spread for the British Isles, using the system of vice-county records, was reported by Conolly (1977), the reproductive biology and fertility by Bailey (1994), control and management by de Waal (1995), Hill (1994) and others, comparative ecology between the native and introduced range by Sukopp (1995), and vegetative regeneration by Brock (1995; 1992).

### 1.3.2 *Further methods*

As time goes on more sophisticated methods are being applied to the study of biological invasions. Computer simulations and modelling, for example, can be used in many different ways. Computer simulations can be used to compare mating simulations within a theoretical population to the genetic structures observed in real population e.g. beechwoods (Thiebaut *et al.*, 1990). Models have also been used to predict the rate of spread of an invading organism.

This can be done in one of two ways. The first is empirical, based on the history of range expansion of the species in other localities or in previous years, the rate of spread is assumed to be similar to the past. The second relies on models, either a detailed model of the biology of the species is constructed, or a dispersal-growth population model is developed, that is then tailored to the species of interest (Andow, 1997). Other authors have used reaction-diffusion equations to model competition for space (Takasu *et al.*, 1997).

### 1.3.3 *Molecular approaches*

In recent years, genetic methods have been used to augment traditional morphological approaches in the study of invasive species (Roderick & Howarth, 1997), however as yet the genetics and evolution of these taxa has received far less attention than their ecology (Sakai *et al.*, 2001). The genetics of an invasion has been studied from two different perspectives; the first examines genetic changes relating to the invasion process itself, while the other uses genetics as a means to provide markers for determining the source and structure of the invading populations (Roderick & Howarth, 1997).

The modern genetic toolbox contains many different markers for cellular components such as proteins, and for the nuclear and organelle (chloroplast and mitochondrial) genomes. The markers differ from each other in many ways, particularly in variability and specificity. Non-coding regions tend to be more variable than coding ones. Analyses that tend to use the amplification of highly variable regions include microsatellites and introns, whereas isozymes for example represent less variable coding regions. Some markers are based on the observed differences in DNA sequences and these include those that use the actual DNA sequence and those that use restriction enzymes to cut the DNA at specific base sequences. Other methods rely solely on differential DNA amplification to detect the presence or absence of alleles (Roderick & Howarth, 1997).

The types of information pertinent to invasions that can be inferred from genetic data include; the origin and time of the invasion, founding population size, current population size, population structure, and rate of population growth (Sakai *et al.*, 2001). The following section is a description of the main molecular techniques that have been, or can be, applied to the study of invasive taxa.

#### 1.3.3.1 <u>Isozymes</u>

Isozymes were originally described by Market & Moller (1959) as different variants of the same enzymes, having identical or similar functions, and present in the same individual.

When these variant electromorphs are encoded by alternative alleles at a single locus they are termed allozymes (Prakash *et al.*, 1969).

In 1966 genetic polymorphisms for isozymes within a population were discovered in both humans (Harris, 1966) and *Drosophila* (Lewontin & Hubby, 1966), thus marking the start of their use for studying population genetics.

Isozymes are revealed when tissue extracts are subjected to electrophoresis in various types of gels and subsequently submersed in enzyme specific staining solutions. Often one or more regions of enzyme activity are revealed. This banding pattern is the electrophoretic phenotype. The phenotype can vary greatly in its complexity depending on numerous factors, including the organism, tissue and enzyme assayed. The range can be from a single invariant band in all samples to phenotypes with 15 or more bands per individual (Soltis & Soltis, 1990).

Because isozymes are proteins, they can directly reflect alterations in the DNA sequence through changes in amino acid composition. Comparisons between individuals and populations based on several gene loci can be made, and if accompanied by progeny analysis, Mendelian segregation ratios can be obtained (Soltis & Soltis, 1990).

Isozyme analysis was one of the earliest methods used to study population genetics, and more recently has been applied to the study of invasive plants. For example, allozyme analysis has been used to assess genetic variation within the introduced, clonal, invasive plant, *Pueraria lobata* (Pappert *et al.*, 2000). Isozyme phenotypes have also been used to both confirm the origin, and to assess the genetic variability of, *Spartina anglica* (Raybould *et al.*, 1991), and in combination with other techniques have been used to assess the origins of *Senecio cambrensis* (Ashton & Abbott, 1992; Lowe & Abbott, 1996). Unfortunately there are many limitations to this technique, including: their inactivity when the tissue is not fresh (Wolff & Morgan-Richards, 1999); the fact that the enzymes extracted are a tiny and probably non-representative sample of the total array of proteins present in an organism; the problem of whether or not differences in isozymes are neutral or adaptive with respect to evolution; and that the electrophoretic differences are only one kind of difference, and on the whole a minor one, of the various differences that exist even between genetically related proteins (Soltis & Soltis, 1990).

1.3.3.2 DNA sequencing

The use of DNA has many advantages over protein analysis, including its independence from environmental conditions, its lack of tissue specificity, and the unlimited number of scorable loci (Torres *et al.*, 1993). DNA sequencing is the direct assessment of the nucleotide sequence of a region of DNA, which can be compared with the sequence of an orthologous region in the genome of a different organism. It is highly reproducible and informative and, since the advent of PCR, homologous DNA sequences from organisms of interest can be isolated with unprecedented speed. Primers are designed which complement areas of the target DNA so that the desired sequence is amplified. In plants, DNA sequencing is mainly used for evaluating phylogenetic relationships, often by sequencing regions of the chloroplast genome or nuclear ribosomal DNA (Weising *et al.*, 1995). Sequencing of the *rbc*L gene has been used to study the invasive red alga *Polysiphonia harveyi* (McIvor *et al.*, 2001), but DNA sequencing is very expensive and therefore often alternative techniques have to be employed, particularly when a large number of individuals are to be studied.

1.3.3.3 Microsatellite DNA

Microsatellites or simple sequence repeats (SSRs) are short tandem repetitive DNA sequences with a repeat length of a few base pairs (1-5). These sequences are abundant, dispersed throughout the genome, and are highly polymorphic in plant genomes (de la Hoz *et al.*, 1996).

Early studies with microsatellites involved digestion of genomic DNA, separation of fragments in agarose gel, transfer of DNA fragments to nylon membrane, and hybridisation with labelled microsatellite probes for visualization of a genetic fingerprint. The arrival of PCR technology enabled the design of sequence-tagged microsatellite (ST-SSR) primers for a particular organism, which are locus specific. In most studies, design of ST-SSR primers involves the construction and screening of a genomic library, isolation and sequencing of DNA from positive clones, followed by primer design from the cloned sequence. Alternative methods for isolation of plant microsatellites are being developed, but currently the design of ST-SSR primers is a costly, time-consuming step (Wolfe & Liston, 1998). Microsatellite analysis has been used to assess levels of genetic variation within invasive Argentine ants, *Linepithema humile* (Tsutsui *et al.*, 2000), to test for isolation by geographical distance in the invasive cane toad, *Bufo marinus* (Leblois *et al.*, 2000), and to study the dispersal strategy of the invasive quagga mussel, *Dreissena bugensis* (Wilson *et al.*, 1999). There are however few, if any, cases of its use for the study of an invasive plant species, possibly as a direct result of the time consuming nature, and high costs, of designing the primers.

Microsatellites have been used extensively in plant and animal ecology and evolutionary biology. These microsatellites have predominantly been found in the nuclear genome, however more recently microsatellites have been found in the chloroplast genome, and these chloroplast microsatellites are showing great potential as a further tool for population studies. Sequence data are used to design the chloroplast microsatellite markers (Provan *et al.*, 2001). The application of this relatively new technique to invasive taxa is lacking.

1.3.3.4 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a means of evaluating variation in DNA sequences. DNA is digested with known restriction endonucleases, which cleave DNA at specific sequence motifs. The resulting fragments may vary in length from plant to plant, and it is these differences that constitute an RFLP (Rajapakse *et al.*, 1992). Traditionally nuclear DNA was used for RFLP analysis, but chloroplast DNA can be used as an alternative. The chloroplast genome is well suited to evolutionary and phylogenetic study for several reasons, including its relative abundance in plant total DNA, the extent of molecular information already available, and the conservative rate of nucleotide substitution. Conservative rates of chloroplast DNA evolution have both a technical and fundamental advantage. From a technical perspective, cloned chloroplast DNA genes can be used as heterologous probes across virtually the entire plant kingdom. The fundamental advantage arises because chloroplast DNA sequence change is ideal to resolve plant phylogenetic relationships to deep levels of evolution (Soltis *et al.*, 1992).

Chloroplast DNA is predominantly uniparentally transmitted in contrast to the biparentally transmitted nuclear DNA (Weising *et al.*, 1995). RFLP analysis of the split *trn*K intron from the chloroplast genome has shown chloroplast inheritance to be maternally transmitted for Japanese Knotweed plants (Hollingsworth *et al.*, 1999). This study also showed that using the restriction enzymes *Cfo*I, *Rsa*I and *Alu*I in combination, it is possible to identify three haplotypes, corresponding to *F. japonica* var. *japonica* (Houtt.) Ronse Decraene, *F. japonica* var. *compacta* (Hook F.) J. P. Bailey and *F. sachalinensis* (F. Schmidt) Ronse Decraene, in Britain. Other studies of invasive species have also benefited from RFLP analysis of both chloroplast DNA, and nuclear ribosomal DNA. Examples include the molecular systematics of the genus *Senecio* (Harris & Ingram, 1992), and the investigation into the origin and evolution of invasive naturalized material of *Rhododendron ponticum* in the British Isles (Milne & Abbott, 2000).

1.3.3.5 Randomly Amplified Polymorphic DNA (RAPD)

RAPD (randomly amplified polymorphic DNA) analysis was first described by Williams *et al*. (1990), and Welsh and McClelland (1990) as an alternative to RFLP analysis. The procedure depends on the differential amplification of DNA fragments using the polymerase chain reaction (PCR) with arbitrary oligonucleotide primers. Polymorphism results from either insertions or deletions in the amplified regions or base changes that alter the primer binding site (Baird *et al.*, 1992). This means that random sequences of DNA can be amplified and used as genetic markers (Brown *et al.*, 1993). The amplification products are separated by gel electrophoresis and detected by staining with ethidium bromide or silver staining (Weising *et al.*, 1995).

There are many advantages of the RAPD technique over RFLP, including the comparatively inexpensive equipment and supplies required, its speed, the small quantities of DNA required, and the high degree of polymorphism that is generated (Torres *et al.*, 1993). RAPD analysis has been used to study the hybridisation between invasive *Spartina alterniflora* and native *S. foliosa* in California (Ayres *et al.*, 1999; Daehler & Strong, 1997), to show the clonal nature of *F. japonica* var. *japonica* in Britain (Hollingsworth & Bailey, 2000a), to assess the levels of variation within a mixed population of Japanese Knotweed along the River Kelvin, Glasgow, Scotland (Hollingsworth *et al.*, 1998), and to a limited extent to assess diversity within *F.* x *bohemica* (Chrtek & Chrtkova) J. P. Bailey and *F. sachalinensis* (Hollingsworth & Bailey, 2000b).

1.3.3.6 Inter Simple Sequence Repeat (ISSR) PCR

In an attempt to design a "taxonomically generalised" approach allowing for simultaneous examination of a variety of genomic loci, Zietkiewicz *et al*. (1994) investigated the possibility of utilising $(CA)_n$ repeats as priming sites for inter-SSR (ISSR) PCR (inter-microsatellite PCR). These inter-SSR primers were anchored by extending outside into a unique sequence with their end two 3' nucleotides, thus avoiding priming from within a $(CA)_n$ element and providing the specificity that reduces the number of targeted genomic loci to those matching the 3'-terminal residues. Amplification with microsatellites anchored at the 5' end displayed broader specificity than all 3'-anchored primers (Zietkiewicz *et al.*, 1994). In several ISSR studies of plant taxa 3' anchored primers have been shown to be more reliable than 5' anchored and non-anchored microsatellites, (Kantety *et al.*, 1995; Parsons *et al.*, 1997; Salimath *et al.*, 1995; Tsumura *et al.*, 1996).

The amplified regions represent the nucleotide sequence between two SSR priming sites orientated on opposite DNA strands. The principle of the assay is that SSR regions are scattered evenly throughout the genome, and the chance of amplifying between two adjacent regions within the limits of *Taq* polymerase processibility is high enough that a large number of polymorphic bands should be generated (Wolfe *et al.*, 1998). The patterns produced are reproducible among experiments and primer lots. Controls that do not contain genomic DNA are reported to be consistently clean (Gupta *et al.*, 1994; Wolfe *et al.*, 1998). Figure 1.1 illustrates the general principle involved when 3'-anchored primers are used.

ISSR markers are inherited in a dominant or codominant Mendelian fashion. They are interpreted as dominant markers similar to RAPD data and are scored as diallelic with 'band present' or 'band absent'. The absence of a band is interpreted as primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement (Wolfe *et al.*, 1998).

ISSR analysis has been shown to be useful for studying genetic variation and molecular biogeography in the North American invasive plant species *Alliaria petiolata* (M. Bieb) Cavara and Grande, commonly known as garlic mustard (Meekins *et al.*, 2001). Hollingsworth *et al*. (1998) compared the efficiency of RAPDs and ISSRs in assessing the patterns of genetic variation in Japanese Knotweed. Split decomposition analysis of the ISSR data for *F.* x *bohemica* showed a major split between the male-sterile plants and the hermaphrodite plants whilst the RAPD data grouped the hermaphrodite individuals together, separate from the male-sterile plants. A putative backcross (*F. japonica* var. *japonica* x *F.* x *bohemica*) showed a greater affinity to *F. japonica* var. *japonica* than to *F.* x *bohemica* based on the RAPD data. A more intermediate position between the putative parental taxa was obtained from the ISSR data set. If the RAPD data were used in isolation they felt it could suggest that this plant was just a different genotype of *F. japonica* var. *japonica*. However, the ISSR data and the intermediate morphology of the plant, provided good evidence for this plant being a backcross. Greater resolution was obtained from the RAPD data set than the ISSRs, although this reflects the fact that twice as many RAPD as ISSR primers were used in the study. On a per-primer basis, they detected no significant difference between the resolving power of the different techniques.

Several other studies have been reported that compare ISSR analysis with techniques such as RFLPs, isozymes and the alternative popular random PCR fingerprinting technique RAPDs. ISSR markers were found to be more powerful than isozymes and RFLPs for fingerprinting

closely related accessions such as the trifoliate orange (*Poncirus trifoliata* (L.) Raf.) (Fang *et al.*, 1997). With Chinese sorghums (*Sorghum bicolor* (L.) Moench), RFLP, RAPD and ISSR markers showed comparable abilities at detecting variation, but the RAPD technique was more likely to generate irreproducible amplified bands than ISSRs and in terms of cost and speed of data generation ISSRs were found to be superior to both RFLPs and RAPDs (Yang *et al.*, 1996). As a genetic marker in wheat, ISSR primers were found to produce several times more information than RAPD markers, with the extent of band polymorphism being similar to that of RFLP markers, and greater than that of RAPDs (Nagaoka & Ogihara, 1997). Pujar *et al*. (2002) compared RAPD markers and ISSR markers in Indian tetraploid wheat germplasm with regard to number of bands amplified, number of polymorphic bands, groupwise percent polymorphism band informativeness, resolving power, average heterozygosity using all bands, and average heterozygosity using only one band and marker index. Barring the number of bands amplified, ISSR markers were found to be superior to RAPD markers in all other parameters. When cultivated barley *Hordeum vulgare* L. and its wild relative were studied using both ISSR and RAPD analysis, the percentage of polymorphic bands was found to be higher for ISSRs than for RAPDs, and both marker systems were more polymorphic than isozymes. The average resolving power of ISSR primers was higher than RAPD primers. The dendrogram generated by the ISSR matrix agreed better with the genealogy of the barley cultivars used than the dendrogram generated by the RAPD results (Fernandez *et al.*, 2002).

ISSR markers offer many advantages over other available techniques including (i) small amounts of DNA may be used; (ii) small reaction volumes and amounts of enzyme are needed for PCR; (iii) the hypervariability of banding patterns; (iv) fresh or large quantities of material for DNA extraction are not required; (v) no specialised apparatus or kits are required other than those needed for standard PCR techniques; and (vi) banding patterns are easily scorable. In addition the higher annealing temperatures used for ISSR reactions may reduce the amount of template-primer mismatch artefact than may be encountered with RAPD markers, which generally rely on lower annealing temperatures. Many researchers who have compared RAPD and ISSR methods have found that ISSR markers exhibit higher levels of polymorphism and/or reproducibility compared to RAPD markers (Nagaoka & Ogihara, 1997; Parsons *et al.*, 1997; Yang *et al.*, 1996). Limitations of the technique are similar to those encountered in the use of RAPD markers: (i) clean DNA template and similar concentrations among accessions are required for standardisation of reactions; (ii) optimisation of initial reactions is needed; (iii) bands are scored as dominant markers; and (iv) genetic diversity estimates are based on diallelic characters (Wolfe *et al.*, 1998).

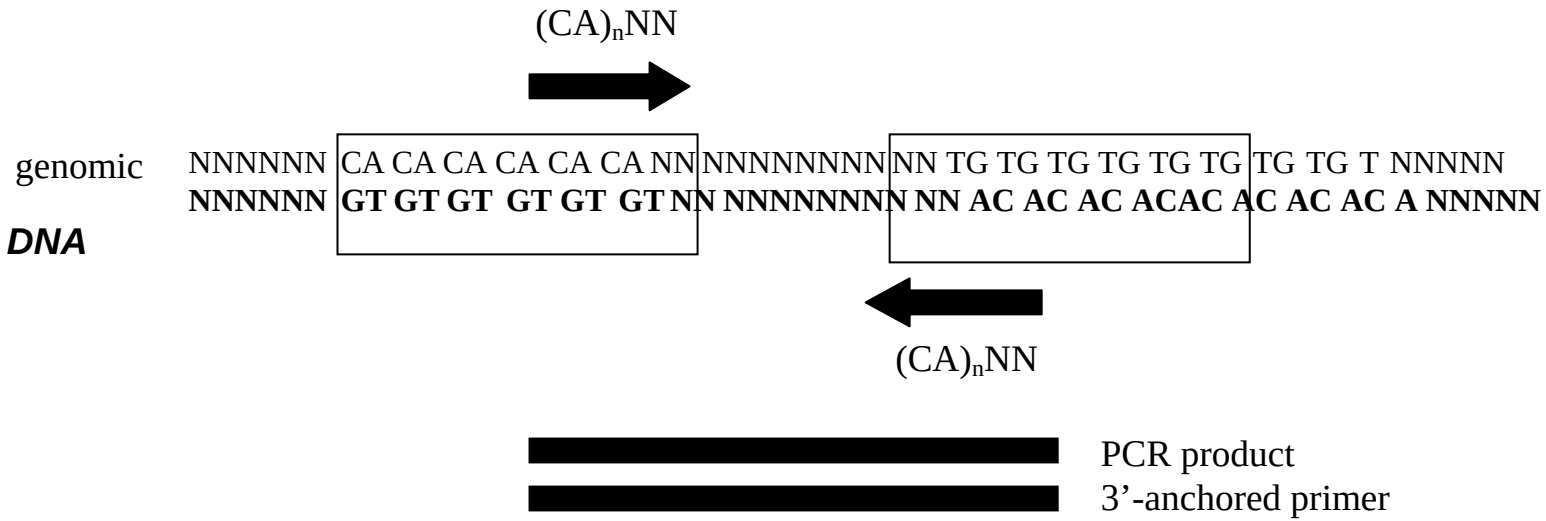



**Figure 1.1** Schematic representation of Inter Simple Sequence Repeat (ISSR) amplification using a 3'-anchored primer.

**Table 1.1:** Standard terminology for plant invasion ecology (Richardson *et al.*, 2000)

| | |
|---|---|
| Introduction | The plant (or its propagule) has overcome, through human agency, a major geographical barrier (intercontinental and/or infra-continental). |
| Alien plants | Plant taxa in a given area whose presence there is due to intentional or accidental introduction as a result of human activity (synonyms: exotic plants; non-native plants; non indigenous plants). |
| Casual alien plants | Alien plants that may flourish and even reproduce occasionally in an area, but which do not form self-replacing populations, and which rely on repeated introductions for their persistence (includes 'waifs', 'transients', 'occasional escapes' and 'persisting after cultivation'). |
| Naturalised plants | Alien plants that reproduce consistently (cf. casual alien plants) and sustain populations over many life cycles without direct intervention by humans (or in spite of human intervention); they often recruit offspring freely, usually close to adult plants, and do not necessarily invade natural, semi-natural or human-made ecosystems. |
| Invasive plants | Naturalised plants that produce reproductive offspring, often in very large numbers, at considerable distances from parent plants (approximate scales: > 100 m; < 50 years for taxa spreading by seeds and other propagules; > 6 m/3 years for taxa spreading by roots, rhizomes, stolons, or creeping stems), and thus have the potential to spread over a considerable area. |
| Weeds | Plants (not necessarily alien) that grow in sites where they are not wanted and which usually have detectable economic or environmental effects (synonyms: plant pests; harmful species; problem plants). 'Environmental weeds' are alien plant taxa that invade natural vegetation usually adversely affecting native biodiversity and/or ecosystem functioning. |
| Transformers | A subset of invasive plants which change the character, condition, form or nature of the ecosystem over a substantial area relative to the extent of that ecosystem. |

# Chapter 2. Introduction to Japanese Knotweed.

## 2.1 Japanese Knotweed *s.l.* in its introduced range

Japanese Knotweed is regarded as one of the most invasive alien plants in Britain. As such it has been subjected to legislation including, 'The wildlife and Countryside Act 1981' which makes it an offence to knowingly introduce Japanese Knotweed into the wild, and 'The Environmental Protection (Duty of Care) Regulations 1991' which states that any material contaminated with Japanese Knotweed is a 'waste' unless treated for re-use, and as such is subject to regulations which require all producers, carriers and disposers of 'waste' to follow a code of practice and keep records.

Japanese Knotweed *sensu lato (s.l.)* comprises taxa from the genus *Fallopia* Adans, section *Reynoutria* (Houtt.) Ronse Decraene. This includes: *F. japonica*, which in Britain can be found as both var. *japonica* and var. *compacta*; *F. sachalinensis*, which is also commonly known as Giant Knotweed; the hybrid between *F. japonica* and *F. sachalinensis* called *F.* x *bohemica*; and any backcrosses these plants may form (Bailey & Conolly, 2000). The different species were introduced to Britain from parts of Asia at various times during the 19$^{th}$ century whilst the hybrids are believed to have arisen since the introduction of the parental species.

The following section will introduce individually this group of taxa as they occur in their introduced range.

### 2.1.1 Fallopia japonica *var*. japonica *(Houtt.) Ronse Decraene*

Synonyms include

*Reynoutria japonica* Houtt

*Polygonum cuspidatum* Sieb. *et* Zucc.

*Polygonum sieboldii* Reinw.

#### 2.1.1.1 Description

*F. japonica* var. *japonica* is a rhizomatous perennial plant, with arching stems that often reach up to 3 m high. The leaves can be 5-12 cm long, 5-8 cm wide, broadly ovate with a cuspidate apex and truncate base (Beerling *et al.*, 1994). An illustration taken from Bailey *et al*. (1996) representing leaves of this, and the related taxa that also comprise Japanese Knotweed *s.l.*, can be found in Figure 2.1. The flowers are small and creamy white in colour and borne in clusters. The inflorescences tend to droop when mature in the male-sterile form (Beerling *et*

*al.*, 1994). Although *F. japonica* var. *japonica* is a gynodioecious species, only male-sterile plants are found in Britain. These are octoploid plants with 2n = 88 (Bailey & Stace, 1992). Molecular evidence suggests that all British, and possibly European, plants of this type are from a single clone (Hollingsworth & Bailey, 2000a).

2.1.1.2 Introduction and use

Bailey & Conolly (2000) go into great detail with regard to the introduction of *F. japonica* var. *japonica* to Britain. To summarise this information, all material of this type is believed to have arisen from a single introduction by Phillipe von Siebold from Japan to his Garden of Acclimatisation in Leiden, Holland in the late 1840s. From here it was sold to gardens throughout Europe, including Britain, under the name *Polygonum sieboldii*, this name being listed as a synonym of *Polygonum cuspidatum*. The earlier date of 1825 given in the literature (Conolly, 1977) relates to a Chinese accession of *F. japonica*, which is morphologically distinct from the *F. japonica* var. *japonica* clone that has invaded so much of Europe (Bailey & Conolly, 2000).

Introduced as an ornamental plant the early sales lists for *F. japonica* var. *japonica* gives a number of uses for this plant besides horticultural ones such as: good forage for livestock; stabilization of sand dunes; nectar for bees; tonic properties in the rhizome; and, for making matches (Bailey, 1994).

2.1.1.3 Escape and naturalisation

Conolly (1977) documents the early escape and colonisation of this species in Britain. The first report of its naturalization was in 1886 in Wales, and by 1920 *F. japonica* var. *japonica* was present in at least 59 of the 10 km square recording areas for the British Isles. In the Czech republic, the phase of exponential growth is listed as starting in 1938 (Pyšek & Prach, 1993). Many of the early reports were from waste-grounds, by railways, or nursery gardens presumably as direct garden escapes by vegetative means, or as outcasts on rubbish heaps (Conolly, 1977). Additionally some of the earliest recorded escapes in Britain and Germany are associated with coal-mining, although as opposed to being genuine escapes these could have been planted deliberately by the colliery owners to stabilize their heaps of colliery waste (Bailey, 1997).

One of the primary reasons given for the highly invasive nature of Japanese Knotweed is its extraordinarily high regeneration potential. The figure most often quoted being that of Brock and Wade (1992) who showed that a new plant can originate from as little as 0.7 g of rhizome

(Bailey *et al.*, 1995; Bímová *et al.*, 2001; Hollingsworth, 1998). Regeneration of shoots and adventitious roots can also occur from stem segments, as long as axillary buds are present. The most successful media for shoot regeneration being water. Dispersal by water of stem material is likely to be a common event in riparian habitats and the additive effect of water transported stems being covered by sediments or debris, during a flood event, greatly increases the chances of further establishment (Brock *et al.*, 1995).

2.1.1.4 Problems

Beerling (1994) summarises an investigation into the type of damage caused by Japanese Knotweed in Wales. Flood protection schemes suffer the most widespread damage, whilst occasional damage also occurs to roads and pavements, with Knotweed actually penetrating tarmac and displacing paving stones and kerb blocks. It is a major problem along watercourses where riparian vegetation can be replaced by dense stands of *F. japonica* var. *japonica* (Brock *et al.*, 1995), and it is also known to have a detrimental effect on native flora and fauna (Beerling & Palmer, 1994).

2.1.1.7 Habitat and distribution

In 1988 *F. japonica* var. *japonica* was present on 84% of rivers surveyed in Wales (Beerling & Palmer, 1994). It is most common on sites disturbed by human activity (Brock *et al.*, 1995).

*F. japonica* var. *japonica* is the most widespread of this group of taxa, being found in over 2761 of the 3859 10 km square recording areas in the British Isles (Preston *et al.*, 2002). A current British distribution map taken from Preston *et al*. (2002) is shown in Figure 2.2. Jalas & Suominen (1979) record the presence of *F. japonica* in most of the countries of mainland North, Central & Southern Europe with the exception of Italy, Albania and European Turkey, although it has since been found in Italy (Bailey, 2003). It also occurs in Australia, Canada, New Zealand, and the U.S.A.

2.1.2 Fallopia japonica *var*. compacta *(Hook F.) J. P. Bailey*

Synonyms include

*Reynoutria japonica* var. *compacta* (Hook. f.) Buchheim

*Polygonum compactum* Hook. f.

*Polygonum pictum* Sieb.

2.1.2.1 Description

A dwarf plant usually less than 1 m tall (Stace, 1997). It has small, broad leaves which compared to *F. japonica* var. *japonica* are thicker and darker green. They are often as broad as they are long, with crimped margins. The flowers tend to be reddish-brown in colour (Lousley & Kent, 1981) particularly in the females. The inflorescences are erect, and scarcely branched (Conolly, 1977). *F. japonica* var. *compacta* is gynodioecious (J. P. Bailey, pers. comm.). All counts for this variety have been tetraploid with 2n = 44 (Bailey & Stace, 1992).

2.1.2.2 Introduction and use

Under the name *Polygonum pictum*, *F. japonica* var. *compacta* was first introduced to Europe in 1841 as part of the collection of Von Siebold. It was grown and sold from his garden in Leiden, Holland. The earliest record for cultivation in the British Isles is given as 1881 (Bailey & Conolly, 2000).

Introduced as a garden ornamental (Bailey & Conolly, 2000), few other uses are listed in the available literature.

2.1.2.3 Escape and naturalisation

According to Bailey and Conolly (2000) the earliest recorded escape was in 1915. The plant is still rather rare, with few instances of naturalisation. It is not considered to be very invasive. The only sizeable naturalized stands in Britain are at Connel Ferry and North Ledaig in Scotland (Bailey, 2003).

2.1.2.4 Distribution

There are currently 30 records mapped on a 10 km square basis, including garden plants (Bailey & Conolly, 2000). Its occurrence on the continent is also extremely rare, with three locations recorded in the Czech Republic (Mandák *et al.*, 2003).

2.1.3 Fallopia sachalinensis *(F. Schmidt) Ronse Decraene*

Synonyms include

*Reynoutria sachalinensis* (F. Schmidt) Nakai

*Polygonum sachalinense* F. Schmidt

*Pleuropterus sachalinensis* (F. Schmidt) Moldenke

*Tiniaria sachalinensis* (F. Schmidt) Janchen

2.1.3.1 Description

*F.sachalinensis* is a stout rhizomatous perennial, whose height can reach up to 4 m (Brabec & Pyšek, 2000). The flowers are functionally dioecious, with male plants having exerted stamens, large anthers with good pollen, and vestigial ovaries. The female plants have included stamens, small, empty anthers and nuts enclosed in well-developed winged fruiting perianths (Conolly, 1977). Bailey (1994) examining *F. sachalinensis* found them to be either male-sterile or hermaphrodite. This pattern of sex expression is known as gynodioecy and is thought by some to represent an intermediate stage in the evolution of dioecy from hermaphroditism (Bailey, 1994).

The rhizomes can attain a diameter of 7 to 8 cm, and can reach 15 to 20 m in length (Marigo & Pautou, 1998). The fully developed leaves tend to be up to 40 cm long and 22 cm wide, ovate-oblong, cordate at the base, and the undersides scattered with long flexuous hairs (Bailey *et al.*, 1996). Flowering starts in late June and reaches an optimum in August (Marigo & Pautou, 1998), and by changing its leaf arrangement and orientation of the inflorescence at the beginning of the reproductive phase, it is able to present the inflorescences in a layer above any competing vegetation (Sukopp and Schick (1993) in Sukopp & Starfinger (1995)).

In most of its introduced range *F. sachalinensis* is found to be tetraploid with 2n=44, the exception being three individuals from the Czech republic with 2n = 66, and a further thirteen with 2n = 88. These are thought to be the result of self-pollination of *F. sachalinensis* with an unreduced gamete to produce the hexaploid, and the result of chromosome doubling in the case of the octoploids (Mandák *et al.*, 2003). Seed collected from male-fertile *F. sachalinensis* at Amroth (S. Wales), and assumed to be *F. sachalinensis* was also found to be hexaploid (Bailey, 2003).

2.1.3.2 Introduction and use

The early literature dealing with the introduction of *F. sachalinensis* has been thoroughly reviewed by Bailey & Conolly (2000). It would appear that there are at least two routes by which it may have arrived in Europe. The first is via St. Petersbourg, where there have been at least two collections from the Sakhalin islands (by Dr H. Weyrich in 1853 and then P. von Glehn in 1861) and also from Hakodate in Japan, collected by C. J. Maximovicz and arriving in St. Petersbourg in 1864. The second route is via Kew botanic gardens. They received plant material from Japan from Wilford in early 1860 and Oldham in 1862-3, although the records don't indicate whether *F. sachalinensis* was among the plants received. There is, however, an 1859 herbarium specimen of *F. sachalinensis* collected by Wilford from

Hakodadi, Japan. Additionally, Charles Maries collected further material from the central mountains of Japan in 1878-9, whilst employed by James Veitch & Son. Although it is not known if live material was brought back there is a herbarium specimen dated 1880 at Kew from this collection (Bailey & Conolly, 2000).

*F. sachalinensis* was initially introduced for forage then subsequently grown as a decorative horticultural plant (Conolly, 1977). Apparently it was popular with huntsman as it was supposed to be more palatable to game than *F. japonica* and also offered good cover (Herberg (1937) in (Sukopp & Starfinger, 1995)). It was also introduced as a riverbank stabiliser (Bailey & Conolly, 2000). Its usage on large estates for scenic plantings, and as cover for shoots, is thought to potentially explain why it is found more often than *F. japonica* well off the beaten track on old estates (Bailey & Conolly, 2000). Other uses listed include as firewood and for the production of matchsticks and medicinal purposes (Sukopp & Starfinger, 1995). The large leaves were also used to protect fruit to be sold in hot climates (Marigo & Pautou, 1998).

In Europe a new use of the plant was found in its efficacy against fungal plant diseases. Leaf extracts are effective against powdery mildew of apple, *Begonia*, cucumber and wheat as well as against grey mould (*Botrytis cinerea*) of sweet peppers (Herger *et al*. (1988) in (Sukopp & Starfinger, 1995)). Recently it was suggested that these plants could be used for the decontamination of soils polluted with heavy metals, since the plants can accumulate such contaminants (Kubota *et al.*, 1988; Nishizono *et al.*, 1989).

2.1.3.3 Escape and naturalisation

The first published account of its naturalisation within the British Isles was Davies (1896) reporting on an extensive stand on waste ground by the Lagan Canal at Lisburn, Antrim (Conolly, 1977). Earlier reports were made from the Czech republic in 1869 (Pyšek & Prach, 1993) and in the same year from Germany (Sukopp & Starfinger, 1995).

The establishment of *F. sachalinensis* is accelerated by human activities such as ploughing and transport of soil from one site to another. However it can also spread in areas unaffected by man such as the islands in alpine rivers. Water erodes the soil and then transports the rhizome sections to a new site where they can form new colonies (Marigo & Pautou, 1998).

The ability of *F. sachalinensis* to colonize the area where it grows is helped by the fact that the seeds produce substances that have allelopathic properties. The substances excreted by

the seeds, during the imbibition stage, affect the rhizogenesis of plants in close proximity (Marigo & Pautou, 1998). Additionally *F. sachalinensis* attracts ants with extrafloral nectaries, which help to protect it against herbivorous insects (Sukopp and Schick (1991) in (Sukopp & Starfinger, 1995)).

2.1.3.4 Problems

*F. sachalinensis* is regarded as being far less invasive than *F. japonica* var. *japonica* or the hybrid *F.* x *bohemica* (Conolly, 1977), and is the easiest to control. Of the three taxa it regenerates the least effectively, although there were still some new shoots emerging at the end of the growing season where the control experiments were being conducted (Bímová *et al.*, 2001). Even so it is regarded as an invasive species and the problems associated with the spread of these plants includes: the restriction of access to stream and river banks; the damage of flood defence structures; the out-competing and exclusion of native vegetation; and, the decrease of potential vegetation development (Marigo & Pautou, 1998).

2.1.3.5 Habitat and distribution

*F. sachalinensis* tends to establish either in locations influenced to some degree by human activities such as gardens, parks or ruderal sites in towns and villages; or along watercourses under near-natural conditions. The conditions can vary from dry and warm for ruderal sites, to wet and cool for riparian sites in the mountains. The stands tend to contain few other species, those that do most frequently accompany it being *Urtica dioica* L., *Artemisia vulgaris* L., *Poa palustris* L., *P. trivialis* L., and *Aegopodium podagraria* L. (Sukopp & Starfinger, 1995). Although it is able to grow in different types of habitat its preference is for low altitude alluvial plains with a constant flow of water & raised temperatures during the period of physiological activity. The presence of a large medullary air space within the internode is claimed by Marigo & Pautou (1998) to be an anatomical indicator of the strong demand of *F. sachalinensis* for water; however, it is capable of adapting to more rigorous conditions.

*F. sachalinensis* is found in most of central, western and eastern European countries though absent from the Mediterranean region (Jalas & Suominen, 1979). It is also reported from USA, Canada, Australia, New Zealand, (Bailey, 2003) and there is an unconfirmed record from a riverside in India (Bailey & Conolly, 2000).

In all these countries it is scattered to fairly common, being locally common in some areas, but always less frequent than *F. japonica* (Sukopp & Starfinger, 1995). Distribution in the Czech Republic is to a large extent confined to the vicinity of rivers & streams (Brabec &

Pyšek, 2000), whilst in France populations are progressing across the region of the Rhône Alpes. In the 1970s, this species was still absent on the islands found in the Rhône between Geneva and Lyon, now there are several colonies scattered along this stretch. In the valley of the Isere, between Montmélian and St-Gervais on the outskirts of Grenoble, the species is very abundant (Marigo & Pautou, 1998). In the British Isles, *F. sachalinensis* now occupies 576 of the 3859 10km squares (Preston *et al.*, 2002). A copy of this map is given in Figure 2.3.

### 2.1.4 Fallopia *x* bohemica *(Chrtek & Chrtkova) J. P. Bailey*

The hybrid between the alien introduced species *F. japonica* and *F. sachalinensis* was first noticed growing in north-eastern Bohemia, Czech Republic (Chrtek & Chrtkova, 1983), and was given the name *Reynoutria* x *bohemica* Chrtek & Chrtková. This work was based on morphological characteristics. Bailey & Conolly (1985), whilst producing chromosome numbers of some of the British Knotweed plants, suggested that six hexaploids and three tetraploids that they had examined were probably also of this same hybrid origin.

#### 2.1.4.1 Description

*F.* x *bohemica* can be readily distinguished from its parental species because it has an intermediate morphology. Bailey *et al* (1995) give a comprehensive guide to the identification of the hybrid. As yet there are no unequivocal characteristics for distinguishing the different ploidy levels other than chromosome number (Bailey *et al.*, 1996).

*F.* x *bohemica* is found at three different ploidy levels in Britain, tetraploid (2n = 44), hexaploid (2n = 66) and octoploid (2n = 88). Given that *F. japonica* var. *compacta* and *F. sachalinensis* are both tetraploid plants (2n = 44), and *F. japonica* var. *japonica* is an octoploid plant (2n=88), the origin of the tetraploid and hexaploid hybrids can be easily explained, as shown in Fig. 2.4, but the origin of the octoploid plant is more speculative (Bailey, 1997). There are at least three potential ways in which an octoploid *F.* x *bohemica* may arise. Firstly a tetraploid *F.* x *bohemica* may undergo autopolyploidy, or alternatively an unreduced gamete from *F. sachalinensis* may pollinate *F. japonica* var. *japonica.* A third albeit less likely, explanation would be the chance occurrence of a euploid F2 or backcrossed hybrid.

#### 2.1.4.2 Problems

The problems associated with the presence of *F.* x *bohemica* are similar to those of the parental taxa. Current control treatments for Japanese Knotweed *s.l.* are currently based on

their effectiveness on *F. japonica* var. *japonica* (Beerling, 1991). However, a recent study has shown *F*. x *bohemica* to be the most resistant to control, with none of the current methods being very successful (Bímová *et al.*, 2001). There is also evidence that *F*. x *bohemica* has a higher regeneration potential than either of its parents (Brabec & Pyšek, 2000) and may in fact be more invasive (Mandak, B. pers. comm.).

2.1.4.3 Distribution

In 1993 a survey of the abundance and distribution of *F*. x *bohemica* in the British Isles was carried out jointly by Leicester and Loughborough Universities (Bailey *et al.*, 1996). The final map they produced had 126 localities that were separable by a six-figure grid reference, which translated to 81 10 km square records on the B.R.C. distribution map. This distribution is believed to be an under-representation of the true picture, and is geographically partially indicative of the number of botanists interested in the hybrid in a given area. For example, no records were received from Ireland, yet the authors were informed that the hybrid is common in parts of Western Ireland (Bailey *et al.*, 1996). In the New Atlas of The British & Irish Flora (Preston *et al.*, 2002) *F*. x *bohemica* was recorded as present in 190 of the 3859 10km recording squares (Preston *et al.*, 2002), shown in Figure 2.5. The majority of these sites represent hexaploid *F*. x *bohemica*, which, although capable of producing F2 and backcrossed seed, are not classed as fertile because the gametes produced, and subsequent plants, are mostly aneuploid. Nevertheless the rarer tetraploid and octoploid hybrids are showing signs of being fully fertile and both sexes of each ploidy level are present within the British Isles (J.P. Bailey, pers. comm.).

As well as the British Isles, *F*. x *bohemica* has now been recorded from Australia, Canada, Czech republic, Denmark, Finland, France, Germany, Guernsey, Hungary, Ireland, the Netherlands, New Zealand, Norway, Poland, Slovakia, Sweden, and the U.S.A. (J.P. Bailey, pers. comm.).

2.1.5 *Further Knotweed hybrids*

The only other Knotweed hybrid to have been given a name is the hybrid between *F. japonica* var. *japonica* and Russian Vine, *F. baldschuanica* (Regel) Holub. Named *F*. x *conollyana* J. P. Bailey, this hybrid is a herbaceous perennial. The young plants are weakly rhizomatous, whilst the long established plants form stout woody rhizomes. Superficially similar to *F. japonica*, but with thinner stems and smaller leaves, the stems bend almost to ground level. The leaves are acuminate, ovate to narrowly ovate-oblong (Bailey, 1988). Vast amounts of seed of this constitution are produced every year throughout Europe, but very few have

germinated or become established (Bailey, 2001). The one renowned stand in Britain is from a railway yard at Haringey, Middlesex v.c. 21 and covers more than 10 square metres (Bailey, 1992). *F.* x *conollyana* has 2n = 54 (Bailey, 2001).

Seed resulting from pollination by *F. baldschuanica* has also been collected from open pollinated plants of *F. sachalinensis*, *F. japonica* var. *compacta* and tetraploid *F.* x *bohemica* growing in the wild in Britain (Bailey, 2003), although there are no records of any plants of this constitution actually growing in the wild. These all have 2n = 32 (Bailey & Stace, 1992).

The intra-specific hybrid between *F. japonica* var. *japonica* and *F. japonica* var. *compacta* has been found twice in Britain and is also known from Germany (Bailey, 2003). These plants can be very tall, up to 3 m, with somewhat leathery leaves with slightly wavy margins (Bailey & Conolly, 1991). These hybrids have 2n = 66 (Bailey & Stace, 1992). A male fertile form of the intra-specific hybrid growing at Buryas Bridge, Cornwall, England, has been shown to pollinate *F. japonica* var. *japonica* (Bailey, unpublished data).

**2.2 Japanese Knotweed in its native range.**

In this thesis the term Japanese Knotweed *s.l.* will only be used when referring to the group of taxa covered in 2.1, when found in their invasive range. With regard to plants originating from the native countries, specific names will be used.

2.2.1 Fallopia japonica

*F. japonica* is native to China, Korea, Japan and Tawain (Bailey, 1989). In this range the external morphology of this species is so variable that several different intraspecific classifications have been proposed (Inamura *et al.*, 2000). There is also some confusion within the literature with regard to the classification of these plants at the generic level. For the most part researchers from the native countries still refer to these plants as belonging to either *Polygonum* L. or *Reynoutria* Houtt. More recently their transfer into *Fallopia* has been accepted (Yonekura, K. pers. comm.) but this is yet to be reflected in the publications or floras.

According to Yonekura and Ohashi (1997) there are three taxonomic varieties of *F. japonica*: var. *japonica*, var. *hachidyoensis* (Makino) K. Yonekura & Hiroyoshi Ohashi and var. *uzenensis* (Honda) K. Yonekura & Hiroyoshi Ohashi, but other researchers recognise other varieties. Many of the less common varieties appear to be endemic to specific islands such as *Reynoutria japonica* var. *insularis* (var. nov., Hotta) which has a thicker leaf than is

considered to be normal and is found on the Southern islands of Kyushu, or *Reynoutria japonica* subsp. *amamiana* (subsp. nov., Hotta) which is endemic to Amami Island and is reported to be a large androdioecious plant (Hotta, M. pers. comm.). *Polygonum cuspidatum* var. *terminalis* Honda is endemic to the Izu Islands and is characterised by the glossy appearance of its leaves (Bailey, 2003). These varieties that are endemic to specific Islands and not found naturalised in the invasive range of this group of plants, will not be considered further.

*F. forbesii* (Hance) Yonekura & H. Ohashi is the name given to the plants that grow on the Chinese mainland and is also used in Korea (Kim & Park, 2000). It is said to be a synonym for *Reynoutria elliptica* (Koidz.) Migo ex Naki. These plants have a very characteristic elliptical leaf shape with an acuminate base (Bailey, 2003). According to Kim and Park (2000) in some of the Korean floras it is treated as a separate taxon to *F. japonica*, whilst in others it is treated as a synonym for *F. japonica.* Using principal components analysis (PCA) of the major morphological characteristics of the leaf, Kim & Park (2000) found Korean *F. forbesii* to be separable from plants they termed Korean *F. japonica* var. *japonica*. *F. forbesii* can be both hexaploid and octoploid (Kim & Park, 2000).

2.2.1.1. *Fallopia japonica* var. *japonica*

*F. japonica* var. *japonica* is rarely used as a taxonomic term in Japan, but when it is used it tends to refer to the taller lowland plants. It is more common for these plants to be referred to by the synonyms *Polygonum cuspidatum* or *Reynoutria japonica*.

The chief habitat for the tall lowland *F. japonica* plants is at the edge of forests and riversides in forests, although it can also be found along canalised rivers and occasionally on urban roadsides (Bailey, 2003). *Miscanthus sinensis* Anderss. Susuki. type grassland is one of the most representative of tall grass type meadows in Japan, and *Reynoutria japonica* is often a component of it (Numata, 1974).

2.2.1.2. *Fallopia japonica* var. *compacta*

In its invasive range, *F. japonica* var. *compacta* can be easily distinguished from *F. japonica* var. *japonica* on a number of characteristics including ploidy level, height, and leaf characteristics. In the native regions the distinctions are not as clear and many researches do not believe *F. japonica* var. *compacta* merits formal taxonomic recognition (Kim & Park, 2000).

Observations on Mt Fuji indicate that the characteristics associated with *F. japonica* var. *compacta* in its introduced range; such as more or less square leaves with crimped edges, red petioles and fruiting perianths, can be found on plants at lower altitudes that can reach heights of up to two metres (Bailey, 2003). Likewise, individuals from other mountains such as those found at the summit of Mt. Aso, Kyushu, do not possess these typical leaf shape characteristics but are clearly dwarf plants (personal observation).

Reciprocal transplantation studies have been conducted between plants from different altitudes by Shisosaka & Shibata (1993) within Japan. Mature plants transplanted from basal level to higher elevations changed their growth form to dwarf with increasing altitude, however the plants from the montane, subalpine, and alpine zones retained their dwarf form when transplanted into basal and montane zones.

Mariko *et al*., (1993) examined altitudinal variation in germination and growth responses of *F. japonica* populations on Mt. Fuji, Japan. The sites on Mt. Fuji ranged from the basal to the alpine region, and were additionally compared to two basal region sites from the prefecture Chiba with altitudes of 10 m and 270 m. They found that the plants could be classified into two ecotypes, whose distribution border lies at an altitude of about 1400 – 1500 m. They found no difference between the lowland Mt. Fuji samples and those obtained in Chiba, these two areas being about 120 km apart. A study by Mariko and Koizumi (1993) comparing respiration in plants from 700 and 2420 m on Mt. Fuji supports the concept of two different ecotypes of plant dependent on altitude. A different altitudinal study of germination characteristics, again using plants from different altitudes of Mt. Fuji and including a lowland meadow field population from Shizuoka for comparison, found no altitudinal differences in germination characteristics (Nishitani & Masuzawa, 1996).

Sequence analysis of a region of the chloroplast genome was conducted on a limited number of Japanese accessions of *F. japonica*, under the synonym *Polygonum cuspidatum* (Inamura *et al.*, 2000). This study analysed four intraspecific taxa, from which *P. cuspidatum* var. *terminalis* and *P. cuspidatum* var. *uzenensis* (Honda) Kitam. were recognized as valid taxa. *P. cuspidatum* var. *compacta* Hiyama was not found to be genetically distinct, suggesting that this taxon is an alpine ecotype rather than a valid taxon, but only one accession of *P. cuspidatum* var. *compacta* was included in the study.

In Korea, a comparison between dwarf plants from Mt. Halla on Cheju Island, and lowland plants termed *F. japonica* var. *japonica*, found the plants to be indistinguishable in all

morphological features other than overall size of the plant. Thus supporting the view of others that it does not merit formal taxonomic recognition (Kim & Park, 2000).

Some authors still separate lowland from highland taxa. For example, recently the name *Reynoutria japonica* var. *monticola* (var. nov., Hotta) has been applied to dwarf plants that grow up to 50cm tall, flower in July to August and are found at the top of volcanic mountains such as Mt Kirishima, Kyushu (Hotta, M. pers. comm.).

2.2.1.3. *Fallopia japonica* var. *uzenensis*

*F. japonica* var. *uzenensis*, distributed in the Hokuriku and Tohoku districts, has many hairs on its leaves (Inamura *et al.*, 2000). Whilst this taxon has not been introduced to Britain and Europe, it is still of interest. It is a tall lowland taxon that is sympatric with part of the *F. sachalinensis* range (Bailey, 2003). It is primarily an octoploid taxon (Bailey, J.P. unpublished) that, apart from the presence of short stiff hairs on the lower leaf epidermis, has other leaf characteristics such as shape, size and colour that are very similar to those found in the invasive *F. japonica* var. *japonica* clone found in Britain and Europe (personal observation).

2.2.2 Fallopia sachalinensis

*F. sachalinensis* is found in the southern part of Sakhalin Island (former USSR), the southern Kurile Islands (Kunashir and Shikotan), and the Japanese Islands Hokkaido and Honshu. On Honshu it is only found on the northwestern side of the Chuba district i.e. the part close to the Japanese Sea (Sukopp & Starfinger, 1995). Whilst it is believed to be native to areas northward of central Honshu, there are also several records of its introduction in Western Japan (Yonekura K, pers. comm.). Additionally it is naturalised on the Island of Yaku-shima, which is found to the South of one of the main Japanese Islands, Kyushu (Hotta, M. pers. comm.), and possesses a national park. *F. sachalinensis* is also reported from the isolated Ullung-do Island between Korea & Japan (Bailey & Conolly, 2000).

There is very little information published in English about the populations from the Sakhalin Island or the southern Kurile Islands, although it is known that specimens were collected from the Sakhalin Island in 1853 (Bailey & Conolly, 2000). This is because more recent collections have been hampered by the political situation in the former USSR. In Korea *F. sachalinensis* is restricted to the Ullung Island. These appear to be dodecaploid with 2n = 132, fully fertile and on the basis of leaf shape and size characteristics, undistinguishable from tetraploid individuals (Kim & Park, 2000).

Sukopp (1995) provides a good description of the Japanese habitats for *F. sachalinensis*, based on information from Miyawaki (1987; 1988). *F. sachalinensis* is found from sea-level to an altitude of ca. 1050 m and forms two different plant communities. The first is from North Honshu and is referred to as Angelico-Polgonetum sachalinensis. This is found along forest edges, roads through forests, in avalanche areas in the mountains and on coastal cliffs. The stands are usually 1.4 to 2.5 m tall and are divided into two sub-associations: one in natural vegetation of coastal cliffs and mountain rivers which are subject to natural earth movements, and the other on road banks and other places with man-induced earth movements. The second community are found only on Hokkaido and are known as Cirsio kamtschatici-Polgonetum sachalinensis. These grow on similar sites to the first community on Honshu, i.e. at forest edges, riverbanks and coastal cliffs as well as on fallow fields and road banks. These can reach the height of 2-3 m, and the total cover can be close to 100%. This plant community can be divided into three sub-associations that differ in species richness and site types (Sukopp & Starfinger, 1995).

On Hokkaido *F. sachalinensis* is strongly implicated in the recovery of vegetation after a volcanic eruption on Mt Uso. It is believed to have predominantly arisen by vegetative reproduction from buried material as opposed to from new seed, although it was also seen to germinate from seed. Of the seedlings that germinated 60 to 80 % died within a year, but once established the survival thereafter was good (Tsuyuzaki, 1989).

From a morphological perspective there appears to be far less variation among native *F. sachalinensis* plants than there is amongst native *F. japonica*. Besides the dodecaploid individuals from the Ullung Island, which may deserve taxonomic recognition (Bailey & Conolly, 2000), there are no varieties or subspecies recognised.

### 2.2.3 Fallopia *x* bohemica

The hybrid between *F. japonica* and *F. sachalinensis* was not described in Japan until 1997, when it was given the name *Reynoutria* x *mizushimae* Yokouchi ex T. Shimizu. This date is later than that of *F.* x *bohemica*. Although both *F. japonica* and *F. sachalinensis* are common on lowlands in northern Honshu, they rarely occur sympatrically in the natural undisturbed habitats. It is in disturbed sites around urban areas that hybridisation is now commonly occurring, and the number of hybrids is increasing rapidly. This situation is being accelerated by the use of both of these taxa for roadside protection from erosion, and several populations of these hybrid plants have been found around Sendai City (northern Honshu) (Yonekura, K. pers. comm.). Dr K. Yonekura of Tohoku University has found intermediate plants that may

be of hybrid origin on Hokkaido, which he has named *F. sachalinensis* var. *intermedia* K. Yonekura & Hiroyoshi Ohashi.

### 2.3 Brief introduction to the geography and climate of Japan

On the basis of morphological and cytological analysis, and historical documentation it is strongly believed that the *F. japonica* var. *japonica* and *F. japonica* var. *compacta*, found naturalised in Britain and Europe originated from Japan, whilst *F. sachalinensis* may have arisen from Japan, or the Sakhalin Islands (Bailey & Conolly, 2000).

The territory of Japan consists of islands that are strung out over a distance of about 2,500 km from the southern end of the Ryukyu Islands, 24º north latitude, to the northern end of Hokkaido, 45.5º north latitude (Minato, 1977). Japan is comprised of about 3,900 islands, with a gross land area of about 372,000 $km^2$. Among these the four main Islands of Honshu, Hokkaido, Kyushu, and Shikoku take up 360,300 $km^2$. There are said to be 186 volcanoes in Japan, of which 60 are still active. The highest point in Japan is a volcano, Mt Fuji, with an elevation of 3,776 m. The highest non-volcanic mountain is Mount Shirane in the Akaishi Mountain Range, with an elevation of 3,192 m (Nishikawa *et al.*, 1980).

The vegetation varies greatly from one end of Japan to the other. The southern Ryukyu Islands are covered with subtropical vegetation and their shores are fringed with coral reefs, whereas in the north Hokkaido belongs to the cool temperate to subarctic zone, and suffers severe cold during the winter months. In spite of the large population some 25 million hectares of the land, 70% of the total area, is still covered with forest (Minato, 1977).

As a region Japan has one of the highest precipitation levels in the world. The mean annual precipitation being about 1,800 mm; though in parts of Hokkaido and the inland basins in central Japan the rainfall does not exceed 1,000 mm a year (Nishikawa *et al.*, 1980). There is a wide difference in climatic characteristics between the northern and southern regions. For example, in winter the areas facing the Japan Sea are subject to heavy snowfall, the snow depth being one of the greatest in the world, whilst the Pacific side is reported to be both cold and very dry (Minato, 1977).

Japan has two main rainy seasons, the *Bai-u* or the *Tsuyu* season in early summer and the *Shurin* season in early autumn. There is a third season of precipitation that affects the areas on the Japan Sea side, which is the season of heavy snowfall that peaks in December or January. Because of these rainy seasons, Japan is deemed to have six seasons as opposed to

the more common four. These are known as winter, spring, Baiu season, midsummer, Shurin season, and late autumn. They are a characteristic that is unique to Japan, not occurring in any other middle latitude country (Minato, 1977).

The variations in atmospheric temperature in a year are more marked in Japan than in other countries of the same latitude. Although Japan is surrounded by sea, it is very cold in winter, whilst the heat of the summer season nearly equals that of the tropics (Minato, 1977).

### 2.4 Thesis Aims

The overall aim of this thesis is to investigate the relationships within and between the taxa of the genus *Fallopia* section *Reynoutria*, through the use of molecular techniques. The project will include investigations into the hybridisation and introgression events occurring between the taxa in Britain where they have been introduced, and will assess the relationship between the introduced and native taxa, concentrating on Japanese plants from the native range.

In particular the areas that will be addressed are:

- The study of a Japanese Knotweed *s.l.* population in North Wales, that is renowned for having a high number of hexaploid *F.* x *bohemica* of both sexes. This chapter will assess the genetic diversity in *F.* x *bohemica* in terms of numbers of genotypes, and look for evidence of backcrossing.
- An analysis of three British Japanese Knotweed *s.l.* populations where both sexes of tetraploid *F.* x *bohemica* occur. Direction of hybridisation, and the number of genotypes of both tetraploid and hexaploid hybrids at these sites will be investigated.
- To estimate levels of genetic variation among British *F. sachalinensis*, to determine whether sexual reproduction or clonal spread best explains the current distribution.
- An investigation to see if the leaf characters that distinguish *F. japonica* from *F. sachalinensis* in the introduced range can be of taxonomic use when studying native material.
- A molecular biogeographical study of Japanese *Fallopia* section *Reynoutria* plants, and to use the results of this study to identify the putative regions where the introduced material of *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and *F. sachalinensis* are most likely to have originated.
- To investigate the genetic relationship between *F. sachalinensis* and *F. japonica*, and in particular to assess the relationship between the introduced and native taxa.

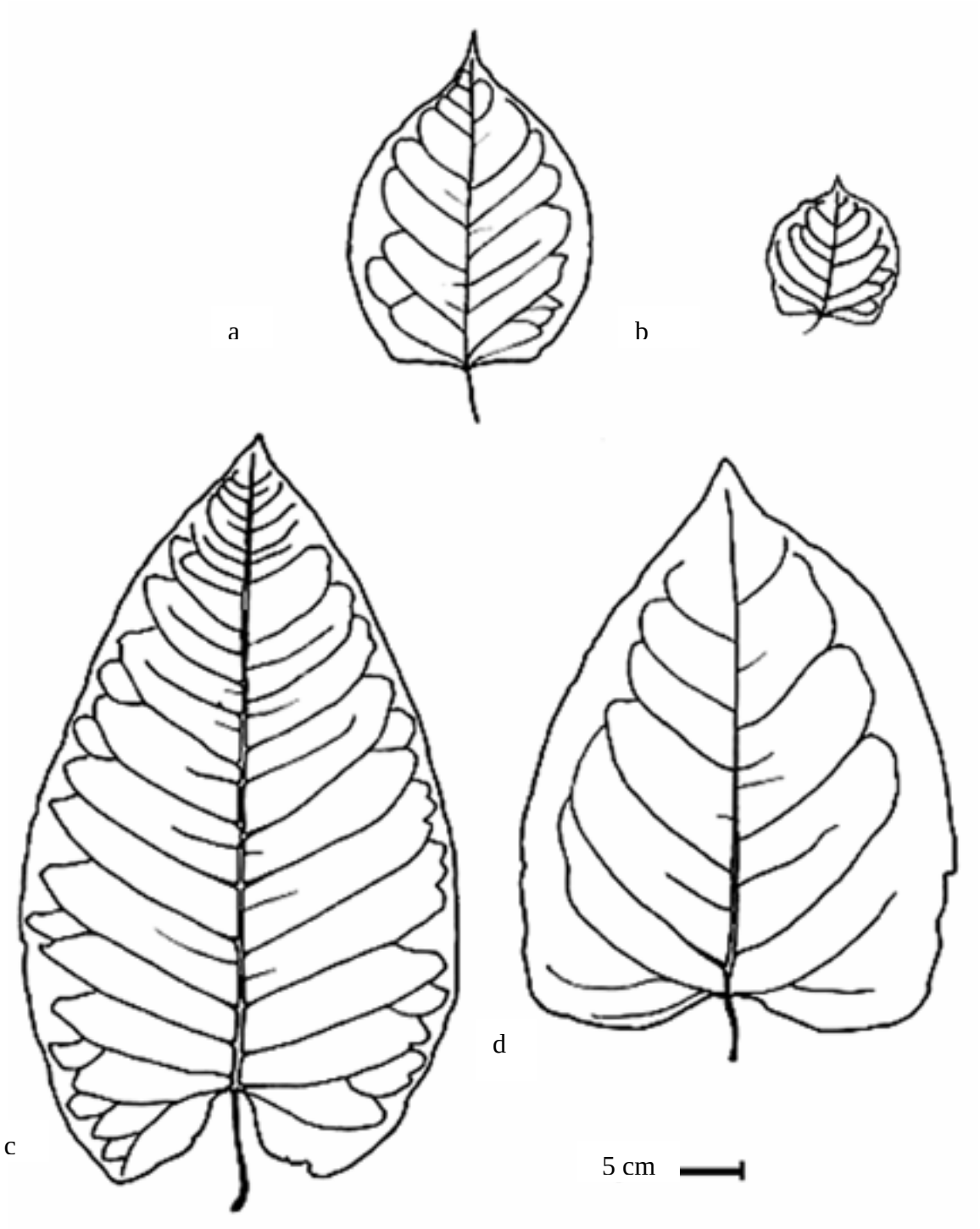



**Figure 2.1** Illustration representing leaves of a) *F. japonica* var. *japonica*, b) *F. japonica* var. *compacta*, c) *F. sachalinensis*, and d) *F.* x *bohemica*. Taken from Bailey *et al*. (1996).

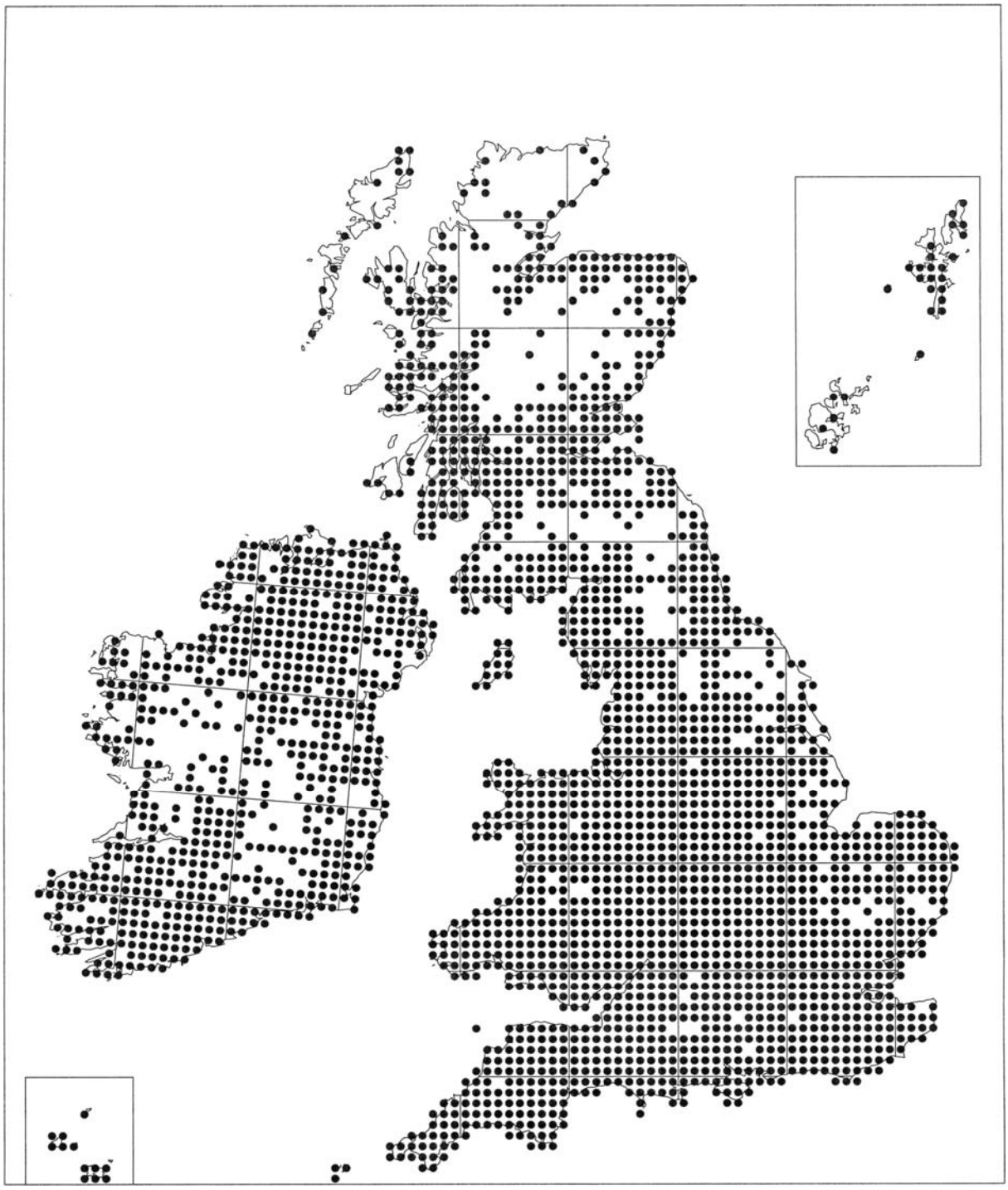


**Figure 2.2** Distribution map of *F.japonica*. Based on records collected for the New Atlas of the British and Irish Flora (Preston *et al.*, 2002). These maps were produced by H. R. Arnold of BRC using Dr. A. J. Morton's DMAP program.

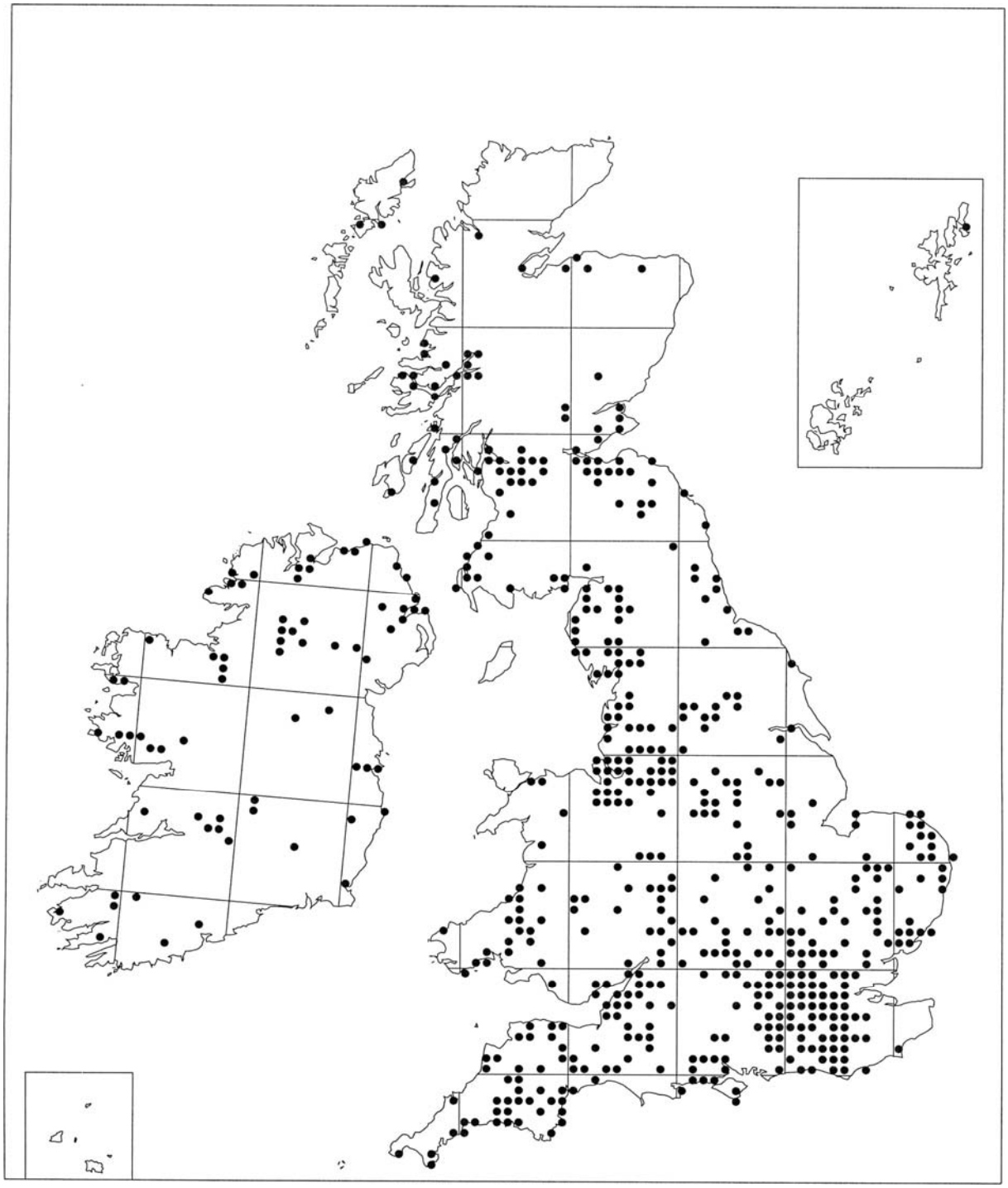


**Figure 2.3** Distribution map of *F.sachalinensis*. Based on records collected for the New Atlas of the British and Irish Flora (Preston *et al.*, 2002). These maps were produced by H. R. Arnold of BRC using Dr. A. J. Morton's DMAP program.

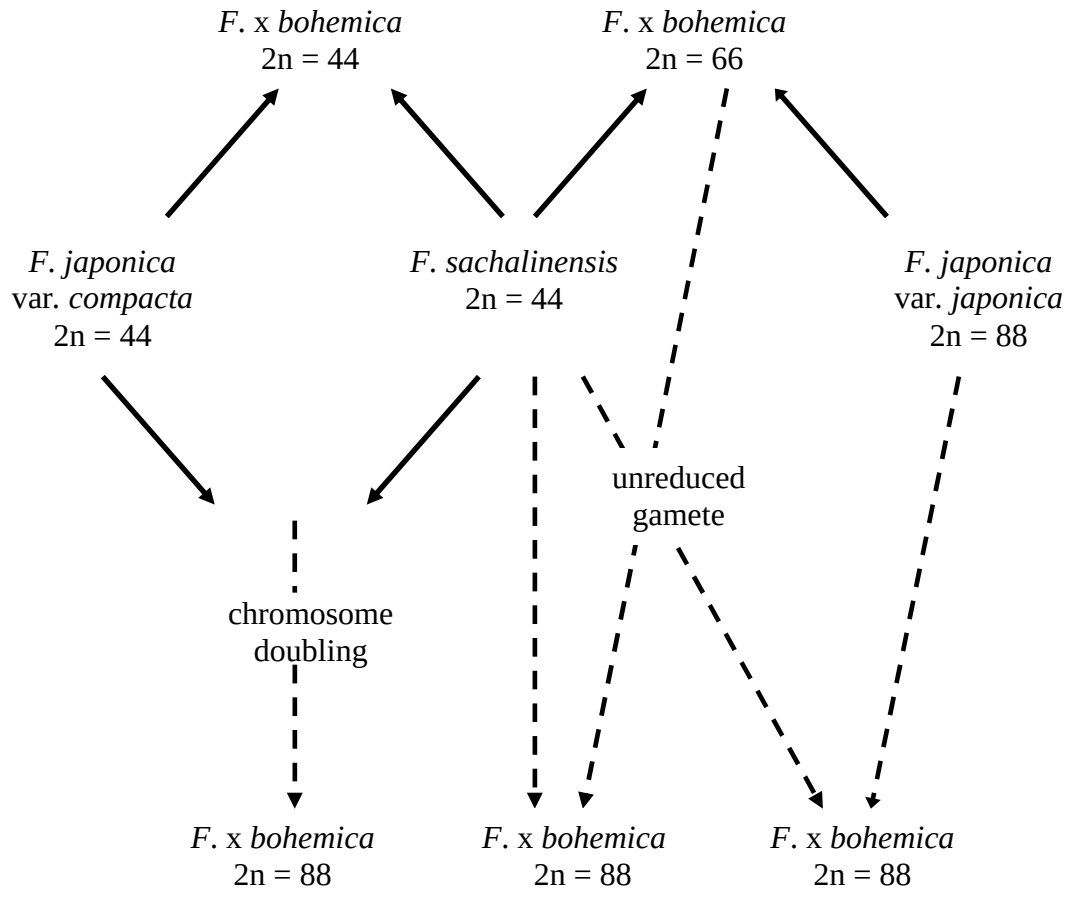



**Figure 2.4** Cytological origins of British *F*. x *bohemica* plants. Known pathways are shown as solid arrows, and the putative ones as dashed arrows. Taken from Pashley *et al*. (2003)

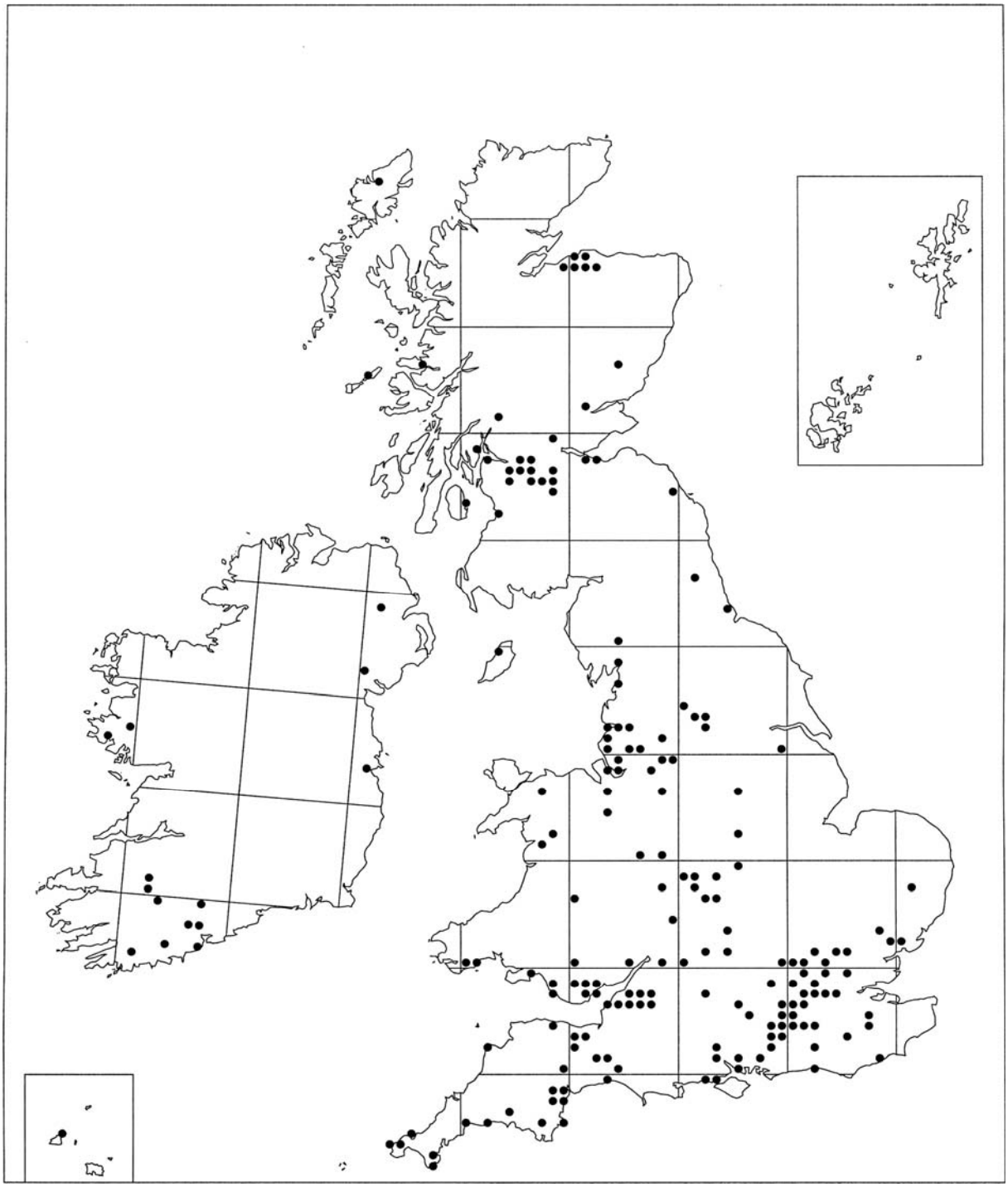


**Figure 2.5** Distribution map of *F*. x. *bohemica*. Based on records collected for the New Atlas of the British and Irish Flora (Preston *et al.*, 2002). These maps were produced by H. R. Arnold of BRC using Dr. A. J. Morton's DMAP program.

# Chapter 3. Materials and Methods.

## 3.1 Materials

A fragment of rhizome, and where possible a voucher specimen, were collected from a representative cane from most stands of Japanese Knotweed used in this study. Exceptions include material grown from seed. Each accession was designated a unique number referred to as a P number. The rhizomes were grown into mature plants in the glasshouses belonging to the University of Leicester, and the voucher specimens have been deposited in the herbarium at the University of Leicester (LTR). Material of Japanese origin was imported under quarantine licence number PHL 58/3112 and held under quarantine conditions at the Botanic Gardens, Leicester.

Details of sampling locations, and specific data such as sex expression and chromosome number, are detailed in the relevant chapters.

The materials, suppliers and formulation details of the reagents used are given in Appendix 1.

## 3.2 Methods

### 3.2.1 *DNA Extractions*

Midribs were removed from leaves for extraction, and the laminae wrapped in aluminium foil, and snap frozen in liquid nitrogen; or stored in a 3% CTAB (cetyltrimethylammonium bromide) saturated sodium chloride (NaCl) solution (Rogstad, 1992). In the first instance material was ground immediately or stored at –20°C until use. If stored in CTAB buffer the material was thoroughly washed, then snap frozen and treated as above. Approximately 100 mg of leaf material was ground in liquid nitrogen using a clean pestle and mortar. Fine sand was used to aid the grinding process, and approximately 50 μg of PVPP (polyvinyl polypyrrolidone) was added to the powdered material to bind and precipitate polyphenolics. The ground mixture was transferred to a 1.5 ml microcentrifuge tube, snap frozen in liquid nitrogen, then stored at – 80°C prior to extraction.

Total genomic DNA was extracted using Qiagen DNeasy plant mini kits, following the manufacturers instructions and including the optional centrifugation step to prevent shearing of DNA. In instances where the DNA was intended to be used for ISSR PCR amplification, a duplicate DNA extraction was completed for each accession.

To estimate the quantity of DNA extracted, 5 µl of each sample was run alongside a λ-DNA (EcoR1/HindIII restricted) ladder on a 1% agarose gel, and the concentrations estimated by visual comparison with the bands of known size and quantity on the λ-DNA ladder. An aliquot of each sample was diluted to 1 ng/µl and stored at – 20°C for subsequent use, whilst the remainder of the stock was stored at – 80°C.

3.2.2 *DNA electrophoresis*

Choice of gel for the visualisation of DNA depends upon the size of the fragments to be visualised. For total genomic DNA and complete chloroplast PCR products a 1% agarose gel was used. For the products of ISSR amplification 1.6% agarose was used whilst restriction digest products were run on 1.6% agarose, 6% polyacrylamide or 3% MetaPhor XR agarose, depending on the chloroplast region that had been digested.

For agarose gels multipurpose agarose was mixed at the appropriate concentration in 1 x TBE buffer, in a 250ml conical flask. The solution was heated at full power in a microwave to yield a clear solution. The molten solution was allowed to cool until lukewarm, and then ethidium bromide was added at 5µl (10mg/ml) per 100 ml of molten agarose. The gel was then poured into a horizontal electrophoresis gel tray, allowed to set for at least 15 minutes at room temperature, and then transferred to 4ºC for a minimum of 10 minutes then run using 0.5 x TBE as running buffer.

For resolution of small DNA fragments a Protean II xi Cell vertical gel rig (Bio-Rad) was used for the running of 6% polyacrylamide gels. Gel plates and clamps were assembled following the manufacturers instructions. The various components of the polyacrylamide gel solution were mixed and the solution poured between the gel plates using a 5,000 µl Gilson pipette. A well-forming comb was placed between the top of the plates and the gel left to set for three hours at room temperature, then kept at 4°C overnight. The gel was clamped in place and run using 1 x TBE as running buffer. This was then stained in 0.1 ng/ml ethidium bromide for 20 minutes.

As an alternative to polyacrylamide, 3% MetaPhor XR agarose, with 1 x TBE as the buffer, was used. The TBE buffer was measured into a 250ml conical flask. The appropriate weight of MetaPhor agarose was then slowly sprinkled into the buffer whilst the mixture was swirled continuously. The conical flask was then covered with a plastic wrap, which was pierced to create a steam vent, and then left for ten minutes to allow the agarose to hydrate. The flask was then heated in a microwave at half power, ensuring the mixture never boiled, to yield a

clear solution. The molten solution was allowed to cool until lukewarm, ethidium bromide added as per agarose gels, and then poured onto a horizontal electrophoresis gel tray. The gel was allowed to set for at least 30 minutes at room temperature, and then transferred to 4ºC for a further 30 minutes prior to use. As with the multipurpose agarose, 0.5 x TBE was used as the running buffer.

3.2.3. *PCR amplifications*

3.2.3.1 Chloroplast DNA

Chloroplast DNA was amplified using the universal primers of Taberlet *et al* (1991), Demesure *et al* (1995) and Dumolin-Lapegue *et al* (1997) and are listed in Table 3.1. A reaction mixture was made up for each pair of primers, sufficient for all samples plus one negative control to which water was added instead of DNA. The reactions were set up on ice. Into each microcentrifuge tube was placed 2.5 µl (approximately 2.5 ng) of the template DNA solution, or distilled water for the negative control, and 22.5 µl of reaction mixture. The reaction was overlaid with two drops of mineral oil to prevent evaporation. The relevant chloroplast PCR cycle on a Perkin Elmer DNA Thermal Cycler was initiated, and once 94°C had been reached, the samples were placed into the machine. Amplification was carried out using the following PCR profile: 1 cycle of 94ºC for 4 min; 32 cycles of 45s at 94°C, 45s at X°C, and Y min at 72°C; 1 cycle of 72°C for 10 min; and finally, 1 cycle of 10°C for 30 min. X and Y are primer specific and listed in Table 3.1.

After amplification, the samples were stored at 4°C. To check that amplification occurred 5 µl of each sample was run on a 1% agarose gel. To estimate the size of the amplified fragment a 1 kb ladder was run simultaneously.

Occasionally a chloroplast PCR amplification would not work. On these occasions repeating the reaction with the addition of 1 µl of Bovine serum Albumin (BSA) per 25 µl reaction would often help. This is presumed to be because BSA will bind to any proteins still in the DNA, which would otherwise interfere with the PCR reaction.

3.2.3.2 Nuclear ISSR amplification

Nuclear DNA was amplified using inter-ISSR primers from either the University of British Columbia set nine, or the laboratories of Dr Mike Wilkinson, University of Reading, as listed in Table 3.2. For each plant accession duplicate reactions were set up using different DNA extractions. The reactions were set up on ice, using 10 µl (approximately 10 ng) of the

template DNA. The relevant ISSR PCR cycle on a Perkin Elmer Gene Amp PCR system 9700 Thermal Cycler was initiated, and once 94°C had been reached, the samples were placed into the machine. Amplification was carried out using the following PCR profile: 1 cycle of 94ºC for 4 min; 40 cycles of 20s at 94°C, 30s at X°C, and 1 min at 72°C; 1 cycle of 72°C for 7 min; and finally, 1 cycle of 4°C for 30 min. X is primer specific and listed in Table 3.2.

After amplification, the samples were stored at 4°C. To visualise the amplified products 10 µl from each reaction, with duplicates placed next to each other, were run on a 1.6% agarose gel with a 1 kb ladder in the outer wells.

### 3.2.4. *RFLP analysis*

Chloroplast PCR product was digested for a minimum of one hour at 37°C, with a single restriction enzyme following the method of Ferris *et al*. (1993). Details of the recognition sites for the restriction enzymes used are found in Table 3.3. Once the incubation period was over, 3 µl of loading buffer was added to each of the samples and they were transferred to 4°C to halt the reaction. The restriction digest products were then loaded into either a 3% MetaPhor XR agarose gel, 1.6% multipurpose agarose gel or a 6% polyacrylamide gel depending on the chloroplast region amplified. Details of the gels used for each chloroplast region can be found in Table 3.1.

### 3.2.5. *Sequencing of PCR products*

#### 3.2.5.1 Purification of PCR products for Sequencing

To sequence regions of the chloroplast, 75 µl of chloroplast PCR product were produced as above. This was then purified to remove excess primer and unincorporated dNTP's using Qiagen's QIAquick PCR Purification Kits, following the manufacturers instructions.

#### 3.2.5.2 Sequencing Reaction

Sequencing was carried out using the dye-deoxy chain termination method. Sequencing reactions in a total volume of 10 µl were set up on ice, and overlaid with mineral oil. These were then amplified in a Perkin Elmer DNA Thermal Cycler, with the samples being added to the machine after it had reached 94°C. Amplification was carried out using the following PCR profile: 1 cycle of 94ºC for 4 min; 25 cycles of 30s at 94°C, 15s at 50°C, and 3 min at 60°C; and finally, 1 cycle of 10°C for 30 min.

3.2.5.3 Purification of Sequencing Reactions

3.2.5.3.1 Ethanol precipitation

The sequencing reaction was removed from under the mineral oil overlay and placed in a labelled 1.5 ml microcentrifuge tube. To this was added 25 µl of 100% ethanol and 1 µl of 2 M sodium acetate (pH 5.2). The tube was mixed and placed on ice for 30 minutes, then centrifuged for 20 minutes at 13,000 RPM. The supernatant was removed using vacuum suction, with due care being taken not to disturb the pellet. The pellet was washed by the addition of 750 µl of 70% ethanol, then centrifuged at 13,000 RPM for a further five minutes. The ethanol was removed by vacuum suction, and the pellets left to dry in a vacuum desiccator for thirty minutes. The ethanol wash was repeated, then the dried pellets were sent to the Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, for sequencing on a Perkin Elmer ABI 377 sequencer.

3.2.5.3.1 DyeEx Spin Kit

In some instances ethanol precipitation was not sufficient to clean the sequencing reaction, and the resultant electropherograms were unclear. For these samples the sequencing reactions were repeated and the products cleaned using Qiagen DyeEx Spin Kits, following the manufacturers instructions.

3.2.5.4. Sequencing by cloning

Sequencing some of the octoploid plants with one particular chloroplast region (*trn*C-*trn*D) was often problematic, therefore a cloning approach was used. As an insert, 1 µl of the cleaned chloroplast PCR product was used. The DNA was ligated into a Promega pGEM®-T Vector System following the manufacturers instructions and left at room temperature for four hours then stored at – 20°C. The ligation was then transformed into DH5∝ competent cells, produced by a standard chemical protocol (Inoue *et al.*, 1990). 70 µl of competent cells were thawed on ice, then added to the ligation-vector mix, gently mixed and left on ice for 20 minutes. The DNA-cell mixture was heat shocked at 42°C for 45 seconds before being left on ice for a further 2 – 3 minutes. These were then added to 900 µl of Luria broth (LB) and grown at 37°C with shaking for one hour. This was then plated onto Luria Agar (LA) plates containing ampicillin to select the plasmid, X-gal (5-Bromo-4-chloro-3-Indolyl-β-D-galactoside) to select the insert and IPTG (Isopropyl-β-D-Thiogalactopyranoside) as an inducer, and incubated at 37°C overnight. This is a standard procedure as found in Sambrook & Russell (2001).

Four white colonies for each plant accession were selected and cultured in LB and carbenicillan (a more stable analogue of ampicillin), and left shaking at 37°C for 16 hours. These were then prepared for sequencing using Qiagen Plasmid Prep Kits following manufacturers instructions, and sent to the Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, for dye terminator sequencing using the original chloroplast primers.

### 3.2.6. *Chromosome counts*

Chromosome numbers were determined using the technique of Bailey & Stace (1992). Actively growing roots were collected from the plants of interest, placed immediately into a bijou bottle containing 0.002M 8OQ (8-Hydroxyquinoline) solution, and stored at 4°C for about 24 hours. After this time the pre-treatment solution was replaced with 3:1 fixative, and stored at 4°C until use.

The root tips were hydrolysed for ten minutes in 5N hydrochloric acid (HCl) at room temperature, then the HCl was replaced by 70% IMS (Industrial methylated spirits). The meristematic regions were dissected out in a drop of 45% acetic acid, then transferred to a drop of aceto-orcein stain and macerated using Tungsten needles. Wet preparations were tapped out, squashed, and examined on a Zeiss universal microscope.

The help of Dr John Bailey, University of Leicester, for providing the majority of chromosome counts is gratefully acknowledged.

### 3.2.7. *Data Analysis*

#### 3.2.7.1 Sequence Analysis

The individual sequence electropherograms were manually checked using Sequence Navigator v. 1.0.1 (Perkin Elmer). Final sequences were exported to EditSeq v. 3.75 (DNAStar) and aligned using MEGALIGN v. 1.0.5 (DNAStar), with manual modification to minimise the number of gaps. Indels (insertion deletion events) that were homologous in length and position were scored as single binary presence or absence characters in a matrix. When the indels were not homologous, for example when more than one variation in length of microsatellite was encountered, no gap was scored as a zero, the first variation was scored as one, the second as two etc. When a small gap was present within sequence data corresponding to a large deletion in one or more other operational taxonomic units (OTUs) then it was considered to be a unique character but scored as missing data within the large deleted OTUs. An inversion of a segment of DNA was coded as a single mutation, with those having the segment in the same orientation as the outgroup being coded 0, and those with the

inverted form, code 1. Any indels within the inverted segment, if present in one orientation only, were treated as an additional mutation. After scoring, the indels matrix was added to the sequence matrix prior to further analysis.

3.2.7.2 ISSR analysis

ISSR bands for use in the analysis were selected on the basis of reproducibility and reliability. They were scored as a binary data matrix table, as presence (1) and absence (0) of bands. The bands were labelled with the primer code they were amplified with and the approximate size of the fragment, as calculated using the molecular weight option of AlphaImager$^{TM}$2200, calibrated against a 1kb ladder.

3.2.7.3 Chloroplast PCR-RFLP analysis

RFLP bands that could be reliably scored on the type of gel selected for the specific chloroplast region were used. Table 3.1 lists the gel types used for each of the amplified regions. The bands were scored by eye and recorded in a binary data matrix table, as presence (1) and absence (0). The bands were labelled with the region of the chloroplast they came from and the approximate size of the fragment, as calculated using the molecular weight option of AlphaImager$^{TM}$2200, calibrated against a 1kb ladder.

3.2.7.4 Methods for analysing the molecular data

Molecular data are often divided based on how the data are treated; distance methods first convert aligned sequences into a pairwise distance matrix, then input that matrix into a tree building method, whereas discrete methods consider each nucleotide site (or some function of each site) directly. Parsimony is a discrete method, whilst similarity measures such as Jaccard's coefficient are distance methods (Page & Holmes, 1998).

3.2.7.4.1 Discrete methods

Discrete methods operate directly on the sequences, or on functions derived from the sequences, rather than on pairwise distances. Hence, they endeavour to avoid the loss of information that occurs when sequences are converted into distances. Discrete methods allow the inference of the attributes of extinct ancestors. These reconstructed ancestors can offer insights about molecular evolution. The two major discrete methods are maximum parsimony and maximum likelihood. The two methods are discussed further in chapter 7.

3.2.7.4.2 Similarity measures

ISSR markers have been shown to be inherited in a dominant or codominant Mendelian fashion (Gupta *et al.*, 1994; Tsumura *et al.*, 1996). As with RAPD data, ISSR bands are interpreted as dominant markers (Wolfe *et al.*, 1998). Three main techniques have been used to calculate similarity measures with RAPD data sets: the simple matching coefficient (SMC), which measures the proportion of shared products and absences; the Jaccard's coefficient (J), which measures the proportion of shared product presences; and the Nei and Li (NL) coefficient, which measures the probability of a product amplified in one sample also being amplified in another sample. The advantages and disadvantages of each of these with respect to RAPD data have been discussed by Harris (1999), however, comparisons between the different measures have found that different similarity measures give essentially the same result (Harris, 1999). The reasons for absence of an ISSR band are not fully understood and, unlike SMC, both J and NL take into account only shared presence of bands. As the software used in this thesis to produce clustering procedures can easily utilise the Jaccard's coefficient, this was used to calculate similarities in ISSR data sets. Jaccard's coefficient is calculated as $D_J=2n_{xy}/n_x=n_y$ where $n_x$ is the number of bands present within accession x, $n_y$ is the number of bands present within accession y, and $n_{xy}$ is the number of bands shared by accessions x and y (Jaccard, 1908).

3.2.7.4.3 Minimum spanning Network

The number of mutational differences between genotypes was calculated and analysed using MINSPNET (Excoffier & Smouse, 1994) to produce a minimum spanning tree. This procedure is used to connect points by direct links having the smallest possible total length. Minimum spanning networks are alternatives to Wagner parsimony trees, but better convey the connections between genotypes (Excoffier & Smouse, 1994).

3.2.7.4.4 Hybrid index scores

Hybrid index scores are often used to identify and quantify introgression and to estimate backcross phenotype frequencies. This method imposes the assumption that hybridisation results in an intermediate condition for each character incorporated in the hybrid index (Hardig *et al.*, 2000). This additive method of character inheritance for morphological characters was challenged in a recent study by Rieseberg and Ellstrand (1993). They reviewed 46 studies reporting morphological character expression in plant hybrids. Their study revealed that only 45% of morphological characters displayed "intermediate" expression in first generation hybrids; the remaining characters were either the same as one

parent or the other (45%), or extreme relative to either parent (10%) (Rieseberg & Linder, 1999).

To calculate the traditional hybrid index using molecular data such as RAPDs, only bands that are monomorphic in the parental population can be used. A "pure" species of one of the parental taxa is given the score 0. The presence of any marker from the other parental taxon increases the index value up to a maximum of 1 for the "pure" alternative parental taxon. $F_1$ plants would theoretically have an index of 0.5 and possess all markers from both parents. Backcrosses are expected to lack a portion of the markers from one species (Fritz *et al.*, 1994). Molecular characters that are polymorphic within both parental populations are not readily exploitable using the conventional means of hybrid index calculation (Hardig *et al.*, 2000).

A novel version of a maximum likelihood (ML) approach to estimate hybrid index scores has been developed that can use both monomorphic and polymorphic RAPD markers (Hardig *et al.*, 2000). It was shown to produce similar graphic results to the traditional arithmetical method, whilst having the advantage of being applicable to a larger class of genetic markers. When one parental type is severely genetically limited, such as is the case of *F. japonica* var. *japonica* in Britain, it is impossible to judge whether a band present would have been monomorphic or polymorphic in a natural population.

### 3.2.8. *Method development experiments*

Several different results chapters use the same suite of primers to analyse the plants relevant to that chapter. Therefore this section, although based on results of experiments, is included in the methods section as the work was completed as part of a method development to choose the most appropriate primers for Japanese Knotweed *s.l.* plants.

#### 3.2.8.1 Selection of chloroplast fragment and restriction enzymes for determining chloroplast haplotype in introduced taxa

Chloroplast DNA (cpDNA) in most plant taxa is uniparentally transmitted in contrast to the biparentally transmitted nuclear DNA (Weising *et al.*, 1995). RFLP analysis of the split *trn*K intron from the chloroplast genome has shown chloroplast inheritance to be maternally transmitted for Japanese Knotweed (Hollingsworth *et al.*, 1999). This study also showed that using the restriction enzymes *Cfo*I, *Rsa*I and *Alu*I it is possible to identify three haplotypes, relating to *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and *F. sachalinensis*, in Britain. No variation was detected within any of these taxa, although variation was detected

when looking at native material (Hollingsworth, 1998). King & Ferris (1998) utilised this technique to study alder populations (*Alnus glutinosa* (L.) Gaertn.). They assayed several regions of the chloroplast genome, and found *trn*C-*trn*D to be more variable than the split *trn*K region.

Two regions of the chloroplast (the split *trn*K intron and *trn*C-*trn*D) were amplified and digested with six different frequent cutting restriction enzymes, the three used by Hollingsworth *et al*. (1999) to detect haplotypes in Japanese Knotweed (*Cfo*I, *Rsa*I and *Alu*I) and three additional ones, *Hae*III, *Hinf*I, and *Mbo*I. The restricted products were run on both 1.6% agarose and 6% polyacrylamide. The aim of this set of experiments was to find an alternative quicker way of distinguishing chloroplast haplotypes in British Japanese Knotweed material, as opposed to requiring three independent restriction digests to be performed. A British accession of *F. japonica* var. *japonica*, *F japonica* var. *compacta* and *F. sachalinensis* were amplified and digested. Additionally a Chinese accession of *F. japonica* was included to assess the potential for identifying native intra-specific variation. Although referred to as *F. japonica* it is decaploid (2n = 110) and was shown to have a different chloroplast haplotype when the *trn*L intron was sequenced (Hollingsworth, 1998).

No variation was detected with *Hae*III or *Mbo*I when restricting the split *trn*K intron. The other four enzymes were able to distinguish one or two from the group of four taxa, however no enzyme was able to distinguish all four so multiple combinations would be required, as used by Hollingsworth *et al*. (1999). The region *trn*C-*trn*D, however, was found to be more variable as seen in Figure 3.1. The restriction enzymes *Alu*I and *Hae*III had no cut sites in this region of the chloroplast. *Cfo*I has a single restriction site and showed a size difference between Chinese *F. japonica* and the other taxa. *Mbo*I showed variation among all four taxa, as did *Rsa*I. However, *Hinf*I was the most informative, particularly when the restriction products were run on a 6% polyacrylamide gel. The restriction digest of the region *trn*C-*trn*D by the enzyme *Hinf*I became the means of identifying the different British haplotypes throughout this thesis, and the fragments were run on either 6% polyacrylamide or 3% MetaPhor agarose.

3.2.8.2. Selection of chloroplast fragments for analysing diversity among native taxa

Restriction site analysis of chloroplast DNA (cpDNA) has become a routinely used technique for both evolutionary and phylogenetic studies, and has been increasingly used to assess intra-specific variation for a wide selection of plants (Soltis *et al.*, 1992). The majority of phylogeographic studies that made use of this technique have used multiple chloroplast

regions and a variety of restriction enzymes. This includes the study of European tree species such as Alder (*Alnus glutinosa*) (King & Ferris, 1998) and Beech (*Fagus sylvatica* L.) (Demesure *et al.*, 1996), and herbaceous plants such as *Tiarella trifoliata* L. (Soltis *et al.*, 1992). An alternative approach appropriate for the argan tree (*Argania spinosa* (L.) Skeels) was the analysis of multiple chloroplast regions with a single restriction enzyme, *hinf*I (El Mousadik & Petit, 1996).

The restriction enzyme *hinf*I was shown to be sufficient for detecting variation within Japanese Knotweed *s.l.* when studying introduced taxa, so the second approach of analysing multiple regions with a single restriction enzyme was employed to study diversity among native taxa.

Seven accessions of native *F. japonica* from different geographical regions were selected for inclusion in a preliminary screen of chloroplast regions, as was the standard British clone of *F. japonica* var. *japonica*. PCR amplification was attempted with eleven different sets of primers as listed in Table 3.1. Nine of these were successful, and the resultant products were digested with the restriction enzyme *hinf*1, then run on both 1.6% agarose, and 6% polyacrylamide. Of the nine regions digested, six regions showed variation on one or other of the gel types, also indicated in Table 3.1. The positions within the chloroplast genome of the six variable regions are shown in Figure 3.2. These six regions were used to determine chloroplast diversity among native material of Japanese Knotweed, and a sub set of them was sequenced.

3.2.8.3. Selection of ISSR primers for analysis of nuclear DNA

Since being introduced in 1994, ISSR PCR amplification has been used for a variety of purposes (for summary see (Wolfe & Liston, 1998)). For this thesis the preliminary use was to determine different genotypes of the various taxa that comprise Japanese Knotweed *s.l.* To this end a series of experiments were conducted to determine a suite of primers that could identify the different key taxa and their hybrids.

Initially a representative British accession of *F. sachalinensis*, *F. japonica* var. *japonica* and *F.* x *bohemica* were used to screen for amplification and variation with thirty primers from the University of British Columbia (UBC) primer set nine. From these a subset of fifteen primers was selected for further screening. Duplicate DNA extractions from a male-fertile *F. sachalinensis*, two *F. japonica* var. *japonica* plants from different geographical locations, and

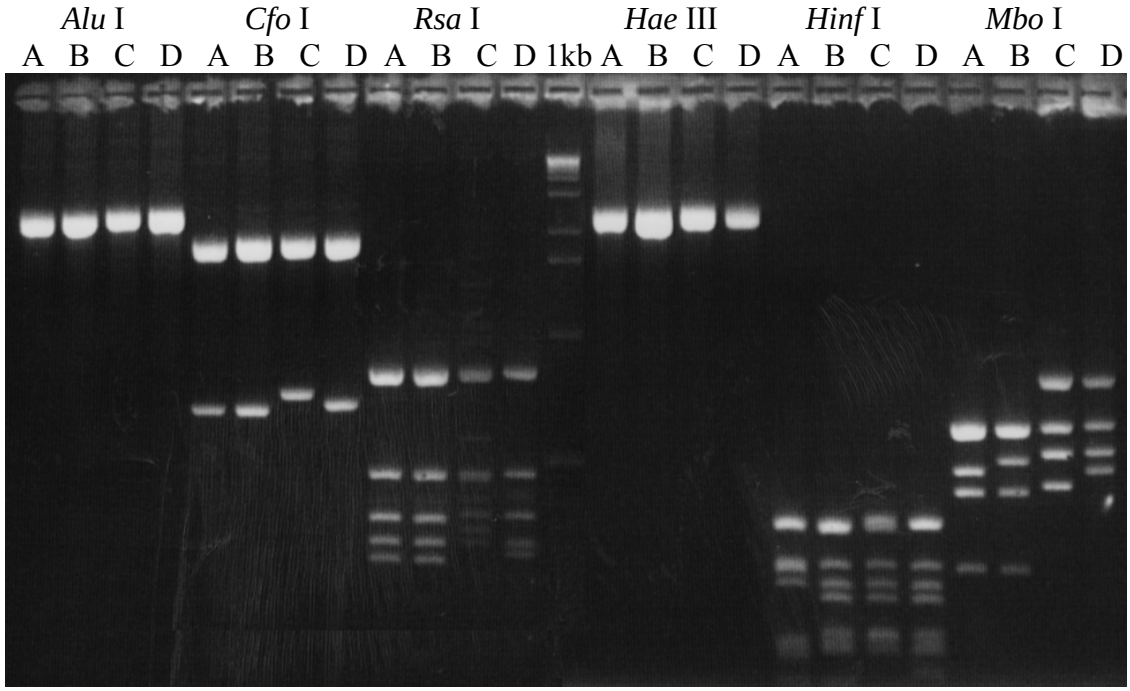
four *F*. x *bohemica* plants from two locations, and including both male fertile and male sterile representatives, were amplified.

A further twelve double anchored primers from the laboratories of Dr Mike Wilkinson (University of Reading) were screened using the same plants. Double-anchored primers are more specific so will amplify fewer bands, and those that do amplify tend to be more robust (M. Wilkinson, pers. comm.).

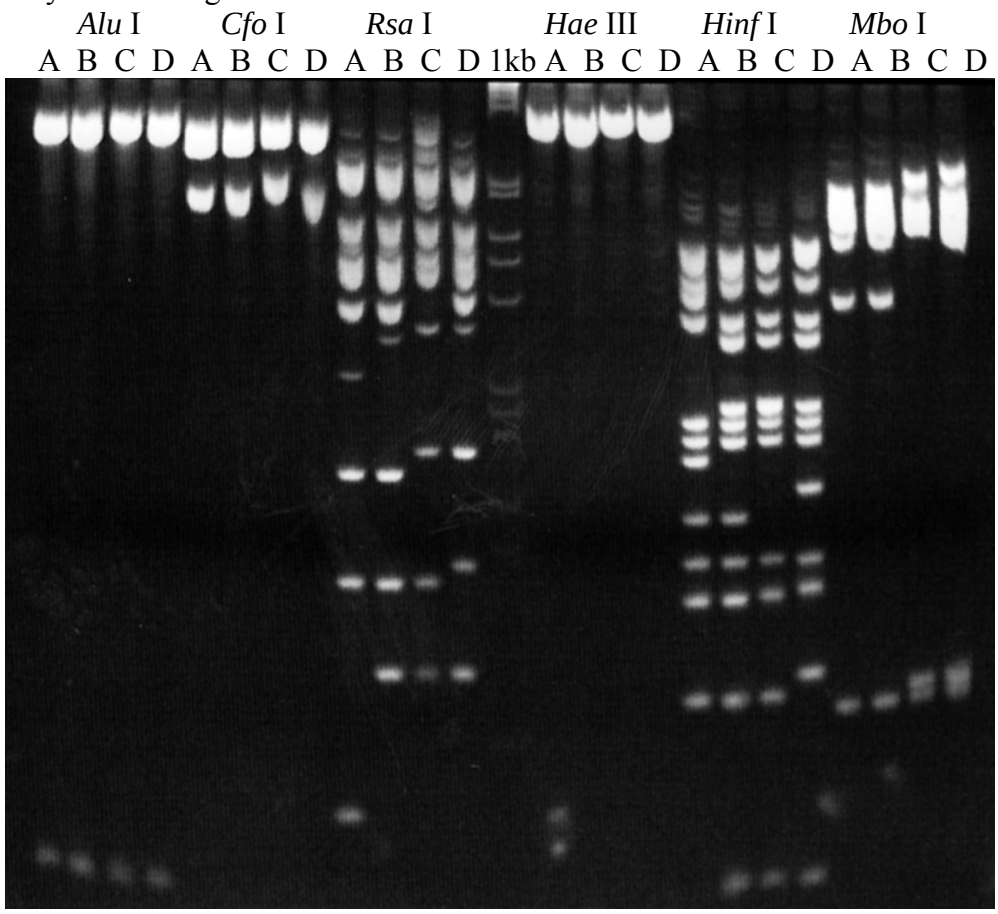
Primers were selected on the basis of reproducibility between duplicate DNA extractions from the same plant, and between different PCR runs. Those that performed best were screened at the recommended annealing temperature and further annealing temperatures, both above and below the recommended. Two different British accessions of *F. japonica* var. *compacta*, and a further *F. sachalinensis* were included in the screen. A final set of seven primers was selected for the determination of different genotypes in this thesis. Table 3.2 lists these primers and the annealing temperature that they worked optimally at.

**Figure 3.1** RFLP analysis of *trn*C-*trn*D.

a) 1.6% Agarose gel




b) 6% Polyacrilamide gel




Key:
A: British *F. japonica* var. *compacta*
B: British *F. sachalinensis*
C: Chinese *F. japonica* var. *japonica*
D: British *F. japonica* var. *japonica*

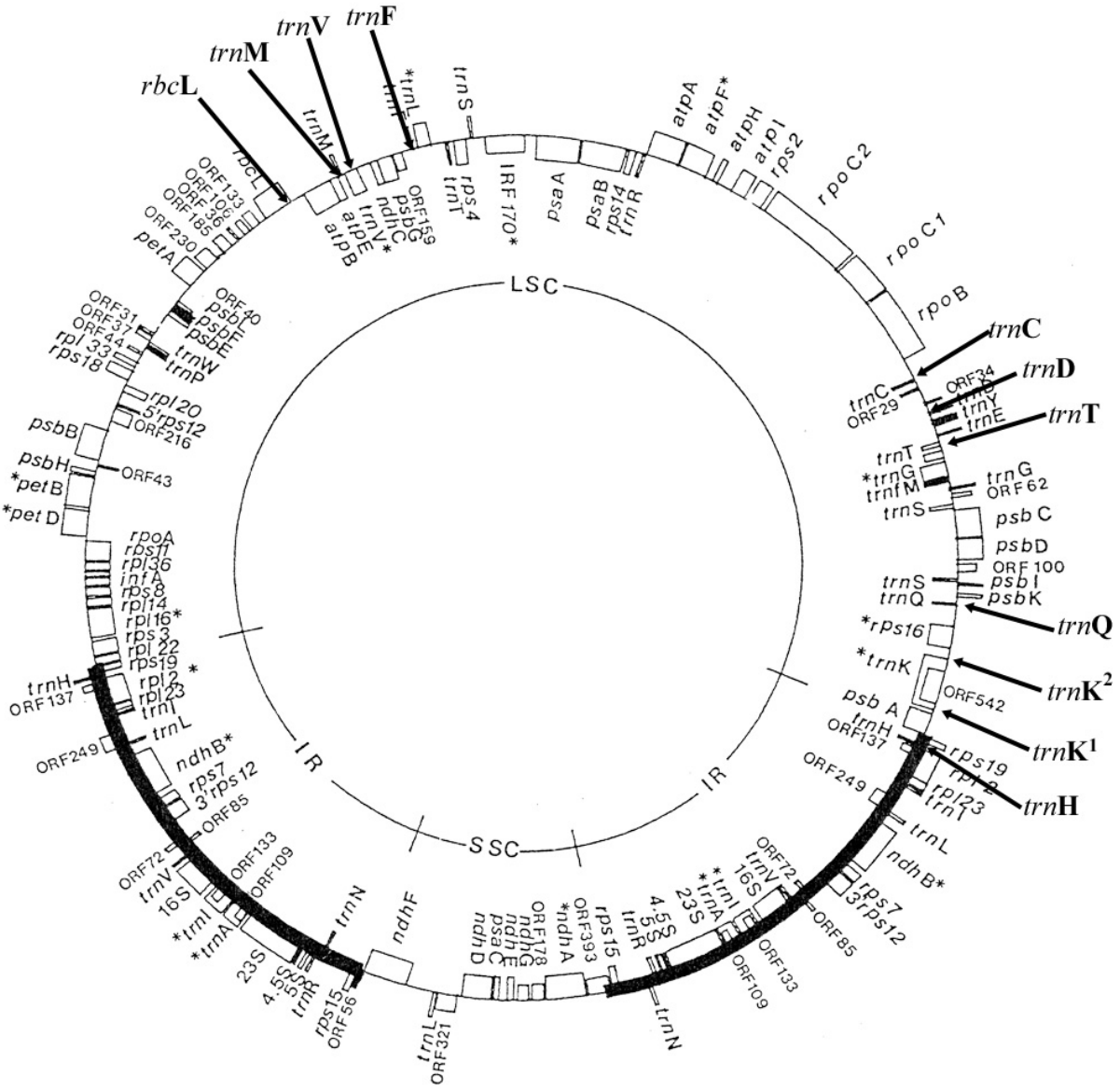



**Figure 3.2** The 134,525 bp chloroplast genome of rice (*Oryza sativa* L.). Genes are located on two strands, with those on the outside strand transcribed anticlockwise and those on the inside strand transcribed in the clockwise direction. Split genes are denoted by asterisks and tRNAs by '*trn*'. Arrows show the six regions found to be variable in Japanese Knotweed *s.l.*, listed in Table 3.1. Image adapted from Page and Holmes (1998).

**Table 3.1** Details of the universal chloroplast primers used in this thesis.

| Primer pair | Primers | Reference | Annealing temp. (X) | Extension time (Y) | Amplification | Variation | Ideal resolution gel for visualising RFLPs |
|---|---|---|---|---|---|---|---|
| *trn*K[1]-*trn*K[2] | tRNA-Lys (UUU) exon 1<br>tRNA-Lys (UUU) exon 2 | Demesure *et al* (1995) | 53°C | 2 $^{1}/_{2}$ minutes. | Yes | Yes | 1.6% agarose |
| *trn*C-*trn*D | tRNA-Cys (GCA)<br>tRNA-Asp (GUC) | Demesure *et al* (1995) | 58°C | 2 $^{1}/_{2}$ minutes | Yes | Yes | 3% metaphor agarose |
| *trn*F-*trn*V | tRNA-phe (GAA)<br>tRNA-Val (UAC) exon 1 | Dumolin-Lapegue *et al*. (1997) | 58°C | 2 $^{1}/_{2}$ minutes | Yes | Yes | 1.6% agarose |
| *trn*H-*trn*K | tRNA-His (GUG)<br>tRNA-Lys (UUU) exon 1 | Demesure *et al* (1995) | 62°C | 2 minutes | Yes | Yes | 3% metaphor agarose |
| *trn*D-*trn*T | tRNA-Asp (GUC)<br>tRNA-Thr (GGU) | Demesure *et al* (1995) | 54°C | 2 minutes | Yes | Yes | 6% polyacrilamide |
| *trn*M-*rbc*L | tRNA-Met (CAU)<br>RuBisCO large subunit | Demesure *et al* (1995) | 59°C | 2 $^{1}/_{2}$ minutes | Yes | Yes | 3% metaphor agarose |
| *psa*A-*trn*S | PSI (P700 apoprotein A1)<br>tRNA-Ser (GGA) | Demesure *et al* (1995) | 58°C | 2 $^{1}/_{2}$ minutes | Yes | No | N/A |
| *psb*C-*trn*S | PSII 44kd protein<br>tRNA-Ser (UGA) | Demesure *et al* (1995) | 57°C | 2 minutes | Yes | No | N/A |
| *trn*L-*trn*F | tRNA-Leu (UAA) exon 1<br>tRNA-phe (GAA) | Taberlet *et al*. (1991) | 58°C | 2 $^{1}/_{2}$ minutes | Yes | No | N/A |
| *trn*S-*trn*fM | tRNA-Ser (UGA)<br>tRNA-fMet (CAU) | Demesure *et al* (1995) | 62°C | 2 minutes | No | N/A | N/A |
| *trn*K[2]-*trn*Q | tRNA-Lys (UUU) exon 2<br>tRNA-Gln(UUG) | Dumolin-Lapegue *et al*. (1997) | 48°C | 2 $^{1}/_{2}$ minutes | No | N/A | N/A |

**Table 3.2** Details of the inter-simple-sequence-repeat (ISSR) primers used in this thesis

| Primer code | Primer sequence | Primer source | Annealing temp. (X) |
|---|---|---|---|
| AAG-1 | $CC(AAG)_5CC$ | Laboratory of Dr Mike Wilkinson (University of Reading) | 47°C |
| AG-4 | $CT(AG)_8GTG$ | Laboratory of Dr Mike Wilkinson (University of Reading) | 60°C |
| CAC-1 | $GT(CAC)_5TG$ | Laboratory of Dr Mike Wilkinson (University of Reading) | 55°C |
| 840 | $(GA)_8YT$ | University of British Columbia set 9 | 55°C |
| 855 | $(AC)_8YT$ | University of British Columbia set 9 | 58°C |
| 864 | $(ATG)_6$ | University of British Columbia set 9 | 55°C |
| 881 | $(GGGTG)_3$ | University of British Columbia set 9 | 58°C |

**Table 3.3** Recognition sites of the restriction enzymes used in section 3.2.8.1

| Restriction Enzyme | Enzyme Recognition Site |
|---|---|
| *Alu*I | ↓<br>A G C T<br>T C G A<br>↑ |
| *Cfo*I | ↓<br>G C G C<br>C G C G<br>↑ |
| *Hae*III | ↓<br>G G C C<br>C C G G<br>↑ |
| *Hinf*I | ↓<br>G A N T C<br>C T N A G<br>↑ |
| *Mbo*I | ↓<br>G A T C<br>C T A G<br>↑ |
| *Rsa*I | ↓<br>G T A C<br>C A T G<br>↑ |

# Chapter 4. Hybridisation, introgression and spread in a Japanese Knotweed *s.l.* hexaploid "hot-spot" in North Wales.

## 4.1. Introduction

Introducing plants to new environments often brings together species that may not have been sympatric in their native range. This, as well as the greatly reduced gene pool associated with an introduced species, often leads to interspecific hybridisation between a native and an invading plant species, or two invading species. Backcrossing to one or both parents leads to the infiltration of genes from one species to another. Such interspecific gene flow is known as introgression. Recent studies have confirmed that interspecific hybridisation following plant invasions may sometimes lead to the rapid evolution of new plant taxa (Abbott, 1992) e.g. *Iris nelsonii* (Arnold *et al.*, 1990). Ellstrand (2000) listed 28 examples representing 12 families where invasiveness was preceded by hybridisation, and listed *F.* x *bohemica* as a noxious weed with clonal growth as its method of stabilisation. "Hybridity is stabilized by a variety of mechanisms, from cytological (polyploidy and permanent translocation heterozygosity) to apomictic (agamospermy and clonal growth)" (Ellstrand & Schierenbeck, 2000).

Although clonal growth is given as its method of stabilisation, *F.* x *bohemica* is also able to reproduce sexually. There are certain areas in Britain that may be regarded as "hot spots" in terms of Japanese Knotweed evolution because of the presence of at least one male-fertile *F. sachalinensis*, male-sterile *F. japonica* stands and the hybrid *F.* x *bohemica*. At these sites there is a strong potential for backcrossing, made possible by such a concentration of genotypes (Bailey *et al.*, 1996). Dolgellau, in the county of Merionethshire (now Gwynedd) North Wales, is one such "hot spot" and as such the Japanese Knotweed population there has been the subject of a long-standing research project. Dolgellau is one of the most complex of the British "hot-spots" due to the presence not only of the British clone of male-sterile *F. japonica* var. *japonica* and several stands of male-fertile *F. sachalinensis*, but numerous stands of both sexes of hexaploid *F.* x *bohemica*. It is also the only site in Britain where octoploid *F.* x *bohemica* has been found, and indeed both a male-fertile and male-sterile stand has been found here. On numerous occasions during the ten years that this site has been studied, germinating seedlings have been found. These seedlings have been both F1 and backcrossed hybrids and their presence indicates that hybridisation is still occurring. Of the fully established hybrid stands one is potentially an aneuploid backcross, although this is yet to be confirmed.

Molecular data are often used for studying hybridisation events (Ayres *et al.*, 1999; Daehler & Strong, 1997; Ferris *et al.*, 1997; Fritz *et al.*, 1994; Harris & Ingram, 1992; Hollingsworth *et al.*, 1999; Rieseberg & Linder, 1999; Wolfe *et al.*, 1998). Reasons given for the use of molecular markers include: the large number of independent markers they generate; their being selectively neutral; and they have simple modes of expression and inheritance (Hardig *et al.*, 2000). When studying hybridisation it is often necessary to have an accurate classification of the hybrids (Rieseberg & Linder, 1999). Frequently a hybrid index score is used to estimate backcross phenotype frequencies, to aid in the identification of any hybrids or backcrosses. Recently the hybrid index score has also been applied to molecular rather than morphological characteristics (Fritz *et al.*, 1994; Hardig *et al.*, 2000).

### 4.2 Aims

The aims of this investigation were to study hybridisation and introgression within Japanese Knotweed *s.l.*, concentrating on a study site in Merioneth, North Wales, to see the impact of sexual reproduction. More specifically: to track the progress of the spread and evolution of *F.* x *bohemica;* to calculate the level of genetic diversity in *F.* x *bohemica* in terms of numbers of genotypes; and, to look for evidence of backcrossing and to investigate its importance in the evolution of Japanese Knotweed.

### 4.3 Study site

Dolgellau is a small market town situated at the foot of the Cader Idris mountain range in southern Snowdonia. As already stated, the Japanese Knotweed population at Dolgellau has been the subject of a long-standing research project. An earlier study using the arbitrary DNA fingerprinting technique RAPDs (Randomly Amplified Polymorphic DNA (Williams *et al.*, 1990)) was completed for some of the plants found in the grounds of Caerynwch Hall and at Dolgellau recreation ground (Hollingsworth & Bailey, 2000b). At the time it was believed that these were the only two sites along the river system where *F.* x *bohemica* was found. *F.* x *bohemica* has since been found along the interconnecting watercourse and the inclusion of these into the study has shown that the situation is even more complex than was originally supposed, and that the potential for the evolution of a more invasive fertile hybrid is more than just an abstract theory. The river course and positions of the three main study sites are shown in Fig. 4.1.

Caerynwch Hall is situated upstream of the start of Torrent Walk, a renowned beauty spot on the river Clwedog, a tributary of the river Wnion. It is in the grounds of this hall that it is suspected that the original plantings of both *F. japonica* and male fertile *F. sachalinensis*

were made, when they were valued as garden plants. At Caerynwch now, one finds further stands of *F. japonica* and *F. sachalinensis* as well as numerous *F.* x *bohemica* stands. The grounds of Caerynwch Hall are in extremely close proximity to the river Clwedog. Just outside the gardens at the start of Torrent Walk can be found a well established male fertile *F. sachalinensis* and further stands of both *F. japonica* and *F.* x *bohemica*.

Torrent Walk, on the Clwedog, is highly turbulent and along its length (~2 km) there is the occasional stand of *F. japonica*, but it is not until the end of the walk, where the waters calm down just prior to joining the River Wnion, that a substantial number of both *F. japonica* and *F.* x *bohemica* plants are found. These are on the banks of the rivers, in gardens of houses that back onto the Clwedog, and also on the small islands that form within the Clwedog. These taxa also stretch for several hundred metres along the River Wnion.

Fourteen *F.* x *bohemica*, three *F. japonica* and three *F. sachalinensis* stands were sampled at Caerynwch. These are shown in Fig. 4.2, as are the positions where five germinating seedlings were collected. The point where the River Clwedog joins the River Wnion is referred to in this study as the Torrent Walk site. Two *F. japonica*, and nine *F.* x *bohemica* stands were sampled here, as shown in Fig. 4.3.

At the recreation ground in Dolgellau numerous stands of both *F.* x *bohemica* and *F. japonica* can be found. Eighteen *F.* x *bohemica*, two *F. japonica* and two seedlings have been sampled (Fig. 4.4).

Japanese Knotweed is reported to grow at various other points along the River Wnion, however not all of it is available for access. Plants that have been collected from areas other than the three main study sites are shown in Fig. 4.5. For ease, bridges have been numbered with no distinction made between footbridges and road bridges.

Approximately 1.5 km down stream from the junction of the Rivers Clwedog and Wnion, at a footbridge (bridge 4) a stand of *F.* x *bohemica* was sampled. Likewise at the next bridge down stream from the recreation ground (bridge 7) a further hybrid plant was collected. On the old Towyn Road a further two stands of *F.* x *bohemica* have been analysed, as have three stands found in a cattle market a short distance away from the river.

## 4.4 Materials and Methods

### 4.4.1. *Plant material*

Due to the importance of Dolgellau as a study site, plant material has been collected on a number of different occasions. Each time the location of the accession was noted, and a voucher specimen was collected and deposited at the University of Leicester herbarium (LTR). Collected material was given a unique P number at time of collection. For the purposes of this thesis a site code has also been allocated to each accession. The code has the letter D, standing for Dolgellau, followed by either the letter J for *F. japonica* var. *japonica*, S for *F. sachalinensis* or B for *F.* x *bohemica*. If at time of collection the hybrid plants were seedlings, then a lowercase "s" follows the letters DB. These seedlings must be hybrids as it is not possible for seedlings of *F. japonica* var. *japonica* or *F. sachalinensis* to be produced at this site. The plants are numbered consecutively starting with the plant furthest upstream. Artificial hybrids were generated from the two octoploid *F.* x *bohemica* plants, four of which were included in this study. They are given the second letter A and are numbered randomly from 1 to 4.

The established parental and hybrid plants were identified on the basis of leaf shape and epidermal trichome characters (Bailey & Conolly, 1991). Sex was also determined for plants that were in flower. Table 4.1 lists the thesis code, P number, taxon, sex, and chromosome number (if known) for all the plants used in this study. Where one plant has two P numbers it has been collected more than once and both samples have been analysed.

### 4.4.2. *Methods*

Duplicate DNA extractions were made for each plant, as detailed in chapter 3 materials and methods. ISSR PCR amplification using the seven primers listed in Table 3.2 was carried out as per chapter 3.

## 4.5 Data analysis

ISSR bands were selected and scored as detailed in chapter 3, section 3.2.7.2.

Maximum Likelihood (ML) estimates of hybrid index scores were calculated using the computer software as described in Hardig *et al.* (2000). The scores were standardised to range between zero and unity. Confidence intervals were assessed using 10,000 bootstrap replicates. The results of a ML hybrid index analysis are dependent in part, on the specific constitution of parental samples (Hardig *et al.*, 2000), therefore the analysis was run initially with both genotypes of *F. sachalinensis*, and then again with just the one most likely to have

been involved in any hybridisation event (S1). To eliminate any variation in banding pattern being a direct result of number of chromosomes the analysis was also run with just the parental taxa and any 2n = c66 hybrid plants.

Winboot (Yap & Nelson, 1996) was used to bootstrap the ISSR binary data 1000 times using the Jaccard's coefficient to produce confidence values. The companion program WinDist was then used to produce a matrix of pair-wise genetic distances between all individuals, using the complement of the Jaccard's similarity coefficient. A Neighbour-Joining tree was drawn from the resulting matrix using the NEIGHBOUR option in PHYLIP version 3.6a3 (Felsenstein, 2002) and TreeView version 1.6.6 (Page, 1996).

The number of differences between each genotype was calculated and analysed using MINSPNET (Excoffier & Smouse, 1994) to produce a minimum spanning tree.

**4.6 Results**

A total of 43 bands were scored, of which 39 were polymorphic. All hybrid bands were attributable to one or other of the parental taxa, as indicated in Table 4.2. Besides the four monomorphic bands there were a further four bands which were present in both parental taxa but absent in one or more of the hybrid plants. *F. japonica* var. *japonica* had nineteen bands not present in *F. sachalinensis*, all of which were present in one or more of the hybrids, three of which were found in all hybrids. The two genotypes of *F. sachalinensis* had fourteen bands common to each other that were not found in *F. japonica* var. *japonica*. The difference between genotypes S1 and S2 was the presence of two bands unique to S2. These bands were also not found in any hybrids. Of the fourteen common bands all bar one were found in one or more of the hybrid plants, but none were found in all of them. The number of bands contributed by each parent to the hybrid taxa is summarised in Figure 4.6. They are arranged in order of their ML hybrid index I score, as explained in section 4.6.2 below. Also given is the chromosome number of each plant, where known.

Including duplicates 76 plants were analysed representing seven stands of *F. japonica* var. *japonica*, three *F. sachalinensis*, 48 established *F.* x *bohemica*, 7 germinating seedlings and four artificial hybrids. Each primer generates a banding pattern referred to as a phenotype. A unique combination of these phenotypes is assumed to be representative of a genotype (also referred to as an individual clone). A total of 42 unique genotypes were detected.

4.6.1 *Distribution of genotypes*

As expected, all *F. japonica* plants sampled in this study were identical (J), whilst there appears to be two genotypes of *F. sachalinensis*, S1 and S2.

The "B number" represents an individual genotype for an established *F.* x *bohemica* plant. They are numbered consecutively starting with the plant furthest upstream. A "Bs number" works using the same principal for the seedlings. Two or more plants sharing the same "B number" are assumed to be a single genotype.

A single genotype ranged from a single stand to five discrete stands within a particular site. Table 4.3 shows the distribution of genotypes between the established *F.* x *bohemica* plants, in terms of numbers of stands they represent for each site. At Caerynwch, out of the fourteen stands sampled, eight different genotypes were found. Five were represented by a single stand, whilst of the three found more than once, two of them (B4 and B8) are represented as adjacent stands. The positions of these can be seen in Fig. 4.7. The genotype referred to as B1 found furthest upstream of all the *F.* x *bohemica* plants sampled, is also present just within the grounds of Caerynwch hall, and at three points outside of the grounds, two on the island and a further one on the bank downstream of the island. It is also represented by an additional stand at both the Torrent Walk site, and in the recreation ground. Caerynwch hall to Dolgellau recreation ground is an overall distance of approximately 4.5 km downstream.

Seedlings DBs1, DBs2 and DBs3 found germinating beneath a *F. japonica* stand were all hexaploid. The most credible explanation being they are F1 *F.* x *bohemica* hybrids. DBs4 and DBs5 were found germinating on the small island just outside of the Caerynwch grounds with both *F. japonica* and *F.* x *bohemica* close by. DBs4 is a hexaploid seedling, whilst DBs5 is a putative backcross with 2n = 64. They are all unique genotypes as expected.

Nine stands of *F.* x *bohemica* were sampled from the Torrent Walk site (Fig. 4.8). Each of these stands is genetically distinct from the others. As has already been mentioned, one of them (B1) was found further upstream at Caerynwch. It is here at Torrent Walk that the only recorded female octoploid *F.* x *bohemica* in Britain can be found, B10 on Fig. 4.8. Seeds germinated by embryo culture from this plant were found to have been pollinated by *F. sachalinensis* and were therefore 2n = 66 (J.P. Bailey, unpublished). Downstream from this point a 2n = 66 hybrid plant could potentially be either an F1 hybrid or a backcross between the octoploid *F.* x *bohemica* and *F. sachalinensis*.

Continuing downstream, a single *F*. x *bohemica* was found at bridge 4. This clone was not found at any other site in the study.

Dolgellau recreation ground has the lowest percentage of unique stands of all the sites studied. As shown in Table 4.3, only five out of the eighteen stands sampled were novel genotypes represented by a single stand. Two genotypes were present as two stands, and a further two genotypes by four. At Caerynwch, with the exception of B1, such stands were found adjacent to each other. As can be seen in Fig. 4.9, at Dolgellau recreation ground these shared genotypes were found interspersed either by other *F*. x *bohemica* genotypes or *F*. *japonica* stands. Seedlings Bs6 and Bs7 were collected from under a *F*. *japonica* var. *japonica* and have 2n = 76 and 2n = 110 respectively. These are most likely backcrosses between a male fertile *F*. x *bohemica* and *F*. *japonica* var. *japonica*. It is assumed that seedling Bs7 arose when an unreduced *F*. x *bohemica* gamete pollinated *F*. *japonica* var. *japonica*. Again the seedlings have unique genotypes.

The only other plant sampled along the watercourse was at bridge 7. As indicated in Table 4.3 this was found to be genotype B9. This genotype had also been found further upstream at the Torrent Walk site. This stand is indicated in Fig. 4.10, as are those plants that were found away from the river itself, at the cattle market and on Towyn road. At the cattle market three stands were sampled and these were represented by two genotypes. Both of these were previously found at Caerynwch hall. Neither of the plants sampled from Towyn road had been found previously. One of them (B27) is the male-fertile clone of octoploid *F*. x *bohemica*.

Fig. 4.11 represents the distribution of the genotypes between the established *F*. x *bohemica* stands for the entire study. As can be seen the vast majority (64%) of genotypes are found at a single stand, whilst only ten genotypes are found at two or more stands. The most dispersed being the seven stands of B1.

### 4.6.2 *Hybrid Index Score*

The mean ML hybrid index score for the *F*. x *bohemica* plants and seedlings was 0.6359 (SD 0.0486) using both *F. sachalinensis* genotypes, and 0.6241 (SD 0.05) with just the genotype S1. In both instances the same thirteen genotypes (marked with an asterisk in Table 4.4 and Figure 4.12) deviated from the mean in excess of a single standard deviation (SD). The lower and upper confidence limits when both *F. sachalinensis* genotypes were included was 0.5488 (2.5% C.I.) and 0.7268 (97.5% C.I.), compared to 0.5337 (2.5% C.I.) and 0.7155 (97.5% C.I.)

with just S1. Of the thirteen genotypes that deviate from the mean in excess of a single SD, only two Bs6 and A3 fall outside of the confidence intervals for the mean hybrid index values, and there is still overlap between their individual confidence values and those of the mean. The two octoploid hybrids came out greater than one standard deviation on either side of the mean ML hybrid index value. The male-sterile octoploid (DB17, genotype B10) fell to the *F. sachalinensis* side of the mean, whilst the male-fertile octoploid (DB47, genotype B27) fell to the *F. japonica* var. *japonica* side. From Figure 4.6 it can be seen that these are the closest hybrids to the parental taxa on both sides with the exception of Bs6 and A3, which were outside of the upper and lower confidence intervals.

Excluding *F. sachalinensis* genotype S2 has the effect of shifting all of the values for the hybrids towards 0 ("pure" *F. sachalinensis*), however, the overall distribution of the various hybrid genotypes remains unchanged.

Table 4.5 and Figure 4.13 show the ML hybrid index scores when only hexaploid *F.* x *bohemica* individuals are included with the parental taxa. As before an asterisk is used to indicate any genotypes that deviate from the mean in excess of a single SD. The mean ML hybrid index score being 0.6317 (SD 0.0382) with a lower confidence interval value of 0.5224 (2.5% C.I.) and upper of 0.7364 (97.5% C.I.), based on 10,000 bootstraps. Any hexaploid genotypes that deviated from the mean in the fuller earlier analysis still did, but an additional five genotypes have now been marked as deviant that previously fell within one SD. These are Bs2 to the *F. japonica* var. *japonica* side, and B6, B7, B12 and B15 to the *F. sachalinensis* side. Even though there were more novel deviant types when just hexaploids are considered, they all fell within the mean hybrid index confidence interval.

Morphological examination of the *F.* x *bohemica* plants *in situ* at the Dolgellau recreation ground site led to the observation that several stands had what appeared to be a larger than average leaf size. These were hypothesised to be a backcross between *F. sachalinensis* and the male-sterile octoploid *F.* x *bohemica* plant (DB17) both of which have leaf sizes larger than the average hexaploid *F.* x *bohemica* plants. DB17 has genotype B10, which according to Figure 4.12 falls to the *F. sachalinensis* side of the mean hybrid index scores, and is further than one SD from the mean. Genetic analysis, of these larger leaved hexaploid individuals, has shown them to all be of a single genotype (B19) that was not one of the deviant genotypes in any of the analyses. When the genotypes are ranked for hybrid index values with the value closest to (0) *F. sachalinensis* being first, the genotype B19 came 7$^{th}$ out of 24 (Table 4.5).

4.6.3 *Neighbour joining tree*

The Neighbour joining tree for all genotypes, based on the Jaccard's similarity coefficient, is shown in Figure 4.14. The internal branches are short and the majority of the distances are confined to the terminal branches. Bootstrapping 1,000 times has shown that only four of the branches hold together over 50% of the time, this includes the two genotypes of *F. sachalinensis* grouping together 100% of the time. The putative backcross Bs6, which has 2n = 76, groups with *F. japonica* var. *japonica*, albeit with a bootstrap value below 50 (45.7). Three of the four artificial hybrids form a clear group with *F. sachalinensis*. The two octoploid hybrids and the large-leaved putative backcross do not occupy significant positions.

4.6.4 *Minimum spanning network*

The minimum-spanning network, Figure 4.15, is based on the number of band differences between each pair of genotypes. There are a high number of alternative links joining the majority of the genotypes leading to a complex inter-connecting network. Again *F. japonica* var. *japonica* comes out closest to Bs6. The *F. sachalinensis* genotypes have at least 17 differences between themselves and the nearest of the hybrids which is the artificial hybrid A3. All four of the artificial hybrids form a cluster very close to *F. sachalinensis*. The male-sterile octoploid genotype (B10) has a terminal position, whereas the male-fertile hybrid and (B27) and the large leaved putative backcross (B19) both fall within a cluster of other hybrid plants.

**4.7 Discussion**

4.7.1 *The origin of Japanese Knotweed* s.l. *at Dolgellau*

The Japanese Knotweed *s.l.* population at Dolgellau represents an evolving system. On three separate years seedlings have been found geminating, and although it has not been proven that these will survive the winter and establish, the very high level of genetic diversity within the Japanese Knotweed *s.l.* population at Dolgellau indicates that this must sometimes happen. Hollingsworth (2000b) reported 14 different genotypes of established *F.* x *bohemica* plants from Dolgellau based upon RAPD fingerprinting, whilst in this current study using ISSR fingerprinting and a larger number of accessions, 28 genotypes have been detected. Although it is theoretically possible that two plants assigned the same genotype may be different, we can only underestimate the amount of variation. Each of the seven seedlings in the study has a unique genotype, which is testament to the sensitivity of the assays. There is obviously overlap between the two studies, but a direct comparison between the actual plants cannot be made as some of the original collections have since died. As a result the rest of this discussion will only deal with the current investigation.

It is highly likely that the parental taxa were originally planted in the grounds of Caerynwch hall. However, the high number of hybrid genotypes and their occurrence at many places along the two river systems is a strong argument for the majority of the hybrids, if not all, having arisen *in situ* at this site rather than having been planted.

### 4.7.2 *Importance of* Fallopia *x* bohemica

Current control treatments for Japanese Knotweed *s.l.* are based on their effectiveness on *F. japonica* var. *japonica* (Beerling, 1991), however a recent study has shown *F.* x *bohemica* to be the most resistant to control, with none of the existing methods being very successful (Bímová *et al.*, 2001). There is also evidence that *F.* x *bohemica* has a higher regeneration potential than either of its parents (Brabec & Pyšek, 2000) and may in fact be more invasive (Mandak, B. pers. comm.). The present study clearly shows that the production of *F.* x *bohemica* is not a rare event, and as such requires more investigation into both its production and its potential as an invasive species.

The hexaploid *F.* x *bohemica* plants, although capable of producing F2 and backcrossed seed, are not capable of reproducing at the hexaploid level because the gametes produced, and subsequent plants, are mostly aneuploid. The presence of octoploid *F.* x *bohemica* plants at Dolgellau is however cause for concern as far as preventing further evolution of these taxa. The fact that this has occurred at least twice, as indicated by the two different genotypes of octoploid *F.* x *bohemica*, is evidence that this is not a rare incident. Octoploid *F.* x *bohemica* plants have also been recorded in other countries (Bailey, 2003).

There are three key ways these octoploid plants can affect the Japanese Knotweed *s.l.* population. As has already been stated, the male sterile octoploid *F.* x *bohemica* has been pollinated *in situ* at Dolgellau by male fertile *F. sachalinensis*. The resultant backcrossed hybrids are hexaploid, with a larger proportion of their genome being attributable to *F. sachalinensis* than the F1 *F.* x *bohemica* plants. Secondly, the *F.* x *bohemica* plants can reproduce with each other, and thirdly a fully fertile hermaphrodite octoploid *F.* x *bohemica* could potentially replace the missing male fertile *F. japonica* var. *japonica* within the British flora, leading to introgression and thereby giving sexual reproduction as an additional means of spread to *F. japonica* var. *japonica*, a plant that has invaded so well by clonal means alone.

### 4.7.3 *Primary and secondary spread*

Japanese Knotweed *s.l.* produces extensive rhizomes that can become disrupted by the action of man or water. These fragments can travel and regenerate (Brock *et al.*, 1995), which could

lead to a patchy distribution such as that seen with clone H1 in this study. It is possible that this widespread clone of *F*. x *bohemica*, H1, represents a more successful invader, then again its current distribution could also have arisen as a matter of chance. Mandak, B. (pers. comm.) found some genotypes of *F*. x *bohemica* to be more invasive than others. It is highly plausible that some genotypes possess a genetic component that allows them to be more invasive than others, and this could even appear as a clone that is more suited to survival and re-establishment after disturbance by man. Alternatively H1, being found the furthest upstream, may have been one of the first *F*. x *bohemica* plant to be fragmented and travel downstream. If this was the case it may have established itself in the available niches before the other hybrid genotypes had the opportunity.

Caerynwch hall is presumed to be the source of the Japanese Knotweed *s.l.* population at Dolgellau. This has been the main site where seedlings have been found in various years, and is the only site in Dolgellau for the male fertile *F. sachalinensis*. It is assumed that the F1 *F*. x *bohemica* plants found further downstream all arose here. Seed could potentially have travelled in the river, but the more plausible hypothesis is that seeds germinated in Caerynwch itself and established along the banks of the river. Fast moving streams caused by natural phenomena such as heavy rain and flash floods can lead to newly established plants being ripped up and carried along with the water and deposited further downstream where the water calms down at places such as the Torrent Walk site. This could easily explain the novel genotypes found at the other sites, yet not at Caerynwch itself.

An important finding is the shared genotype of the plants at the cattle market with Caerynwch, which gives some indication of secondary spread. The implication being that the primary dispersal is from Caerynwch via the river to Dolgellau, followed by subsequent movement by man. There have clearly been a lot of earthworks in the area, for example when the disused railway was converted to a main road or during the production of the flood defences, which may have facilitated the secondary dispersal. Similarly it is quite possible that the Towyn road plants originated by such a process. These genotypes not having been found at Caerynwch does not detract from this argument, as we have already seen novel genotypes at sites along the river that most likely originated at Caerynwch.

### 4.7.4 *Genetic variation*

At the Torrent Walk site each stand is genetically distinct. This is in clear contrast to Dolgellau recreation ground where only five of the eighteen stands sampled are novel genotypes represented by a single stand. The stands that share genotypes are also more

widely distributed than those at Caerynwch. At Caerynwch, with the exception of H1, such stands were found adjacent to each other, whereas at Dolgellau recreation ground these shared genotypes were found interspersed either by other *F.* x *bohemica* genotypes or *F. japonica* stands. This level of disruption at Dolgellau is presumably an effect of the level of disturbance by man.

As would be expected from a plant reputed to be a single clone throughout Britain (Hollingsworth & Bailey, 2000a) each accession of *F. japonica* var. *japonica* analysed was identical and matched the ISSR genotype for the same taxa from other sites in Britain (chapter 4). Two genotypes of *F. sachalinensis* were discovered, S1 which was at two sites within the grounds of Caerynwch hall, and S2 which was found at one site just outside of the grounds at the start of Torrent Walk. As stated, the difference between genotypes S1 and S2 was the presence of two bands unique to S2. Bands that were not found in any of the hybrids analysed. Given its position within the grounds it is more likely that S1 was the original genotype of *F. sachalinensis* that was introduced, and that S2 is a somatic mutation that has arisen from it. Additionally the genotype S1 is shared by other male-fertile *F. sachalinensis* plants in Britain (chapter 8), unlike S2.

The mean ML hybrid index score when only hexaploid plants were analysed was 0.6317. Given that 66% of the genome of an F1 hexaploid *F.* x *bohemica* comes from *F. japonica* var. *japonica* that seems quite plausible, and is also reflected in the percentage of bands that arise from *F. japonica* var. *japonica* as shown in Figure 4.6. The plants that deviate from the mean are a direct reflection on the amount of variation within the hybrid group, hence why bootstrapping to produce confidence intervals is also required. The only plants that were outside of the mean confidence interval were an octoploid artificial backcross between two octoploid *F.* x *bohemica* plants, and a plant with 2n= 76 that is presumed to be a backcross between a hexaploid *F.* x *bohemica* and *F. japonica* var. *japonica*. Even these had overlapping confidence intervals. When only hexaploid hybrids were analysed no significantly different genotypes were found. Whilst it was disappointing not to find any obvious hexaploid backcrosses it does not mean that they don't occur. Hybrid and parental genotypic classes often differ minimally in terms of expected marker proportions. By chance some F2s are expected to have the same multilocus molecular genotype as F1s, and likewise certain backcrosses are likely to be identical in molecular constitution to F1s or parental individuals (Rieseberg & Linder, 1999). Even with a higher number of molecular markers, genealogical classification has not always been successful. Rieseberg and Linder (1999) used a large number of RAPD markers to analyse known pedigree hybrids between two wild

sunflower species, *Helianthus annuus* L. and *H. petiolaris* Nutt. They found a significant deviation in the expected patterns of marker inheritance.

One of the factors that may cause problems when trying to infer hybrid genealogies on Japanese Knotweed *s.l.* plants could be the variation in ploidy levels. *F. japonica* var. *japonica* is octoploid, and *F. sachalinensis* is tetraploid. It is not known if these arose as auto or allopolyploids, a factor that could possibly affect the number of copies of each band in the parental taxa and therefore the subsequent hybrids. It is possible that an ISSR band arising from a single copy of a locus may be masked by a stronger signal arising from an ISSR band with multiple copies of the same locus. There could even be problems arising from DNA competition occurring during the PCR amplification itself.

Neither the neighbour joining tree nor the minimum spanning network detected any significant clustering within the hexaploid hybrids. A similar lack of clustering was found in the earlier study of Hollingsworth (1998). This could be due to all of the hexaploid *F.* x *bohemica* genotypes being F1 progeny from the same cross, or simply that there is very little genotypic difference between an F1 and a backcrossed hybrid. From a cytological perspective none of the hybrids in this study were thought to be F2 plants, as these tend to be aneuploids (Bailey, pers. comm.). The mature plants in this study were all euploid apart from the one individual that was 2n = 65, which is more likely to be an F1 that has lost a chromosome than an F2 (Bailey, J.P. pers. comm.) The seedlings that were aneuploid were found growing under *F. japonica* var. *japonica* stands and are much more likely to be backcrosses. Interestingly one of these aneuploid seedlings Bs6 (2n=76) came out next to *F. japonica* var. *japonica* in all three analyses, whereas the second seedling Bs7 (2n = c110) whilst still to the *F. japonica* var. *japonica* side of the ML hybrid index score, was not significantly so and did not have a significant position in either the neighbour joining tree or the minimum spanning network. To have 2n = c110, it most likely arose from an unreduced gamete of *F.* x *bohemica* pollinating *F. japonica* var. *japonica*. A larger proportion of its genome is attributable to *F. japonica* var. *japonica* but it still contains as many *F. sachalinensis* chromosomes as a hexaploid hybrid, and, since its *F. japonica* var. *japonica* chromosomes occurred through natural segregation from both the maternal and paternal side, there is no reason why it should have more ISSR bands than a hybrid with fewer *F. japonica* var. *japonica* chromosomes. This also applies to the one with 2n = 76, however it would appear that either chance or the loss of *F. sachalinensis* chromosomes during the production of an aneuploid *F.* x *bohemica* gamete, has made it genetically closer to *F. japonica* var.

*japonica*. There is no significant evidence to prove or refute the hypothesis that the larger leaved individuals are a backcross between the octoploid *F*. x *bohemica* and *F. sachalinensis*.

As detailed in chapter 2, Figure 2.1, there are at least three potential ways in which an octoploid *F*. x *bohemica* may arise. A tetraploid *F*. x *bohemica* may undergo autopolyploidy, an unreduced gamete from *F. sachalinensis* may pollinate *F. japonica* var. *japonica*, or through the chance occurrence of a euploid F2 or backcrossed hybrid, such as would occur if an unreduced gamete from a hexaploid *F*. x *bohemica* pollinated a *F. sachalinensis*. Both of the octoploid hybrids have been shown to have *F. japonica* var. *japonica* as the maternal parent (Pashley, C.H., unpublished) which rules out both a tetraploid hybrid undergoing autopolyploidy, and an unreduced gamete from a hexaploid *F*. x *bohemica* pollinating *F. sachalinensis*. This leaves the possibility of an unreduced gamete from *F. sachalinensis* pollinating *F. japonica* var. *japonica* or the less plausible explanation of a chance occurrence of a euploid F2 or backcrossed hybrid involving the hexaploid *F*. x *bohemica* or *F. japonica* var. *japonica* as the maternal parent. The first of these two scenarios would result in a plant with 50% *F. sachalinensis* rather than the usual 33%. The second scenario would probably result in a lower proportion of *F. sachalinensis* in the genome, although there is no way of predicting which chromosomes would be lost during the production of a euploid F2 or backcross involving hexaploid *F*. x *bohemica*. The ML hybrid index showed the male-sterile octoploid (DB17, genotype B10) falling to the *F. sachalinensis* side of the mean, which supports the first scenario, however the male-fertile octoploid (DB47, genotype B27) fell to the *F. japonica* var. *japonica* side. The artificial hybrids generated from these two octoploids clustered closer to *F. sachalinensis* than *F. japonica* var. *japonica* in all three methods of analysis. An unreduced gamete can occur through non-segregation at either the first or second meiotic division. One would expect a plant produced from an unreduced gamete at the first meiotic division to possess all the bands of the parent, but not so at the second. Neither of the octoploid hybrids from Dolgellau possess all of the *F. sachalinensis* bands therefore it is more likely that if an unreduced gamete occurred it was at the second meiotic division.

### 4.7.5 *Implications for management*

Utilising molecular markers has played an important role in aiding our understanding of both the production of *F*. x *bohemica* hybrids and the dispersal mechanisms. This is important in understanding the invasive potential in this species. The value of such molecular markers is also exemplified by the study in San Francisco Bay where they are trying to control the invading species *S. alterniflora* (Ayres *et al.*, 1999). A standard means of control is to

remove the invading plant, but in this situation this failed because of the extensive rhizomes produced by the plants. The RAPD study by Ayres (1999) of these plants has shown that there is now *S. alterniflora* influence in almost every individual; therefore selectively pulling won't work and complete removal of populations containing the alien is required. Any study of invasive species, especially where hybridisation or dispersal/invasive potential are important, could greatly benefit from the use of molecular markers.

Understanding the population structure can also have a role in the management of invasive plants. In this particular situation at Dolgellau the local council have been herbicide-treating the populations along the recreation ground, and in summer 2001 it appears to be working quite successfully. However, the fact the Clwedog and Caerynwch are untouched means that Japanese Knotweed *s.l.* could reinvade!

**4.8 Conclusions**

There is clearly a high level of genetic diversity of *F.* x *bohemica* at Dolgellau. There is evidence that unique genotypes of *F.* x *bohemica* have established themselves further along the river system, and that both primary and secondary spread of genotypes already established at Caerynwch hall are likely to have occurred. There is cytological evidence to show that backcrossing does occur, even if there is no significant genetic data to prove or refute the presence of balanced hexaploid hybrids. The existence of octoploid hybrids is evidence in itself that backcrossing may become a significant factor in the evolution of Japanese Knotweed *s.l.*

Dolgellau is a unique site within Britain for many reasons, including that it is the only confirmed site for the octoploid *F.* x *bohemica* in Britain, and that germinating seedlings have been found. This could partially be due to its similarity with the native habitat of these plants (Bailey, 2003), but the prerequisites needed, i.e. presence of at least one male-fertile *F. sachalinensis*, male-sterile *F. japonica* stands and the hybrid *F.* x *bohemica*, has been shown to exist at other places within Britain. It could only be a matter of time before the Japanese Knotweed *s.l.* plants at one of the other "hot spots" evolves in a similar way.

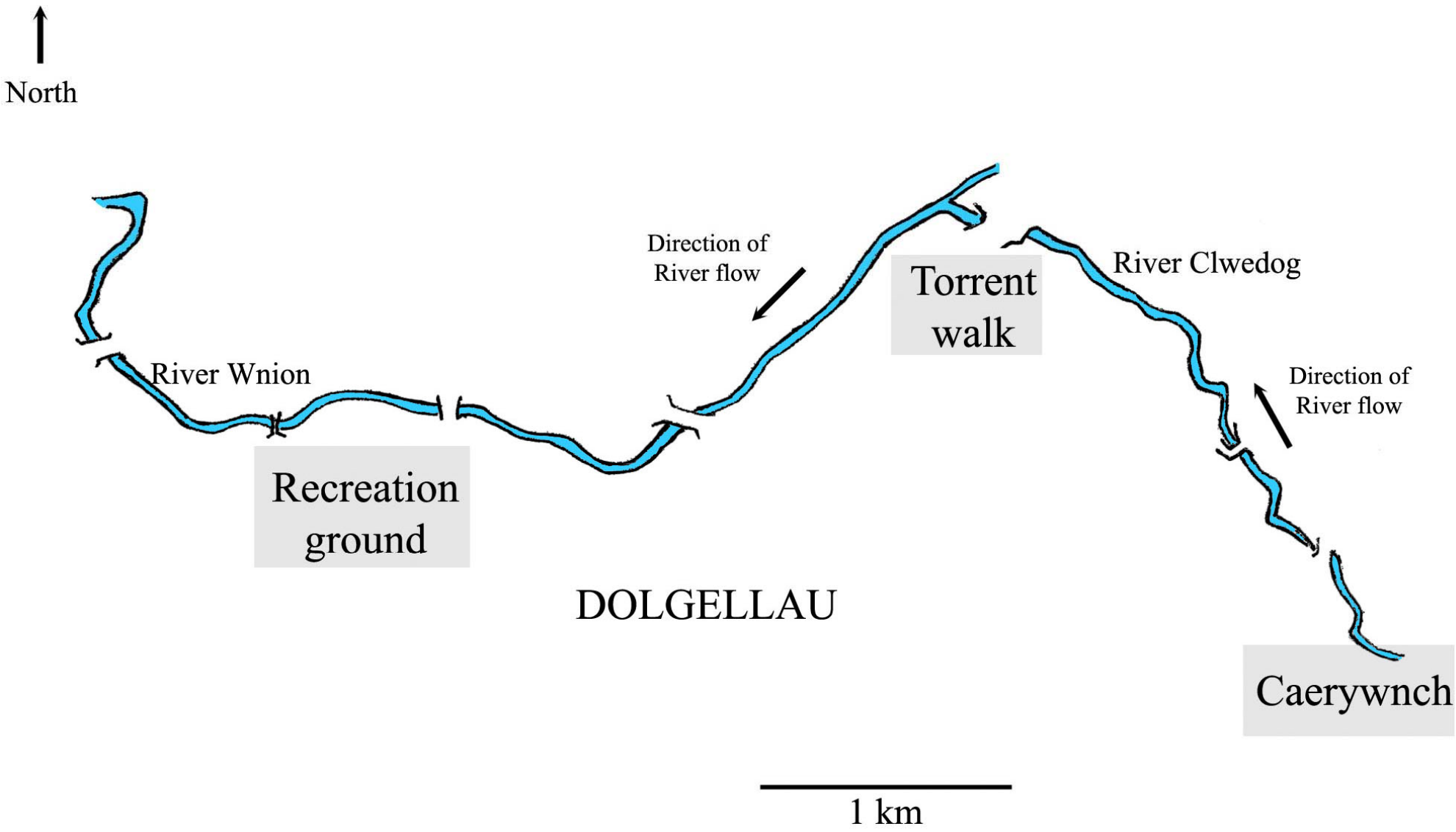



**Figure 4.1** Dolgellau river systems showing the positions of the three main study sites, Caerynwch, Torrent walk, and Dolgellau recreation ground.

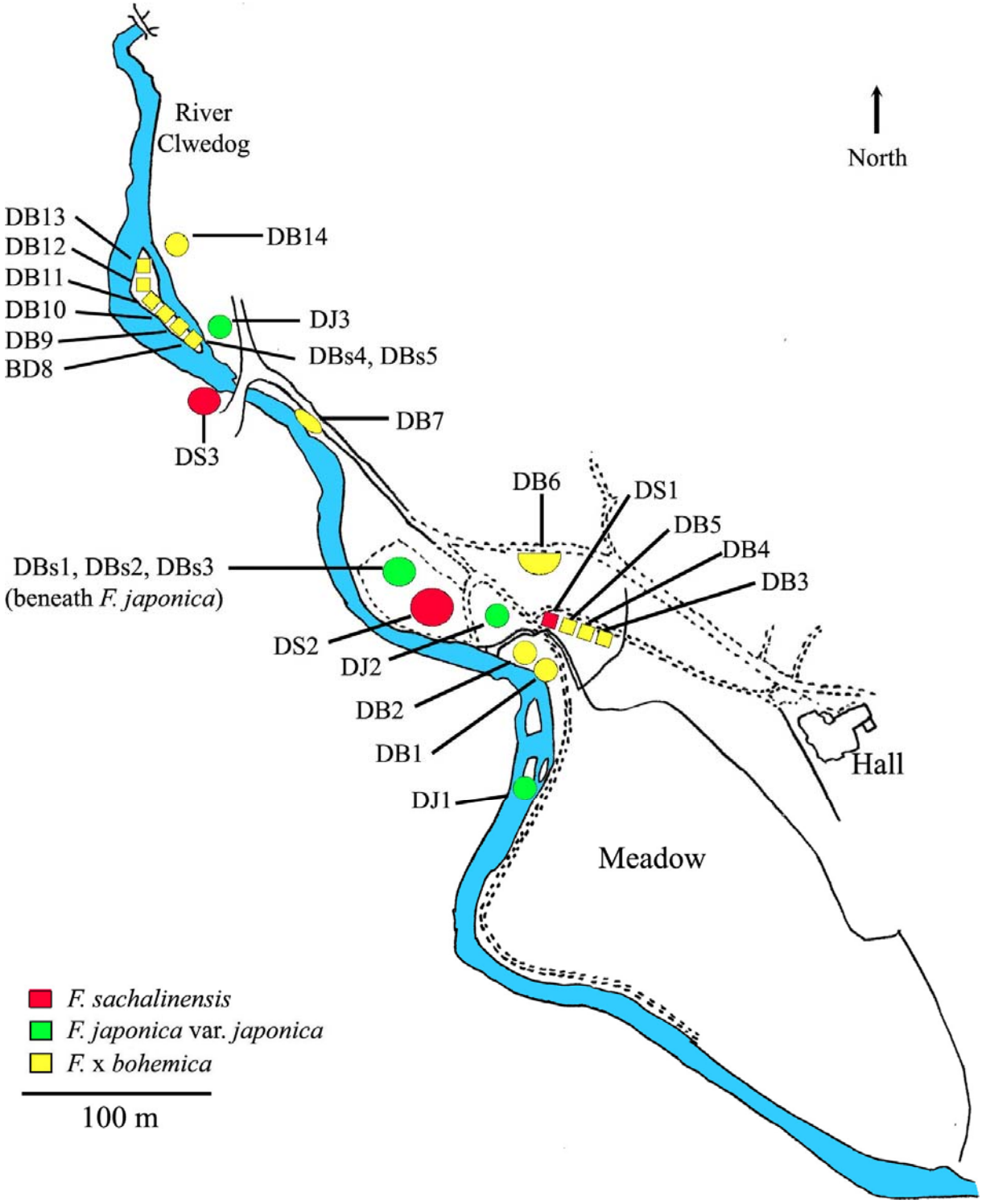



**Figure 4.2** Map showing the location of the plants at the Caerynwch site. Plants are given a thesis code which is the letter D, standing for Dolgellau, followed by either the letter J for *F. japonica* var. *japonica*, S for *F. sachalinensis* or B for *F.* x *bohemica*. If at time of collection the hybrid plants were seedlings, then a lowercase "s" follows the letters DB. The plants are numbered consecutively starting with the plant furthest upstream.

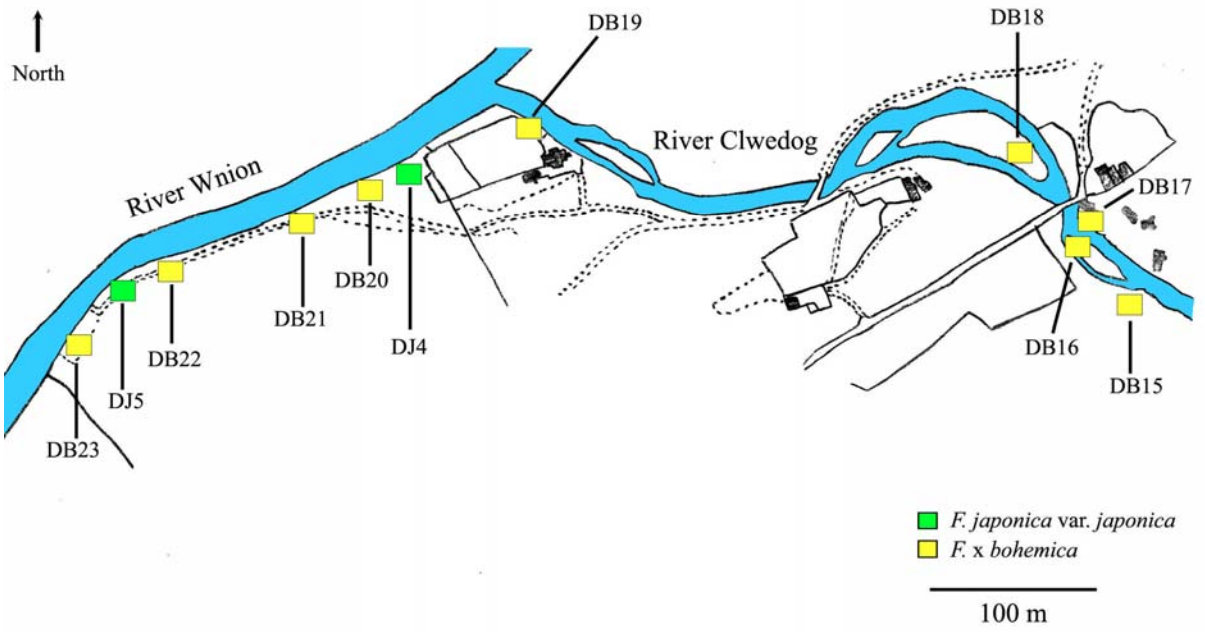



**Figure 4.3** Map showing the location of the plants at the Torrent walk site. Plants are given a thesis code which is the letter D, standing for Dolgellau, followed by either the letter J for *F. japonica* var. *japonica*, or B for *F.* x *bohemica*. The plants are numbered consecutively starting with the plant furthest upstream.

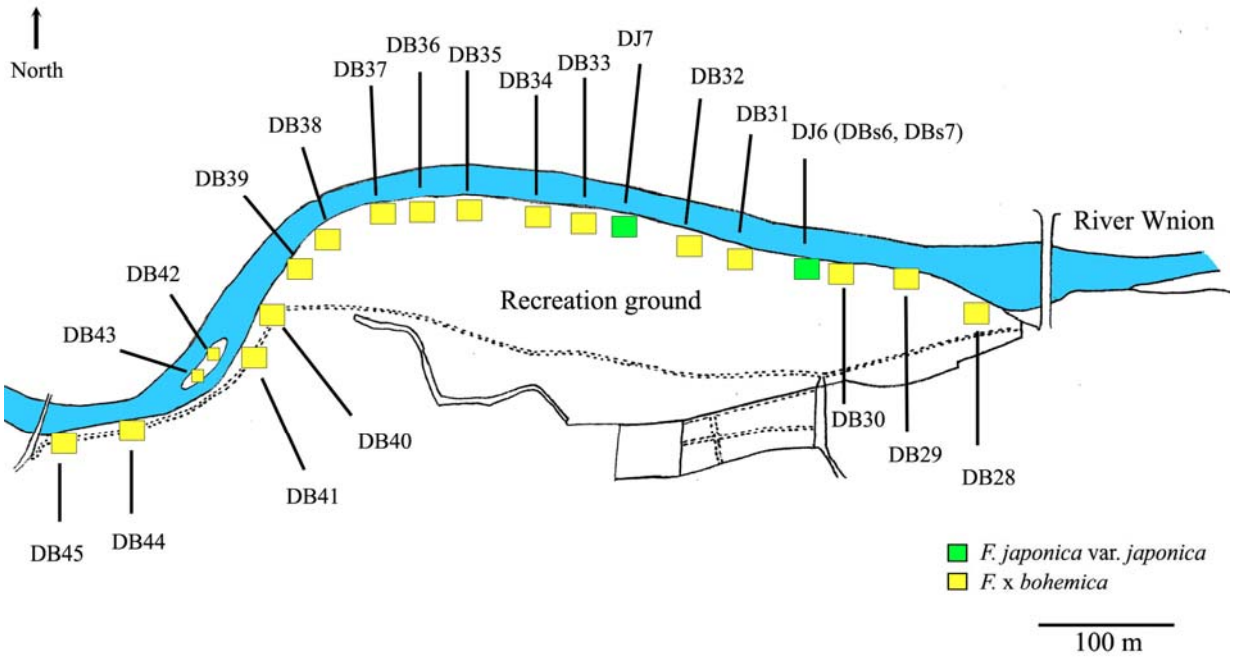



**Figure 4.4** Map showing the location of the plants at the Dolgellau recreation ground site. Plants are given a thesis code which is the letter D, standing for Dolgellau, followed by either the letter J for *F. japonica* var. *japonica* or B for *F.* x *bohemica*. If at time of collection the hybrid plants were seedlings, then a lowercase "s" follows the letters DB. The plants are numbered consecutively starting with the plant furthest upstream.

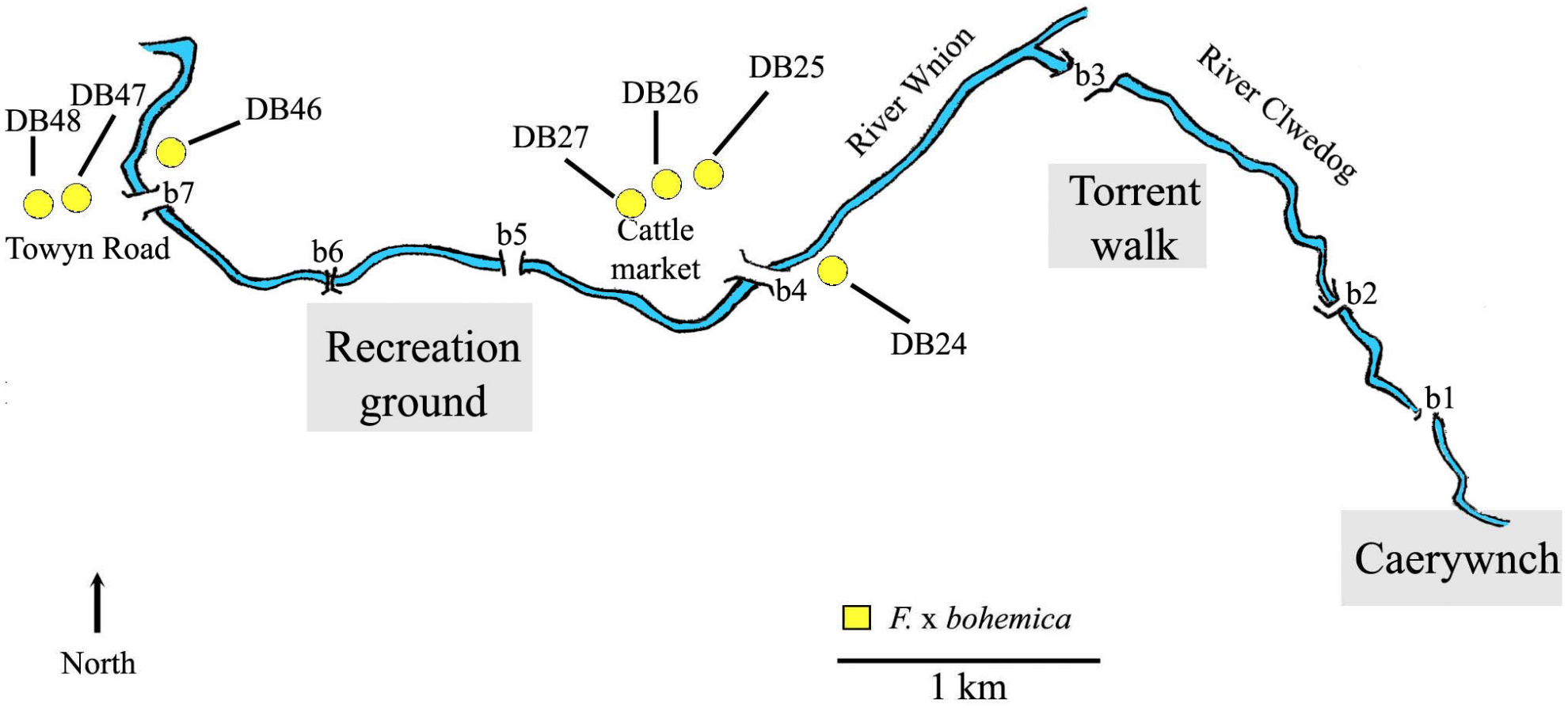



**Figure 4.5** Dolgellau river systems showing the positions of the three main study sites and the additional plants included in the study. Bridges are numbered b1 to b7. Plants are given a thesis code which is the letter D, standing for Dolgellau, followed by the letter B for *F*. x *bohemica*. The plants are numbered consecutively starting with the plant furthest upstream.

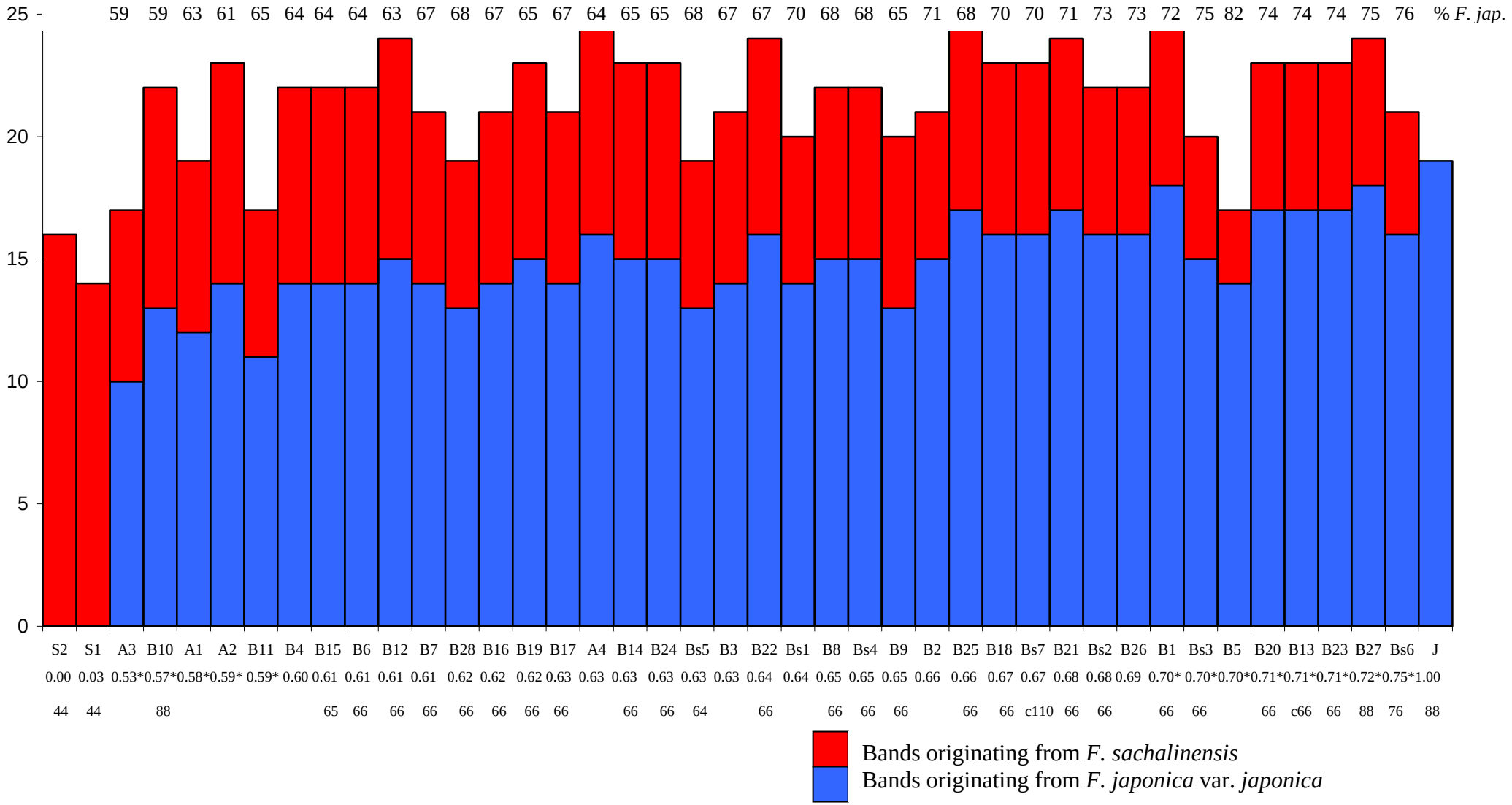



**Figure 4.6** Graph showing distribution of *Fallopia japonica* var. *japonica* and *F. sachalinensis* ISSR bands between the hybrids, ML hybrid index score and chromosome number. Percentage of bands arising from *F. japonica* var. *japonica* is written above the chart for each hybrid.

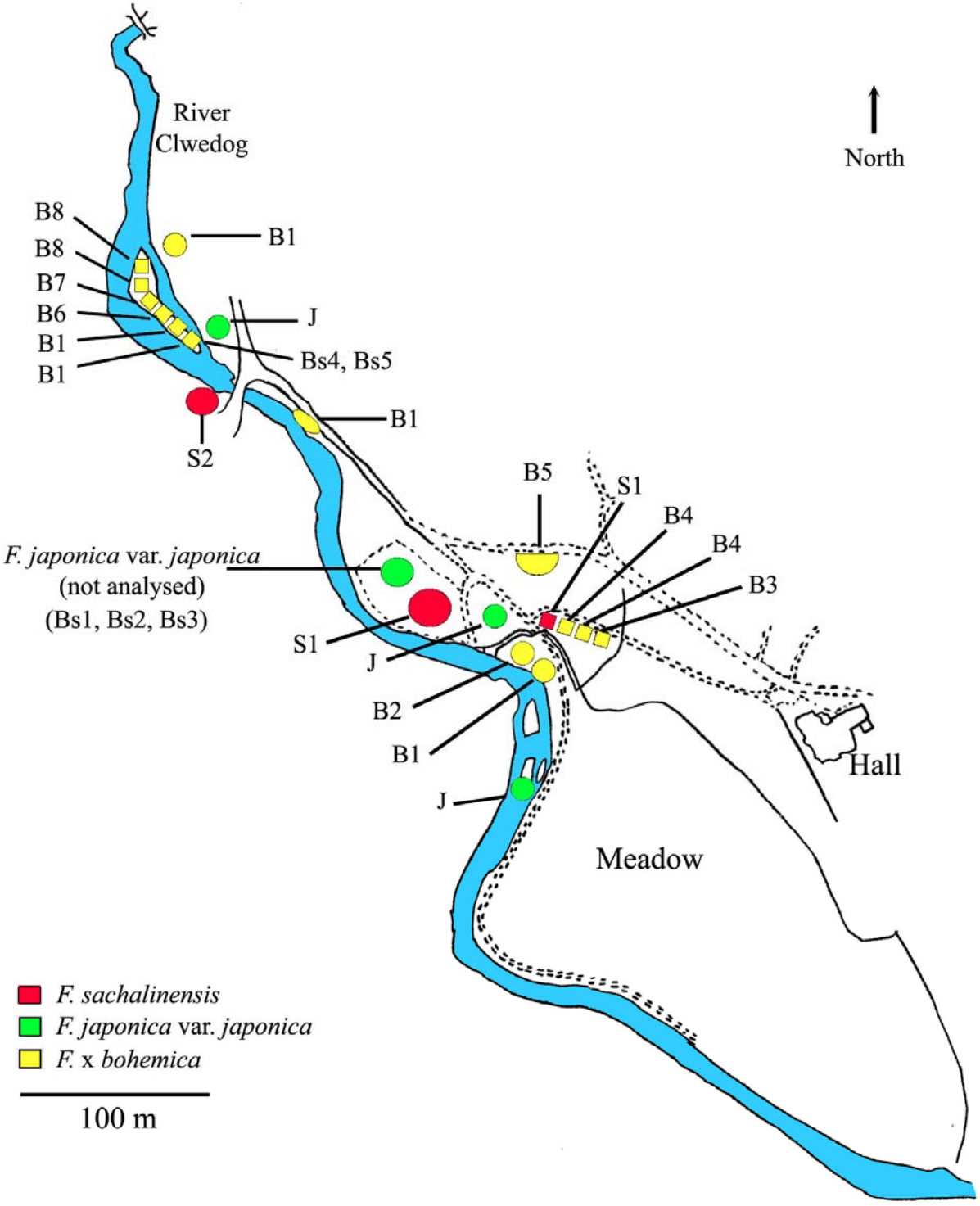



**Figure 4.7** Map showing the different genotypes detected at the Caerynwch site. The "B number" represents an individual genotype for an established *F*. x *bohemica* plant. They are numbered consecutively starting with the plant furthest upstream. A "Bs number" works using the same principal for the seedlings. Two or more plants sharing the same "B number" are assumed to be a single genotype. The letter J represents *F. japonica* var. *japonica*, and the codes S1 and S2 stand for the genotypes of *F. sachalinensis*

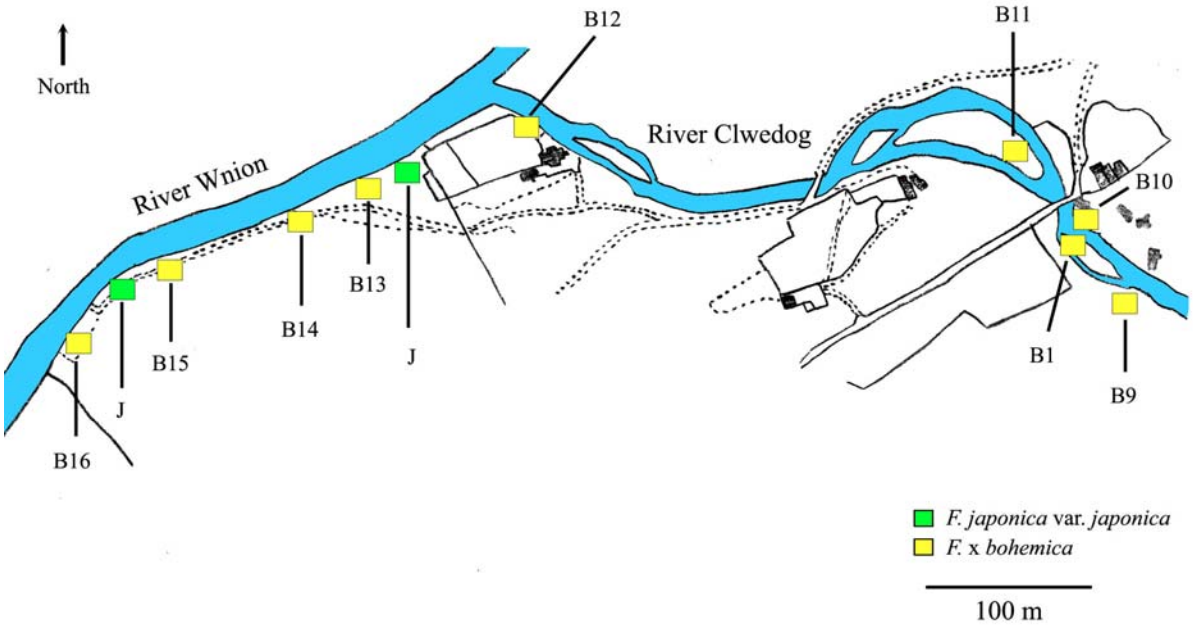



**Figure 4.8** Map showing the different genotypes detected at the Torrent Walk site. The "B number" represents an individual genotype for an established *F*. x *bohemica* plant. They are numbered consecutively starting with the plant furthest upstream. Two or more plants sharing the same "B number" are assumed to be a single genotype. The letter J represents *F. japonica* var. *japonica*.

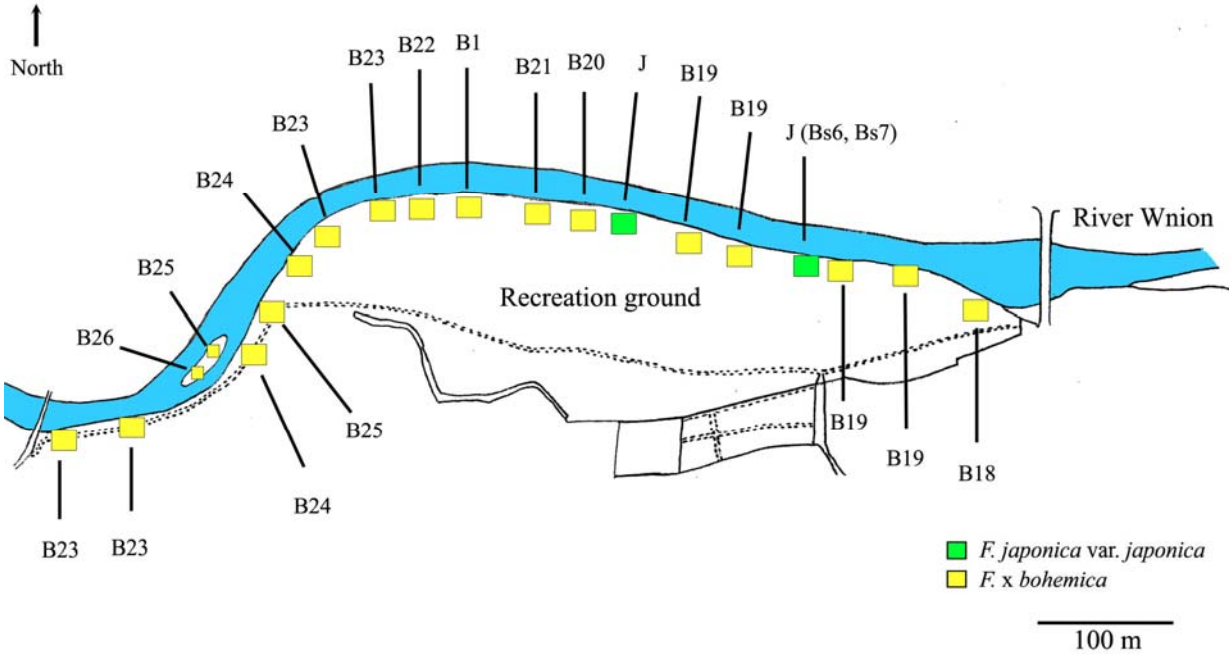



**Figure 4.9** Map showing the different genotypes detected at the Dolgellau recreation ground site. The "B number" represents an individual genotype for an established *F*. x *bohemica* plant. They are numbered consecutively starting with the plant furthest upstream. A "Bs number" works using the same principal for the seedlings. Two or more plants sharing the same "B number" are assumed to be a single genotype. The letter J represents *F. japonica* var. *japonica*.

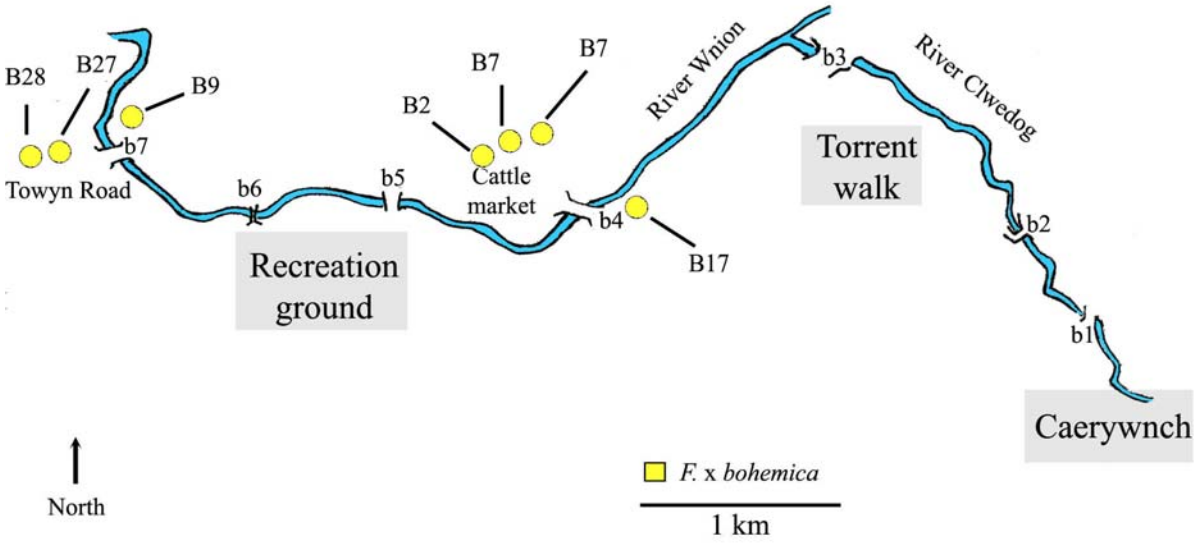



**Figure 4.10** Dolgellau river systems showing the positions of the three main study sites and the genotypes of the additional plants included in the study. Bridges are numbered b1 to b7. The "B number" represents an individual genotype for an established *F*. x *bohemica* plant. They are numbered consecutively starting with the plant furthest upstream. Two or more plants sharing the same "B number" are assumed to be a single genotype.

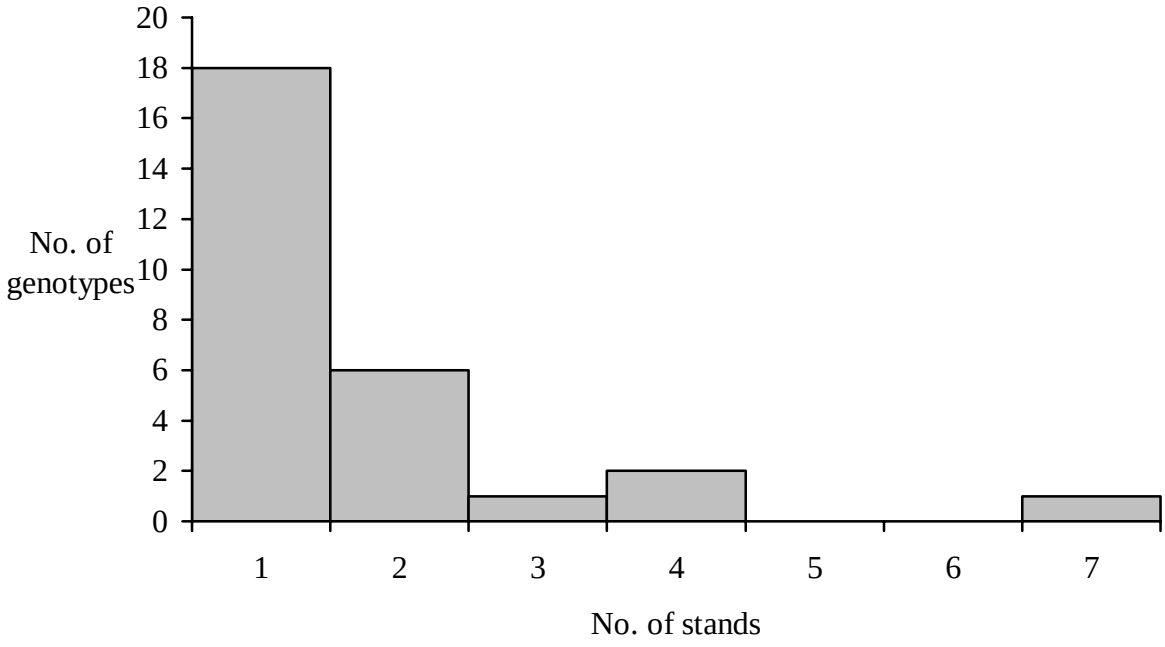



**Figure 4.11** Summary data showing the frequency distribution of number of genotypes against number of stands for the entire study.

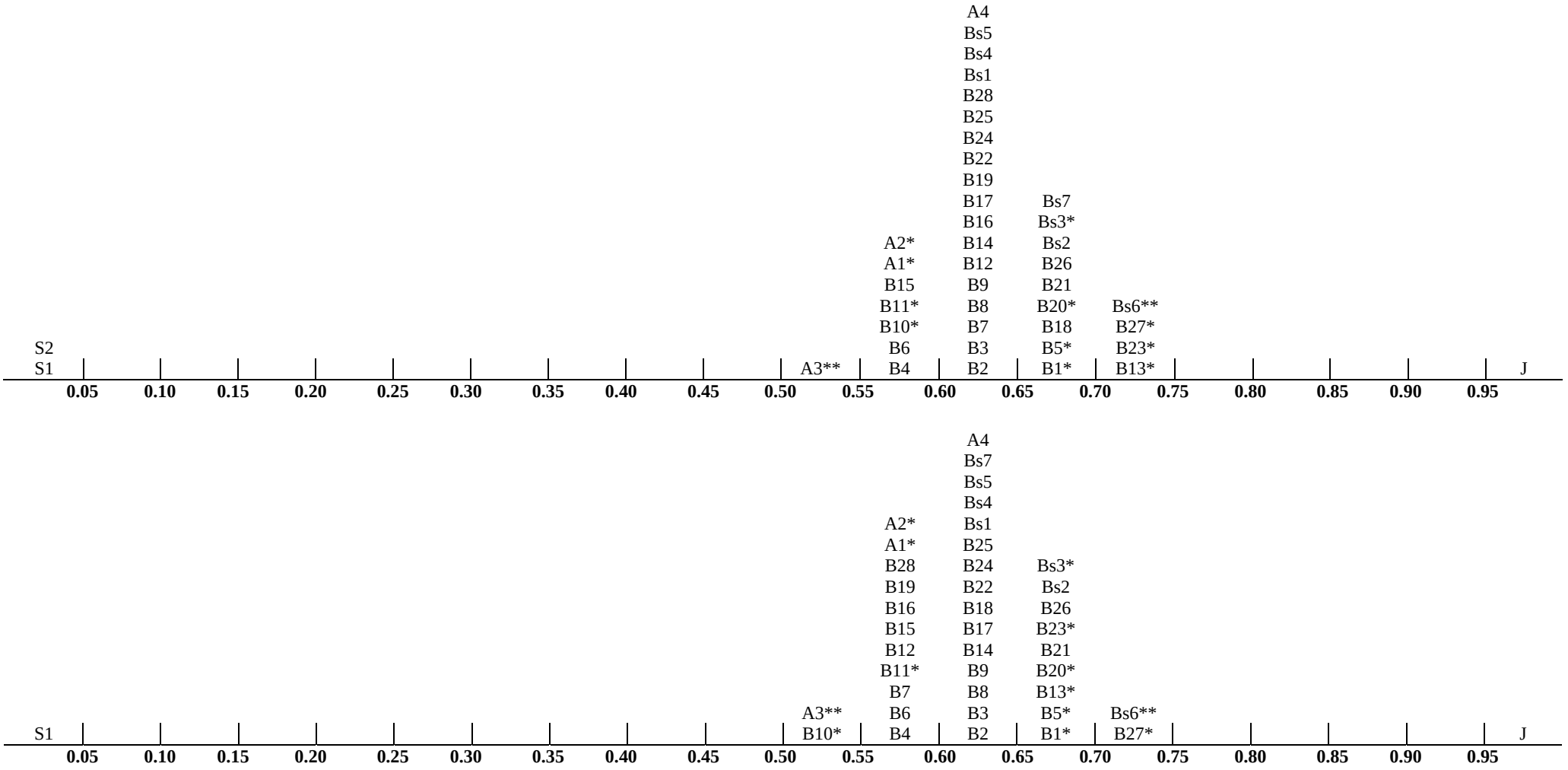



**Figure 4.12** Histograms of standardized maximum likelihood index scores, by individual genotypes. An asterisk (*) indicates hybrid index scores that deviated below the mean in excess of a single standard deviation, whilst two asterisks indicate a hybrid index value that is also outside of the mean hybrid index score confidence interval. Above histogram has both S1 and S2 as second parent genotypes, whilst below has just S1 as second parent.

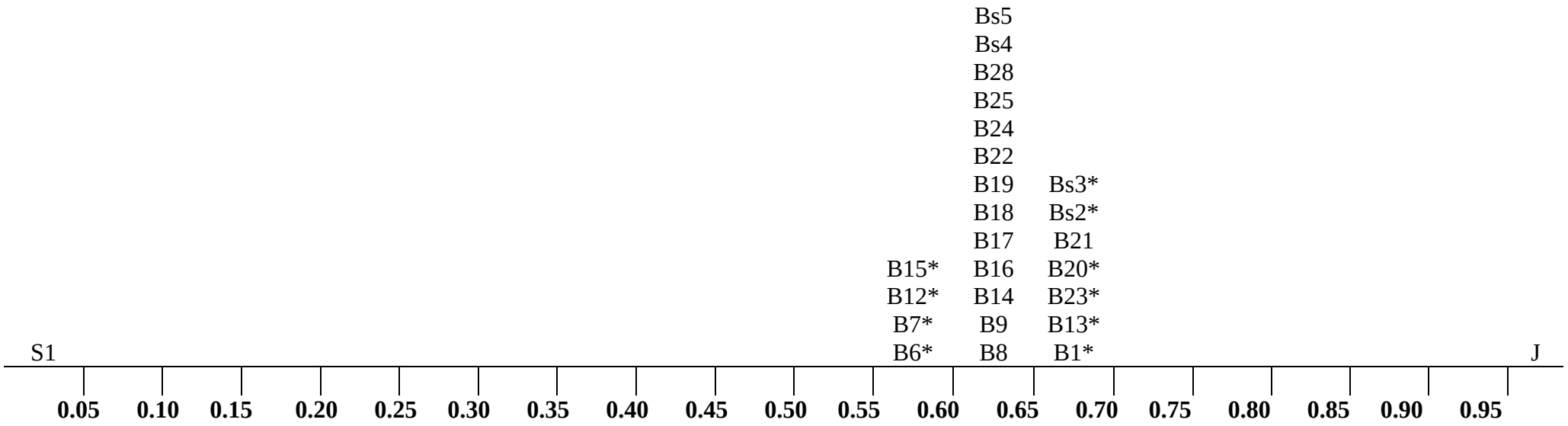



**Figure 4.13** Histograms of standardized maximum likelihood index scores, by individual genotypes, for hexaploid hybrids and the parental taxa only. An asterisk (*) indicates hybrid index scores that deviated from the mean in excess of a single standard deviation.

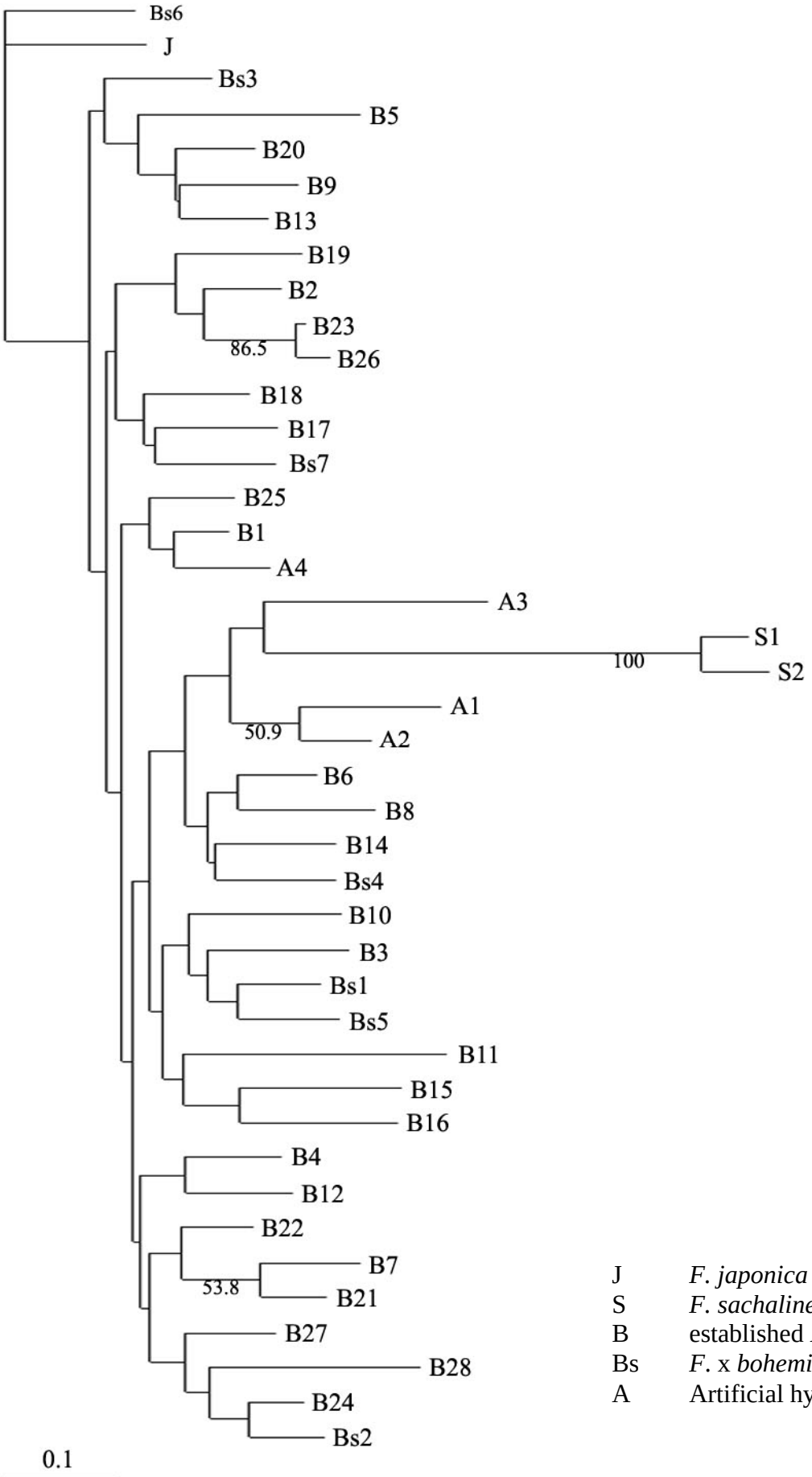
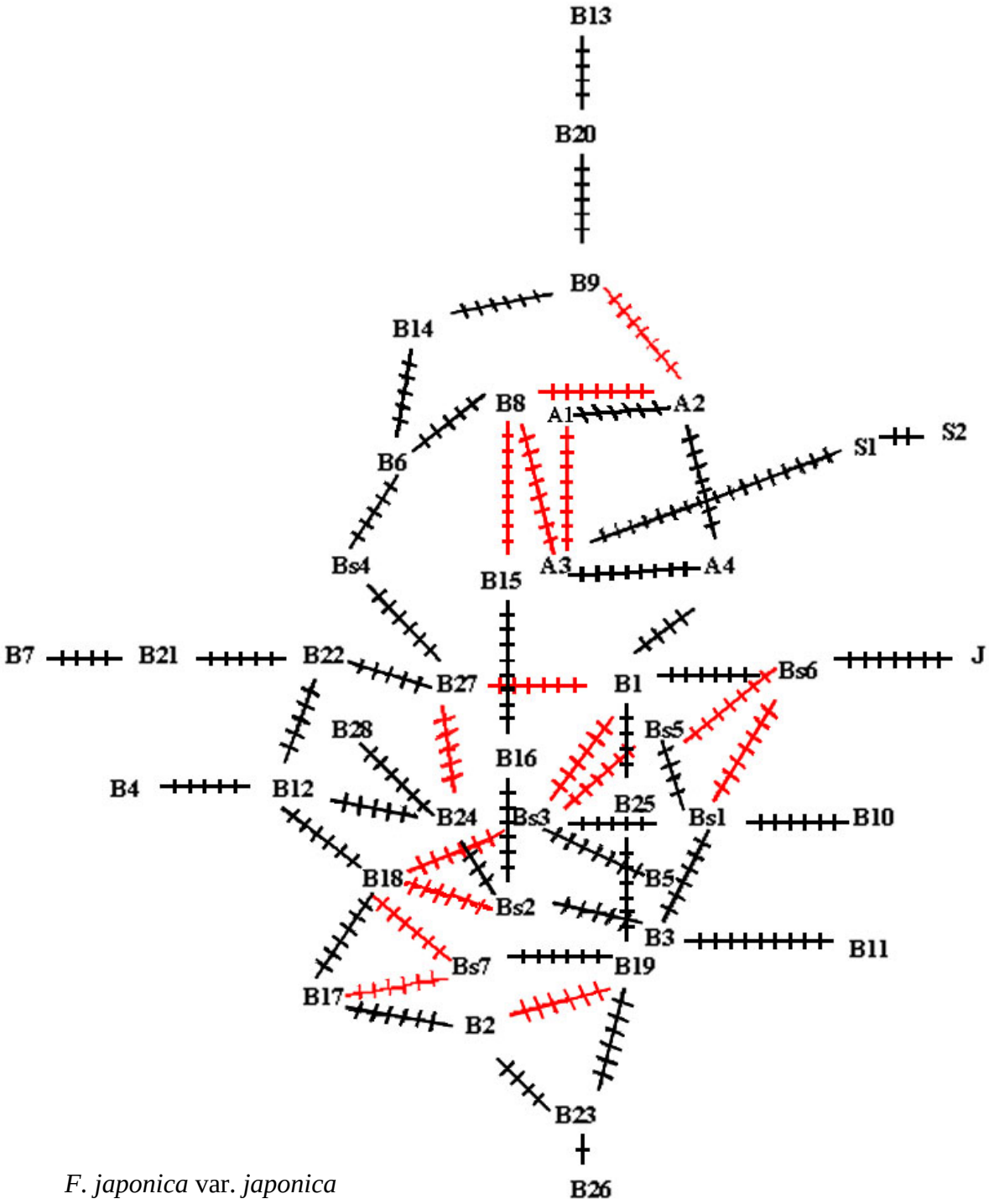



**Figure 4.14** Neighbour Joining tree showing all genotypes found at Dolgellau, including the artificial hybrids. Only bootstrap values greater than 50 are shown.




J *F. japonica* var. *japonica*
S *F. sachalinensis*
B established *F.* x *bohemica*
Bs *F.* x *bohemica* seedling
A Artificial hybrid

**Figure 4.15** Minimum spanning network showing the number of differences between genotypes. The cross-links show the number of differences. The red links are equally likely alternative links between genotypes.

**Table 4.1.** Details of the Japanese Knotweed *s.l.* plants studied from Dolgellau, North Wales.

| Thesis code | P number | Taxon | Sex expression | Chromosome number |
|---|---|---|---|---|
| DJ1 | P334 | *F. japonica* var. *japonica* | | |
| DB1 | P332 | *F.* x *bohemica* | | |
| DB2 | P331 | *F.* x *bohemica* | | |
| DB3 | P330 | *F.* x *bohemica* | | |
| DB4 | P329 | *F.* x *bohemica* | | |
| DB5 | P328 | *F.* x *bohemica* | | |
| DS1 | P326 | *F. sachalinensis* | | |
| DB6 | P333 | *F.* x *bohemica* | | |
| DJ2 | P315 | *F. japonica* var. *japonica* | | |
| DS2 | P327 / P624 | *F. sachalinensis* | | |
| DBs1 | P390 | *F.* x *bohemica* seedling | | |
| DBs2 | P644 | *F.* x *bohemica* seedling | | 2n = 66 |
| DBs3 | P646 | *F.* x *bohemica* seedling | | 2n = 66 |
| DB7 | P429a, P429b | *F.* x *bohemica* | | 2n = 66 |
| DS3 | P896 | *F. sachalinensis* | | 2n = 44 |
| DJ3 | P888 | *F. japonica* var. *japonica* | | |
| DBs4 | P1018 | *F.* x *bohemica* seedling | | 2n = 66 |
| DBs5 | P1019 | *F.* x *bohemica* seedling | | |
| DB8 | P890 | *F.* x *bohemica* | | 2n = 66 |
| DB9 | P892 | *F.* x *bohemica* | | 2n = 66 |
| DB10 | P893 | *F.* x *bohemica* | | 2n = 66 |
| DB11 | P891 | *F.* x *bohemica* | | 2n = 66 |
| DB12 | P894 | *F.* x *bohemica* | | 2n = 66 |
| DB13 | P895 | *F.* x *bohemica* | | |
| DB14 | P889 | *F.* x *bohemica* | | |
| DB15 | P897 | *F.* x *bohemica* | | 2n = 66 |
| DB16 | P898 | *F.* x *bohemica* | | 2n = 66 |
| DB17 | P899a, P899b | *F.* x *bohemica* | male-sterile | 2n = 88 |
| DB18 | P1005 | *F.* x *bohemica* | | |
| DB19 | P1012 | *F.* x *bohemica* | | 2n = 66 |
| DJ4 | P1011a, P1011b | *F. japonica* var. *japonica* | | 2n = 88 |
| DB20 | P1010a, P1010b | *F.* x *bohemica* | | 2n = 66 |
| DB21 | P1009 | *F.* x *bohemica* | | 2n = 66 |

| | | | | |
|---|---|---|---|---|
| DB22 | P1008 | *F*. x *bohemica* | | 2n = 65 |
| DJ5 | P1007 | *F. japonica* var. *japonica* | | |
| DB23 | P1006 | *F*. x *bohemica* | | 2n = 66 |
| DB24 | P1013 | *F*. x *bohemica* | | 2n = 66 |
| DB25 | P1017 | *F*. x *bohemica* | | 2n = 66 |
| DB26 | P1016 | *F*. x *bohemica* | | 2n = 66 |
| DB27 | P1015 | *F*. x *bohemica* | | 2n = 66 |
| DB28 | P740 | *F*. x *bohemica* | male-sterile | 2n = 66 |
| DB29 | P741 | *F*. x *bohemica* | male-fertile | |
| DB30 | P742 | *F*. x *bohemica* | male-fertile | |
| DJ6 | P743 | *F. japonica* var. *japonica* | male-sterile | 2n = 88 |
| DBs6 | P1021 | *F*. x *bohemica* seedling | | 2n = 76 |
| DBs7 | P1020 | *F*. x *bohemica* seedling | | 2n = c110 |
| DB31 | P744 | *F*. x *bohemica* | male-fertile | |
| DB32 | P745 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DJ7 | P747 | *F. japonica* var. *japonica* | male-sterile | 2n = 88 |
| DB33 | P746 | *F*. x *bohemica* | male-sterile | 2n = 66 |
| DB34 | P748 | *F*. x *bohemica* | | 2n = 66 |
| DB35 | P749 | *F*. x *bohemica* | male-sterile | 2n = 66 |
| DB36 | P750 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB37 | P751 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB38 | P752 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB39 | P753 | *F*. x *bohemica* | male-sterile | 2n = 66 |
| DB40 | P754 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB41 | P755 | *F*. x *bohemica* | male-sterile | 2n = 66 |
| DB42 | P621 | *F*. x *bohemica* | | |
| DB43 | P619 | *F*. x *bohemica* | | |
| DB44 | P756a, P756b | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB45 | P757 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB46 | P827 | *F*. x *bohemica* | male-fertile | 2n = 66 |
| DB47 | P51b, P826 | *F*. x *bohemica* | male-fertile | 2n = 88 |
| DB48 | P49 | *F*. x *bohemica* | male-sterile | 2n = 66 |
| DA1 | P1157a | Artificial hybrids | | |
| DA2 | P1157b | 8x *F*. x *bohemica* F2's | | |
| DA3 | P1157c | P826 x P899 | | |
| DA4 | P1157d | | | |

**Table 4.2** ISSR banding pattern for each genotype. Bands marked blue are inherited from *F. japonica* var. *japonica*, and red from *F. sachalinensis*.

| | AAG-1 | | | | | 855 | | | 881 | | | | | | | | | CAC-1 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | 1573 | 1510 | 1364 | 1321 | 1064 | 1005 | 815 | 420 | 2036 | 1772 | 1752 | 1713 | 1634 | 1414 | 1117 | 1053 | 978 | 1556 | 1425 | 1336 | 1156 | 1129 |
| J | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| S1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| S2 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| B1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B2 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| B3 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| B4 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B5 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| B6 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B7 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B8 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B9 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B10 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B11 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| B12 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B13 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B14 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B15 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| B16 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| B17 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| B18 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B19 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |

| Genotype | AAG-1 | | | | | 855 | | | 881 | | | | | | | | | CAC-1 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1573 | 1510 | 1364 | 1321 | 1064 | 1005 | 815 | 420 | 2036 | 1772 | 1752 | 1713 | 1634 | 1414 | 1117 | 1053 | 978 | 1556 | 1425 | 1336 | 1156 | 1129 |
| B20 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B21 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B22 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| B23 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| B24 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| B25 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| B26 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| B27 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| B28 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| Bs1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| Bs2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 |
| Bs3 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| Bs4 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Bs5 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| Bs6 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| Bs7 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| A1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| A2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| A3 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 |
| A4 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

| Genotype | 840 | | | | | | | AG-4 | | | | | 864 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1360 | 1275 | 1168 | 1026 | 1011 | 962 | 574 | 1531 | 1362 | 1285 | 907 | 840 | 1790 | 1725 | 1530 | 1502 | 1450 | 1227 | 1182 | 897 | 844 |
| J | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| S1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| S2 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| B1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B2 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B3 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B4 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B5 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B6 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B7 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B8 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B9 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| B10 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| B11 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| B12 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B13 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B14 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 |
| B15 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| B16 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| B17 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B18 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B19 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| B20 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B21 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |

| Genotype | 840 | | | | | | | AG-4 | | | | | 864 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1360 | 1275 | 1168 | 1026 | 1011 | 962 | 574 | 1531 | 1362 | 1285 | 907 | 840 | 1790 | 1725 | 1530 | 1502 | 1450 | 1227 | 1182 | 897 | 844 |
| B22 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B23 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B24 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B25 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B26 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B27 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| B28 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| Bs1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| Bs2 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| Bs3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| Bs4 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| Bs5 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| Bs6 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| Bs7 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| A1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| A2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| A3 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 |
| A4 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |

**Table 4.3**. Distribution of established *F*. x *bohemica* genotypes within each site at Dolgellau, Wales.

| | Genotypes found as a single stand | Genotypes found as two stands | Genotypes found as four stands | Genotypes found as five stands | Total number of stands | Total number of genotypes |
|---|---|---|---|---|---|---|
| **Caerynwch** | B2*, B3, B5, B6, B7* | B4, B8 | - | B1* | 14 | 8 |
| **Torrent walk** | B9*, B10, B11, B12, B13, B14, B15, B16, B1* | - | - | - | 9 | 9 |
| **Recreation ground** | B18, B20, B21, B22, B26, B1* | B24, B25 | B19, B23 | - | 18 | 10 |
| **Bridge 4** | B17 | - | - | - | 1 | 1 |
| **Bridge 6** | B9* | - | - | - | 1 | 1 |
| **Cattle market** | B2* | B7* | - | - | 3 | 2 |
| **Towyn road** | B27, B28 | - | - | - | 2 | 2 |

* Clones that appear in more than one site at Dolgellau.

*Table 4.4* ***Maximum likelihood (ML) hybrid index scores for all genotypes, and the confidence interval generated by bootstrapping 10,000 times. An asterisk (*) indicates specimens that deviate from the mean in excess of a single standard deviation. Index I includes both genotypes of*** **F. sachalinensis,** ***whilst index II only uses genotype S1***

| Genotype | Plants | ML Index I. | 2.5%CI | 97.5%CI | ML index II. | 2.5%CI | 97.5%CI | 2n = |
|---|---|---|---|---|---|---|---|---|
| J | DJ1, DJ2, DJ3, DJ4, DJ5, DJ6, DJ7 | 1 | 1 | 1 | 1 | 1 | 1 | 88 |
| S1 | DS1, DS2, DS2 | 0.0297 | 0 | 0.0743 | 0 | 1 | 1 | 44 |
| S2 | DS3 | 0 | 0 | 0 | N/A | N/A | N/A | 44 |
| B1 | DB1, DB7, DB8, DB9, DB14, DB16, DB35 | 0.6913* | 0.5913 | 0.7908 | 0.6813* | 0.5774 | 0.7836 | 66 |
| B2 | DB2, DB27 | 0.6496 | 0.5231 | 0.7709 | 0.6384 | 0.5095 | 0.7621 | |
| B3 | DB3 | 0.6189 | 0.4857 | 0.7492 | 0.6064 | 0.4715 | 0.7403 | |
| B4 | DB4, DB5 | 0.5938 | 0.4702 | 0.7218 | 0.5809 | 0.4512 | 0.7085 | |
| B5 | DB6 | 0.6971* | 0.5554 | 0.8337 | 0.6869* | 0.5453 | 0.8239 | |
| B6 | DB10 | 0.5982 | 0.4702 | 0.7232 | 0.5854 | 0.4540 | 0.7113 | 66 |
| B7 | DB11, DB25, DB26 | 0.6030 | 0.4743 | 0.7312 | 0.5903 | 0.4580 | 0.7166 | 66 |
| B8 | DB12, DB13 | 0.6356 | 0.4979 | 0.7730 | 0.6236 | 0.4826 | 0.7572 | 66 |
| B9 | DB15, DB46 | 0.6390 | 0.5120 | 0.7679 | 0.6270 | 0.4975 | 0.7527 | 66 |
| B10 | DB17 | 0.5568* | 0.4304 | 0.6842 | 0.5428* | 0.4132 | 0.6703 | 88 |
| B11 | DB18 | 0.5790* | 0.4322 | 0.7218 | 0.5656* | 0.4171 | 0.7073 | |
| B12 | DB19 | 0.6011 | 0.4776 | 0.7271 | 0.5879 | 0.4636 | 0.7119 | 66 |
| B13 | DB20 | 0.7031* | 0.5901 | 0.8146 | 0.6930* | 0.5763 | 0.8064 | c66 |
| B14 | DB21 | 0.6172 | 0.4963 | 0.7369 | 0.6050 | 0.4808 | 0.7224 | 66 |
| B15 | DB22 | 0.5973 | 0.4659 | 0.7299 | 0.5841 | 0.4506 | 0.7143 | 65 |
| B16 | DB23 | 0.6115 | 0.4829 | 0.7392 | 0.5991 | 0.4659 | 0.7241 | 66 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| B17 | DB24 | 0.6149 | 0.4863 | 0.7416 | 0.6026 | 0.4698 | 0.7335 | 66 |
| B18 | DB28 | 0.6560 | 0.5382 | 0.7707 | 0.6449 | 0.5257 | 0.7616 | 66 |
| B19 | DB29, DB30, DB31, DB32 | 0.6116 | 0.4890 | 0.7283 | 0.5992 | 0.4751 | 0.7171 | 66 |
| B20 | DB33 | 0.6980* | 0.5815 | 0.8140 | 0.6877* | 0.5703 | 0.8027 | 66 |
| B21 | DB34 | 0.6706 | 0.5573 | 0.7842 | 0.6596 | 0.5406 | 0.7736 | 66 |
| B22 | DB36 | 0.6306 | 0.5163 | 0.7437 | 0.6188 | 0.5021 | 0.7330 | 66 |
| B23 | DB37, DB38, DB44, DB45 | 0.7047* | 0.5855 | 0.8271 | 0.6942* | 0.5720 | 0.8167 | 66 |
| B24 | DB39, DB41 | 0.6181 | 0.4918 | 0.7442 | 0.6055 | 0.4778 | 0.7308 | 66 |
| B25 | DB40, DB42 | 0.6499 | 0.5414 | 0.7575 | 0.6387 | 0.5258 | 0.7481 | 66 |
| B26 | DB43 | 0.6817 | 0.5511 | 0.8119 | 0.6705 | 0.5397 | 0.7985 | |
| B27 | DB47 | 0.7184* | 0.6113 | 0.8245 | 0.7088* | 0.5983 | 0.8166 | 88 |
| B28 | DB48 | 0.6104 | 0.4714 | 0.7465 | 0.5976 | 0.4565 | 0.7370 | 66 |
| Bs1 | DBs1 | 0.6314 | 0.5009 | 0.7602 | 0.6196 | 0.4810 | 0.7468 | |
| Bs2 | DBs2 | 0.6769 | 0.5560 | 0.7990 | 0.6661 | 0.5423 | 0.7875 | 66 |
| Bs3 | DBs3 | 0.6928* | 0.5717 | 0.8065 | 0.6828* | 0.5581 | 0.7990 | 66 |
| Bs4 | DBs4 | 0.6383 | 0.5098 | 0.7662 | 0.6264 | 0.4932 | 0.7535 | 66 |
| Bs5 | DBs5 | 0.6187 | 0.4777 | 0.7580 | 0.6062 | 0.4587 | 0.7486 | 64 |
| Bs6 | DBs6 | 0.7431** | 0.6274 | 0.8488 | 0.7346** | 0.6208 | 0.8453 | 76 |
| Bs7 | DBs7 | 0.6601 | 0.5437 | 0.7692 | 0.6492 | 0.5297 | 0.7629 | c110 |
| A1 | DA1 | 0.5711* | 0.4340 | 0.7092 | 0.5575* | 0.4175 | 0.6947 | |
| A2 | DA2 | 0.5784* | 0.4483 | 0.7072 | 0.5646* | 0.4346 | 0.6937 | |
| A3 | DA3 | 0.5171** | 0.3780 | 0.6620 | 0.5019** | 0.3566 | 0.6470 | |
| A4 | DA4 | 0.6154 | 0.5044 | 0.7281 | 0.6032 | 0.4879 | 0.7182 | |

*More than 1 SD from the class average. ** More than 1 SD from the class average, and outside the confidence limits for the mean ML hybrid index score.

**Table 4.5** Maximum likelihood (ML) hybrid index scores for all hexaploid hybrid genotypes, and the confidence interval generated by bootstrapping 10,000 times. An asterisk (*) indicates specimens that deviate from the mean (0.6317) in excess of a single standard deviation (0.0382).

| Genotype | Rank | ML Index III. | 2.5%CI | 97.5%CI | 2n = |
|---|---|---|---|---|---|
| J | - | 1 | 1 | 1 | 88 |
| S1 | - | 0 | 0 | 0 | 44 |
| B1 | 20 | 0.6847* | 0.5646 | 0.7943 | 66 |
| B6 | 2 | 0.5863* | 0.4405 | 0.7240 | 66 |
| B7 | 4 | 0.5897* | 0.4471 | 0.7263 | 66 |
| B8 | 13 | 0.6215 | 0.4691 | 0.7706 | 66 |
| B9 | 14 | 0.6266 | 0.4863 | 0.7659 | 66 |
| B12 | 3 | 0.5884* | 0.4439 | 0.7252 | 66 |
| B13 | 23 | 0.6965* | 0.5679 | 0.8230 | 66 |
| B14 | 9 | 0.6074 | 0.4703 | 0.7365 | c66 |
| B15 | 1 | 0.5850* | 0.4400 | 0.7255 | 66 |
| B16 | 5 | 0.6014 | 0.4574 | 0.7383 | 65 |
| B17 | 8 | 0.6043 | 0.4583 | 0.7424 | 66 |
| B18 | 17 | 0.6476 | 0.5156 | 0.7731 | 66 |
| B19 | 7 | 0.6040 | 0.4692 | 0.7330 | 66 |
| B20 | 22 | 0.6895* | 0.5571 | 0.8173 | 66 |
| B21 | 18 | 0.6628 | 0.5351 | 0.7868 | 66 |
| B22 | 12 | 0.6215 | 0.4892 | 0.7476 | 66 |
| B23 | 24 | 0.6982* | 0.5639 | 0.8314 | 66 |
| B24 | 11 | 0.6082 | 0.4638 | 0.7446 | 66 |
| B25 | 16 | 0.6427 | 0.5185 | 0.7614 | 66 |
| B28 | 6 | 0.6024 | 0.4479 | 0.7519 | 66 |
| Bs2 | 19 | 0.6707* | 0.5303 | 0.8009 | 66 |
| Bs3 | 21 | 0.6854* | 0.5499 | 0.8110 | 66 |
| Bs4 | 15 | 0.6274 | 0.4827 | 0.7661 | 66 |
| Bs5 | 10 | 0.6080 | 0.4499 | 0.7594 | 64 |

*More than 1 SD from the class average.

# Chapter 5. Hybridisation, introgression, and spread in three Japanese Knotweed *s.l.* tetraploid "hot-spots" in England.

## 5.1. Introduction

*F.* x *bohemica* can be found at three different ploidy levels, tetraploid, hexaploid and octoploid. The octoploid is so far only known from one region of Wales, although octoploid *F.* x *bohemica* plants have also been recorded from Germany, France and the Czech republic (Bailey, 1997). The hexaploid is by far the most common of the *F.* x *bohemica* plants, recorded as present in 190 of the 3859 10km recording squares in the New Atlas of The British & Irish Flora (Preston *et al.*, 2002). Although capable of producing F2 and backcrossed seed, it is not capable of reproducing at the hexaploid level because the gametes produced, and subsequent plants, are mostly aneuploid. The tetraploid *F.* x *bohemica,* which arises from a cross between *F. sachalinensis* and the dwarf *F. japonica* var. *compacta,* has been given very little attention, but is thought to be fully fertile. There are no published accounts referring to the invasive ability of the tetraploid hybrid, unlike the hexaploid hybrid that is proving to be more of a problem than either of its parents (Bímová *et al.*, 2001; Brabec & Pyšek, 2000).

In Britain *F. sachalinensis* is regarded as being far less invasive than *F. japonica* var. *japonica* (Conolly, 1977), and occupies only 576 of the 3859 10km squares in the New Atlas of The British & Irish Flora (Preston *et al.*, 2002). *F. japonica* var. *compacta* is rare, with few instances of naturalisation. It is not considered to be very invasive. The only sizeable naturalized stands in Britain are at Connel Ferry and North Ledaig in Scotland (Bailey, 2003). There are currently 30 records mapped on a 10 km square basis, including garden plants (Bailey & Conolly, 2000).

Flowering in *F. japonica* var. *compacta* begins early in July but can continue into September. *F. sachalinensis* does not tend to flower until late August, but there is still a clear overlap between the flowering times for hybridisation to occur (Bailey, J.P. unpublished). Both taxa are gynodioecious with male-fertile and male-sterile individuals being present in Britain, giving the opportunity for hybridisation to occur in both directions in contrast to the hexaploid *F.* x *bohemica.* Chloroplast DNA is maternally inherited in Japanese Knotweed *s.l.* and can be used to show the direction of hybridisation (Hollingsworth *et al.*, 1999). Tetraploid hybrids show bi-directional hybridisation, with wild hybrids in Britain having one or other of the parental chloroplast types, whilst all hexaploid hybrids have *F. japonica* var. *japonica* as the maternal parent (Hollingsworth *et al.*, 1999).

On a European basis, tetraploid *F*. x *bohemica* is relatively rare; there are seven known locations for this plant in Britain and at least one in the Czech Republic (Bailey J.P. pers. comm.). Four of these British locations have only a single sex of the tetraploid hybrid and are therefore not going to have an immediate effect on the evolution of this taxon. Gomshall in Surrey (v.c. 17) and Tottenham Marshes in Middlesex (v.c. 21) have only male-sterile plants, whilst South Wylam in Durham (v.c. 66) is known to have a male-fertile specimen. The fourth site Cheshunt, Hertfordshire (v.c. 20) has both tetraploid *F*. x *bohemica* and *F. sachalinensis*, but both are male-sterile so will not backcross. Seed obtained from open pollinated male-sterile stands in both Surrey and Hertfordshire had been pollinated by the commonly grown garden plant *F. baldschuanica* (Bailey & Stace, 1992). *F. baldschuanica* often pollinates male-sterile members of Japanese Knotweed *s.l.* in the absence of other members of the group (Bailey & Stace, 1992). The other three sites in Britain have both male-fertile and male-sterile stands and it is these sites that this study will concentrate on. These sites are Preston, W. Lancashire, (v.c. 60); Leeds, Yorkshire, (v.c 64); and, Cirencester, E. Gloucestershire, (v.c. 33). Cirencester was the first of these sites to be discovered, but the tetraploid plants here occupy less ground than the hexaploid hybrids (Bailey *et al.*, 1996). It was the discovery of the sites in Leeds and Preston that inspired the current study. In Leeds tetraploid *F*. x *bohemica* is the dominant Knotweed taxon, whilst at Preston one of the stands of tetraploid *F*. x *bohemica* covers an area of approximately 2,250 $m^2$ and grows 4m tall. Figure 5.1 shows Dr John Bailey and Mr Peter Jepson standing in this large tetraploid stand, which appears to have as strong an invasive potential as its hexaploid relative.

At sites where the tetraploid hybrid is found in Britain there is no evidence of *F. japonica* var. *compacta*. It is not known if it once did occur at these sites but has since disappeared, and that the tetraploid hybrids arose *in situ*, or whether the hybridisation event occurred elsewhere, for example at nursery gardens where the two taxa were often cultivated together, and it is these hybrids that have been introduced to the sites.

### 5.2 Aims

This study aims to analyse the genetic diversity of *F*. x *bohemica* plants found at three sites in Britain where both sexes of tetraploid *F*. x *bohemica* occur. Direction of hybridisation of the tetraploid hybrids will be determined using chloroplast PCR RFLPs. ISSR PCR will be used to calculate number of genotypes of both tetraploid and hexaploid hybrids at these sites, and to see if any genotypes are shared between the three sites studied. This study also aims to discuss whether the distribution within a site is likely to be the result of sexual reproduction or clonal spread.

## 5.3 Study sites

### 5.3.1 *Cirencester*

Cirencester, Gloucestershire is an historic Roman town in the heart of the Cotswolds. Cirencester is known to have both male-fertile and male-sterile hexaploid *F.* x *bohemica* and male-sterile tetraploid *F*. x *bohemica*. In addition it has both male-fertile *F. sachalinensis* and the *male-sterile* clone of *F. japonica* var. *japonica*, and as such was one of the first British locations to be given the term a "hot-spot" in terms of Japanese Knotweed evolution (Bailey *et al.*, 1996). The study by Bailey *et al*. (1996) recorded combined tetraploid and hexaploid *F*. x *bohemica* ground coverage of approximately 925 $m^2$. Most of the stands they recorded in September 1993 were still present in October 1995 and June 1999 when fresh collection trips were made, and have therefore been used in this study, as well as additional stands added in 1999 that includes male-fertile tetraploid *F*. x *bohemica*. The Cirencester plants are found in two locations referred to as area A and area B in Figure 5.2.

#### 5.3.1.1 Cirencester area A

A number of linear stands of *F*. x *bohemica* were growing along the road verge of the A429 on the approach to Cirencester. Originally 12 separate stands could be distinguished, although by 1999 only eleven of these were found. Figure 5.3 is a sketch of the road showing the relative positions of these plants, all of which were male-sterile.

#### 5.3.1.2 Cirencester area B

The main area plants were found was in and around the Abbey Grounds, as shown in Figure 5.4. The Abbey was dissolved in 1539, however the grounds have been maintained for public use. Alongside the feeder stream for the lake, which was once the Abbey Fishpond, were numerous stands of *F*. x *bohemica*. The two samples furthest from the lake were collected outside of the grounds, one from the garden of a local house and the other on the roadside. This area had previously been a part of the Abbey Grounds. Six stands of *F*. x *bohemica* were found inside the grounds along the feeder stream.

Also in the Abbey Grounds, near to the main entrance by a stream, were several stands of Japanese Knotweed *s.l.* To one side of the stream was a stand a male-fertile *F. sachalinensis* and two stands of *F*. x *bohemica*. On the other side of the stream was a stand of *F. japonica* var. *japonica* and three stands of *F*. x *bohemica*.

A new area of Japanese Knotweed *s.l.* was found in 1999, outside of the Abbey grounds just past the Spital gate. There was a lot of *F.* x *bohemica* and *F. japonica* var. *japonica*. Four samples of *F.* x *bohemica* and one sample of *F. japonica* var. *japonica* were collected.

Along the A435 dual carriageway, Grove Lane, was a stand approximately 35 metres in length, of both male-fertile and male-sterile *F.* x *bohemica*. Both sexes were sampled in an early collection, and a further sample was taken in 1999.

South of the Abbey Grounds site, growing on an embankment near the footbridge at a roundabout, A417/A435, was an additional stand of male-fertile *F. sachalinensis*.

5.3.2 *Preston*

Open pollinated seed collected from a male-sterile tetraploid *F.* x *bohemica* in Preston was found to be 2n = 44. This was surprising as the assumption had been that it would be 2n = 32, pollinated by *F. baldschuanica,* as was the case with similar stands in Surrey and Hertfordshire. This meant that the seed was in fact either the result of backcrossing with one of the parental taxa, presumably *F. sachalinensis* given how scarce *F. japonica* var. *compacta is,* or was F2 hybrid seed. For this reason a wider study of the Japanese Knotweed *s.l.* population in Preston was initiated, the results of which are given in this chapter. When Preston was visited no *F. sachalinensis* was found but male-fertile tetraploid *F.* x *bohemica* was found.

There appears to be a strong connection with the current and old railway system in Preston and the location of the Japanese Knotweed *s.l.* stands. This is not unusual; Japanese Knotweed *s.l.* is often found growing in disturbed sites such as along road verges and railway embankments (Child *et al.*, 1992).

Within Preston there are three main areas where *F.* x *bohemica* was collected. These are referred to as areas A, B and C and are shown in Figure 5.5.

5.3.2.1 Preston area A

Area A encompasses Avenham Park, Frenchwood Park, Miller Park and the Preston railway station and is shown in detail in Figure 5.6. The first collection was at the SWS Depot, EastCliff railway yard. This is very near to Preston railway station and is the spot where the tetraploid seed had previously been collected. The spot where the original seed was collected had been weed killed (Jepson P., pers. comm.), but a large area close by, presumed to be part

of the same stand, was still present. Three specimens were collected. Two seedlings were also found growing in this area, but unfortunately these did not survive the transplantation. It is quite likely that they had been affected by herbicide.

The brick building in the vicinity of the car parks and railway yard was once the old railway hotel and was connected to the railway by a footbridge. It is believed that the plants may have originally been planted in the grounds of this hotel. This building is now the Lancashire County Council (L.C.C) Social Services. Between the L.C.C building, the East-Cliff car park and the bank above the main railway line was a male-fertile stand of *F*. x *bohemica*.

There used to be two railway stations in Preston (Jepson P., pers. comm.). The line that used to run from East Lancashire to Blackpool via the second Railway station is now disused. There is a very large stand of male-sterile *F*. x *bohemica* that can be seen clearly from Bailey bridge. The stand spreads along the old line for a distance of about 90 m and is 25 m wide, and is the stand shown in Figure 5.1. The plants are all male-sterile, in full flower and setting a high quantity of seed. These plants are also very tall, over 4 m.

Further stands of *F*. x *bohemica* were sampled within area A. One *F*. x *bohemica* stand was found by the gateway into Miller Park from the East Cliff offices (old railway hotel). Another was a male-sterile stand next to the river by the Tramway Bridge in Avenham Park. Frenchwood recreation ground had a large stand of *F*. x *bohemica* growing for 90 m along the River Ribble, and additionally stands of *F. japonica* var. *japonica,* one of which was also sampled.

5.3.2.2 Preston area B

Area B is comprised of two sites one on either side of the River Ribble as shown in Figure 5.7. To the South of the River is found Penwortham golf course, and here, in the car park near to the club house, was found a specimen of *F*. x *bohemica* that was not in flower. To the North of the River is the Riversway duel carriageway. On one side of this road was found *F. japonica* var. *japonica* and on the other side was a stretch of several hundred metres of *F*. x *bohemica*. A specimen was taken from each end of this stretch.

5.3.2.3 Preston area C

This is along one of the main routes into Preston, the A6. As can be seen in Figure 5.8 *F*. x *bohemica* was found on both sides of the road. The plant on the left hand side of the road as you leave Preston from the South was behind some railings that backed onto the Athletics

track, whilst the stand on the other side of the A6 was roadside. Both stands were male sterile.

### 5.3.3 *Leeds*

The Japanese Knotweed *s.l.* population in Leeds is spread over several discrete sites. Three of these sites have been included in this study, the first being along the River Aire at the site of Kirkstall Abbey (area A), the second is in the vicinity of the old Wood's Hardy Plants Club (area B) and the third being the Woodside Quarry (area C). These are shown in Figure 5.9.

#### 5.3.3.1 Leeds area A

Kirkstall Abbey lies on the outskirts of Leeds city centre. It was founded in 1147 by Henry de Lacy, Lord of Pontefract, for an order of Cistercian monks. They remained until its dissolution in 1539. To prevent the monks from returning, the main road into Leeds was diverted through the Nave and great East window. This road is now known as the A65 Kirkstall Road. The grounds and the remains of the Abbey are now a preserved site, and it is believed that the plants would have been planted here when they were still a desirable plant for large gardens. In the car park belonging to the Abbey, on the North bank of the River Aire, was found a very large stand (approx. 30m x 30m) of male-fertile *F. sachalinensis*. This plant was about 4m tall and covered the river embankment. On the same side of the River, moving downstream, were several stands of *F. japonica* var. *japonica* that on morphological grounds appeared to be the common *F. japonica* var. *japonica* clone. A further stand of *F. japonica* var. *japonica* was present by the weir. Approximately 100 m further away, growing along the path leading to the bridge, were two stands of *F.* x *bohemica.* These plants are shown in Figure 5.10.

#### 5.3.3.2 Leeds area B

In the early 1900's John Wood of Woodville, Kirkstall, Leeds ran the Wood's Hardy Plant Club. Although most of the plant lists associated with this club are both unnumbered and undated, this club was known to have been selling *F. sachalinensis* in July 1902, describing it as "a most majestic and effective plant for the wild garden, lake or pond side" (Wood, 1902). A further undated, unnumbered list from the same club shows *F. sachalinensis*, *F. japonica* var. *japonica* and *F. japonica* var. *compacta* for sale, under the synonyms *Polygonum sachalinense*, *P. sieboldii* and *P. sieboldii compactum* respectively (Bailey, J.P., pers. comm.). These plants growing together in close proximity would have given ample opportunity for both tetraploid and hexaploid hybrids to have arisen, and are believed to be the origin of the Japanese Knotweed *s.l.* plants in Leeds. Although the sex of the *F.*

*sachalinensis* and *F. japonica* var. *compacta* is not given, the *F. sachalinensis* still in existence in Leeds is known to be male-fertile. There is no current evidence of the dwarf variety still existing in Leeds. Whilst the nursery garden is no longer around, its location is shown as a red X in Figure 5.11. About 200 m from the location of the original nursery garden is an abandoned Quarry (Vesper Road Quarry). Both male-fertile and male-sterile stands of *F*. x *bohemica* were found and collected. On the other side of the quarry, about 30 m apart were two further stands.

5.3.3.3 Leeds area C

The final site that plants were collected in Leeds is shown in Figure 5.12, the Woodside Quarry. A sample of *F. japonica* var. *japonica* and an *F*. x *bohemica* of unknown sex were collected here. This site was rumoured to have once had *F. japonica* var. *compacta* but no evidence of this taxon was found.

## 5.4 Materials and Methods

5.4.1. *Plant material*

In a similar manner to the Dolgellau study (chapter 4), at each of the three sites the location of the plant accession was noted, and a voucher specimen was collected and deposited at the University of Leicester herbarium (LTR). Collected material was given a unique P number at time of collection. For the purposes of this thesis a site code has also been allocated to each accession. The first letter of the code indicates the site it was collected from, C for Cirencester, P for Preston and L for Leeds. This is followed by either the letter J for *F. japonica* var. *japonica*, S for *F. sachalinensis* or B for *F*. x *bohemica*. The plants are then numbered consecutively within each site. Five samples of *F*. *japonica* var. *compacta* from locations in England, Scotland and Ireland, have been included in the study. These are given the code FC, and are numbered from 1 to 5.

The parental and hybrid plants were identified on the basis of leaf shape and epidermal trichome characters (Bailey & Conolly, 1991). Generally it is not possible to distinguish the tetraploid from the hexaploid hybrids except by chromosome number (Bailey *et al.*, 1995). When plants were in flower their sex was determined. Table 5.1 lists the thesis code, P number, taxon, location they were collected from, sex, and chromosome number (if known) for all the plants used in this study.

5.4.2. *Methods*

Duplicate DNA extractions were made for each plant, as detailed in chapter 3 materials and methods. ISSR PCR amplification using the seven primers listed in Table 3.2 was carried out and additionally the chloroplast region *trn*C-*trn*D was amplified for each plant, and restricted with the enzyme *Hinf*I, as per chapter 3.

## 5.5 Data analysis

The maternal parent for each hybrid plant was determined by the visualisation of the *Hinf*I restriction digest products of the chloroplast region *trn*C-*trn*D, and comparison against the parental types, shown in Figure 5.13.

ISSR bands were selected, scored and named as per chapter 3, section 3.2.7.2. The *F. sachalinensis* plants collected from these sites were male-fertile with the common genotype (S1). *F. sachalinensis* can be the paternal or maternal parent, therefore an example of a wide-spread British male-sterile *F. sachalinensis* genotype (S3) was included in the ISSR analyses.

The ISSR data were bootstrapped 10,000 times, and a Neighbour-Joining tree using the Jaccard's similarity coefficient was produced, as per chapter 4.

For the tetraploid genotypes only, the number of differences between each genotype was calculated and analysed using MINSPNET (Excoffier & Smouse, 1994) to produce a minimum spanning tree.

## 5.6 Results

5.6.1 *Chloroplast haplotypes*

Figure 5.14 is a gel, showing the *Hinf*1 RFLP fingerprint of most of the plants from Cirencester. Table 5.2 gives the chloroplast type that was determined from this gel and others like it, for the three sites and the *F. japonica* var. *compacta* plants. All tetraploids analysed from Cirencester and Leeds had *F. japonica* var. *compacta* with chloroplast type C as their maternal parent, whilst those found in Preston had *F. sachalinensis* with chloroplast type D. The hexaploids from all three sites had *F. japonica* var. *japonica,* chloroplast type A, as would be expected given the lack of an octoploid male-fertile *F. japonica* var. *japonica* in Britain.

5.6.2 *ISSR genotypes*

Including duplicates 64 plants were analysed representing five accessions of *F. japonica* var. *compacta,* five of *F. japonica* var. *japonica*, three of male-fertile *F. sachalinensis*, 29 of tetraploid *F*. x *bohemica,* and 22 of hexaploid *F*. x *bohemica*. Each ISSR primer generates a banding pattern referred to as a phenotype. A unique combination of these phenotypes is assumed to be representative of a genotype. A total of 24 unique genotypes were detected, of which ten were from tetraploid hybrids and eight from hexaploids.

Including the 24 genotypes detected in this study and the additional male-sterile *F. sachalinensis* (S3), a total of 55 ISSR bands were scored, of which 53 were polymorphic. These are shown in Table 5.3. There were four bands in *F. japonica* var. *japonica* that were not present in the other potential parental taxa, but were found in some of the hexaploid hybrids; these are coloured blue. Bands assigned as green were found in one or more *F. japonica* var. *compacta* genotypes. There were seven of these, of which four were also found in tetraploid hybrids whilst the other three were not found in any other taxa in the study. There were twelve bands coloured red which were found in one or both of the *F. sachalinensis* genotypes but not the other potential parents. Of these twelve, nine were found in one or more of the hybrid taxa. There was also five bands coloured pink that were found in some of the tetraploid hybrids but none of the putative parents.

5.6.3 *Distribution of genotypes*

As expected all *F. japonica* var. *japonica* individuals were identical (J). The three stands of male-fertile *F. sachalinensis*, two from Cirencester and one from Leeds, were identical to each other and to the genotype S1 found in Dolgellau, Wales (chapter 4). Of the five samples of *F. japonica* var. *compacta* included in the study, the two individuals from Brecon Rd and Newbridge were genetically identical with chloroplast type C. These are referred to as genotype C2. The sample from Connel Ferry had the same chloroplast type (C), but had a unique genotype (C3). The other two *F. japonica* var. *compacta* individuals from Bracken Hill and Blarney Castle had unique genotypes (C1 and C4) but shared the chloroplast type (B).

The *F*. x *bohemica* genotypes have been split into two categories. Those found in hexaploid individuals have been given the first letter H, whilst those from tetraploid individuals are given the first letter T. The genotype for each plant is given in Table 5.2.

5.6.3.1 Distribution of genotypes in Cirencester

In Cirencester, of the 31 stands of *F*. x *bohemica* sampled, eighteen were hexaploid and the remaining thirteen were tetraploid. The eighteen hexaploid stands were represented by five different genotypes and the thirteen tetraploids by four.

As can be seen in Figure 5.15, the majority of the stands along the A429 are dominated by a single genotype of hexaploid *F*. x *bohemica*. This genotype is unique to this site. The only other genotype present at this site is represented by a single stand of tetraploid *F*. x *bohemica*, genotype T1.

Genotype T1 was only found as a single stand in area A, but it is also found in vast quantities within the Abbey Grounds, area B. As seen in figure 5.16, seven of the stands found along the feeder stream that leads to the lake are all this same genotype of male-sterile tetraploid *F*. x *bohemica*. The only exception to this is a unique genotype of hexaploid *F*. x *bohemica* found furthest up the feeder stream, just before the lake.

The plants found near the stream by the main entrance to the Abbey Gardens are comprised of two stands of a single genotype of male-fertile tetraploid *F*. x *bohemica*, the British clone of *F. japonica* var. *japonica*, a common male-fertile genotype of *F. sachalinensis*, and three stands of hexaploid *F*. x *bohemica*. The hexaploid hybrids are made up of a single stand with a unique genotype and two adjacent stands that share a genotype that is not found anywhere else.

From the area outside the grounds, beyond the Spital Gate, a single unique genotype of hexaploid *F*. x *bohemica* was detected, along with the *F. japonica* var. *japonica* clone.

The male-fertile and male-sterile stands of *F*. x *bohemica* growing along the A435 dual carriageway, Grove Lane, were both tetraploid hybrids with unique genotypes. Judging by the shared genotype, it was the male-sterile stand that was re-sampled during the collection in 1999 (plant CB31).

5.6.3.2 Distribution of genotypes in Preston

In Preston of the 13 stands of *F*. x *bohemica* sampled, ten were tetraploid and only three were hexaploid. The three hexaploid stands were represented by two different genotypes, as were the ten tetraploid stands. The two tetraploid genotypes represent a male-sterile genotype and

a male-fertile genotype. The sex of the hexaploid individuals is not known, although an earlier collection from Preston was male-fertile (Bailey J.P. pers. comm.).

Hexaploid *F*. x *bohemica* are limited to area B in Preston as shown in Figure 5.17. The two stands to the North of the Riversway dual carriageway have different genotypes. One is a unique genotype, whilst the other is shared by the third Preston hexaploid *F*. x *bohemica* stand that is also found in area B, but South of the River Ribble in the car park of the Penwortham golf course.

There were only two genotypes of tetraploid *F*. x *bohemica* detected. These two genotypes appear to be somewhat separated geographically within area A, as shown in Figure 5.17. The male-fertile genotype (T5) is restricted to the area near to the Railway station and the L.C.C building, spreading as far as the gateway into Miller Park. The male-sterile genotype (T6) is found to the east of this area, being near the Tramway Bridge in Avenham Park and the Frenchwood Park. The closest the male-sterile genotype gets to the male-fertile genotype is the large stand found near the disused second railway line. This is found near to both Avenham Park and the L.C.C building.

Both tetraploid stands found along the A6 in area C of Preston were the male-sterile T6 genotype.

5.6.3.3 Distribution of genotypes in Leeds

Seven *F*. x *bohemica* plants were collected in Leeds, six of which were tetraploid. The hexaploid individual had a unique genotype. Of the six tetraploid stands, four genotypes were detected.

The hexaploid individual was the only *F*. x *bohemica* collected from the Woodside Quarry, also referred to as Leeds area C. Tetraploid *F*. x *bohemica* was found in both of the other Leeds areas. As indicated in Figure 5.18, the two stands analysed from area A were of a single genotype that was not found in area B. Four stands of *F*. x *bohemica* were sampled in area B, and these were represented by three different genotypes.

5.6.4 *Neighbour joining tree*

The Neighbour joining tree for all genotypes based on the Jaccard's similarity coefficient, is shown in Figure 5.19. There is clear separation between the different ploidy levels of *F*. x *bohemica*, which is supported when the data are bootstrapped 10,000 times. The hexaploid

hybrids form a cluster with *F. japonica* var. *japonica* with a bootstrap value of 92.6, but there is very little structure within this group. The only grouping within the hexaploid cluster that holds together with a bootstrap value over 50 is that of the two Preston genotypes, but that bootstrap value is only 53.3. The tetraploid hybrids form a group that has a bootstrap value of 59.6, but has far more structure within the cluster. The two genotypes from Preston hold together with a bootstrap value of 99.2. The four genotypes from Cirencester cluster close to each other, although are strongly associated with two of the four Leeds genotypes. The four Leeds genotypes are split between two at one extreme of the cluster and two at the other.

The two *F. sachalinensis* genotypes form a group with a bootstrap value of 96.3. They are positioned closest to the tetraploid *F.* x *bohemica* group. The four *F. japonica* var. *compacta* genotypes form a cluster with a bootstrap value of 74.9. Within this cluster, genotypes C2 and C3 hold together with a value of 67.6. These are the two genotypes that possess chloroplast type C. The other two genotypes have chloroplast type B but do not group with each other any closer than they do with the other two of the different chloroplast type.

### 5.6.5. *Minimum spanning network*

The minimum spanning network, Figure 5.20, shows the number of differences between each of the tetraploid genotypes. Next to the genotype for the *F.* x *bohemica* tetraploids is a symbol to denote the sex. There were more male-sterile genotypes than male-fertile detected. There were only three differences between the male-sterile and male-fertile *F.* x *bohemica* genotypes from Preston, and as with the neighbour joining analysis these group together in a central position within the rest of the tetraploid *F.* x *bohemica* group. There are twelve differences between the Preston genotypes and the next nearest. The Cirencester genotypes form a group at the centre of network, with the two male-fertile genotypes being the closest of the four. The four Leeds genotypes have the highest number of differences between them, with two male-sterile genotypes coming close to the male-fertile *F. sachalinensis* genotype, albeit with a minimum of fourteen differences. The other male-sterile and the male-fertile genotype are closer to the *F. japonica* var. *compacta* genotypes being closest to the genotype C3 which is one of the ones with the chloroplast type B, the other being C1. There are at least 16 differences between these *F.* x *bohemica* genotypes and those of the closest *F. japonica* var. *compacta*.

## 5.7 Discussion

### 5.7.1 *Origin of* Fallopia *x* bohemica *at the different sites*

#### 5.7.1.1. Cirencester

Four different genotypes of tetraploid *F*. x *bohemica* were found in Cirencester, all of which had *F. japonica* var. *compacta* as their maternal parent. The Abbey Grounds, being a maintained garden, is presumed to be the original source of these plants. As seen in Figure 5.16, the tetraploid hybrid plants within the grounds were of two genotypes, a male-fertile clone and a male-sterile clone, found in separate areas. It is likely that these were introduced as garden plants and have subsequently spread by clonal means around the surrounding area. The male-sterile clone is the most common genotype, being found in seven stands along the feeder stream to the lake including the two stands outside of the grounds. This area outside the grounds was once a part of the gardens and the *F*. x *bohemica* was presumably already *in situ* and the houses built on top of the contaminated soil. This common male-sterile genotype was also found as a single stand along the A429, Figure 5.15. This is probably a case of secondary spread through human interference as opposed to planting. The male-fertile genotype is restricted to two adjacent stands near to the main entrance of the Abbey grounds. The limited number of genotypes of tetraploid *F*. x *bohemica* and the lack of an *F. japonica* var. *compacta* implies that the tetraploid hybrids from the Abbey grounds were introduced into the gardens from a nursery as opposed to having arisen *in situ*.

The other two tetraploid *F*. x *bohemica* genotypes were those found along the A435 dual carriageway, Grove Lane. These genotypes were found as a mixed stand of both sexes. Whether these plants were planted is difficult to say. That they are different genotypes from those found within the grounds could imply they were not planted by the same gardener, and also that they were not the result of material from within the grounds being thrown out. That they do appear to cluster closely with the other two Cirencester genotypes, on both the neighbour joining analysis (Figure 5.19) and the minimum spanning network (Figure 5.20), could mean they are F2 progeny of the original two planted genotypes, or it could mean they are siblings produced from the same cross at the nursery gardens.

As has already been mentioned, the Cirencester Abbey Grounds is a maintained garden. As a result of this each year the Japanese Knotweed plants are cut back, often before they have a chance to flower. This could explain the limited number of genotypes within the grounds, as new variation through seed germination would be prevented. The specimens along the A435 however are less well maintained. Given that both sexes are present and the plants are not cut

back, tetraploid F2 seed is much more likely to arise from these plants. In fact seed has been found on these plants.

The presence of both male-fertile *F. sachalinensis* (genotype S1) and male-fertile tetraploid *F.* x *bohemica* (genotype T2) in close proximity to the *F. japonica* var. *japonica* could mean that the hexaploids, assuming they arose *in situ*, could be either true F1 hybrids or a hybrid with genetic material from all three parental taxa, as shown in Figure 5.21. Table 5.4 shows the putative parental distribution of ISSR bands from the five hexaploid genotypes found in Cirencester. As can be seen there are 7 bands that have not been included as they were present in all three putative parental taxa. The majority of bands in all of the hexaploid hybrids appear to have come from *F. japonica* var. *japonica*; unfortunately, as both hybridisation events lead to approximately 67% *F. japonica* var. *japonica,* this does not help elucidate the male parent. No bands were attributable exclusively to the putative tetraploid *F.* x *bohemica* parent. Only one band in two of the hexaploids could be attributed to *F. sachalinensis*, which again is unhelpful given that *F. sachalinensis* would contribute either 16.5% or 33% of the chromosomes of the hybrid depending on the cross. For this type of analysis to indicate the male parent further molecular markers would need to be used, specific to the two male parents. Figure 5.19 shows the Cirencester genotypes forming a group immediately around *F. japonica* var. *japonica* but not significantly separated from the hexaploid hybrids from the two other sites according to the bootstrap analysis.

The high number of hexaploid genotypes in Cirencester, and the presence of both parental taxa, means that it is quite likely that at least some of these hexaploids have arisen *in situ*. The genotype found along the A429, Figure 5.15, was not found within the Abbey grounds and where it originated is not clear. Its current distribution in discrete stands along a long stretch of the road is quite likely a result of road works having gone through a stand that was possibly discarded from a garden. The road works would create the disturbed land for the rhizomes to establish. The other four genotypes of hexaploid hybrid are all shown in Figure 5.16. They tend to be found as single-stands, the exception being genotype H4, which is found as two adjacent stands. The presence of the male-fertile stand of *F. sachalinensis* at the roundabout away from the grounds, but still within pollinating distance of the *F. japonica* var. *japonica* plants, could be the source of pollen if in fact the *F. sachalinensis* and male-fertile tetraploid *F.* x *bohemica* are indeed cut back every year. There is no information about how often these plants are cut back, only personal observation that in 1999 this was the case within the grounds of the old Abbey.

5.7.1.2. Preston

The majority of the *F.* x *bohemica* plants examined in Preston were tetraploids, hexaploids being restricted to three stands in area B. As indicated in Figure 5.17, these three stands are comprised of two different genotypes of unknown sex. Their presence by the Riversway dual carriageway is more likely a result of tipping and spread through road works than planting. Likewise the stand in the car park of the Penwortham golf course is presumably a result of secondary movement by man from either the stand on the Riversway or the site that these Riversway plants originated. The lack of *F. sachalinensis* from these sites in Preston implies the hexaploid hybrids would not have arisen *in situ*.

There were only two genotypes of tetraploid *F.* x *bohemica* detected in Preston, both of which had *F. sachalinensis* as the maternal parent. These plants were much taller than those found in Cirencester and Leeds, growing up to 4m tall as shown in Figure 5.1. This could be a result of having the giant Knotweed as the maternal parent, as opposed to the paternal parent as was the case in Cirencester and Leeds. Alternatively it may have found a more successful niche, although this is a less likely explanation.

As with Cirencester, there is no evidence of the *F. japonica* var. *compacta* parent. Additionally no *F. sachalinensis* was found so it is highly likely these hybrids arose in a nursery gardens and were then introduced into Preston, presumably not the same nursery as the other northern England site Leeds, given that in Leeds the maternal parent was *F. japonica* var. *compacta*. The two genotypes represent a male-fertile and a male-sterile genotype. Interestingly there is very little difference between the ISSR banding patterns of the two genotypes. They differ from each other by a single band with three of the seven ISSR primers, primers AAG-1, 855 and 881. Whether this level of similarity would cause problems with the detection of F2s is unclear without the production and analysis of controlled crosses. They have shared unique bands that were not found in any of the other tetraploid hybrids or in the putative parental taxa.

The genotype T5 was found only in Area A. It is restricted to the area close to the Railway station and therefore the site of the original Railway Hotel, the grounds of which it is presumed the plants were originally introduced into. Of the five stands sampled that were found to have this genotype, only one was in flower and it was male-fertile. Three of these stands were found near to the site of the original *F.* x *bohemica* stand, from which the tetraploid seed that inspired the further collection of plants from Preston were obtained. This original stand has been weed killed since the seed was collected. It was thought that these

stands might have been a continuation of that original stand. Given that the original stand was male-sterile and these plants have the male-fertile genotype, this is unlikely. The fact that two seedlings were also found in this area would indicate that there was a male-sterile stand in the vicinity, but that it was not sampled in this study.

The genotype T6 is male-sterile and has the widest geographical spread. As shown in Figure 5.17, three stands were found in Area A, and a further two either side of the A6 in area C. The large male-sterile stand occupying about 2,250 $m^2$ was of this genotype. At the time of collection these were setting a high number of seeds, presumed to be F2. Although there is very little genetic variation within the Preston tetraploid *F*. x *bohemica* population, the presence of both sexes of hybrid and the high production of seed means that this situation may change in the near future. Given that these stands appear to be successful invaders this situation could be problematic for those trying to control this weed that is already highly successful through clonal means.

5.7.1.3 Leeds

In Leeds the majority of *F*. x *bohemica* plants were tetraploid, as was the case in Preston. Only a single stand of hexaploid *F*. x *bohemica* with a unique genotype was detected. This was found in the Woodside Quarry, area C on Figure 5.18. This location means it is highly likely to be a result of the tipping of contaminated soil.

The tetraploids have *F. japonica* var. *compacta* as the maternal parent, as did those in Cirencester, but there were no common genotypes between the two different Knotweed populations. As shown in Table 5.3, there are bands that are unique to the tetraploid hybrids that are found in some of the Leeds and Cirencester hybrids but not in the Preston samples. For example, the bands sized 1309 and 1076 with ISSR primer 864. This could imply a shared parentage for at least one of the parents involved in the hybridisation; however, as some of the plants from the same location lack these bands it could be a matter of chance that the two from Preston are lacking them also.

The Japanese Knotweed *s.l.* plants found in the Kirkstall Abbey grounds, area A on Figure 5.18, are believed to have been planted. Both male-fertile *F. sachalinensis* and *F. japonica* var. *japonica* were found but as yet no hexaploid *F*. x *bohemica* stands. How often the Abbey grounds gardeners cut these plants back is unclear, but may be a reason for the lack of the hexaploid hybrids in the area.

The tetraploid hybrids in area A are restricted to two adjacent stands of the same male-sterile genotype. This genotype is unique to these stands. As has already been stated, it is believed that these were most likely planted here. Of more interest is area B as this is very close to the original site of the Wood's Hardy Plant Club, where the hybridisation is believed to have occurred. Four different tetraploid *F*. x *bohemica* stands from the abandoned Vesper Road Quarry were analysed. Of these four stands three genotypes were detected, two male-sterile and a male-fertile. As shown by the minimum spanning network in Figure 5.20, two of these genotypes, T9 and T10, are genetically distant from T8 and the fourth Leeds tetraploid from area A, T7. This high level of variation in Leeds is more than that of either Cirencester or Preston. There is also less evidence of clonal spread in Leeds than at the other two sites. If indeed the tetraploid hybrids arose in the vicinity this could explain the diversity, and if the Quarry is no longer used then one assumes the disturbance is minimal, hence the lack of clonal spread. In fact the tetraploids may have arrived in the abandoned Quarry as a result of tipping from the old Wood's Hardy Plant Club nursery, but this would be hard to prove without material from the original Club.

### 5.7.2 *Importance of tetraploid* Fallopia *x* bohemica

Tetraploid *F*. x *bohemica* is relatively rare in Britain compared to the hexaploid hybrid which is gaining in notoriety. However, given the difference in fertility between the two ploidy levels of *F*. x *bohemica,* the tetraploid should not be dismissed as unimportant in terms of the further evolution of this invasive group of plants.

As has been shown by both Hollingsworth *et al*. (1999) and this study, hybridisation between *F. japonica* var. *compacta* and *F. sachalinensis* can occur in both directions. The hybrids appear to be fully capable of hybridisation and seed production, and seedlings presumably of this F2 constitution were found growing in Preston showing that germination in the British climate is possible. Whether these seedlings would establish is not known, but is indeed plausible. Ten different genotypes of established tetraploid *F*. x *bohemica* were detected by this study and there are other known tetraploids in Britain that have not yet been analysed. This implies that the hybridisation event is not a rare occurrence. The limiting factor to the number of such hybrids is more likely a result of the scarcity of the *F. japonica* var. *compacta* parent, and the careful management of the plants found in maintained gardens such as the Cirencester and Kirkstall Abbey grounds.

The tetraploid hybrid is believed to be fully fertile (Bailey J.P. pers. comm.) as opposed to the hexaploid hybrid, which tends to produce aneuploid offspring. The traditional view of

hybridisation between different species is that the F1 progeny will be less fit in terms of fertility than either of its parents, often as a result of the production of sterile or semi-sterile F1 hybrids. However, there are many hybrids that are fully fertile. Even in those instances where F1s are partially sterile, full fertility is often achieved in later hybrid generations. In studies where a fitness parameter has been reported, there are studies where the hybrids can be more fit, less fit, or equivalent in fitness to both parents (Rieseberg, 1995). The hybrid *F.* x *bohemica* appears to be one of the examples where the hybrid is more fit. In the case of the hexaploid hybrid there is already evidence that it may be more invasive than either of its parents even if it is only partially fertile (Bímová *et al.*, 2001; Brabec & Pyšek, 2000). As for the tetraploid *F.* x *bohemica,* it appears to be fully fertile and potentially more invasive.

In Cirencester, in a highly managed garden in the grounds of the old Abbey, the tetraploid hybrid did not appear to be that invasive. It was much shorter in stature than the stand in Preston that was about 4m tall, and occupied less ground space than the hexaploid hybrid (Bailey *et al.*, 1996). In both Preston and Leeds however tetraploid *F.* x *bohemica* is in the majority, even though some of the other Knotweed taxa are present. In Preston the two genotypes occupy numerous stands and the stands occupy a large ground area. They are also taller than those found at the other sites and it is interesting to speculate whether having *F. sachalinensis* as the maternal parent gives an advantage in terms of invasive ability to those plants compared to those with *F. japonica* var. *compacta* as the maternal parent. *F. sachalinensis* is considered to be an invasive taxon, albeit less so than the *F. japonica* var. *japonica* or hexaploid *F.* x *bohemica,* whilst *F. japonica* var. *compacta* is not (Bailey, 2003). In Leeds the stands are shorter than those in Preston and cover less ground space, but they do still appear to be fully established where they are found and more invasive than those at Cirencester. The high level of genetic diversity among the plants at Leeds could give scope for a more invasive genotype to evolve.

The production of a successful invader, capable of sexual reproduction, is of great concern to the many people who are struggling to control the highly invasive relative *F. japonica* var. *japonica* that invades so well by clonal means alone. To fully establish the risk factor caused by these tetraploid *F.* x *bohemica* plants, better estimates of the fertility of these hybrids should be conducted. It would also be interesting to compare the invasive ability of the hybrids that have *F. sachalinensis* as the maternal parent with those that have *F. japonica* var. *compacta*. The analysis of the other known British tetraploid hybrids not included in this study would also be of interest. They were not included because there is only known to be a single sex present at the various locations and therefore no immediate risk of further evolution

through hybridisation. However, if the analyses showed these other hybrids to be of the same genotype as each other or some of the genotypes in this study, it may help to determine where these hybrids arose. If they are different it gives further strength to the concern that their production is not an isolated event, and that these hybrids are cause for concern from an invasive point of view.

### 5.8 Conclusions

This study has shown that hybridisation between *F. sachalinensis* and *F. japonica* can occur in both directions, with the plants at Preston having *F. sachalinensis* as the maternal parent, and those at Cirencester and Leeds having *F. japonica* var. *compacta*. Ten different genotypes of tetraploid *F.* x *bohemica* were detected, with no shared genotypes between the different sites. A total of nine genotypes of hexaploid *F.* x *bohemica* were also found at these three sites, with no genotypes shared between the sites and no genotypes shared with those found at Dolgellau (chapter 4). The distribution of tetraploid hybrids at Cirencester and Preston is more a product of the clonal spread of a few genotypes rather than a result of sexual reproduction. At Leeds however there was little evidence of clonal spread but a high level of genetic variation between the different genotypes.

**Figure 5.1** Dr John Bailey and Mr Peter Jepson standing in a large male-sterile tetraploid *F*. x *bohemica* stand in Preston, W. Lancashire. The plant grew up to 4m tall and occupied about 2,250 $m^2$ of land.

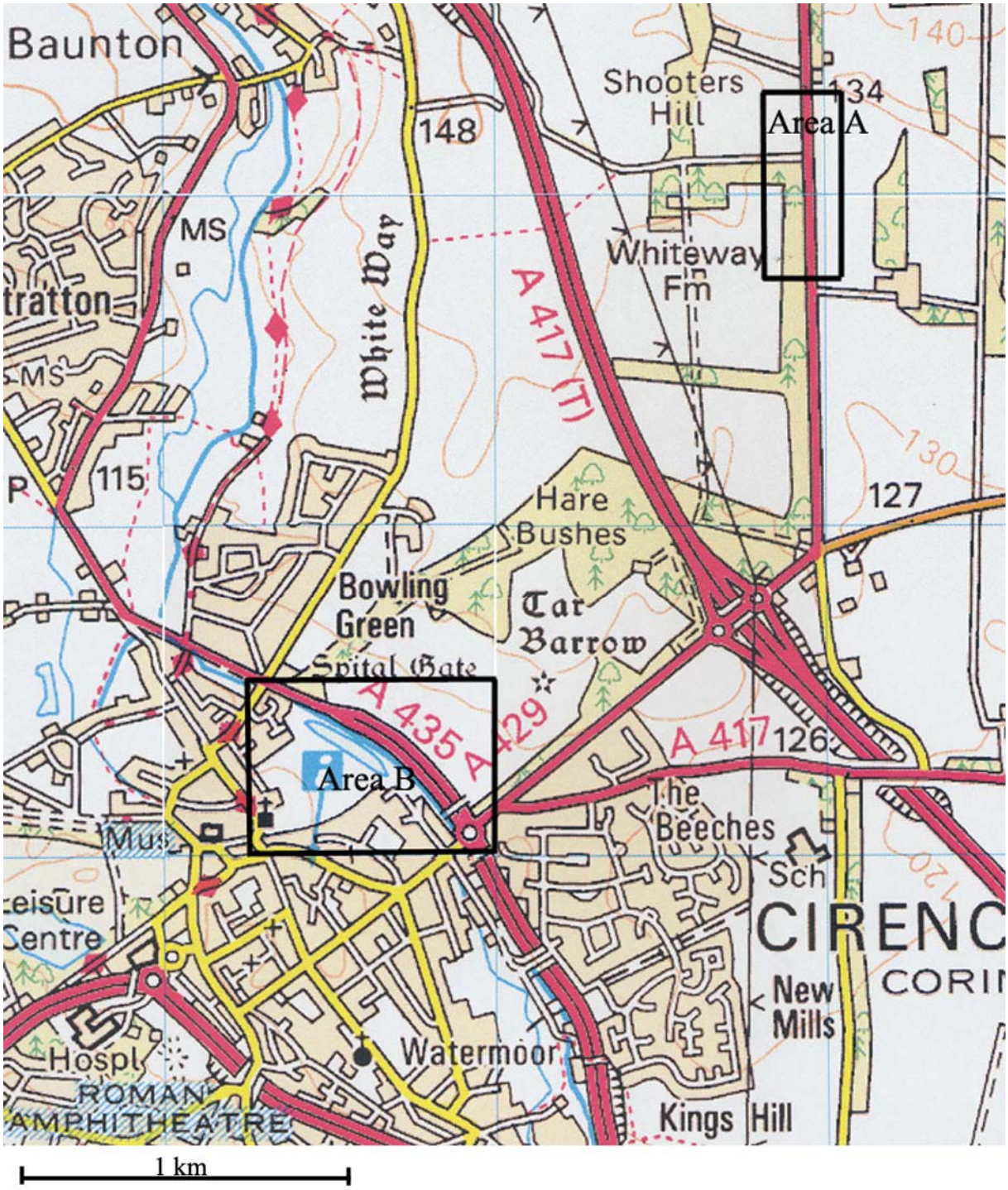



**Figure 5.2** Map of Cirencester, taken from the Ordnance Survey map, showing the two areas where plants were collected for analysis.

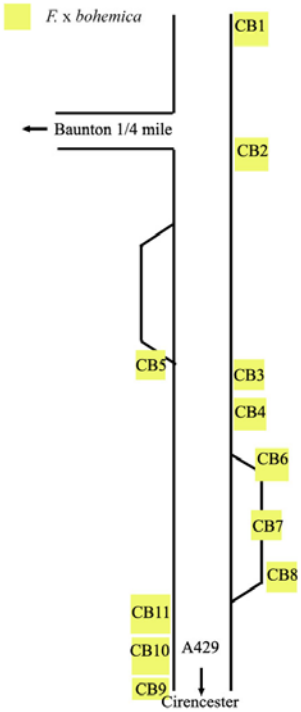



**Figure 5.3** Cirencester area A. Diagrammatic representation of the *F*. x *bohemica* plants growing along the A429, not drawn to scale. The first letter of the code indicates that the plant was collected from Cirencester. This is followed by the letter B for *F*. x *bohemica*.

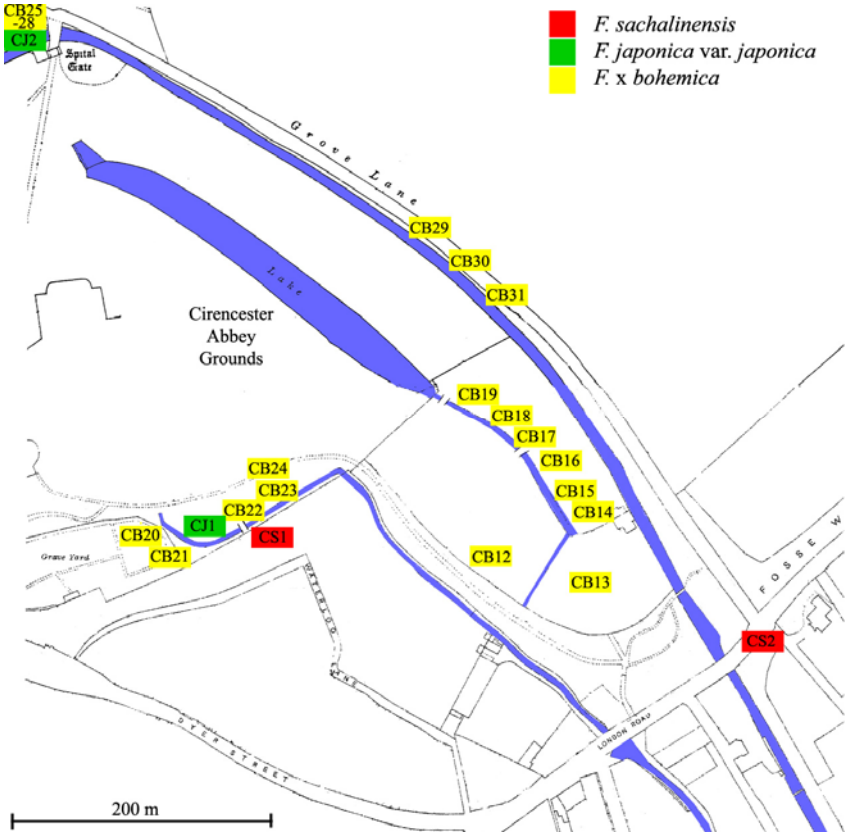



**Figure 5.4** Cirencester area B. Diagrammatic representation of the area in and around the Cirencester Abbey grounds, showing the positions the plants were collected. The first letter of the code indicates that the plant was collected from Cirencester. This is followed by either the letter J for *F. japonica* var. *japonica*, S for *F. sachalinensis* or B for *F*. x *bohemica*.

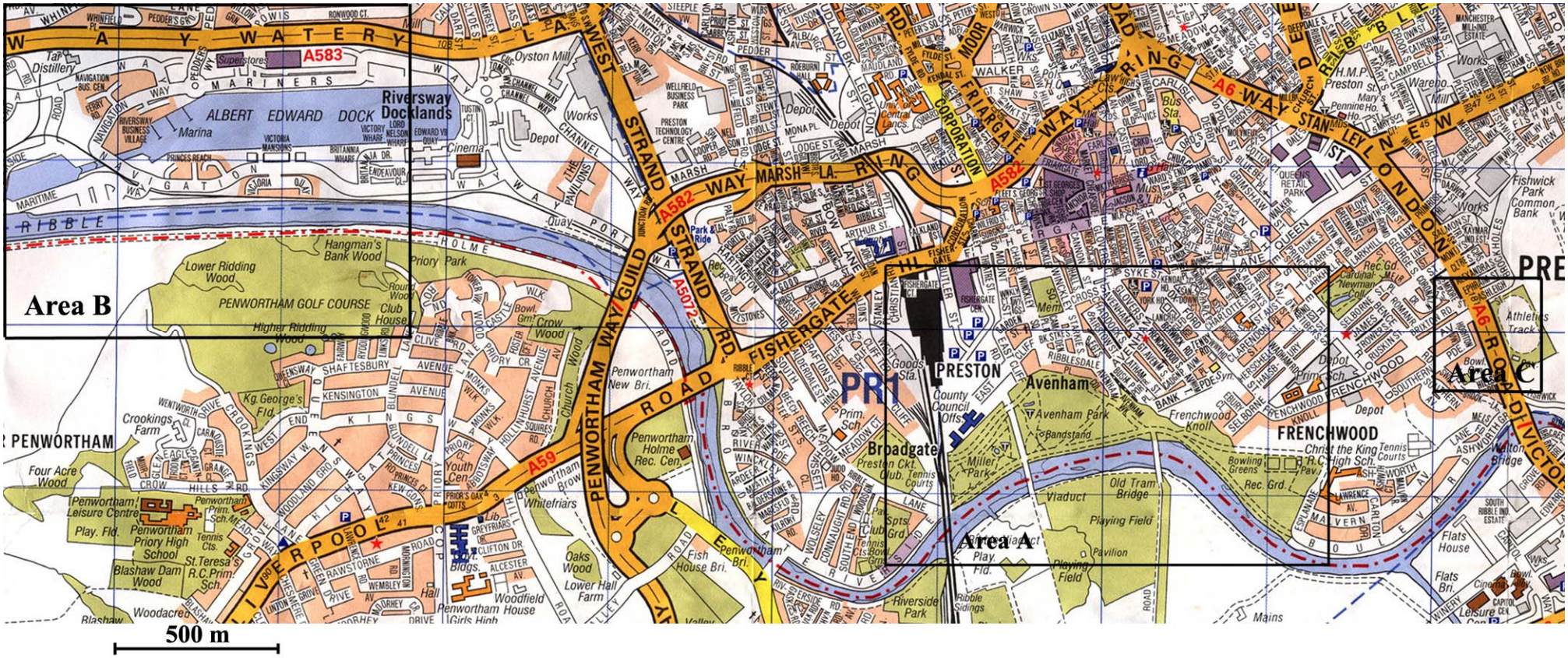



**Figure 5.5** Map taken from the A-Z of Preston (Edition 2, 2000) indicating the three main areas where plants were collected.

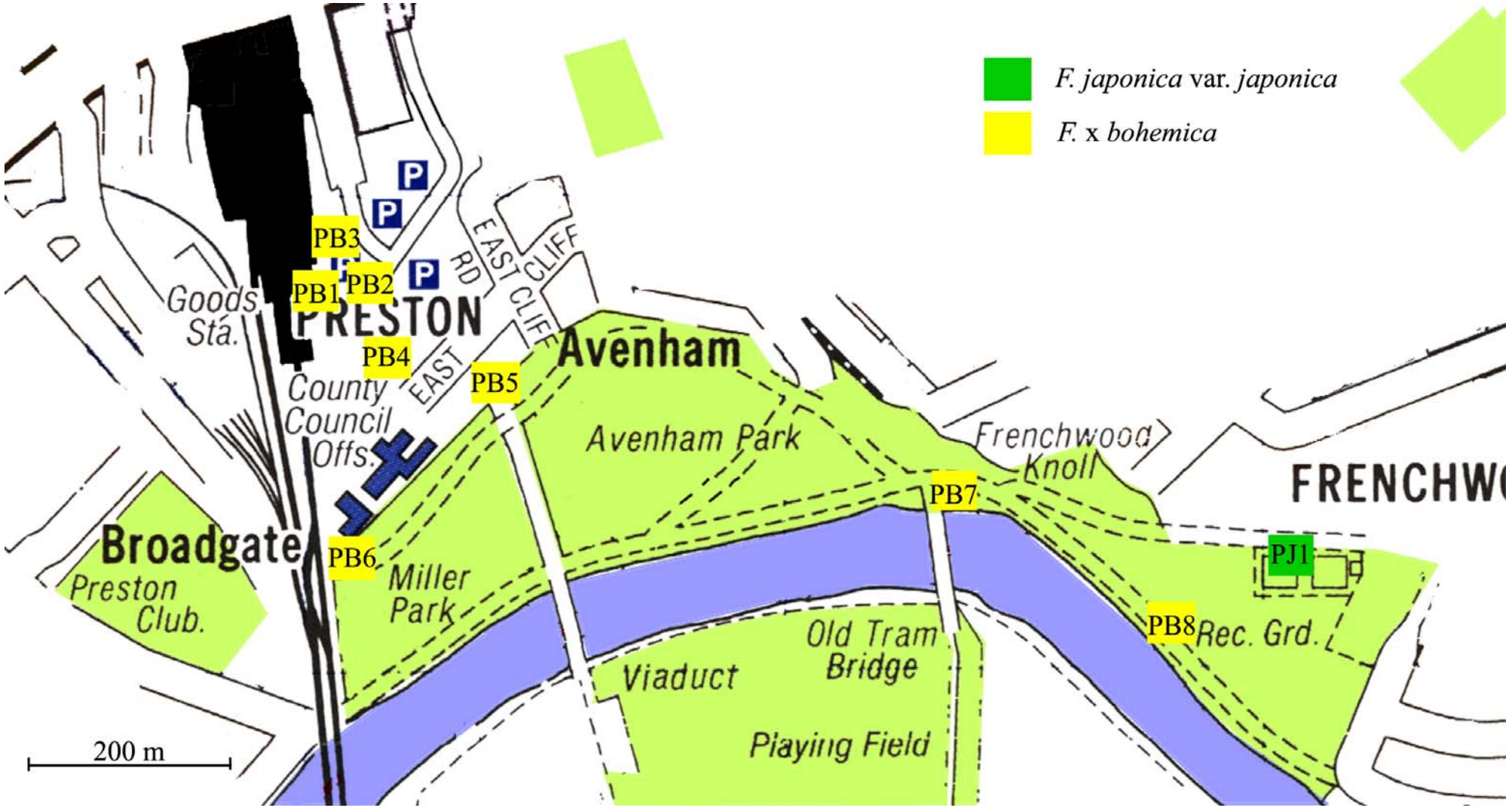



**Figure 5.6** Preston area A, showing positions plants were collected from. The first letter of the code indicates that the plant was from Preston. This is followed by either the letter J for *F. japonica* var. *japonica* or B for *F.* x *bohemica*.

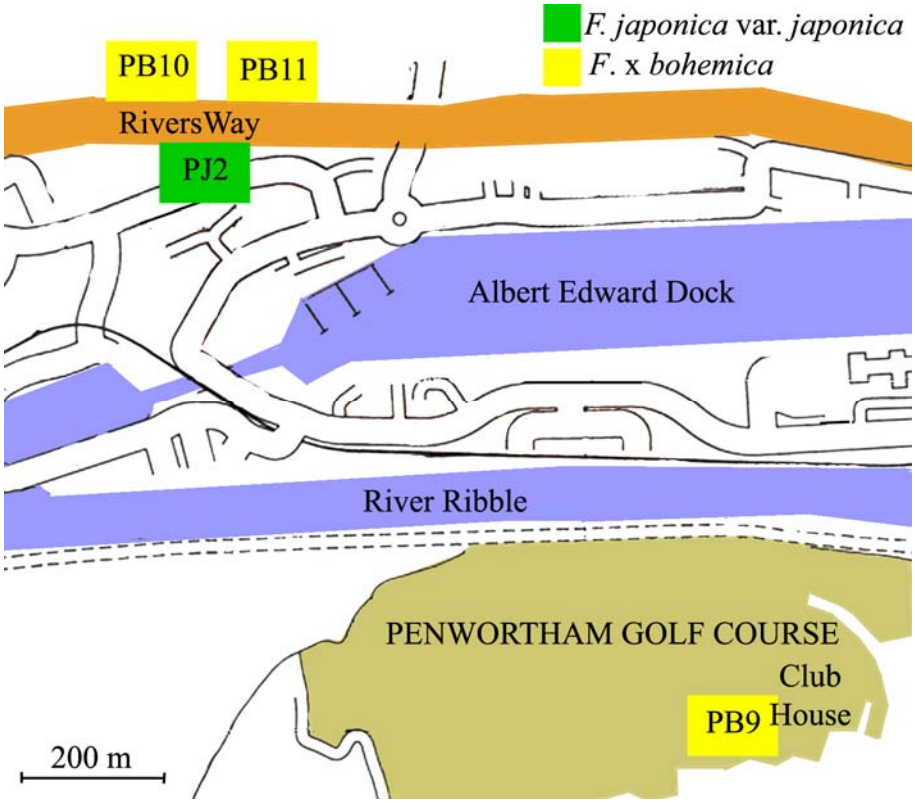



**Figure 5.7** Preston area B. Location of plants collected along the Riversway Dual carriageway and on the Penwortham Golf Course. The first letter of the code indicates that the plant was from Preston. This is followed by either the letter J for *F. japonica* var. *japonica* or B for *F*. x *bohemica*.

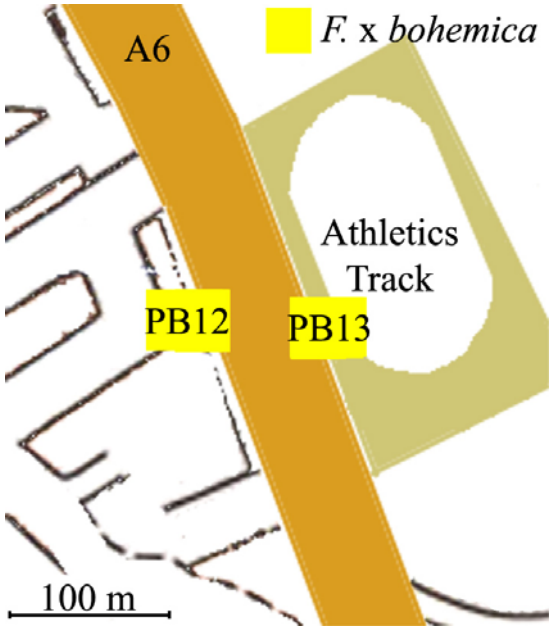



**Figure 5.8** Preston area C, showing the location of the two plants growing along the A6. Plants coded P for Preston and B for *F*. x *bohemica*.

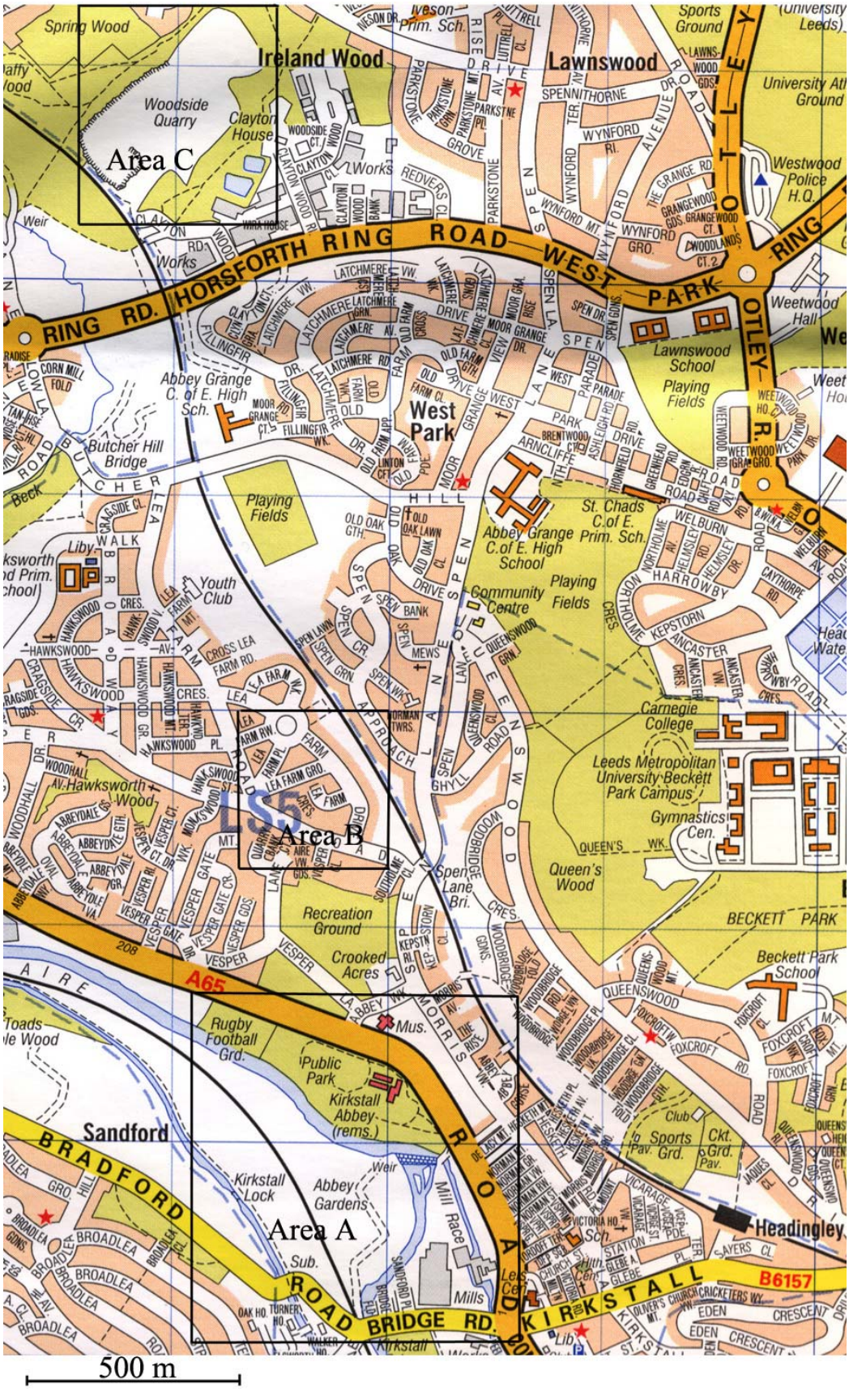



**Figure 5.9** Map of Leeds taken from the A-Z of Leeds (1999) showing the location of the three sites from where plants were collected for analysis.

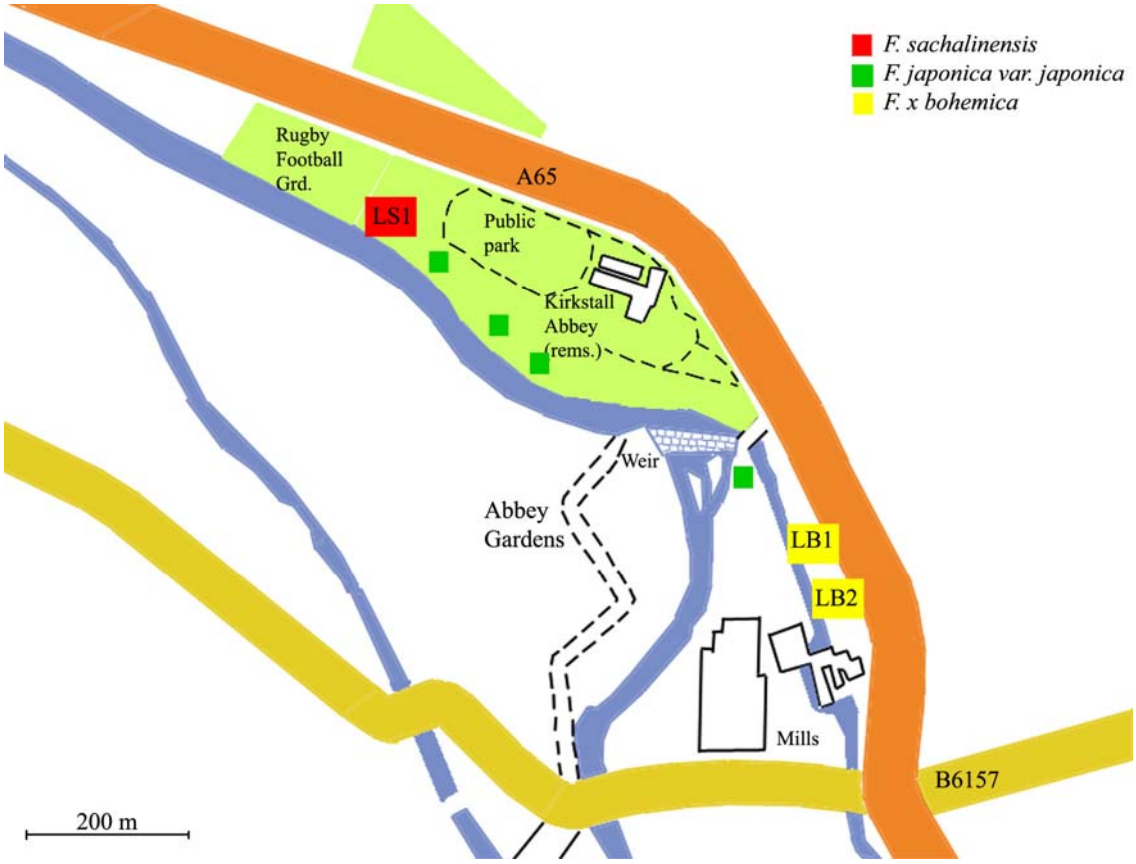



**Figure 5.10** Leeds area A, Kirkstall Abbey grounds showing the location of the plants collected.

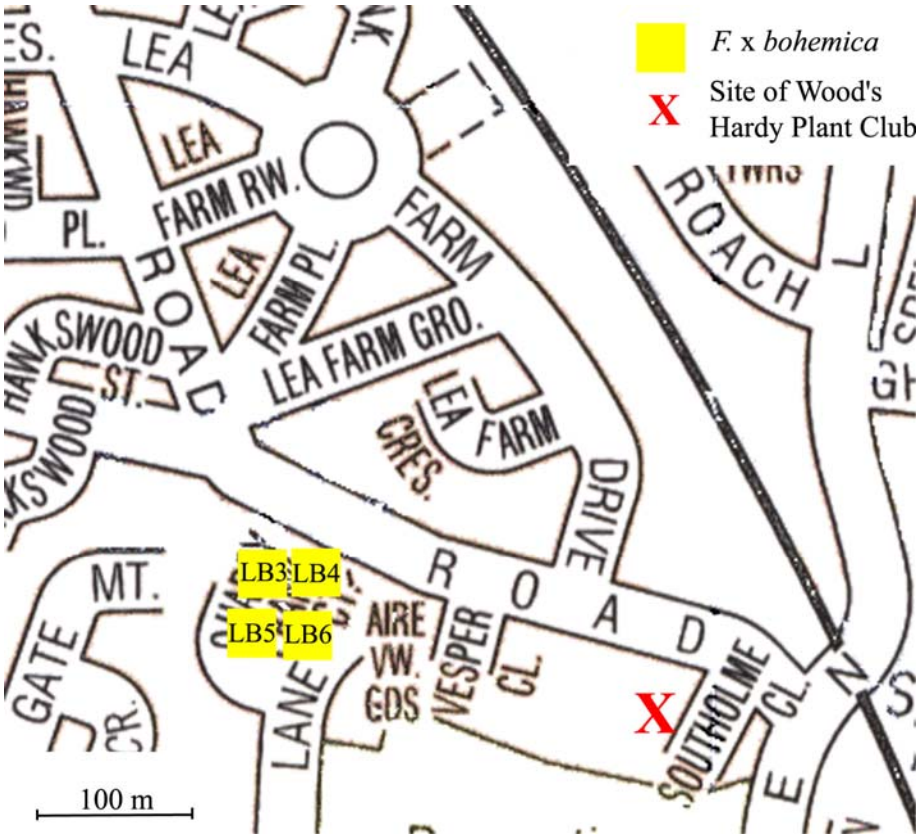



**Figure 5.11** Leeds area B, showing the location of the original Wood's Hardy Plant club Nursery (X) and the location of the four *F.* x *bohemica* plants collected from the abandoned Vesper Road Quarry.

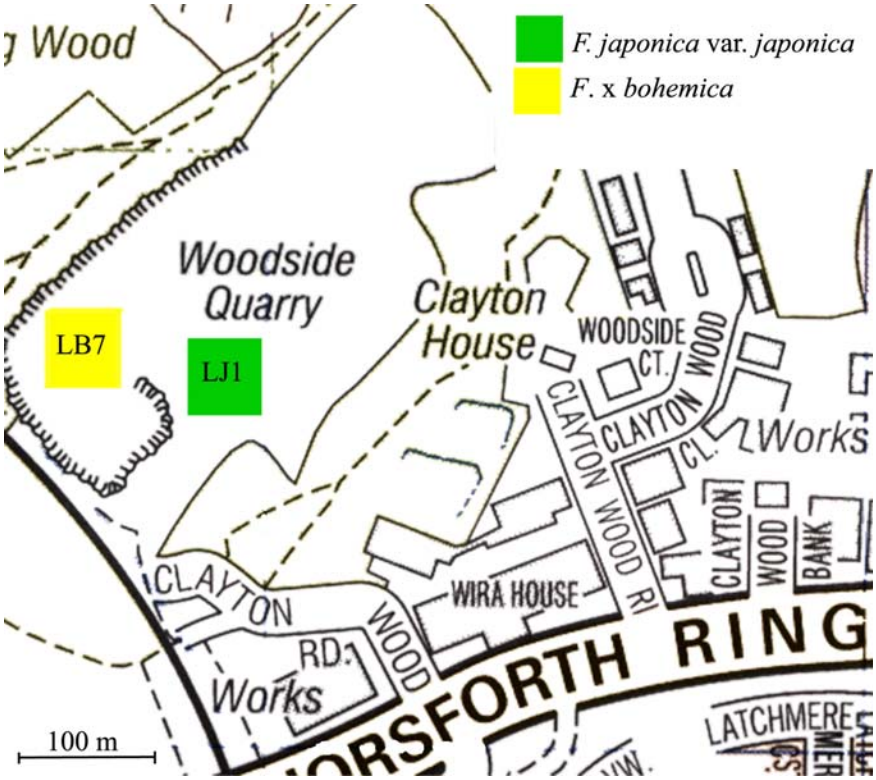



**Figure 5.12** Leeds area C, showing plants collected from the Woodside Quarry.

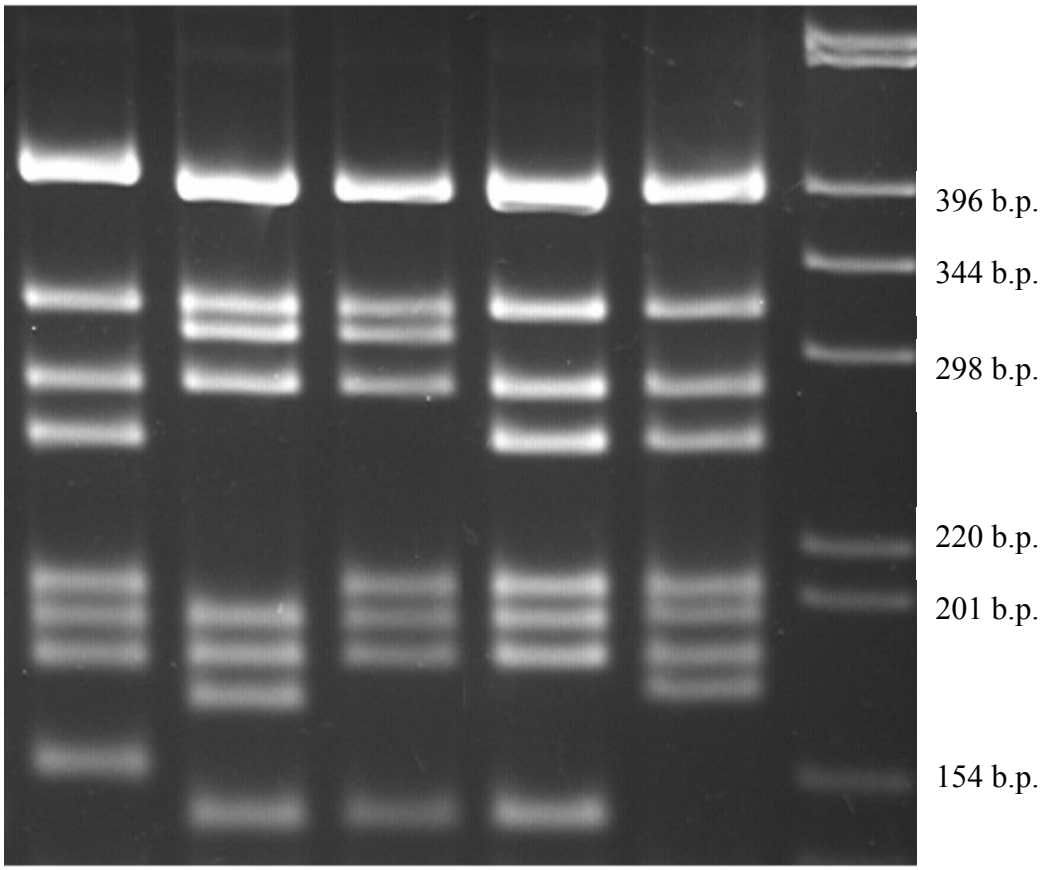



**Figure 5.13** Gel showing the five chloroplast haplotypes detected within Japanese Knotweed *s.l.* in Britain. From left to right: lane 1, type A - *F. japonica* var. *japonica*; lane 2, type B - *F. japonica* var. *compacta*; lane 3, type C - *F. japonica* var. *compacta*; lane 4, type D - *F. sachalinensis;* lane 5, type E - *F. sachalinensis*; lane 6, 1kb ladder.

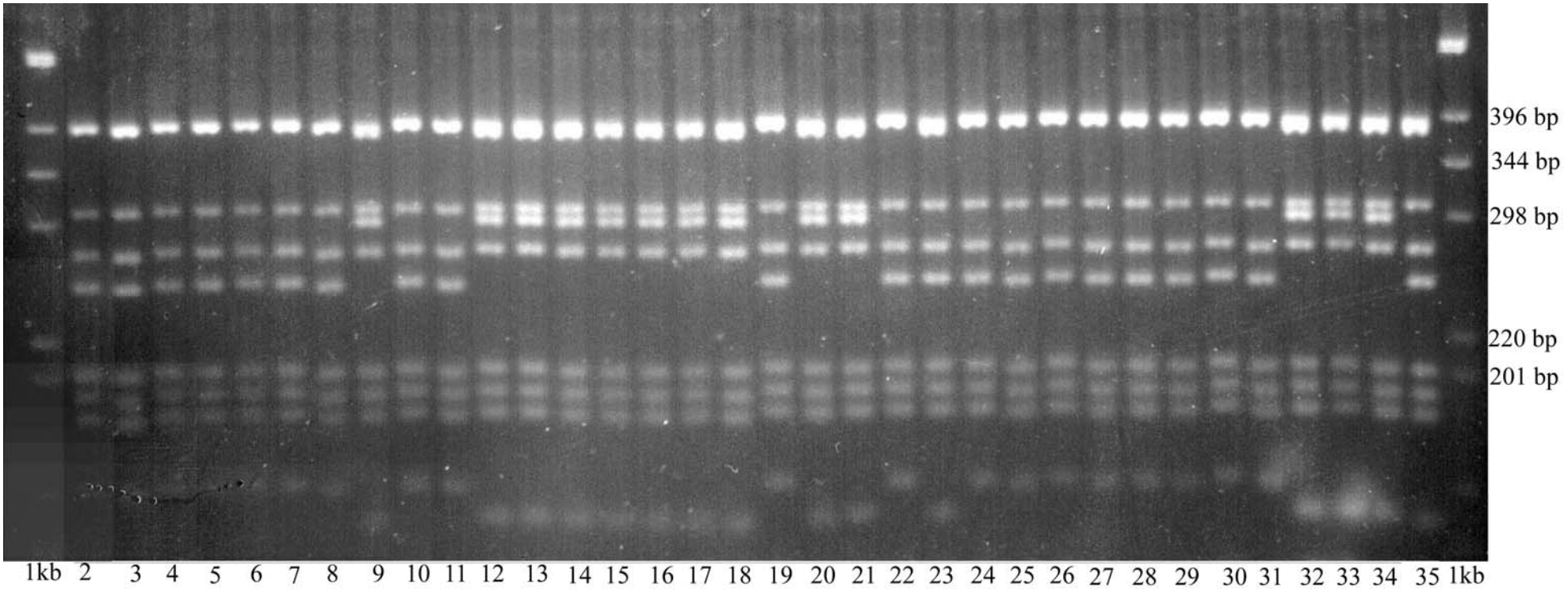



**Figure 5.14** Gel showing chloroplast (Cp) haplotypes for most of the plants from Cirencester. Lanes from left to right: 1, 1kb ladder; 2, CB1 (Cp type A); 3, CB2 (A); 4, CB3 (A); 5, CB4 (A); 6, CB5 (A); 7, CB6 (A); 8, CB7 (A); 9, CB8 (Cp type C); 10, CB9 (A); 11, CB11 (A); 12, CB12 (C); 13, CB13 (C); 14, CB14 (C); 15, CB15 (C); 16, CB16 (C); 17, CB17 (C); 18, CB18 (C); 19, CB19 (A); 20, CB20 (C); 21, CB21 (C); 22, CJ1 (A); 23, CS1 (Cp type B); 24, CB22 (A); 25, CB23 (A); 26, CB24 (A); 27, CB25 (A); 28, CB26 (A); 29, CB27 (A); 30, CB28 (A); 31, CJ2 (A); 32, CB29 (C); 33, CB30 (C); 34, CB31 (C); 35, CS2 (B); 36, 1kb ladder. Sizes of the 1kb ladder fragments are written to the right of the gel.

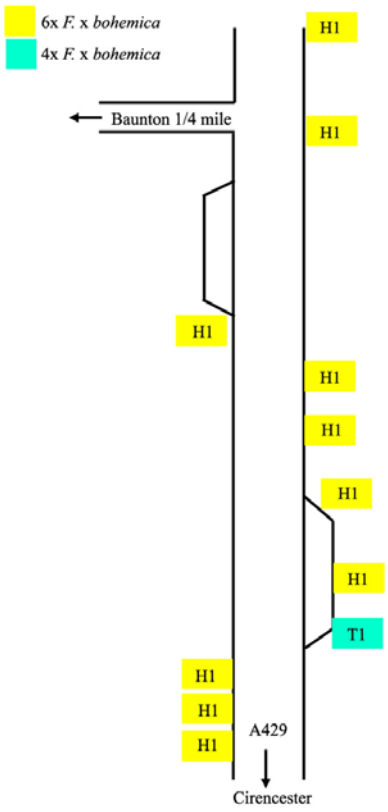



**Figure 5.15** Cirencester area A, showing the distribution of the genotypes of *F*. x *bohemica*. H stands for hexaploid *F*. x *bohemica* and T for tetraploid. The number specifies the genotype.

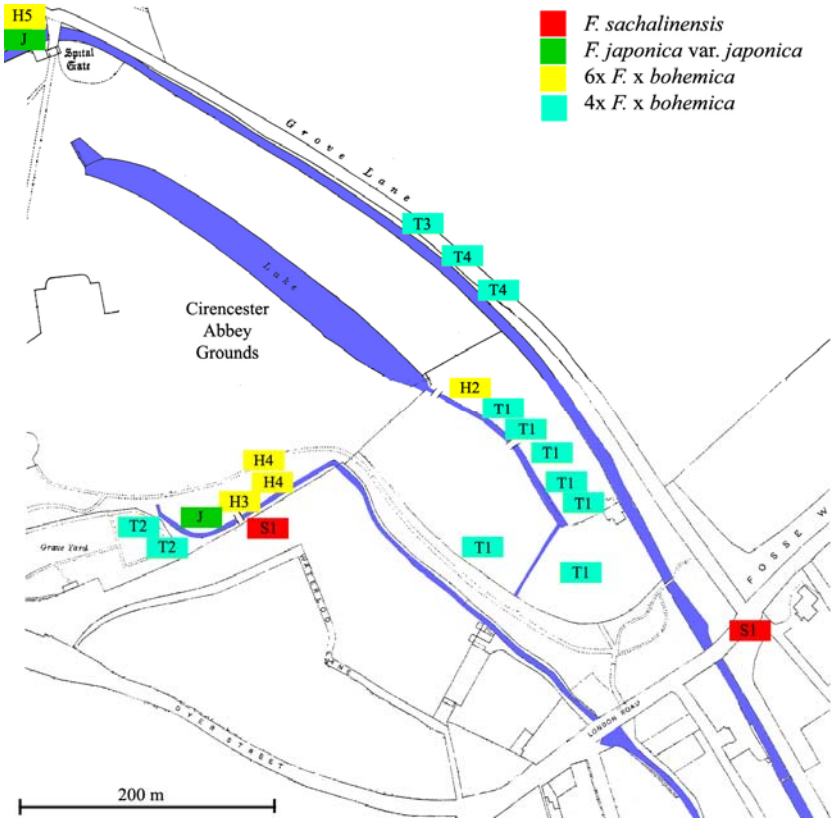



**Figure 5.16** Cirencester area B, showing the distribution of the various genotypes of Japanese Knotweed *s.l.* S stands for an *F. sachalinensis*, J for *F. japonica* var. *japonica*, H for hexaploid *F*. x *bohemica* and T for tetraploid *F*. x bohemica. The number specifies the genotype.

Area A


Area B

Area C

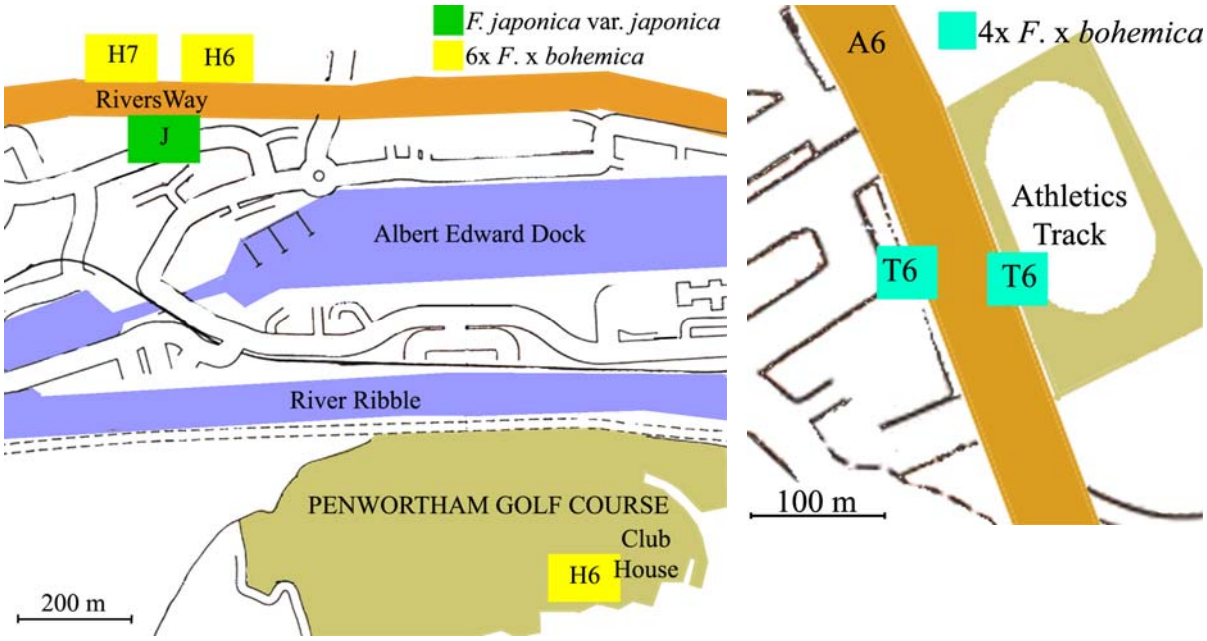
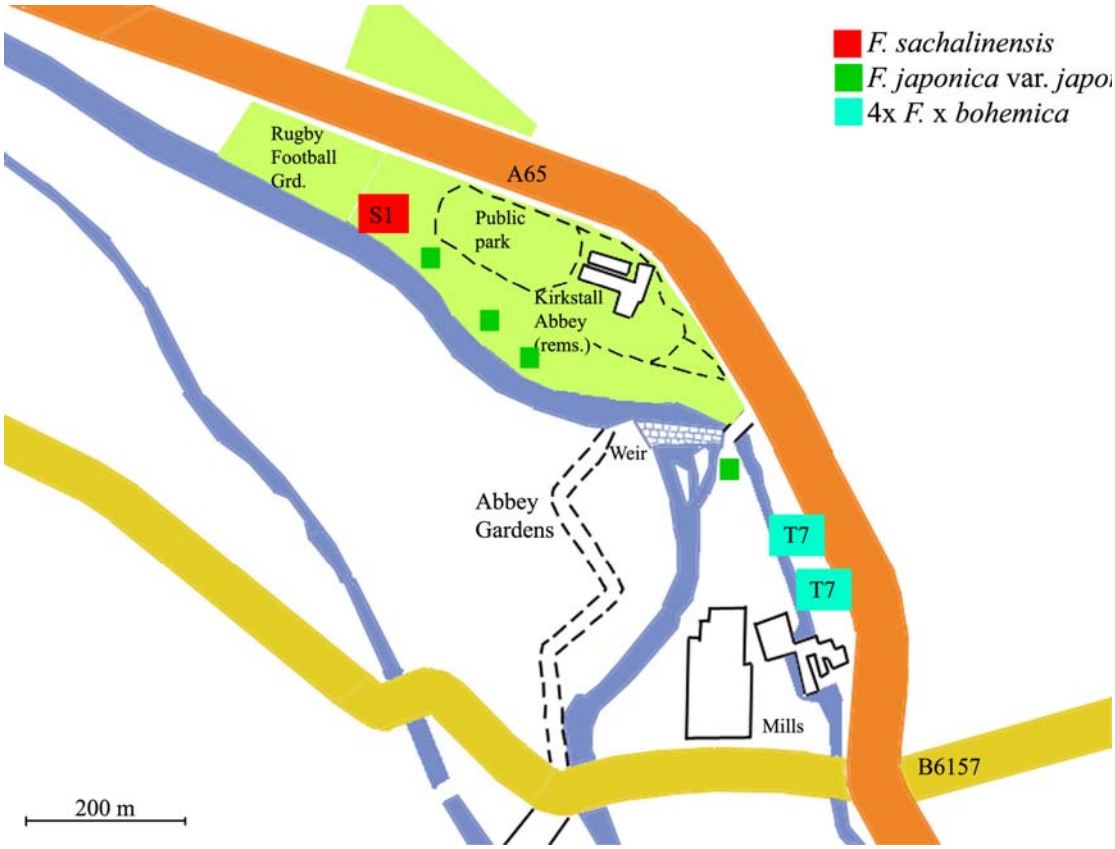
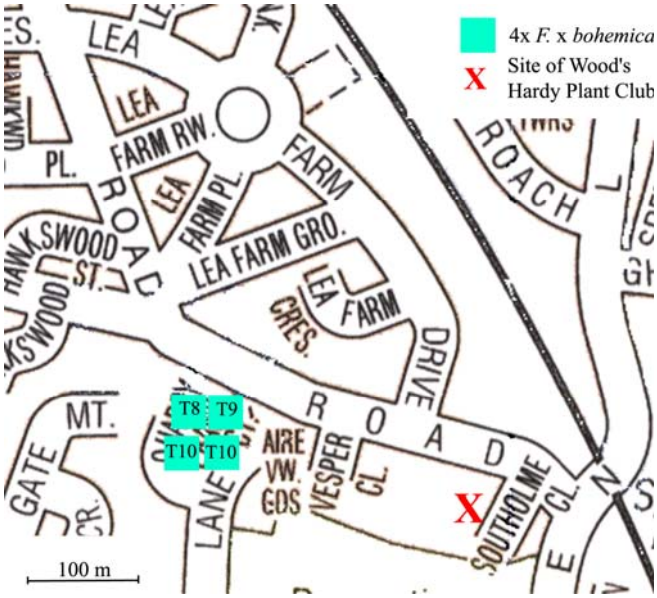



**Figure 5.17** Distribution of the *F*. x *bohemica* genotypes in areas A, B and C of Preston. T refers to tetraploid *F*. x *bohemica* genotypes, H to hexaploid. J represents analysed *F. japonica* var. *japonica*. The number specifies the genotype.

Area A




Area B




Area C

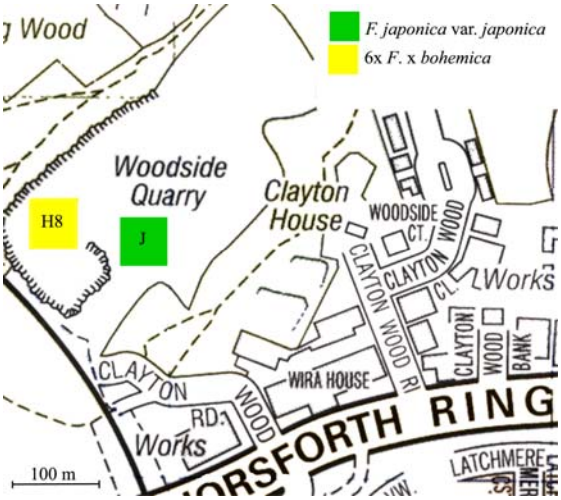



**Figure 5.18** Distribution of the *F.* x *bohemica* genotypes in areas A, B and C of Leeds. T refers to tetraploid *F.* x *bohemica* genotypes, H to hexaploid. J represents analysed *F. japonica* var. *japonica*, and S *F. sachalinensis*. The number specifies the genotype.

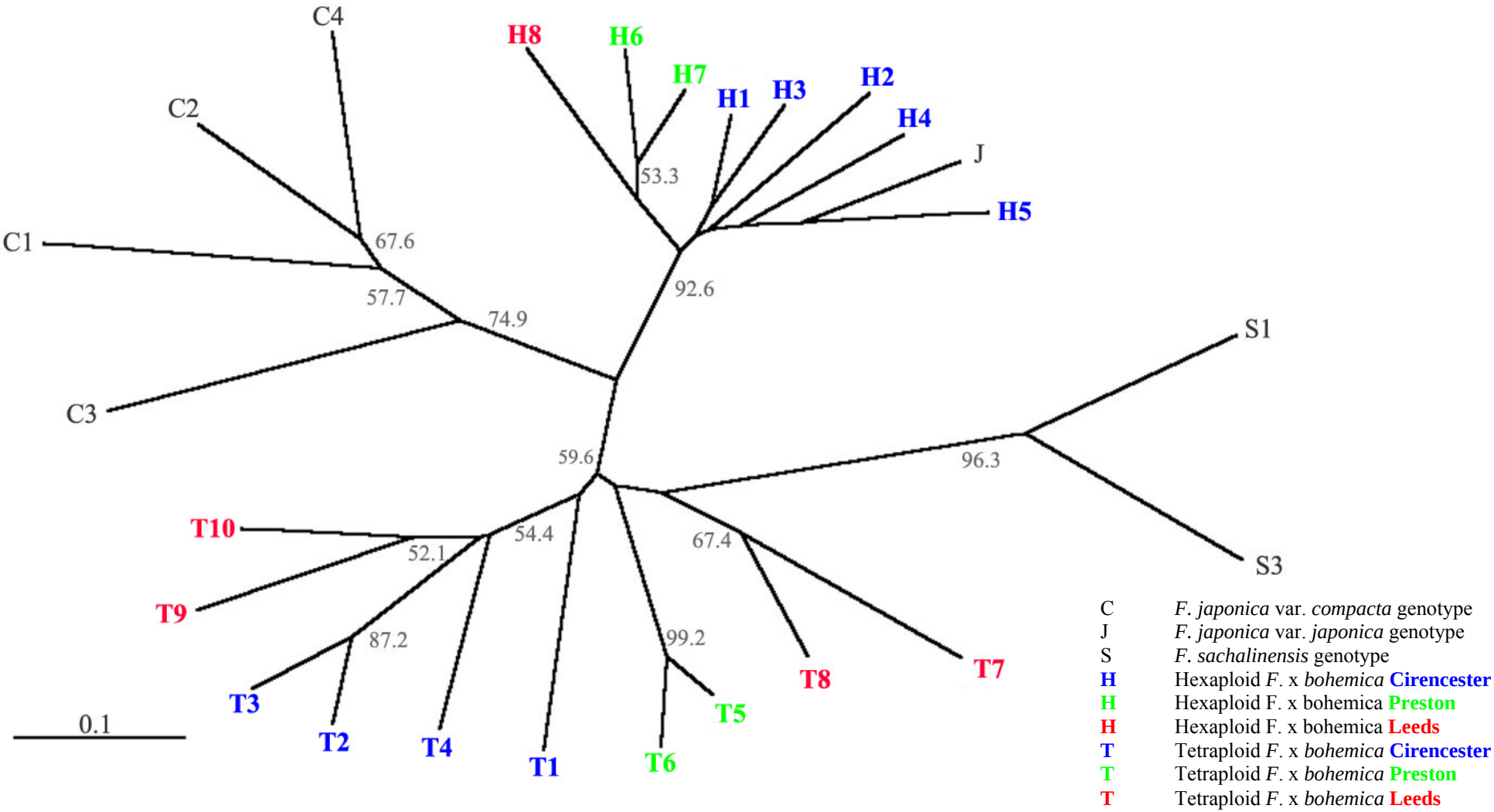



**Figure 5.19** Neighbour-joining tree depicting relationships between the various genotypes, based on the Jaccards similarity coefficient. Numbers in grey are confidence values based on bootstrapping 10,000 times. Only bootstrap values greater than 50 are shown.

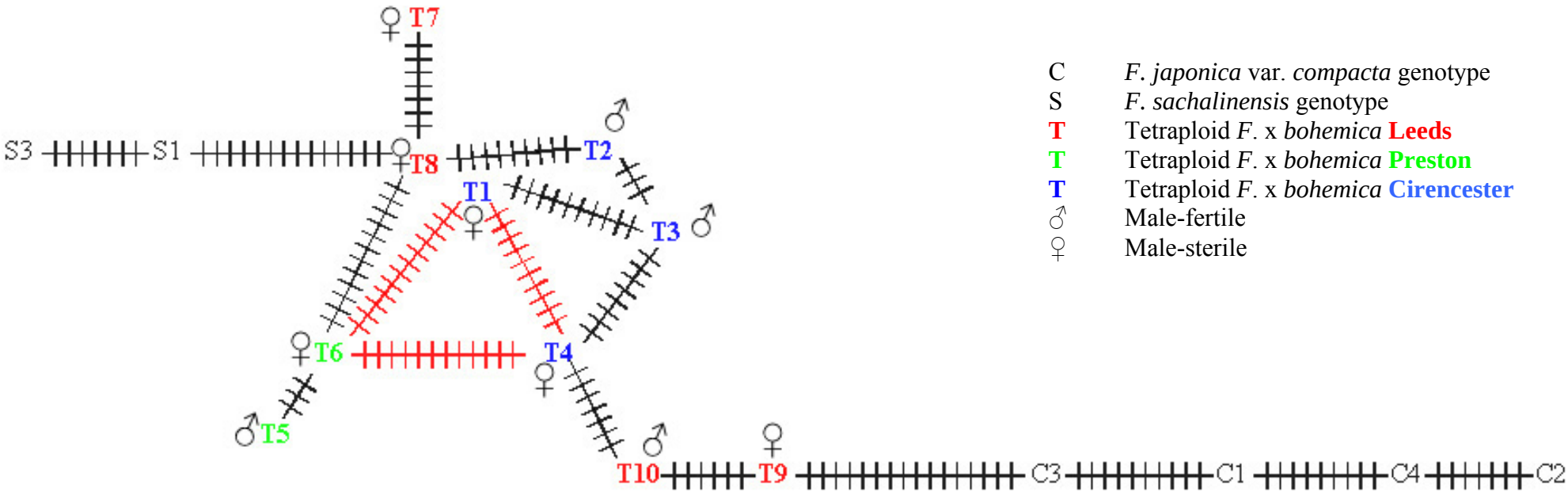



**Figure 5.20** Minimum spanning network showing the number of differences between tetraploid genotypes. The cross-links show the number of differences. The red links are equally likely alternatives between genotypes.

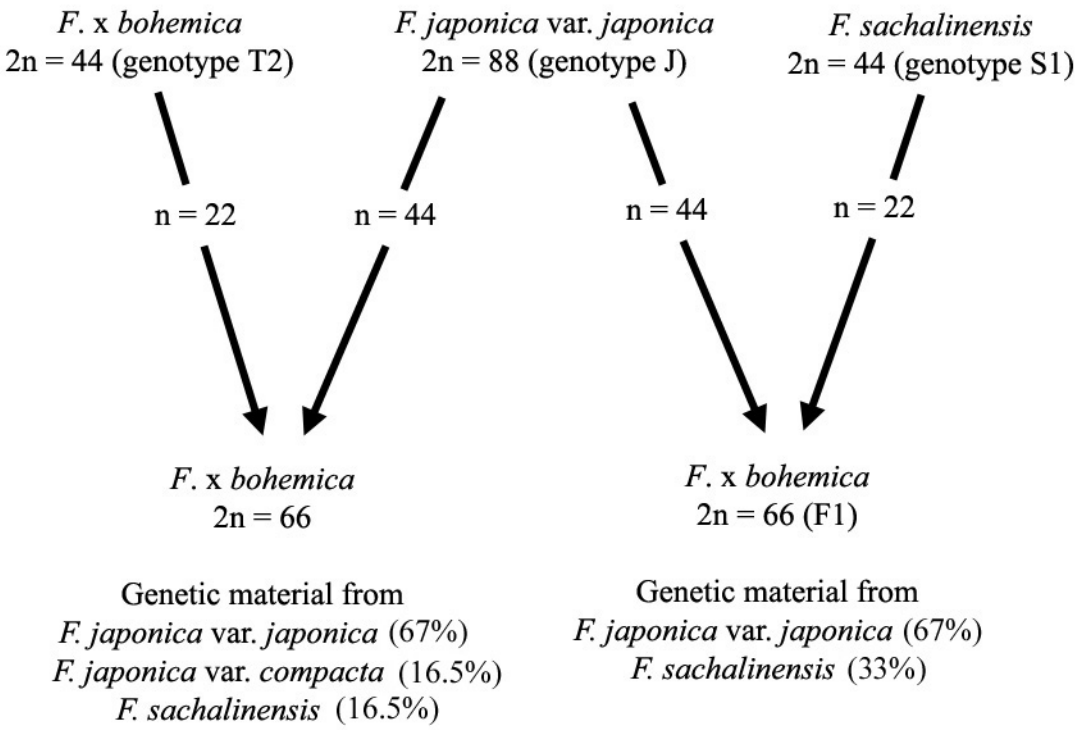



**Figure 5.21** Potential crosses that would lead to a hexaploid *F*. x *bohemica* in Cirencester.

**Table 5.1.** Details of the Japanese Knotweed *s.l.* plants studied from the three sites.

| Thesis code | P number | Taxon | Location | Sex expression | Chromosome number |
|---|---|---|---|---|---|
| CB1 | P218 | *F.* x *bohemica* | Cirencester | male-sterile | 66 |
| CB2 | | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB3 | P219 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB4 | P220 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB5 | P221 | *F.* x *bohemica* | Cirencester | male-sterile | 66 |
| CB6 | P223 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB7 | P224 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB8 | P225 | *F.* x *bohemica* | Cirencester | male-sterile | 44 |
| CB9 | P226 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB10 | P227 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB11 | P228 | *F.* x *bohemica* | Cirencester | male-sterile | c66 |
| CB12 | P860 | *F.* x *bohemica* | Cirencester | | 44 |
| CB13 | P861 | *F.* x *bohemica* | Cirencester | | |
| CB14 | P862 | *F.* x *bohemica* | Cirencester | | 44 |
| CB15 | P863 | *F.* x *bohemica* | Cirencester | | 44 |
| CB16 | P864 | *F.* x *bohemica* | Cirencester | male-sterile | |
| CB17 | P865 | *F.* x *bohemica* | Cirencester | male-sterile | 44 |
| CB18 | P866 | *F.* x *bohemica* | Cirencester | male-sterile | 44 |
| CB19 | P867 | *F.* x *bohemica* | Cirencester | male-fertile | 66 |
| CB20 | P868 | *F.* x *bohemica* | Cirencester | male-fertile | 44 |
| CB21 | P869 | *F.* x *bohemica* | Cirencester | | 44 |
| CJ1 | P870 | *F. japonica* var. *japonica* | Cirencester | male-sterile | 88 |
| CS1 | P871 | *F. sachalinensis* | Cirencester | male-fertile | |
| CB22 | P872 | *F.* x *bohemica* | Cirencester | male-fertile | 66 |
| CB23 | P873 | *F.* x *bohemica* | Cirencester | | 66 |
| CB24 | P874 | *F.* x *bohemica* | Cirencester | male-fertile | |
| CJ2 | | *F. japonica* var. *japonica* | Cirencester | male-sterile | |
| CB25 | P875a | *F.* x *bohemica* | Cirencester | | c66 |
| CB26 | P875b | *F.* x *bohemica* | Cirencester | | c66 |
| CB27 | P875c | *F.* x *bohemica* | Cirencester | | |
| CB28 | P875d | *F.* x *bohemica* | Cirencester | | c66 |
| CB29 | P462 | *F.* x *bohemica* | Cirencester | male-fertile | |
| CB30 | P463 | *F.* x *bohemica* | Cirencester | male-sterile | |

| Thesis code | P number | Taxon | Location | Sex expression | Chromosome number |
|---|---|---|---|---|---|
| CB31 | P876 | *F.* x *bohemica* | Cirencester | | 44 |
| CS2 | P877 | *F. sachalinensis* | Cirencester | | |
| PB1 | P1031 | *F.* x *bohemica* | Preston | | 44 |
| PB2 | P1032 | *F.* x *bohemica* | Preston | | |
| PB3 | P1033 | *F.* x *bohemica* | Preston | | |
| PB4 | P1034 | *F.* x *bohemica* | Preston | male-fertile | 44 |
| PB5 | P1035 | *F.* x *bohemica* | Preston | male-sterile | 44 |
| PB6 | P1036 | *F.* x *bohemica* | Preston | | 44 |
| PB7 | P1037 | *F.* x *bohemica* | Preston | male-sterile | 44 |
| PB8 | P1038 | *F.* x *bohemica* | Preston | male-sterile | 44 |
| PJ1 | P1039 | *F. japonica* var. *japonica* | Preston | male-sterile | c88 |
| PB9 | P1040 | *F.* x *bohemica* | Preston | | 66 |
| PB10 | P1041 | *F.* x *bohemica* | Preston | | 66 |
| PJ2 | P1042 | *F. japonica* var. *japonica* | Preston | | |
| PB11 | P1043 | *F.* x *bohemica* | Preston | | 66 |
| PB12 | P1044 | *F.* x *bohemica* | Preston | male-sterile | 44 |
| PB13 | P1045 | *F.* x *bohemica* | Preston | male-sterile | 44 |
| LS1 | P1117 | *F. sachalinensis* | Leeds | male-fertile | 44 |
| LB1 | P1119 | *F.* x *bohemica* | Leeds | male-sterile | 44 |
| LB2 | P1120 | *F.* x *bohemica* | Leeds | male-sterile | 44 |
| LB3 | P1121 | *F.* x *bohemica* | Leeds | male-sterile | 44 |
| LB4 | P1122 | *F.* x *bohemica* | Leeds | male-sterile | 44 |
| LB5 | P1123 | *F.* x *bohemica* | Leeds | | 44 |
| LB6 | P1124 | *F.* x *bohemica* | Leeds | male-fertile | 44 |
| LB7 | P1125 | *F.* x *bohemica* | Leeds | | c66 |
| LJ1 | P1126 | *F. japonica* var. *japonica* | Leeds | male-sterile | |
| FC1 | P002 | *F. japonica* var. *compacta* | Bracken Hill | | 44 |
| FC2 | P174 | *F. japonica* var. *compacta* | Brecon rd | | |
| FC3 | P413 | *F. japonica* var. *compacta* | Blarney castle | | 44 |
| FC4 | P175 | *F. japonica* var. *compacta* | Newbridge | | |
| FC5 | P1226 | *F. japonica* var. *compacta* | Connel ferry | | |

**Table 5.2.** Chloroplast type, genotype and chromosome number for the Japanese Knotweed *s.l.* plants included in the study.

| Thesis code | Taxon | Location | Chloroplast type | Geno-type | 2n= |
|---|---|---|---|---|---|
| CB1 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | 66 |
| CB2 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB3 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB4 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB5 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | 66 |
| CB6 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB7 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB8 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | 44 |
| CB9 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB10 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | |
| CB11 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H1 | c66 |
| CB12 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | 44 |
| CB13 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | |
| CB14 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | 44 |
| CB15 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | 44 |
| CB16 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | |
| CB17 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | 44 |
| CB18 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T1 | 44 |
| CB19 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H2 | 66 |
| CB20 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T2 | 44 |
| CB21 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T2 | 44 |
| CJ1 | *F. japonica* var. *japonica* | Cirencester | *japonica* (A) | J | 88 |
| CS1 | *F. sachalinensis* | Cirencester | *sachalinensis* (D) | S1 | |
| CB22 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H3 | 66 |
| CB23 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H4 | 66 |
| CB24 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H4 | |
| CJ2 | *F. japonica* var. *japonica* | Cirencester | *japonica* (A) | J | |
| CB25 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H5 | c66 |
| CB26 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H5 | c66 |
| CB27 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H5 | |
| CB28 | *F.* x *bohemica* | Cirencester | *japonica* (A) | H5 | c66 |
| CB29 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T3 | |

| Thesis code | Taxon | Location | Chloroplast type | Geno-type | 2n= |
|---|---|---|---|---|---|
| CB30 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T4 | |
| CB31 | *F.* x *bohemica* | Cirencester | *compacta* (C) | T4 | 44 |
| CS2 | *F. sachalinensis* | Cirencester | *sachalinensis* (D) | S1 | |
| PB1 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T5 | 44 |
| PB2 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T5 | |
| PB3 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T5 | |
| PB4 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T5 | 44 |
| PB5 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T6 | 44 |
| PB6 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T5 | 44 |
| PB7 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T6 | 44 |
| PB8 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T6 | 44 |
| PJ1 | *F. japonica* var. *japonica* | Preston | *japonica* (A) | J | c88 |
| PB9 | *F.* x *bohemica* | Preston | *japonica* (A) | H6 | 66 |
| PB10 | *F.* x *bohemica* | Preston | *japonica* (A) | H7 | 66 |
| PJ2 | *F. japonica* var. *japonica* | Preston | *japonica* (A) | J | |
| PB11 | *F.* x *bohemica* | Preston | *japonica* (A) | H6 | 66 |
| PB12 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T6 | 44 |
| PB13 | *F.* x *bohemica* | Preston | *sachalinensis* (D) | T6 | 44 |
| LS1 | *F. sachalinensis* | Leeds | *sachalinensis* (D) | S1 | 44 |
| LB1 | *F.* x *bohemica* | Leeds | *compacta* (C) | T7 | 44 |
| LB2 | *F.* x *bohemica* | Leeds | *compacta* (C) | T7 | 44 |
| LB3 | *F.* x *bohemica* | Leeds | *compacta* (C) | T8 | 44 |
| LB4 | *F.* x *bohemica* | Leeds | *compacta* (C) | T9 | |
| LB5 | *F.* x *bohemica* | Leeds | *compacta* (C) | T10 | |
| LB6 | *F.* x *bohemica* | Leeds | *compacta* (C) | T10 | |
| LB7 | *F.* x *bohemica* | Leeds | *japonica* (A) | H8 | |
| LJ1 | *F. japonica* var. *japonica* | Leeds | *japonica* (A) | J | |
| FC1 | *F. japonica* var. *compacta* | Bracken Hill | *compacta* (B) | C1 | 44 |
| FC2 | *F. japonica* var. *compacta* | Brecon rd | *compacta* (C) | C2 | |
| FC3 | *F. japonica* var. *compacta* | Blarney castle | *compacta* (B) | C3 | 44 |
| FC4 | *F. japonica* var. *compacta* | Newbridge | *compacta* (C) | C2 | |
| FC5 | *F. japonica* var. *compacta* | Connel ferry | *compacta* (C) | C4 | |

**Table 5.3** ISSR banding pattern for each genotype. J stands for *F. japonica* var. *japonica;* S for *F. sachalinensis*; C for *F. japonica* var. *compacta;* T for tetraploid *F.* x *bohemica;* H for hexaploid *F.* x *bohemica;* and, the number specifies the genotype. Bands originating from *F. japonica* var. *japonica* are coloured blue; those from *F. sachalinensis* are coloured red, and those from *F. japonica* var. *compacta* are coloured green. Bands coloured pink were not found in any of the putative parental taxa.

| | AAG-1 | | | | | 855 | | | | 881 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | **1573** | 1510 | 1364 | **1321** | 1064 | 1005 | 815 | **480** | 420 | 2036 | **1888** | **1772** | **1752** | 1713 | **1634** | **1480** | **1414** | **1220** | 1117 | 1053 | 978 |
| J | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | **1** | 0 | **1** | 0 | **1** | 0 | 0 | 1 | 0 |
| S1 | **1** | 0 | 0 | **1** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| S3 | **1** | 0 | 0 | **1** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| C1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | **1** | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 1 | 0 |
| C2 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | **1** | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 1 | 0 | 0 |
| C3 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 1 | 1 |
| C4 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | **1** | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 1 | 0 |
| T1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 1 | 1 |
| T2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| T3 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| T4 | **1** | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 1 | 1 | 0 |
| T5 | **1** | 0 | 1 | 0 | 1 | 1 | 1 | **1** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| T6 | **1** | 0 | 1 | 0 | 0 | 1 | 0 | **1** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 1 | 1 | 1 |
| T7 | 0 | 0 | 1 | **1** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| T8 | **1** | 0 | 1 | **1** | 0 | 1 | 0 | 0 | 1 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| T9 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 1 | 1 |
| T10 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| H1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 1 | 1 | 0 |
| H2 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 1 | 0 |
| H3 | **1** | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | 1 | 0 |
| H4 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | 1 | 0 |
| H5 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | **1** | 0 | **1** | 0 | **1** | 0 | 0 | 1 | 1 |
| H6 | **1** | 1 | 1 | **1** | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 1 | 1 | 1 |
| H7 | **1** | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 1 | 1 | 0 |
| H8 | **1** | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 1 | 1 | 1 |

| Genotype | CAC-1 | | | | | 840 | | | | | | AG-4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1556 | 1425 | 1336 | **1156** | 1129 | **1360** | 1275 | **1238** | 1168 | 1026 | **574** | **1531** | 1362 | **1328** | 1285 | **1209** | 907 | **867** | 840 |
| J | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| S1 | 1 | 1 | 0 | **1** | 0 | **1** | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| S3 | 1 | 1 | 1 | **1** | 0 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 0 | **1** | 1 | 0 | 0 | 0 | 0 |
| C1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 1 | **1** | 1 |
| C2 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | **1** | 1 | 1 | 0 | 0 | 1 | 0 | 0 | **1** | 1 | **1** | 1 |
| C3 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | **1** | 0 |
| C4 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | **1** | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | **1** | 1 |
| T1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | **1** | 1 | **1** | 0 |
| T2 | 1 | 1 | 1 | 0 | 1 | **1** | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 0 | 0 | 1 | **1** | 0 |
| T3 | 1 | 1 | 1 | 0 | 1 | **1** | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 1 | **1** | 0 |
| T4 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 1 | **1** | 0 |
| T5 | 1 | 1 | 1 | **1** | 1 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 1 | **1** | 0 |
| T6 | 1 | 1 | 1 | **1** | 1 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 1 | **1** | 0 |
| T7 | 1 | 1 | 1 | **1** | 1 | 0 | 0 | 0 | 1 | 0 | **1** | 0 | 1 | 0 | 1 | **1** | 1 | **1** | 0 |
| T8 | 1 | 1 | 1 | **1** | 1 | **1** | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | **1** | 1 | **1** | 0 |
| T9 | 1 | 1 | 1 | **1** | 1 | 0 | 0 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 0 | 0 | 1 | **1** | 0 |
| T10 | 1 | 1 | 1 | **1** | 1 | **1** | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 0 | 0 | 1 | **1** | 0 |
| H1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | **1** | **1** | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| H2 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | **1** | **1** | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| H3 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | **1** | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| H4 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| H5 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | **1** | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| H6 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| H7 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | **1** | **1** | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| H8 | 1 | 1 | 1 | **1** | 1 | **1** | 1 | 0 | 1 | 0 | **1** | **1** | 0 | 0 | 0 | 0 | 1 | 0 | 1 |

| Genotype | 864 | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1790 | 1725 | 1530 | 1502 | 1450 | 1400 | 1309 | 1227 | 1182 | 1128 | 1076 | 924 | 897 | 884 | 844 |
| J | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| S1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| S3 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| C1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C2 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| C3 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C4 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| T1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| T2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| T3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| T4 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| T5 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| T6 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| T7 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| T8 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| T9 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| T10 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| H1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H2 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H3 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H4 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H5 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H6 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H7 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| H8 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |

**Table 5.4** Distribution of bands in the hexaploid *F*. x *bohemica* genotypes from Cirencester.

| | J | S1 | T2 | J & T2 | S1 & T2 | J & S1 | no. accounted for / total no. of bands |
|---|---|---|---|---|---|---|---|
| H1 | 9 | 1 | 0 | 6 | 3 | 0 | 19/26 |
| H2 | 10 | 0 | 0 | 6 | 3 | 1 | 19/26 |
| H3 | 10 | 1 | 0 | 6 | 2 | 0 | 19/26 |
| H4 | 8 | 0 | 0 | 6 | 2 | 1 | 17/23 |
| H5 | 11 | 0 | 0 | 5 | 3 | 1 | 20/27 |

## Chapter 6. Chloroplast DNA variation and molecular biogeography of Japanese populations of *Fallopia japonica* and *Fallopia sachalinensis*.

### 6.1. Introduction

*Fallopia japonica* var. *japonica* is currently regarded as the most problematic of the group of invasive weeds referred to as Japanese Knotweed *s.l.* Whilst there is mounting evidence that the hybrid *F.* x *bohemica* is as invasive as its parental taxa, if not more so (Brabec & Pyšek, 2000; Pashley *et al.*, 2003) and harder to control (Bímová *et al.*, 2001), at the moment it is less widespread than its maternal parent and therefore less of an immediate threat. *F. japonica* var. *japonica* is found in over 71% of the 10 km square recording areas in the British Isles (Preston *et al.*, 2002), and current control techniques used for the group as a whole are based on their effectiveness against this taxon. It is the extensive rhizome system that makes control of these taxa difficult and therefore whatever control is used, it is the killing of the rhizomes that must be achieved for control to be successful. Control of these plants is both difficult and very expensive, and any management programmes must be planned over a number of years (Child & Wade, 2000). Researchers trying to control the spread of Japanese Knotweed are looking towards biological control as a possible future aid in the battle against the spread of these plants, and to that end in 1999 a proposal to find a biological control for *F. japonica* was put forward (Shaw, 1999) and phase 1 of the programme has already been completed (Shaw, 2001). Given the clonal nature of the plant and the limited genetic diversity it is a prime target for biological control. Identifying the precise native origin of the invasive taxon could aid the search for a highly specific control organism (Shaw, 1999).

Biological control, which is often shortened to biocontrol, is the purposeful use of an organism or organisms to reduce a plant or animal population that is unfavourable to man. This is achieved by the deployment of natural enemies against specific animal pests or weeds. Whilst complete eradication of the pest is seldom achieved, the numbers of pests can be suppressed to a level where they are no longer a nuisance nor cause economic damage. Classical biocontrol involves the search for natural enemies of the pest in their native home where these natural enemies are exerting a regulatory pressure upon the pest organism. These natural enemies are collected and sent to the country or area where the pest is lacking them and where it is in outbreak numbers (Samways, 1981). In the introduced range virtually nothing infects Japanese Knotweed *s.l.*, however in its native range it is highly susceptible to predation from a variety of organisms (Bailey, 2003; Child & Wade, 2000; Hollingsworth & Bailey, 2000b; Shaw, 1999; 2001).

Molecular biogeography can be used to help elucidate the origin of a species. Biogeography is the study of the geographical distribution, both past and present, of plants, animals and other organisms across the globe. Biogeography is a broad subject area that includes more specialised areas such as historical, analytical, ecological, and applied biogeography (Spellerberg & Sawyer, 1999). Phylogeography is the study of the relationship between the phylogeny of variants and their geographic distribution (Dumolin-Lapegue *et al.*, 1997). The term was initially used on studies assessing mitochondrial DNA variation in animals (Avise *et al.*, 1987). The technique has since been applied to the study of plant variation, with the chloroplast genome being the most popular organelle to study. Molecular biogeography is a more general term applied when molecular markers are used to assess variation amongst samples across a geographical area, but without a phylogeny being inferred.

The chloroplast genome is well suited to evolutionary and phylogenetic studies and as such has become the organelle of choice for many plant phylogeographic studies. Two of the first organisms to have their entire chloroplast genomes sequenced were an angiosperm (*Nicotinia tabacum* L. (Shinozaki *et al.*, 1986)) and a liverwort (*Marchantia polymorpha* L. (Ohyama *et al.*, 1986)). Comparisons between these, and others since, have revealed much information with regard to structure and sequence homology. The chloroplast genome is a circular double-stranded molecule, which in tobacco is about 156 kilo bases (kb) in size, and is arranged into four parts; two identical 25-kb segments that form an inverted repeat separating the rest of the molecule into single-copy regions of 87 kb and 18 kb. The typical chloroplast genome is densely packed with about 120 genes, with both strands being actively expressed. It is believed to evolve quite slowly, with the gene content being highly conserved. Only two known gene differences between tobacco and *Marchantia* have been found, even though they diverged about 400 million years ago. Additionally the order of genes is also highly conserved. Sequence comparisons have revealed a low rate of nucleotide substitution, although rate differences exist among specific chloroplast genes (Palmer *et al.*, 1988).

Initially it was believed that the chloroplast genome was ideally suited for interspecific analyses, but its utility as an intraspecific marker was in question. This was primarily because intraspecific variation can be limited due to the conservative nature of the chloroplast genome (Palmer *et al.*, 1988). A review by Soltis *et al*., (1992) revealed nearly 60 examples of intraspecific variation from over 15 different plant families, many of the studies of which were not designed to look for this type of variation. Since then numerous studies have been published where chloroplast DNA variation has been actively used to assess intraspecific variation. One such study looked at the phylogeography of the argan tree, *Argania spinosa* of

Morocco. This particular study found sufficient intraspecific variation for a phylogeographical study by analysing multiple chloroplast regions with a single restriction enzyme (El Mousadik & Petit, 1996).

In the introduced range there are clear morphological differences between the various members of the group of plants referred to as Japanese Knotweed *s.l.* A number of these are tabulated in Bailey *et al.*, (1996). As shown in Figure 2.1, leaf shape is one of the major features that can be used to distinguish between *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and *F. sachalinensis*, with the hybrid *F.* x *bohemica* having an intermediate shape. In the wild the vast majority of hybrids appear to be F1 hybrids. There is very little evidence of introgression (chapters 4 & 5), which could otherwise potentially lead to a more normal distribution of leaf characteristics, covering the range from one parental extreme to the other.

As is often the case when plants are introduced to a new environment, only a small sub-set of the native variation is introduced. This is particularly true of Japanese Knotweed. As discussed in chapter 2.2, there is a much greater range of morphology in the native distribution, and this is also shown in the greater number of intraspecific classifications that have been proposed. *F. japonica* is native to China, Korea, Japan and Taiwan (Bailey, 1989). Two varieties of *F. japonica* are recognised in the introduced range, var. *japonica* and var. *compacta,* both of which are believed to have been imported from Japan (Bailey & Conolly, 2000). *F. sachalinensis* is found in the southern part of Sakhalin Island (former USSR), the southern Kurile Islands (Kunashir and Shikotan), and the Japanese Islands Hokkaido and Honshu (Sukopp & Starfinger, 1995). Historical records indicate that it may have been introduced from either the Sakhalin islands or Japan, or indeed have been introduced from both (Bailey & Conolly, 2000).

Given the wider range of morphological characteristics, leaf shape alone cannot be relied upon to accurately identify the different taxa. Bailey (1989) studied the epidermal characteristics of a number of taxa from the genus *Fallopia*. Specifically *F. baldschuanica*, *F. multiflora* (Thunb.) Haraldson, *F. cilinodis* (Michaux) Holub, *F. cynanchoides* (Hemsl.) Haraldson, *F. sachalinensis*, and *F. japonica*. He found two characters in particular that were useful for distinguishing between some of the taxa, those characters being the cuticular patterning and the trichomes of the lower epidermis.

The epidermal cell-wall outlines of the lower leaf surface were found to be generally rather undulate, with the degree of cuticular ornamentation varying from just a few striae in the

region of a stoma to heavily folded cuticles that obscure the epidermal cell outline. The degree of striation referred to, being a result of folding of the cuticular layer. The range in the degree of cuticular ornamentation can be observed in Figure 6.2. Three different types of trichome were found, characterized as types A, B and C. Type A referred to a single swollen oval epidermal cell that protrudes above the surrounding cells, B referred to a uniseriate multicellular hair comprising of between 4 and 20 cells, whilst C was a stiff unicellular trichome with a papillate surface that arises from the base without accessory cells (Bailey, 1989). Types A and B can be seen in Figure 6.3, however it should be noted that type C in this figure does not refer to the type C trichome from Bailey (1989).

As can be seen in Table 6.1, these two characteristics are unable to distinguish *F. cilinodis* from *F. cyanchoides*, but these were clearly separated from the rest of the group. Additionally *F. japonica*, *F. baldschuanica* and *F. multiflora* were not readily distinguished from each other. However, the important feature relevant to this present study is that *F. sachalinensis* and *F. japonica* could be readily distinguished on the basis of both of these characters. Additionally, their hybrid *F.* x *bohemica* was found to have intermediate characters (Bailey, 1989).

Preliminary genetic analysis of native *F. japonica* plants from Asia was initiated by Hollingsworth & Bailey (2000b). In this study twelve individuals were analysed for variation using RAPD PCR analysis, ten from the main island Honshu, Japan and two from China. Considerable diversity was found among the native samples, with greater genetic distance between Japanese and Chinese materials than there was between the two named varieties that occur in Britain. Five of these samples were analysed for sequence variation for a single region of the chloroplast (*trn*L intron) with no matches to the British accessions. A larger study of Japanese material was conducted by Inamura (2000). Using sequence analysis of a single region of the chloroplast (*rbc*L to *acc*D) Inamara *et al*. analysed 65 *F. japonica* accessions and 3 *F. sachalinensis* samples from the four main Islands, Kyushu, Shikoku, Honshu and Hokkaido. Geographical clustering of the clades based on Inamura's chloroplast haplotypes was found, and two *F. japonica* varieties were differentiated genetically, those being var. *terminalis* (Honda) J. P. Bailey and var. *uzenensis*. The authors had intended to use *F. sachalinensis* as an outgroup, but found their three samples were not only dispersed within their ingroup, but also fell within two different *F. japonica* clades. Further work is obviously required with larger sample sizes and preferably more regions of the chloroplast genome.

## 6.2 Aims

To see if the leaf characters that distinguish *F. japonica* from *F. sachalinensis* in the introduced range can be of use when studying native material. To investigate the chloroplast RFLP variation among plants collected from the four main islands of Japan, and to use this to identify the regions where the introduced material of *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and *F. sachalinensis* are most likely to have originated.

## 6.3 Materials and Methods

### 6.3.1. *Plant material*

The majority of plants were collected during trips to Japan in 1999 (C. H. Pashley, and J. P. Bailey) and 2000 (J. P. Bailey, R. Shaw and H. Evans). At the time of collection, material was given a unique P number. A preliminary identification of the plant was noted, the location recorded using a hand held Global Positioning System (GPS), and brief notes relating to the habitat type were made. For specimens that were in flower, sex was also noted. Voucher specimens were collected and deposited at the University of Leicester herbarium (LTR). The rhizomes were imported under licence number PHL 58/3112 and grown under quarantine conditions at the Botanic Gardens, Leicester. Other specimens were sent from various sources as either seed or rhizome, and in some cases were missing some information such as plant height, but were still used in the study. These plants are now housed in the greenhouses belonging to The University of Leicester, Department of Biology.

Each site in Japan from which plants were received or collected, has been given a unique site number. Table 6.2 lists these sites and information pertaining to them including the number of plants obtained from each site. The distribution of sites is shown in Figure 6.1. The dot that appears to be in the sea to the south of Honshu represents site number 43, Hachijo Islands, which is administratively part of Tokyo, and from where *F. japonica* var. *terminalis* was received.

Additional plants were used in this study, including representatives from other Asiatic countries where Japanese Knotweed is native, and also specimens from the introduced range. These are listed in Table 6.3.

In total 246 plants of Asiatic origin (240 from Japan) were included in this study and a further 39 plants from the introduced range.

6.3.2. *Methods*

6.3.2.1 Plant identification

Leaf characters were used to aid taxonomic identification. Besides noting whether the base of the leaf was acuminate, truncate or cordate, two lower epidermal characters were analysed, trichome type and degree of cuticular striation. A subset of the Japanese plants was analysed for these three characters. These were selected randomly to give a good representation of the variation across Japan. Further plants from sites where the possibility of hybridisation was high were assessed for trichome type and leaf shape, and for those that appeared to have a strong potential of being of hybrid origin, degree of striation was also included.

Where possible fully expanded mature leaves were used. For fresh leaf material an epidermal peel was used as per Bailey (1989). This involved a fine cut parallel to the leaf surface being made with a razor. The epidermis was then peeled back with fine forceps and mounted on a microscope slide in a drop of 50% glycerol. When fresh material was not available herbarium specimens were examined. A thin layer of clear nail varnish was applied to the lower epidermal surface and allowed to dry. This replica was then peeled off with fine forceps and mounted as per fresh material.

The mounted specimens were observed at x16 magnification on a Zeiss universal light microscope. Images were captured digitally. Degree of cuticular striation was determined by comparison against standards as shown in Figure 6.2a for fresh material, and Figure 6.2b when a replica was used.

To determine trichome characteristics the lower epidermal surface of either a fresh leaf or herbarium specimen was observed using a dissection microscope with the sample illuminated at an angle of approximately 90º. As far as possible fully expanded mature leaves were used. Leaves were broadly grouped as possessing one of four trichome types, which are also shown in Figure 6.3.

A Trichome; single-celled, swollen oval cell, usually striate, protrudes above the surrounding cells, sometimes joining with neighbouring cells to form ridges along the vein. Giving a rough appearance to the leaf veins.

B Trichome, uniseriate multicellular, 4 -20 cells in length. Very thin walled, often collapsed in herbarium specimen. Leaf veins very smooth. Distribution of hairs variable, ranging from heavily pubescent to scarce.

C Trichome, uniseriate multicellular, 3 – 5 cells in length, perpendicular to the vein, with basal cell an almost equilateral triangle. Leaf veins otherwise smooth, trichomes densely distributed along the veins.

D Trichome, uniseriate multicellular, 1 – 6 cells in length, usually striate at the base, basal cell asymmetrical. Trichomes emerging at less than 90°, distribution of trichomes varies from heavily pubescent to scarce.

6.3.2.2 Molecular analysis

A single DNA extraction was made for each plant, as detailed in chapter 3 materials and methods. Chloroplast DNA PCR amplification of the six regions listed in Table 3.1 as being variable was completed, followed by RFLP analysis of the fragments using the restriction enzyme *Hin*fI, as per chapter 3.

## 6.4 Data analysis

RFLP bands were scored in the manner detailed in chapter 3, section 3.2.7.3. Figure 6.4 shows a sample gel for each chloroplast region depicting most of the chloroplast haplotypes.

PAUP 4.0 beta version 10 (Swofford, 2003) was used to perform a parsimony analysis on the binary RFLP data. The search strategy was heuristic, based on 1000 replicates, and confidence was assessed using 1,000 fast "pairwise-step" bootstrap replicates. No outgroup was used because the potential outgroups *F. baldschuanica*, *F. multiflora*, and *F. convolvulus* were too different from the ingroup to be reliably scored as indicated in Figure 6.5.

Winboot (Yap & Nelson, 1996) was used to bootstrap the RFLP binary data 1,000 times using the Jaccard's coefficient to produce confidence values. The companion program WinDist was then used to produce a matrix of pair-wise genetic distances between all individuals, using the complement of the Jaccard's similarity coefficient. A Neighbour-Joining tree was drawn from the resulting matrix using the NEIGHBOUR option in PHYLIP version 3.6a3 (Felsenstein, 2002) and TreeView version 1.6.6 (Page, 1996).

## 6.5 Results

6.5.1 *Plant identification*

Characteristics that were used to help identify the taxa, such as degree of epidermal striation, trichome type and leaf base shape, are found in Table 6.4 along with chromosome number, sex of plant, height of plant and the altitudinal vegetation zone the plant came from. This

table only contains the 240 plants that came from Japan. For ease the plants have been grouped according to their multi-primer-haplotype (MPH), as defined in 6.5.2.

All plants identified as *F. japonica* were found to have very little cuticular ornamentation, with the majority of the plants having level 0 to 1/2, and a minority having level 2. Those with trichome type C were identified as *F. japonica* var. *uzenensis*. The remainder all had trichome type A and, with the exception of *F. japonica* var. *terminalis*, were grouped together as *F. japonica* var. *japonica*. To distinguish native *F. japonica* var. *japonica* from the British *F. japonica* var. *japonica* clone, native material shall be referred to as *F. japonica* var. '*japonica'*, in accordance with Hollingsworth and Bailey (2000). *F. japonica* var. *terminalis* appeared to have neither trichome (B,C,D) nor swollen cell (A). The *F. japonica* plants primarily had truncate leaves, although some appeared slightly cordate or slightly acuminate. Both 2n = 44 and 2n = 88 *F. japonica* var. '*japonica'* plants were found in Japan, with 2n = 44 being the most common.

*F. sachalinensis* plants were identified by their cordate leaf and long uniseriate trichome (B). They all possessed a highly striate cuticle layer.

In the majority of cases the *F. sachalinensis* plants were 2n = 44. There were two cases from Aomori, where *F. sachalinensis* plants are sympatric with *F. japonica* var. *uzenensis*, that both had 2n = 66. They had highly ornamented lower cuticles as would be expected of *F. sachalinensis*, but their trichomes appeared to be a cross between type B and C, which potentially indicates a hybrid between the two taxa. They both had MPH 2, which apart from these potential hybrids was only found in *F. sachalinensis*.

All plants identified as *F. japonica* var. *uzenensis* that were counted had 2n = 88. There were some 2n = 66 plants with C-like trichomes but with a slight resemblance of type D (the type found on *F.* x *bohemica*, the hybrid between *F. sachalinensis* with type B and *F. japonica* with type A trichomes) but being slightly less folded and more dense as normally found with type C. It was assumed that these were of hybrid origin. Those with a greater level of ornamentation possibly being interspecific hybrids between *F. japonica* var. *uzenensis* and *F. sachalinensis*, and those with almost no ornamentation being intraspecific hybrids between tetraploid *F. japonica* var. '*japonica'* and *F. japonica* var. *uzenensis*.

For *F. japonica* plants the term tall has been given to any plant that has a height of greater than or equal to 150 cm. The term dwarf is harder to apply given that sometimes plants may

not have been at their growth peak at time of collection or may have been cut down. Whilst it may be true that some tall plants will not be at their growth peak and would be excluded from the group of tall plants, the term tall is a generalisation whereas the term dwarf is one of the features that characterises the variety *F. japonica* var. *compacta*. In general the term dwarf has been applied to plants that are less than or equal to 80 cm in height for *F. japonica*, including those that have been described as being var. *uzenensis*. For lowland specimens they have only been termed dwarf if they were in flower at time of collection as that indicates a later stage of development. Neither term has been applied to any *F. sachalinensis* plants or putative hybrids. As seen in Table 6.4, *F. japonica* plants defined as dwarf have shared chloroplast types with plants defined as tall in seven different chloroplast MPH groups.

The altitudinal vegetation zones for central Japan were defined by Takeda (Takeda, in Ishizuka (1974)). These have been adopted for this study and are summarised as follows:

1) The Hilly Zone – The area from sea level up to an altitude of 500 – 600 m in central Japan. Assumed to be warm-temperate broad-leaved evergreen forest, however most of the original forests have been transformed by human activity. In their place paddy fields, deciduous coppice forests, and grasslands now occupy the hills and lower mountainsides. Referred to in this thesis as lowland.
2) The Montane Zone – This zone lies between 500 and 1500 metres above sea level (masl) in central Japan, and is dominated by cool-temperate broad-leaved deciduous forest.
3) The Subalpine Zone – This zone lies between 1500 and 2500 masl in central Japan and is characterized by evergreen coniferous forests. The Subalpine forest altitude drops towards the north, i.e. to between 1000 – 1300 and 2000 masl in northern Honshu, and on Hokkaido starting between 1 – 500 masl and stopping between 100 – 1500 masl. In southwestern Japan, in the mountains of Shikoku and the Kii Peninsula, the lower limit of the subalpine zone rises to an altitude of about 1700 masl. The subalpine zone is completely absent from the mountains of Kyushu and the Chugoku district of southwest Japan.
4) The Alpine Zone –The alpine zone embraces all areas extending from the subalpine coniferous forest limit (about 2500 masl) to the summits of the mountains.

As shown in Table 6.4, the majority of plants in this study were from the lowland or montane vegetation zones. There were no plants analysed from the alpine zone. In all cases except one, the plants from the higher altitudes shared chloroplast type with plants collected from the

lowlands. The exception being MPH 24, which was a rare chloroplast type found in a single plant from the subalpine vegetation zone on Mt. Tateyama (site no. 49 Table 6.2).

In the introduced range, one of the features that distinguish the dwarf variety *F. japonica* var. *compacta* from *F. japonica* var. *japonica* is that it possesses leaves that are often as broad as they are long, with crimped margins. In Japan, as seen in Table 6.4, dwarf, intermediate and tall plants from Mt. Fuji and the surrounding prefectures with MPH 3 and MPH 4 are found with this characteristic leaf shape. There are other plants that share these two MPHs, which do not possess this characteristic leaf shape. There are dwarf plants with these MPHs, and dwarf plants with other MPHs, that do not have this characteristic leaf shape.

### 6.5.2 *Distribution of taxa*

The prefectures each of the taxa has been identified from are shown in Figure 6.6. Putative hybrids have been excluded but could be expected in any area where two or more taxa are sympatric. Prefectural boundaries are of course political not biological so plants growing at the boundary of one prefecture could easily be pollinated by nearby plants from the neighbouring prefecture. This map is not intended to be a complete distribution map for the taxa, but to be a guide to where the taxa used in this study were from. Octoploid *F. japonica* var. 'japonica' plants were primarily found in the southern prefectures of Honshu, and the closest of the prefectures to Honshu from the island Kyushu, on the Japan Sea side of the islands. In three of these prefectures both tetraploid and octoploid plants were found, and it is believed that in at least one of the prefectures with only octoploids, tetraploids would have also been found if further samples had been counted. Only tetraploids were found in the prefectures to the south of these, on both Kyushu and Honshu, including all of the island of Shikoku, towards the Pacific Sea side of Japan.

In the central region of Honshu, only tetraploid *F. japonica* var. '*japonica*' individuals have been found. To the north of this area *F. japonica* var. *uzenensis* was found. On the Japan Sea side of this region tetraploid *F. japonica* var. '*japonica*', octoploid *F. japonica* var. *uzenensis* and *F. sachalinensis* were sympatric. To the Pacific Ocean side, with the exception of Fukushima, tetraploid *F. japonica* var. '*japonica*' was not found and the predominant taxon was *F. japonica* var. *uzenensis*, although *F. sachalinensis* was also found in the more northerly prefecture.

Aomori is the most northern of the Honshu prefectures. *F. japonica* var. *uzenensis* and *F. sachalinensis* are sympatric, but an octoploid *F. japonica* var. '*japonica*' was also found. Only *F. sachalinensis* was analysed from the northern island Hokkaido.

### 6.5.3 *Chloroplast haplotypes*

The chloroplast regions amplified ranged in size from approximately 1.7 to 3.2 kb, as shown in Table 6.5. Between four and ten bands were scored from each chloroplast region after digestion with the restriction enzyme *Hin*f1. Not all bands generated were scored, as some were either too large or too small for the resolution of the gel they were run on. Each combination of these scored bands is referred to as a haplotype and the number of haplotypes found in the study for each region is shown in Table 6.5.

A letter is used to represent each haplotype. The bands that comprise each of the haplotypes are shown in Table 6.6. A combination of these haplotypes is referred to as a multi-primer-haplotype (MPH) and is intended to represent the chloroplast type of the plants in which it was found. From these six chloroplast regions forty different chloroplast types were found. The chloroplast types (MPHs) are numbered from 1 to 40 and are shown in Table 6.7.

### 6.5.4 *Genetic relationship between the haplotypes*

Two methods of analysis were used to determine the relationship between the haplotypes. The first was PAUP, which produced 1377 shortest trees of length 76. From these trees a majority rule consensus tree was produced. The mid-point rooted version of this consensus tree is shown in Figure 6.7. The second was a neighbour joining tree based on the Jaccard's similarity co-efficient. An unrooted tree was produced which is shown in Figure 6.8. The two trees were compared to determine clades.

The chloroplast types 1,5,9,12,17 & 18 have been called clade A and are coloured red on both trees. They come together as a group 100% of the time on the majority rule consensus tree (Figure 6.7), and as a group have a bootstrap value of 72. On the neighbour joining tree (Figure 6.8) they also form a clear group with a bootstrap value of 65. Chloroplast type 14 has been excluded from the group as it lies closer to those chloroplast types coloured black on the neighbour joining tree, and whilst it falls between the clade A and the rest of the chloroplast types on the majority rule tree, it does not do so significantly when bootstrapping is applied to the data.

Chloroplast types 2,3,4,8,25 and 28 are coloured dark blue, 11,33 and 37 are light blue. Together they have been called clade B. They have been grouped together as they form clear groups on both trees, with high majority rule consensus values, but with bootstrap values of less than 50, which is why they are not shown on the tree. On both trees 11,33 and 37 fell outside of the main group, which is why they have been coloured a lighter shade of blue. Chloroplast type 39 was excluded because, although it appears to join the group in the majority rule tree (Figure 6.7), on the neighbour joining tree (Figure 6.8) it appears to be more closely related to the chloroplast types 13 and 16, but with a long branch indicating it is genetically very different to most of the chloroplast types included in the study.

Clade C consists of chloroplast types 6,20, 24 and 38 coloured dark green, and 21 coloured a lighter green. The four dark green chloroplast types group together 100% of the time on the majority rule consensus tree (Figure 6.7) and have a bootstrap value of 74. Chloroplast type 21 forms a group with the others 100% of the time according to the consensus, but is not supported by bootstrapping. On the neighbour joining tree (Figure 6.8) the four dark green types form a group with a bootstrap value of 60, with type 21 falling just outside of this group. Chloroplast type 19 is the next closest to the group on this tree but has been coloured black and is one of the unresolved chloroplast types according to the majority rule tree.

Clade D is comprised of chloroplast types 10, 26, 32, 34 and 36 and is coloured purple. On the majority rule consensus tree (Figure 6.7) in 95% of the 1377 trees they form a group. This group has a bootstrap value of 51. In the neighbour joining tree (Figure 6.8) they form a clear group away from the other chloroplast types, although bootstrapping does not support this. The nearest chloroplast type is 29 on both trees, but as it is further from the group than they are from each other it has been excluded and coloured black with the rest of the unresolved chloroplast types. This applies also to the group of two, made up of chloroplast types 16 and 13.

The remainder of the chloroplast types are coloured black. With the exception of those already mentioned previously (13 & 16, 14, 29, and 39) they are unresolved on the majority rule consensus tree (Figure 6.7) implying they are no closer to each other than to any of the afore mentioned clades. According to the neighbour joining tree though, they fall into two areas. The majority of them form a group that sits between clade A and clade C. The others fall between clade B and clade D. For future mentioning in this results section and in the discussion they shall be referred to as the unresolved group.

### 6.5.5 *Geographical distribution of chloroplast haplotypes*

The clades show some degree of geographical structuring, and their maximum distribution is summarised in Figure 6.9. There is some overlap between the distributions, with clade B having the widest distribution covering Hokkaido and Honshu, whilst clade C has the smallest being found only in two prefectures, Niigata and Toyama, in the centre of Honshu. Plants on the islands Kyushu, and Shikoku are found only in clade D or as part of the unresolved group. A more detailed distribution for each of the clades is shown in Figures 6.10a-e. Table 6.8 shows the numbers of accessions of each taxon that share a chloroplast type, and this is also ordered according to the clades. There are several cases of both tetraploid and octoploid plants having the same chloroplast type, and also incidences of different taxa sharing a chloroplast type.

#### 6.5.5.1 Clade A

As seen in Table 6.8, clade A is comprised of six chloroplast types, including one that is shared by the invasive clone of *F. japonica* var. *japonica* found in Europe and the USA (MPH 1). As far as the Japanese plants are concerned all are either *F. japonica* var. *uzenensis* or putative hybrids, with one exception. The one exception is a single accession of octoploid *F. japonica* var. '*japonica*'. The distribution of clade A is shown in more detail in Figure 6.10a. The vast majority of these plants are found in the prefectures Niigata, Yamagata, Miyagi and Fukushima, just north of the centre of the Island Honshu. This region is also referred to as the Tōhoku and Horuriku districts of Japan. The only sample found away from this region was found in Osaka and is the only non *F. japonica* var. *uzenensis*/hybrid in this clade. Chloroplast types 17 and 18 are represented by a single accession each. As shown in Figure 6.10a the other chloroplast types, in particular number 12, appear to be distributed evenly across the area covered rather than being concentrated at a single site.

#### 6.5.5.2 Clade B

This is a very mixed group of taxa comprising both *F. sachalinensis* and *F. japonica* var. '*japonica*', as seen in Table 6.8. In fact the majority of the invasive *F. sachalinensis* plants found in Europe and the USA have chloroplast type 2 which is found in this clade, as are both chloroplast types (3 and 4) that are found in British samples of *F. japonica* var. *compacta*. The plants can be found from the furthest south prefecture of Honshu, Yamaguchi, to the furthest north Aomori, and also on the island Hokkaido. However they are not found on either Shikoku or Kyushu. Their distribution is shown in detail in Figure 6.10b. Unlike the chloroplast types found in clade A, those in clade B do exhibit a higher level of clustering around smaller geographical localities.

Chloroplast type 2 is only found on Hokkaido and in the most northern of the Honshu prefectures Aomori. It was the only chloroplast type found in the *F. sachalinensis* plants analysed from two distinct areas of Hokkaido. The two putative hybrids of this chloroplast type were found in Aomori where *F. sachalinensis* is sympatric with both the pubescent octoploid *F. japonica* var. *uzenensis* and the non-pubescent octoploid *F. japonica* var. '*japonica*'. The other main chloroplast type in this clade that is only found in Aomori is type 8. This chloroplast type was found in three specimens identified as octoploid *F. japonica* var. *uzenensis*, two specimens of *F. sachalinensis* and one specimen of octoploid *F. japonica* var. '*japonica*'. Besides these it was also found in fourteen putative hybrids, twelve of which were hexaploids and the remaining two being an octoploid and a tetraploid. Chloroplast type 28 is also restricted to Aomori, but it is a rare chloroplast type found only in one putative hexaploid hybrid.

Forming a group of three chloroplast types within the group 2, 8 and 28 on the majority rule tree (Figure 6.7), but being found on a separate branch within the same clade on the neighbour joining tree (Figure 6.8), are chloroplast types 3, 4 and 25. As seen on Figure 6.10b, they have a completely separate distribution from 2, 8 and 28. Chloroplast type 3 is the only type found in this study from Mt. Fuji, and is found in many sites in the surrounding prefectures but is also found as far south as the most southern prefecture of Honshu, Yamaguchi. It was found in two of the four British samples of *F. japonica* var. *compacta* analysed, and in Japan was only found in tetraploid specimens of *F. japonica*. Chloroplast type 4 was found in the other two British *F. japonica* var. *compacta* plants analysed. In Japan it was mainly found in tetraploid *F. japonica* var. '*japonica*' plants, but was also found in two specimens of each of *F. sachalinensis*, *F. japonica* var. *uzenensis* and putative hybrid plants. Like chloroplast type 3, it was found in the prefectures surrounding Mt. Fuji and as far south as Yamaguchi, however it was also found further north as far as Yamagata and Miyagi. Chloroplast type 25 is represented by a single tetraploid *F. japonica* var. '*japonica*' specimen, found on Mt. Tateyama in Toyama in the central region of Honshu.

Chloroplast types 11, 33 and 37 are those coloured a lighter shade of blue in both of the trees. Type 33 was only found in a hexaploid putative hybrid in the region around Yamagata and Miyagi. In the same region and found in four accessions of *F. sachalinensis* was type 37. Chloroplast type 11 was also found in this region, but was additionally found further north in Aomori. Primarily found in *F. sachalinensis* (twelve accessions) it was also found in two accessions of *F. japonica* var. *uzenensis* and one accession of tetraploid *F. japonica*.

6.5.5.3 Clade C

This clade is comprised of 5 chloroplast types that between them contain both *F. sachalinensis* and tetraploid *F. japonica* var. '*japonica*', as seen in Table 6.8. Plants found within clade C occupy a restricted area of Japan, being found only from Toyama and Niigata on the island Honshu (Figure 6.9). Chloroplast types 6, 20, 24 and 38 are held together 100% of the time according to the consensus majority rule tree, and have a bootstrap value of 74. Of this smaller group 7 accessions were found to be *F. sachalinensis*, and 3 were tetraploid *F. japonica* var. '*japonica*'. One of the accessions, MPH 38, has also been found in three different British accessions of *F. sachalinensis*. The fifth chloroplast type within this clade (MPH 21) falls outside of the rest of the group on both the majority rule tree (Figure 6.7) and the neighbour joining tree (Figure 6.8). Geographically, as shown in Figure 6.10c, it was found at two adjacent sites slightly further north than the rest of the group, and was found at both of these sites in a single accession of tetraploid *F. japonica* var. '*japonica*'.

6.5.5.4 Clade D

Clade D contains both tetraploid and octoploid *F. japonica* var. '*japonica*' as seen in Table 6.8. With the exception of MPH 10, the rest of the clade was restricted geographically to the island of Shikoku (Figure 6.10d). All the plants on Shikoku were tetraploid as seen also in Figure 6.6. MPH 10 was found on Kyushu, Shikoku and the southern prefecture of Honshu, Yamaguchi, and was found in 12 octoploids and 28 tetraploids.

6.5.5.5 Unresolved group

The group of plants referred to as the unresolved group, comprises all those coloured black on both the majority rule and neighbour joining trees (Figures 6.7 and 6.8). On the majority rule tree most of these chloroplast types form an unresolved section at the base of the tree, the exceptions being those found at the base of the various clades as mentioned in section 6.5.3. Also mentioned in 6.5.3 was the fact that they fall into two separate areas of the neighbour joining tree.

Within the clades A-D were chloroplast types that matched the British invasive plants MPHs. The other non-Japanese plants included in the study were from China and Korea, where they are native. As shown in Table 6.8, these plants fell within this unresolved section, with no exact matches found from within Japan itself. Also within this group were *F. japonica* var. *terminalis* and *F. elliptica* (*comb. nov.*). The rest of the group was made up of both tetraploid and octoploid *F. japonica* var. '*japonica*', and two accessions of *F. japonica* var. *uzenensis*.

Figure 6.10e shows the distribution of the Japanese samples, and the relationship between these taxa and the other clades according to the neighbour joining tree. Chloroplast types 35, 31, 22, 7 and 30 all arise from a single point on the tree. MPH 35 at the base of this branch is found in both tetraploid and octoploid *F. japonica* var. '*japonica*'. It is found on Shikoku, Kyushu and two southern prefectures of Honshu, Tottori and Osaka. Genetically very close to MPH 35 is MPH 22, which is found in a single accession of tetraploid *F. japonica* var. '*japonica*' collected in Osaka. Chloroplast type 31 branches at a similar point to 35 and 22, and was found in six different accessions of tetraploid *F. japonica* var. '*japonica*' collected from three different sites in the most southern Honshu prefecture, Yamaguchi, and from a site on Shikoku. The other two chloroplast types in this group are MPH 7 and MPH 30. On the majority rule consensus tree they formed a group with a consensus value of 100, which was unsupported by bootstrapping. Each was only found in a single accession, type 7 was found in an *F. japonica* var. *uzenensis* collected from Niigata, which is north of the centre of Honshu, whilst MPH 30 was found in an *F. japonica* var. '*japonica*' on Shikoku.

On a separate branch, very close to the previous group was found three of the non-Japanese native Asian chloroplast haplotypes. These were 27, 23 and 40 and were found in the Chinese octoploid and decaploid accessions of *F. japonica* var. '*japonica*' and the Korean plant sent as *F. elliptica*.

Chloroplast type 19 was found very close to clade C on the neighbour joining tree but in the unresolved section of the majority rule consensus tree. This was found in a single accession of *F. japonica* var. *uzenensis* from Niigata, north of the centre of Honshu. This is within the geographical area occupied by the other members of clade C.

Chloroplast types 14 and 15 branched at close points, 15 in particular was on a long-branch. They were situated part way between the non-Japanese branch and clade A. Both were tetraploid *F. japonica* var. '*japonica*', MPH 14 being found in a single accession from Shikoku whilst MPH 15 was found in five accessions found at two sites on Shikoku and two sites in the southern Honshu prefecture Yamaguchi.

The two samples of *F. sachalinensis* from Korea with 2n = 102, were found to have MPH 29. This was positioned near to the clade D branch but with a relatively long terminal branch. Between this branch and clade B was the longest terminal branch on the tree that belonged to *F. japonica* var. *terminalis* sent from the isolated Hachijo islands to the south of Tokyo. Branching from a low point on this branch was another branch that terminated with MPH 13

and MPH 16. These two chloroplast types also grouped together on the majority rule consensus tree with a consensus value of 93% and a bootstrap value of 62. Both of these chloroplast types were found in single accessions of tetraploid *F. japonica* var. '*japonica*', MPH 13 from a plant collected from Shikoku and MPH 16 from the southern part of the island Kyushu.

### 6.5.6 *Biogeography of the main taxa*

#### 6.5.6.1 Tetraploid *Fallopia japonica*

In the study, 108 tetraploid *F. japonica* var. '*japonica*' accessions were analysed, including those presumed to be tetraploid due to similarity to known tetraploids from the same site with the same MPH. Of these plants 40 were recorded as being dwarf, 38 tall and the remaining 30 were either of intermediate or unknown height. Dwarf tetraploid *F. japonica* var. '*japonica*' plants were found at eight sites, as shown by yellow circles on Figure 6.11, situated on Kyushu, Shikoku and Honshu. As seen in Table 6.9 the majority of these were from the montane vegetation zone or higher, with only 1 lowland dwarf accession recognised in this study. Tall tetraploid *F. japonica* var. '*japonica*' plants were found at 25 sites, as shown by red triangles on Figure 6.11, situated on Kyushu, Shikoku and Honshu. Of those where the vegetation zone was known, the majority were found in lowland areas, with a few from montane zones and none from higher altitudes. The plants of intermediate height, or unknown height, were collected from 19 sites covering Kyushu, Shikoku and Honshu. These are shown as X symbols on Figure 6.11. Where the vegetation zone was known they were primarily lowland and montane accessions.

#### 6.5.6.2 Octoploid *Fallopia japonica*

In total, 57 octoploid *F. japonica* accessions were studied. Of these, 15 were *F. japonica* var. '*japonica*' which has trichome type A, as found in the introduced invasive clone of *F. japonica* var. *japonica*, 41 were classified as *F. japonica* var. *uzenensis* which has trichome type C and one appeared to be a hybrid between *F. japonica* var. *uzenensis* and an octoploid *F. japonica* var. '*japonica*'. Four MPHs were found to be present in the octoploid *F. japonica* var. '*japonica*', as shown in Table 6.10. As clearly seen the vast majority were MPH 10, with the remaining MPHs being found in only one accession each. These plants were distributed between 8 different sites in Japan, although due to close proximity of two of these, only seven can be distinguished in Figure 6.12. Shown as yellow circles, they are primarily found in the southern prefectures of Honshu, and the northern prefecture of Kyushu. The exception to this distribution is the spot in Aomori in the northern part of Honshu, which represents a single accession. The *F. japonica* var. *uzenensis* plants were collected from 24 sites, 21 of which

can be distinguished in Figure 6.12, and are shown as red triangles. These plants were found in the region to the north of the centre of Honshu, and growing as far north as the most northern Honshu prefecture, Aomori. The suspected hybrid was found in Aomori and is shown as a cross in Figure 6.12. Nine different MPHs were found in *F. japonica* var. *uzenensis*, and unlike the octoploid *F. japonica* var. '*japonica*', these were distributed more evenly across the accessions with only two MPHs being found in single accessions, as shown in Table 6.10.

#### 6.5.6.3 *Fallopia sachalinensis*

Forty-four *F. sachalinensis* accessions were analysed. These plants possessed eight different MPHs with a minimum of two accessions per haplotype as seen in Table 6.11. When analysed these fell in two very distinct clades, B and C (Figures 6.7 and 6.8). Those in clade B were found at ten different sites, including Hokkaido and northern Honshu, as indicated by the red triangles on Figure 6.13. The majority of the *F. sachalinensis* plants (37of the 44) possessed one of the 5 MPHs from this clade. The remaining 7 accessions were represented by 3 MPHs found in clade C. Shown as yellow circles on Figure 6.13, these plants were found at three sites that were restricted to Niigata, which is on the Japan Sea coastline just north of the central region of Honshu. The MPHs were not restricted to one MPH per site; at two of the three sites two of the three MPHs were found.

## 6.6 Discussion

### 6.6.1 *Plant identification*

A molecular study without proper identification of the study species can often lead to confusion and misinterpretation of data, particularly if hybridisation has occurred but not been recorded. Native Japanese Knotweed is renowned for its highly variable morphology resulting in taxonomic confusion (Inamura *et al.*, 2000; Kim & Park, 2000) however, there is clearly a need for an anatomical and morphological approach to complement the molecular and cytological analysis. In this study epidermal striation, trichome type, leaf shape characteristics, and plant height have been used to aid identification. These characteristics were not always easy to determine. Ideally for identification large fully mature leaves should be used but these were not always available. In many instances the native plants were found to have suffered greatly from predation leading to leaf damage, sometimes to the point of having almost no leaves to study. Specimens imported and grown in quarantine were often kept in small pots due to restricted greenhouse space and this may have affected the leaf shapes. New leaves sometimes appeared to be hastate until the plant grew to a larger size. Based on a number of these characteristics the majority of the plants used in this study could

be classified as *F. japonica*, within which one variety, *F. japonica* var. *uzenensis,* was recognised, or *F. sachalinensis*. Those of intermediate morphology were assumed to be of hybrid origin, although exact parental combinations could not always be determined.

The combined use of cuticular ornamentation and trichome type used by Bailey (1989) for plants in the introduced range does appear to be a useful tool in differentiating between the taxa in their native range. As is the case in the introduced range, *F. japonica* was found to be far less striate than *F. sachalinensis*. Young fresh *F. sachalinensis* leaves were found to be as striate as the more mature leaves from the herbarium specimens, which meant that the inclusion of younger material was not detrimental to this aspect of the morphological analysis. Trichome characters, as determined from the introduced plants, were also applicable to the native material. All *F. japonica* tetraploids and some of the octoploids had type A trichomes, defined as swollen oval cells as opposed to multicellular trichomes. This is in accordance with *F. japonica* var. *japonica* and *F. japonica* var. *compacta* in the introduced range. The remainder of the *F. japonica* octoploids were found to have uniseriate multicellular trichomes of 3 – 5 cells in length. These are referred to as type C trichomes and define *F. japonica* var. *uzenensis*. Unlike the thin-walled often collapsed trichomes found in *F. sachalinensis*, the *F. japonica* var. *uzenensis* trichomes are rigid and arise perpendicular to the vein. The majority of *F. japonica* var. *uzenensis* trichomes are more densely distributed than any found on *F. sachalinensis*, and their trichomes are present in very young fresh material as well as the more mature leaves. Mature *F. sachalinensis* leaves were found to possess long uniseriate trichomes (type B) although the distribution of these ranges from dense to very scarce. This is also true in introduced plants. Younger *F. sachalinensis* leaves are often missing these trichomes, however the veins are smooth on both mature and young *F. sachalinensis* leaves. In *F. japonica* var. '*japonica*' the veins appear to be bumpy or ridged because of the presence of the swollen cells. Therefore, even in younger specimens *F. sachalinensis* and *F. japonica* var. '*japonica*' individuals can be distinguished.

In the introduced range intraspecific hybrids between *F. japonica* var. *japonica* and *F. japonica* var. *compacta* cannot be distinguished on the basis of trichome type and cuticular striation, but the interspecific hybrid *F.* x *bohemica* can be clearly recognised. *F.* x *bohemica* has an intermediate degree of striation (normally level 2 – 3, Figure 6.2) and trichome type D. Two hundred and forty six native accessions of Japanese Knotweed were analysed for this study. From these 27 were identified as putative hybrids, 25 hexaploids, 1 octoploid and 1 tetraploid. There was a much greater range in trichome type and striation in these hybrids than is experienced in the introduced range. Table 6.12 lists these putative hybrids and also

includes information about where they were found growing, their chromosome number, lower epidermal characteristics and putative parentage. As can be seen, very few of these plants had trichome type D, which in the introduced range is found on all interspecific hybrids. This could be because octoploid *F. japonica* var. '*japonica*' was almost never found growing sympatrically with *F. sachalinensis*. In this study only one such specimen was found in Aomori. For the majority of these hybrids *F. japonica* var. *uzenensis* appeared to be one of the parents. This taxon is often found to be sympatric with *F. sachalinensis*. As no controlled crosses have been studied with this parentage it is hard to predict what the trichomes of a hybrid between plants with trichome type C and those with type B would look like.

The putative parents on Table 6.12 fell into three categories, interspecific hybrids (a, b and c), intraspecific hybrids (d and e) and non-hybrids (f and g). The non-hybrid category was used for hexaploid individuals that appeared to have the normal characteristics associated with the tetraploid *F. sachalinensis* (f) or the octoploid *F. japonica* var. *uzenensis* (g). Hexaploid *F. sachalinensis* can be produced when an unreduced gamete pollinates or is pollinated by a normal reduced gamete, and is found naturalised in the Czech republic. Hexaploid *F. japonica* var. *uzenensis* is more difficult to explain. There are no reports of tetraploid *F. japonica* var. *uzenensis*, and no such specimens were found during this study. Whilst unreduced gametes are known to occur, for a gamete to be produced with a quarter of the sporophytic number of chromosomes rather than the normal half is very unlikely. Seed from native Japanese *F. japonica* var. *uzenensis* counted in Leicester by Bailey (pers. comm.) was found to contain both hexaploids and octoploids. So either some mechanism is occurring that produces a diploid gamete from an octoploid or these plants are being pollinated by a tetraploid. Either a tetraploid *F. japonica* var. *uzenensis* exists but has not been recorded or the hexaploid *F. japonica* var. *uzenensis* accessions are of hybrid origin. Given the very low level of striation, one would predict they were intraspecific hybrids with tetraploid *F. japonica* var. '*japonica*'. Nineteen plants with this potential parentage were indicated in Table 6.12, however in thirteen of these cases no tetraploid *F. japonica* var. '*japonica*' has so far been found in the same prefecture. These were all collected in Aomori where the only recorded tetraploid was *F. sachalinensis*. So either tetraploid *F. japonica* var. '*japonica*' exists here but was not found in this study, or *F. sachalinensis* was involved in these hybridisation events, but the resultant hybrids were far less striate than the hybrids found in the introduced range with *F. sachalinensis* as a parent. An alternative theory is that there are cases of *F. sachalinensis* not having a highly striate lower epidermis. This could explain the tetraploid hybrid shown in Table 6.12 that appeared to have type B (*F. sachalinensis*) trichomes but almost no (level 1) epidermal striation. This plant was predicted to be a cross

between *F. sachalinensis* and tetraploid *F. japonica* var. '*japonica*' but is in fact less striate than usually associated with this combination of parentage, and has hairs more like pure *F. sachalinensis* than tetraploid *F.* x *bohemica*. Additionally it was found in Aomori where no tetraploid *F. japonica* var. '*japonica*' has been recorded. It should however be noted that this survey was not exhaustive, and in Aomori no site above 258 masl was investigated. Tetraploid *F. japonica* var. '*japonica*' is often found at higher altitudes than this and therefore could have been missed. In the past only *F. sachalinensis* was found growing on Mt. Hakkoda in Aomori, but in recent years *F. japonica* has been introduced as roadside cuttings and is reported to be growing gregariously (Yonekura, K. pers. com.). It is not known whether the introduced *F. japonica* was pubescent or not, nor at what ploidy level it is found. It is also not known where the cuttings have been brought in from.

Assuming the hexaploids that could be pure *F. sachalinensis* were hybrid, the most plausible parentage, given their overall characteristics, was *F. sachalinensis* and *F. japonica* var. *uzenensis*. These plants were more striate than would be expected from this combination. It is clear that epidermal striation and trichome type are hard to predict in these putative hybrids. A study of these characteristics in controlled reciprocal crosses would be advisable, to investigate any effect caused by the direction of hybridisation.

Leaf shape was less useful than epidermal characteristics in identifying the main taxa. The leaves of many of the plants were recorded as approximately truncate, or approximately cordate as it was often difficult to judge. Two leaves from the same stem would appear to be different from each other, so an average impression would be recorded. The characteristic shapes from the introduced range as depicted in Figure 2.1 were not as applicable in the native range. Whilst it held that *F. sachalinensis* leaves were larger and broader with basal leaves being ovate to oblong with cordate base (Bailey *et al.*, 1996; Bailey *et al.*, 1995), the reference specimens from the introduced range of *F. japonica* var. *japonica* and *F. japonica* var. *compacta* were of less use. For example, in general the leaf-shape for dwarf plants growing on Mt. Fuji was very similar to the type specimen of *F. japonica* var. *compacta.* The leaves of the dwarf plants growing on Mt. Aso, however, were very small and proportionately longer compared to their width, than the reference specimens of either variety found in the introduced range. Additionally these leaves were closer to being cordate than truncate. Some leaves collected from plants on Shikoku had characteristics reminiscent of *F. forbesii* found in Korea. Unfortunately a confirmed specimen of Korean *F. forbesii* was not available for this study, but its specific status has often been questioned and some authors believe it to be a synonym for *F. japonica* (Kim & Park, 2000). Kim and Park (2000) studied 18 leaf size and

shape characters, six of which were derived ratios, for *Fallopia* plants collected in Korea. They used a principal components analysis (PCA) to compare the measurements and found *F. forbesii* to be clearly distinguishable from *F. japonica* and *F. sachalinensis*, but no differences between tetraploid, hexaploid and octoploid *F. japonica*. They concluded that *F. forbesii* was a genuine species. There is some contention regarding the use of derived ratios and original measurements in a single analysis, the argument being that doing so gives extra weight to certain characteristics. The authors found hexaploid and octoploid *F. forbesii* but no tetraploid, leading to the question if *F. forbesii* is a genuine species how did the hexaploid arrive without a tetraploid, unless the tetraploid was around until relatively recently but is now either extinct or very rare. Another feature of note is that the octoploid *F. forbesii* grouped closer to *F. japonica* than did the hexaploids. The type specimen of *F. forbesii* is characterised by relatively small orbicular leaves with round bases, and was found in all hexaploids and one population of octoploids in Korea (Kim & Park, 2000). The remainder of the Korean octoploids were found to be intermediate between the type specimen of *F. forbesii* and *F. japonica*, having the rounded base characteristic of *F. forbesii* but slightly narrower tapering upper leaf portions similar *to F. japonica*. Genetic analysis of *F. forbesii* may help to answer the question regarding its specific status and help to elucidate whether the Shikoku specimens with similar features are genetically similar.

Plant height was a feature that could only be recorded in the field. To describe an individual as tall was relatively straight forward, although plants that had not reached their maximum growth could have been omitted from the group. To describe a plant as dwarf was a harder process. In many instances the plants had the appearance of having been cut down, and the regrowth may have been less than the arbitrary dwarf cut off point of 80 cm when the plant itself, if allowed to grow without interference, may have grown a lot higher. Restricting the categorisation to plants that have reached the stage of flowering, as was done in this study, can mean that genuine dwarf plants were not recognised. The other factor that needs to be considered is whether plant height is recorded as how high the highest point is from the ground, or how long is the longest stem. Often stems are not fully erect, and this could lead to confusion particularly when different people have been collecting the specimens and no standard was set. Some of the specimens on Mt. Aso were relatively long and if measured after being removed from the ground may not have been classed as dwarf, but if measured *in situ* as how high the clump arises from the soil, they would. Regardless of the difficulties, plant height is still an important character to measure, given that having a dwarf stature is one of the characteristics that distinguish *F. japonica* var. *compacta* in the introduced range.

6.6.2 *Distribution of the taxa*

6.6.2.1 *Fallopia sachalinensis*

As seen in both Figure 6.6 and 6.13, *F. sachalinensis* was collected from the northern part of Japan only. This represents the majority of its natural distribution for Japan, although it does extend slightly further to the south. The southernmost natural habitat for the distribution of *F. sachalinensis* is Fukui, where 23 records have been recorded in the north of this prefecture (Kanai, 1992). Moving east across Honshu, *F. sachalinensis* is found in the northernmost part of Gifu (11 records) (Kanai, 1993), the northernmost part of Gunma (22 records) (Kanai, 1996), and the western side of Fukushima (47 records) (Kanai, 2000).

As seen in Figure 6.13 there is a clear geographical separation between the *F. sachalinensis* plants with chloroplast haplotypes within clade C, and those found in clade B. Those with MPHs within clade C were only found in Niigata, whilst those from clade B were found further north, from Yamagata/Miyagi up to and including Hokkaido. The native distribution of *F. sachalinensis* is known to continue north of Hokkaido to include the southern part of Sakhalin Island and the southern Kurile Islands (Kunashir and Shikotan); the latter are part of the former USSR (Sukopp & Starfinger, 1995). As plants from these regions were not sampled for this study it is impossible to state whether they would have had MPHs that would have been within clade B, or would have been genetically distinct from the Japanese material. In a study of chloroplast diversity within Japanese *Primula cuneifolia* Ledab, plants from the north of Hokkaido were distinct from those in the main part of Hokkaido (Fujii *et al.*, 1999), where the Hokkaido specimens used in this study came from.

Ullung Island is the only area of Korea known to have *F. sachalinensis.* This island is situated 150 km east of peninsula Korea, and is about 1.8 million years old. There are reported to be 37 endemic angiosperm taxa, representing 25 different families. The presumptive progenitors to the endemic taxa are believed to have come mainly from peninsula Korea and Japan, with lesser connections to the Sakhalin Islands and other northern areas (Weiss *et al.*, 2002). In the case of *F. sachalinensis*, given its absence from mainland Korea, it presumably came from either northern Japan, or the Sakhalin Islands. The *F. sachalinensis* on Ullung Island is normally dodecaploid (2n = 132) although the sample included in this study was 2n = 102. This is discussed further in section 6.6.5.3 below.

6.6.2.2 *Fallopia japonica*

*F. japonica* is found growing on three of the four main islands of Japan, Shikoku, Kyushu and Honshu. Besides *F. japonica* var. '*japonica*', one additional variety has been recognised in

this study from the plants collected on these islands and that is *F. japonica* var. *uzenensis*. *F. japonica* var. *terminalis* has also been included in the molecular analysis but this was not found on the main Japanese islands. For the purposes of discussing the distribution of *F. japonica* var. '*japonica*' the classifications tall and dwarf, and tetraploid and octoploid, have been used, and references to the altitude at which the plants were found have also been made.

*F. japonica* var. *'japonica'* was found throughout Shikoku, Kyushu, and Honshu, although in the north of Honshu it was less common, *F. japonica* var. *uzenensis* being more common. Samples have also been obtained from Korea and China, but none was available from Taiwan, where it is also considered to be native. Given the limited number of samples from non-Japanese native countries this section of the discussion will deal only with Japanese *F. japonica* var. '*japonica'*. Given that the introduced clone of *F. japonica* var. *japonica* is octoploid, it is of interest to compare the distribution of octoploid and tetraploid *F. japonica* var. '*japonica'*. Only fifteen octoploid *F. japonica* var. '*japonica'* plants were found in this study, from 7 sites in Japan as shown in Figure 6.12. Primarily found in the southern prefectures of Honshu, and the northern prefecture of Kyushu, there was an exception in a single accession from Aomori. Most of the *F. japonica* plants in this northern region were *F. japonica* var. *uzenensis*, so this single accession of octoploid *F. japonica* var. '*japonica'* may have arisen in this location due to the actions of man. In comparison to the relatively rare octoploid, 108 tetraploid *F. japonica* var. '*japonica'* plants were found in this study. The greater number of tetraploid plants is not surprising, as it is presumed that the octoploids arose as a result of chromosome doubling from a tetraploid, and unless these have some sort of selective advantage that would cause them to out-compete the tetraploids, one would expect to find more at the tetraploid level. These tetraploids were found all over Shikoku, Kyushu and southern and central Honshu as seen in Figure 6.11. In fact, as seen in Figure 6.6, in many of these prefectures and for the whole of Shikoku, tetraploid *F. japonica* var. '*japonica'* was the only taxon found in this study.

Tetraploid *F. japonica* var. '*japonica'* appears to be well adapted to a variety of Japanese habitats. It can be found growing in both lowland and high altitudinal areas, with the upper distribution limit on Mt. Fuji being 3776 masl (Mariko *et al.*, 1993). The numbers of accessions were split relatively evenly by height between plants considered to be dwarf, intermediate (includes unknown), and tall, 40, 30 and 38 respectively. The dwarf plants however were only found at 8 sites, whilst the intermediates came from 19 different sites and the tall plants from 25 (see Figure 6.11). This reflects two factors, the first being that primarily dwarf plants were only found at high altitudes and fewer sites of high altitude were

visited for this study. The second factor is that at the higher altitude sites there were large numbers of what appeared to be genetically diverse populations of *F. japonica* var. '*japonica'*. At the lowland sites, however, where the tall and intermediate plants tended to be found, *F. japonica* var. '*japonica'* tended to be found as single well-separated stems (Bailey, 2003), in very low numbers. Geographically these sites were spread evenly throughout the area of study, with dwarf, intermediate and tall plants being found on all three islands. There may appear to be a dominance of tall plants from Shikoku, with 12 out of the fifteen plants from here falling into this category, but this merely reflects the fact that the collector was asked to specifically look out for taller specimens.

*F. japonica* var. *uzenensis* (*Polygonum cuspidatum* var. *uzenensis*) is naturally distributed in the Hokuriku and Tōhoku districts of Honshu (Inamura *et al.*, 2000). As seen in Figure 6.12, specimens from a large part of this range were collected and analysed for this study, and no specimens were found outside of these districts. For most of this distribution it is sympatric with *F. sachalinensis*.

*F. japonica* var. *terminalis* is endemic to a chain of islands to the south of Honshu, known as the Izu islands (Bailey, 2003). Synonyms include *Reynoutria hachidyoensis* var. *terminalis* Honda, fide Ohwi and *Polygonum cuspidatum* var. *terminalis*. Hachijō-jima is one of the larger of the Izu islands. It is approximately 70 $km^2$ in area, and is located about 300 km south of Tokyo, with a population of about 9,500. The island has two large volcanoes, Mt. Nishiyama and Mt. Higashiyama (Yamashita *et al.*, 2000). The specimen in this chapter referred to as *F. japonica* var. *terminalis* was sent as seed from Hachijō-jima. This taxon has also been recorded from two of the other Izu islands, Oshima and Miyakejima (Inamura *et al.*, 2000).

6.6.2.3 Hybrids

Hybrid plants can arise anywhere where the parental taxa are sympatric. In Japan *F. sachalinensis* and *F. japonica* are found in most of the northern prefectures that make up the Hokuriku and Tōhoku districts of Honshu. For the best part of this range it is the octoploid *F. japonica* var. *uzenensis* that is present, meaning the hybrids would most likely be hexaploid. Whilst both species are common in the lowlands in northern Honshu, in the natural undisturbed habitat they rarely occur together. It is in the disturbed areas around urban developments that mixed populations occur and hybridisation has occurred. The hybrid was recently (1997) described as *Reynoutria* x *mixushimae*. It is believed that the hybrid rarely occurred in the past in Japan (Yonekura, K., pers. com.).

In this present study twenty-seven putative hybrids were identified, although as discussed above some of these may have been intraspecific *F. japonica* hybrids as opposed to the interspecific hybrid between *F. japonica* and *F. sachalinensis*. The majority of these were found growing in Aomori prefecture as seen in Table 6.12. Twenty-three of these plants were confirmed hexaploids, with two more being suspected hexaploids. Only two of the suspected hybrids were found from the more southern part of the Hokuriku and Tōhoku districts of Honshu, from the prefecture Niigata, and both were collected from the same site, site 50. As seen in Table 6.2, this site was a scrubland area from which four plants were collected. From Table 6.4 it can be deduced that the other two accessions were *F. sachalinensis* plants with MPH 6. The putative hybrids from this site had MPHs 9 and 18. Both MPHs are found within clade A, which is mainly comprised of *F. japonica* var. *uzenensis* plants. Moving north of Niigata into the prefectures Miyagi and Yamagata, three were found in Yamagata each from different sites (67, 69 and 70), whilst five were found from the same site in Miyagi. The Miyagi site (site 77) represents a streamside population in a river floodplain from which ten plants were collected. The other five plants collected here were all tetraploid *F. sachalinensis* accessions with MPH 11. None of the hybrids had inherited this MPH; in fact all five hybrids had chloroplast MPHs that fell within clade A (MPHs 5, 9 and 17), which is mainly comprised of *F. japonica* var. *uzenensis*. This would imply that *F. japonica* var. *uzenensis* was the maternal parent for the hybrids from both Niigata and Miyagi, even though none was found at the sites. This could mean that the hybrid plants had been introduced from another area. With regards to the Yamagata putative hybrids, two of the three sites in which they were found were roadside, and the third was a garage forecourt. All of these are disturbed sites. Only one other accession was found at one of these sites (site 70) and that was an *F. sachalinensis* with MPH 37. Two of the three hybrids had MPH 4, the third MPH 33. All three MPHs, including that of the *F. sachalinensis,* fell within clade B, which is comprised of *F. japonica* var. '*japonica', F. japonica* var. *uzenensis* and *F. sachalinensis* plants.

The remaining seventeen of the twenty-seven putative hybrids were all collected in Aomori, from six different sites (sites 79 – 84 inclusive). There was only one Aomori site that did not yield a putative hybrid, and that was site 85 from which a single plant was collected, the sole octoploid *F. japonica* var. '*japonica'* found in Aomori. These six sites include a riverside that has been disturbed by man, a park, and several roadside sites all of which can be classed as disturbed. One of these six sites was a woodland margin, which was believed to be a natural habitat for these plants. Of the putative hybrids fourteen had MPH 8, as did the sole octoploid *F. japonica* var. '*japonica',* three octoploid *F. japonica* var. *uzenensis* accessions and four *F.*

*sachalinensis* from Aomori. One of the hybrids had MPH 28, which from Tables 6.6 and 6.7 can be seen to differ from MPH 8 by a probable length polymorphism of one band in one of the six regions (*trn*H-*trn*K haplotype A (MPH 8) has a band of length 209 bp, whilst C (MPH 28) has a band of length 222 bp). MPH 28 was not found in any other accession in this study. The final two putative hybrids found in Aomori had MPH 2, as did two *F. sachalinensis* accessions also found in Aomori. Besides MPHs 2, 8 and 28 the only other MPH found in Aomori was MPH 11 found in a single accession of *F. japonica* var. *uzenensis*. Unlike MPH 28, MPH 11 was also found in other accessions in other prefectures of Honshu.

There are a number of issues that this concentration of hybrids in Aomori, and the MPHs that they possess raises. Why were there more putative hybrids in Aomori than any other prefecture? Why do most of them have MPH 8? Is there a reason why MPH 8 is also found in what is believed to be *F. japonica* var. '*japonica*', *F. japonica* var. *uzenensis* and *F. sachalinensis*? With regards to the first issue, there is probably a higher incidence of hybrids in Aomori than the other prefectures due to the fact that both *F. sachalinensis* and *F. japonica* are being actively planted in this prefecture (Yonekura, K., pers. com.). This planting brings the two species together, and creates a disturbed habitat for any hybrids produced to establish themselves in. That is assuming the hybrids are produced *in situ*. There is also the possibility that instead of planting the species, hybrid plants are being planted. As for the predominance of MPH 8, there could be many explanations. If the hybrid plants are indeed being planted, they may all have originated from the same nursery from the same hybridisation event. They may even represent different accessions of the same clone. This does not explain how both of the parental types also possess this MPH. As seen in Figure 6.10b, MPH 8 was only found within Aomori, and it is possible that a selective sweep of this chloroplast type has occurred. Selective sweeps of chloroplast types will be discussed more fully later. Alternatively what have been taken to be pure samples of the parental species may have arisen through a series of hybridisation and backcrossing events leading to the introgression of one chloroplast type into what appears to be "pure" representatives of the parental taxa. To fully understand the relationship between these plants in Aomori further molecular and morphological analysis would be required.

### 6.6.3 *Relationships between the taxa*

The relationships between the taxa have been determined by the RFLP analysis of six different regions of the chloroplast genome. For each region only a sub-set of the bands produced by the digest was scored due to the limitations of the resolution system used. This meant that restriction fragments rather than restriction sites were used as characters for the

analyses, which does not distinguish between length mutations (i.e., insertions, deletions and microsatellites) and substitutions in restrictions sites. Length mutations in restriction enzyme studies usually occur in spacers between genes and pose problems for phylogenetic analysis because they tend to cluster in "hotspot" regions having high level of variability. These can often result in a high level of homoplasy within a data set and are therefore usually excluded from formal phylogenetic analysis (Palmer *et al.*, 1988).

The restriction enzyme selected for this study was *Hin*fI. *Hin*fI recognizes a 5 bp sequence starting with GA, ending in TC, and having any base between. It is often referred to as a "frequent-cutter". It was selected for this study because it was shown in preliminary experiments to detect more variation than some of the other available restriction enzymes. The results of these preliminary experiments were described in chapter 3. This investigation is concerned with detecting variation in the chloroplasts of native material, and aimed among other things to determine the origin of the introduced material. For this purpose the high levels of variation detected by *Hin*fI was ideal. The negative side to the use of *Hin*fI is that it cuts so frequently, that a large number of fragments with a wide range of size are produced. It was not possible to resolve all bands on one simple gel system; therefore it is impossible to state with any conviction what mutation occurred that led to the different bands that were scored.

Whilst a formal phylogenetic analysis cannot be made from the data in this study, and a suitable outgroup that would be required to infer evolutionary information is missing, it is still possible to gain some insight into the relationships between the different taxa. But before discussing the relationships between the different taxa, the MPHs and the clades must be considered.

Two different means of analysing the data were used to produce the trees, from which the clades were inferred. Neighbour joining analysis based on the Jaccard's similarity co-efficient, and a heuristic parsimony analysis performed by PAUP. In both instances bootstrapping was completed to produce confidence values. Bootstrapping involves random sampling with replacement of a set of characters until a replicate data set of the same size as the original is constructed. This replicate data set is then analysed, and a phylogenetic tree reconstructed. This process is repeated a specified number of times, and the results are then summarised as a bootstrap consensus tree. Unfortunately bootstrapping of large data sets can often be problematic. These analyses if done thoroughly and with a large number of replicates, can be very time consuming and in some cases may not be practical (Mort *et al.*,

2000). One of the alternative ways of assessing support is to use a simpler search strategy that does not perform branch swapping, such as the fast "pairwise-step" analysis performed in this study for the parsimony analysis, using PAUP 4.0 beta version (Swofford, 2003). Mort *et al*. (2000) compared three methods for estimating internal support of phylogenetic trees, including the fast bootstrapping method used in this study. They found that fast bootstrapping provided results that were similar to, although statistically less than, the values resulting from bootstrap analyses with branch swapping. The differences in the values were greater at nodes that received less support. For nodes receiving support of 75 % or greater, the difference between fast bootstrapping and bootstrap analyses with branch swapping was often within a few percent.

The two trees produced were compared to determine the clades used in this study. The major structure of the tree is consistent for both trees, with the main discrepancies being the positioning of certain MPHs. For example, on the majority rule consensus tree (Figure 6.7) MPH 39 is at the base of clade B and the group consisting of MPHs 13 and 16 is at the base of clade D. On the neighbour joining tree (Figure 6.8), these three MPHs are positioned on a single branch that lies between clades B and D, albeit closer to clade B. It is possible that it is the inclusion of MPHs such as these that has led to the low levels of support for these two clades. That aside there is relatively strong support for both clades A and C (bootstrap values of 72 and 74 respectively on the majority rule consensus tree), which also show stronger geographical clustering (Figure 6.9).

Excluding the MPHs assigned to plants from China and Korea, there were seventeen MPHs that were only found in single plant accessions. In numerical order they were 7, 13, 14, 16, 17, 18, 19, 22, 24, 25, 26, 28, 30, 32, 33, 36, and 39. Apart from number 39 that was found in *F. japonica* var. *terminalis*, the remainder were found in accessions of *F. japonica* var. '*japonica'* (10), *F. japonica* var. *uzenensis* (2) and putative hybrids (4). This high proportion of unique MPHs may lead to the suspicion that this variation is caused by a high number of, what are considered in evolutionary terms, minor mutations such as length mutations. Whilst it is true that some of these rare MPHs may be caused by a single simple mutation from their genetically nearest MPH, that does not mean they are not significant, and many of them have more than one difference between themselves and the next nearest MPH.

There were five cases in this study of MPHs being shared by more than one taxon. Whilst there is the possibility that the different taxa with the same MPH have variations in regions of the chloroplast that were not screened, it would still imply a high level of similarity. One of

these five is MPH 1, which had a single accession of *F. japonica* var. '*japonica'* and five accessions of *F. japonica* var. *uzenensis* as well as the British clone of *F. japonica* var. *japonica*. These represent different varieties of the same species and the extent of the difference between them is not known. The remaining four represent cases of different species sharing a MPH. These are MPHs 4, 8, 11 and 20. MPH 4 is predominately comprised of *F. japonica* var. '*japonica'* with the addition of two accessions of *F. sachalinensis* and two of *F. japonica* var. *uzenensis.* MPH 8 was mainly found in putative hybrid plants from Aomori, but additionally three accessions of *F. japonica* var. *uzenensis* and two of *F. sachalinensis,* also from the prefecture Aomori. MPH 11 was predominantly *F. sachalinensis* but was also found in one accession of *F. japonica* var. '*japonica'*, and two accessions of *F. japonica* var. *uzenensis.* MPH 20 was found in two accessions of *F. japonica* var. '*japonica'* and two of *F. sachalinensis*. This sharing of MPHs between closely related species that readily hybridise is not uncommon and a number of hypotheses to explain this phenomenon have been proposed. The most common reason given is chloroplast capture, without transfer of nuclear genes, via introgressive hybridisation. Cases of chloroplast capture have been recorded in *Alnus* (King & Ferris, 2000), *Quercus* (Dumolin-Lapegue *et al.*, 1997; Whittemore & Schaal, 1991), *Salix* (Brunsfeld *et al.*, 1992) and others. The study by Inamura *et al*. (2000) that looked at sequence variation within *F. japonica* but under the name *Polygonum cuspidatum*, only analysed three accessions of *F. sachalinensis*. Whilst these three accessions did not share chloroplast haplotypes with *F. japonica*, the *F. sachalinensis* haplotypes were dispersed among those of *F. japonica* and fell into two different clades. They believed this was the result of cytoplasmic gene flow between the two species.

Whilst there are instances of shared MPH between the different taxa, there are many MPHs that appear to be taxon specific. Table 6.13 shows the distribution of the MPHs between the three main taxa, and the clades. There are four MPHs that are only found in *F. sachalinensis*, five in *F. japonica* var. *uzenensis* and seventeen in *F. japonica* var. '*japonica'*. Whilst it may appear that there is more variation therefore in *F. japonica* var. '*japonica'*, the number of accessions sampled also plays a part. There were 127 accessions of *F. japonica* var. '*japonica'* in this study, but only 41 *F. japonica* var. *uzenensis* and 44 *F. sachalinensis*.

In this study the *F. japonica* plants from Honshu, Shikoku, and Kyushu have been split into two varieties on the basis of trichome type, *F. japonica* var. '*japonica'* with trichome type A and *F. japonica* var. *uzenensis* with trichome type C. In the introduced range another variety is recognised, *F. japonica* var. *compacta.* In the introduced range this taxon can be clearly

distinguished from the octoploid *F. japonica* var. *japonica* clone. The study by Kim and Park (2000) looking at morphological variation in native Korean specimens does not recognise *F. japonica* var. *compacta* as deserving taxonomic recognition and quotes three other authors who share this view. Likewise the molecular study by Inamura *et al*. (2000) looking at Japanese accessions found it not to be differentiated genetically, and suggested that it was merely an alpine ecophene. From a native plant perspective this study would be inclined to agree with their position. Both dwarf and tall plants share the leaf-shape that characterise *F. japonica* var. *compacta*, and there are many dwarf specimens that do not possess these characteristics. From a molecular perspective there was no genetic distinction found between the dwarf and tall plants, the majority of which are found to share MPHs.

Octoploid plants are generally believed to have arisen from a chromosome-doubling event in a tetraploid. As seen in Table 6.10, four different MPHs were found in octoploid *F. japonica* var. '*japonica'*. In terms of numbers of accessions the majority of the octoploid *F. japonica* var. '*japonica'* plants (12 out of 15) had MPH 10, which is also found in a further 28 tetraploid *F. japonica* var. '*japonica'* accessions. One octoploid *F. japonica* var. '*japonica'* accession had MPH 35, which is also found in 9 tetraploid accessions. These results would indicate that chromosome doubling has probably occurred on at least two occasions. The remaining two accessions had MPH 1 and MPH 8 respectively. No tetraploid plants were found in this study with MPH 1, and the only tetraploids with MPH 8 were *F. sachalinensis*.

The other octoploids in this study were accessions of *F. japonica* var. *uzenensis*. As seen in Table 6.10, these plants were divided between eight different MPHs. Five of these MPHs are considered to be specific to *F. japonica* var. *uzenensis* and were therefore not found in any tetraploid plants. One of the nine was MPH 1, which was also not found in any tetraploids. The remaining three were MPHs 4, 8, and 11. MPHs 4, 8 and 11 were found in both tetraploid *F. japonica* var. '*japonica'* and *F. sachalinensis*. MPH 4 was predominantly tetraploid *F. japonica* var. '*japonica'* (21 accessions), with only two accessions of *F. japonica* var. *uzenensis* and two of *F. sachalinensis*. MPH 8 is the chloroplast type found exclusively in Aomori, and predominantly in putative hybrid plants. As explained above it was also found in octoploid *F. japonica* var. *japonica*, but the only tetraploids in which it was found were *F. sachalinensis*. MPH 11 was found in two accessions of *F. japonica* var. *uzenensis*, one accession of tetraploid *F. japonica* var. '*japonica'* and 12 accessions of *F. sachalinensis*. The shared MPHs would suggest that either the MPH got into the *F. japonica* var. *uzenensis* by chloroplast capture, or that the octoploid *F. japonica* var. *uzenensis* arose from a chromosome doubling event from one of the tetraploid taxa or a hybrid between the two

species, then a further mutation occurred which lead to the production of the type C trichomes characteristic of *F. japonica* var. *uzenensis.* The five MPHs specific to *F. japonica* var. *uzenensis* may have evolved since the event that produced one of the shared MPHs found in *F. japonica* var. *uzenensis* and other taxa, or may represent the true chloroplast types for this variety of *F. japonica*, whilst the shared ones are the product of hybridisation and introgression.

Not enough is known about *F. japonica* var. *uzenensis* to lead to any firm answers as to how this taxon has evolved. Plants of this morphological type have been accorded either specific or varietal status depending on the researcher. How genetically different this taxon is from *F. japonica* var. '*japonica'* is not known. The study by Inamura *et al*. (2000) found this taxon to form a monophyletic group within their consensus tree, with the exception of one accession, which shared a chloroplast haplotype with an accession of *Polygonum cuspidatum* (*F. japonica* var. '*japonica'*) and formed a clade with two accessions of *Polygonum sachalinense* (*F. sachalinensis*). They proposed that *F. japonica* var. *uzenensis* was a valid taxon since it formed a monophyletic group, and that the one aberrant accession, was the result of cytoplasmic gene flow. The current study, which includes far more accessions than that of Inamura *et al*. (2000), portrays a similar picture. Clade A is comprised predominantly of *F. japonica* var. *uzenensis* and may represent a monophyletic group. The majority of *F. japonica* var. *uzenensis* accessions (32 out of 41 accessions) were found within this clade. However, unlike the Inamura *et al*. (2000) study, which found one exception to this trend, this study had 9 exceptions, which represents 22% of the total number of accessions of *F. japonica* var. *uzenensis* analysed. Two MPHs specific to single accessions of *F. japonica* var. *uzenensis* were found within the unresolved group. Three MPHs representing 7 accessions of *F. japonica* var. *uzenensis*, but also found within other taxa, were found within clade B.

*F. japonica* var. *terminalis* was found to be genetically distinct from the other *F. japonica* accessions in this study. In three of the six chloroplast regions listed in Table 6.7, *F. japonica* var. *terminalis* had a unique chloroplast haplotype. In the neighbour joining tree, MPH 39 found in *F. japonica* var. *terminalis* had the longest terminal branch indicating that it has been separate from the other taxa for a long period. This is in agreement with the study by Inamura *et al*. (2000).

The plants of Chinese and Korean origin have been known by many names. Sometimes they are grouped with *F. japonica*, on other occasions they are given a different varietal name or have been treated as a different species. In Korea, one taxon in particular that there is some

contention over the naming of is *F. forbesii*. *F. forbesii* is given as a synonym for *Reynoutria elliptica* (Kim & Park, 2000). This study included a specimen of *R. elliptica*, although it was recorded as *F. elliptica* on Table 6.3. Whilst having a unique MPH (40) it formed part of the unresolved group (Figure 6.8) and was on the same branch as the Chinese octoploid and decaploid accessions of *F. japonica*. The *F. elliptica* used in this study was octoploid and differed from the octoploid Chinese *F. japonica* (MPH 27) in only one of the six chloroplast regions (*trn*M-*rbc*L, Table 6.7). The octoploid Chinese *F. japonica* differed from the decaploid Chinese *F. japonica* in one region (*trn*C-*trn*D), meaning there were two chloroplast regions that differed between *F. elliptica* and Chinese decaploid *F. japonica*. From Table 6.7 it can be seen that the difference between the Chinese accession of octoploid *F. japonica* and the Korean *F. elliptica* (*trn*M-*rbc*L haplotypes B and F) was a single band about 5 base pairs longer in haplotype F. This could be caused by a mono-nucleotide repeat or microsatellite. The difference between the Chinese accessions of *F. japonica* (*trn*C-*trn*D haplotypes D and G) is the absence of a 172 bp band in G (the decaploid), which could be due to an extra restriction site in this fragment resulting in two fragments smaller than the region bands were scored from. So it is clear that there is very little difference in the chloroplast genomes of *F. elliptica* and both Chinese and Japanese *F. japonica*, which would support the idea that the three names *F. elliptica*, *F. japonica* and *F. forbesii* are all conspecific.

*F. sachalinensis* has always been regarded as a separate species from *F. japonica*. It is clearly morphologically distinct, with larger obviously cordate leaves, trichomes of type B, and a highly striate lower epidermis. As well as this it is often associated with a bluish appearance of the lower epidermis, although this characteristic was omitted from this study because of the difficulty of observing this in herbarium material, and because the intensity can vary between different fresh specimens. The study of Inamura *et al*. (2000) had intended to use *F. sachalinensis* as an outgroup for variation in *F. japonica*, however the three accessions they analysed were found to be dispersed among the *F. japonica* OTUs (operational taxonomic units) from two different clades. Two accessions in one clade were from Hokkaido, whilst the single accession within a different clade was from Toyama. They gave two possible explanations for this. One was that *F. sachalinensis* was a polyphyletic species derived from *F. japonica*. The other explanation that Inamura *et al*. (2000) preferred was that this was the result of cytoplasmic gene flow. In this study 44 accessions of *F. sachalinensis* were analysed. As with the study of Inamura *et al*., (2000) instead of forming an outgroup these plants had chloroplast haplotypes that fell within the OTUs for *F. japonica*. Also similar to the study by Inamura *et al*. (2000), these haplotypes were from two different clades. In clade C, 7 accessions represented by 3 MPHs were all found from the prefecture Niigata. This

prefecture is the neighbouring prefecture to Toyama where one of the accessions of Inamura *et al*. (2000) originated. The majority of the *F. sachalinensis* in this study were part of clade B, and as seen in Figure 6.13 were found in the northern part of Honshu and Hokkaido. Hokkaido being where the other two accessions analysed by Inamura *et al*. (2000) came from.

Clade C consists of a small number of plants, all collected from Toyama and Niigata. The main four MPHs in this clade are 6, 20, 24 and 38, which are held together 100% of the time according to the consensus majority rule tree (Figure 6.7), and have a bootstrap value of 74, indicating strong support. On the neighbour joining tree (Figure 6.8) they also form a group with significant support, although in this case the bootstrap value is 60. MPH 6 consists of three *F. sachalinensis* accessions, MPH 20 two *F. sachalinensis* and two tetraploid *F. japonica* var. '*japonica'*, MPH 24 one tetraploid *F. japonica* var. '*japonica',* and MPH 38 two *F. sachalinensis* from Japan and three British accessions of *F. sachalinensis*. This means in total there were 7 Japanese *F. sachalinensis* accessions and three tetraploid *F. japonica* var. '*japonica'*. Given that the plants are sympatric there is a high chance that hybridisation has occurred which may have led to the introgression of the chloroplast type from one taxon going to the other, although only two putative hybrids were detected, most likely with *F. japonica* var. *uzenensis* as a maternal parent. If introgression has occurred it is unclear as to the direction. Three of the four MPHs contained *F. sachalinensis*, whilst only two contained tetraploid *F. japonica* var. '*japonica',* however the next nearest MPH on both trees was MPH 21 which belongs to tetraploid *F. japonica* var. '*japonica'*. The clade itself according to the majority rule consensus tree is a sister clade to the other three clades and the unresolved section.

Clade B was the most complicated of the four clades, and had the least support, but it held together a very high proportion of the time in the majority rule consensus tree. The plants within this clade had the widest overall geographical distribution as seen in Figure 6.9, and was comprised of *F. sachalinensis*, tetraploid and octoploid *F. japonica* var. '*japonica'*, *F. japonica* var. *uzenensis* and many putative hybrids as listed in Table 6.8. Additionally the MPHs found within the majority of *F. sachalinensis* found in the introduced range and all the *F. japonica* var. *compacta* from the introduced range were also within this clade. As seen in Table 6.13, there were five MPHs containing *F. sachalinensis* accessions within clade B, of which two were specific to *F. sachalinensis*. One of these, MPH 2, was the only MPH found from the two sites in Hokkaido, and in a few individuals in Aomori. *F. japonica* has only recently been introduced into this area, and therefore if introgression is the cause of this high degree of chloroplast similarity between *F. sachalinensis* and *F. japonica* it must have been

from a much earlier time. That is assuming they were sympatric in the past, an issue that will be discussed further in the next section. As for both Hokkaido sites, which are some distance apart, having plants possessing only the one MPH, two possible explanations can be invoked. Often when an entire geographical area has only a single chloroplast haplotype a selective sweep is proposed. A mutation in any coding region of the chloroplast molecule that confers some selective advantage can give rise to a selective sweep, resulting in the dominance of just a single haplotype in a geographical area, while maintaining variation in the nuclear genome (King & Ferris, 2000). Alternatively, this lack in chloroplast variation in plants from Hokkaido may reflect a lack of diversity within the plants. Fujii *et al*. (1997; 1999) found lower chloroplast diversity but larger geographical range in both *Primula cumeifolia* and *Pedicularis chamissonis* Steven from the northern part of Honshu and Hokkaido, compared to plants from the southern part of their Japanese distribution. They believed this reflected the results of a cycle of glacial advance and retreat in Japan, with the northern plants having arrived in Japan at a later glacial event than those from the southern regions. A study of nuclear variation among *F. sachalinensis* individuals from these two Hokkaido sites may help to shed some light upon this situation.

Besides MPH 2, there were four other MPHs found in *F. sachalinensis* plants in clade B, MPHs 4, 8, 11 and 37. MPH 37 was the only other MPH within this clade that was exclusively found in *F. sachalinensis*. Four accessions were found between two different sites in Yamagata. According to the neighbour joining tree, MPH 37 is genetically close to MPH 11 and 33. On the majority rule consensus tree these three accessions were found to be no nearer to each other than to the rest of clade B. MPH 33 was only found in a hybrid. MPH 11 was one of the other clade B *F. sachalinensis* haplotypes, albeit one that was also found in other taxa. Twelve *F. sachalinensis* accessions were found with MPH 11, and were collected from four sites in Yamagata and Miyagi as listed in Table 6.4. It was also present in an accession of tetraploid *F. japonica* var. '*japonica*' from Yamagata, an accession of *F. japonica* var. *uzenensis* in Miyagi and a further accession of *F. japonica* var. *uzenensis* from Aomori. So for the most part this appears to be a predominantly *F. sachalinensis* chloroplast haplotype. So that makes three different MPHs predominantly or exclusively found in *F. sachalinensis* and representing 33 accessions. The chances of each of these being caused by recent chloroplast capture would appear remote, particularly given the geographic location of the plants from MPH2.

The final two MPHs found in clade B *F. sachalinensis* individuals and other taxa are 4 and 8. Only two accessions of *F. sachalinensis* were found with MPH 4 and a further two with MPH

8. MPH 8 is the haplotype found only in Aomori and predominantly in putative hybrids. Therefore it is unclear as to whether this would be predominantly found in *F. japonica* or *F. sachalinensis*. Besides the putative hybrids, which were found at the tetraploid, hexaploid and octoploid levels, and the *F. sachalinensis,* it was found in three accessions of *F. japonica* var. *uzenensis* and one accession of octoploid *F. japonica* var. '*japonica'*. MPH 4 was found in two accessions of *F. japonica* var. *uzenensis,* two putative hybrids and twenty-one accessions of tetraploid *F. japonica* var. '*japonica'*. Presumably chloroplast capture from tetraploid *F. japonica* var. '*japonica'* accessions was the reason MPH 4 was found in the other taxa.

So to summarise, there is evidence that some of the MPHs found in *F. sachalinensis* may be the result of cytoplasmic gene flow from *F. japonica*. However, in terms of recent hybridisation and introgression events this does not explain the presence of some of the chloroplast haplotypes, in particular MPH 2, within clade B. So we should look again at the two hypotheses proposed by Inamura *et al*. (2000): the first being that *F. sachalinensis* was a polyphyletic species derived from *F. japonica*; and the second being that cytoplasmic gene flow had occurred. This study does not refute or support either proposal. Cytoplasmic gene flow as the only mechanism behind the high genetic similarity between the chloroplast genomes of *F. sachalinensis* and *F. japonica* seems unlikely. However so does a polyphyletic origin. It is more likely that the accessions found within clade C found in Toyama and Niigata are the result of cytoplasmic gene flow, where both taxa are tetraploid and sympatric. The *F. sachalinensis* in clade B are predominantly sympatric with octoploid *F. japonica.* Gene flow between tetraploid and octoploid plants is less likely due to the presence of 'sterile' hexaploids. Those within clade B either represent an ancient case of chloroplast capture, or indicate that *F. sachalinensis* may be more closely related to *F. japonica* than was originally proposed, maybe even a giant variety as opposed to a separate species. There have been similar cases of different ecological races within a single species, such as the dwarf subalpine tetraploid *Achillea millefolium* var. *alpicola* Garrett, and the tall tetraploid *Achillea millefolium* var. *puberla* (Rhydb.) Nobs. These two varieties hybridise readily and the F1 hybrids are highly fertile. Hybridisation has been shown to readily occur between the giant *F. sachalinensis* and the dwarf *F. japonica* var. *compacta* in the introduced range, with the F1 hybrids showing signs of full fertility. The majority of the *F. japonica* accessions within clade B were of the tetraploid *F. japonica* var. '*japonica'* type and many of them were dwarf.

6.6.4 *Relationship between taxa and geography*

In general the MPHs detected in this study were good indicators of geographical distribution. Whilst there is obviously variation among the specificity of the MPHs, most of them were localised to some extent within a geographical area. MPHs within clade A (Figure 6.10a) were predominantly found in northern Honshu in the Tōhoku and Horuriku districts, but not found as far north as Aomori. The prefectures they were found in were Niigata, Miyagi and Yamagata. The one exception to this distribution was the octoploid *F. japonica* var. '*japonica*' found in Osaka with MPH 1.

Clade B has the widest overall distribution (Figure 6.9), but the MPHs within this clade do show some geographical clustering (Figure 6.10b). MPHs 3 and 4 have the widest geographical distribution found in the whole study, being found as far south as Yamaguchi, and as far north as the prefectures around Tokyo and Mt. Fuji, or as is the case of MPH 4 as far north as Yamagata and Miyagi. However, in both cases they were only found on the Honshu. The remainder of the MPHs within clade B exhibit a far higher degree of geographical clustering, being found mainly in neighbouring prefectures.

Clade C was only found in Toyama and Niigata (Figure 6.10c). The MPHs within clade D were found on three of the main islands, Shikoku, Kyushu and the southern part of Honshu (Figure 6.10d). Clade D is comprised of five MPHs, four of which were only found on Shikoku. The fifth, MPH 10, was the sole MPH found in plants at the summit of Mt. Aso, and was also found at three sites in Shikoku, two other prefectures on Kyushu and the westernmost prefecture of Honshu, Yamaguchi.

The MPHs that form the unresolved group from the majority rule tree (Figure 6.7) but cluster in two different areas of the neighbour joining tree (Figure 6.8) were found on three of the main islands, Shikoku, Kyushu and Honshu. Three were specific to single sites on Shikoku, one was specific to a single locality in southern Kyushu and three were found at single sites on Honshu. Of those that were found at multiple sites, only one (MPH 35) was found on all three islands; MPH 15 was found at three sites on Honshu (but from two different prefectures); and finally MPH 31 was found from three sites in the westernmost Honshu prefecture, Yamaguchi and from one site on Shikoku.

Whilst ideally a molecular biogeographic study would concentrate only on plants that have arisen from the natural distribution of the taxa concerned, in this study it was not so easy to define natural from those translocated by man so both were included. Obviously the plants

collected from the summit of the Mt. Aso, and the plants collected on Mt. Fuji, fit into the category natural, whilst those collected from a park or the roadside probably were there due to man. However, when the roadside meets the edge of natural woodland, normally considered a natural habitat for the lowland plants, would plants found there be considered natural or not? Unless a plant is being transplanted because of its aesthetic characteristics or medicinal properties, which roadside specimens are not typically chosen for, the plants used are likely to have arisen locally and therefore still represent the chloroplast type for that region.

The taxa in this study are found in a variety of native habitats ranging from the alpine region of mountains to lowland areas. These plants are also rather unusual in that they are often primary colonisers of volcanoes. For the most part it is a dwarf form of *F. japonica* var. '*japonica'* that is associated with the primary colonisation of volcanoes, for example Mt. Fuji and Mt. Aso that were included in this study. However, on Hokkaido it is *F. sachalinensis* that is associated with the primary colonisation of volcanoes. For example, after the eruptions in 1977 and 1978 of Mt. Usu, Hokkaido, *F. sachalinensis* played a major factor contributing to the recovery of plant vegetation (Tsuyuzaki, 1987; Tsuyuzaki, 1989).

Many of the Japanese alpine plant species are considered relics of those that had migrated, during the glacial periods, from the Asian or North American continents over land bridges to Japan (Fujii *et al.*, 1999). During the ice age the Sea of Japan and Yellow Sea were about 100 m lower than at present and a land connection existed between Korea and Japan. Likewise the four main islands that comprise Japan were all interconnected during this time. Figure 6.14 adapted from Chung and Chung (2000) shows the land-mass that is now Japan and Korea, and how it was in the middle Pleistocene. Glaciation itself was restricted to the northernmost mountains of Korea and northern and central Japan, above 2,000 masl (Chung & Chung, 2000).

Very few phylogeographical or molecular biogeographical studies for Japanese plant taxa are published in English. Exceptions include the soybean (*Glycine max* (L.) Merr.) (Shimamoto, 2001), Japanese beech (*Fagus crenata* Blume) (Tomaru *et al.*, 1997), *Pedicularis chamissonis* (Fujii *et al.*, 1997), and *Primula cuneifolia* (Fujii *et al.*, 1999).

Two of these Japanese plant species that have undergone chloroplast DNA phylogenetic analysis are perennial herbs that occur in the subalpine and alpine areas in Japan. They are *Pedicularis chamissonis,* which is a "sunny meadow" plant (Fujii *et al.*, 1997), and *Primula cuneifolia,* which is a wet meadow plant (Fujii *et al.*, 1999). For both species the analysis

revealed major clades with geographical distributions. *Pedicularis chamissonis* was made up of two clades, referred to as the Southern clade and the Northern clade. *Primula cuneifolia* was made up of three clades, the Northern, Hokkaido and Southern. Both studies showed the cpDNA haplotypes within each clade diverged from a common ancestral genome. In both studies the haplotypes of the Northern (and Hokkaido in *Primula*) clades had wider distribution areas than that of the southern clade. However both studies also showed the genetic diversification of the Northern (and Hokkaido) clades was lower than that of the Southern. They concluded that their results indicated that geographical isolation had helped to effectively diversify the haplotypes of the Southern clade, and/or that the start of diversification was earlier than that of the Northern and Hokkaido clades (Fujii *et al.*, 1997; Fujii *et al.*, 1999).

Fujii *et al* (1997; 1999) proposed three hypotheses that could explain the evolutionary patterns of the three major clades in *Primula cuneifolia* and the two major clades in *Pedicularis chamissonis*. The first hypothesis was that the clades arose from long-term extrinsic barriers to gene flow (e.g., volcanoes or tectonic line). The border between the two clades in *Pedicularis chamissonis* was located at northern Honshu, and the borders for *Primula cuneifolia* were northern Honshu and northern and eastern Hokkaido. However there are no known barriers to gene flow over a long period in either of those regions. Their second hypothesis was that the major lineages entered the Japanese Archipelago over different land bridges. In the Pleistocene, Japan was connected to Sakhalin and the Kuriles, and western Japan was connected to the Korean Peninsula. The hypothesis being the Northern (and Hokkaido) haplotypes entered from the Sakhalin/Kuriles whilst the Southern clade may have entered from the Korean Peninsula. As neither of their species was found in western Japan, the Korean Peninsula, or China, the authors dismissed this hypothesis. The third hypothesis proposed in both papers and believed to be the most likely for their study species, was that the major lineages arose and assumed their present distribution area through a number of cycles of glacial advance and retreat. During the Pleistocene, the suggestion is that glacial advances and retreats occurred at least four times in the Northern hemisphere (Minato & Ljiri, 1976 in (Fujii *et al.*, 1997; Fujii *et al.*, 1999)). Fujii *et al*., propose that the greater diversity in the Southern clade for both species was caused by the ancestral genomes of the Southern clade during climatic cooling travelling southwards to central Honshu, Japan, and remaining alive only on high mountains due to climatic warming. When it was cool again, the ancestral genomes of the Northern clade moved southwards to northern Honshu. The ancestral genomes of the Southern clade that had been distributed in northern Honshu and northward

would have been replaced by the Northern clade through competitive exclusion of local populations.

The land bridges present in the Pleistocene may in some way have lead to the current distribution of the taxa in this study. The northern part of Honshu represents the northern end of the natural distribution for *F. japonica,* whilst to the south it is found in both Korea and China. In contrast the northern part of Honshu represents the southern end of the natural distribution for *F. sachalinensis,* which extends northwards into the Sakhalin and Kurile islands. It is possible that during the Pleistocene *F. sachalinensis* entered Japan from the northern land bridge, and *F. japonica* from the southern land bridge. However in this scenario *F. japonica* would not have been sympatric in the past with *F. sachalinensis* in Hokkaido and therefore an ancient common ancestor would not be a plausible explanation for the high degree of similarity between the chloroplast types of these two taxa.

An alternative hypothesis could be that the plants found in clade B, which consists primarily of tetraploid *F. japonica* var. '*japonica'* and *F. sachalinensis,* do have a common ancestor and both arose in the region to the north of Japan. Assuming they arrived in Japan during several different glaciation cycles, the tetraploid *F. japonica* var. '*japonica'* plants may have arrived during one of the earlier climatic warming then cooling periods, and during a later cycle when *F. sachalinensis* arose would have been replaced by competitive exclusion of local populations. This could explain the low level of variation in the *F. sachalinensis* plants that are found over a larger range, however in that scenario the tetraploid *F. japonica* var. '*japonica'* individuals would be expected to have evolved further and therefore not still be within the same clade. This also does not explain how the *F. japonica* plants found in the other clades arrived and diverged further.

In this study four major clades were found, and a further group of OTUs that formed an unresolved polytomy at the base of the majority rule consensus tree, but formed two groups, one more distinct than the other, on the neighbour joining tree. Unlike some of the other studies looking at the biogeography of Japanese species, there was no distinct northern and southern divide, although geographic clustering was found. One possible reason for this could be that these taxa were less affected by the glacial cycles than most taxa, due to their affinity with volcanoes.

There were more than 200 volcanoes active in the middle and late Quaternary periods (Machida, 1980). The Quaternary and Tertiary periods occupy the last 65 million years.

Table 6.14 taken from Lincoln *et al*. (1998) shows how these periods are further divided. In the middle Miocene the southern half of Japan belonged to a tropical zone. As the atmospheric temperature gradually decreased and the Quaternary started, Japan experienced various climatic changes throughout the glacial and interglacial periods (Sakaguchi, 1980). In the full glacial stage of the latest glacial age about 20,000 years ago, the whole of Hokkaido and the northern part of Northeast Japan became a periglacial area, right down to the lowlands. The distribution of glacial landforms in Japan is limited to the mountain ranges of Hida, Kiso and Akaishi, the Japan Alps, and the Hidaka Mountain Range in Hokkaido. The lower limit of distribution of the glacial topography for the southern mountain range is about 2,600 m, while for Hidaka it is about 1,300 m. No glacier is recognised on such high, but new, volcanoes as Mount Fuji. It is estimated that the transformation of Japan into mountainous land commenced at the beginning of the Quaternary period (Minato, 1977).

The land connection shown in Figure 6.14 between the Korean peninsula and southern Japanese archipelagos no longer existed after the middle Pleistocene, but the frequent typhoons that hit north-east Asia can carry a lot of things between populations in the two countries (Chung & Chung, 2000). This could explain why there is higher variation in the plants from the southern islands of Kyushu and Shikoku, and in the southern part of Honshu. However no matches of material from China or Korea were found in Japan in this study, so if this is a factor it is not likely to be a current one. Man's interference may have played a greater part in the mixing of material from Japan and China in the past. The Japanese governed Korea for a long period, and during that time plants may have been transferred between the two countries. *F. japonica* is often used in both Japanese and Chinese traditional medicine, and may have therefore been cultivated outside of its natural distribution. The cultivated plants may have escaped and become naturalised allowing them to hybridise with the plants naturally found in the area and therefore increase the genetic diversity.

One hypothesis that could explain some of the variation is that during the glacial periods the volcanoes became refugia for the early progenitors of the present taxa. Mt. Aso or another nearby volcano may have been the refugium for the plants on Kyushu, Mt. Fuji for some of the plants on Honshu, and other volcanoes in other regions for the other districts. The plants on Mt. Aso probably had MPH 10, and at the end of the glacial period the plants may have moved down from the volcano into the surrounding prefectures, some becoming taller but remaining tetraploid, others undergoing chromosome doubling to produce the lowland octoploids, and some mutating into the other MPHs found within clade D, that were found on the nearby islands of Shikoku and southern Honshu. Likewise the same could explain the *F.*

*japonica* plants in clade B, with Mt. Fuji being the refugium and the plants having MPH 3. MPH 4 probably arose from a single mutation early after the plants started moving into the lowland areas, which could explain why both of these MPHs have a very similar distribution and why the plants found with MPH 4 furthest from Mt. Fuji are the taller specimens. *F. sachalinensis*, which may be a true species or an ecological race of *F. japonica*, could have had a refugium on one of the volcanoes on Hokkaido. Most likely these were MPH 2 and as the plants moved away from whichever volcano they were on, MPH 11 and the other related MPHs evolved. Clade A may represent a more recent group of plants that have evolved from a common ancestor that arose after a chromosome doubling event from a tetraploid progenitor. Presumably a tetraploid progenitor would have come from the Tōhoku or Horuriku districts of Japan, although whether it would have had trichomes of type C is unclear. The plants that form clade C, which are few in number, may represent a more recent evolutionary step, possibly including a progenitor that arose from the hybridisation and introgression of a chloroplast type into either *F. sachalinensis* or *F. japonica*.

Clearly the relationship between the taxa in this study is confusing and, whilst this study represents an increase in the understanding of the relationships between them, many questions remain to be answered.

### 6.6.5 *Geographical origin of the introduced plants.*

One of the main aims of this study was to identify probable native regions from which the introduced taxa may have originated. Exact matches to the chloroplast MPH discovered in plants from the introduced range have been found in plants from the native range. All the matches were found in Japan, with no matches between the introduced material and China or Korea, and no matches between China, Korea and Japan.

#### 6.6.5.1 *Fallopia japonica* var. *japonica*

The chloroplast haplotype from the introduced invasive clone of *F. japonica* var. *japonica* has been numbered MPH 1. This MPH was found within clade A, and was present in plants from six different sites in Japan as indicated in Figure 6.10a. With the exception of the accession found in Osaka, all of these plants were found in the region to the north of central Japan in the prefectures Niigata, Yamagata and Miyagi. Again with the exception of the Osaka individual, all of these plants were *F. japonica* var. *uzenensis*. Given that the British clone of *F. japonica* var. *japonica* has trichomes of type A, as does the Osaka accession but none of the others, it would be possible to say that this is where it arose, however this may not be the case for several reasons. Firstly this specimen was found growing along the roadside in Osaka, and

we have been informed that both *F. japonica* and *F. sachalinensis* are frequently used to protect roadside cutting from erosion in Japan (Yonekura K. pers. com). Secondly this individual was octoploid, whilst all other specimens from Osaka were tetraploid. Finally, it is genetically distinct from the other plants from this region that had either MPH 35, found within the unresolved group, or MPHs 3 or 4, which form part of clade B.

If Osaka is not where the British clone and the Osaka roadside specimen originate, the question still remains as to where they are from. As seen in Table 6.8, there were five other plants that possessed MPH 1, all of which were *F. japonica* var. *uzenensis*. This MPH was within clade A, which had a further five MPHs consisting of plants that were either *F. japonica* var. *uzenensis* or putative hybrids. In overall leaf shape and colour some of these *F. japonica* var. *uzenensis* plants were strongly reminiscent of the British *F. japonica* var. *japonica* clone (personal observation), with the obvious exception of possessing dense uniseriate trichomes of between 3 and 5 cells in length (trichome type C). One or two genes frequently govern many morphological character differences, particularly those of presence versus absence. Pubescence is often thought to be one of these presence versus absence characters, the variations in which are presumed to have arisen as a means to reduce or prevent herbivore damage, or for the maintenance of proper leaf temperature for photosynthesis (Gottlieb, 1984). For example, the difference between glandular trichomes and non-glandular trichomes in *Datura wrightii* Regel is controlled by a single gene (Elle & Hare, 2002). Another example is Cotton (*Gossypium hirsutum* L.), which displays varying densities of trichomes, ranging from none (smooth) to moderate (hirsute) to densely pubescent (pilose). Using a detailed RFLP map, five genes associated with pubescence of cotton leaves and/or stems were found, with a single allele on chromosome 6 being responsible for dense leaf pubescence (Wright *et al.*, 1999). Therefore it is not unreasonable to suggest that there may be only a single or a few genes that differentiate between plants with trichome type A and those with trichome type C. The clone that we are familiar with in Britain may have arisen from one of these *F. japonica* var. *uzenensis* plants found in the Tōhoku and Horuriku districts of Japan. A further possibility could be that chloroplast capture between *F. japonica* var. *uzenensis* and *F. japonica* var. '*japonica'* has occurred. To clarify this situation more plants from the Osaka region should be analysed to see if this specimen with MPH 1 is indeed an exception to the typical plants found in the area. Furthermore the Osaka individual, the British clone, and the *F. japonica* var. *uzenensis* specimens should be analysed with a non chloroplast marker such as a nuclear single copy gene or ribosomal marker that shows bi-parental inheritance which should help indicate if chloroplast capture has occurred.

6.6.5.2 *Fallopia japonica* var. *compacta*

*F. japonica* var. *compacta* is rarely found naturalised within the introduced range (Bailey & Conolly, 2000). Nonetheless four such accessions were included in this study, and each was shown in chapter 5 to have unique a genotype. These accessions possessed two different chloroplast haplotypes, MPH 3 and MPH 4. Both of these MPHs were found within clade B, the distribution of the plants in Japan from this clade being found in Figure 6.10b. Both of these chloroplast haplotypes were found to have a very wide distribution. MPH 3, the only chloroplast haplotype found in plants from Mt. Fuji, was found in many of the prefectures surrounding Mt. Fuji; but was also found as far south as Yamaguchi, the most southern prefecture on Honshu. MPH 4 was also found in the prefectures surrounding Mt. Fuji and as far south as Yamaguchi, but it was also found further north as far as Yamagata and Miyagi.

The leaf characteristic "crimped edge" was found in dwarf, intermediate and tall plants from Mt. Fuji and the surrounding prefectures with MPH 3 and MPH 4 (Table 6.4). The only confirmed dwarf plants with this characteristic were some of those from Mt. Fuji with MPH 3, and it is highly likely that this is where the British *F. japonica* var. *compacta* with that chloroplast type originated. As for the British *F. japonica* var. *compacta* with MPH 4, whilst no confirmed dwarf plants with crimped edges of that chloroplast type were found in this study, it is still highly likely that they also originated from the vicinity around Mt. Fuji. Especially considering how Mt. Fuji is a popular destination for visitors to Japan. The difference between these two MPHs in this study is found only in the chloroplast region *trn*C-*trn*D (Table 6.7) implying that the two MPH are indeed very similar and the plants are likely to have originated from the same common ancestor. It is not possible to say whether one type has evolved from the other using this study alone, although one may predict that these plants originated as dwarf plants of MPH 3 on Mt. Fuji itself and that as the plants progressed down and away from the mountain some lost their dwarf growth habit, others lost their crimped edges and at some point MPH 4 evolved and also spread into the surrounding areas. The tallest lowland *F. japonica* var. '*japonica'* with MPH 3 being 3m high and found in Yamaguchi, the southernmost prefecture in which that MPH was found.

6.6.5.3 *Fallopia sachalinensis*

Seventeen accessions of *F. sachalinensis* from the UK, one from the USA and one from the Czech Republic were analysed from the introduced range, as was a specimen sent from Germany as *Reynoutria* x *vivax* J. Schmitz & Strank, which is a synonym for *F. sachalinensis*. The *R.* x *vivax* and sixteen of the other introduced *F. sachalinensis* plants were all found to have MPH 2. The remaining three specimens were from two sites within the UK

and possessed MPH 38. Both of these chloroplast haplotypes were found within Japan, MPH 2 being part of clade B and MPH 38 being found as part of clade C.

As shown in Figure 6.10b, MPH 2 was found on Hokkaido and northern Honshu. It was the only chloroplast type found in the *F. sachalinensis* plants analysed from two distinct areas of Hokkaido, whilst in Aomori other chloroplast types were found as well. As seen in Table 6.2, seven sites were sampled in Aomori (sites 79–85), with a mixture of *F. sachalinensis*, *F. japonica* and putative hybrids sampled. In total 26 plants were sampled. After morphological analysis it was discovered that only four of these plants were *F. sachalinensis*, and that only two of the seven Aomori sites had *F. sachalinensis* as seen in Figure 6.13. From Table 6.4 it can be seen that there were two *F. sachalinensis* accessions from site 82 with MPH 2, and two from site 79 with MPH 8. Whilst there is a possibility that the introduced *F. sachalinensis* with MPH 2 came from Aomori, it is more likely that it came from Hokkaido where all accessions shared the same MPH.

MPH 38 is part of clade C and as shown in Figure 7.10c representatives of the clade were only found in Niigata and Toyama, whilst MPH 38 was only found at one site in Niigata. It is therefore highly likely that the three accessions of *F. sachalinensis* in Britain with this MPH originated from this prefecture.

The Korean specimen of *F. sachalinensis* originated from the Ullung Island, the only place where *F. sachalinensis* is found in Korea. A specimen was collected, counted as 2n = 102 by Lee (1972), and sent to the botanic gardens in Seoul, Korea. Seed was collected from this plant and sent to Leicester, UK, where it was grown. These plants grown from seed were 2n = 102 (Bailey, 1989) and presumably arose following a balanced reduction after the loss of some of the chromosome pairs, or by apomixis (Bailey, pers. com.). It was one of these 2n = 102 plants grown from seed that was analysed for this study. More typically *F. sachalinensis* from this island is 2n = 132, the highest chromosome number known in the genus (Kim & Park, 2000). Whilst 2n = 102 may not be truly representative of *F. sachalinensis* plants from Ullung, the maternally inherited chloroplast should still be representative of the chloroplast types on the island. On the grounds of chloroplast type (MPH 29) and cytology (2n = 102 for our sample, 2n = 132 otherwise) the *F. sachalinensis* from the Ullung island of Korea can be ruled out as a potential source of the introduced *F. sachalinensis*. Unfortunately in spite of our efforts, no material was available from the other countries to which *F. sachalinensis* is native, those being Sakhalin Island (former USSR), and the southern Kurile Islands (Kunashir and Shikotan) (Sukopp & Starfinger, 1995). Given that an exact match was found on

Hokkaido, and that one would expect plants originating at some geographical distance from the sites on Hokkaido to be genetically distinct, this study would indicate that the *F. sachalinensis* that is most common in Britain with the MPH 2 originated from Hokkaido. However, for this statement to have more validity material originating from both the Sakhalin and Kurile Islands, and a wider range of samples from Hokkaido, would need to be obtained and analysed.

### 6.7 Conclusions

The combined use of cuticular ornamentation and trichome type does appear to be a useful tool in differentiating between the taxa in their native range, whilst leaf shape is less useful. To fully utilize these characteristics a study of the interspecific and intraspecific native hybrids is required. Although only recently recognised in the Japanese Flora, hybridisation is clearly occurring between these native taxa, particularly in prefectures such as Aomori where the different taxa are being planted in disturbed habitats.

Tetraploid *F. japonica* var. '*japonica'* was the most common taxon found in this study, although *F. japonica* var. *uzenensis* dominates the northern Honshu prefectures, where it is sympatric with *F sachalinensis*. Octoploid *F. japonica* var. '*japonica'* was relatively rare with limited chloroplast diversity. However, there appear to have been at least two separate chromosome-doubling events that have lead to the production of octoploid *F. japonica* var. '*japonica'*. No genetic difference was found between high altitude dwarf plants and the tall lowland taxon, leading to the conclusion that the dwarf high altitude plants do not deserve formal recognition, for example by the use of the name *F. japonica* var. *compacta*. Conversely *F. japonica* var. *terminalis* was found to be genetically distinct. *F. japonica* var. *uzenensis* appears to be a distinct taxon, albeit one that readily hybridises with other sympatric taxa.

From the limited number of Korean and Chinese accessions of *F. japonica* var. '*japonica'* included in this study, these plants do not appear to be significantly diverged enough from the Japanese accessions to warrant specific status. However the Korean *F. sachalinensis* from Ullung Island does appear to have diverged some time ago from the Japanese *F. sachalinensis* plants. The relationship between *F. sachalinensis* and *F. japonica* is still unclear, but they are clearly very closely related.

In general the MPHs detected in this study were good indicators of geographical distribution, and the inclusion of plants from disturbed habitats has not detracted from the overall

distribution portrayed. The evidence presented here would suggest that the introduced *F. japonica* var. *compacta* plants originated from Mt. Fuji on Honshu, and *F. sachalinensis* from Hokkaido. The origin of the introduced *F. japonica* var. *japonica* is less easy to determine. As far as a chloroplast match was concerned these plants would appear to come from the Tōhoku and Horuriku district in Honshu. These plants, however, were of *F. japonica* var. *uzenensis*. Dependent on the genetic basis for the different trichome types this could be an accurate picture of where they came from. The alternative is Osaka, but this is based on a single sample that may have been transplanted by human activity, and does not fit in with the ploidy level or chloroplast type of other local plants.

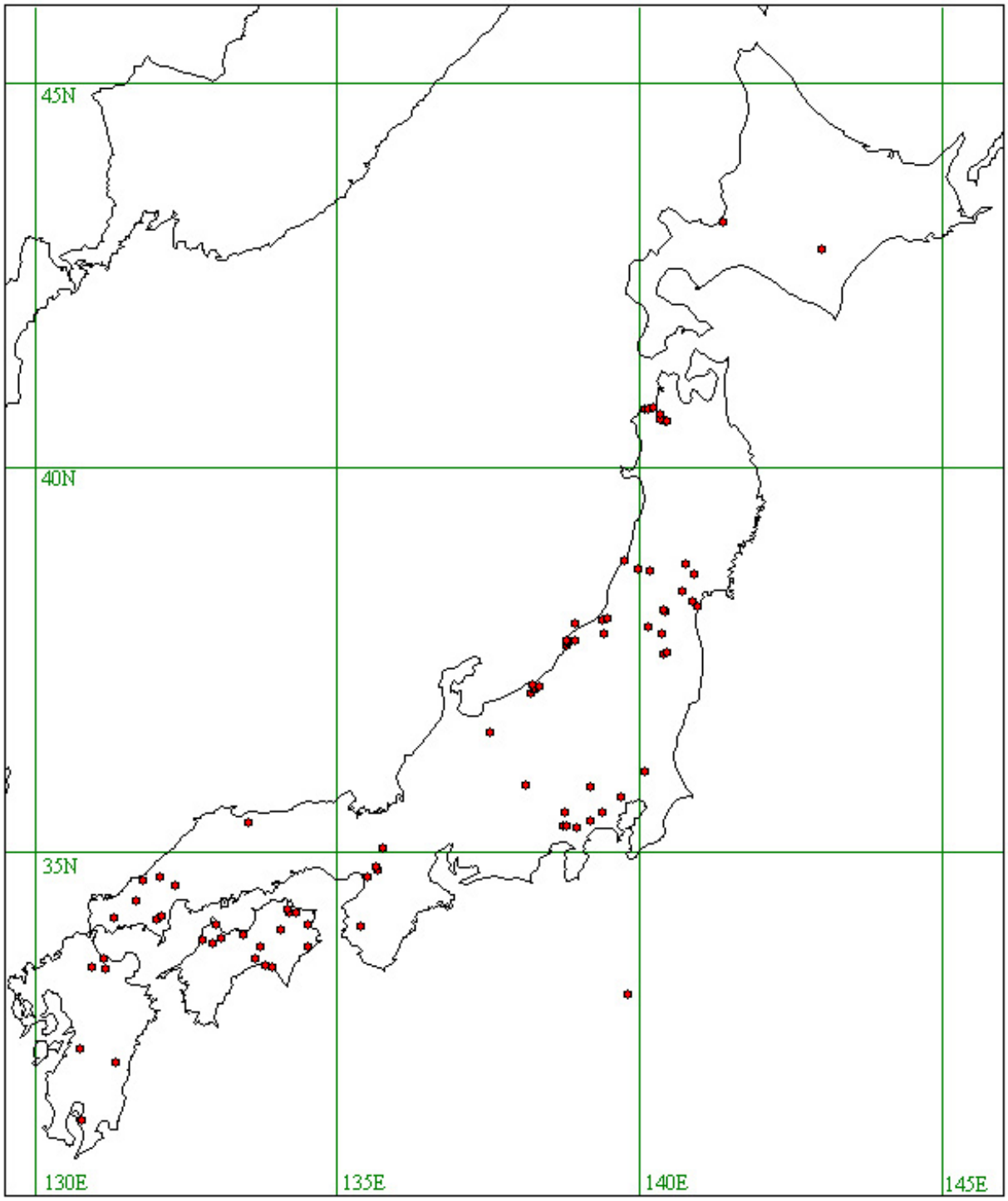



**Figure 6.1** Distribution of sites in Japan from which Japanese Knotweed specimens have been analysed. Map was produced using Dr. A. J. Morton's DMAP program.

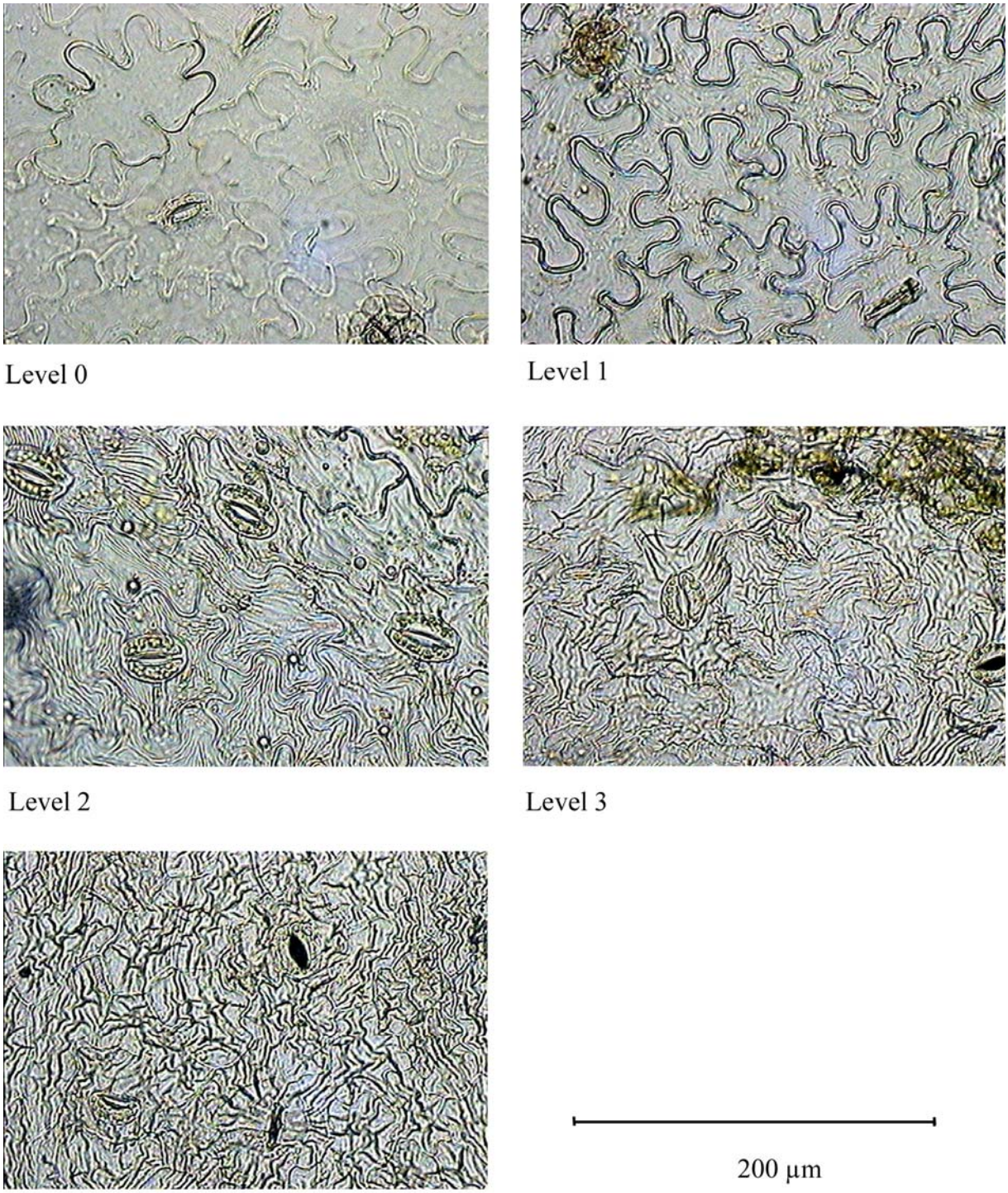



**Figure 6.2a** Epidermal peels taken from fresh leaf material. Images show the various levels of cuticular striation on the lower epidermis.

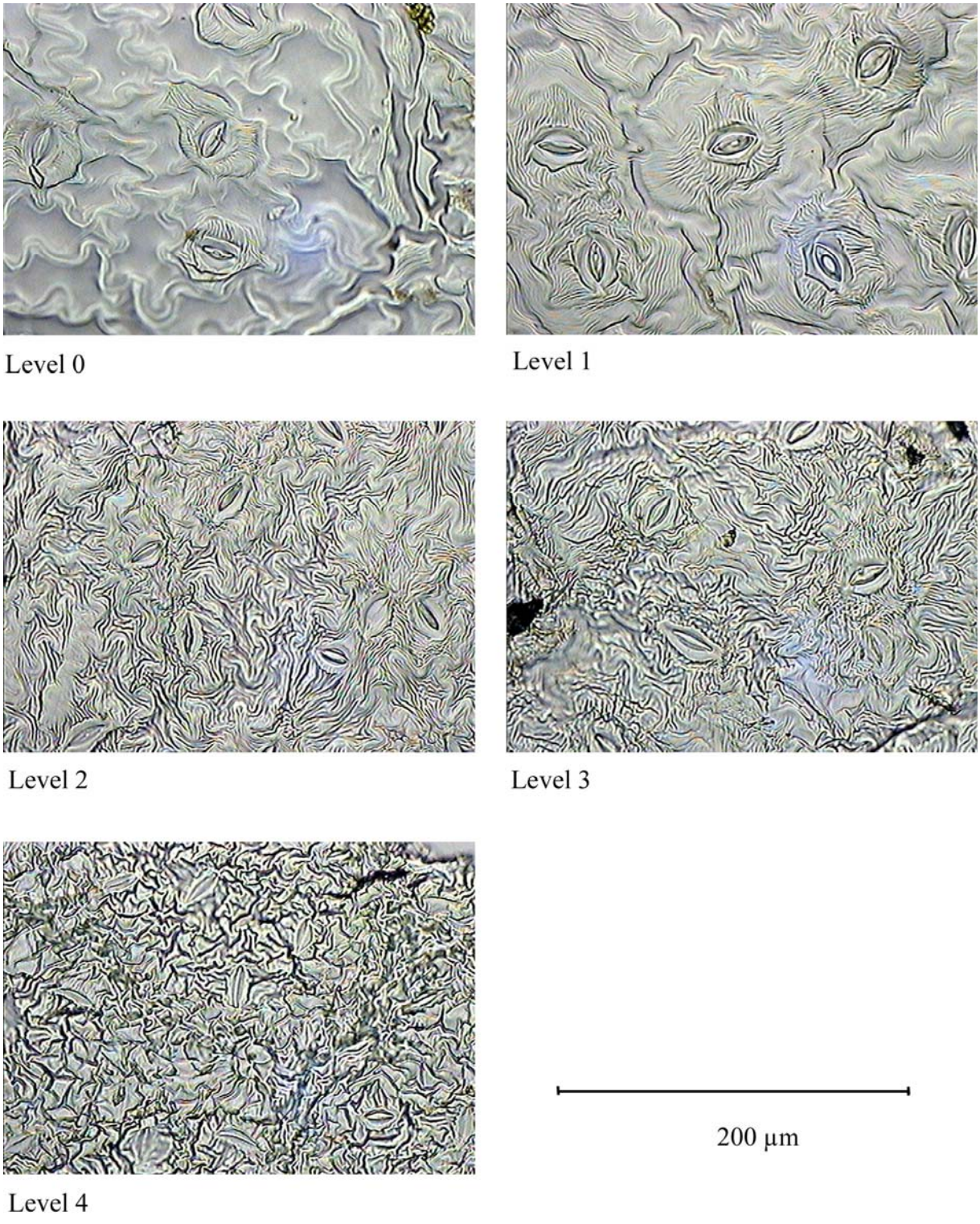



**Figure 6.2b** Epidermal peel replicas made from herbarium material. Images show the various levels of cuticular striation on the lower epidermis.

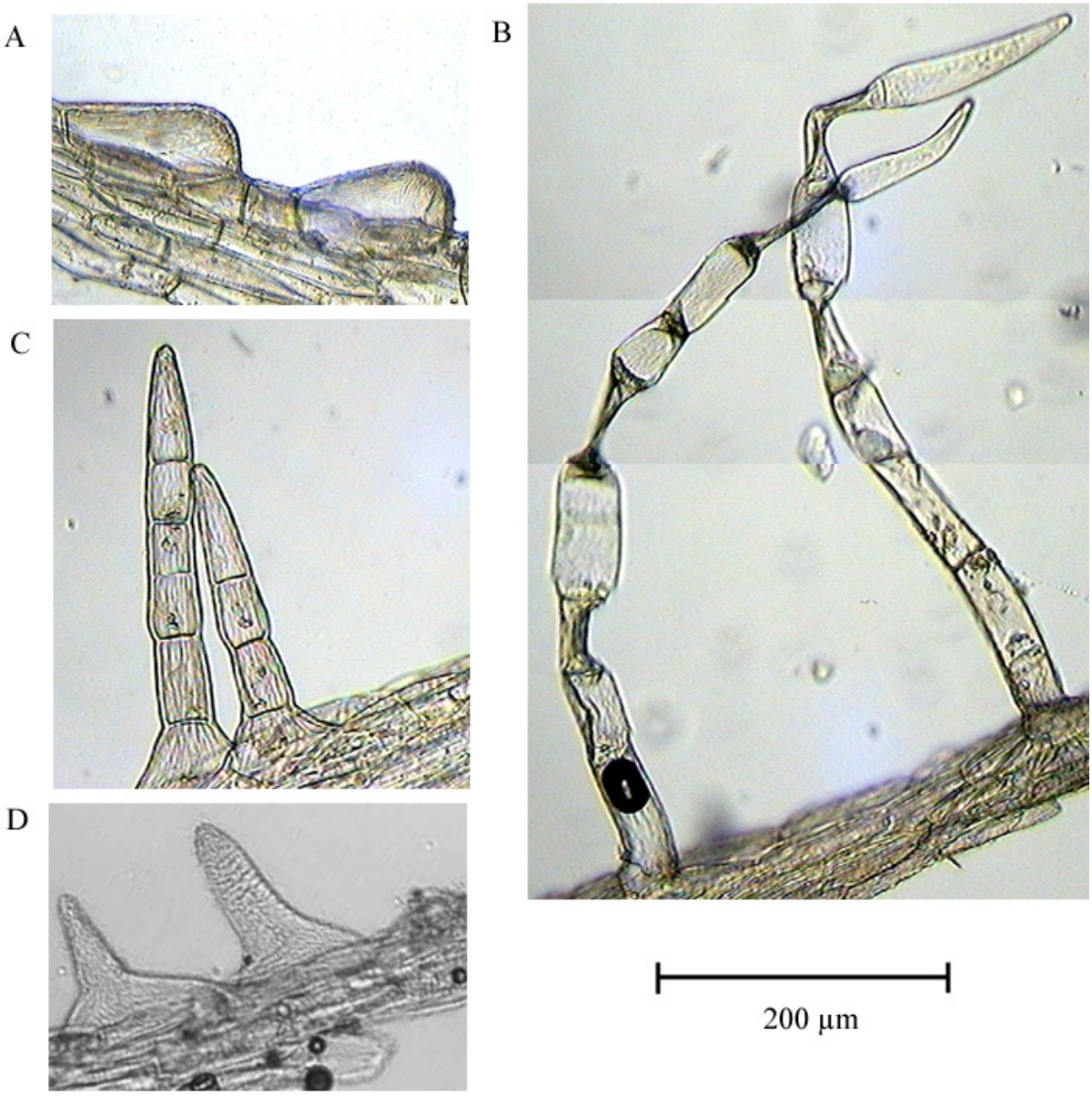



**Figure 6.3** Images showing trichome classifications. A) single-celled, swollen oval cell, usually striate, protrudes above the surrounding cells. B) uniseriate multicellular, 4 -20 cells in length, very thin walled, often collapsed in herbarium specimen. C) uniseriate multicellular, 3 – 5 cells in length, perpendicular to the vein, with basal cell an almost equilateral triangle. D) uniseriate multicellular, 1 – 6 cells in length, usually striate at the base, basal cell asymmetrical, emerging at less than 90°.

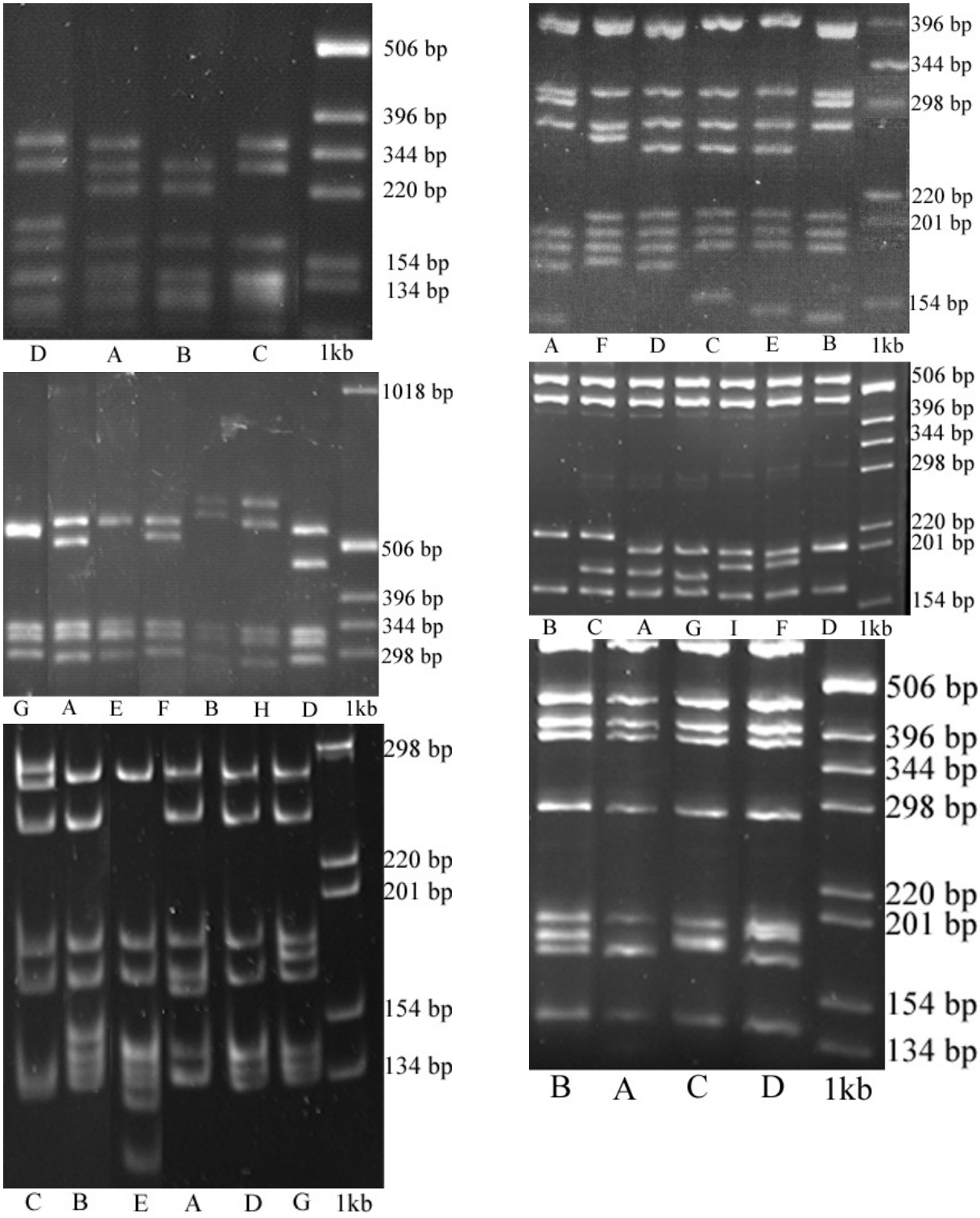



**Figure 6.4** Photographs illustrating some of the PCR-RFLP derived chloroplast haplotypes for the regions, *trn*K[1]-*trn*K[2], *trn*C-*trn*D, *trn*F-*trn*V, *trn*H-*trn*K, *trn*D-*trn*T, and *Trn*M-*rbc*L. Photographs from left to right, top to bottom. The letters indicate the chloroplast haplotypes as shown in Table 6.6.

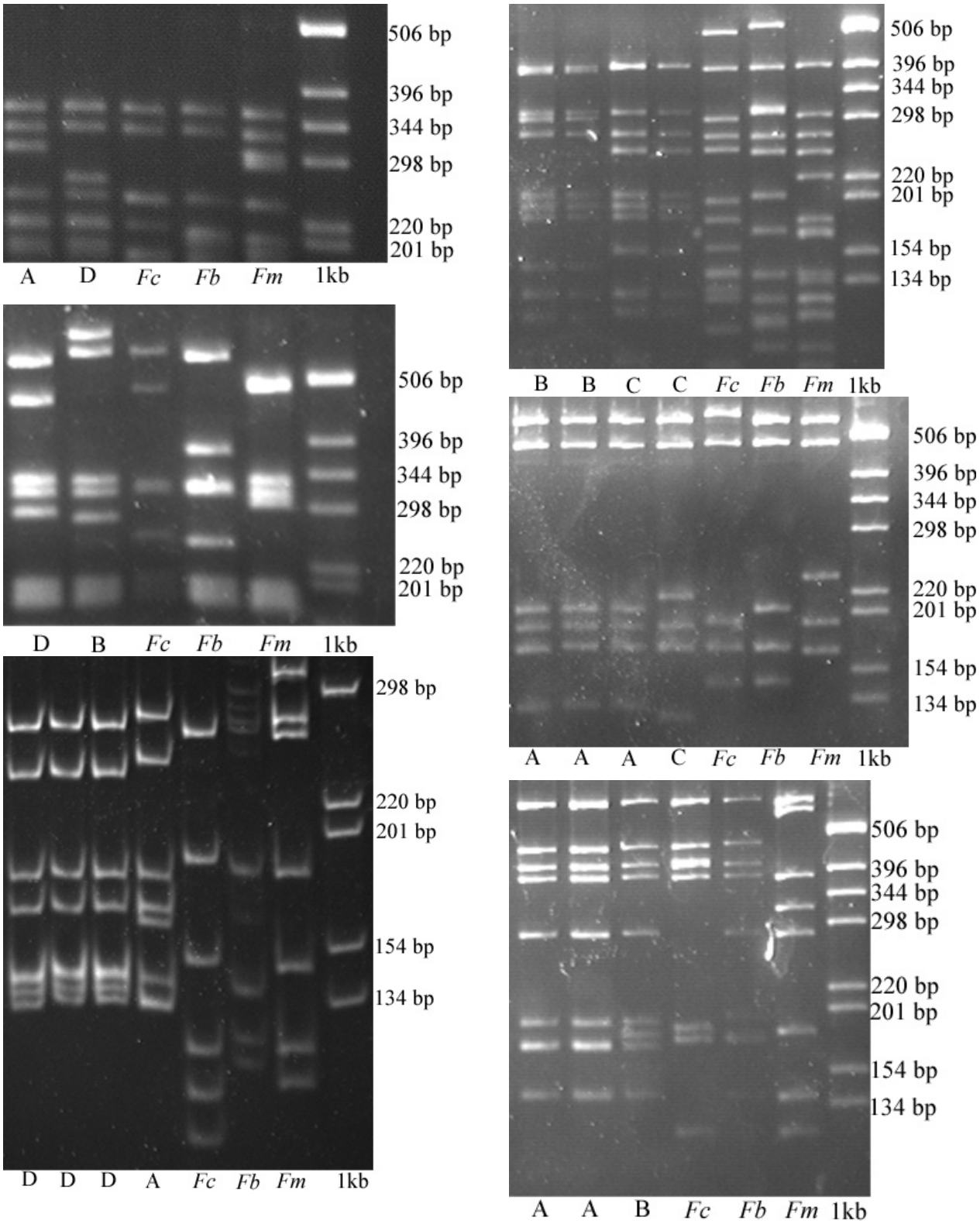



**Figure 6.5** Photographs illustrating the difference in banding patterns between the plants from *Fallopia* section *Reynoutria*, and the closely related taxa *F. convolvulus* (*Fc*), *F. baldschuanica* (*Fb*) and *F. multiflora* (*Fm*). Non-italic letters under lanes represent the chloroplast haplotype as per Table 6.6. From left to right, top to bottom the photographs show the RFLP banding patterns for the chloroplast regions *trn*K[1]-*trn*K[2], *trn*C-*trn*D, *trn*F-*trn*V, *trn*H-*trn*K, *trn*D-*trn*T, and *Trn*M-*rbc*L.

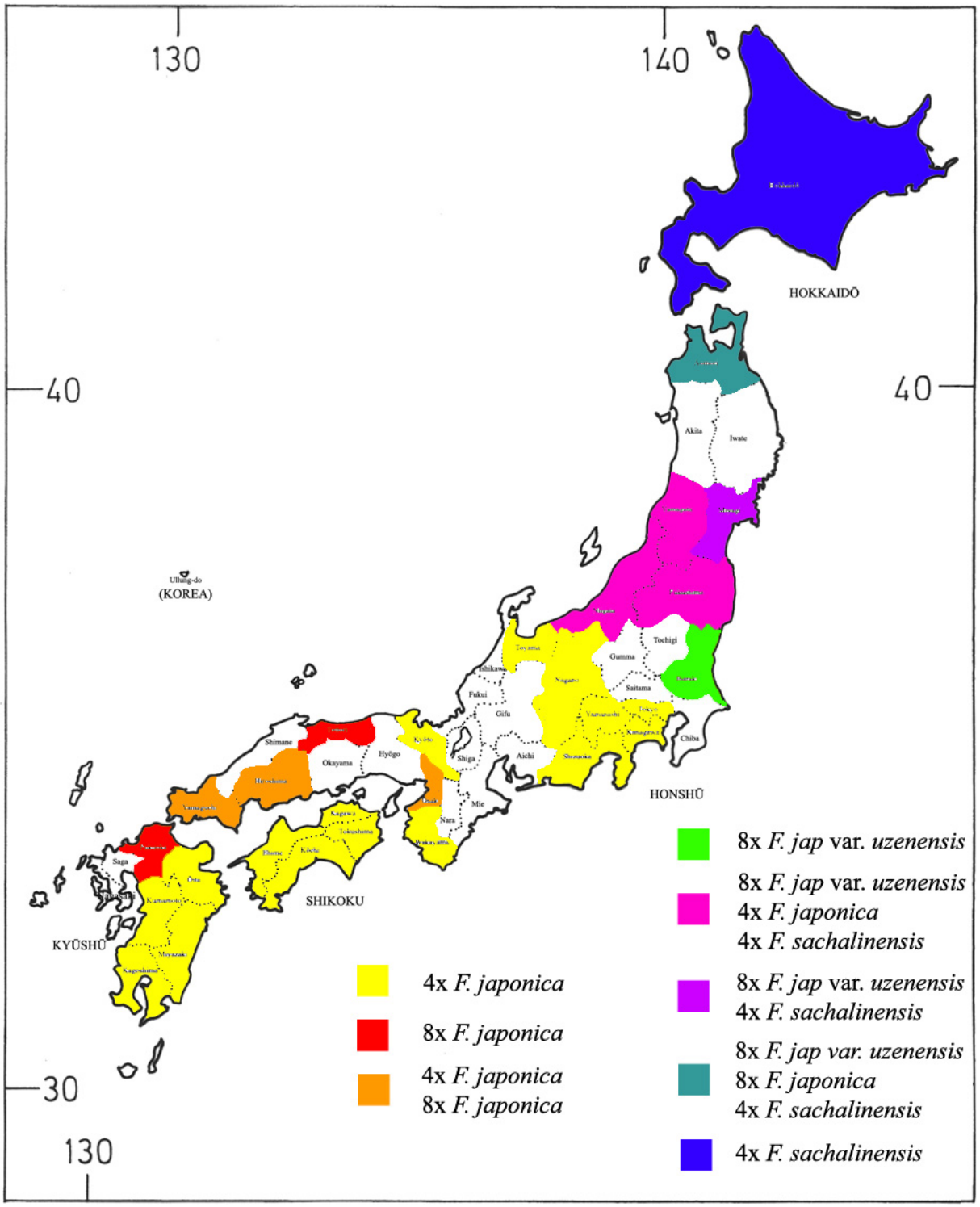



**Figure 6.6** Map of Japan showing the different taxa sampled from each prefecture. Represents only the plants used in this study and is not a complete distribution map for the different taxa. Putative hybrids have been excluded.

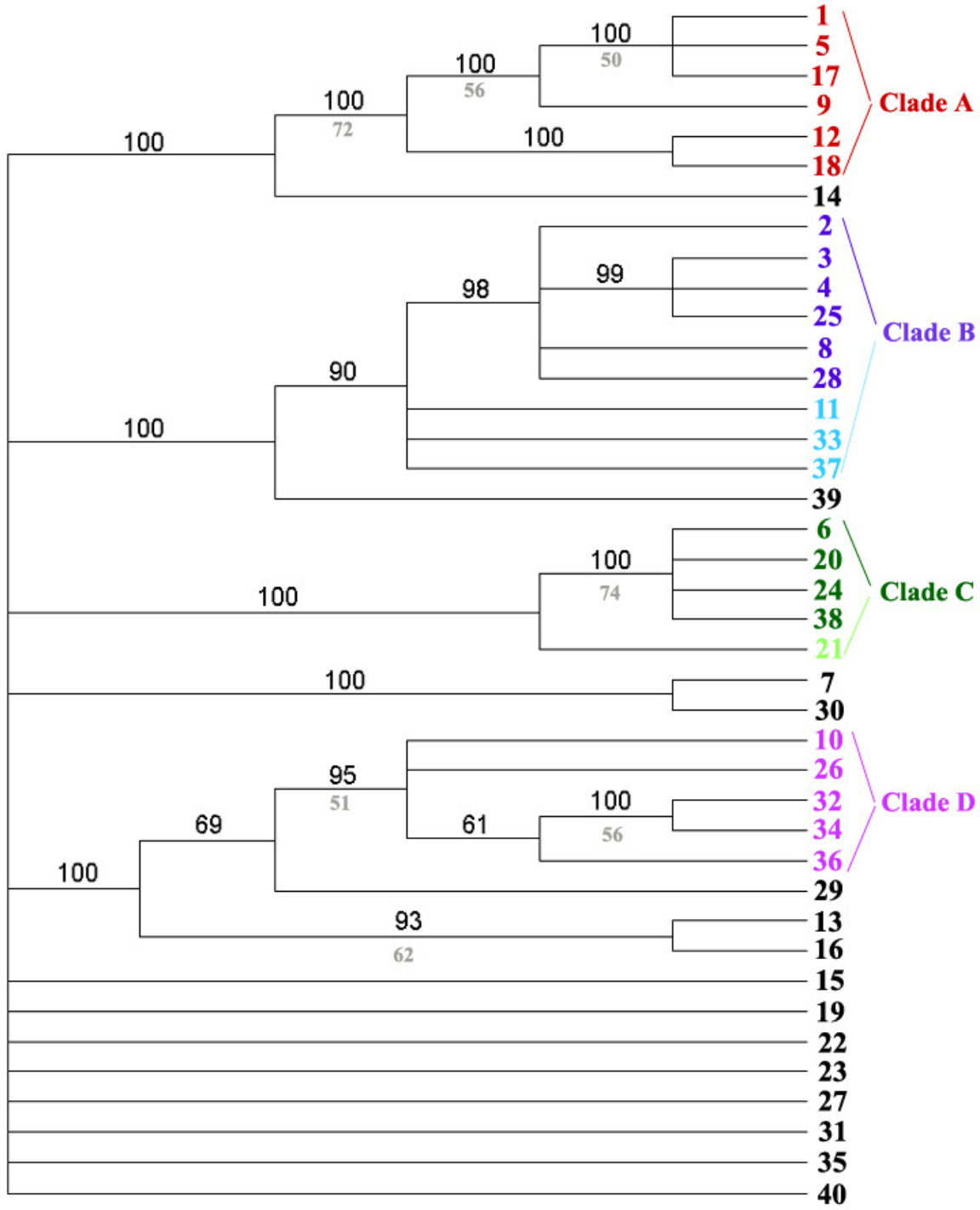



**Figure 6.7** Majority rule tree drawn with mid-point rooting, based on the consensus of 1377 shortest trees with a length of 76. Large numbers above the branch are the consensus values whilst below the branch, shown in small grey numbers, are the bootstrap values obtained by fast "pairwise-step" bootstrapping 1,000 replicates.

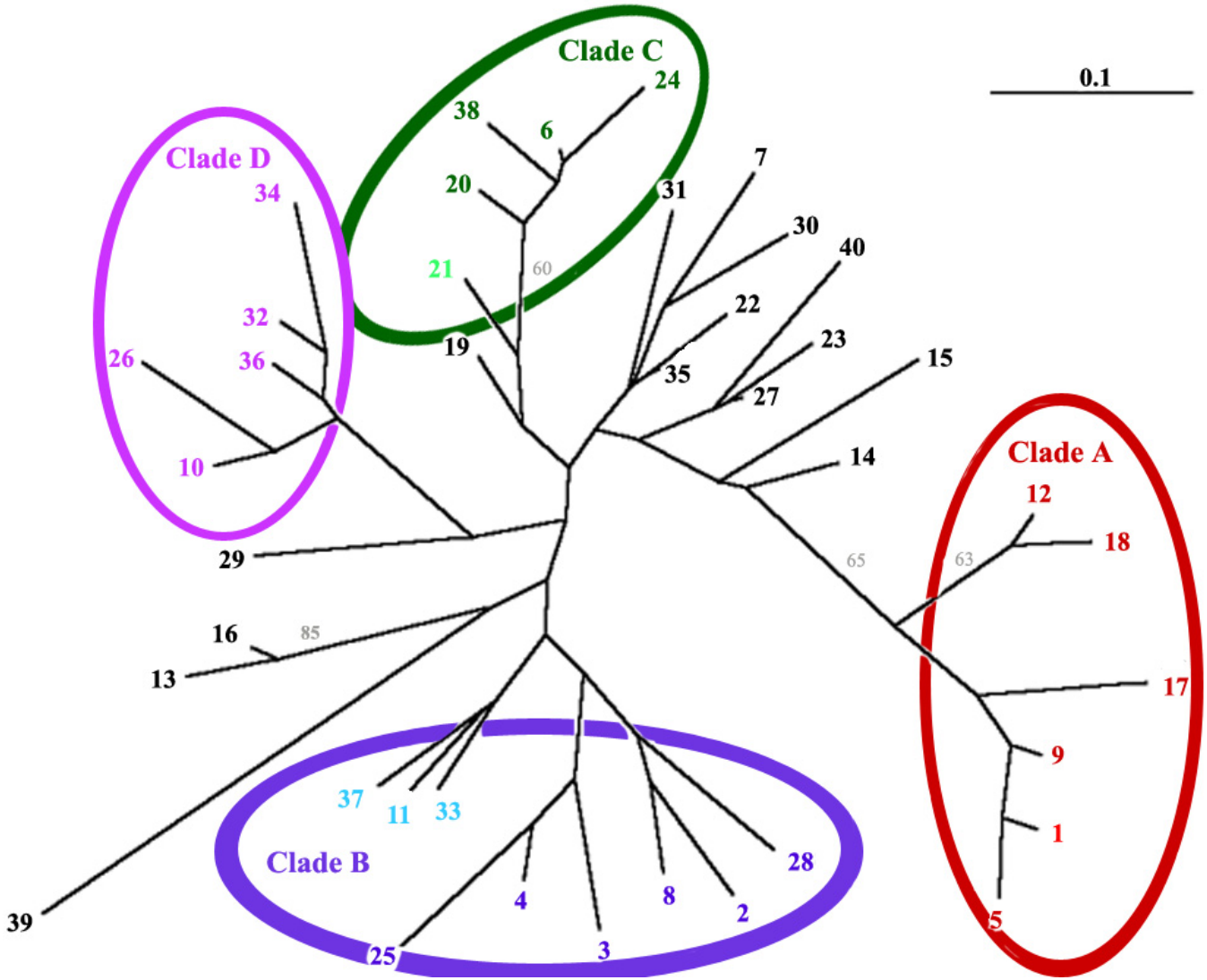



**Figure 6.8** Neighbour joining analysis unrooted tree, based on the Jaccard's similarity co-efficient. Numbers in grey are bootstrap values based on 1,000 repetitions.

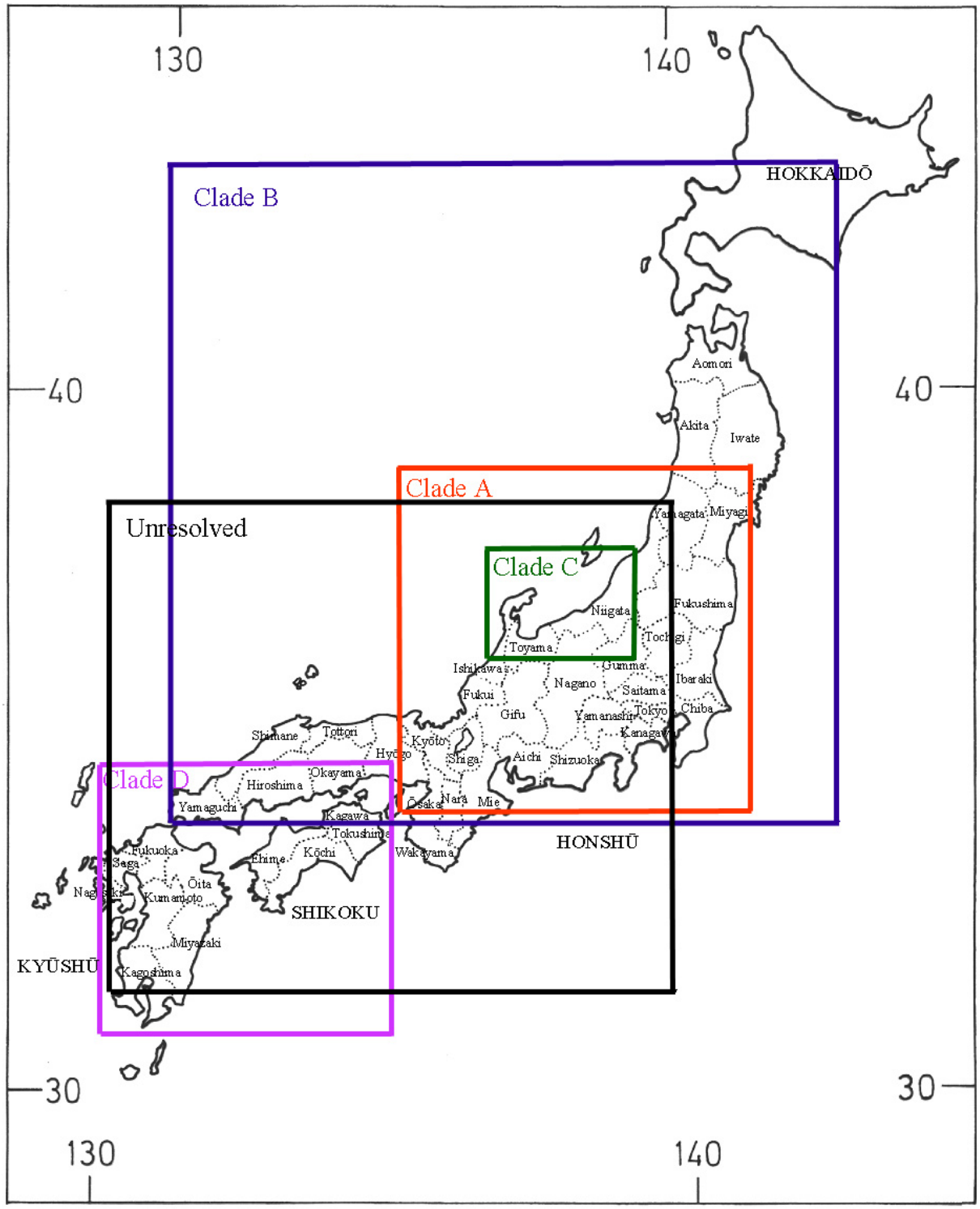



**Figure 6.9** Map showing the widest distribution the individual plants that make up the various clades can be found in Japan.

*Figure 6.10a*

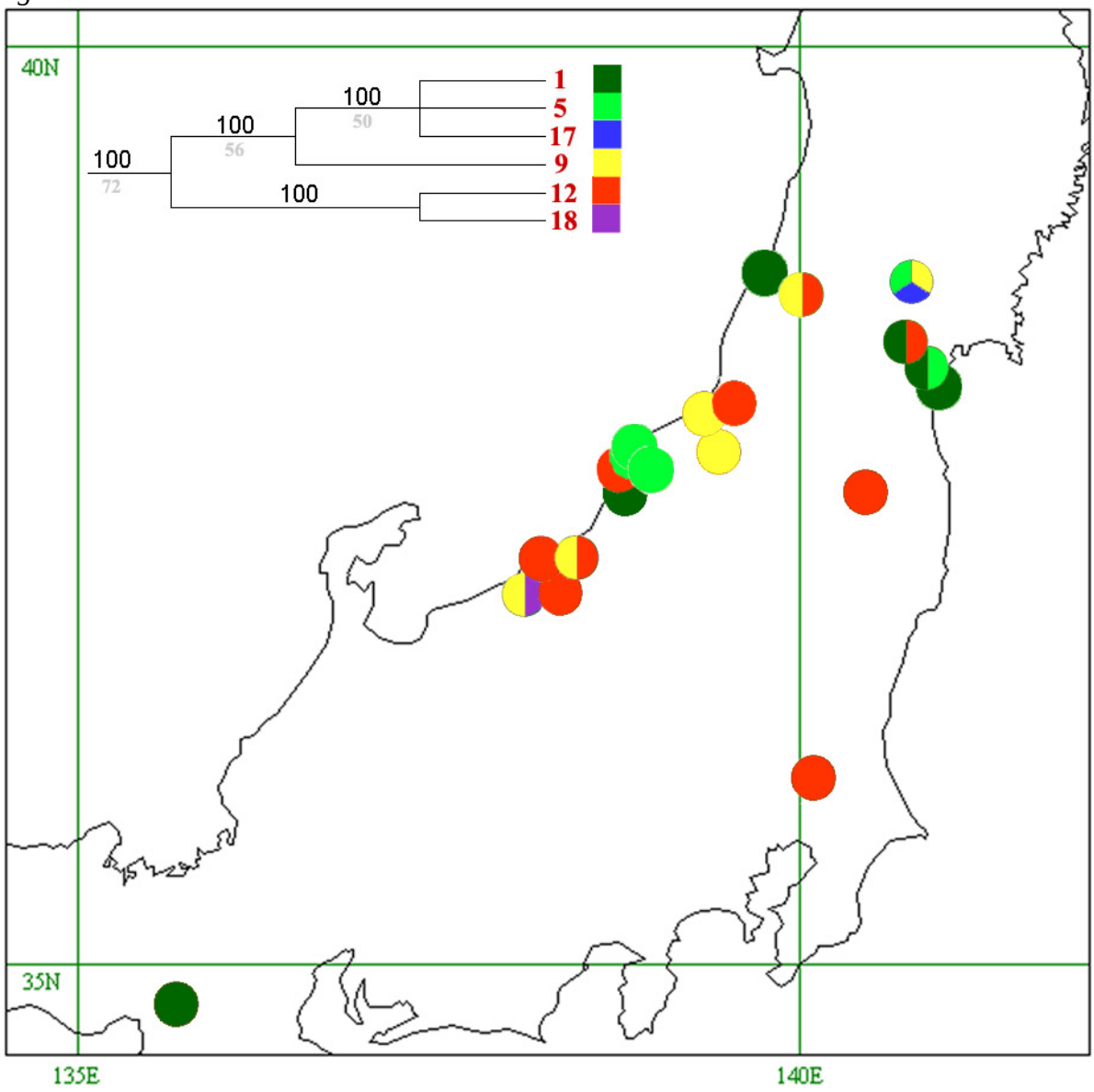



**Figure 6.10** Distribution of the plants found in the various clades. The base maps were obtained from Dr. A. J. Morton's DMAP program. The area the maps cover is as per Figure 6.9. Each pie chart represents a site as described in Table 6.2. The segments represent the presence of a chloroplast type but not the number of individuals with that chloroplast type.
a) clade A; b) clade B; c) clade C; d) clade D; e) unresolved group.
On Figure 6.10e numbers with red rings around them represent chloroplast haplotypes found in plants not found on one of the four main islands of Japan.

*Figure 6.10b*

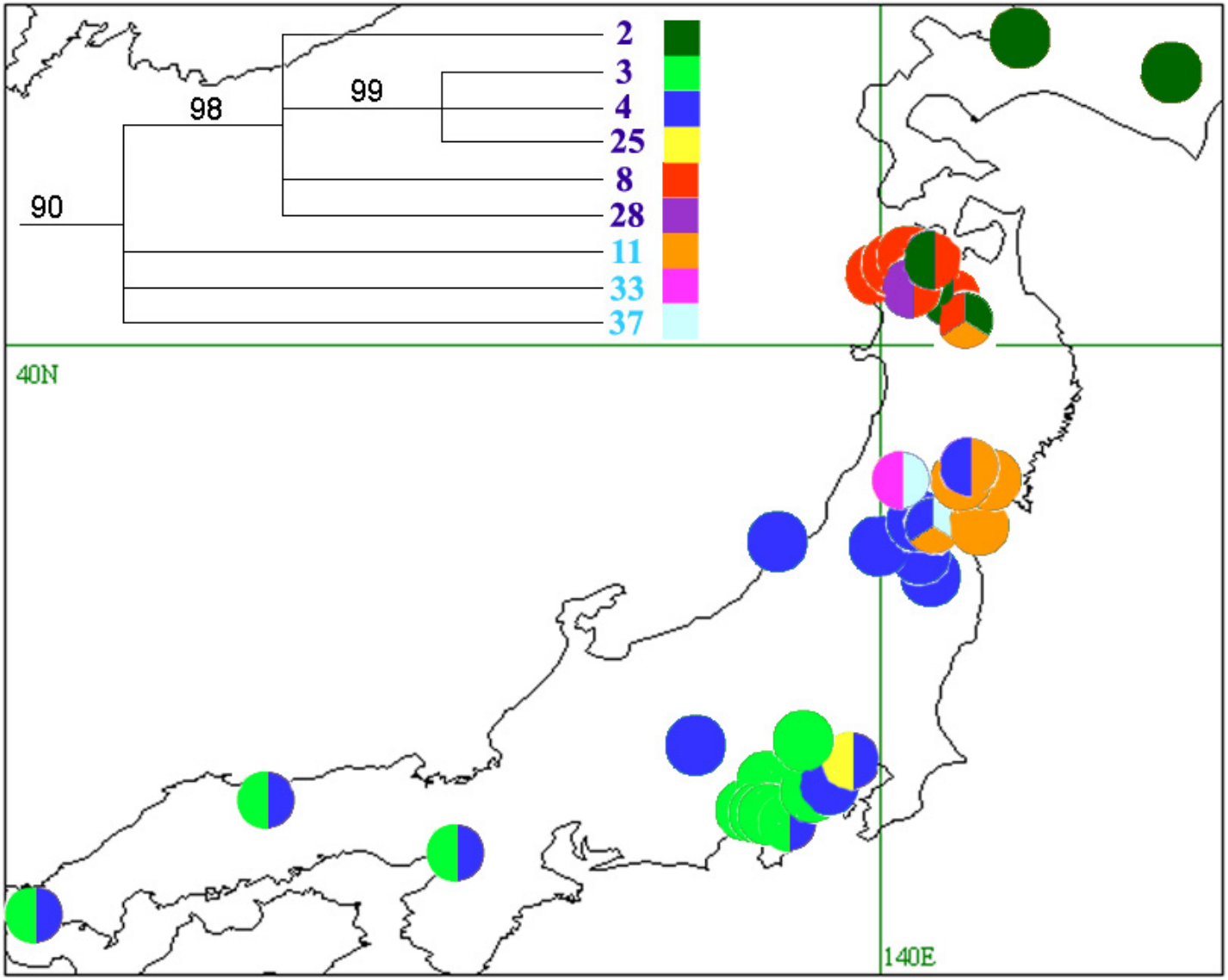


**Figure 6.10c**

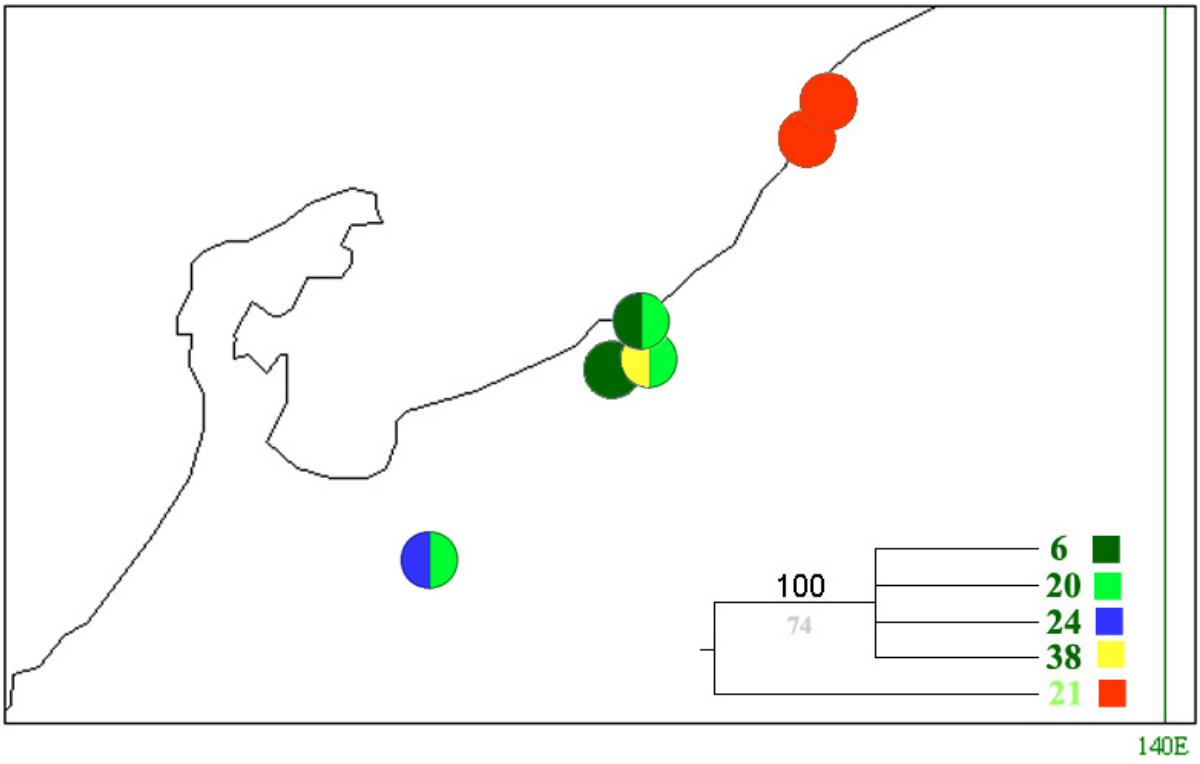



*Figure 6.10d*

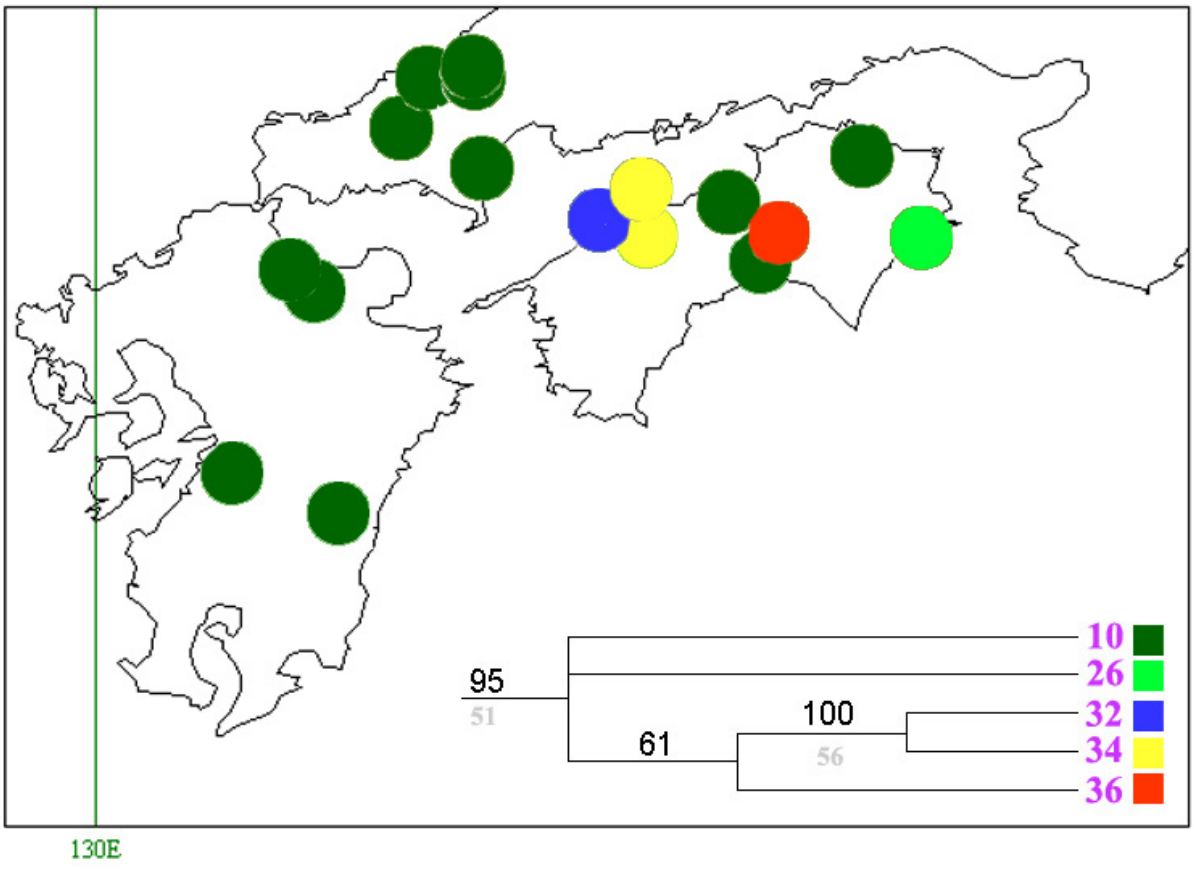
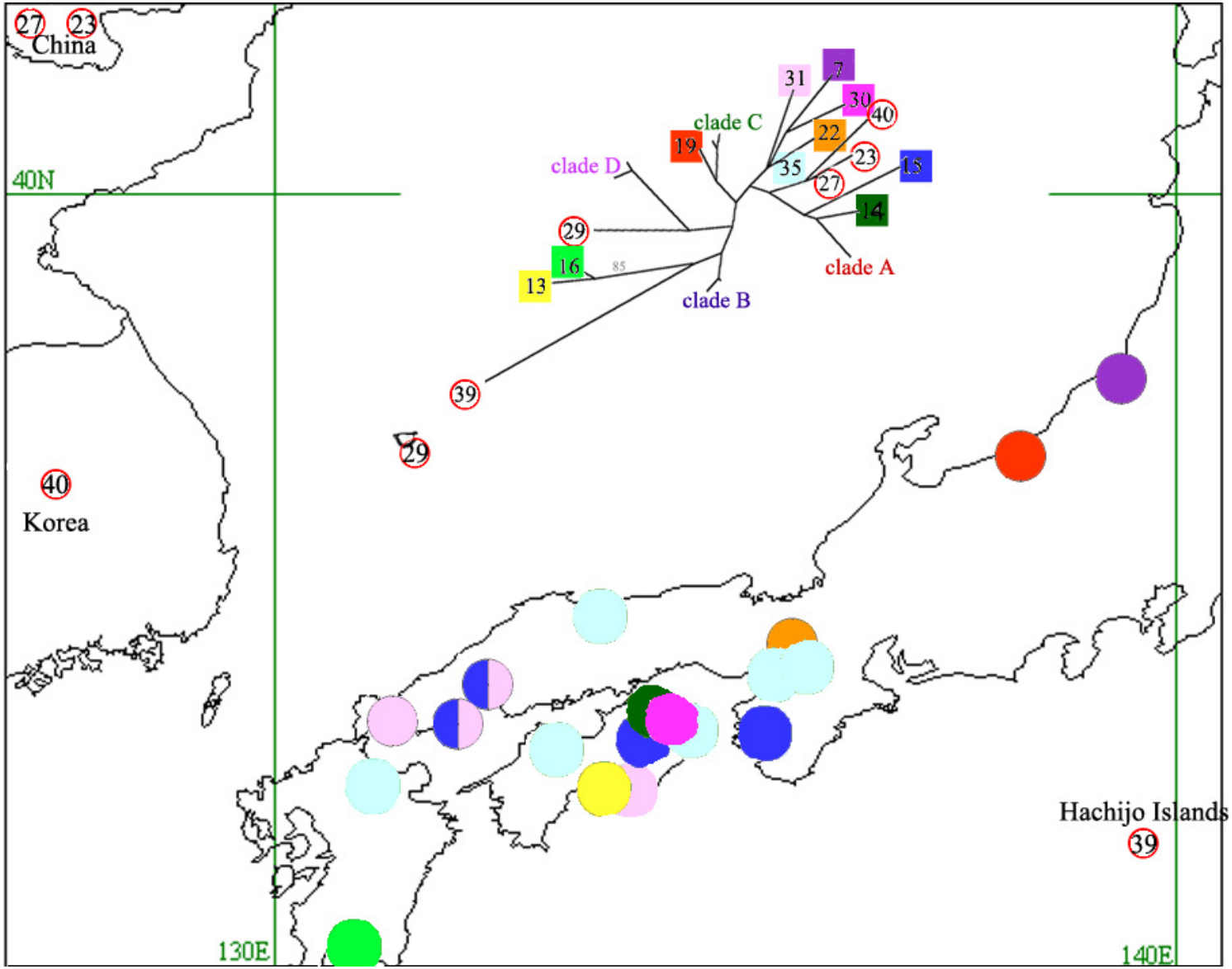


**Figure 6.10e**


27
23
China
40N
clade C
clade D
clade A
clade B
31
7
30
40
22
19
23
35
15
27
14
29
16
85
13
39
29
40
Korea
Hachijo Islands
39
130E
140E

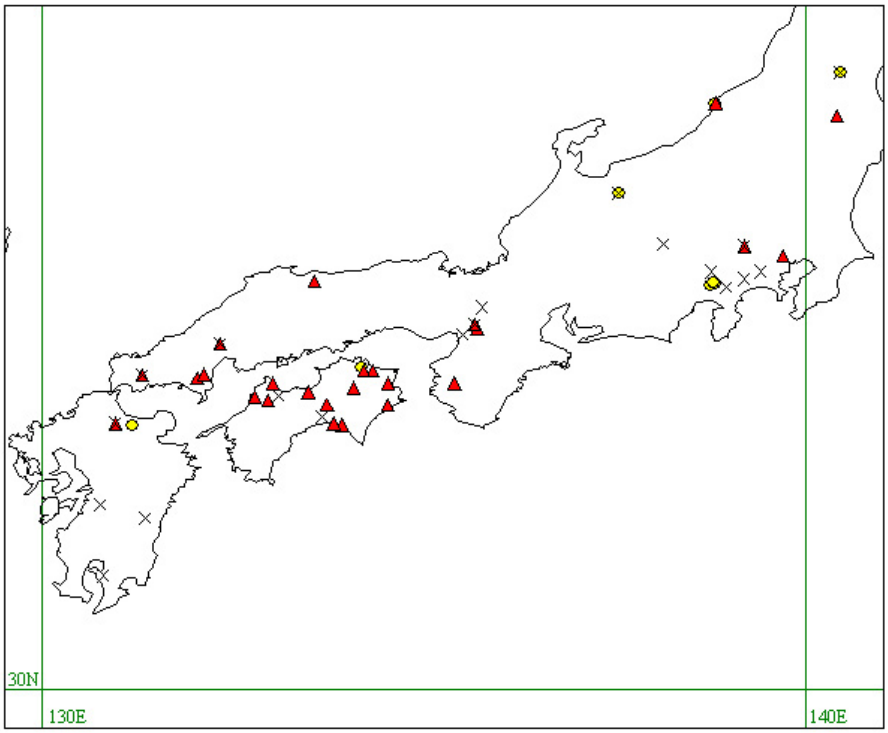



**Figure 6.11** Distribution of tetraploid *F. japonica* var. '*japonica*' plants. Yellow circles represent sites where dwarf plants (less than or equal to 80 cm in height at their peak) were collected, red triangles represent sites where tall plants (greater than or equal to 150 cm) were found, and crosses represent sites where plants of intermediate or unknown height were from.

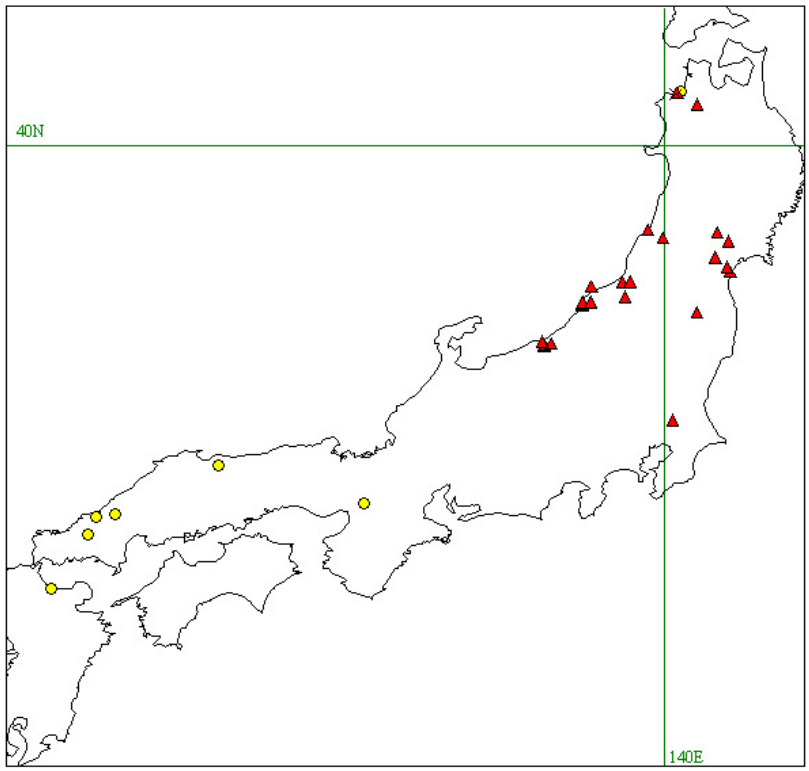



**Figure 6.12** Distribution of octoploid *F. japonica* plants. The yellow circles represent sites where *F. japonica* var. '*japonica*' was found. Red triangles represent sites where *F. japonica* var. *uzenensis* was found, whilst X shows the site where a putative hybrid between the two varieties of *F. japonica* was found.

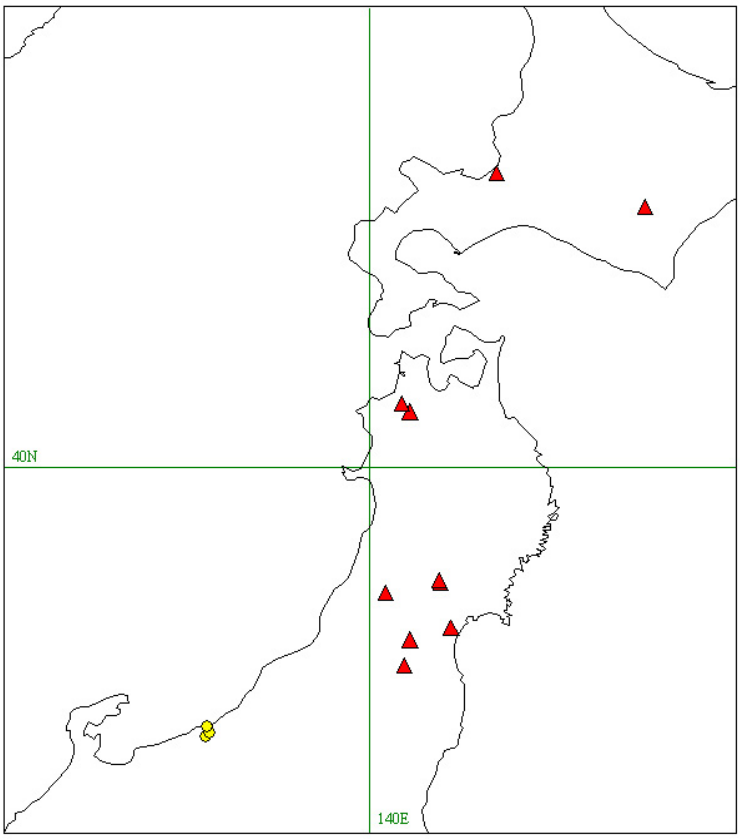



**Figure 6.13** Distribution of sites from which *F. sachalinensis* was found. Yellow circles represent *F. sachalinensis* accessions with an MPH found in clade C in Figures 6.7 and 6.8, whilst red triangles represent accessions with an MPH found in clade B of the same figures.


**Figure 6.14** Map of Japan and Korea, the broken lines indicate a coastal line or land connection in the middle Pleistocene. Adapted from Chung and Chung (2000).

**Table 6.1** Summary of lower epidermal characteristics for some *Fallopia* species from the study by Bailey (1989).

| Species | Degree of cuticular striation | Trichome type |
|---|---|---|
| *F. baldschuanica* | ± | A |
| *F. multiflora* | – | A |
| *F. cilinodis* | + + | C |
| *F. cyanchoides* | + + | C |
| *F. sachalinensis* | + + + | B |
| *F. japonica* | ± | A |

**Table 6.2** Site and plant details for Japanese Knotweed plants collected or sent from within Japan. When there is the possibility that the plants may be of hybrid origin a letter H is used in parenthesis after the taxon name. This applies to any plant growing in a prefecture where the two species are known to be sympatric, even if only one of the taxa was found at the actual site.

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Kyushu | Kagoshima | Tarumizu | *F. japonica* | 1 | Not Known | N 31 30, E 130 47 | Not Known | Not known |
| 2 | Kyushu | Miyazaki | Not known | *F. japonica* | 3 | Not known | N 32 15, E 131 20 | Not known | Not known |
| 3 | Kyushu | Kumamoto | Hitoyoshi | *F. japonica* | 3 | Not known | N 32 26, E 130 45 | Not known | Not known |
| 4 | Kyushu | Kumamoto | Mt. Aso | Dwarf *F. japonica* | 18 | 20 cm - 90 cm | N 32 53, E 131 06 | 1300 | Volcano summit |
| 5 | Kyushu | Oita | Hiko-san | *F. japonica* | 2 | 300 cm | N 33 30.110, E 130 57.870 | 720 | Roadside in forest |
| 6 | Kyushu | Fukuoka | Nakatsu | *F. japonica* | 3 | 150 cm | N 33 35.681, E 131 09.180 | 110 | Riverside |
| 7 | Shikoku | Kochi | Kono, Achi city | *F. japonica* | 1 | 150 – 200 cm | N 33 29.73, E 133 55.43 | Not known | Hillside |
| 8 | Shikoku | Kochi | Okatoyo-cho, Nankoku city | *F. japonica* | 1 | 80 – 150 cm | N 33 35.95, E 133 39.03 | Not known | Near Railway |
| 9 | Shikoku | Kochi | Near Osugi Shrine, Ootoyo | *F. japonica* | 1 | 150 – 200 cm | N 33 45.68, E 133 43.55 | Not known | Near Paddy field |
| 10 | Shikoku | Tokushima | Urushiyawa, Ikeda town | *F. japonica* | 1 | 150 – 200 cm | N 33 30.27, E 133 48.71 | Not known | Riverside |
| 11 | Shikoku | Tokushima | Hiwada town | *F. japonica* | 1 | 150 – 200 cm | N 33 45.68, E 134 31.29 | Not known | Riverside |
| 12 | Shikoku | Tokushima | Kawauchi, Ichiu village | *F. japonica* | 1 | 150 – 200 cm | N 33 58.09, E 134 04.62 | Not known | Riverside |

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 13 | Shikoku | Tokushima | Mukaeda, Tokushima city | *F. japonica* | 1 | 150 – 200 cm | N 34 02.45, E 134 31.94 | Not known | Riverside |
| 14 | Shikoku | Ehime | Ochide kawauchi cho, Onsen county | *F. japonica* | 1 | 150 – 200 cm | N 33 48.55, E 132 57.14 | Not known | Hillside |
| 15 | Shikoku | Ehime | Iwaidani cho, Dogo, Matsuyama city | *F. japonica* | 1 | 150 – 200 cm | N 33 51.08, E 132 46.43 | Not known | Hillside |
| 16 | Shikoku | Ehime | Myoguchi, Komatsu town, Shuso county | *F. japonica* | 1 | 80 – 150 cm | N 33 52.16, E 133 05.52 | Not known | Riverside |
| 17 | Shikoku | Ehime | Okunodaira Tachikawa, Nihama city | *F. japonica* | 1 | 150 – 200 cm | N 33 55.41, E 133 28.00 | Not known | Riverside |
| 18 | Shikoku | Ehime | Katayama, Imabari city | *F. japonica* | 1 | 150 – 200 cm | N 34 02.73, E 133 00.00 | Not known | Riverside |
| 19 | Shikoku | Kagawa | Ikejiri, Ouchi town | *F. japonica* | 1 | 150 – 200 cm | N 34 12.00, E 134 19.78 | Not known | Roadside |
| 20 | Shikoku | Kagawa | Sukemitu, Tawa, Nagao town | *F. japonica* | 1 | 150 – 200 cm | N 34 12.55, E 134 12.20 | Not known | Roadside |
| 21 | Shikoku | Kagawa | Nagao town, Kita county | *F. japonica* | 1 | < 80 cm | N 34 15.00, E 134 10.55 | Not known | Roadside |
| 22 | Honshu | Wakayama | Not known | F. japonica | 2 | 2 m | N 34 02, E 135 23 | 300 | Not known |
| 23 | Honshu | Yamaguchi | Noguchi Kano | *F. japonica* | 3 | 300 cm - 400 cm | N 34 06.613, E 132 01.574 | 170 | Roadside near stream |
| 24 | Honshu | Yamaguchi | Tano-Tokuji | *F. japonica* | 4 | 140 cm - 300 cm | N 34 08.624, E 131 18.063 | 400 | Roadside |
| 25 | Honshu | Yamaguchi | Nishiki Gawa, nr Iwakuni | *F. japonica* | 1 | 400 cm | N 34 09.546, E 132 06.118 | 220 | Wooded roadside |

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 26 | Honshu | Yamaguchi | Abu gawa | *F. japonica* | 2 | 300 cm | N 34 21.823, E 131 40.774 | 380 | Riverside |
| 27 | Honshu | Yamaguchi | Shimane | *F. japonica* | 3 | Regrowth | N 34 37.405, E 131 48.325 | 140 | Riverside |
| 28 | Honshu | Yamaguchi | Shimane | *F. japonica* | 1 | Regrowth 150 cm | N 34 39.872, E 132 04.229 | 680 | Roadside in forest |
| 29 | Honshu | Hiroshima | Chugoku | *F. japonica* | 3 | 20 cm - 150 cm | N 34 33.372, E 132 19.824 | 140 | Riverside, growing in sand |
| 30 | Honshu | Hiroshima | Tigouichi | *F. japonica* | 3 | 100 cm - 200 cm | N 34 39.286, E 132 04.710 | 650 | Open area off road |
| 31 | Honshu | Osaka | Yamanoue, Hirokuta city | *F. japonica* | 1 | 300 cm | N 34 45.988, E 135 41.189 | Not known | Hill in national park |
| 32 | Honshu | Osaka | Yamanoue, Hirokuta city | *F. japonica* | 8 | 95 cm - 160 cm | N 34 48.181, E 135 39.677 | Not known | Ruderal |
| 33 | Honshu | Osaka | Osaka castle | *F. japonica* | 1 | 80 cm | N 34 40.23, E 135 30.00 | Not known | Growing inside castle |
| 34 | Honshu | Kyoto | University | F. japonica | 1 | Not Known | N 35 02, E 135 45 | Not Known | Not known |
| 35 | Honshu | Shizuoka | Mukai village, Gotenba city | *F. japonica* | 3 | 30 cm - 145 cm | N 35 18.534, E 138 57.149 | 415 | Ruderal |
| 36 | Honshu | Shizuoka | Mt. Fuji | Dwarf *F. japonica* | 8 | c50 cm | N 35 19.862, E 138 44.059 | 2245 | Volcano |
| 37 | Honshu | Shizuoka | Mt. Fuji | Dwarf *F. japonica* | 3 | c30 cm | N 35 20.165, E 138 47.966 | 1452 | Volcano |
| 38 | Honshu | Shizuoka | Mt. Fuji | Dwarf *F. japonica* | 1 | 35 cm | N 35 20.165, E 138 47.966 | 1282 | Volcano |
| 39 | Honshu | Tottori | Mt. Daisen | F. japonica | 6 | 2 m | N 35 22, E 133 33 | 700 | Not known |
| 40 | Honshu | Kanagawa | Hadano | *F. japonica* | 3 | 1.2m – 1.5 m | N 35 24, E 139 12 | 780 | Not known |

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 41 | Honshu | Kanagawa | Not known | *F. japonica* | 1 | Not known | N 35 30, E 139 24 | 650 | Not known |
| 42 | Honshu | Yamanashi | Kawaguchi | *F. japonica* | 2 | Not known | N 35 30.34, E 138 45.60 | 1000 | Mountain |
| 43 | Honshu | Tokyo | Hachijo Islands | *F. japonica*. var. *terminalis* | 1 | Not known | N 33 08.00, E 139 48.09 | Not known | Not known |
| 44 | Honshu | Tokyo | Shinjuku | F. japonica | 3 | 1.5 m | N 35 42, E 139 42 | Not known | Ruderal |
| 45 | Honshu | Tokyo | Tamagawa | *F. japonica* | 2 | 1 m - 2 m | N 35 50, E 139 12 | Not known | River floodplain |
| 46 | Honshu | Tokyo | Not known | *F. japonica* | 1 | Not known | N 35, E 139 | Not known | Not known |
| 47 | Honshu | Nagano | River Fuji | *F. japonica* | 1 | Not known | N 35 52, E 138 07 | Not known | Not known |
| 48 | Honshu | Ibaraki | Tsukuba | *F. japonica* | 1 | 50 cm | N 36 02.111, E 140 06.203 | 50 | Roadside beneath pines |
| 49 | Honshu | Toyama | Mt. Tateyama | F. japonica | 3 | Not known | N 36 33, E 137 32 | 1980 | Mountain |
| 50 | Honshu | Niigata | Mt. Namba, N. of Joetsu city | *F. japonica* (H), *F. sachalinensis* (H) | 4 | 122 cm - 262 cm | N 37 04.180, E 138 12.429 | 370 | Scrubland |
| 51 | Honshu | Niigata | Joetsu | *F. japonica* (H), *F. sachalinensis* (H) | 5 | Not Known | N 37 06, E 138 15 | Not Known | Not known |
| 52 | Honshu | Niigata | Aota-gawa (river) | *F. sachalinensis* (H) | 1 | 186 cm | N 37 07.604, E 138 15.066 | 0 | Riverside |
| 53 | Honshu | Niigata | Kanda, Samua village, | *F. japonica* (H) | 3 | Regrowth, 20 cm - 270 cm | N 37 08.570, E 138 21.351 | 20 | Roadside |
| 54 | Honshu | Niigata | Naoetsu city | *F. japonica* (H), *F. sachalinensis* (H) | 3 | 70 cm - 224 cm | N 37 10.285, E 138 13.796 | 5 | Coastline |
| 55 | Honshu | Niigata | Mt. Yahiko | *F. japonica* (H) | 1 | 154cm regrowth | N 37 41.091, E 138 48.751 | 280 | Woodland margins |

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 56 | Honshu | Niigata | Mt. Yahiko | *F. japonica* (H) | 3 | 140 cm - 206 cm | N 37 43.141, E 138 48.806 | 470 | Woodland margins |
| 57 | Honshu | Niigata | Mt. Yahiko | *F. japonica* (H) | 2 | c60 cm - 290 cm | N 37 43.154, E 138 48.701 | 436 | Woodland margins |
| 58 | Honshu | Niigata | Mt Yahiko | *F. japonica* (H) | 2 | 260 cm - 280 cm | N 37 44.182, E 138 48.619 | 150 | Stream in dense Woodland |
| 59 | Honshu | Niigata | Mt. Yahiko | *F. japonica* (H) | 2 | 186 cm - 278 cm | N 37 44.207, E 138 49.397 | 120 | Woodland margins |
| 60 | Honshu | Niigata | Akadani valley | *F. japonica* (H) | 3 | 120 cm - 150 cm | N 37 49.839, E 139 25.435 | 230 | Roadside in woodland |
| 61 | Honshu | Niigata | Niigata town | *F. japonica* (H) | 1 | Not known | N 37 58.187, E 138 56.477 | Not known | Bridge outside town |
| 62 | Honshu | Niigata | Mt Omineyama | *F. japonica* (H) | 2 | 150 cm - 350 cm | N 38 01.071, E 139 23.844 | 150 | Riverside in woodland |
| 63 | Honshu | Niigata | Mt Tainai | *F. japonica* (H) | 2 | 270 cm - 300 cm | N 38 02.253, E 139 29.071 | 220 | *Cryptomeria* woodland |
| 64 | Honshu | Fukushima | Sagita, Nihonmatsu city | *F. japonica* (H) | 2 | 300 cm | N 37 33.818, E 140 24.858 | 290 | Railway side |
| 65 | Honshu | Fukushima | Nihonmatsu | *F. japonica* (H) | 2 | 70 cm | N 37 35.657, E 140 27.424 | 220 | Roadside at supermarket |
| 66 | Honshu | Fukushima | Yonezawa Rd | *F. sachalinensis* (H) | 2 | 250 cm | N 37 50.365, E 140 22.360 | 360 | Quarry edge |
| 67 | Honshu | Yamagata | Yonezawa Town | *F. japonica* (H) | 1 | 200 cm | N 37 54.702, E 140 08.071 | 320 | Garage forecourt |
| 68 | Honshu | Yamagata | Zao Quasi National Park | *F. japonica* (H), *F. sachalinensis* (H), mostly dwarves | 11 | 30 cm - 200 cm | N 38 07.725, E 140 26.187 | 1500 | Heathland |
| 69 | Honshu | Yamagata | Zao Quasi National Park, Zao-zan | *F. japonica* (H) | 1 | 50 cm | N 38 08.466, E 140 23.543 | 900 | Roadside |

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 70 | Honshu | Yamagata | Shinjo – Sakata | *F. japonica* (H), *F. sachalinensis* (H) | 2 | 300 cm (*F. sachalinensis*) | N 38 38.967, E 140 10.359 | 330 | Roadside |
| 71 | Honshu | Yamagata | Haguro san (Mt.) | *F. japonica* (H) | 2 | 150 cm | N 38 40.061, E 139 58.979 | 450 | *Cryptomeria* woodland |
| 72 | Honshu | Yamagata | Yunahama Onsen | *F. japonica* (H) | 1 | 160 cm | N 38 47.071, E 139 45.802 | 25 | Roadside |
| 73 | Honshu | Miyagi | River Nanakita Gamou, Sendai | *F. japonica* (H) | 1 | 200 cm | N 38 11.071, E 140 56.943 | 0 | Riverside |
| 74 | Honshu | Miyagi | Mt. Aoba-yama, nr. Sendai | *F. japonica* (H), *F. sachalinensis* (H) | 9 | 80 cm - 157 cm | N 38 15.00, E 140 53.14 | Foot of mt. | Ruderal |
| 75 | Honshu | Miyagi | Mt Izumi,Sendai | *F. japonica* (H) | 2 | 100 cm - 150 cm | N 38 23.420, E 140 43.311 | 550 | Roadside |
| 76 | Honshu | Miyagi | Tohoku district | *F. japonica* (H) | 1 | 300 cm | N 38 37.065, E 140 54 698 | Not known | Roadside |
| 77 | Honshu | Miyagi | Eai River Valley | *F. japonica* (H), *F. sachalinensis* (H) | 10 | 115 cm - 337 cm | N 38 44.053, E 140 46.122 | 80 | Streamside in River floodplain |
| 78 | Honshu | Miyagi | Naruko | *F. japonica* (H), *F. sachalinensis* (H) | 2 | 90 cm - 270 cm | N 38 45.006, E 140 45.713 | 160 | Roadside / Streamside |
| 79 | Honshu | Aomori | Hirosaki City, Iwaki river | *F. japonica* (H), *F. sachalinensis* (H) | 6 | 110 cm - 300 cm | N 40 36.004, E 140 26.366 | 0 | Riverside, growing through flood rivetments |
| 80 | Honshu | Aomori | Hirosaki City Castle park | *F. japonica* (H), *F. sachalinensis* (H) | 6 | 100 cm - 238 cm | N 40 36.285, E 140 27.863 | 60 | Public park |
| 81 | Honshu | Aomori | Mt. Iwaki | *F. japonica* (H), *F. sachalinensis* (H) | 3 | Regrowth, *F. sachalinensis* 3m | N 40 37.302, E 140 21.353 | 258 | Woodland margins |
| 82 | Honshu | Aomori | Road from Mt. Iwaki to coast | *F. japonica* (H), *F. sachalinensis* (H) | 5 | 75 cm - 170 cm | N 40 41.764, E 140 21.125 | 208 | Roadside |
| 83 | Honshu | Aomori | Coastal road, Japan sea side | *F. japonica* (H) | 1 | Regrowth | N 40 44.891, E 140 05.401 | 5 | Coastal Roadside |

| Site | Island | Prefecture | Locality | Taxa collected | No. of plants | Height of plants | Latitude Longitude | Altitude (masl) | Habitat |
|---|---|---|---|---|---|---|---|---|---|
| 84 | Honshu | Aomori | Ajiga-sawa, Japan sea side | *F. japonica* (H) | 4 | 60 cm - 175 cm | N 40 45.763, E 140 09.684 | 5 | Coastal Roadside |
| 85 | Honshu | Aomori | Road from Mt. Iwaki to coast | *F. japonica* (H) | 1 | 40 cm | N 40 46.166, E 140 13.094 | 21 | Roadside |
| 86 | Hokkaido | N/A | Memuro | *F. sachalinensis* | 8 | Not known | N 42 50.12, E 143 00.44 | Not known | Not known |
| 87 | Hokkaido | N/A | Ishikari | *F. sachalinensis* | 7 | Not known | N 43 12.24, E 141 23.11 | Not known | Not known |

**Table 6.3** Non Japanese plants included in the study.

| Taxon | Country | 2n = | No. of specimens | P number |
|---|---|---|---|---|
| *F. japonica* | China | 88 | 1 | 113 |
| *F. japonica* | China | 110 | 2 | 583, 584 |
| *F. sachalinensis* | Korea | 102 | 2 | 476, 477 |
| *F. elliptica* | Korea | 88 | 1 | 555 |
| *F. japonica* var. *japonica* | UK | 88 | 12 | 192,195,199, 334, 415, 447, 743, 823, 853, 870, 1039, 1126, |
| *F. japonica* var. *japonica* | Holland | 88 | 2 | 1025, 1026 |
| *F. japonica* var. *japonica* | Germany | 88 | 1 | 848 |
| *F. japonica* var. *compacta* | UK | 44 | 4 | 2, 174, 193, 413 |
| *F. sachalinensis* | UK | 44 | 17 | 55, 57, 310, 327, 618, 624, 626, 706, 710, 824, 871, 1117, 877, 1227, 716, 851, 852. |
| *F. sachalinensis* | USA | 44 | 1 | 834 |
| *F. sachalinensis* | Czech republic | 44 | 1 | 573 |
| *Reynoutria* x *vivax* | Germany | 44 | 1 | 204 |

**Table 6.4** Individual plant information for plants originating from Japan, grouped according to shared chloroplast multi primer haplotype (MPH). Includes site number, P number, leaf characteristics; chromosome number; sex of plant; plant height and the altitudinal vegetation zone. Gaps indicate missing data. A hyphen indicates a plant whose height was known but was not classed as a tall or dwarf *F. japonica.*

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 32 | 987 | 88 | 1 | A | ≈truncate | | | 100 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 1 | 55 | 983a | 88 | 1 | C | ≈truncate | | male fertile | 154 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 1 | 72 | 1078 | c88 | | C | ≈truncate | | | 65 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 1 | 73 | 1074 | 88 | | C | ≈truncate | | male sterile | 200 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 1 | 74 | 924 | 88 | | C | truncate | | male sterile | 80 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 1 | 75 | 1073 | 88 | | C | ≈truncate | | male fertile | 100–150 cm | - | Montane | *F. japonica.* var. *uzenensis* |
| 2 | 79 | 940 | 66 | 3/4 | B (B/C) | cordate | | male fertile | 110 cm | - | Lowland | *F. sachalinensis* or hybrid |
| 2 | 80 | 935 | c66 | 3 | B (B/C) | cordate | | male fertile | 188 cm | - | Lowland | *F. sachalinensis* or hybrid |
| 2 | 82 | 954 | 44 | 3 | B | cordate | | male fertile | 122 cm | - | Lowland | *F. sachalinensis* |
| 2 | 82 | 955 | 44 | | B | cordate | | male sterile | 170 cm | - | Lowland | *F. sachalinensis* |
| 2 | 86 | 1167 | | | | | | | | | | *F. sachalinensis* |
| 2 | 86 | 1168 | | | | | | | | | | *F. sachalinensis* |
| 2 | 86 | 1169 | | 4 | B | cordate | | | | | | *F. sachalinensis* |
| 2 | 86 | 1170 | | | | | | | | | | *F. sachalinensis* |
| 2 | 86 | 1171 | | 3/4 | B | ≈cordate | | | | | | *F. sachalinensis* |
| 2 | 86 | 1172 | | 4 | B | cordate | | | | | | *F. sachalinensis* |
| 2 | 86 | 1173 | | | | | | | | | | *F. sachalinensis* |
| 2 | 86 | 1174 | | | | | | | | | | *F. sachalinensis* |
| 2 | 87 | 1160 | | | | | | | | | | *F. sachalinensis* |
| 2 | 87 | 1161 | | 4 | B | cordate | | | | | | *F. sachalinensis* |
| 2 | 87 | 1162 | | 4 | B | cordate | | | | | | *F. sachalinensis* |
| 2 | 87 | 1163 | | | | | | | | | | *F. sachalinensis* |
| 2 | 87 | 1164 | | | | | | | | | | *F. sachalinensis* |
| 2 | 87 | 1165 | | | | | | | | | | *F. sachalinensis* |
| 2 | 87 | 1166 | | | | | | | | | | *F. sachalinensis* |
| 3 | 24 | 1096 | 44 | 1 | A | ≈truncate | | male sterile | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 3 | 24 | 1098 | 44 | 1 | A | ≈truncate | | male sterile | 150 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 3 | 32 | 988 | 44 | 1 | A | truncate | | | 95 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 3 | 35 | 909 | 44 | 1/2 | A | ≈truncate | | | 30 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 3 | 36 | 901 | c44 | 0 | A | ≈truncate | Yes | male fertile | 50 cm | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 36 | 902 | 44 | 0/1 | A | ≈truncate | Yes | male sterile | 50 cm | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 36 | 903 | | 1 | A | ≈truncate | Yes | male sterile | 50 cm | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 36 | Jap 5 | | | | | | male fertile | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 36 | Jap 6 | | | | | | male fertile | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 36 | Jap 7 | | | | | | male fertile | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 36 | Jap 8 | | | | | | male fertile | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 36 | Jap 14 | | | | | | male sterile | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 3 | 37 | 904 | c44 | 0/1 | A | ≈truncate | Yes | male sterile | 30 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 3 | 37 | 905 | 44 | 0/1 | A | ≈truncate | | male sterile | 20 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 3 | 37 | 906 | 44 | | A | ≈truncate | Yes | male fertile | 30 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 3 | 38 | Jap 32 | | | | | | male fertile | 35 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 3 | 39 | 801a | | | A | ≈accuminate | | | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 3 | 40 | 783a | | | A | ≈truncate | | | 120–150 cm | - | Montane | *F. japonica* var.*'japonica'* |
| 3 | 40 | 784a | | | A | ≈truncate | Yes | | 120–150 cm | - | Montane | *F. japonica* var.*'japonica'* |
| 3 | 40 | 786c | | | A | ≈truncate | | | 120–150 cm | - | Montane | *F. japonica* var.*'japonica'* |
| 3 | 42 | 595 | | | | | | | | | Montane | *F. japonica* var.*'japonica'* |
| 3 | 42 | 673 | 44 | 0/1 | A | ≈truncate | Yes | | | | Montane | *F. japonica* var.*'japonica'* |
| 3 | 45 | 733 | 44 | 0/1 | A | ≈truncate | Yes | | 100 cm | - | | *F. japonica* var.*'japonica'* |
| 3 | 45 | 734 | 44 | 1/2 | A | ≈truncate | | | 200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 3 | 46 | 114b | 44 | | | | | | | | | *F. japonica* var.*'japonica'* |
| 4 | 24 | 1094 | 44 | 1/2 | A | ≈truncate | | male sterile | 140 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 4 | 32 | 986 | 44 | | | | | | 160 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 4 | 32 | 820b | 44 | 1 | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 4 | 32 | 821a | 44 | 0/1 | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 4 | 32 | 821b | 44 | 1 | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 4 | 32 | 821c | 44 | 0/1 | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 4 | 35 | 907 | 44 | 1 | A | ≈truncate | | male fertile | 145 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 4 | 35 | 908 | | | A | ≈truncate | | | regrowth | | Lowland | *F. japonica* var.*'japonica'* |
| 4 | 39 | 798c | | | A | truncate | | | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 4 | 41 | 594 | 44 | 1/2 | A | ≈truncate | Yes | | | | Montane | *F. japonica* var.*'japonica'* |
| 4 | 44 | 730 | 44 | 1 | A | ≈truncate | Yes | | 150 cm | Tall | | *F. japonica* var.*'japonica'* |
| 4 | 44 | 731 | | 1 | A | truncate | Yes | | 150 cm | Tall | | *F. japonica* var.*'japonica'* |
| 4 | 47 | 736 | 44 | | A | ≈cordate | | | | | | *F. japonica* var.*'japonica'* |
| 4 | 61 | 984 | 88 | 1/2 | C | truncate /cordate | | | | | Lowland | *F. japonica.* var. *uzenensis* |
| 4 | 64 | 1051 | 44 | 2 | A | ≈truncate | | male fertile | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 4 | 64 | 1052 | 44 | 2 | A | truncate | | male fertile | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 4 | 66 | 1055 | 44 | 4 | B | cordate | | | 250 cm | - | Lowland | *F. sachalinensis* |

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | 66 | 1056 | 44 | 4 | B | cordate | | male fertile | 250 cm | - | Lowland | *F. sachalinensis* |
| 4 | 67 | 1057 | 66 | 2 | C/B | ≈truncate | | male fertile | 200 cm | | Lowland | Hybrid |
| 4 | 68 | 1059 | 44 | 1 | A | ≈truncate | | male sterile | 85 cm | | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 4 | 68 | 1060 | 44 | | A | ≈truncate | | male sterile | 3 cm | Dwarf | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 4 | 68 | 1063 | 44 | 0 | A | ≈truncate | | | 45 cm | Dwarf | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 4 | 68 | 1064 | 44 | 1/2 | A | ≈truncate | | male fertile | 45 cm | Dwarf | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 4 | 68 | 1066 | 44 | 0/1 | A | ≈truncate | | | 25 cm | Dwarf | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 4 | 68 | 1068 | | | A | ≈truncate | | | 10 cm | Dwarf | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 4 | 69 | 1058 | 66 | 1/2 | A/D | ≈truncate | | male sterile | 50 cm | - | Montane | Hybrid |
| 4 | 78 | 910 | 88 | | C | ≈truncate | | | 90 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 57 | 980 | 88 | 1 | C | ≈truncate | | male fertile | 290 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 58 | 1088 | | 1 | C | ≈truncate | | male fertile | 260 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 58 | 1089 | c88 | 0 | C | truncate | | male sterile | 280 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 59 | 977 | 88 | 1 | C | ≈truncate | | male fertile | 186 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 59 | 978 | cc88 | 1 | C | ≈truncate | | male fertile | 278 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 74 | 928 | 88 | | C | ≈truncate | | | | | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 74 | 929 | 88 | | C | ≈truncate | | male sterile | 100 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 74 | 930 | 88 | 0 | C | ~truncate | | | | | Lowland | *F. japonica.* var. *uzenensis* |
| 5 | 77 | 922 | 66 | 2 | A/D | ≈truncate | | | 115 cm | - | Lowland | Hybrid |
| 5 | 77 | 923 | 66 | 2 | C/D | ≈truncate | | | 135 cm | - | Lowland | Hybrid |
| 6 | 50 | 965 | 44 | 4 | B | cordate | | | 225 cm | - | Lowland | *F.sachalinensis* |
| 6 | 50 | 966 | 44 | 2/3 | B | cordate | | | 242 cm | - | Lowland | *F. sachalinensis* |
| 6 | 54 | 976 | 44 | 3 | B | cordate | | | 70 cm | - | Lowland | *F. sachalinensis* |
| 7 | 62 | 1084 | c88 | | C | ≈truncate | | male sterile | 150 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 8 | 79 | 939 | 66 | 1/2 | C (B/C) | ≈truncate | | | 120 cm | - | Lowland | Hybrid |
| 8 | 79 | 941 | 66 | 1 | C/D | ≈cordate | | | regrowth | | Lowland | Hybrid |
| 8 | 79 | 942 | 44 | | B | cordate | | male sterile | 300 cm | - | Lowland | *F. sachalinensis* |
| 8 | 79 | 943 | 44 | 4 | B | cordate, | | male fertile | 170 cm | - | Lowland | *F. sachalinensis* |
| 8 | 79 | 944 | c66 | 1 | C/D | ≈cordate | | | 110 cm | - | Lowland | Hybrid |
| 8 | 80 | 932 | c66 | 1/2 | C/D | truncate | | | regrowth | | Lowland | Hybrid |
| 8 | 80 | 933 | 66 | 2 | C/D | ≈truncate | | | regrowth | | Lowland | Hybrid |
| 8 | 80 | 934 | c66 | 1 | D | ≈truncate | | | regrowth | | Lowland | Hybrid |
| 8 | 80 | 938 | 66 | 1 | C | cordate | | | 100 cm | - | Lowland | Hybrid |
| 8 | 81 | 950 | 66 | 1/2 | B/C | cordate | | male fertile | 300 cm | - | Lowland | Hybrid |
| 8 | 81 | 951 | 66 | 2 | D | ≈truncate | | | 27cm | - | Lowland | Hybrid |
| 8 | 82 | 953 | 66 | 1/2 | D/C | ≈truncate | | | 115 cm | - | Lowland | Hybrid |
| 8 | 82 | 956 | | 1/2 | D/C | ≈truncate | | | 165 cm | - | Lowland | Hybrid |

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | 82 | 957 | 66 | 1 | C | ≈cordate | | | 75 cm | - | Lowland | Hybrid |
| 8 | 83 | 959 | 44 | 1 | B | cordate | | | regrowth | | Lowland | Hybrid |
| 8 | 84 | 961 | 85 | | C | truncate | | male sterile | 60 cm | Dwarf | Lowland | *F. japonica.* var. *uzenensis* |
| 8 | 84 | 962 | c88 | 1/2 | C | truncate | | male fertile | 175 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 8 | 84 | 963 | cc88 | 2 | A/D | truncate | | male fertile | 62 cm | - | Lowland | Hybrid |
| 8 | 84 | 964 | 88 | | C | truncate | | male fertile | 120 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 8 | 85 | 958 | c88 | 1/2 | A | ≈cordate | | | regrowth | - | Lowland | *F. japonica* |
| 9 | 50 | 969 | 66 | 0/1 | C/D | truncate/cordate | | | 122 cm | - | Lowland | Hybrid |
| 9 | 53 | 970 | 88 | | C | truncate | | | regrowth | | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 53 | 972 | c88 | | C | ≈truncate | | | 270 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 60 | 1085 | 88 | | C | ≈truncate | | male fertile | 150 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 60 | 1086 | c88 | | C | ≈truncate | | male fertile | 150 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 60 | 1087 | 88 | | C | ≈truncate | | male sterile | 120 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 62 | 1083 | 88 | | C | ≈truncate | | male sterile | 350 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 71 | 1080 | 88 | 1 | C | ≈truncate | | male fertile | 150 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 9 | 77 | 915 | 66 | 3/4 | B (B/C) | cordate | | | 254 cm | - | Lowland | Hybrid |
| 9 | 77 | 916 | | 3/4 | B (B/C) | cordate | | | 380 cm | - | Lowland | Hybrid |
| 10 | 2 | 770a | 44 | | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 10 | 2 | 788d | | | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 10 | 2 | 789a | | | A | ≈accuminate | | | | | | *F. japonica* var.*'japonica'* |
| 10 | 3 | 771a | 44 | | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 10 | 3 | 790a | | | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 10 | 3 | 791e | | | A | ≈truncate | | | | | | *F. japonica* var.*'japonica'* |
| 10 | 4 | 990 | 44 | | | | | male fertile | 80 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 991 | 44 | | | | | male fertile | 45 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 992 | 44 | 0 | A | ≈truncate | | male sterile | 28 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 993 | 44 | | | | | male fertile | 23 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 994 | 44 | | | | | male sterile | 30 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 995 | cc44 | | | | | male sterile | 45 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 996 | c44 | 0/1 | A | ≈truncate | | male sterile | 25 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | 997 | 44 | | | | | male sterile | 30 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap115 | | | | | | male fertile | 75 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap116 | | | | | | male fertile | 20 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap117 | | | | | | male sterile | 70 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap118 | | | | | | male sterile | 55 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap119 | | | | | | male sterile | 35 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap120 | | | | | | male sterile | 50 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 4 | Jap121 | | | | | | male fertile | 60 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap122 | | | | | | male sterile | 30 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap123 | | | | | | male fertile | 80 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 4 | Jap124 | | | | | | male fertile | 40 cm | Dwarf | Montane | *F. japonica* var.*'japonica'* |
| 10 | 6 | 1099 | c88 | 1 | A | ≈truncate | | male fertile | 150 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 6 | 1100 | 88 | 1 | A | truncate / cordate | | male sterile | 150 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 6 | 1101 | 88 | 1 | A | ≈truncate | | | 150 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 8 | 1137 | 44 | 0/1 | A | ≈truncate | | | 80–150 cm | - | | *F. japonica* var.*'japonica'* |
| 10 | 17 | 1138 | 44 | 1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 10 | 20 | 1128 | 44 | 0/1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 10 | 25 | 1090 | cc44 | 0 | A | ≈truncate | | male fertile | 400 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 26 | 1104 | 88 | 1 | A | accuminate | | male sterile | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 26 | 1105 | 88 | 1 | A | ≈truncate | | male sterile | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 27 | 1107 | c88 | 1 | A | truncate | | male sterile | 100 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 27 | 1108 | 88 | 1 | A | truncate | | male fertile | 90 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 27 | 1109 | 88 | 1 | A | accuminate | | male sterile | | | Lowland | *F. japonica* var.*'japonica'* |
| 10 | 28 | 1110 | 88 | 1 | A | ≈truncate | | male fertile | 150 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 10 | 30 | 1111 | c88 | 1/2 | A | ≈truncate | | male fertile | 100 cm | - | Montane | *F. japonica* var.*'japonica'* |
| 10 | 30 | 1112 | 88 | 1 | A | ≈truncate | | male sterile | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 10 | 30 | 1113 | 88 | 1 | A | ≈truncate | | male fertile | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 11 | 68 | 1061 | 44 | 0/1 | A | ≈truncate | | male fertile | 30 – 40 cm | Dwarf | Subalpine/Montane | *F. japonica* var.*'japonica'* |
| 11 | 68 | 1065 | 44 | 4 | B | cordate | | male fertile | 100 cm | - | Subalpine/Montane | *F. sachalinensis* |
| 11 | 74 | 925 | 44 | | B | cordate | | | 157 cm | - | Lowland | *F. sachalinensis* |
| 11 | 74 | 926a | 44 | 4 | B | cordate | | male fertile | | | Lowland | *F. sachalinensis* |
| 11 | 74 | 926b | | | B | cordate | | male fertile | | | Lowland | *F. sachalinensis* |
| 11 | 74 | 927 | 44 | | B | cordate | | | 130 cm | - | Lowland | *F. sachalinensis* |
| 11 | 74 | 931 | 44 | | B | cordate | | male fertile | | | Lowland | *F. sachalinensis* |
| 11 | 76 | 1075 | | 2 | C | ≈truncate | | male fertile | 300 cm | Tall | | *F. japonica.* var. *uzenensis* |
| 11 | 77 | 913 | c44 | | B | cordate | | | 240 cm | - | Lowland | *F. sachalinensis* |
| 11 | 77 | 918 | 44 | | B | cordate | | | 337 cm | - | Lowland | *F. sachalinensis* |
| 11 | 77 | 919 | 44 | 3/4 | B | cordate | | male sterile | 254 cm | - | Lowland | *F. sachalinensis* |
| 11 | 77 | 920 | 44 | | B | cordate | | male sterile | 180 cm | - | Lowland | *F. sachalinensis* |
| 11 | 77 | 921 | c44 | 3/4 | B | ≈cordate | | male fertile | 170 cm | - | Lowland | *F. sachalinensis* |
| 11 | 78 | 912 | 44 | | B | cordate | | | 270 cm | - | Lowland | *F. sachalinensis* |
| 11 | 80 | 936 | 88 | 1 | C | ≈cordate | | male sterile | 238 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 48 | 1050 | 88 | 0 | C | truncate / cordate | | male fertile | 50 cm | Dwarf | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 51 | 772c | 88 | | C | ≈truncate | | | | | | *F. japonica.* var. *uzenensis* |

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12 | 51 | 792a | | | C | ≈truncate | | | | | | *F. japonica.* var. *uzenensis* |
| 12 | 53 | 971 | cc88 | | C | ≈truncate | | | 20 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 54 | 974 | 88 | 0/1 | C | truncate / cordate | | | 75 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 56 | 982 | c88 | 0/1 | C | truncate / cordate | | male fertile | 140 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 63 | 1081 | c88 | 0/1 | C | ≈truncate | | male fertile | 270 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 63 | 1082 | 88 | 0 | C | ≈truncate | | male sterile | 300 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 65 | 1053 | c88 | | C | ≈truncate | | | 70 cm | - | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 65 | 1054 | c88 | | C | ≈truncate | | male fertile | 180 cm | Tall | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 71 | 1079 | c88 | | C | ≈truncate | | male fertile | | | Lowland | *F. japonica.* var. *uzenensis* |
| 12 | 75 | 1072 | c88 | 0/1 | C | ≈truncate | | male sterile | 100–150 cm | - | Montane | *F. japonica.* var. *uzenensis* |
| 13 | 10 | 1130 | 44 | 1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 14 | 21 | 1127 | 44 | 0/1 | A | ≈truncate | | | < 80 cm | Dwarf | | *F. japonica* var.*'japonica'* |
| 15 | 12 | 1129 | 44 | 0/1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 15 | 22 | 766e | 44 | | A | truncate | | | 200 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 15 | 22 | 767d | 44 | | A | ≈truncate | | | 200 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 15 | 23 | 1093 | cc44 | 1 | A | ≈truncate | | male sterile | 400 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 15 | 29 | 1115 | 44 | | | | | | 100 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 16 | 1 | 769a | 44 | | A | ≈accuminate | | | | | | *F. japonica* var.*'japonica'* |
| 17 | 77 | 914 | 66 | 2 | D/A | ≈truncate | | | | | Lowland | Hybrid |
| 18 | 50 | 967 | 66 | 2 | C/B | ≈truncate | | | 262 cm | Tall | Lowland | Hybrid |
| 19 | 52 | 973 | 88 | | C | ≈truncate | | | 186 cm | Tall | Lowland | *F. jap.* var. *uzenensis* |
| 20 | 49 | 726 | 44 | | A | truncate | | | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 20 | 49 | 727 | 44 | | | | | | | Dwarf | Subalpine | *F. japonica* var.*'japonica'* |
| 20 | 51 | 768a | 44 | | B | cordate | | | | | | *F.sachalinensis* |
| 20 | 54 | 975 | 44 | 4 | B | cordate | | | 224 cm | - | Lowland | *F.sachalinensis* |
| 21 | 56 | 981a | 44 | 1 | A | ≈truncate | | male sterile | 206 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 21 | 56 | 981b | | | | | | male sterile | 206 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 21 | 57 | 979 | 44 | 0 | A | truncate / cordate | | male fertile | c60 cm | Dwarf | Lowland | *F. japonica* var.*'japonica'* |
| 22 | 34 | 773d | 44 | | A | ≈cordate | | | | | | *F. japonica* var.*'japonica'* |
| 24 | 49 | 728 | 44 | | A | ≈truncate | | | | | Subalpine | *F. japonica* var.*'japonica'* |
| 25 | 44 | 732 | 44 | | | | | | 150 cm | Tall | | *F. japonica* var.*'japonica'* |
| 26 | 11 | 1134 | 44 | 1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 28 | 81 | 952 | 66 | 1/2 | D | ≈truncate | | | regrowth | - | Lowland | Hybrid |
| 30 | 19 | 1132 | | 0/1 | A | truncate /cordate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 31 | 7 | 1136 | 44 | 0/1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 31 | 23 | 1091 | 44 | 1 | A | ≈truncate | | | 400 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |

| mph | Site no. | P no. | 2n = | Epidermal striation | Trichome type | Leaf base | Crimped margins | Sex of plant | Plant height | Dwarf/ Tall | Altitudinal vegetation zone | Taxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 31 | 23 | 1092 | 44 | 2 | A | ≈truncate | | male fertile | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 31 | 24 | 1095 | 44 | 1/2 | A | truncate | | | 150 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 31 | 29 | 1114 | c45 | | A | ≈truncate | | | 20 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 31 | 29 | 1116 | 44 | | A | ≈truncate | | male sterile | 150 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 32 | 15 | 1141 | 44 | 0 | A | cordate | | | 150 – 200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 33 | 70 | 1077 | 66 | 1/2 | C/D | ≈truncate | | male fertile | 160 cm | - | Lowland | Hybrid |
| 34 | 14 | 1140 | 44 | 1 | A | ≈accuminate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 34 | 18 | 1142 | 44 | 1 | A | ≈truncate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 35 | 5 | 1102 | 44 | 1 | A | ≈cordate | | | 300 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 35 | 5 | 1103 | 44 | 1 | A | truncate / cordate | | | | | Montane | *F. japonica* var.*'japonica'* |
| 35 | 13 | 1133 | 44 | 1 | A | truncate / cordate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 35 | 16 | 1139 | 44 | 0 | A | truncate / cordate | | | 80–150 cm | - | | *F. japonica* var.*'japonica'* |
| 35 | 31 | 989 | 44 | | | | | | 300 cm | Tall | Lowland | *F. japonica* var.*'japonica'* |
| 35 | 33 | 985 | 44 | 0/1 | A | truncate / cordate | | | 80 cm | - | Lowland | *F. japonica* var.*'japonica'* |
| 35 | 39 | 765c | 88 | | A | truncate | | | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 35 | 39 | 796a | | | A | ≈truncate | | | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 35 | 39 | 799a | | | | | | | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 35 | 39 | 800a | | | A | ≈truncate | | | 200 cm | Tall | Montane | *F. japonica* var.*'japonica'* |
| 36 | 9 | 1131 | 44 | 0/1 | A | ≈cordate | | | 150–200 cm | Tall | | *F. japonica* var.*'japonica'* |
| 37 | 68 | 1062 | 44 | 3 | B | cordate | | | 85 cm | - | Subalpine/Montane | *F. sachalinensis* |
| 37 | 68 | 1069 | 44 | 4 | B | cordate | | | 150 cm | - | Subalpine/Montane | *F. sachalinensis* |
| 37 | 68 | 1070a | 44 | 3 | B | cordate | | male sterile | 200 cm | - | Subalpine/Montane | *F. sachalinensis* |
| 37 | 70 | 1076 | 44 | 4 | B | cordate | | male fertile | | | Lowland | *F. sachalinensis* |
| 38 | 51 | 794a | | | B | cordate | | | | | | *F. sachalinensis* |
| 38 | 51 | 795a | | | B | cordate | | | | | | *F. sachalinensis* |
| 39 | 43 | 134 | 44 | 0/1 | None | truncate | | | | | | *F. japonica.* var. *terminalis* |

**Table 6.5** The size of each chloroplast region analysed. Table also shows which gel type the restriction digests were run on, the number of bands scored and the number of haplotypes detected.

| Chloroplast Region | Approximate size of PCR product (bp) | Gel Type | No. of bands scored | No. of haplotypes |
|---|---|---|---|---|
| $trnK^1$-$trnK^2$ | 2816 | 1.6% agarose | 4 | 4 |
| trnC-trnD | 2768 | 3% metaphor agarose | 8 | 8 |
| trnF-trnV | 3197 | 1.6% agarose | 7 | 8 |
| trnH-trnK | 1927 | 3% metaphor agarose | 8 | 9 |
| trnD-trnT | 1685 | 6% polyacrylamide | 10 | 7 |
| trnM-rbcL | 3279 | 3% metaphor agarose | 5 | 6 |

**Table 6.6** Haplotypes generated from each region of the chloroplast. Each haplotype is represented by a letter, and each band by the name of the region it came from and its approximate size in base pairs (bp).

| | trnK[1]-trnK[2] | | | | trnC-trnD | | | | | | | | trnF-trnV | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 350 | 324 | 296 | 262 | 255 | 204 | 197 | 193 | 182 | 172 | 154 | 141 | 590 | 547 | 539 | 514 | 503 | 476 | 452 |
| A | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| B | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| C | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| D | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| E | | | | | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| F | | | | | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| G | | | | | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| H | | | | | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |

| | trnH-trnK | | | | | | | | trnD-trnT | | | | | | | | | | trnM-rbcL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 222 | 215 | 209 | 198 | 194 | 190 | 185 | 170 | 188 | 182 | 173 | 167 | 150 | 143 | 138 | 134 | 127 | 109 | 195 | 190 | 187 | 178 | 170 |
| A | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| B | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| C | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| D | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| E | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| F | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| G | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | | | | | |
| H | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | | | | | | | | | | | | | | | |
| I | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | | | | | | | | | | | | | | | |

**Table 6.7** The combination of haplotypes that make up the multi-primer-haplotype (MPH). The table is ordered alphabetically starting with the region on the furthest left of the table.

| trnK[1]-trnK[2] | trnC-trnD | trnF-trnV | trnH-trnK | trnD-trnT | trnM-rbcL | mph |
|---|---|---|---|---|---|---|
| A | A | A | A | D | A | 3 |
| A | B | A | A | D | A | 4 |
| A | B | A | F | D | A | 25 |
| A | C | B | C | A | B | 9 |
| A | D | B | A | C | B | 15 |
| A | D | B | C | D | B | 14 |
| A | D | E | B | D | A | 21 |
| A | D | E | B | E | A | 6 |
| A | D | E | B | F | A | 24 |
| A | D | E | E | D | B | 19 |
| A | D | E | E | E | A | 20 |
| A | D | E | H | E | A | 38 |
| A | D | H | A | D | B | 27 |
| A | D | H | A | D | F | 40 |
| A | D | H | B | D | B | 22 |
| A | D | H | C | D | B | 35 |
| A | D | H | C | D | D | 31 |
| A | D | H | C | G | C | 30 |
| A | E | B | C | A | A | 18 |
| A | E | B | C | A | B | 12 |
| A | E | H | C | G | B | 7 |
| A | F | A | D | B | B | 16 |
| A | F | A | I | B | B | 13 |
| A | F | C | A | B | B | 10 |
| A | F | C | A | D | B | 36 |
| A | F | C | A | G | B | 32 |
| A | F | C | F | B | B | 26 |
| A | F | F | C | B | B | 29 |
| A | G | H | A | D | B | 23 |
| B | C | B | A | A | B | 17 |
| B | C | B | B | A | B | 5 |
| B | C | B | C | A | B | 1 |
| C | D | A | A | D | A | 33 |
| C | D | F | A | D | A | 11 |
| C | D | G | A | D | A | 37 |
| C | E | A | A | D | A | 8 |
| C | E | A | C | D | A | 28 |
| C | E | D | A | D | A | 2 |
| D | F | C | A | G | B | 34 |
| D | H | A | G | D | E | 39 |

**Table 6.8** Summary table showing the number of accessions of each taxon at the different ploidy levels that share a multi-primer-haplotypes (MPH). The table is organised according to the clade that the mph are grouped into. Taxa not originating from Japan have their origin written in bold next to their name.

| Clade | mph | Taxon | Ploidy | No. of accessions |
|---|---|---|---|---|
| A | 1 | *F. japonica* var. *uzenensis* | 8x | 5 |
| | | *F. japonica* var. *'japonica'* | 8x | 1 |
| | | *F. japonica* var. *japonica* **– Europe** | 8x | 15 |
| | 5 | *F. japonica* var. *uzenensis* | 8x | 8 |
| | | Hybrid | 6x | 2 |
| | 9 | *F. japonica* var. *uzenensis* | 8x | 7 |
| | | Hybrid | 6x | 3 |
| | 12 | *F. japonica* var. *uzenensis* | 8x | 12 |
| | 17 | Hybrid | 6x | 1 |
| | 18 | Hybrid | 6x | 1 |
| B | 2 | *F. sachalinensis* | 4x | 17 |
| | | *F. sachalinensis* **– Europe and USA** | 4x | 17 |
| | | Hybrid or *F. sachalinensis* | 6x | 2 |
| | 3 | *F. japonica* var. *'japonica'* | 4x | 25 |
| | | *F. japonica* var. *compacta* **- UK** | 4x | 2 |
| | 4 | *F. japonica* var. *'japonica'* | 4x | 21 |
| | | *F. japonica* var. *compacta* **- UK** | 4x | 2 |
| | | *F. japonica* var. *uzenensis* | 8x | 2 |
| | | *F. sachalinensis* | 4x | 2 |
| | | Hybrid | 6x | 2 |
| | 8 | *F. japonica* var. *uzenensis* | 8x | 3 |
| | | *F. sachalinensis* | 4x | 2 |
| | | *F. japonica* var. *'japonica'* | 8x | 1 |
| | | Hybrid | 6x | 12 |
| | | Hybrid | 8x | 1 |
| | | Hybrid | 4x | 1 |
| | 25 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 28 | Hybrid | 6x | 1 |

| | | | | |
|---|---|---|---|---|
| | 11 | *F. sachalinensis* | 4x | 12 |
| | | *F. japonica* var. *uzenensis* | 8x | 2 |
| | | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 33 | Hybrid | 6x | 1 |
| | 37 | *F. sachalinensis* | 4x | 4 |
| C | 6 | *F. sachalinensis* | 4x | 3 |
| | 20 | *F. sachalinensis* | 4x | 2 |
| | | *F. japonica* var. *'japonica'* | 4x | 2 |
| | 24 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 38 | *F. sachalinensis* | | 2 |
| | | *F. sachalinensis* - **UK** | 4x | 3 |
| | 21 | *F. japonica* var. *'japonica'* | 4x | 3 |
| D | 10 | *F. japonica* var. *'japonica'* | 8x | 12 |
| | | *F. japonica* var. *'japonica'* | 4x | 28 |
| | 26 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 32 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 34 | *F. japonica* var. *'japonica'* | 4x | 2 |
| | 36 | *F. japonica* var. *'japonica'* | 4x | 1 |
| Unresolved | 14 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 39 | *F. japonica* var. *terminalis* | 4x | 1 |
| | 7 | *F. japonica* var. *uzenensis* | 8x | 1 |
| | 30 | *F. japonica* var. *'japonica'* | - | 1 |
| | 29 | *F. sachalinensis* - **Korea** | 2n = 102 | 2 |
| | 13 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 16 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 15 | *F. japonica* var. *'japonica'* | 4x | 5 |
| | 19 | *F. japonica* var. *uzenensis* | 8x | 1 |
| | 22 | *F. japonica* var. *'japonica'* | 4x | 1 |
| | 23 | *F. japonica* var. *'japonica'* **-China** | 10x | 2 |
| | 27 | *F. japonica* var. *'japonica'* **-China** | 8x | 1 |
| | 31 | *F. japonica* var. *'japonica'* | 4x | 6 |
| | 35 | *F. japonica* var. *'japonica'* | 4x | 6 |
| | | *F. japonica* var. *'japonica'* | 8x | 1 |
| | | *F. japonica* var. *'japonica'* | - | 3 |
| | 40 | *F. elliptica* - **Korea** | 8x | 1 |

**Table 6.9** Summary showing the number of tetraploid *F. japonica* accessions within each height group, for each multi-primer-haplotype (MPH). Also indicated is the altitudinal vegetation zone they were found in.

| Plant Height | MPH | Lowland | Montane | Montane - subalpine | Subalpine | Unknown | Total no. of accessions |
|---|---|---|---|---|---|---|---|
| | 3 | | 4 | | 8 | | 12 |
| **Dwarf** | 4 | | | 5 | | | 5 |
| | 10 | | 18 | | | | 18 |
| | 11 | | | 1 | | | 1 |
| | 14 | | | | | 1 | 1 |
| | 20 | | | | 2 | | 2 |
| | 21 | 1 | | | | | 1 |
| Total | | 1 | 22 | 6 | 10 | 1 | 40 |
| | 3 | 2 | 1 | | | 1 | 4 |
| **Tall** | 4 | 3 | 1 | | | 2 | 6 |
| | 10 | 1 | | | | 2 | 3 |
| | 13 | | | | | 1 | 1 |
| | 15 | 3 | | | | 1 | 4 |
| | 21 | 2 | | | | | 2 |
| | 25 | | | | | 1 | 1 |
| | 26 | | | | | 1 | 1 |
| | 30 | | | | | 1 | 1 |
| | 31 | 4 | | | | 1 | 5 |
| | 32 | | | | | 1 | 1 |
| | 34 | | | | | 2 | 2 |
| | 35 | 1 | 4 | | | 1 | 6 |
| | 36 | | | | | 1 | 1 |
| Total | | 16 | 6 | 0 | 0 | 16 | 38 |
| | 3 | 2 | 5 | | | 2 | 9 |
| **Intermediate or unknown** | 4 | 3 | 1 | 1 | | 5 | 10 |
| | 10 | | | | | 3 | 3 |
| | 15 | 1 | | | | | 1 |
| | 16 | | | | | 1 | 1 |
| | 22 | | | | | 1 | 1 |
| | 24 | | | | 1 | | 1 |
| | 31 | 1 | | | | | 1 |
| | 35 | 1 | 1 | | | 1 | 3 |
| Total | | 8 | 7 | 1 | 1 | 13 | 30 |

**Table 6.10** Number of accessions of each multi-primer haplotype (MPH) found in octoploid *F. japonica*, grouped according to their trichome type with *F. japonica* var. '*japonica*' having trichome type A, *F. japonica* var. *uzenensis* having trichome type C, and the putative hybrid having a trichome type that appears to be somewhat between the two.

| MPH | Taxon | Trichome type | 2n = | No of accessions |
|---|---|---|---|---|
| 1 | *F. japonica* var. '*japonica*' | A | 88 | 1 |
| 8 | *F. japonica* var. '*japonica*' | A | 88 | 1 |
| 10 | *F. japonica* var. '*japonica*' | A | 88 | 12 |
| 35 | *F. japonica* var. '*japonica*' | A | 88 | 1 |
| 1 | *F. japonica* var. *uzenensis* | C | 88 | 5 |
| 4 | *F. japonica* var. *uzenensis* | C | 88 | 2 |
| 5 | *F. japonica* var. *uzenensis* | C | 88 | 8 |
| 7 | *F. japonica* var. *uzenensis* | C | 88 | 1 |
| 8 | *F. japonica* var. *uzenensis* | C | 88 | 3 |
| 9 | *F. japonica* var. *uzenensis* | C | 88 | 7 |
| 11 | *F. japonica* var. *uzenensis* | C | 88 | 2 |
| 12 | *F. japonica* var. *uzenensis* | C | 88 | 12 |
| 19 | *F. japonica* var. *uzenensis* | C | 88 | 1 |
| 8 | Hybrid | A/C | 88 | 1 |

**Table 6.11** Number of accessions of each MPH of *F. sachalinensis* and the clade to which the MPH belonged according to Figures 6.7 and 6.8.

| | MPH | Taxon | No of accessions |
|---|---|---|---|
| Clade B | 2 | *F. sachalinensis* | 17 |
| | 4 | *F. sachalinensis* | 2 |
| | 8 | *F. sachalinensis* | 2 |
| | 11 | *F. sachalinensis* | 12 |
| | 37 | *F. sachalinensis* | 4 |
| Clade C | 6 | *F. sachalinensis* | 3 |
| | 20 | *F. sachalinensis* | 2 |
| | 38 | *F. sachalinensis* | 2 |

**Table 6.12** Putative hybrids, their location, chromosome number, lower epidermal characteristics and predicted parentage.

| MPH | Site no. | Prefecture | 2n = | Epidermal striation | Trichome type | *Putative parentage* |
|---|---|---|---|---|---|---|
| 2 | 79 | Aomori | 66 | 3/4 | B (B/C) | a(SxU) or f(S) |
| | 80 | Aomori | *ca.*66 | 3 | B (B/C) | a(SxU) or f(S) |
| 4 | 67 | Yamagata | 66 | 2 | C/B | a(SxU) |
| | 69 | Yamagata | 66 | 1/2 | A/D | c(SxJ)* or d(JxU) |
| 5 | 77 | Miyagi | 66 | 2 | A/D | c(SxJ)*or d(JxU) |
| | 77 | Miyagi | 66 | 2 | C/D | a(SxU) or d(JxU) |
| 8 | 79 | Aomori | 66 | 1/2 | C (B/C) | d(JxU)* |
| | 79 | Aomori | 66 | 1 | C/D | d(JxU)* |
| | 79 | Aomori | *ca.*66 | 1 | C/D | d(JxU)* |
| | 80 | Aomori | *ca.*66 | 1/2 | C/D | d(JxU)* |
| | 80 | Aomori | 66 | 2 | C/D | d(JxU)* |
| | 80 | Aomori | *ca.*66 | 1 | D | d(JxU)* |
| | 80 | Aomori | 66 | 1 | C | d(JxU)* or g(U) |
| | 81 | Aomori | 66 | 1/2 | B/C | a(SxU) or d(JxU)* |
| | 81 | Aomori | 66 | 2 | D | a(SxU) or d(JxU)* |
| | 82 | Aomori | 66 | 1/2 | C/D | d(JxU)* |
| | 82 | Aomori | | 1/2 | C/D | d(JxU)* |
| | 82 | Aomori | 66 | 1 | C | d(JxU)* or g(U) |
| | 83 | Aomori | 44 | 1 | B | c(SxJ)* |
| | 84 | Aomori | *ca.*88 | 2 | A/D | e(JxU) |
| 9 | 50 | Niigata | 66 | 0/1 | C/D | d(JxU) |
| | 77 | Miyagi | 66 | 3/4 | B (B/C) | a(SxU)or f(S) |
| | 77 | Miyagi | | 3/4 | B (B/C) | a(SxU)or f(S) |
| 17 | 77 | Miyagi | 66 | 2 | A/D | b(SxJ)* or d(JxU) |
| 18 | 50 | Niigata | 66 | 2 | B/C | a(SxU) |
| 28 | 81 | Aomori | 66 | 1/2 | D | d(JxU)* |
| 33 | 70 | Yamagata | 66 | 1/2 | C/D | d(JxU) |

a(SxU) *F. sachalinensis* x *F. japonica* var. *uzenensis*

b(SxJ) *F. sachalinensis* x 8x *F. japonica*

c(SxJ) *F.sachalinensis* x 4x *F. japonica*

d(JxU) 4x *F. japonica* x *F. japonica* var. *uzenensis*

e(JxU) 8x *F. japonica* x *F. japonica* var. *uzenensis*

f(S) Hexaploid *F. sachalinensis* (unreduced gamete x normal gamete)

g(U) Hexaploid *F. japonica* var. *uzenensis*

* One of the putative parental taxa not found in this prefecture during this study.

**Table 6.13** Distribution of the multi-primer-haplotypes (MPHs) between the three main taxa, and the various clades. An asterisk denotes a MPH that is shared between more than one taxon. The numbers in parenthesis after the MPH refers to the numbers of accessions of that taxon found with that MPH.

| | Clade A | Clade B | Clade C | Clade D | Unresolved group |
|---|---|---|---|---|---|
| *F. japonica* var. '*japonica'* | 1*(1) | 3 (25) | 20*(2) | 10 (40) | 13 (1) |
| | | 4*(21) | 21 (3) | 26 (1) | 14 (1) |
| | | 8*(1) | 24 (1) | 32 (1) | 15 (5) |
| | | 11*(1) | | 34 (2) | 16 (1) |
| | | 25 (1) | | 36 (1) | 22 (1) |
| | | | | | 30 (1) |
| | | | | | 31 (6) |
| | | | | | 35 (10) |
| *F. japonica* var. *uzenensis* | 1*(5) | 4*(2) | | | 7 (1) |
| | 5 (8) | 8*(3) | | | 19 (1) |
| | 9 (7) | 11*(2) | | | |
| | 12 (12) | | | | |
| F. sachalinensis | | 2 (17) | 6 (3) | | |
| | | 4*(2) | 20*(2) | | |
| | | 8*(2) | 38 (2) | | |
| | | 11*(12) | | | |
| | | 37 (4) | | | |

**Table 6:14** Geological time scale for the Quaternary and Tertiary periods. Taken from Lincoln *et al*. (1998).

| Geological time (million years before present) | Period | Epoch | Duration (million years) |
|---|---|---|---|
| 0.01 to present | Quaternary | Holocene | 0.01 |
| 1.6 to 0.01 | | Pleistocene | 1.59 |
| 5 to 1.6 | Tertiary | Pliocene | 3.4 |
| 26 to 5 | | Miocene | 21 |
| 38 to 26 | | Oligocene | 12 |
| 54 to 38 | | Eocene | 16 |
| 65 to 54 | | Palaeocene | 11 |

# Chapter 7. Chloroplast DNA phylogeography of native Japanese *Fallopia* section *Reynoutria* taxa.

## 7.1. Introduction

In chapter 6, the molecular biogeography of Japanese populations of *F. japonica* and *F. sachalinensis* was investigated through the use of chloroplast RFLP variation. The relationship between the native plants and the taxa that were introduced to Britain and other countries was also considered. In that study 285 plants were analysed for six different chloroplast regions. Chloroplast PCR-RFLP variation is ideal for investigating a high number of individuals due to the relatively low costs involved when compared to techniques such as traditional RFLP analysis or DNA sequence studies. The choice of PCR-RFLP enabled a lot of variation to be found to determine the molecular biogeography of these plants. However, the choice of technique meant that genetic relationships between the plants could only be generally discussed due to the lack of knowledge as to what was creating the variation between the plants, and the lack of a suitable outgroup.

Chloroplast DNA sequence analysis is a powerful tool for estimating phylogenetic relationships in plants (Gielly & Taberlet, 1994). DNA sequence analysis allows single base comparisons which results in a lower level of homoplasy when compared to RFLP analysis where a mutation in any of the four to six enzyme recognition bases can lead to the loss of a restriction site (Palmer *et al.*, 1988). Sequence analysis enables the exact nature of the mutations that create the variation to be determined and an appropriate outgroup to be chosen.

Two factors need to be taken into consideration when using chloroplast DNA sequence variation, the regions to be sequenced and the method of analysing the final data. The use of non-coding sequences of cpDNA for the establishment of plant phylogenies can lead to incorrect phylogenetic inferences due to the presence of numerous length mutational events, however some of them can provide useful phylogenetic information (Gielly & Taberlet, 1996). This study does not intend to produce a complete phylogeny for the genus *Fallopia*, but instead intends to gain some insight into the chloroplast variation found in native individuals of *F. japonica* and *F. sachalinensis.* To this end non-coding sequences known to be variable within this group of plants should be useful.

There is much contention about the best method for analysing sequence data. It is generally accepted that discrete methods are better than distance methods. In contrast to distance methods, discrete methods operate directly on the sequences, or on functions derived from the

sequences, rather than on pairwise distances. The two major discrete methods for analysis are maximum parsimony and maximum likelihood. Maximum parsimony chooses the tree (or trees) that requires the fewest evolutionary changes. Maximum likelihood chooses the tree (or trees) that of all trees is the one that is most likely to have produced the observed data (Page & Holmes, 1998).

The data for maximum parsimony comprise individual nucleotide sites. The aim of maximum parsimony is to reconstruct the evolution of a nucleotide site on a tree whilst invoking the fewest possible evolutionary changes. Parsimony is relatively straightforward to understand, and makes few assumptions about the evolutionary process. However the justification for choosing the most parsimonious tree as the best estimate of phylogeny is the subject of considerable controversy. The principal objection to parsimony is that under some models of evolution it is not consistent, that is, even if more data are added it is possible to obtain the wrong tree. Consistency, in terms of molecular evolutionary tree-building, is the ability of a tree to converge on the right answer given enough data (Page & Holmes, 1998).

Likelihood is the probability of observing the data given a particular model. Different models may make the observed data more or less probable. Whilst probabilities add up to 1, likelihoods do not. Given a tree and a model of sequence evolution, it is theoretically possible to work out the probability of obtaining all possible data sets, the sum of which would equal 1. Maximum likelihood, however, is interested in the probability of obtaining just one of those possible data sets (the observed data set). The likelihood is not the probability that the tree is the true tree, but the probability that the tree has given rise to the data collected. The appeal of maximum likelihood is that it can incorporate explicit models of sequence evolution, and also permits statistical tests of evolutionary hypotheses. Likelihood requires an explicit model for evolution, which may be seen as both its strength and its weakness. It is a strength because it makes one aware that assumptions are being made, but it is a weakness in that it raises the question of which model to use, and what values of the parameters, such as transition/transversion ratio, should be employed (Page & Holmes, 1998).

### 7.2 Aims

To investigate the relationship between *F. sachalinensis* and *F. japonica* through the use of chloroplast DNA sequence variation, and in particular to assess the relationship between the introduced and native taxa.

## 7.3 Materials and Methods

### 7.3.1. *Plant material*

Twenty-five plants of Japanese origin were selected for sequence analysis, as listed in Table 7.1. Additionally four British, two Chinese and two Korean plants were selected (Table 7.2), as well as a further three related species to be used as outgroups, namely *F. multiflora*, *F. baldschuanica* and *F. convolvulus*. All of the plants selected excluding the outgroup taxa had previously undergone chloroplast RFLP analysis as detailed in chapter 6, and were chosen to cover as much of the native variation as possible, including samples from China and Korea, as well as British samples for comparative purposes. Figure 7.1 shows where the Japanese accessions were from.

### 7.3.2. *Methods*

A single DNA extraction was made for each plant, as detailed in chapter 3, materials and methods. Initially three British plants representing *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and the more common chloroplast haplotype of *F. sachalinensis* (MPH 2), underwent chloroplast DNA PCR amplification of the six regions used in chapter 6, as did the three plants that comprise the outgroup. These were then sequenced in both directions effectively generating twelve fragments, and the three most informative were selected. The six chloroplast regions range in size from approximately 1685 to 3279 base pairs, as detailed in chapter 6, Table 6.5. The size of the fragments generated by sequencing were too short to cover the entire region, even when sequencing was attempted from both ends, as indicated by the number of character analysed shown in Table 7.3. The remaining thirty plants were then amplified and sequenced using the three regions selected. Chloroplast amplification and sequencing were completed as per chapter 3.

## 7.4 Data analysis

The individual sequence electropherograms were manually checked and aligned as detailed in chapter 3, section 3.2.7.1.

Phylogenetic relationship among samples was estimated individually for each fragment, and then the data were combined. PAUP 4.0 beta version 10 (Swofford, 2003) was used to perform the analysis, although branch lengths were checked using an earlier version of PAUP (version 3.1.1) due to occasional discrepancies in the tree drawing output. With version 4.0 beta 10 tree scores are correctly calculated, however branch lengths may be exaggerated so that they sum to a value greater than the tree length. To construct the trees the parsimony method was used, and the equally most parsimonious trees were identified using the "branch

and bound" option of PAUP 4.0 beta 10. No weighting or ordering was performed on the characters and any gaps were treated as missing data. Multistate taxa were interpreted as uncertainty. The initial upper bound was computed heuristically. The options: compute via stepwise; addition sequence furthest; and, save all optimal trees (MulTrees), were selected. The COLLAPSE but not the STEEPEST DESCENT options were used. *F. multiflora*, *F. baldschuanica* and *F. convolvulus* were specified as the outgroup. When more than one tree of equal length was found, a strict consensus was constructed to summarise nodes that did not conflict in alternative trees. Confidence was assessed using 1,000 bootstrap replicates, using the full heuristic bootstrapping with general search options of PAUP 4.0 beta 10. For the combined data set the analysis was repeated without the inclusion of any variation caused by the presence of microsatellites within the data.

Finally, for the combined data set without microsatellite variation only, a maximum likelihood analysis was performed for comparative purposes. Modeltest version 3.06 (Posada & Crandall, 1998) was used to survey the sequence data to select the best model for the maximum likelihood analysis from fifty-six different models of evolution. The selected model was then used as the basis of a maximum likelihood analysis using the heuristic general search option of PAUP 4.0 beta 10 (Swofford, 2003). The starting tree was obtained via stepwise. The options: addition sequence as-is; and, save all optimal trees (MulTrees), were selected. The COLLAPSE but not the STEEPEST DESCENT options were used. The swapping algorithm TBR (tree-bisection-reconnection) was used, and only one tree was held at each step. Confidence was assessed using 100 bootstrap replicates.

## 7.5 Results

### 7.5.1 *Selection of regions for sequencing*

To select which of the chloroplast regions used in chapter 6 for the RFLP analysis would be sequenced in this study, a subset of DNA samples was used. *F. multiflora*, *F. baldschuanica* and *F. convolvulus* were selected to form the outgroup, and a British sample of each of *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and *F. sachalinensis* were used as the ingroup. These six plants were sequenced for all six chloroplast regions in both directions. From Table 7.3 it can be seen that the fragments with the highest number of mutations between the ingroup taxa were *trn*D-*trn*T when sequenced from the *trn*T end, and *trn*F-*trn*V when sequenced from the *trn*F end. These two fragments were therefore selected for the full study and shall hereafter be referred to as fragments T and F respectively. From the remaining ten fragments in Table 7.3, those that showed a higher level of resolution within the ingroup were; *trn*C-*trn*D when sequenced from the *trn*D end, and *trn*F-*trn*V when

sequenced from the *trn*F end. Of these the fragment from *trn*C-*trn*D had a slightly longer length of readable sequence than that from *trn*F-*trn*V, and came from a region that had not already been selected, so was selected as the third and final fragment for the study. Hereafter this fragment will be referred to as fragment D.

### 7.5.2 *Fragment D*

Sequence analysis of fragment D detected 14 haplotypes from the 33 taxa that comprise the ingroup. Each haplotype number has been preceded with the letters CD to represent the region of the chloroplast they were obtained from. When aligned with the outgroup taxa, a consensus sequence of 787 bases was produced which is shown in Figure 7.2. Table 7.4 details the mutations and their position in relation to the consensus sequence for all the plants sequenced. As far as possible plants that share a mutation have been listed together within the table. The types of mutations found include single base changes, indels ranging from one base to 32 bases, microsatellites, repeated segments of DNA, and a 27 base pair inversion. Three of the 14 haplotypes share this inverted region that starts at base 559 on the consensus sequence. In the indels matrix for this fragment the presence of the inversion was coded for as one, whilst the outgroup and remaining 11 haplotypes were coded as zero. The indels matrix for this fragment is shown in Table 7.5.

Phylogenetic analysis produced a single most parsimonious tree of length 86, as shown in Figure 7.3. *F. multiflora* holds a position that is no closer to the ingroup than it is the rest of the outgroup taxa, comprising *F. baldschuanica* and *F. convolvulus*. The ingroup is shown to be a monophyletic group held together and away from the outgroup by 9 mutations within fragment D, with 100% bootstrap support for the clade. *F. sachalinensis* and *F. japonica* were found interspersed within the tree and therefore do not appear to be phylogenetically diverse species, at least not according to the chloroplast genome.

Low levels of resolution were found within the ingroup for fragment D. Two haplotypes were found outside of the main clade (CD9 and CD10), but with a very low bootstrap support value of 58, whilst the clade itself was produced through a single shared mutation. CD9 was found in only one plant, a Korean *F. sachalinensis* with 2n = 102. CD10 was found in a number of native *F. japonica* var. '*japonica*' plants, namely a Chinese octoploid, a Japanese octoploid, two tall Japanese tetraploids and one dwarf Japanese tetraploid.

Within the main clade two supported groups were found, as was an unsupported group consisting of three haplotypes and an unresolved haplotype CD4. CD4 was shared by the

British *F. sachalinensis* with the chloroplast haplotype more commonly found in British *F. sachalinensis* plants, and six Japanese accessions, three *F. sachalinensis* and three *F. japonica*. The three *F. japonica* plants consisted of a tall tetraploid, a dwarf tetraploid and an octoploid. One of the three *F. sachalinensis* plants was a dwarf, whilst the other two were the more common tall form. The three haplotypes that formed an unsupported group were CD1, CD2 and CD3. CD1 was found in the British *F. japonica* var. *compacta*, and both a tall and dwarf Japanese tetraploid. CD2 and CD3 were both found in single accessions of *F. sachalinensis*, one of which was a dwarf.

The first of the two supported groups consisted of CD5, CD6, CD7 and CD8, and had a bootstrap value of 60. As a group they shared a single mutation, however within this group was a sub-group that excluded CD8, and was held together by four mutations and a bootstrap value of 94. CD8 had three unique mutations, giving it the longest terminal branch of any of the ingroup, and was found only in *F. japonica* var. *terminalis*. The subgroup had the highest support of any of the ingroup. The subgroup consisted of a tall and a dwarf Japanese tetraploid *F. japonica* var. '*japonica'* and three *F. sachalinensis* plants, one of which was the British *F. sachalinensis* with the chloroplast haplotype that is rarely found in Britain.

The other supported group had a bootstrap value of 75 and consisted of four chloroplast haplotypes that were fully resolved, but with relatively low bootstrap values of 57 and 61. This group was made up of CD11, CD12, CD 13 and CD14. The three accessions included in this study of *F. japonica* var. *uzenensis*, the British clone of *F. japonica* var. *japonica*, and an octoploid accession of *F. japonica* var. '*japonica'* shared CD14. This chloroplast haplotype possessed two unique mutations and shared two mutations with the rest of the group, which consisted of the Korean accession of *F. elliptica*, a Chinese decaploid *F. japonica* var. '*japonica',* and two tall Japanese tetraploid *F. japonica* var. '*japonica'*.

There were two instances of a chloroplast haplotype being found in *F. japonica* and *F. sachalinensis*, three instances of tall and dwarf tetraploid *F. japonica* var. '*japonica'* individuals possessing the same chloroplast type, two of octoploid and tetraploid *F. japonica* var. '*japonica*' individuals sharing a chloroplast type, and additionally a chloroplast haplotype shared between octoploid *F. japonica* var. '*japonica'* and *F. japonica* var. *uzenensis*.

### 7.5.3 *Fragment F*

Sequence analysis of fragment F detected 13 haplotypes from the 33 taxa that comprise the ingroup. Each haplotype number has been preceded with the letters FV to represent the

region of the chloroplast they were obtained from. When aligned with the outgroup taxa, a consensus sequence of 695 bases was produced which is shown in Figure 7.4. Table 7.6 details the mutations and their position in relation to the consensus sequence for all the plants sequenced. As with fragment D, as far as possible plants that share a mutation have been listed together within the table. The types of mutations found include single base changes, indels ranging from one to eight bases in length, microsatellites, and repeated segments of DNA. The indels matrix for this fragment is shown in Table 7.7.

Phylogenetic analysis resulted in eleven equally most parsimonious trees of length 73. To demonstrate the branch lengths, an example of one of the eleven trees is shown in Figure 7.5, as is the strict consensus tree that summarises the nodes that did not conflict in the alternative trees. Consistent with fragment D, *F. multiflora* holds a position that is no closer to the ingroup than it is the rest of the outgroup taxa, comprising *F. baldschuanica* and *F. convolvulus*. . The ingroup is monophyletic, held together by 11 mutations, with 100% bootstrap support for the clade and *F. sachalinensis* and *F. japonica* were found interspersed within the tree.

The ingroup formed two clades with bootstrap values of 54 and 63. The clade supported by the bootstrap value of 54 has three subgroups, although one contains only a single haplotype FV3. The other two subgroups have bootstrap values of 66 and 87. All nine accessions of tetraploid *F. sachalinensis* were found within this clade, as was the British *F. japonica* var. *compacta* and three accessions each of Japanese tall and dwarf tetraploid *F. japonica* var. '*japonica',* and an accession of octoploid *F. japonica* var. '*japonica'*.

The second clade, which had a bootstrap value of 63, had very little resolution. One group of two chloroplast haplotypes was supported within this clade. These were chloroplast haplotypes FV10 and FV11, which were comprised of the three accessions of *F. japonica* var. *uzenensis*, the British clone of *F. japonica* var. *japonica*, and an octoploid accession of *F. japonica* var. '*japonica'*. With fragment D they had all shared a haplotype, but with fragment D one *F. japonica* var. *uzenensis*, P1082, had a unique chloroplast haplotype formed by a single mutation. The remainder of the clade was unresolved and contained all Chinese and Korean plants, *F. japonica* var. *terminalis*, and a number of Japanese *F. japonica* var. '*japonica'* plants including dwarf and tall tetraploids, and an octoploid. With fragment D the Korean *F. elliptica* was indistinguishable from the Chinese decaploid *F. japonica* var. '*japonica'*. With fragment F the Korean *F. elliptica* shared a chloroplast haplotype with the octoploid Chinese *F. japonica* var. '*japonica', F. japonica* var. *terminalis* and a tall tetraploid

*F. japonica* var. *'japonica'*. These last two were distinguishable independently with fragment D. With this fragment the Chinese decaploid *F. japonica* var. '*japonica'* shared a chloroplast haplotype with the Korean *F. sachalinensis*.

Whilst with fragment D there were two cases of a chloroplast haplotype being found in *F. japonica* and tetraploid *F. sachalinensis*, with fragment F there was only one. Fragment F had two instances of tall and dwarf tetraploid *F. japonica* var. '*japonica'* individuals possessing the same chloroplast type, fragment D had had three. Both fragments had two cases of octoploid and tetraploid *F. japonica* var. '*japonica*' individuals sharing a chloroplast haplotype. As with fragment D, fragment F had a chloroplast haplotype shared between octoploid *F. japonica* var. '*japonica'* and *F. japonica* var. *uzenensis*.

7.5.4 *Fragment T*

Sequence analysis of fragment T detected 21 haplotypes from the 33 taxa that comprise the ingroup. Each haplotype number has been preceded with the letters DT to represent the region of the chloroplast they were obtained from. When aligned with the outgroup taxa, a consensus sequence of 667 bases was produced which is shown in Figure 7.6. Table 7.8 details the mutations and their position in relation to the consensus sequence for all the plants sequenced. As with fragments D and F, as far as possible plants that share a mutation have been listed together within the table. The types of mutations found include single base changes, indels ranging from one base to ten bases, microsatellites, and repeated segments of DNA of up to 19 bases in length. The indels matrix for this fragment is shown in Table 7.9.

Phylogenetic analysis resulted in twenty-four equally most parsimonious trees of length 85. To demonstrate the branch lengths, an example of one of the twenty-four trees is shown in Figure 7.7, as is the strict consensus tree that summarises the nodes that did not conflict in alternative trees. This fragment shows the ingroup to form a monophyletic group. However in both previous fragments D and F this group had bootstrap values of 100, whereas with fragment T the support is 79. *F. baldschuanica* and *F. convolvulus* still remained together with a bootstrap value of 100, with *F. multiflora* again having a position that is no closer to the ingroup than it is the other two outgroup taxa. The ingroup is a polytomy consisting of one clade with a bootstrap value of 60, three groups consisting of two haplotypes each, and seven haplotypes that do not resolve. The clade possesses exactly the same plants as the first clade found with fragment F, although with slightly different structuring within the clade.

The three accessions of *F. japonica* var. *uzenensis*, the British clone of *F. japonica* var. *japonica*, and an octoploid accession of *F. japonica* var. '*japonica'* shared a haplotype in fragment D. In fragment F they formed a group of two haplotypes with one of the *F. japonica* var. *uzenensis* plants, P1082, having a unique chloroplast haplotype formed by a single mutation. In fragment T they form a group of two haplotypes with a bootstrap value of 88. This time it is the British clone of *F. japonica* var. *japonica* that has a unique haplotype (DT21), whilst the rest share a haplotype (DT20) however there were no phylogenetically informative characters involved with haplotype DT21. From Table 7.8 it can be seen that the British clone has its own haplotype because of ambiguous nucleotides at positions 292, 492 and 508. All three positions could be an adenine or a guanine, whilst for the other plants they appeared to always be adenine at those positions.

The Korean *F. elliptica* shared a chloroplast haplotype with the Chinese decaploid *F. japonica* var. '*japonica'* with fragment D, and with the Chinese octoploid *F. japonica* var. '*japonica', F. japonica* var. *terminalis* and a tall tetraploid *F. japonica* var. *'japonica'* with fragment F. With fragment T, the Korean *F. elliptica* possessed a unique haplotype but grouped with the Chinese decaploid *F. japonica* var. '*japonica'*. The remainder of the tree was mainly unresolved and did not conflict with either of the trees produced by the other two fragments.

Fragment T generated more haplotypes than the previous two fragments with seventeen of the 33 plants analysed having unique haplotypes. There were only two cases of dwarf and tall tetraploid *F. japonica* var. '*japonica'* sharing a chloroplast haplotype and no instances of tetraploid and octoploid *F. japonica* var. '*japonica'*. There were two cases of a chloroplast haplotype being shared by *F. japonica* and *F. sachalinensis*, and one of *F. japonica* var. '*japonica'* and *F. japonica* var. *uzenensis* sharing a chloroplast haplotype.

### 7.5.5 *Combined data*

The three fragments sequenced vary slightly in the grouping of the taxa that produced the haplotypes. Table 7.10 lists the haplotypes from each fragment and the combined multi-primer-haplotype from sequence data (sMPH). For the most part the sMPHs that were determined in this study clustered within the same groups as with the previous study based on chloroplast PCR-RFLP data. Some individuals that shared a MPH in chapter 6 were found to have different sMPHs in this study, and likewise some individuals that previously were found to have different MPHs were now found to share an sMPH. This variation, however, was never between plants that were previously found in different clades.

The conflict that arises between the three fragments in this study arose in two areas of Table 7.10, among samples within clade A, and among some of the taxa that previously fell within the group referred to as the unresolved group that are now split between the unresolved group and clade C. There was no conflict among plants in clades B and D. When the three data sets were combined a total of 27 sMPHs were found among the 33 taxa that comprise the ingroup.

The hypervariability of simple sequence repeats suggests that caution should be exercised in inferring relationships from them. In a study of two chloroplast microsatellites Doyle *et al*. (1998) found that, whilst able to reveal previously undetected chloroplast variation, these markers did not faithfully reflect the relationships between the species studied. Some of the variation within the total data set in this study was due to the six microsatellites listed in Table 7.11. The variation caused by these microsatellites was not found to affect the sMPHs. As a result of this phylogenetic parsimonious analysis was conducted on the data set both with and without the inclusion of variation resulting from microsatellites.

Phylogenetic analysis with all the data resulted in twenty-one equally most parsimonious trees of length 252. To demonstrate the branch lengths, an example of one of the twenty-one trees is shown in Figure 7.8, and the strict consensus tree that summarises the nodes that did not conflict in alternative trees is shown in Figure 7.9. Phylogenetic analysis without the microsatellite variation resulted in 175 equally most parsimonious trees of length 231. To demonstrate the branch lengths, an example of one of the 175 trees is shown in Figure 7.10, and the strict consensus tree that summarises the nodes that did not conflict in alternative trees is shown in Figure 7.11.

Consistent with all three fragments and for the trees both with and without microsatellite variation, *F. multiflora* holds a position that is no closer to the ingroup than it is the rest of the outgroup taxa, *F. baldschuanica* and *F. convolvulus,* which form a group held together by a bootstrap value of 100. The ingroup is shown to be a monophyletic group held together by 24 mutations, with 100% bootstrap support for the clade. *F. sachalinensis* and *F. japonica* were found interspersed throughout the tree.

The full data set supports two clades within the ingroup, one with a bootstrap value of 89 and the other with a value of 62. The first clade contains all tetraploid *F. sachalinensis* plants. Those shown in Table 7.10 to have been part of clade B in the PCR-RFLP study form a group with a bootstrap value of 53 that was not picked up on the consensus tree. This includes the British *F. sachalinensis* with the common chloroplast type. The other plant that grouped with

these *F. sachalinensis* plants is an octoploid *F. japonica* var. '*japonica',* P958. According to the consensus tree they form an unresolved polytomy at the base of a group that also includes a subgroup held together by a bootstrap value of 98. This unresolved section and the subgroup share three mutations and are supported by a bootstrap value of 94. The subgroup consists of the British *F. japonica* var. *compacta* and two tall and two dwarf tetraploid Japanese *F. japonica* var. '*japonica'*. This group of twelve plants were all found in clade B in the PCR-RFLP study in chapter 6. No other plants from clade B were found in an alternative part of the tree, nor were plants from other clades in chapter 6 found within this group.

Besides this group of plants from clade B, there is a second subclade within the first major clade, which shares five mutations and is supported by a bootstrap value of 98. This group forms a polytomy of four chloroplast haplotypes. Five plants are found having one of these four chloroplast haplotypes, and this includes the British *F. sachalinensis* with a rare chloroplast haplotype, P852, and two other Japanese *F. sachalinensis* plants that according to the PCR-RFLP study all were found within clade C. The other two plants were a tall and a dwarf *F. japonica* var. '*japonica'* that also previously came from clade C.

This major clade consisting of two subclades, one from clade B and the other from clade C, still holds together when the microsatellite variation is removed. By comparing the two consensus trees (Figures 7.9 and 7.11) it can be seen that their topography is identical and the bootstrap support is very similar. By looking at the number of mutations shown on Figures 7.8 and 7.10 it becomes clear that the relationship between those that were clade C was formed by non-microsatellite mutations, as the branch values are identical on both trees, whereas those that originated from clade B have a lot fewer mutations between them once microsatellite variation has been eliminated.

The second major clade shown on Figures 7.8 and 7.9 with a bootstrap value of 62 does not hold together once microsatellites are removed, Figures 7.10 and 7.11, and becomes three separate groups. With the microsatellite data included, the Korean *F. sachalinensis* and *F. japonica* var. *terminalis* are unresolved and the remaining haplotypes form two groups supported by bootstrap values of 67 and 76. In contrast once the microsatellite variation is removed, *F. japonica* var. *terminalis* becomes unresolved from the entire ingroup, whilst the Korean *F. sachalinensis* becomes a part of the subgroup that was part of the second major clade held together with a bootstrap value of 67, but instead now forms its own group at the base of the tree with a higher bootstrap value of 71, but without the internal structure that was found with the microsatellite variation. This group that the Korean *F. sachalinensis* joined

consists of Japanese tall and dwarf tetraploid *F. japonica* var. '*japonica'*, and both Japanese and Chinese octoploid *F. japonica* var. '*japonica'*. With the microsatellite variation, Figures 7.8 and 7.9, the Japanese accessions form a subgroup without the Chinese accession.

The other subgroup within the second major clade holds together as a group with a bootstrap value of 76 with microsatellite data and 79 without. The topography is consistent between the two methods of analysis, however the group consisting of *F. japonica* var. *uzenensis,* the British clone of *F. japonica* var. *japonica* and a Japanese octoploid *F. japonica* var. '*japonica'* loses its resolution after the microsatellites are removed. These plants shared 6 mutations, none of which were due to microsatellite variation and have a bootstrap value of 100. The Korean *F. elliptica* comes out closest to the Chinese decaploid *F. japonica* var. '*japonica'* with a bootstrap value of 83. The remainder of this subclade consists of two tall tetraploid *F. japonica* var. '*japonica'* plants.

All of the previous phylogenies were based on a parsimony analysis using the "branch and bound" option of PAUP 4.0 beta 10 (Swofford, 2003). An alternative method of analysis to parsimony is maximum likelihood. As there are many different models of evolution that can be used for a maximum likelihood analysis Modeltest version 3.06 (Posada & Crandall, 1998) was used to determine which of the 56 different models it assesses was best suited for the sequence data generated in this study. Modeltest suggests two different models the first derived from hierarchical Likelihood Ratio Tests (hLRT) and the second using the Akaike Information Criterion (AIC). The hLRT gives the model that is overall the best for the data and in this instance that was the model F81 + G. Some nucleotide substitutions may be more common than others due to variation in base composition. Felsenstein's model (F81) addresses this concern by allowing the frequencies of the four nucleotides to be different (Felsenstein, 1981). The G stands for a shape parameter of the gamma distribution. Among-site rate heterogeneity in the data is often assumed to fit either a negative binomial or gamma distribution, and confirmation can be assessed statistically. Rates of variation at sites are usually expected to fit a gamma distribution model and a parameter can be determined to define the shape of that underlying function in a maximum likelihood analysis (Kelchner, 2000).

A heuristic maximum likelihood analysis based on this model was completed. In total 8,635 rearrangements were tried, and the best tree was found to have a score of 4,013.87678. This tree is shown in Figure 7.12. As with the parsimony analyses, the ingroup forms a monophyletic clade supported by a bootstrap value of 100. *F. multiflora* holds a position that

is no closer to the ingroup than it is the rest of the outgroup taxa, comprising *F. baldschuanica* and *F. convolvulus,* which form a group with a bootstrap value of 100. As can be seen on the bootstrap consensus tree, Figure 7.13, the ingroup possesses two supported clades and an unresolved polytomy at the bottom of the tree. The first major clade is consistent with the first major clade in the parsimony analyses both with and without microsatellite data, resulting in two groups within the major clade, which from now will be referred to as clade A and clade B, clade A being the plants that belonged to clade B in the PCR-RFLP study, and clade B being those that were in clade C. The subgroup consisting of British *F. japonica* var. *compacta* and other Japanese tetraploid *F. japonica* var. '*japonica'* plants will now be referred to as the sub-group of clade A.

The second supported clade can likewise be divided into two clades, hereafter referred to as clades C and D. The plants that made up clades C and D formed a group on both parsimony analyses with the same topography as found with maximum likelihood. Clade C consists of the Korean *F. elliptica,* the Chinese decaploid *F. japonica* var. '*japonica'* and two tall tetraploid *F. japonica* var. '*japonica'* plants. Clade D is comprised of the three *F. japonica* var. *uzenensis* plants, the British clone of *F. japonica* var. *japonica* and an octoploid Japanese *F. japonica* var. '*japonica'*.

The remainder of the samples form an unresolved polytomy at the base of the tree made up of the Korean *F. sachalinensis*, the Chinese octoploid *F. japonica* var. '*japonica*', *F. japonica* var. *terminalis* and Japanese accessions of *F. japonica* var. '*japonica'* consisting of two tall tetraploids, a dwarf tetraploid and an octoploid. The Japanese accessions do form a group on the maximum likelihood tree but bootstrapping does not support it.

### 7.5.6 *Geographical origin of the clades*

The combined data were analysed in three ways: firstly a parsimony analysis using "branch and bound" on all the data; secondly a parsimony analysis using "branch and bound" on all the data excluding characters relating to variation caused by microsatellites; and finally by a maximum likelihood analysis on all the data excluding characters relating to variation caused by microsatellites. Of the three, the bootstrap tree generated for the third shows the groups that were consistent by all three means of analysis and leaves as an unresolved polytomy at the base of the clade the sMPHs that conflicted between the three previous trees. As sisters to the unresolved section were two major clades, each of which were divided into two sister clades, referred to as clades A and B from the first major clade, and C and D from the second major clade. Within clade A, a sub-group was found that has been coloured red as opposed to

pink like the rest of clade A. The geographical origins of the plants that make up these groups have been plotted on Figure 7.14.

Clade A (excluding the subgroup) consisted predominantly of *F. sachalinensis* from the north of Honshu and Hokkaido. There was one *F. japonica* var. '*japonica'* within this clade and this originated from the most northern Honshu prefecture, Aomori. The clade A subgroup has a much wider distribution but was only found on Honshu, and not to the north of the island. Clade B consisted of both *F. sachalinensis* and *F. japonica* var. '*japonica'* plants found only in Niigata prefecture on Honshu. Clade C plants were found in the south west of Japan and both China and Korea, as were the plants that form the unresolved group. Clade D was made up of *F. japonica* var. *uzenensis* from the region to the north of central Honshu known as the Tōhoku and Horuriku district, and an octoploid *F. japonica* var. '*japonica'* from Osaka.

## 7.6 Discussion

### 7.6.1 *Fragments sequenced and methods of analysing the data*

There have been many studies published that demonstrate the potential phylogenetic utility of non-coding regions in the chloroplast (see (Kelchner, 2000)). Unfortunately these studies have contradicted the initial assumptions of constraint-free evolution in non-coding regions. Difficulties include alternative alignment possibilities of indels, regions of length mutation in which homology is questionable or impossible, and the occurrence of localised "hot spots" of inferred excessive mutation, frequently to the point of saturation and loss of a phylogenetic signal (Kelchner, 2000). In an attempt to limit the impact of these, all sequences in this study were manually checked and indels aligned taking a conservative approach, and additionally so that repeated units could be recognised if they occurred in tandem. In addition to this, analysis was completed with and without the inclusion of mono- and dinucleotide repeats.

Strings of mononucleotide repeats, particularly but not exclusively those of A or T, appear frequently in non-coding cpDNA, and slipped-strand mispairing may potentially generate length mutations within these strings. There is an increase in potential for further length mutation relative to string length, making it difficult to assess homology. Uncertainty of homology is made worse by potential inaccuracies of enzymatic processes during PCR amplification and sequencing which can also generate variable-length repeat strings independent of the template's sequence constitution. For this reason the removal of such regions is recommended (Kelchner, 2000).

The three regions used in this study were selected based on their potential for generating informative characters. The selected sequences were from the regions *trn*C-*trn*D, *trn*D-*trn*T and *trn*F-*trn*V. Due to the length of the fragments the whole region could not be sequenced without the use of internal primers, so the regions were sequenced in the direction that appeared most useful.

The chloroplast genome is inherited as a unit and is not subject to recombination; therefore chloroplast DNA sequences are often combined. The advantages of this include greatly shortened run times, enhanced resolution, increased internal support for clades, and the presence of uniquely supported clades when compared to the separate data sets (Soltis & Soltis, 1998). All three fragments used in this study were first analysed independently. Whilst there was slight variation between the relationships determined by the fragments independently, the contentious chloroplast haplotypes, such as that belonging to the Korean *F. sachalinensis,* became part of the unresolved group. The plants associated with the clades predicted by the combined data set were found associated in all fragments analysed independently.

Two of the three fragments used in this study (*trn*C-*trn*D and *trn*D-*trn*T) were found to be useful in a screen of chloroplast regions for use in a phylogenetic analysis in *Allium* L. (Mes *et al.*, 1997). The region *trn*D-*trn*T has also been used to study the phylogeography of the coniferous *Cunninghamia konishii* Hayata (Lu *et al.*, 2001) and to find variation between different walnut species, *Juglans* L. (Potter *et al.*, 2002). This study found the fragments from the chloroplast regions *trn*C-*trn*D (fragment D) and *trn*F-*trn*V (fragment F) led to trees with better resolution, whilst the fragment from the region *trn*D-*trn*T (fragment T) generated the most chloroplast haplotypes. For most of the plants used in this study there was no conflict between the chloroplast haplotypes determined by the individual fragments and the combined sMPH. The exception to this being some plants within clade A being grouped with different clade A plants depending on the fragment analysed. Likewise, some of those that had previously been in the PCR-RFLP study unresolved group grouped with different plants from the PCR-RFLP study unresolved group depending on the fragment analysed. As seen from Table 7.10, the plants that were previously in the PCR-RFLP study unresolved group, in this study were found in both the unresolved section and clade C. Fragment T was found to distinguish all the plants that comprised clade C and the unresolved section. Fragment D can distinguish those in clade C from those in the unresolved section. It is fragment F that appears to have caused the conflict, haplotype FV8 being found in two plants from clade C and two from the unresolved section, and haplotype FV7 being shared by one from clade C

and one from the unresolved section. The only difference between FV7 and FV8 was caused by a mononucleotide A repeat at base number 583-585 on the consensus sequence, this variation therefore being omitted from two of the combined analyses.

There was no conflict between the plants that made up the four clades and the unresolved group resulting from the choice of analysis. Additionally all three methods had clade A and B as sister clades, and C and D as sister clades, and the combined clade A and B being a sister to the combined clade formed by C and D. The main effect the choice of method for analysing the data had was on the positioning of the seven plants that form the unresolved group. According to the ML analysis the seven plants formed an unresolved polytomy at the base of the clade; a supported group of six plants on the parsimony analysis without microsatellite data, that was a sister to the two major clades, with the seventh being found at the base of the tree; and a supported group of five that was a sister clade to the major clade formed by clades C and D, with the remaining two taxa being unresolved at the base of clades C & D and the group of five.

### 7.6.2 *Relationships between the taxa in* Fallopia *section* Reynoutria

The genus *Fallopia* is divided into four sections: *Fallopia*, to include the annual climbers *F. convolvulus* (L.) Löve, *F. dumetorum* (L.) Holub, and *F. scandens* (L.) Holub; *Parogonum* Haraldson, to include the perennial climbers *F. cilinodis* and *F. cynanchoides*; *Sarmentosae* (Grintz) Holub to include the woody climbers *F. multiflora* and *F. baldschuanica*; and *Reynoutria* to include the robust rhizomatous herbaceous perennials *F. japonica* and *F. sachalinensis* (Bailey & Conolly, 1985).

Two taxa from section *Sarmentosae* and one from section *Fallopia* were selected to form the outgroup to section *Reynoutria*. The plants from section *Reynoutria* were found to form a monophyletic group supported by a bootstrap value of 100, regardless of the means of analysing the data. *F. multiflora*, from section *Sarmentosae,* was found to take a position no closer to the plants from section *Reynoutria* than to the other two plants that made up the outgroup taxa. *F. multiflora* is a rhizomatous herbaceous plant with the same base number as the ingroup (n = 11). It is believed to be either a diploid progenitor to the ingroup, or at least a close relative of the progenitor (J. P. Bailey, pers. comm.). The other two plants that made up the outgroup taxa were *F. baldschuanica,* which is found in the same section as *F. multiflora*, and *F. convolvulus* from section *Fallopia*. These two taxa formed a group of their own supported by a bootstrap value of 100. A total of 49 mutations (47 without microsatellites) were shared by these two taxa, and a further 24 mutations were shared

between the three outgroup taxa. *F. baldschuanica* is a woody perennial with 2n = 20. *F. convolvulus* is an annual plant with the same base number as *F. baldschuanica* but can be found with 2n = 20 and 2n = 40. All three plants that made up the outgroup taxa were climbing plants unlike the ingroup. These data challenge the current taxonomic segregation that puts *F. multiflora* and *F. baldschuanica* together in section *Sarmentosae*, with *F. convolvulus* being placed in section *Fallopia*.

The accessions from the genus *Fallopia* section *Reynoutria* form a monophyletic group. Four distinctive groups of plants can be found which have been referred to as clades A, B, C and D, which represent 26 of the 33 accessions in the study. Clades A and B combine to form a major clade, as did clades C and D, the two major clades being sister clades. The remaining seven accessions formed an unresolved polytomy at the base of the ML analysis; a supported group of six plants on the parsimony analysis without microsatellite data, that was a sister to the two major clades, with the seventh being found at the base of the tree; and a supported group of five that was a sister clade to the major clade formed by clades C and D, with the remaining two taxa being unresolved at the base of clades C & D and the group of five.

Support for the two major clades (the combined A and B clades, and the combined C and D clades) varies dependent on method of analysis. The parsimony analysis using all data has bootstrap support for the two major clades at 89 and 76 respectively. Without microsatellite data the support decreases to 79 for the first clade and increases slightly to 79 for the second, and finally the ML analysis gives the lowest bootstrap values of 60 and 61.

All *F. sachalinensis* plants except the one from Korea were found within the major clade comprising clades A and B. The Korean *F. sachalinensis* used in this study was aneuploid having 2n = 102. The *F. sachalinensis* on Ullung Island is normally dodecaploid (2n = 132). However, an Ullung specimen was collected, counted as 2n = 102 by Lee (1972), and sent to the botanic gardens in Seoul, Korea. Seed was collected from this plant and sent to Leicester, UK, where it was grown. These plants grown from seed were 2n = 102 (Bailey, 1989) and presumably arose following a balanced reduction after the loss of some of the chromosome pairs, or by apomixis (Bailey, pers. comm.). It was one of these 2n = 102 plants grown from seed that was analysed for this study. Whilst 2n = 102 may not be truly representative of *F. sachalinensis* plants from Ullung, the maternally inherited chloroplast should still be representative of the chloroplast types on the island. From a cytological perspective the Korean *F. sachalinensis* differs from other native *F. sachalinensis*. The chloroplast haplotypes as determined by PCR-RFLP analysis in chapter 6, and by sequence analysis in

the current study, show the Korean *F. sachalinensis* to be highly divergent from other *F. sachalinensis* plants. A study by Kim and Park (2000) based solely on characteristics derived from size and shape of leaves found no difference between Korean dodecaploid and Japanese tetraploid *F. sachalinensis* plants. The 2n = 102 Korean *F. sachalinensis* had large leaves with cordate bases, characteristics associated with tetraploid *F. sachalinensis*. However, inspection of the lower epidermis as described in chapter 6 reveals discrepancies between the two. Typically *F. sachalinensis* plants have long uniseriate trichomes and a highly striate cuticle layer. No trichomes were found on the Korean 2n = 102 *F. sachalinensis,* and the cuticle layer was not found to be striate. The combination of the cytological, chloroplast and morphological evidence would suggest that some level of taxonomic recognition be required for these plants from Ullung. The chloroplast DNA sequence data showed the Korean *F. sachalinensis* to be genetically closer to the other non-Japanese native plants, compared to the other *F. sachalinensis* plants from this study. Fragment F found no differences between the Korean *F. sachalinensis* and the Chinese decaploid *F. japonica* var. '*japonica',* and only a single difference caused by a microsatellite A repeat between their chloroplast haplotype and that of the other Korean and Chinese plants, *F. japonica* var. *terminalis* from the Hachijo islands, and a Japanese tall tetraploid *F. japonica* var. '*japonica*' from the island Shikoku.

As already stated, the remaining *F. sachalinensis* plants were found within the major clade comprising clades A and B. There was very strong support for both clades A and B, with bootstrap values of 91 and 98 respectively according to the ML analysis; 96 and 97 for the parsimony analysis without microsatellite data; and 94 and 98 for the parsimony analysis on all data. Clades A and B are both made up of chloroplast haplotypes found in *F. sachalinensis* and *F. japonica* plants, indicating a very close relationship between the two species. The PCR-RFLP analysis in chapter 6 found the majority of the plants had chloroplast haplotypes that could be grouped into one of four clades consistent with both a parsimony analysis and a neighbour joining analysis based on the Jaccard's similarity coefficient. The remaining chloroplast haplotypes formed an unresolved polytomy at the base of the group by the parsimony analysis and were found in two areas of the neighbour joining matrix, Figures 6.7 and 6.7. In the PCR-RFLP study *F. sachalinensis* was found in two clades, B and C, however these two clades were no closer to each other than the other two clades that did not contain *F. sachalinensis*. In the current study chloroplast haplotypes from *F. sachalinensis* were found in clades A and B which were sister clades and therefore more closely related to each other than to clades C and D and the unresolved section. In Britain two different chloroplast types have been found in *F. sachalinensis* by chloroplast PCR-RFLP analysis. The common chloroplast type found in British *F. sachalinensis* was called MPH 2 in chapter

6, and the rare one MPH 38. In the current study one of the British plants that had MPH 38 was found to contain sMPH s10, which falls within clade B. A British plant that had MPH 2 was found to have sMPH5, found in clade A.

Clade A (excluding the subgroup) consists predominantly of *F. sachalinensis* from the north of Honshu and Hokkaido. There is one *F. japonica* var. '*japonica'*, P958, within this clade and this originated from the most northern Honshu prefecture, Aomori. P958 was the sole representative in this chapter from the PCR-RFLP MPH 8. It was included in this study as an example of an octoploid *F. japonica* var. '*japonica'*. As seen in all analyses in this chapter, it occupies a very different part of the *Fallopia* section *Reynoutria* clade to the other two Japanese octoploid *F. japonica* var. '*japonica'* plants, P987 and P1113, which were found in clade D and the unresolved section respectively. Retrospectively, on closer inspection of P958 after completion of the current study and the PCR-RFLP analysis, the identification of P958 as *F. japonica* var. '*japonica'* may be spurious. It was identified as such because it was lacking any obvious trichomes, and had low levels of striation on the lower epidermis. In total in the previous study twenty different plants were found to possess this chloroplast type; P958 was the only one classified as octoploid *F. japonica* var. '*japonica'*. The remaining octoploids were *F. japonica* var. *uzenensis*, of which there were three, and a plant thought to be a putative hybrid between *F. japonica* var. '*japonica'* and *F. japonica* var. *uzenensis.* Additionally two *F. sachalinensis* and thirteen other hybrids, 12 hexaploid and 1 tetraploid, shared MPH 8. No other *F. japonica* var. '*japonica'* regardless of ploidy level was found this far north. All of the plants with MPH 8 came from Aomori and clearly many hybridisation events have occurred. It is possible that P958 gained its chloroplast type through past introgression, or that it may be a misidentified hybrid between *F. japonica* var. *uzenensis* and an unreduced gamete from a tetraploid source.

There were six *F. sachalinensis* plants, including a British accession, whose chloroplast haplotypes formed clade A. These were found to possess three different MPHs according to PCR-RFLP, whilst in the current study they were divided into four different sMPHs. *F. sachalinensis* is normally typified by having the greatest height of all the *Fallopia* section *Reynoutria* taxa. In Japan, growing at high altitude, were found two *F. sachalinensis* that were dwarf compared to the more normal plants, although not as short as would be associated with dwarf *F. japonica,* but possessed the trichome type, leaf shape and degree of cuticular striation that characterises *F. sachalinensis*. These were P1062 and P1065, and were found by PCR-RFLP to have different chloroplast haplotypes MPH 37 and 11 respectively. One of the other Japanese *F. sachalinensis* plants included in the current study was P919, which was

one of the eleven *F. sachalinensis* of the more normal tall form that were previously found to share MPH 11. The other two Japanese *F. sachalinensis* plants selected for sequence analysis were found to have MPH 2 which matches the MPH of the most common chloroplast haplotype found in British *F. sachalinensis* (MPH 2). One of these came from Aomori, P954, whilst the other came from Hokkaido (P1169). Both were included in an attempt to see which the British chloroplast was genetically closer to.

Sequencing data were not able to distinguish between P1062, P919 and P954, three of the four *F. sachalinensis* that came from the north of Honshu, and including one of the dwarfs. These had previously been separated by PCR-RFLP analysis as MPH 37, 11 and 2 respectively. The other dwarf was found to have a unique sMPH, as were the *F. sachalinensis* from Hokkaido and the British *F. sachalinensis*. The Hokkaido *F. sachalinensis* only varied from the Aomori *F. sachalinensis* in one fragment, fragment D. That variation was caused by two sites at base numbers 559 and 717 where it was impossible to determine the base so was recorded as N, meaning this study was unable to distinguish Aomori from Hokkaido as the probable source of the British invasive *F. sachalinensis*. Although the British *F. sachalinensis* was found to have a unique sMPH, none of the mutations was of phylogenetic relevance. The dwarf *F. sachalinensis* had chloroplast types similar to and matching other *F. sachalinensis* plants, and it is presumed that the dwarf form resulted from the environmental effect of the altitude at which they were found. In both studies they were found to have different chloroplast haplotypes, so cannot be two stands of the same clone, even though they were found at the same location and shared the dwarf characteristic.

Within clade A was a subgroup consisting of tetraploid *F. japonica* var. *japonica*. This was a well-supported group within clade A, with bootstrap values of 98, 97 and 88 dependent on the method with which data were analysed. This subgroup was comprised of five plants, one of which was a British *F. japonica* var. *compacta*, providing evidence that the British *F. sachalinensis* and British *F. japonica* var. *compacta* are genetically more similar, at least according to their chloroplast types, to each other than either of them are to the British *F. japonica* var. *japonica*. In Britain the dwarf tetraploid *F. japonica* is recognised as a separate variety to the octoploid highly invasive clone *F. japonica* var. *japonica*. It is known as *F. japonica* var. *compacta*. In the introduced range the two varieties can be clearly distinguished by a number of morphological features and ploidy level, but as discussed in chapter 6 these features are less applicable to native material. In Britain *F. japonica* var. *compacta* is rarely found naturalised, but two chloroplast haplotypes have been distinguished from British

material, referred to in chapter 6 as MPH 3 and MPH 4. All of the plants within the subgroup of clade A were previously determined as either MPH 3 or 4. The Japanese plants from these chloroplast haplotypes selected for sequencing in this study included a dwarf and a tall of each. The dwarf with MPH 3 came from Mt. Fuji, and the tall with MPH 4 came from a lowland area from the same prefecture. The tall plant that had MPH 3 came from Yamaguchi and represented the most southern plant with chloroplast MPHs 3 or 4, whilst the dwarf with MPH 4 represented the furthest north these MPHs were found.

In the current study the British *F. japonica* var. *compacta* differed from the two Japanese plants that previously shared MPH 3 with it, in fragment T only. Besides having one base at position 49 that could not be determined, at position 83 the British *F. japonica* var. *compacta* had a T rather than a G, as did the British *F. sachalinensis* from clade B, but no other plants sequenced. The two Japanese plants could not be distinguished from each other even though one was tall and the other dwarf, and they came from geographically distant sites. The two plants that previously had MPH 4, the other chloroplast haplotype found in British *F. japonica* var. *compacta*, were found to have unique sMPHs. In fragment D they shared an sMPH with *F. sachalinensis* plants also in clade A, which differed from the other tetraploid *F. japonica* plants within the subgroup by a single mutation. The mutation was a 32 base pair deletion found in the three *F. japonica* plants that had previously had MPH 3, which was not found in those that previously were MPH 4, or in the *F. sachalinensis* plants. In chapter 6 the difference between MPH 3 and MPH 4 was found only in this same chloroplast region, i.e. *trn*C-*trn*D, (Table 6.7). The PCR-RFLP results were unable to say whether one type had evolved from the other, however the prediction was that these plants originated as dwarf plants of MPH 3 on Mt. Fuji and that as the plants progressed down and away from the mountain, MPH 4 evolved. The plants with MPH 4 lacked the 32 bp deletion, as did the rest of the plants found in *Fallopia* section *Reynoutria*, suggesting that absence of the deletion is the plesiomorphic (primitive) state and that therefore MPH 3 evolved more recently. However, an interesting feature that may challenge this statement is that one of the three outgroup taxa, *F. convolvulus*, shared this 32 bp deletion. With regard to the other two fragments in this study, those previously with MPH 4 differed from each other and those with MPH 3 in fragment F, and in fragment T one of them shared a MPH with the two Japanese plants with MPH 3, whilst the other had a unique haplotype. The variation in fragment F was generated by a 6 base CCGTGC repeat starting at position 60 unique to P907, and a single nucleotide mutation of T to C transition at base 471, unique to P1066 within the ingroup, but shared by *F. baldschuanica* and *F. convolvulus* from the outgroup, although the latter is quite

likely to be a case of homoplasy. The variation in fragment T was caused by an 8 base TTATAATA repeat starting at position 580 and unique to P907.

The plants within clade A excluding the subgroup were mainly *F. sachalinensis*, and those in the subgroup were *F. japonica*. They were not found to be sympatric for the majority of their geographic range, therefore recent hybridisation events are very unlikely to be the cause of the high degree of chloroplast similarity. Three mutations excluding microsatellite variation held clade A together. These were a C to T transition at base 376, an A to G transition at base 490, and an A to C transversion at base 629. All these mutation events were within fragment T.

Besides the three fragments shared by all of clade A there were a further two mutations that were shared by all taxa in clade A, and all taxa in the sister clade B. These consisted of an 8 base deletion in fragment F starting at base 342 that was shared by the plants in both clade A and its sister clade B. This was an apomorphic (derived) mutation as it was not found in the rest of *Fallopia* section *Reynoutria* sequenced or the outgroup taxa. The second mutation was a synapomorphic C to T transition at base 463 of fragment T.

The sister clade, clade B consisted of chloroplast haplotypes that were found in two Japanese *F. sachalinensis* plants, a tall and a dwarf Japanese tetraploid *F. japonica* var. '*japonica'* and a British accession of *F. sachalinensis* with a chloroplast haplotype rarely found in Britain. In the PCR-RFLP study, all of these taxa were found in clade C. One of the two Japanese *F. sachalinensis* shared MPH 38 with the British *F. sachalinensis*. The other shared a MPH with the dwarf Japanese tetraploid *F. japonica* var. '*japonica'*. The tall Japanese tetraploid *F. japonica* var. '*japonica* had a MPH unique to these five plants but found in two other *F. japonica* in the previous study. In terms of sequence data all five plants shared the same chloroplast haplotype in fragment F, which appeared to be the ancestral type to the combined clade A and clade B group, given that they had no unique mutations to distinguish them from the rest of the group. In fragment T they were all identical except for the British *F. sachalinensis* that varied due to the presence of two ambiguous bases, one at position 370 and the other at base 376. In fragment T they again appeared to be ancestral to the rest of the combined clade A and clade C group. Unlike both fragments T and F, in which there are no unique mutations distinguishing these plants from the rest of the combined clade, with fragment D there were four shared mutations that held the chloroplast haplotypes found in these plants that form clade B together. The two Japanese *F. sachalinensis* share a chloroplast haplotype that appears to be the ancestral form to that shared by the British *F. sachalinensis* and the dwarf tetraploid *F. japonica* var. '*japonica'*, and to the tall tetraploid *F.*

*japonica* var. '*japonica'* that possesses a unique chloroplast haplotype. An A to C transversion at base 178 distinguished the tall tetraploid *F. japonica* var. '*japonica'* from the others. The dwarf tetraploid *F. japonica* var. '*japonica'* and the British *F. sachalinensis* differ from the rest of the group due to a shared T to A transversion, a synapomorphic mutation found also in the chloroplast haplotypes of the plants in clade A, and that of P1136 which falls within clade C. This mutation may be a source of homoplasy within the data.

The plants that were found in clade B originated from Niigata prefecture, as shown by the blue symbols on Figure 7.14. In this prefecture *F. sachalinensis* and *F. japonica* var. '*japonica*' were found to be both tetraploid and sympatric. This meant that hybridisation had a higher chance of occurring compared to between non sympatric taxa, or sympatric taxa with different ploidy levels. The latter does happen, but often does not have a long term effect on the evolution of the taxa due to the production of hexaploid hybrids that are unable to reproduce at the hexaploid level. These factors mean there is a high probability that introgression or chloroplast capture has led to the high degree of chloroplast similarity between the two taxa in this clade. The ancestral nature of the two Japanese *F. sachalinensis* plants could indicate that the chloroplast capture or introgression was from *F. sachalinensis* to *F. japonica*. This theory could suggest a polyphyletic origin for *F. sachalinensis*, which seems unlikely, or that the plants in this clade were also the ancestors to the *F. sachalinensis* in clade A. This also seems unlikely given the restricted distribution of the *F. sachalinensis* plants from clade A at the southernmost edge of their range, compared to the distribution of those in clade B. *F. sachalinensis* is found at a lower density in this district than those further north as indicated by the comparison of the distribution of these plants produced for selected prefectures by Kanai (1991; 1992; 1993; 1996; 2000). Regardless of the confusion over the relationship between the taxa, the British *F. sachalinensis* with the rare chloroplast haplotype is most likely to have originated from Niigata.

The second major group determined in this study was that of the combined C and D clades. Depending on the method of analysis these clades were often found associated with some of the unresolved group. Clade C was comprised of plants that in the PCR-RFLP study were part of the unresolved group. There were four plants that had chloroplast haplotypes found within clade C, two from Japan and two that were non-Japanese native plants. Each plant was found to have its own unique sMPH. This was the only clade where the chloroplast haplotypes were fully resolved by all three means of analysing the combined data and where the support as determined by bootstrapping was greater without microsatellites than with, and greater when the data without microsatellites were analysed by ML instead of parsimony.

Within clade C the Korean *F. elliptica* was found closely related to the Chinese decaploid *F. japonica* var. '*japonica'*. The Korean *F. elliptica* appears to only have one unique mutation, found within fragment T, thus supporting the argument that *F. elliptica* does not deserve taxonomic recognition at the species level, although further molecular studies analysing non-chloroplast regions such as single copy nuclear genes would need to be completed to ensure the close relatedness is genuine and not an artefact caused by a phenomenon such as chloroplast capture. This result also indicates that the event that produced the decaploid *F. japonica* var. '*japonica'* may have occurred relatively recently as only two unique mutations were found in the chloroplast haplotype of this plant, again both within fragment T. The unique mutation in the Korean *F. elliptica* was a five base pair GGATT insertion starting at position 593. The decaploid Chinese *F. japonica* var. '*japonica'* is missing a 6 base pair TTTAGA repeat found in the rest of the ingroup and two of the three outgroup taxa. The repeat was also missing in *F. baldschuanica*, suggesting homoplasy. The other 'unique' mutation was a T to G transversion at base 575, which was unique to the clade but was also found in P1065 from clade B and in one of the outgroup *F. multiflora*. This character was likely to have arisen through homoplasy. The other two taxa in this clade were P1093 and P1136, both of which were tall tetraploid *F. japonica* var. '*japonica'* plants that came from the southwestern part of Japan. P1093 was from Yamaguchi prefecture, in the southwestern part of Honshu, and P1136 was from the prefecture Kochi on the island of Shikoku.

The Korean plants came from Seoul in South Korea, which is over 300 km from the North Korea/China border. Given the high degree of similarity between the Korean and Chinese plants, and their affinity to the Japanese plants found in south-western Japan, one could predict that Japan is the centre of diversity for *F. japonica* and that the plants found in Korea and China may prove to contain limited genetic variation, having originated from the Japanese plants. Limited genetic diversity could also explain why so many of them have similar morphological appearances to the extent where the Korean plants have been classed as a separate species to those found on Japan. During the Ice Age the Sea of Japan and Yellow Sea were about 100 m lower than at present and a land connection existed between Korea and Japan giving plenty of opportunity for the plants to have moved from Japan to Korea, and from there up into China. The land connection no longer existed after the middle Pleistocene, but the frequent typhoons that hit north-east Asia can carry a lot of things between populations in the two countries (Chung & Chung, 2000).

A limited number of Chinese *F. japonica* examined at Leicester were found to be octoploid or decaploid (Bailey, 2003). Kim and Park (2000) report Korean *F. japonica* var. '*japonica'* as

being tetraploid, hexaploid and octoploid. *F. forbesii* is given as a synonym for *Reynoutria elliptica* (*F. elliptica* in this study) and is found from south-western China through Manchuria to Korea (Kim & Park, 2000). Manchuria is officially known as the Northeast by the Chinese and has been under the political control of China, Japan and Soviet Russia during different time periods. Currently Outer Manchuria is Russian while Inner Manchuria is Chinese. The Korean *F. forbesii* have been reported to be hexaploid and octoploid (Kim & Park, 2000). Octoploids are believed to arise from chromosome doubling events in tetraploids. The lack of tetraploids from the, admittedly limited number of, Chinese accessions analysed supports Chinese material having originated from either Korea or Japan.

Found as a sister clade to clade C was clade D. This group comprised of chloroplast haplotypes found in five plants, three of which were found in *F. japonica* var. *uzenensis*; the remaining two being found in octoploid *F. japonica* var. '*japonica,*' one from Japan and the other being found in the British invasive clone of *F. japonica* var. *japonica*. The significance of the British clone of *F. japonica* var. *japonica* being associated so strongly with *F. japonica* var. *uzenensis* and found in only one anomalous accession of Japanese octoploid *F. japonica* var. '*japonica'*, and its bearing on finding the origin of the invasive clone of *F. japonica* var. *japonica,* were discussed in detail in chapter 6 so will not be repeated here. However the relationship between the chloroplast haplotypes will be discussed. In the PCR-RFLP study the British clone of *F. japonica* var. *japonica* represented in this study by P823, an aberrant *F. japonica* var. '*japonica'* P987, and several accessions of *F. japonica* var. *uzenensis* all shared MPH 1. In addition to sequencing P823 and P987 one accession of *F. japonica* var. *uzenensis* found to contain MPH 1 was selected for the current study, as was an *F. japonica* var. *uzenensis* that was found to possess MPH 9, and a further one that had chloroplast MPH 12. These combined MPHs were selected as between them they represented the majority of chloroplast haplotypes found in *F. japonica* var. *uzenensis* in the PCR-RFLP study.

In the current study the chloroplast genomes from the two Japanese plants that were previously classified as MPH 1, and the accession that previously had MPH 9 could not be distinguished whilst the British clone that also used to be classified as MPH 1 was found to be different in one fragment, T. This variation however was due to the presence of three ambiguity codes in the sequence and was not of phylogenetic significance. These ambiguities were picked up through cloning, with one in four of the clones having a G when the others had an A. Three ambiguity sites were detected from different clones. The other *F. japonica* var. *uzenensis* that had previously been found to have MPH 12, was found in the current study to have a unique sMPH s25. The variation here was due to a G to A transition at base 208 in

fragment F. The support for clade D was attributed to 6 mutations, two from each fragment, none of which were caused by microsatellites. In fragment D there was an A to C transversion at position 629, and a 10 base pair CAAATTATAT insertion beginning at base 719. In fragment F there was a C to T transition at base 654 and a 10 base AATATATATT repeat starting at position 669. Finally in fragment T there was a 19 base ACTATACTATATAATAAAG repeat starting at position 84, and a C to T transition at base 393. Simplified, the six mutations can be summarised as an indel and either a single base transition or transversion event relatively close to the indel, found in each fragment. There is the possibility that these types of mutations were the result of secondary structure in the form of stem-loops within the non-coding chloroplast sequence, and if this were the case some caution would be required in incorporating them into a phylogenetic analysis (Kelchner, 2000). Stem-loops are believed to occur during single-stranding events when inverted repeats meet to form a region of pairing (the stem) surmounted by their interceding sequence (the loop). Probable stem-loop secondary structure is commonly reported in non-coding regions of chloroplast genomes. Loop regions of stem-loop secondary structure are often associated with hot spots for mutation in non-coding regions, both of nucleotide substitutions and indel events. Indels located in probable loop sequence are frequently inserted repeat units likely the result of slipped-strand mispairing (SSM). However, length mutations not attributable to SSM often occur within loop sequences as well and may be remnants of recombination events (Kelchner, 2000).

The sister clades C and D were found to be grouped because of two shared mutations in fragment D. One of these was the presence of a 27 base pair inversion, which in the plesiomorphic state was found between base number 552 and 578 in the consensus sequence, with the apomorphic inversion being aligned to positions 579 to 605. The second synapomorphic mutation was a T to C transition at position 649. Inversions are also sometimes associated with stem-loop secondary structure in non-coding chloroplast regions, and therefore the phylogenetic implications of these mutations must be treated with caution. An interesting feature of these inversions was that there were two mutations found within this region that only affected a subset of the taxa in only one orientation implying these mutations have occurred since the inversion event, and therefore the mutation may be of more phylogenetic use than a standard inversion. In the plesiomorphic orientation the plants from clade B have a deletion of the C at position 567. There is no evidence of a deletion in the inverted section. However, within the inverted section the Chinese decaploid and Korean *F. elliptica* both had a T whilst the rest of the taxa with the inverted section had G, and the corresponding plesiomorphic taxa had the complement to G, C. This would support the

theory that the Chinese and Korean samples originated from a common ancestor in Japan, presumably after the inversion had occurred.

There were seven plants that formed the unresolved section of this study. They formed an unresolved polytomy at the base of the ML analysis; a supported group of six plants on the parsimony analysis without microsatellite data, that was a sister to the two major clades, with the seventh being found at the base of the tree; and a supported group of five that was a sister clade to the major clade formed by clades C and D, with the remaining two taxa being unresolved at the base of clades C & D and the group of five.

The most divergent accession in the ingroup was *F. japonica* var. *terminalis*. Sequencing fragment D revealed three unique mutations, and a mutation that *F. japonica* var. *terminalis* shared with clade B that resulted from a G to A transition at position 401. The three unique mutations were A to T transversions at positions 403, 446, and 501. With fragment F, it was found to share a chloroplast haplotype with Korean *F. elliptica*, Chinese octoploid *F. japonica* var. '*japonica',* and a Japanese tall tetraploid *F. japonica* var. *'japonica'*. The shared haplotype appeared to be ancestral to clade D. Fragment T identified four mutations unique to the chloroplast haplotype of *F. japonica* var. *terminalis*, a C to T transition at base 7, an A to G transition at base 13, a T to G transversion at position 34 (also found in *F. baldschuanica* but probably due to homoplasy) and a C to T transition at base 268. In the combined study, *F. japonica* var. *terminalis* was found to have the longest terminal branch, which is consistent with the PCR-RFLP analysis. This study therefore confirms the genetically distinct nature of *F. japonica* var. *terminalis,* which is endemic to the Hachijo islands to the south of Honshu.

In the parsimony study with all data, both Korean *F. sachalinensis* and *F. japonica* var. *terminalis* were unresolved at the base of a group made up of the combined C and D clades, and the remaining five plants from the unresolved section that formed a group by this analysis supported by a bootstrap value of 67 (Figure 7.9). The Korean *F. sachalinensis* was discussed above.

As already mentioned the remaining five plants formed a group that was a sister to the combined C and D clades according to the parsimony study with all data. This group was created by the accessions within it sharing a single mutation, whereas the four Japanese plants formed a group within the group of five produced through two mutations, with a bootstrap value of 74. These four also formed a group on the ML analysis although bootstrapping did not support this. The four Japanese plants were all obtained from the southwestern region of

Japan, and as with clade C, there appear to be links between the plants from this region and those from Korea and China.

### 7.6.2 *Phylogeographical relationships*

The clades indicated by the sequence analysis show a high degree of geographical clustering. However, it should be noted that the plants selected for sequencing were chosen because of the relationships between chloroplast genomes as determined by PCR-RFLP analysis in chapter 6. These MPHs were found to be good indicators of geographical distribution, therefore it is unsurprising that sequencing these same regions created the same effect, especially when the accessions chosen for sequencing were purposefully selected as the most representative samples to clarify some of the questions raised by the PCR-RFLP analysis.

Sequencing is a better method than PCR-RFLP analysis for understanding the types of mutations that cause variation in chloroplast haplotypes, and the relationship between the haplotypes. For identifying variation between plants and general clustering of related haplotypes the two methods were found to be congruent, with PCR-RFLP being the quicker and cheaper method to collect the data and less time consuming to analyse the raw data.

## 7.7 Conclusions

Some of the mutations that grouped some of the taxa were found to be of questionable phylogenetic use, a fact that would not have been obvious from PCR-RFLP analysis.

The method used to analyse the data does have an effect on the phylogenetic structure of the ingroup. The removal of microsatellites caused by mono and di-nucleotide repeats had no effect on the main relationships determined by the study, but did have an effect on those that were less strongly associated, leading, at the extreme, to an unresolved polytomy at the base of the ingroup. However, the main clades predicted by the current study were consistent between the three ways of analysing the combined data.

The chloroplast haplotypes found in *F. sachalinensis* were found associated with those from *F. japonica* in two different clades, A and B, as was the case in the PCR-RFLP analysis detailed in chapter 6, and in the study by Inamura (2000). The reason for this is unclear although a number of hypotheses were raised in the two studies.

The British tetraploids *F. japonica* var. *compacta* and *F. sachalinensis* have chloroplast haplotypes that according to sequence data were genetically closer to each other than to that of the British octoploid *F. japonica* var. *japonica*.

As with the previous study, *F. japonica* var. *terminalis* and Korean *F. sachalinensis* merit some level of taxonomic recognition, but a taxonomic distinction between dwarf and tall plants within *F. japonica* var. '*japonica'* is not supported. The relationship between *F. japonic*a var. *uzenensis* and octoploid *F. japonica* var. '*japonica'* has not been clarified in this study beyond what was determined in the PCR-RFLP study.

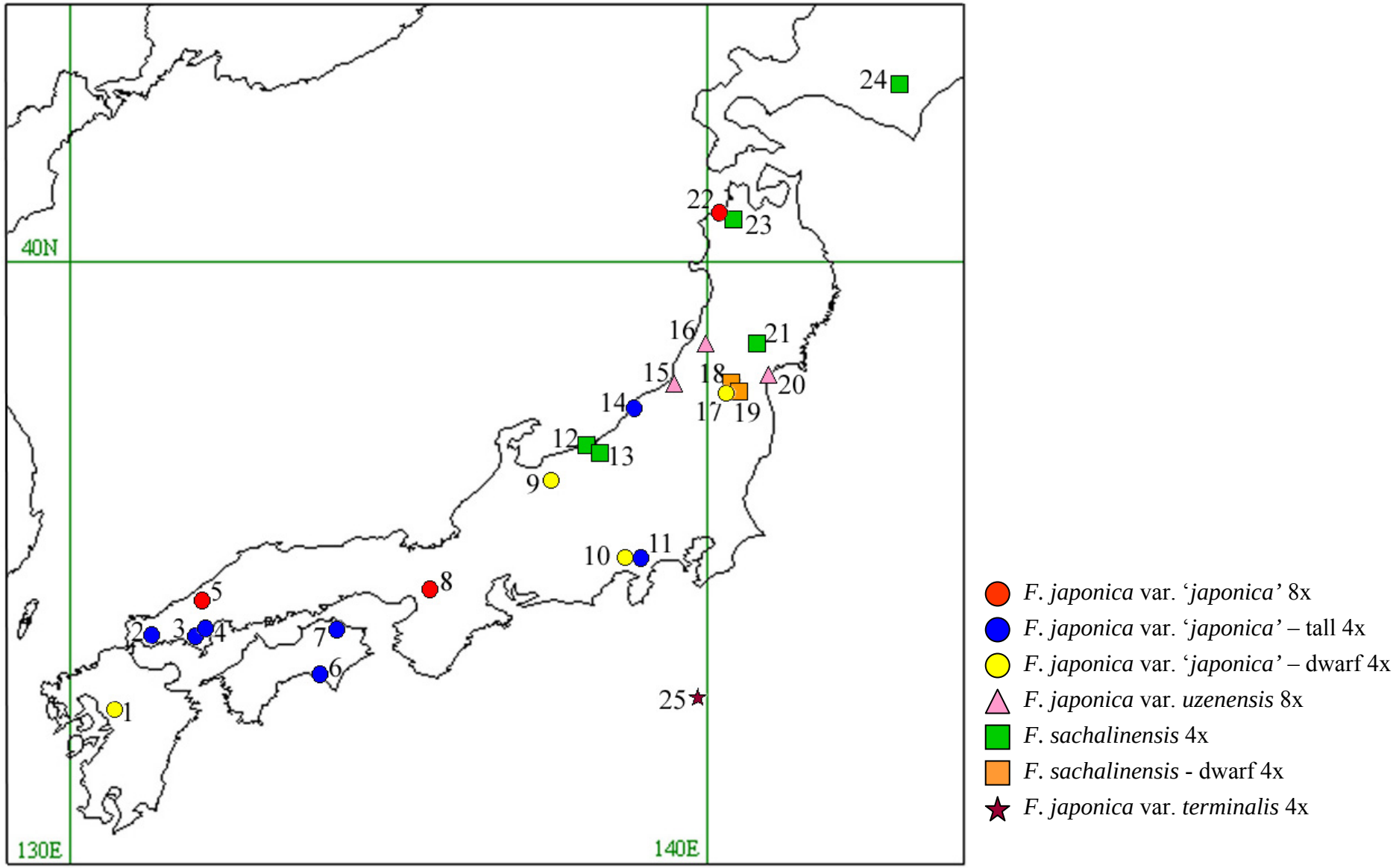



**Figure 7.1** Map depicting the sites from which each of the Japanese plants sequenced originated. Numbers relate to those shown in table 7.1.

**Figure 7.2** Consensus sequence of the chloroplast region *trn*C-*trn*D when sequenced from the *trn*D end (fragment D), for the ingroup that comprises thirty-three plants, and the outgroup taxa, *F. multiflora*, *F. baldschuanica,* and *F. convolvulus*.

```
        10         20         30         40         50
GCGGAAGCTG CGGGTTCGAG CCCCGTCAGT CCCGACGGAT CCAATAACCA
        60         70         80         90        100
CATCAACTCA CCTCTCCATT TTCATTTTAT GGCAAAAGAG GCACAATGAA
       110        120        130        140        150
- - - - - - - ATG AATATAATTT AGGTCATTCT CAATAGGAAT TTTTTTATTT
       160        170        180        190        200
TTTCGTTATT TCTCATCAAA CACAAACAAA TATATATA - - - - - - AATTTT
       210        220        230        240        250
CATTACTTCA ATGAGTATCT ATTGGTGGGA GAGAGATATA ATTGGATTAG
       260        270        280        290        300
TCCAATTATT GGGAACATCA TATTCAGGAT TCCGAGGGAT AGCGTACTGG
       310        320        330        340        350
GTCTGGTCTT TCAATTTATT GCCTCTATCT AATGGAATAG AGTATCATTT
       360        370        380        390        400
CTCGGGAGCA CATACACAAC TAGAATTCAT TTCTTGTACG CTCCAAAATC
       410        420        430        440        450
GAATTTGAGT TACCCCGAAA AAGGCTTTTC AGATTAAAAA CCTCTCCCCT
       460        470        480        490        500
TCTAGTTCCG ATTTTTTGTA GTCTCAATGA CTCAAACTAA CTCCTTTTTT
       510        520        530        540        550
AAAATCTATC TCATTCAAAT TTTACACACCTT TCTTTTTTTA C - TAT - - - - -
       560        570        580        590        600
 - ATGCTAGTA CTAATCCAAG AAAAGAGA - - - - - - - - - - - - - - - - - - - - -
       610        620        630        640        650
 - - - - - ATAGT AAAAAAAGAA AGGTTTTTAT AGAAGA - - - - - - - TTAGATC
       660        670        680        690        700
TTTTATAC - - - - - CGGAATT TGGCTTTTTC ACAATTTTCT TTTTCTTACA
       710        7250       730        740        750
AGAAAAAGAA AATTATAT - - - - - - - - - CA TAAAAAAATA GGAGTTTCGA
       760        770        780        790
GTTTAACTCC CGCCCCCTTT TCGTTTACTT CTATTGG
```

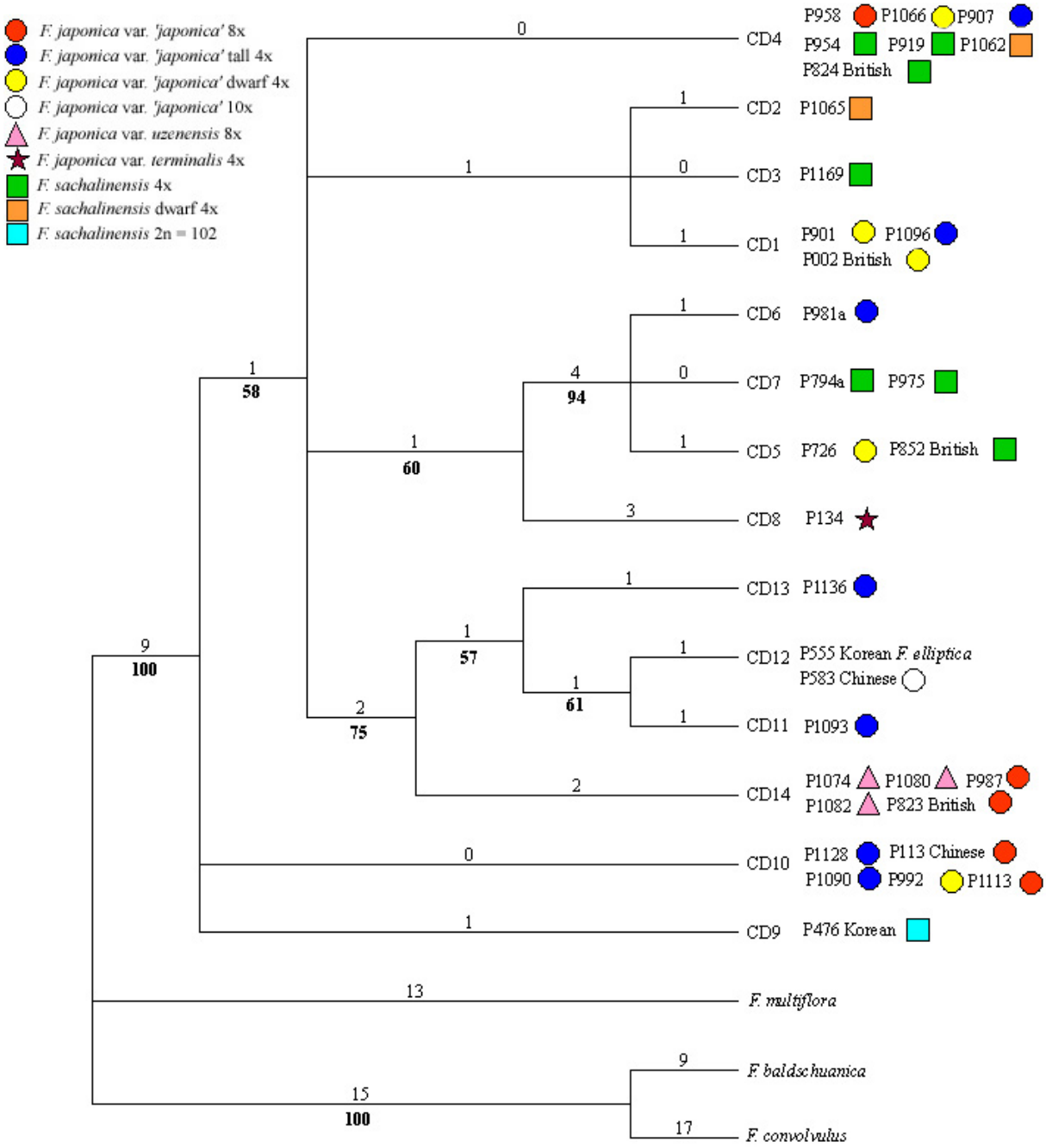



**Figure 7.**3 Phylogeny for fragment D (chloroplast region *trn*C-*trn*D sequenced from the *trn*D end). Numbers of mutations are given above the branches, and bootstrap values where they occur are below in bold.

**Figure 7.4** Consensus sequence of the chloroplast region *trn*F-*trn*V when sequenced from the *trn*F end (fragment F), for the ingroup that comprises thirty-three plants, and the outgroup taxa, *F. multiflora*, *F. baldschuanica,* and *F. convolvulus*.

```
        10         20         30         40         50
ATCTATTTTA CAAATGAATT GATATAGATC GATATTCAT - - - - - GTATAG
        60         70         80         90        100
CCCCCGTGC - - - - - - ACAGA CGTAACTTAT TTCTCTAGGT - - - - - - - - GT
       110        120        130        140        150
CTAGAGATAT ACCCCACCTA TAAAATAGAT GGGTAAAGAG TAGATATAAA
       160        170        180        190        200
AA- TGTAAAA GAGTTAGGTT TTCTTTTCGT TTTTTATTTG TTGTTTAT - -
       210        220        230        240        250
- - - - AATGTG TCTCCTCTCA TTGAAAAAGA ATATTTACTTC TTCATGTGGA
       260        270        280        290        300
TTCGAAAAAG GGTTTAATTA GGTAAA - - - - - - - - - - GTAAA TTAGTTGCAA
       310        320        330        340        350
GACGAAAAAA GGGGAGTTAA AGTAAA - - - - - - GGATGAGA ATAGACAAAA
       360        370        380        390        400
TGTATCTCAG ATACAGTACA AATAGAATTC GACCTCCTTT CATTTCTTTT
       410        420        430        440        450
TTTCTATTTC TTCATTTCCC CCCC - - - ACA TTACGTGACT TTCTACAAAC
       460        470        480        490        500
CATCTAAGTG ATACTCGATG TT - - - - - CGC GGTACAAAGT TCATGATACA
       510        520        530        540        550
AAAATTTTTT GGTTCATTCT ATTGGCTTGG CTAATCAAAA ATCCAATTGT
       560        570        580        590        600
TCCTTTTTAA CCAACCATAA TCCG - AAAAA AAAA - TGTCA ATTACTCCGA
       610        620        630        640        650
TTGTTTGATC TAGAACAGAG TGTACAAATA TTCTTTATAC TTATAACTTT
       660        670        680        690
ATACTTATAA TATATATT - - - - - - - - - TG TTTTATTTGA ATTTG
```

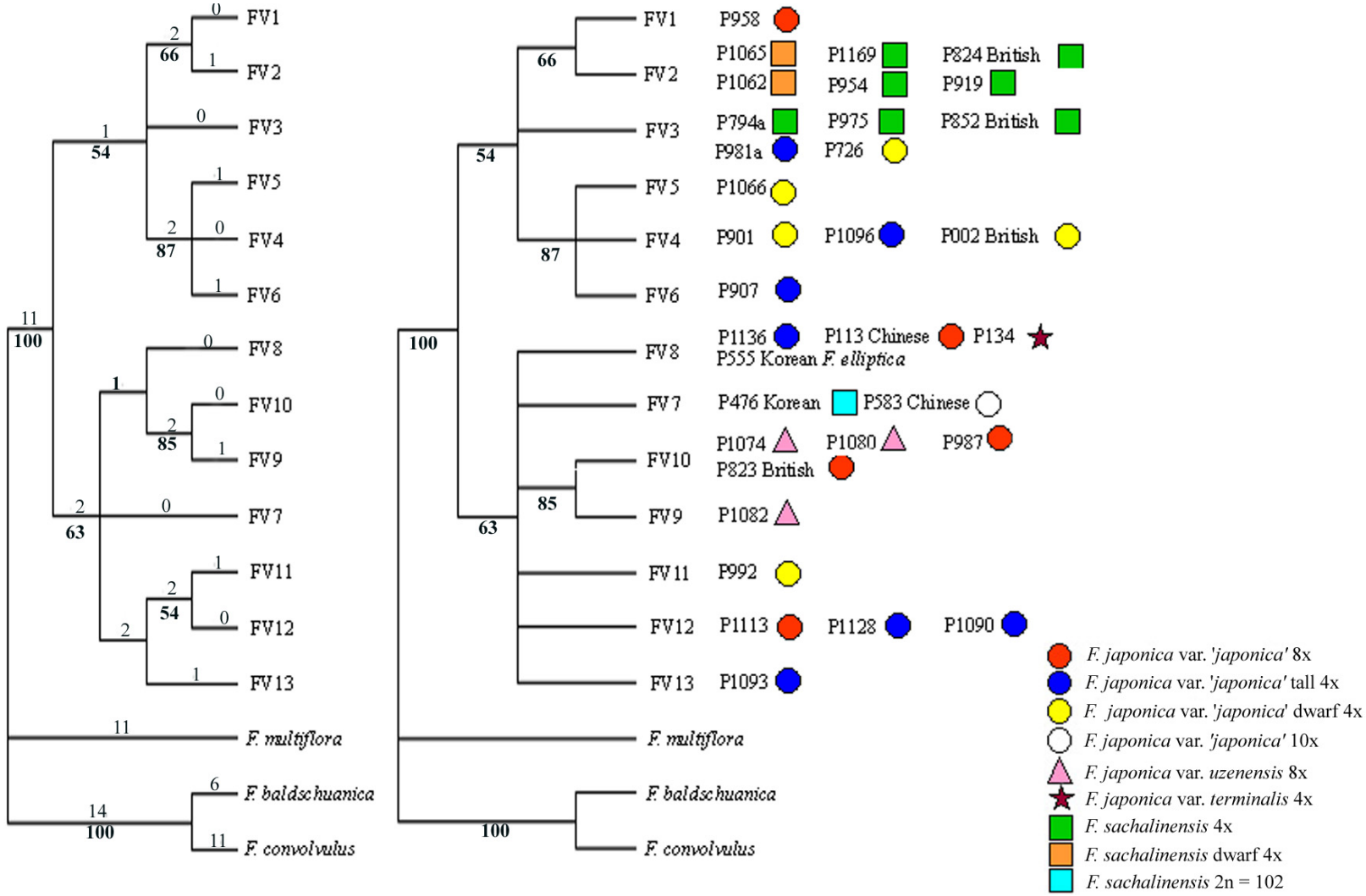



**Figure 7.5** Phylogeny for fragment F (chloroplast region *trn*F-*trn*V sequenced from the *trn*F end). To the left is an example of one of the eleven equally most parsimonious trees. Numbers of mutations are given above the branches, and bootstrap values where they occur are below in bold. To the right is the strict consensus tree, which summarises the nodes that did not conflict in alternative trees.

**Figure 7.6** Consensus sequence of the chloroplast region *trn*D-*trn*T when sequenced from the *trn*T end (fragment T), for the ingroup that comprises thirty-three plants, and the outgroup taxa, *F. multiflora*, *F. baldschuanica,* and *F. convolvulus*.

```
                10                  20                  30                  40                  50
ATCTATCTCC      CTATATTAC -         - - - - TATTAT      ATATAATATG      TTATTATAGA
                60                  70                  80                  90                  100
CT - - - - - - - -          - - ATACTATA        TAA - - - - - TA        AAG - - - - - - -           - - - - - - - - - -
                110                 120                 130                 140                 150
 - - TAAATTCA       TAGCGGCGGG      TGGCTCTTTC      CGGAATTATA      AAGTTGATTT
                160                 170                 180                 190                 200
ACTG - - - - - -            - - TCGAGTCT        CGGATCAAAC      GATTTATTGA      TCTTTTAAAA
                210                 220                 230                 240                 250
CTATCACTTC      AATGAACCAA      GCCGACCCTC      ATTTTTTT – C        TTTTATTAAA
                260                 270                 280                 290                 300
TTG - - - - - - -           - - - - - ATCCC     TATCAGAACA      AAAATCACTT      GAGACATGTA
                310                 320                 330                 340                 350
CCTACCAATT      CGACATAGAT      TCAAGATATT      T - - - - - - - - -         - - - CATTAAA
                360                 370                 380                 390                 400
TCAT - - - - - -            - GTGATGGAG         AAGTTCGATT      TGTCCCCTTA      ATC - - - - - - C
                410                 420                 430                 440                 450
ATAAAAAAGA      ATTTCCGAAA      TTTTATTGAT      CCCCTTTATC      ATCCCGGATT
                460                 470                 480                 490                 500
TCT - - - - - - -           - CCTTTATAA         ATTTTCTGGT      TTACTACGAA      GACGTATTTC
                510                 520                 530                 540                 550
GGCTTAAAAA      AATGAATGAA      TCAAGAAAGT      AGAAGAAATG      GAGTTGAAAG
                560                 570                 580                 590                 600
TTTTATTTTT      A - - - - - - - - T      ATTATAATA -         - - - - - - - GTA        TT - - - - - CTA
                610                 620                 630                 640                 650
GTAATCGA - -            - - - - - - GACC        TTTTTTTCAT      ATTTAATTTA      GATTTAGATA
                660
GAATTTTCTT      AGATATT
```

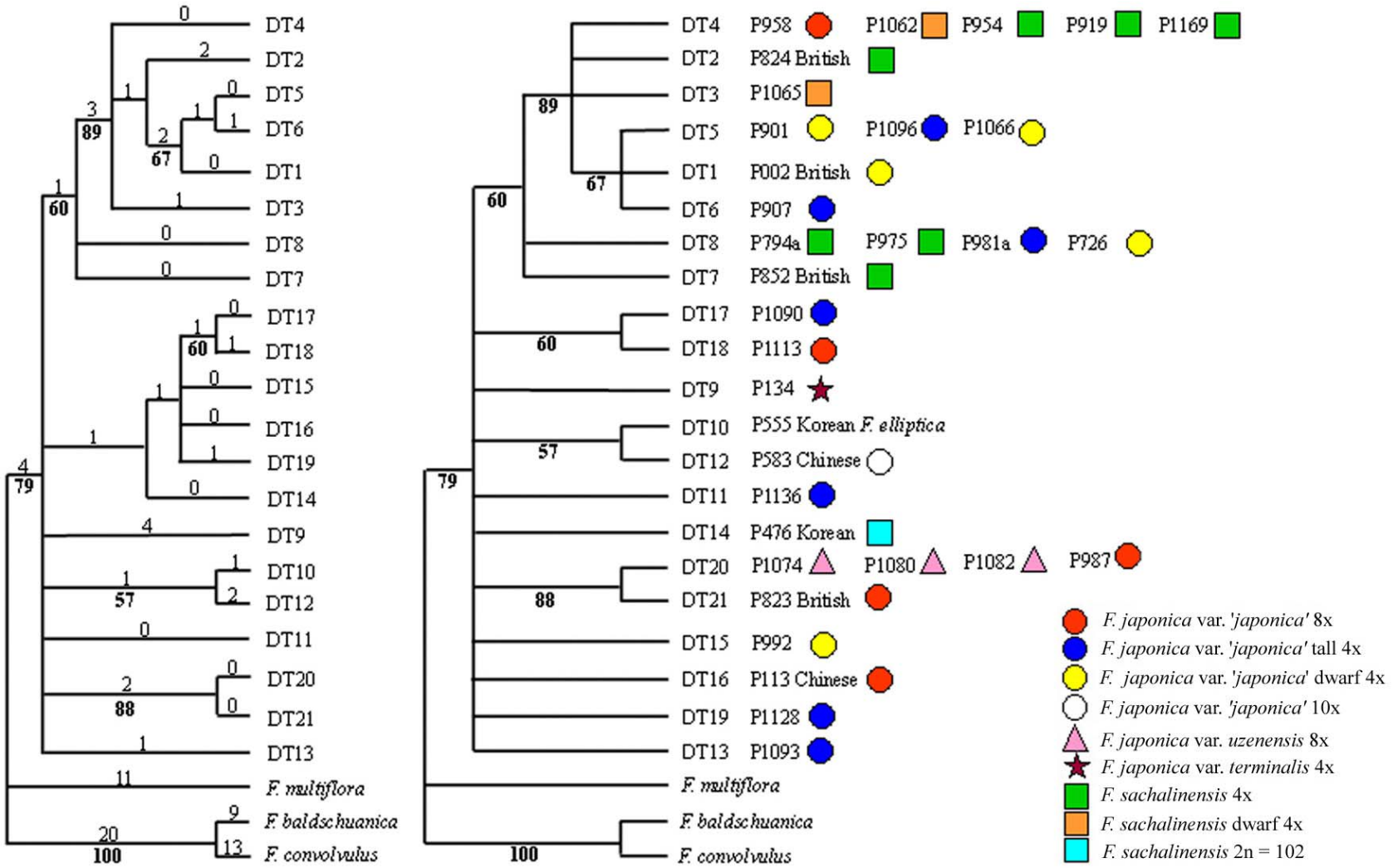



**Figure 7.7** Phylogeny for fragment T (chloroplast region *trn*D-*trn*T sequenced from the *trn*T end). To the left is an example of one of the twenty-four equally most parsimonious trees. Numbers of mutations are given above the branches, and bootstrap values where they occur are below in bold. To the right is the strict consensus tree, which summarises the nodes that did not conflict in alternative trees.

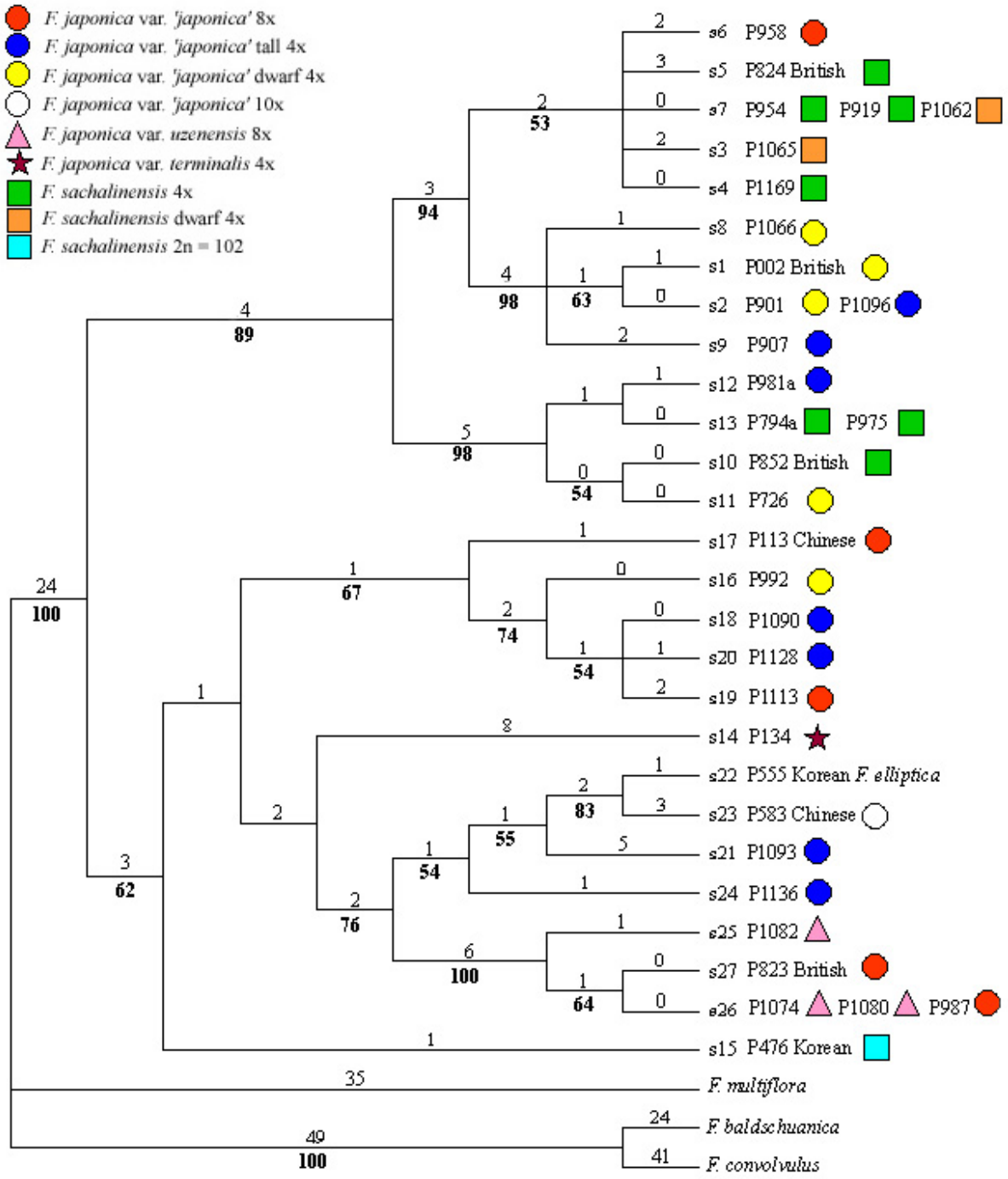



**Figure 7.8** An example of one of the twenty-one equally most parsimonious trees found when all data from the three fragments D, F and T were combined. Numbers of mutations are given above the branches, and bootstrap values where they occur are below in bold.

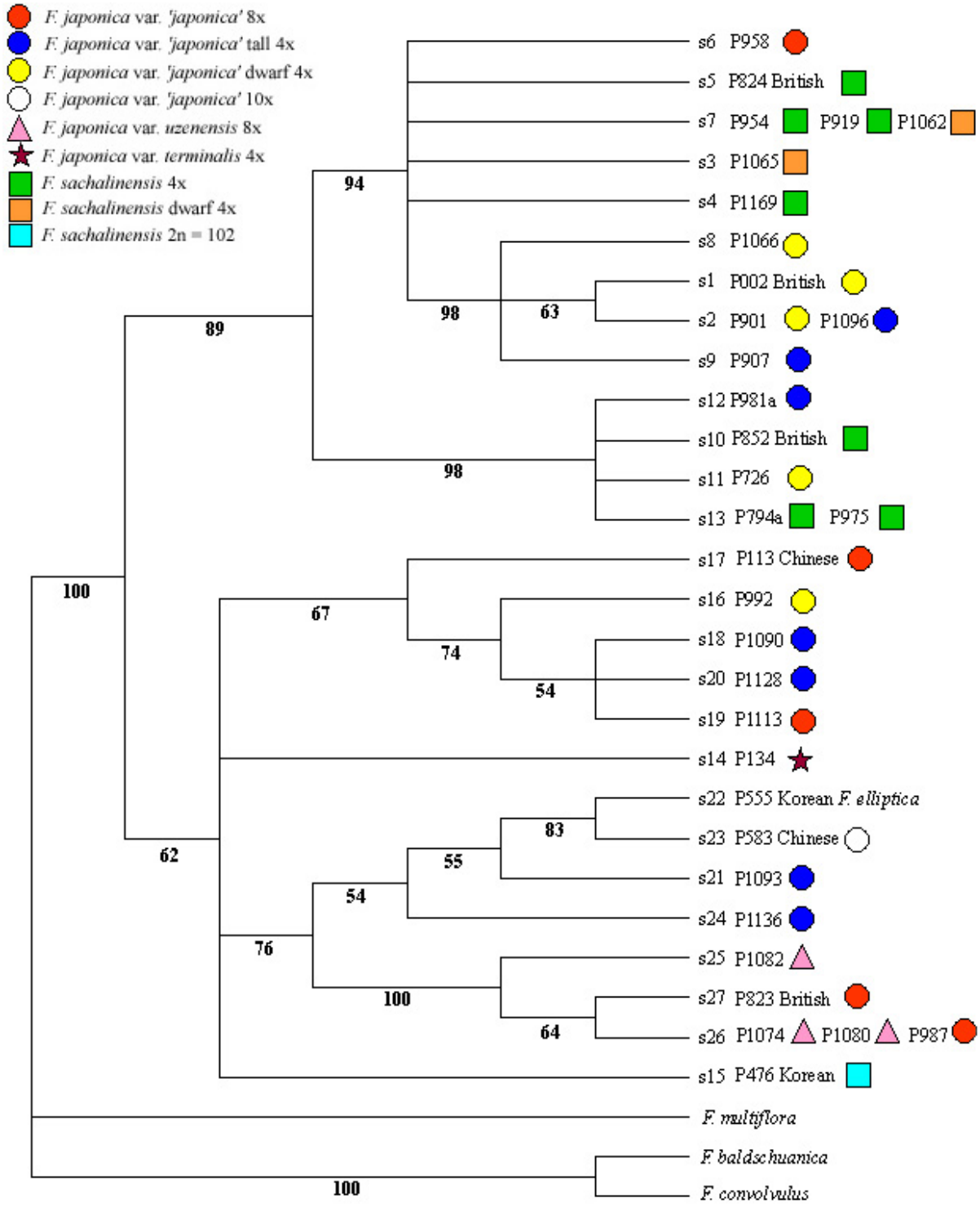



**Figure 7.9** The strict consensus tree that summarises the nodes that did not conflict in the twenty-one alternative equally most parsimonious trees found when all data from the three fragments D, F and T were combined. Bootstrap values are shown below the branches in bold.

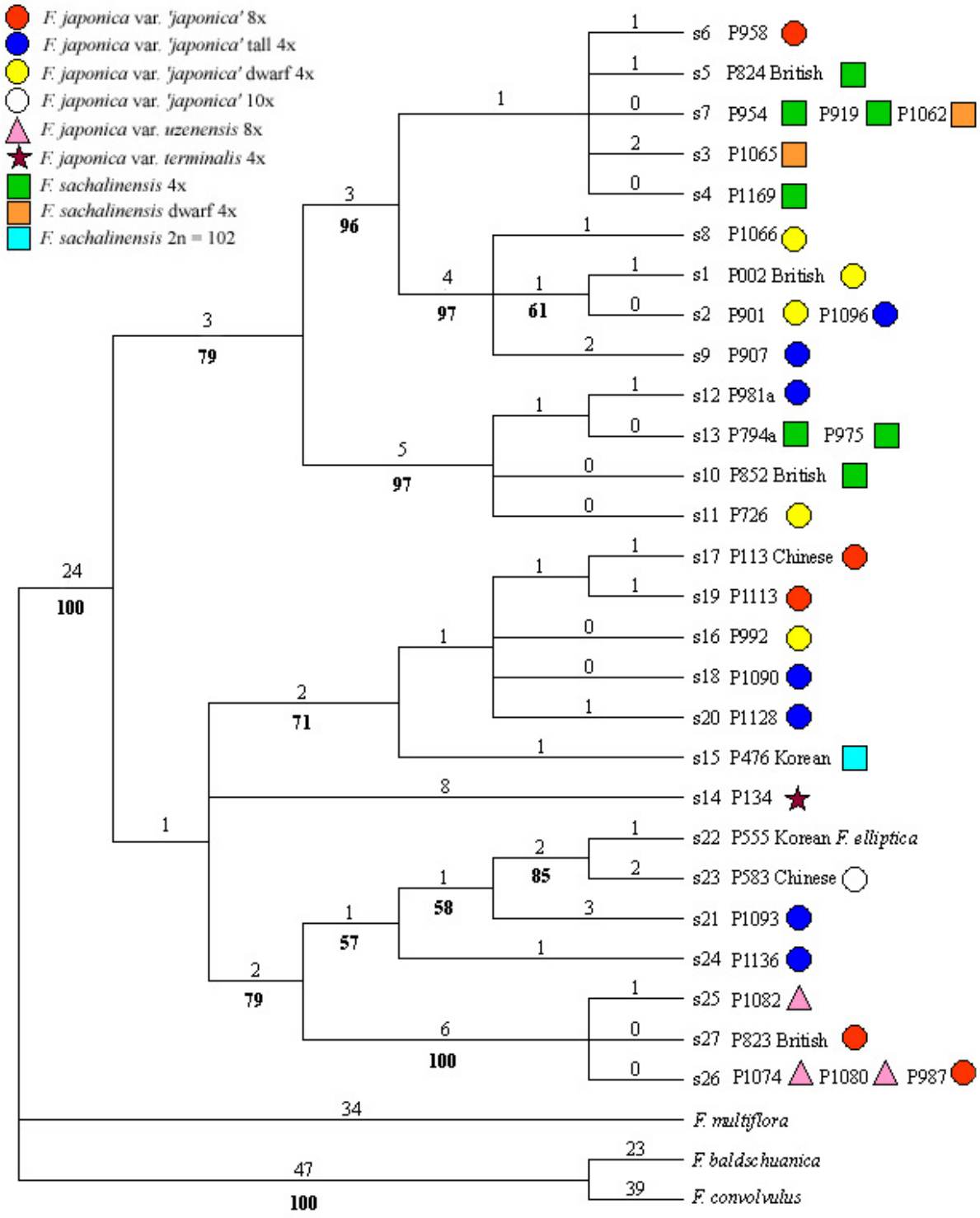



**Figure 7.10** An example of one of the 175 equally most parsimonious trees found when all data from the three fragments D, F and T were combined, but excluding characters that arose from variation caused by microsatellites. Numbers of mutations are given above the branches, and bootstrap values where they occur are below in bold.

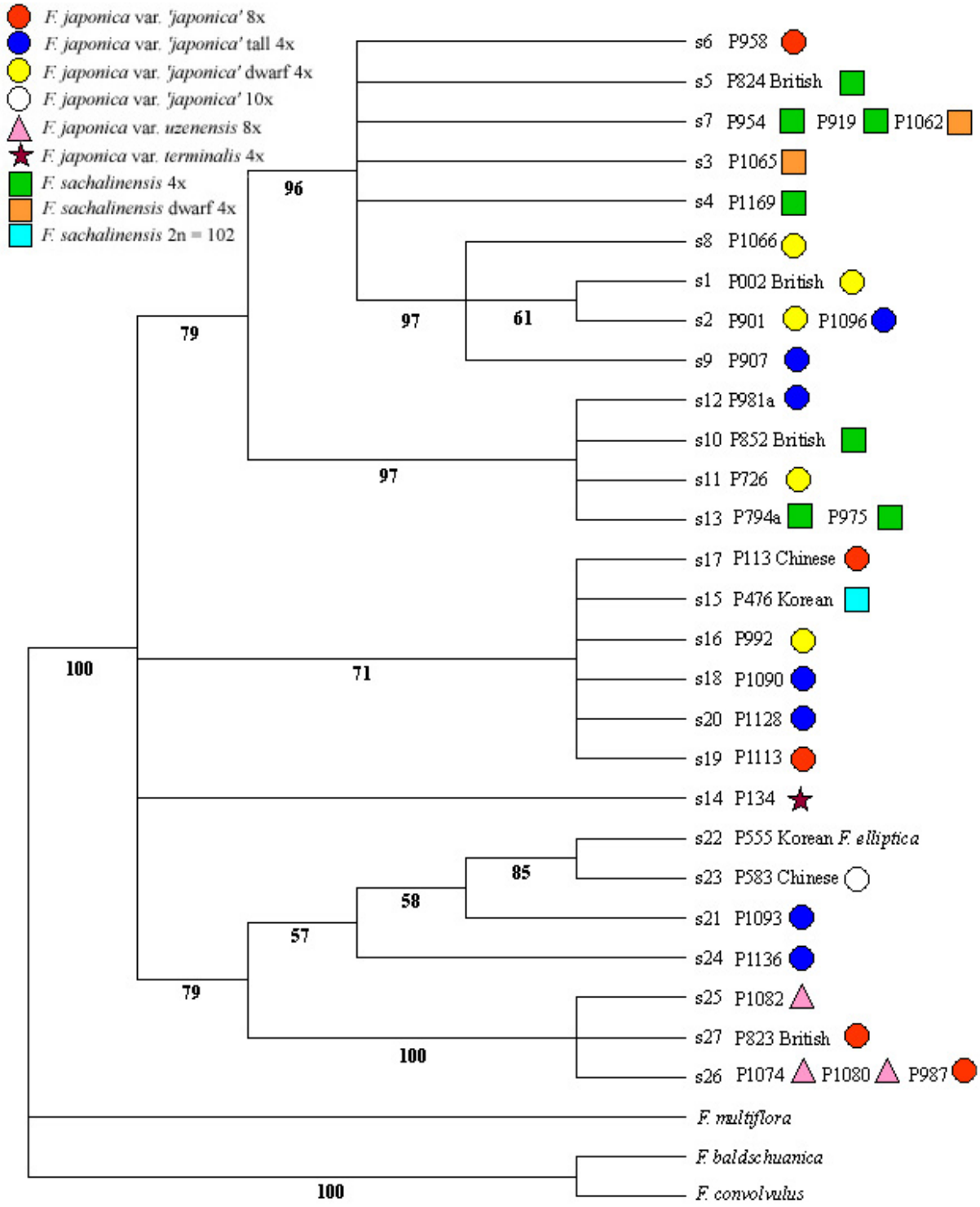



**Figure 7.11** The strict consensus tree that summarises the nodes that did not conflict in the 175 alternative equally most parsimonious trees found when all data from the three fragments D, F and T were combined, but excluding characters that arose from variation caused by microsatellites. Bootstrap values are shown below the branches in bold.

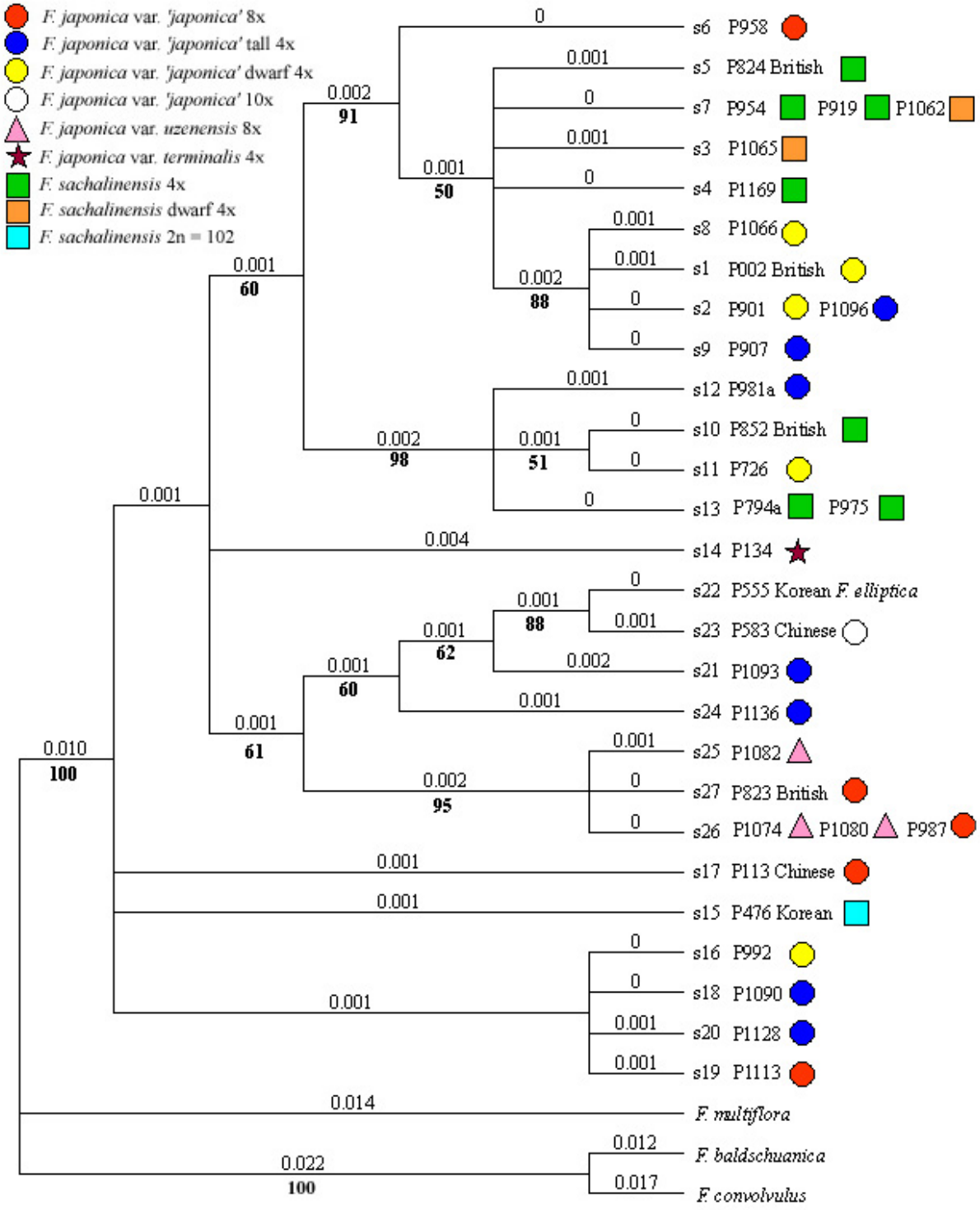



**Figure 7.12** The maximum likelihood tree based on a heuristic search using the model F81 + G, for all data from the three fragments D, F and T, but excluding characters that arose from variation caused by microsatellites. Maximum likelihood values are given above the branches, and bootstrap values where they occur are below in bold.

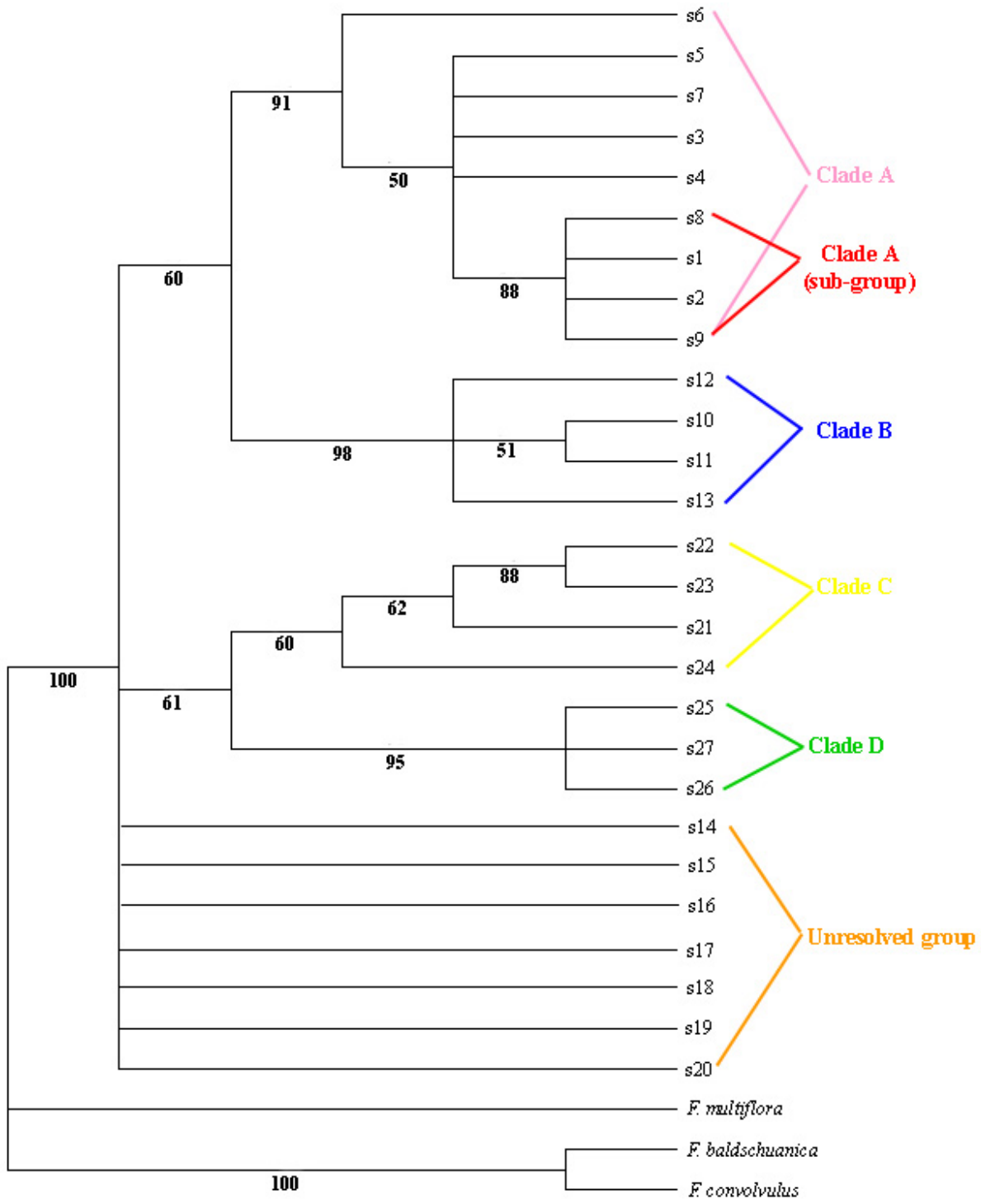



**Figure 7.13** The bootstrap consensus tree for the maximum likelihood analysis shown in Figure 7.12, showing the different groups in which the multi-primer-haplotypes derived from sequence data (sMPHs) were found. Bootstrap values are given in bold.

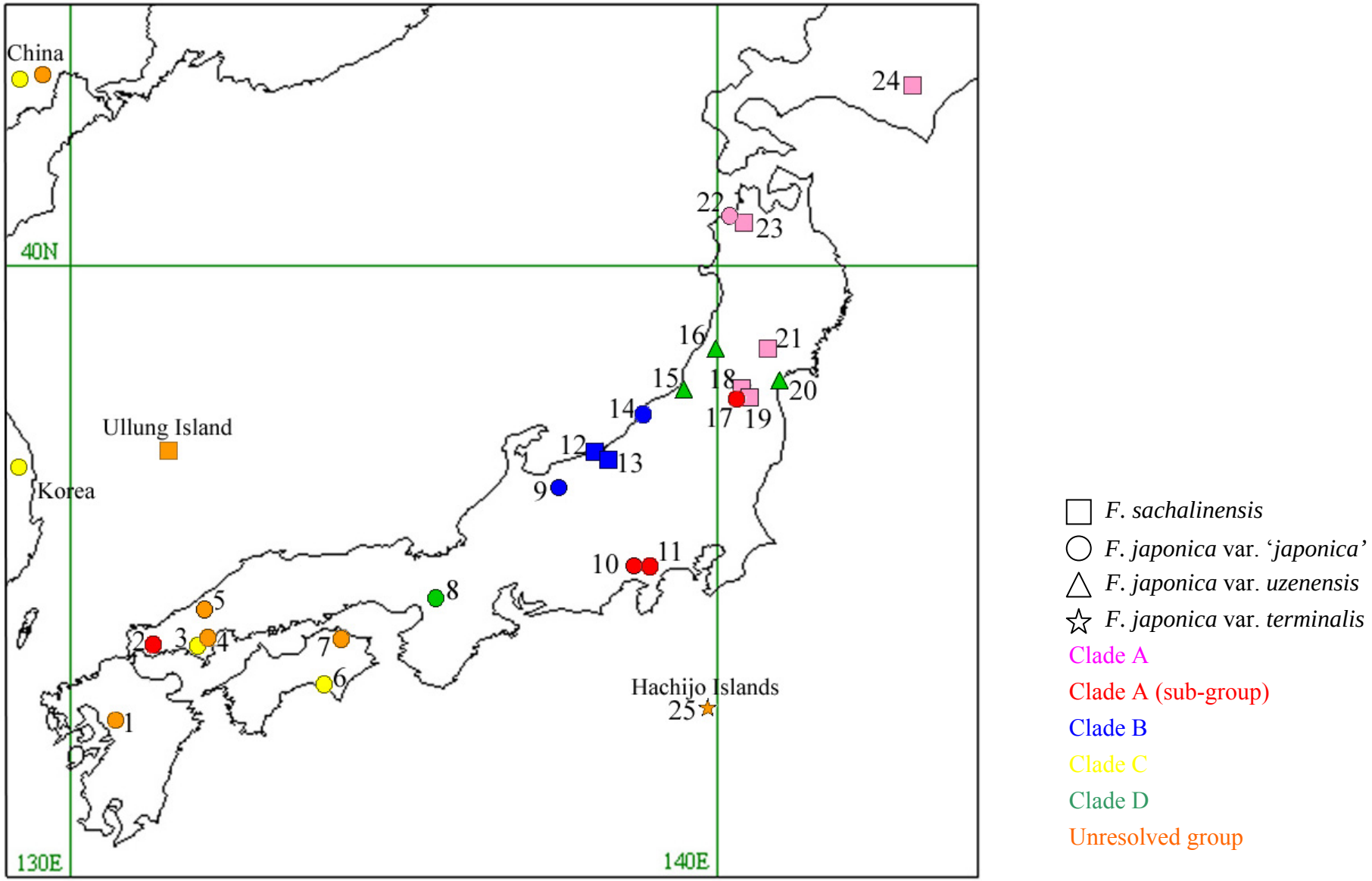



**Figure 7.14** Map depicting the sites from which each of the Japanese plants that formed the clades originated. Numbers relate to those shown in table 7.1.

**Table 7.1** Information relating to the Japanese plants selected for sequence analysis. Also included in the table is the chloroplast multi-primer-haplotype (MPH) and clade as determined by restriction in chapter 6.

| P no. | Taxon | Country | Prefecture | Location Figure 7.1 | Ploidy | Tall/ dwarf | Altitudinal vegetation zone | MPH (from chapter 6) | Clade |
|---|---|---|---|---|---|---|---|---|---|
| P987 | *F. japonica* var. '*japonica*' | Japan | Osaka | 8 | 8x | | Lowland | 1 | A |
| P1113 | *F. japonica* var. '*japonica*' | Japan | Hiroshima | 5 | 8x | Tall | Montane | 10 | D |
| P958 | *F. japonica* var. '*japonica*' | Japan | Aomori | 22 | 8x | | Lowland | 8 | B |
| P1074 | *F. japonica* var. *uzenensis* | Japan | Miyagi | 20 | 8x | Tall | Lowland | 1 | A |
| P1080 | *F. japonica* var. *uzenensis* | Japan | Yamagata | 16 | 8x | Tall | Lowland | 9 | A |
| P1082 | *F. japonica* var. *uzenensis* | Japan | Niigata | 15 | 8x | Tall | Lowland | 12 | A |
| P1093 | *F. japonica* var. '*japonica*' | Japan | Yamaguchi | 3 | 4x | Tall | Lowland | 15 | U |
| P981a | *F. japonica* var. '*japonica*' | Japan | Niigata | 14 | 4x | Tall | Lowland | 21 | C |
| P1090 | *F. japonica* var. '*japonica*' | Japan | Yamaguchi | 4 | 4x | Tall | Lowland | 10 | D |
| P1128 | *F. japonica* var. '*japonica*' | Japan | Kagawa | 7 | 4x | Tall | | 10 | D |
| P1136 | *F. japonica* var. '*japonica*' | Japan | Kochi | 6 | 4x | Tall | | 31 | U |
| P1096 | *F. japonica* var. '*japonica*' | Japan | Yamaguchi | 2 | 4x | Tall | Lowland | 3 | B |
| P907 | *F. japonica* var. '*japonica*' | Japan | Shizuoka | 11 | 4x | | Lowland | 4 | B |
| P992 | *F. japonica* var. '*japonica*' | Japan | Kumamoti | 1 | 4x | Dwarf | Montane | 10 | D |
| P901 | *F. japonica* var. '*japonica*' | Japan | Shizuoka | 10 | 4x | Dwarf | Subalpine | 3 | B |
| P1066 | *F. japonica* var. '*japonica*' | Japan | Yamagata | 17 | 4x | Dwarf | Subalpine-Montane | 4 | B |
| P726 | *F. japonica* var. '*japonica*' | Japan | Toyama | 9 | | Dwarf | Subalpine | 20 | C |
| P794a | *F. sachalinensis* | Japan | Niigata | 13 | | N/A | | 38 | C |
| P954 | *F. sachalinensis* | Japan | Aomori | 23 | 4X | N/A | Lowland | 2 | B |
| P1169 | *F. sachalinensis* | Japan | Memuro | 24 | | N/A | | 2 | B |
| P919 | *F. sachalinensis* | Japan | Miyagi | 21 | 4X | N/A | Lowland | 11 | B |
| P975 | *F. sachalinensis* | Japan | Niigata | 12 | 4X | N/A | Lowland | 20 | C |
| P1062 | *F. sachalinensis* | Japan | Yamagata | 18 | 4X | (Dwarf) | Subalpine-Montane | 37 | B |
| P1065 | *F. sachalinensis* | Japan | Yamagata | 19 | 4X | (Dwarf) | Subalpine-Montane | 11 | B |
| P134 | *F. japonica* var. *terminalis* | Japan | Hachijo Island | 25 | 4x | | | 39 | U |

**Table 7.2** British and Asiatic, but non-Japanese, plants included in the study. Also included in the table is the chloroplast multi-primer-haplotype (MPH) and clade as determined by restriction in chapter 6.

| **P no.** | **Taxon** | **Country** | Chromosome no. | **MPH (from chapter 6)** | **Clade** |
|---|---|---|---|---|---|
| P476 | *F. sachalinensis* | Korea (Ullung Island) | 2n = 102 | 29 | U |
| P555 | *F. elliptica* | Korea | 8x | 40 | U |
| P583 | *F. japonica* var. '*japonica*' | China | 10x | 23 | U |
| P113 | *F. japonica* var. '*japonica*' | China | 8x | 27 | U |
| P823 | *F. japonica* var. *japonica* | British | 8x | 1 | A |
| P002 | *F. japonica* var. *compacta* | British | 4x | 3 | B |
| P824 | *F. sachalinensis* | British | 4x | 2 | B |
| P852 | *F. sachalinensis* | British | | 38 | C |

**Table 7.3** Summary of the sequence data analysis for each of the twelve fragments analysed. A British sample of each of *F. japonica* var. *japonica*, *F. japonica* var. *compacta* and *F. sachalinensis* were used as the ingroup, and *F. multiflora*, *F. baldschuanica* and *F. convolvulus* were selected to form the outgroup

| Chloroplast region | Sequencing primer | No. of shortest trees | Shortest tree length | No. of characters analysed | No. of mutations between ingroup | Comments |
|---|---|---|---|---|---|---|
| *trn*K[1]-*trn*K[2] | *trn*K[1] | 2 | 31 | 552 | 5 | Ingroup taxa resolved. *F. multiflora* was no closer to the ingroup than it was the rest of outgroup. |
| | *trn*K[2] | 1 | 32 | 647 | 1 | No resolution of ingroup. *F. multiflora* was no closer to the ingroup than it was the rest of outgroup. |
| *trn*C-*trn*D | *trn*C | N/A | N/A | N/A | N/A | Ingroup aligned between themselves, and outgroup aligned between themselves, but unable to align ingroup with outgroup. |
| | *trn*D | 1 | 70 | 800 | 6 | Ingroup taxa resolved. *F. multiflora* took an intermediate position between ingroup and rest of outgroup. |
| *trn*F-*trn*V | *trn*F | 2 | 63 | 704 | 9 | Ingroup taxa resolved. *F. multiflora* took an intermediate position between ingroup and rest of outgroup. |
| | *trn*V | 1 | 52 | 718 | 8 | Ingroup taxa resolved *F. multiflora* took an intermediate position between ingroup and rest of outgroup. |
| *trn*H-*trn*K | *trn*H | 15 | 40 | 328 | N/A | Unable to separate the ingroup from *F. multiflora*. |
| | *trn*K | 1 | 30 | 614 | 4 | No resolution of ingroup. *F. multiflora* was no closer to the ingroup than it was the rest of outgroup. |
| *trn*D-*trn*T | *trn*D | 1 | 42 | 690 | 5 | Ingroup taxa resolved. *F. multiflora* took an intermediate position between ingroup and rest of outgroup. |
| | *trn*T | 1 | 68 | 667 | 11 | Ingroup taxa resolved. *F. multiflora* took an intermediate position between ingroup and rest of outgroup. |
| *trn*M-*rbc*L | *trn*M | 1 | 34 | 734 | 1 | No resolution. *F. multiflora* was no closer to the ingroup than it was the rest of outgroup. |
| | *rbc*L | 2 | 32 | 622 | 4 | Ingroup taxa resolved. *F. multiflora* was no closer to the ingroup than it was the rest of outgroup. |

**Table 7.4** Table showing mutations and their positions in relation to the consensus sequence shown in Figure 7.2 for fragment D (chloroplast region *trn*C-*trn*D sequenced from the *trn*D end), for the ingroup that comprises thirty-three accessions from *Fallopia* section *Reynoutria*, and the outgroup taxa *F. multiflora*, *F. baldschuanica*, and *F. convolvulus*.

| | 36 | 38 | 41 | 48 | 49 | 73 | 79 | 92 | 96 | 98 | 101-107 | 130 | 146 | 147 | 175 | 177-180 | 187 | 188 | 189-194 | 200 | 205 | 218 | 219 | 235 | 238 | 263 | 268 | 282 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | C | G | C | C | C | C | A | C | A | G | - - - - - - - | T | T | A | A | CAAA | T | A | - - - - - - | T | A | T | C | G | A | G | T | C |
| P002 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1096 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P901 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1065 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · |
| P1169 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P958 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P824 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P907 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P726 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · |
| P852 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · |
| P981a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CCAA | · | C | · | · | · | · | · | · | · | · | · | · | · |
| P975 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · |
| P794a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · |
| P134 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P476 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | G | · | · | · | · | · | · | · | · | · | · | · | · |
| P113 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P992 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1090 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1128 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1113 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1093 | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P583 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P555 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P823 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| *F. multiflora* | · | · | · | A | · | · | · | · | · | · | · | · | · | C | T | - - - - | · | · | · | · | · | C | · | · | C | · | G | · |
| *F. baldschuanica* | · | T | T | A | · | A | T | T | · | C | · | G | G | C | · | · - - | · | · | TATA*- - | · | G | C | T | · | · | · | · | T |
| *F. convolvulus* | T | · | · | A | T | · | T | T | C | C | CACTCAA | G | · | C | · | · | · | · | TATATA* | G | · | C | T | · | · | C | · | T |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data

**Table 7.4 cont.**

| | 310-312 | 340 | 343 | 344 | 348-379 | 353 | 359 | 367 | 372 | 390 | 400 | 401 | 402 | 403 | 422 | 431 | 446 | 500 | 501 | 529-531 | 540 | 541 | 542 | 546-551 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | TTC | G | T | A | | C | C | C | A | G | C | G | A | A | A | A | C | T | A | TTT | A | C | - | - - - - - - |
| P002 | · | · | · | · | 32 bp deletion | ? | ? | ? | ? | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P1096 | · | · | · | · | 32 bp deletion | ? | ? | ? | ? | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P901 | · | · | · | · | 32 bp deletion | ? | ? | ? | ? | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P1065 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P1169 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P958 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P824 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P907 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P726 | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | A | · | · | · | · | · | · |
| P852 | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | A | · | · | · | · | · | · |
| P981a | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · | · | · | · | · | · | · |
| P975 | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · | · | · | · | · | · | · |
| P794a | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · | · | · | · | · | · | · |
| P134 | · | · | · | · | · | · | · | · | · | · | · | A | · | T | · | · | T | · | T | · | · | · | · | · |
| P476 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P992 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1090 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1128 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1093 | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P583 | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P555 | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P823 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| *F. multiflora* | · | · | · | C | · | · | T | A | · | A | G | · | · | · | · | · | · | · | · | GAA | · | - | A | · |
| *F. baldschuanica* | · | A | G | · | · | T | · | · | · | A | T | T | · | · | · | · | · | · | · | GAA | · | - | A | ATATAT |
| *F. convolvulus* | GGA | A | · | · | 32 bp deletion | ? | ? | ? | ? | A | T | · | G | · | G | C | · | · | · | GAA | - | · | A | ATCTAT |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data

**Table 7.4 cont.**

| | 552-605 | | | | | 609 | 611 | 615 | 622/3 | 629 | 637-643 | 649 | 659-663 | 672 | 698/9 | 702 | 717 | 719-728 | 750 | *trn*C-*trn*D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 552-578 | | | 579-605 | | | | | | | | | | | | | | | | |
| | 559 | 560 | 567 | inversion | 582 | | | | | | | | | | | | | | | |
| Consensus | T | A | C | N/A | G | G | A | A | GG | A | - - - - - - - | T | - - - - - | G | AC | G | A | - - - - - - - - - - | A | haplotype |
| P002 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD1 |
| P1096 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD1 |
| P901 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD1 |
| P1065 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD2 |
| P1169 | N | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | N | · | · | CD3 |
| P958 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P824 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P907 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD4 |
| P726 | · | · | - | · | · | · | · | · | · | · | · | · | · | · | GT | · | · | · | · | CD5 |
| P852 | · | · | - | · | · | · | · | · | · | · | · | · | · | · | GT | · | · | · | · | CD5 |
| P981a | · | · | - | · | · | · | · | · | · | · | · | · | · | · | GT | · | · | · | · | CD6 |
| P975 | · | · | - | · | · | · | · | · | · | · | · | · | · | · | GT | · | · | · | · | CD7 |
| P794a | · | · | - | · | · | · | · | · | · | · | · | · | · | · | GT | · | · | · | · | CD7 |
| P134 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD8 |
| P476 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD9 |
| P113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD10 |
| P992 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD10 |
| P1090 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD10 |
| P1128 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD10 |
| P1113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | CD10 |
| P1093 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | · | · | C | · | T | · | · | · | · | · | CD11 |
| P583 | “.” | “.” | “.” | inversion | T | · | · | · | · | · | · | C | · | T | · | · | · | · | · | CD12 |
| P555 | “.” | “.” | “.” | inversion | T | · | · | · | · | · | · | C | · | T | · | · | · | · | · | CD12 |
| P1136 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | · | · | C | · | T | · | · | · | · | · | CD13 |
| P1082 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | C | · | C | · | · | · | · | · | CAAATTATAT | · | CD14 |
| P1080 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | C | · | C | · | · | · | · | · | CAAATTATAT | · | CD14 |
| P987 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | C | · | C | · | · | · | · | · | CAAATTATAT | · | CD14 |
| P823 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | C | · | C | · | · | · | · | · | CAAATTATAT | · | CD14 |
| P1074 | “.” | “.” | “.” | inversion | “.” | · | · | · | · | C | · | C | · | · | · | · | · | CAAATTATAT | · | CD14 |
| *F. multiflora* | · | C | · | · | · | T | T | · | · | · | TAGAAGA* | · | TATAT | · | · | · | · | · | G | X |
| *F. baldschuanica* | · | · | · | · | · | T | T | G | · | · | · | · | · | · | ? | ? | ? | ? | ? | Y |
| *F. convolvulus* | | | | | | T | T | G | TT | · | · | · | · | · | GT | T | · | · | · | Z |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data, N A/C/G/T

“.” Base present is the inversion of the consensus. There is a 27 base pair inversion. Bases 552 to 578 are the same orientation as the three taxa that comprise the outgroup, and is considered to be the consensus. Bases 579 to 605 represent the inverted sequence. Mutations are only shown that differ from the sequence once the inversion has been taken into account.

**Table 7.5** The indels matrix for fragment D (chloroplast region *trn*C-*trn*D sequenced from the *trn*D end). The base numbers indicated correspond to the position in the consensus sequence that represents the start of the indel.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Base number | 101 | 177 | 189 | 348 | 540 | 541 | 542 | 546 | 551 | 567 | 637 | 659 | 719 |
| P002 | 1 | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1096 | 1 | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P901 | 1 | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1065 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1169 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P958 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P824 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1062 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P919 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1066 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P907 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P954 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P726 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| P852 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| P981a | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| P975 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| P794a | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| P134 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P476 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P113 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P992 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1090 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1128 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1113 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| P1093 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| P583 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| P555 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| P1136 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| P1082 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| P1080 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| P987 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| P823 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| P1074 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| *F. multiflora* | 1 | 1 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| *F. baldschuanica* | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | ? |
| *F. convolvulus* | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |

? Data missing

**Table 7.6** Table showing mutations and their positions in relation to the consensus sequence shown in Figure 7.4 for fragment F (chloroplast region *trn*F-*trn*V sequenced from the *trn*F end), for the ingroup that comprises thirty-three accessions from *Fallopia* section *Reynoutria*, and the outgroup taxa *F. multiflora*, *F. baldschuanica*, and *F. convolvulus*.

| | 16 | 27 | 32 | 40-44 | 51 | 54-55 | 60-65 | 81 | 91-98 | 111 | 140 | 143 | 147 | 153 | 199-204 | 208 | 212 | 223 | 236 | 241 | 252/3 | 255-262 | 265 | 272 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | G | G | A | - - - - - | C | CC | - - - - - - | T | - - - - - - - - | A | G | G | T | - | - - - - - - | G | C | G | A | T | TC | AAAAAGGG | T | G |
| P958 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P824 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1062 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1065 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1169 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P919 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P954 | · | · | · | · | · | · | · | · | CTCTAGGT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P981a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P852 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P975 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P726 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P794a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P002 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1096 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P901 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P907 | · | · | · | · | · | · | CCGTGC* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P476 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P583 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P113 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P134 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P555 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1136 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1082 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | A | · | · | · | · | · | · | · | · |
| P1080 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P987 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P823 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1074 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P992 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1090 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1128 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1113 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1093 | · | · | C | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| *F. multiflora* | T | · | · | TAATC | · | - - | · | · | · | · | · | T | A | · | GTTTAT* | · | · | · | C | · | - G | - - - - - - - - | G | · |
| *F. baldschuanica* | T | A | · | TAATC | T | - - | · | A | · | · | · | .T | A | · | · | · | T | A | C | G | · | - - - - - - - - | · | · |
| *F. convolvulus* | T | A | · | TAATC | · | - - | · | · | · | C | A | .T | A | A* | -TTTAT* | · | T | A | C | G | · | - - - - - - - - | · | A |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data

**Table 7.6 cont.**

| | 277-285 | 286-290 | 298 | 309/310 | 314-316 | 327-332 | 342-349 | 360 | 375 | 385 | 406 | 410-417 | 421 | 425-427 | 429 | 435 | 463 | 471 | 473-477 | 504 | 510 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | - - - - - - - - - | GTAAA | C | AA | GAG | - - - - - - | TAGACAAA | G | G | T | A | CTTCATTT | C | - - - | C | G | A | T | - - - - - | A | T |
| P958 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P824 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P1062 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P1065 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P1169 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P919 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P954 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P981a | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P852 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P975 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P726 | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P794a | . | . | . | . | . | . | - - - - - - - - | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| P002 | . | . | . | . | . | . | - - - - - - - - | . | . | C | . | . | . | . | . | . | . | . | . | C | . |
| P1096 | . | . | . | . | . | . | - - - - - - - - | . | . | C | . | . | . | . | . | . | . | . | . | C | . |
| P901 | . | . | . | . | . | . | - - - - - - - - | . | . | C | . | . | . | . | . | . | . | . | . | C | . |
| P1066 | . | . | . | . | . | . | - - - - - - - - | . | . | C | . | . | . | . | . | . | . | C | . | C | . |
| P907 | . | . | . | . | . | . | - - - - - - - - | . | . | C | . | . | . | . | . | . | . | . | . | C | . |
| P476 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P583 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P113 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P134 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P555 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P1136 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P1082 | . | . | . | . | . | . | . | . | . | . | . | . | . | C* - - | . | . | . | . | . | . | . |
| P1080 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | . | . | . | . | . | . | . |
| P987 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | . | . | . | . | . | . | . |
| P823 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | . | . | . | . | . | . | . |
| P1074 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | . | . | . | . | . | . | . |
| P992 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | T | . | . | . | . | . | . |
| P1090 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | T | . | . | . | . | . | . |
| P1128 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | T | . | . | . | . | . | . |
| P1113 | . | . | . | . | . | . | . | . | . | . | . | . | . | CC* - | T | . | . | . | . | . | . |
| P1093 | . | . | . | . | . | . | . | . | . | . | . | . | . | CCC* | . | A | . | . | . | . | . |
| *F. multiflora* | . | - - - - - | T | . | . | AGTAAA* | . | . | . | . | C | . | . | . | . | . | G | . | . | . | G |
| *F. baldschuanica* | . | - - - - - | . | GG | . | . | . | A | T | . | . | - - - - - - - - | T | . | . | . | G | C | ATGCT* | . | . |
| *F. convolvulus* | TTTAATTAG | . | . | GG | - - - | . | . | A | . | . | . | - - - - - - - - | T | . | . | . | G | C | ATCCT | . | . |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data

**Table 7.6 cont.**

| | 518 | 525 | 528 | 536 | 543 | 547 | 549 | 553 | 575 | 583-585 | 607 | 621 | 624 | 643 | 654 | 669-678 | *trn*F-*trn*V |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | T | G | T | C | C | T | G | C | - | AA*- | G | T | A | A | C | - - - - - - - - - - | haplotype |
| P958 | · | · | · | · | · | · | · | · | · | AAA* | · | · | · | · | · | · | FV1 |
| P824 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV2 |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV2 |
| P1065 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV2 |
| P1169 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV2 |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV2 |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV2 |
| P981a | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV3 |
| P852 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV3 |
| P975 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV3 |
| P726 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV3 |
| P794a | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV3 |
| P002 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV4 |
| P1096 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV4 |
| P901 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV4 |
| P1066 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV5 |
| P907 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV6 |
| P476 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV7 |
| P583 | · | · | · | · | · | · | · | · | · | A* - - | · | · | · | · | · | · | FV7 |
| P113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV8 |
| P134 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV8 |
| P555 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV8 |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV8 |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | AATATATATT* | FV9 |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | AATATATATT* | FV10 |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | AATATATATT* | FV10 |
| P823 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | AATATATATT* | FV10 |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | AATATATATT* | FV10 |
| P992 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | FV11 |
| P1090 | · | · | · | · | · | · | · | · | · | AAA* | · | · | · | · | · | · | FV12 |
| P1128 | · | · | · | · | · | · | · | · | · | AAA* | · | · | · | · | · | · | FV12 |
| P1113 | · | · | · | · | · | · | · | · | · | AAA* | · | · | · | · | · | · | FV12 |
| P1093 | · | · | · | · | · | · | · | · | · | AAA* | · | · | · | · | · | · | FV13 |
| *F. multiflora* | · | A | C | · | · | · | · | T | · | - - - | · | C | · | · | ? | ? | X |
| *F. baldschuanica* | C | · | C | A | · | A | T | T | · | A* - - | A | · | · | · | ? | ? | Y |
| *F. convolvulus* | · | · | C | · | T | A | T | T | C | A* - - | A | · | C | C | ? | ? | Z |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data

**Table 7.7** The indels matrix for fragment F (chloroplast region *trn*F-*trn*V sequenced from the *trn*F end). The base numbers indicated correspond to the position in the consensus sequence that represents the start of the indel.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Base number | 40 | 54 | 60 | 91 | 153 | 199 | 252 | 255 | 277 | 314 | 327 | 341 | 410 | 425 | 473 | 575 | 583 | 669 |
| P958 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 0 | 1 |
| P824 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| P1062 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| P1065 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| P1169 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| P919 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| P954 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| P981a | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P852 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P975 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P726 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P794a | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P1066 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P002 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P1096 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P901 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P907 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 | 1 | 1 | 2 | 1 |
| P113 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 1 |
| P134 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 1 |
| P555 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 1 |
| P1136 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 1 |
| P476 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 2 | 1 |
| P583 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 2 | 1 |
| P1080 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 0 |
| P1082 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 1 | 1 | 0 |
| P987 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| P823 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| P1074 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| P992 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| P1090 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| P1128 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| P1113 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 |
| P1093 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |
| *F. multiflora* | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 1 | 1 | 3 | ? |
| *F. baldschuanica* | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 3 | 0 | 1 | 2 | ? |
| *F. convolvulus* | 0 | 1 | 1 | 1 | 0 | 2 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 3 | 0 | 0 | 2 | ? |

? Data missing

**Table 7.8** Table showing mutations and their positions in relation to the consensus sequence shown in Figure 7.6 for fragment T (chloroplast region *trn*D-*trn*T sequenced from the *trn*T end), for the ingroup that comprises thirty-three accessions from *Fallopia* section *Reynoutria*, and the outgroup taxa *F. multiflora*, *F. baldschuanica*, and *F. convolvulus*.

| | 5 | 7 | 13 | 20-24 | 25-32 | 34 | 35 | 49 | 53-62 | 68 | 74-78 | 83 | 84-102 | 116 | 120 | 121 | 130 | 133 | 138 | 139 | 140 | 143 | 152 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | A | C | A | - - - - - | TATTATAT | T | A | G | - - - - - - - - - - | A | - - - - - | G | - - - - - - - - - - - - - - - - - - | G | G | T | C | G | A | T | A | G | C |
| P002 | · | · | · | · | · | · | · | N | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · |
| P824 | · | · | · | · | · | · | · | N | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · |
| P1065 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P958 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1169 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1096 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P901 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P907 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P852 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P981a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P975 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P726 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P794a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P134 | · | T | G | · | · | G | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P555 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P583 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1093 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P476 | · | · | · | · | · | · | · | · | · | · | TATAA* | · | · | · | · | · | · | · | · | · | · | · | · |
| P992 | · | · | · | · | · | · | · | · | · | · | TATAA* | · | · | · | · | · | · | · | · | · | · | · | · |
| P1090 | · | · | · | · | · | · | · | · | · | · | TATAA* | · | · | · | · | · | · | · | · | · | · | · | · |
| P113 | · | · | · | · | · | · | · | · | · | · | TATAA* | · | · | · | · | · | · | · | · | · | · | · | · |
| P1113 | · | · | · | · | · | · | · | · | · | · | TATAA* | · | · | · | · | · | · | · | · | · | · | · | · |
| P1128 | · | · | · | · | · | · | · | · | · | · | TATAA* | · | · | · | · | · | · | · | · | G | · | · | · |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | ACTATACTATATAATAAAG* | · | · | · | · | · | · | · | · | · | · |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | ACTATACTATATAATAAAG* | · | · | · | · | · | · | · | · | · | · |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | ACTATACTATATAATAAAG* | · | · | · | · | · | · | · | · | · | · |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | ACTATACTATATAATAAAG* | · | · | · | · | · | · | · | · | · | · |
| P823 | · | · | · | · | · | · | · | · | · | · | · | · | ACTATACTATATAATAAAG* | · | · | · | · | · | · | · | · | · | · |
| *F. multiflora* | C | · | · | · | - - - - - - - - | · | · | · | ATACTATATA | · | · | · | · | · | N | · | · | T | · | · | C | · | · |
| *F. baldschuanica* | C | · | · | · | - - - - - - - - | G | G | · | · | C | · | · | · | T | · | C | A | · | C | · | · | · | T |
| *F. convolvulus* | C | · | · | ATTAC* | - - - - - - - - | · | · | · | · | C | · | · | · | T | · | C | A | · | C | · | · | N | T |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data, N A/C/G/T

**Table 7.8 cont.**

| | 155-162 | 165 | 180 | 181 | 186 | 237-239 | 247 | 254-265 | 268 | 276 | 286 | 292 | 305 | 321 | 327 | 329 | 332-343 | 355-361 | 370 | 376 | 393 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | - - - - - - - - | G | C | G | A | TT* - | T | - - - - - - - - - - - - | C | G | C | A | C | T | T | T | - - - - - - - - - - - - | - - - - - - - | G | C | C |
| P002 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P824 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P1065 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P958 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P1169 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P1096 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P901 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P907 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T | · |
| P852 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | N | Y | · |
| P981a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P975 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P726 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P794a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P134 | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · |
| P555 | · | · | · | · | · | · | · | · | · | · | Y | · | · | · | · | · | · | · | · | · | · |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P583 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1093 | · | A | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P476 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P992 | · | · | · | · | · | TTT* | · | · | · | R | · | · | · | · | · | · | · | · | · | · | · |
| P1090 | · | · | · | · | · | TTT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P113 | · | · | · | · | · | TTT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1113 | · | · | · | · | · | TTT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1128 | · | · | · | · | · | TTT* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T |
| P823 | · | · | · | · | · | · | · | · | · | · | · | R | · | · | · | · | · | · | · | · | T |
| *F. multiflora* | · | · | · | · | · | TTT* | · | · | · | · | · | · | · | G | · | G | · | · | · | · | · |
| *F. baldschuanica* | · | · | G | · | T | T* - - | A | TTTATTAAATTG* | · | · | · | · | T | C | · | · | CCCAAGATATGT | · | · | · | · |
| *F. convolvulus* | ATTTATTG* | · | · | A | T | - - - | A | TTTATTAAATTG* | · | · | · | · | · | C | N | · | TCCAAGATATTT* | AAATCAT* | · | · | · |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data, N A/C/G/T, Y C/T, R A/G

**Table 7.8 cont.**

| | 394-399 | 403 | 409 | 417 | 419-422 | 432 | 452 | 454-461 | 462 | 463 | 469 | 475 | 476 | 477 | 490 | 491 | 492 | 494 | 496-505 | 499 | 508 | 512 | 521 | 544 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | - - - - - - | A | G | G | AATT | C | C | - - - - - - - - | C | C | A | T | C | T | A | G | A | G | ATTTCGGCTT | T | A | A | T | T |
| P002 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | G | · | · | · | · |
| P824 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P1065 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P958 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P1062 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P1169 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P919 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P954 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | · | · | · | · | · |
| P1066 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | G | · | · | · | · |
| P1096 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | G | · | · | · | · |
| P901 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | G | · | · | · | · |
| P907 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | C | · | · | · | · | G | · | · | · | · |
| P852 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P981a | · | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P975 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P726 | · | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P794a | · | · | · | · | · | · | · | · | · | T | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P134 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P555 | · | · | · | · | · | · | · | · | Y | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P583 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1093 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P476 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P992 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | R | · | · | · | · | · | · | · | · |
| P1090 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | M | · | · | · | · | · | · |
| P1113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | M | · | · | · | · | · | C |
| P1128 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · |
| P823 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | R | · | · | · | · | R | · | · |
| *F. multiflora* | · | · | · | A | · | · | · | · | · | · | C | · | A | · | · | · | · | · | · | · | · | · | · | · |
| *F. baldschuanica* | TTAATC* | C | T | · | - - - - | | T | · | · | · | · | · | · | A | · | · | · | · | · | · | · | - | G | · |
| *F. convolvulus* | · | C | · | · | - - - - | T | A | GGATTTAT* | · | · | · | G | · | A | · | · | · | · | - - - - - - - - - - | ? | · | - | G | · |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data, N A/C/G/T, Y C/T, R A/G, M A/C

**Table 7.8 cont.**

| | 554 | 555 | 562-569 | 572 | 575 | 577-579 | 580-587 | 588/9 | 593-597 | 600 | 605 | 609-616 | 617 | 627 | 629 | 640 | 641 | 643-648 | 646 | 652 | 658 | *trn*D-*trn*T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | T | A | - - - - - - - - | T | T | ATA | - - - - - - - - | GT | - - - - - | A | T | - - - - - - - - | G | T* | A | A | G | TTTAGA* | A | A | C | haplotype |
| P002 | · | · | · | · | · | · | · | · | · | · | · | GTAATCGA | · | · | C | · | · | · | · | · | · | DT1 |
| P824 | · | · | · | · | · | · | · | · | · | · | · | · | C | - | C | · | · | · | · | · | · | DT2 |
| P1065 | · | · | · | · | G | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | DT3 |
| P958 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | DT4 |
| P1062 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | DT4 |
| P1169 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | DT4 |
| P919 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | DT4 |
| P954 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | · | DT4 |
| P1066 | · | · | · | · | · | · | · | · | · | · | · | GTAATCGA | · | · | C | · | · | · | · | · | · | DT5 |
| P1096 | · | · | · | · | · | · | · | · | · | · | · | GTAATCGA | · | · | C | · | · | · | · | · | · | DT5 |
| P901 | · | · | · | · | · | · | · | · | · | · | · | GTAATCGA | · | · | C | · | · | · | · | · | · | DT5 |
| P907 | · | · | · | · | · | · | TTATAATA* | · | · | · | · | GTAATCGA | · | · | C | · | · | · | · | · | · | DT6 |
| P852 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT7 |
| P981a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT8 |
| P975 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT8 |
| P726 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT8 |
| P794a | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT8 |
| P134 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT9 |
| P555 | G | · | · | · | · | · | · | · | GGATT | · | N | · | · | · | · | · | · | · | · | · | · | DT10 |
| P1136 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT11 |
| P583 | G | · | · | · | G | · | · | · | · | · | · | · | · | · | · | · | · | - - - - - - | · | · | · | DT12 |
| P1093 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT13 |
| P476 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT14 |
| P992 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT15 |
| P1090 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT16 |
| P113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT17 |
| P1113 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | N | DT18 |
| P1128 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT19 |
| P1080 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT20 |
| P1082 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT20 |
| P987 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT20 |
| P1074 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT20 |
| P823 | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | DT21 |
| *F. multiflora* | · | · | GATTTTTA | · | G | · | · | · | · | · | · | · | · | · | · | C | · | · | · | · | · | X |
| *F. baldschuanica* | · | G | · | C | · | - - - | · | - - | · | · | · | · | · | · | · | · | · | - - - - - - | · | C | · | Y |
| *F. convolvulus* | · | G | · | · | · | - - - | · | - - | · | T | · | · | · | · | · | · | A | · | C | C | · | Z |

. Matches the consensus sequence, * DNA repeat /microsatellite, - DNA missing, ? Missing data, N A/C/G/T, R A/G

**Table 7.9** The indels matrix for fragment T (chloroplast region *trn*D-*trn*T sequenced from the *trn*T end). The base numbers indicated correspond to the position in the consensus sequence that represents the start of the indel.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Base number | 20 | 25 | 53 | 74 | 84 | 155 | 237 | 254 | 332 | 355 | 394 | 419 | 454 | 497 | 562 | 580 | 593 | 609 | 627 | 643 |
| P002 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| P824 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| P1065 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P958 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| 1062 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1169 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P919 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P954 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1066 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| P1096 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| P901 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| P907 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| P852 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P981a | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P975 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P726 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P794a | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P134 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P555 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| P1136 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P583 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 |
| P1093 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P476 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P992 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1090 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P113 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1113 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1128 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1080 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1082 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P987 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P1074 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| P823 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| *F. multiflora* | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| *F. baldschuanica* | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 2 | 1 | 1 | 0 | 1 |
| *F. convolvulus* | 0 | 1 | 1 | 1 | 1 | 0 | 3 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 1 | 0 | 0 |

**Table 7.10** Summary of the haplotypes found when sequencing the three fragments, and the combined sequence multi-primer-haplotype (sMPH). Included for comparative purposes are the multi-primer-haplotypes (MPHs) and clades as determined by RFLP analysis in chapter 6.

| | trnC-trnD | trnF-trnV | trnD-trnT | sMPH | Clade | MPH (from chapter 6) | Clade |
|---|---|---|---|---|---|---|---|
| P002 | CD1 | FV4 | DT1 | s1 | A | 3 | B |
| P1096 | CD1 | FV4 | DT5* | s2 | A | 3 | B |
| P901 | CD1 | FV4 | DT5* | s2 | A | 3 | B |
| P1065 | CD2 | FV2* | DT3 | s3 | A | 11 | B |
| P1169 | CD3 | FV2* | DT4* | s4 | A | 2 | B |
| P824 | CD4 | FV2* | DT2 | s5 | A | 2 | B |
| P958 | CD4 | FV1 | DT4* | s6 | A | 8 | B |
| P1062 | CD4 | FV2* | DT4* | s7 | A | 37 | B |
| P919 | CD4 | FV2* | DT4* | s7 | A | 11 | B |
| P954 | CD4 | FV2* | DT4* | s7 | A | 2 | B |
| P1066 | CD4 | FV5 | DT5* | s8 | A | 4 | B |
| P907 | CD4 | FV6 | DT6 | s9 | A | 4 | B |
| P852 | CD5 | FV3 | DT7 | s10 | B | 38 | C |
| P726 | CD5 | FV3 | DT8 | s11 | B | 20 | C |
| P981a | CD6 | FV3 | DT8 | s12 | B | 21 | C |
| P794a | CD7 | FV3 | DT8 | s13 | B | 38 | C |
| P975 | CD7 | FV3 | DT8 | s13 | B | 20 | C |
| P134 | CD8 | FV8* | DT9 | s14 | U | 39 | U |
| P476 | CD9 | FV7* | DT14 | s15 | U | 29 | U |
| P992 | CD10 | FV11 | DT15 | s16 | U | 10 | D |
| P113 | CD10 | FV8* | DT16 | s17 | U | 27 | U |
| P1090 | CD10 | FV12 | DT17 | s18 | U | 10 | D |
| P1113 | CD10 | FV12 | DT18 | s19 | U | 10 | D |
| P1128 | CD10 | FV12 | DT19 | s20 | U | 10 | D |
| P1093 | CD11 | FV13 | DT13 | s21 | C | 15 | U |
| P555 | CD12 | FV8* | DT10 | s22 | C | 40 | U |
| P583 | CD12 | FV7* | DT12 | s23 | C | 23 | U |
| P1136 | CD13 | FV8* | DT11 | s24 | C | 31 | U |
| P1082 | CD14 | FV9 | DT20 | s25 | D | 12 | A |
| P1074 | CD14 | FV10 | DT20 | s26 | D | 1 | A |
| P1080 | CD14 | FV10 | DT20 | s26 | D | 9 | A |
| P987 | CD14 | FV10 | DT20 | s26 | D | 1 | A |
| P823 | CD14 | FV10 | DT21 | s27 | D | 1 | A |

* Haplotypes that appear in more than one area of the table.

**Table 7.11** Microsatellites found to cause variation within the sequence data set, and their positions within the respective fragments.

| Fragment | Type of microsatellite | Position within consensus sequence where variation occurred | Number of the corresponding indel |
|---|---|---|---|
| D | TA repeat | 189-194 | 3 |
| F | A repeat | 153 | 5 |
| | C repeat | 425-427 | 14 |
| | A repeat | 583-585 | 17 |
| T | T repeat | 237-239 | 7 |
| | T repeat | 617 | 19 |

# Chapter 8. Comparison of clonal diversity in British populations of the introduced Giant Knotweed, *Fallopia sachalinensis,* with a limited number of European and native Japanese plants.

## 8.1. Introduction

Clonal growth is a widespread phenomenon in the plant kingdom, which can also occur in lichens, fungi and some groups of lower animals. Plants can reproduce clonally in many ways, including the formation of ramets on above or belowground creeping stems, and the vegetative production of plantlets and bulbils on aerial plants (Stuefer *et al.*, 2001). In the case of Japanese Knotweed, clonal spread is achieved through large underground rhizomes, from which as little as 0.7g is required to give rise to a new plant (Brock & Wade, 1992). Well-established plants develop woody stocks, with a central taproot that penetrates vertically into the ground. Buds form on the stock and the woody rhizomes between autumn and winter and emerge to give vertical shoots the following spring. Woody stocks continue to increase in width by secondary thickening with age, and also produce lateral creeping rhizomes within their first year (Beerling *et al.*, 1994). The rhizomes of a mature plant can extend up to 7m away from the parent plant (Child & Wade, 2000).

*F. japonica* var. *japonica*, the most common of the plants that comprise Japanese Knotweed *s.l.* in Europe, was shown to be represented in Britain by a single male-sterile octoploid clone. This clone was also found in France, Germany, the Czech Republic and the U.S.A. (Hollingsworth & Bailey, 2000a). In contrast there appeared to be high levels of genetic variation in the related Giant Knotweed, *F. sachalinensis* (Hollingsworth, 1998; 2000b).

*F. sachalinensis*, in comparison with *F. japonica* var. *japonica*, has not spread to anything like the same extent. Its occurrence on distribution maps reflects mainly the incidence of many independent primary escapes (Conolly, 1977). *F. sachalinensis* may not be as invasive as *F. japonica* var. *japonica*, but it can be a significant pollen source for the male-sterile *F. japonica* var. *japonica*, producing the hybrid *F.* x *bohemica*. *F.* x *bohemica* is gaining in notoriety, and is thought to be at least as invasive as *F. japonica* var. *japonica* (Bímová *et al.*, 2001; Brabec & Pyšek, 2000). Given the lack of diversity in *F. japonica* var. *japonica*, and the rarity of *F. japonica* var. *compacta,* the alternative *F. japonica* parent for *F.* x *bohemica*, the genetic composition of *F. sachalinensis* is an important factor for potentially introducing novel variation into *F.* x *bohemica*.

Molecular studies of British sites where *F. sachalinensis*, *F. japonica* var. *japonica* and *F.* x *bohemica* were sympatric revealed a common genotype of male-fertile *F. sachalinensis*. These sites were geographically diverse, being found in: Merioneth, North Wales (chapter 4); Cirencester in the middle of England; Leeds in the North of England (chapter 5); and Hempstead, to the East of England (Pashley, unpublished). This is in stark contrast to the studies of Hollingsworth (1998) and Hollingsworth & Bailey (2000b) that found no shared *F. sachalinensis* genotypes between twelve different sites.

Colonization events may involve a bottleneck because the number of plants introduced is often small. Genetic drift during colonization may also reduce genetic variation in the newly established population, thus a newly established population is likely to be much less genetically diverse than the population from which it is derived (Sakai *et al.*, 2001). When an introduced ornamental plant can be easily propagated by vegetative means, such as is the case with Japanese Knotweed *s.l.*, the likelihood is that very little material is introduced at any one time.

*F. sachalinensis* was first recorded in the wild in Germany in 1869, in the Czech republic in 1869, and in Great Britain in 1896 (Sukopp & Starfinger, 1995). As detailed further in chapter 2, it would appear that there were at least two routes by which *F. sachalinensis* may have arrived in Europe. The first is via St. Petersbourg, and the second route is via Royal Botanic Gardens Kew (Bailey & Conolly, 2000). This could mean that there have been multiple introductions of *F. sachalinensis* into Britain, as was suggested by the presence of two different chloroplast types in British *F. sachalinensis* accessions (Chapters 6 and 7). The presence of both male-fertile and male-sterile individuals of both chloroplast haplotypes would indicate that at a minimum there must be four genotypes. The existence of these two routes may also result in variation between *F. sachalinensis* plants found in Britain, when compared to plants found in mainland Europe.

### 8.2 Aims

To estimate levels of genetic variation among British *F. sachalinensis*, to determine whether sexual reproduction or clonal spread best explains the current distribution. To compare the British plants to a limited number of plants from other countries where *F. sachalinensis* has been introduced, namely Germany, the Czech Republic, and the USA, and to native plants from Japan.

## 8.3 Materials and Methods

### 8.3.1. *Plant material*

The thirty-one *F. sachalinensis* accessions, representing 21 populations from Britain, are listed in Table 8.1; and their distribution is mapped in Figure 8.1. A further nine samples from the introduced range, five from Germany, two from the Czech republic and two from the USA, were included in the study. One of the German samples was supplied under the name *Reynoutria* x *vivax*, a synonym for *F. sachalinensis.*

Sixteen native Japanese *F. sachalinensis* were also analysed. These were chosen to represent both the geographical distribution of the plants, and the variation in chloroplast haplotypes as determined by PCR-RFLP analysis in chapter 6. The chloroplast haplotype for each of the Japanese plants is given in parentheses after the P number. Figure 8.2 shows the locations of the Japanese accessions.

### 8.3.2. *Methods*

Duplicate DNA extractions were made for each plant, as detailed in chapter 3 materials and methods. ISSR PCR amplification using the seven primers listed in Table 3.2 was carried out, and additionally the chloroplast regions *trn*C-*trn*D and *trn*F-*trn*V were amplified for each plant, and restricted with the enzyme *Hinf*I, as per chapter 3.

In chapter 6, variation in six different regions of the chloroplast was analysed. The combination of just two of these regions, *trn*C-*trn*D and *trn*F-*trn*V, was sufficient for distinguishing between the chloroplast haplotypes of the Japanese accessions that have been included in this study.

## 8.4 Data analysis

The chloroplast haplotypes were analysed and defined as per chapter 6. The results obtained from Chapter 6 showed that two fragments combined, *trn*C-*trn*D and *trn*F-*trn*V, were sufficient to detect the same multi-primer-haplotypes for *F. sachalinensis* as obtained from all six regions, therefore only those two fragments were used to determine the MPHs of the other plants used in this study.

As with previous studies, ISSR bands were selected, scored and named as per Chapter 3, section 3.2.7.2.

The ISSR data were bootstrapped 1,000 times, and a Neighbour-Joining tree using the Jaccard's similarity coefficient was produced, as per chapter 4.

The number of differences between each genotype was calculated and analysed using MINSPNET (Excoffier & Smouse, 1994) to produce a minimum spanning tree.

## 8.5 Results

### 8.5.1 *Chloroplast haplotypes*

As seen from Table 8.2, with the exception of the three plants already determined as having MPH 38, the rest of the plants from Britain, Germany (including *R*. x *vivax*), the Czech Republic and the USA were all found to have MPH 2.

### 8.5.2 *ISSR genotypes*

Each ISSR primer generated a banding pattern referred to as a phenotype. From five to thirteen bands were scored per primer, producing between five and twenty-eight phenotypes depending upon the primer used. The bands and phenotypes are listed in Table 8.3. A total of fifty-nine bands were scored, of which 52 were polymorphic. An example of the banding patterns produced in *F. sachalinensis* with one of the ISSR primers, primer 840 from Table 3.2, is shown in Figure 8.3.

A unique combination of these phenotypes is assumed to be representative of a genotype. A total of 32 genotypes were detected. Sixteen genotypes were found in plants from the introduced range; these have been given the letter I followed by a unique number. The remaining sixteen genotypes were from native Japanese plants; these have been given the letter N followed by a number. Table 8.4 lists the genotypes, what plants were of each genotype, the sex of the plants and the corresponding MPH.

### 8.5.3 *Distribution of genotypes*

Sixteen native *F. sachalinensis* plants were analysed. As seen in Figure 8.4, each plant possessed a unique genotype, indicating a high level of genetic diversity among native plants. Plants from the same geographical area were already known to share the same chloroplast haplotype (chapter 6), however none from the same area shared a genotype, and whilst some plants clustered with plants from the same population on the ISSR-PCR generated network shown in Figure 8.4, this was not true for all of them.

In contrast to this, in Britain only eight genotypes were found from the thirty-one plants analysed. The location where each genotype was found has been plotted in figure 8.5. As can be seen there were two widespread clones, I1 shared by eleven accessions and I4 by thirteen. From Table 8.4 these can be seen to represent a male-fertile and a male-sterile clone respectively, both with MPH 2. The remainder of the genotypes were found at single localities, with a maximum of two plants sharing the genotype. The three accessions with MPH 38 were found to have individual genotypes. The other three genotypes with MPH 2 were represented by; a male-fertile plant from South Hampshire (I13), two male-sterile stands from Warwick (I6), and a single stand from Merioneth (I2).

There were nine *F. sachalinensis* accessions analysed from the introduced range that were not from Britain. The two plants from the U.S.A had unique genotypes (I7 and I12). They represented a male-sterile plant from the state of Washington, and a male-fertile plant from the state of Seattle. Five plants came from Germany, two male-fertile, one male-sterile and two of unknown sex. Three of the plants were from the same area of Germany, Aachen, but all five were found to have unique genotypes (I15, I14, I9, I3 and I10). Two plants were analysed from the Czech Republic, both male-sterile. Whilst one had a unique genotype, I5, the other shared the male-sterile genotype, I4, that was common in Britain.

### 8.5.4 *Neighbour joining tree*

The Neighbour joining tree for all genotypes based on the Jaccard's similarity coefficient, is shown in Figure 8.6. The internal branches are short and the majority of the distances are confined to the terminal branches. Bootstrapping 1,000 times has shown that only four of the branches hold together over 50% of the time. In each case the support was for a group of two genotypes. There was support of 68.0% for the two genotypes with MPH 4, native plants from Fukushima (N10 and N11), and a bootstrap value of 91.5% for the two native genotypes with MPH 38, which came from Niigata. The other support was for introduced plants. The genotypes I8 and I11 had a bootstrap value of 76.9% and consisted of two of the three British plants with MPH 38, the male-sterile plant from Colchester and a plant of unknown sex from Glasgow. The other introduced genotypes that were grouped together were I1 and I2 and these were supported with a value of 99.2%. These genotypes represented the common male-fertile genotype (I1) and an individual stand from Merioneth.

The majority of the introduced genotypes, twelve of the sixteen, were all found to diverge from a single branch with no native genotypes forming part of the group. In contrast the native genotypes appeared to arise from four main branches, with two of the branches closer

to the branch with the majority of the introduced genotypes than the other two. One of the four branches was further divided into two clear groups, one comprising N13 and N2, and the other made up of N7, N8, N1 and N6. The three introduced genotypes from plants with MPH 38 were found intermingled with native genotypes, two of them grouping with the Japanese plants with MPH 38 as well as a genotype I9, that came from a plant with MPH 2. The other genotype that was found mixed in with the native genotypes was the British male-fertile genotype with MPH 38.

### 8.5.5 *Minimum spanning network*

The minimum-spanning network, Figure 8.7, is based on the number of band differences between each pair of genotypes. Next to the genotype is a symbol to represent whether the genotype is male-fertile or male-sterile. Unlike the study of Hollingsworth (1998) there was no obvious separation between male-fertile and male-sterile genotypes. The network is almost linear in shape, with only seven equally likely alternative links throughout the whole network. The genotypes found in the introduced plants occupy either end of the network, with six native genotypes found between the nearest of the introduced genotypes at either end. These six genotypes represent 65 band differences between I16 and I13, which were the closest of the genotypes from the two ends. The two common British genotypes (I1 and I4) were found next to each other to the left side of the network as portrayed in Figure 8.7, and were found to have only ten band differences. The group of genotypes to the left of the network were the same twelve genotypes that came from the single branch in the neighbour joining analysis. The minimum spanning network showed a closer relationship between the four introduced genotypes that were not grouped with the other introduced genotypes than did the previous analysis. According to the minimum spanning network they were all interconnected by genotype I11, to the right of the network.

## 8.6 Discussion

### 8.6.1 *Comparison between this study and those of Hollingsworth (1998) and Hollingsworth & Bailey (2000b)*

Hollingsworth (1998) examined fourteen *F. sachalinensis* accessions from twelve localities in Britain using RAPD analysis. All samples showed different genotypes, which when analysed formed two clusters, one consisting of plants from Scotland, the North of England and Wales, the other plants from England and Wales. There was some correlation between sex and genetic relationship. In Hollingsworth & Bailey (2000b) the same study was summarised, but with an additional 15 plants from the Glasgow location, and one extra from Merioneth. No new variation was detected in the additional plants.

This study examined thirty-one *F. sachalinensis* accessions from twenty-one localities in Britain using ISSR analysis. Only eight genotypes were detected. The majority of British plants were found to be one of two clones, either a widespread male-fertile clone, or a widespread male-sterile clone. At Glasgow a second genotype was detected, from a plant with the less common chloroplast haplotype MPH 38. This is consistent with the two genotypes detected by Hollingsworth & Bailey from Glasgow, one being found in only a single stand (Hollingsworth & Bailey, 2000b). In Merioneth a second genotype was detected, again consistent with Hollingsworth & Bailey (2000b). The remaining four genotypes were represented by one or two stands from single localities, none of which were examined by Hollingsworth (1998) or Hollingsworth & Bailey (2000b). There was no obvious correlation between sex and genetic relationship found in this study.

Different molecular markers were used to produce the data for the two studies, RAPD and ISSR analysis. Previous comparisons between the levels of variation detected in Japanese Knotweed *s.l.* using these markers have given consistent results e.g. levels of variation in *F.* x *bohemica* in Merioneth using RAPDs (Hollingsworth, 1998; Hollingsworth & Bailey, 2000b) and the same site using ISSRs (Pashley *et al.*, 2003) and chapter 4. Whilst it is theoretically possible that ISSRs are less sensitive to variation in *F. sachalinensis* than they are to variation in *F.* x *bohemica*, it is unlikely given the very high level of genetic diversity found in native *F. sachalinensis*, and the high levels in introduced non-British accessions, in this study.

Due to time constraints Hollingsworth (1998) was unable to perform repeat DNA extractions, and as stressed in the discussion to the study "care is required relying solely on repeated amplification as evidence of reliability of results. There is the possibility that the large number of genotypes detected is in part attributable to PCR artefacts". It was the recommendation of Hollingsworth (pers. comm.) that duplicate DNA extractions be used for random fingerprinting techniques such as RAPD and ISSR analysis, as was done for this study. Hollingsworth (1998) includes a photograph of one of the gels used in the RAPD *F. sachalinensis* study, with the corresponding presence/absence matrix. Whilst it is not clear exactly which bands were scored, it does appear that the majority of the variation was caused by the presence/absence of faint bands, which could well be PCR artefacts or the result of different intensities of bands. These dubious band differences could easily cause the correlation between sex and genetic relationship seen, as this study would predict that there was primarily two clones found in the study of Hollingsworth (1998), one of each sex, and only a small number of other genotypes.

The remainder of this discussion will deal only with the results of the current ISSR study.

### 8.6.2 *Distribution of genotypes*

#### 8.6.2.1 Native genotypes

Sixteen native *F. sachalinensis* plants were analysed each of which possessed a unique genotype. The genotypes appeared to arise from four main branches, with two of the branches closer to the branch with the majority of the introduced genotypes than the other two. As seen in Figure 8.4, the two branches closest to that of the majority of the introduced genotypes, was made up of one branch that grouped the genotypes N3 and N4, and another that grouped N14, N5 and N16, with N5 having the longest terminal branch. N4 and N16 were both from Hokkaido, N3 and N14 were both from Aomori, and N5 from Yamagata. Given the match in chloroplast type and relatively close link between the introduced genotypes and N4 and N16, it could imply that the majority of the introduced *F. sachalinensis* were more closely related to the samples from the East of Hokkaido than the west of Hokkaido or from Honshu, however this is a very speculative statement given that N3 and N14 from Aomori, Honshu were found to have a similar level of genetic relatedness to the introduced group, however they did not share the chloroplast haplotype.

Of the other two branches, one was further divided into two clear groups, one comprising N13 and N2, and the other made up of N7, N8, N1 and N6. N13 and N2 both had MPH 38, and it was from their combined branch that the introduced branch comprising the German sample with genotype I9 and MPH 2, and the British samples with genotypes I8 and I11, and MPH 38 arose (see Figures 8.5 and 8.6). N2 and N13 were from Niigata and it is highly likely that this is where the British samples with MPH 38 arose, but why the German genotype with I9 would cluster with these is less clear. The second group consisted of the three genotypes found in the population on the west side of Hokkaido and one of the genotypes from Miyagi.

The final branch consisted of a genotype from Yamagata (N15), one from Miyagi (N12) and two from Fukushima (N10 and N11), all of which are in the Hokuriku and Tōhoku district of Japan. There is one introduced genotype found within this cluster, I16, the male-fertile genotype from Colchester, England, with MPH 38. According to the minimum spanning network, this genotype occupies an intermediate position between the group comprising N10, N11, N12 and N15, and the group consisting of I8, I9, I11, N2 and N13, and is therefore not as different from the rest of the British samples with the same MPH as the neighbour-joining tree would suggest.

Whilst the native genotypes do not always cluster with those they share a chloroplast haplotype with, e.g. the two genotypes arising from plants with MPH 37 that occupy different branches in Figure 8.6, for the most part there does appear to be some correlation between genetic relationship and geographical distribution.

8.6.2.2 Introduced genotypes

The majority of the introduced genotypes, twelve of the sixteen, were all found to diverge from a single branch with no native genotypes forming part of the group. The other four genotypes have already been discussed and consist of the three British genotypes with MPH 38 and a male-sterile German plant with genotype I9 and MPH 2, P522.

Within the introduced group three subgroups were formed. The first consisted of two German genotypes and one from the USA (Figure 8.5). These equate to genotype I7, found in P834 from Washington, and genotypes I3 and I10, found in P527 and P532 respectively. The second subgroup consisted of a British sample with genotype I13 (P1210), a German genotype I15, and I12 that was found in P1176 from Seattle, USA. The third group consisted of the common British genotypes I1 and I4, the British genotypes I6 and I2, the Czech genotype that was not shared by any of the British samples, I5 found in P572, and the German sample with genotype I14, P521. Whether this implies a closer relationship between the German plants and those found in the USA is unclear, but it is clear that the majority of *F. sachalinensis* from Britain, Germany, the Czech Republic and the USA are very closely related, and were probably introduced from a similar area of Japan.

There appears to be a higher level of genetic variation in the other countries to which *F. sachalinensis* was introduced, in particular Germany, than was found in Britain. This could imply that the plants were introduced earlier to Germany than they were Britain, a theory that is complemented by the dates the plants first appeared in the wild. They were first recorded wild in both Germany and the Czech Republic in 1869 but not in Great Britain until 1896 (Sukopp & Starfinger, 1995).

The sharing of the male-sterile genotype between Britain and the Czech Republic implies the plants were introduced from the same collection, presumably from St. Petersbourg botanic garden. It is possible that three different collections have made it to St. Petersbourg: that of Dr H. Weyrich, from Sakhalin in September 1853; that of P. von Glehn in 1861, also from Sakhalin that arrived in St. Petersbourg in early 1863; and material of Japanese origin collected by C. J. Maximovicz during 1859-1864 (Bailey & Conolly, 2000). Whilst it is not

possible to determine whether the plants came from Sakhalin or Japan due to a lack of material from the Sakhalin island, data from this chapter, and chapter 6 and 7, would suggest that if the plants with MPH 2 did come from Japan it would have been from the northern island Hokkaido. This is in contrast to the results discussed in the previous section (8.6.2.1) that suggested that the rare British *F. sachalinensis* accessions with MPH 38 originated from Niigata. Given that there has been multiple introductions of *F. sachalinensis* into countries where it is not native, these findings do not contradict each other (Bailey & Conolly, 2000).

8.6.3 *Relationships between the introduced and native plants*.

The low level of resolution and support found with the neighbour joining analysis means conclusive statements about the relationships between the native Japanese and introduced genotypes of *F. sachalinensis* cannot be made. Even so, some observations can be made, and tentative suggestions towards how the current distribution arose can be proposed.

Whether the genotypes found in the introduced *F. sachalinensis* were present in the native range before the plants were collected, or have arisen by sexual reproduction since their introduction is unclear. It is likely that the situation has arisen from a combination of the two factors. A limited number of genotypes were probably introduced from Japan, most likely Hokkaido, or the Sakhalin Island into St. Petersbourg. In the light of the results in this chapter, it is more likely that a minimum of a male-fertile and a male-sterile clone were probably sent to Britain from St. Petersbourg as established plants rather than seed. These were then distributed throughout Britain to various nursery gardens, and from there to many stately homes and manors, eventually leading to the escapes reflected in the distribution noted by Conolly (1977). Given how easy *F. sachalinensis* is to propagate from the rhizome there would be little point in the gardeners germinating and growing seed for distribution. At the same time, or earlier, plants arising from the same original collection were probably being distributed to Germany, the Czech Republic and other European countries. *F. sachalinensis* was recommended, especially on the continent, as a forage plant for cattle, as well as being introduced as a riverbank stabiliser (Bailey & Conolly, 2000). As a riverbank stabiliser mature plants were most likely used, which would presumably originate from vegetative reproduction. As well as for a forage plant *F. sachalinensis* was also used on large estates for scenic plantings and cover for shoots (Bailey & Conolly, 2000). As with riverbank stabilising, the plants introduced for scenic plantings were probably introduced as mature plants. However, seed may have been used to produce the plants for forage, which could explain why there was found to be higher levels of genetic variation on the continent, but

these plants all cluster together away from the native possibly indicating that they were produced from the original introductions by sexual reproduction.

The three genotypes found in Britain in plants that have MPH 38 probably went straight from Niigata on Honshu, in Japan, to Britain. This explanation could explain why no plants with this chloroplast type have been found anywhere besides Niigata and Britain. The two sites in Britain where these were found were Glasgow and Colchester. The plants in Colchester were found growing on the site of an old nursery garden reported to have sold *F. sachalinensis* (Bailey & Conolly, 2000). Both a male-fertile and a male-sterile *F. sachalinensis* with MPH 38 were found in Colchester. The site in Glasgow is close to a botanic garden, and the suggestion is that the male-sterile plant there with MPH 38 may have been sent from Colchester.

### 8.7 Conclusions

The majority of British *F. sachalinensis* were found to be one of two genotypes, either a widespread male-fertile, or a widespread male-sterile clone. The remaining British genotypes that came from plants that share the chloroplast MPH 2 were presumed to have arisen from the same introduction, as were most of the plants in the other introduced countries included in the study. These were hypothesised as having been introduced via St. Petersbourg. Only one genotype was found shared between Britain and the any of the other countries to which *F. sachalinensis* was introduced. That genotype was the widespread male-sterile clone, and it was found in a single accession from The Czech Republic.

The three genotypes found in Britain in plants that have chloroplast MPH 38 probably went straight from Niigata on Honshu, in Japan, to Britain, and were not found in any other country to which *F. sachalinensis* was introduced that have been included in this study.

A high level of genetic diversity was found among native *F. sachalinensis*, with each of the sixteen plants analysed having a unique genotype. There was some correlation between genetic relationship and the geographical distribution of these native plants.

There is clearly a higher level of genetic diversity in *F. sachalinensis* than there is in *F. japonica* var. *japonica*. However, there is strong evidence that a lot of the current British distribution has resulted from the spread of two clones rather than via sexual reproduction.

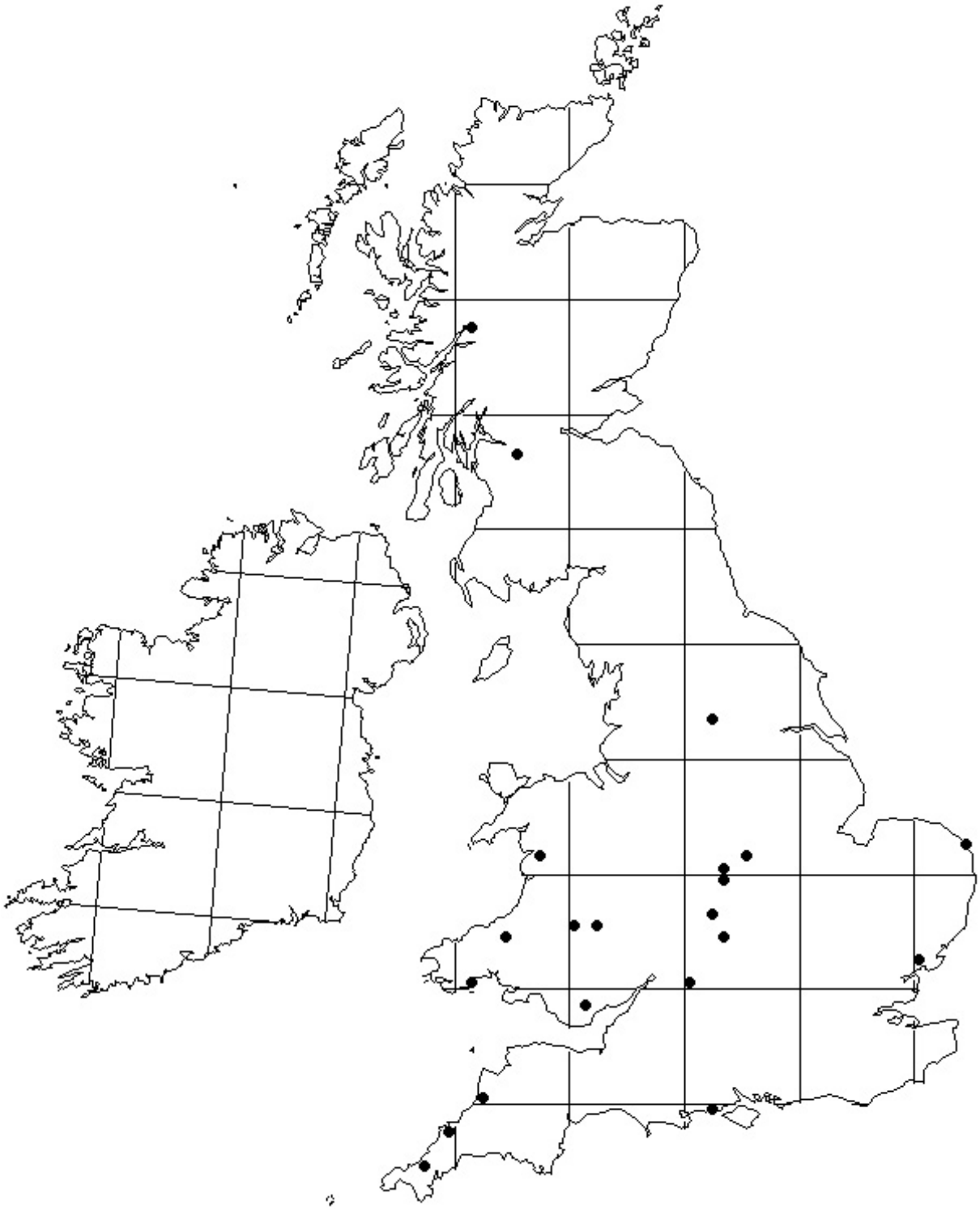


**Figure 8.1** Sample localities of British *F. sachalinensis*. The map was produced using Dr. A. J. Morton's DMAP program.

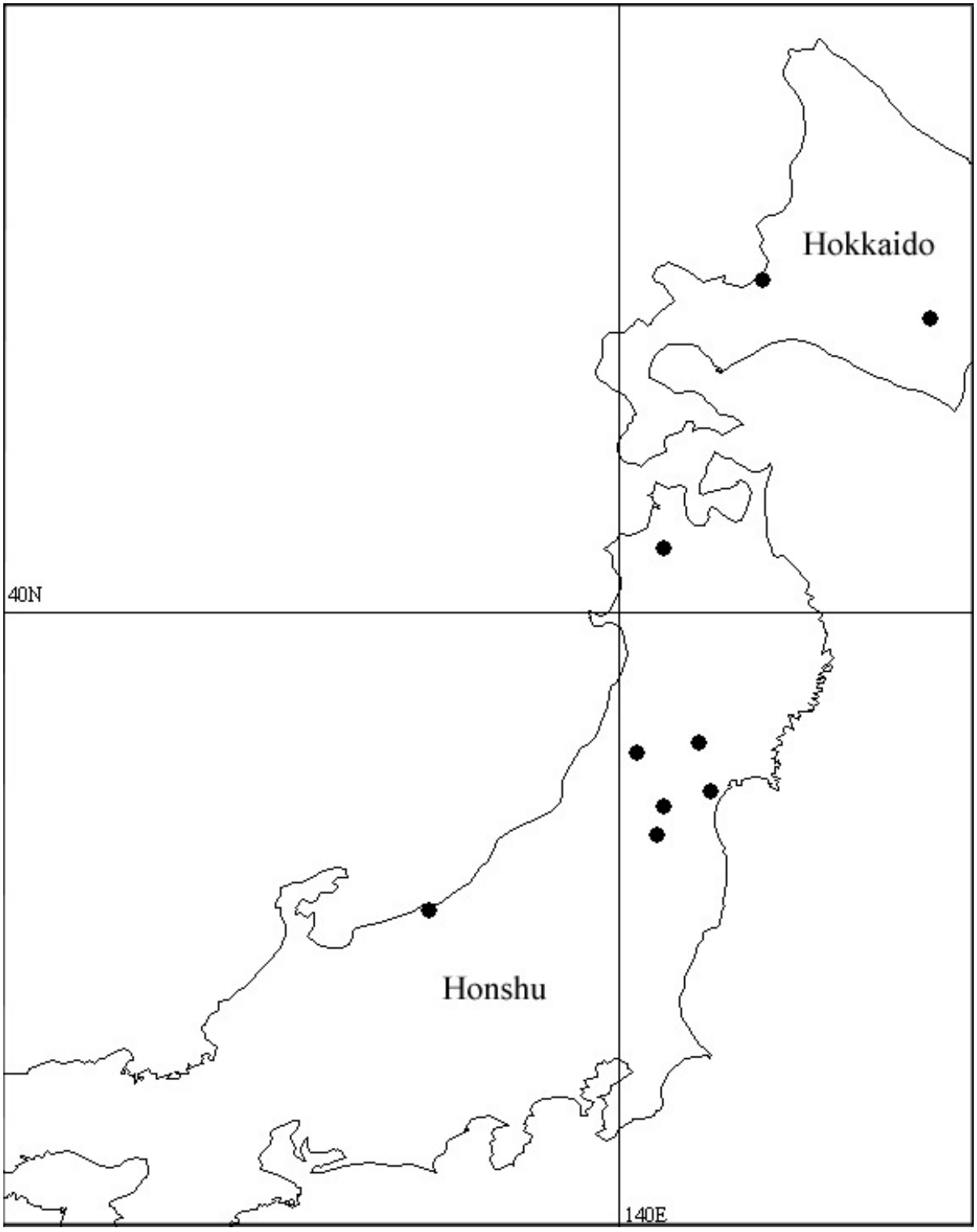



**Figure 8.2** Sample localities of Japanese *F. sachalinensis*. The map was produced using Dr. A. J. Morton's DMAP program.

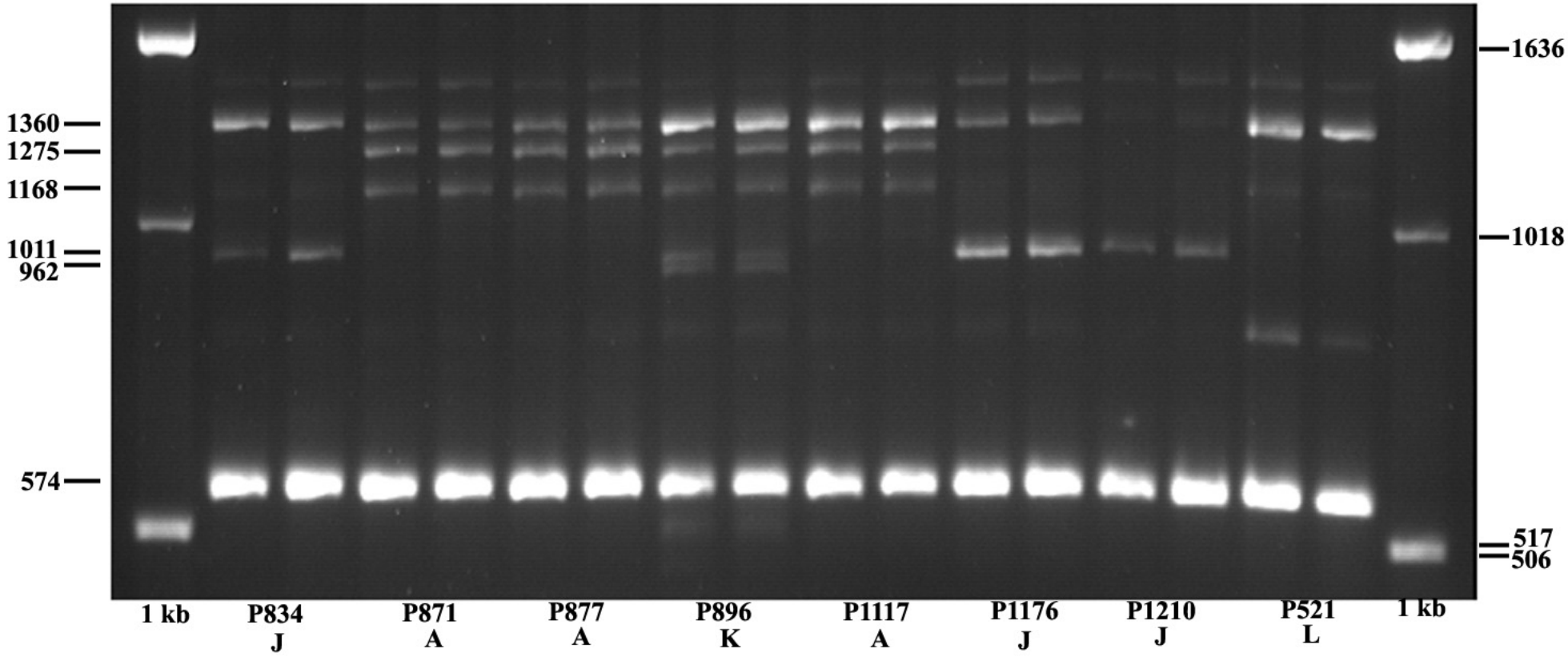



**Figure 8.3** An ISSR photograph showing some of the phenotypes detected in *F. sachalinensis* with primer 840 from the University of British Columbia set nine. Plant identification number and phenotypes are written under the lanes. Duplicate DNA samples were amplified and ran next to each other in the 1.6% agarose gel. Phenotypes codes are as given in Table 8.3.

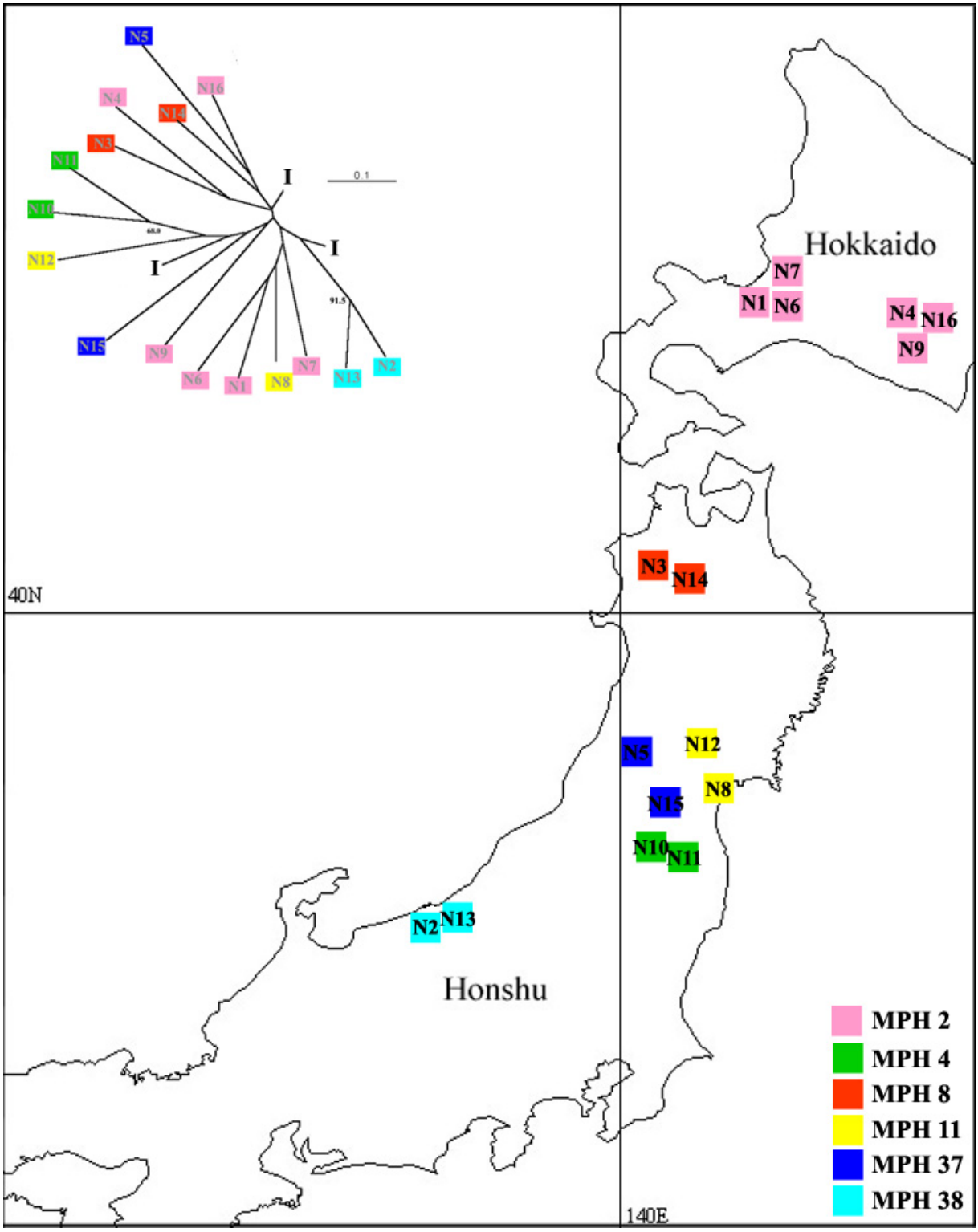



**Figure 8.4** Localities of Japanese *F. sachalinensis* genotypes. The map was produced using Dr. A. J. Morton's DMAP program.

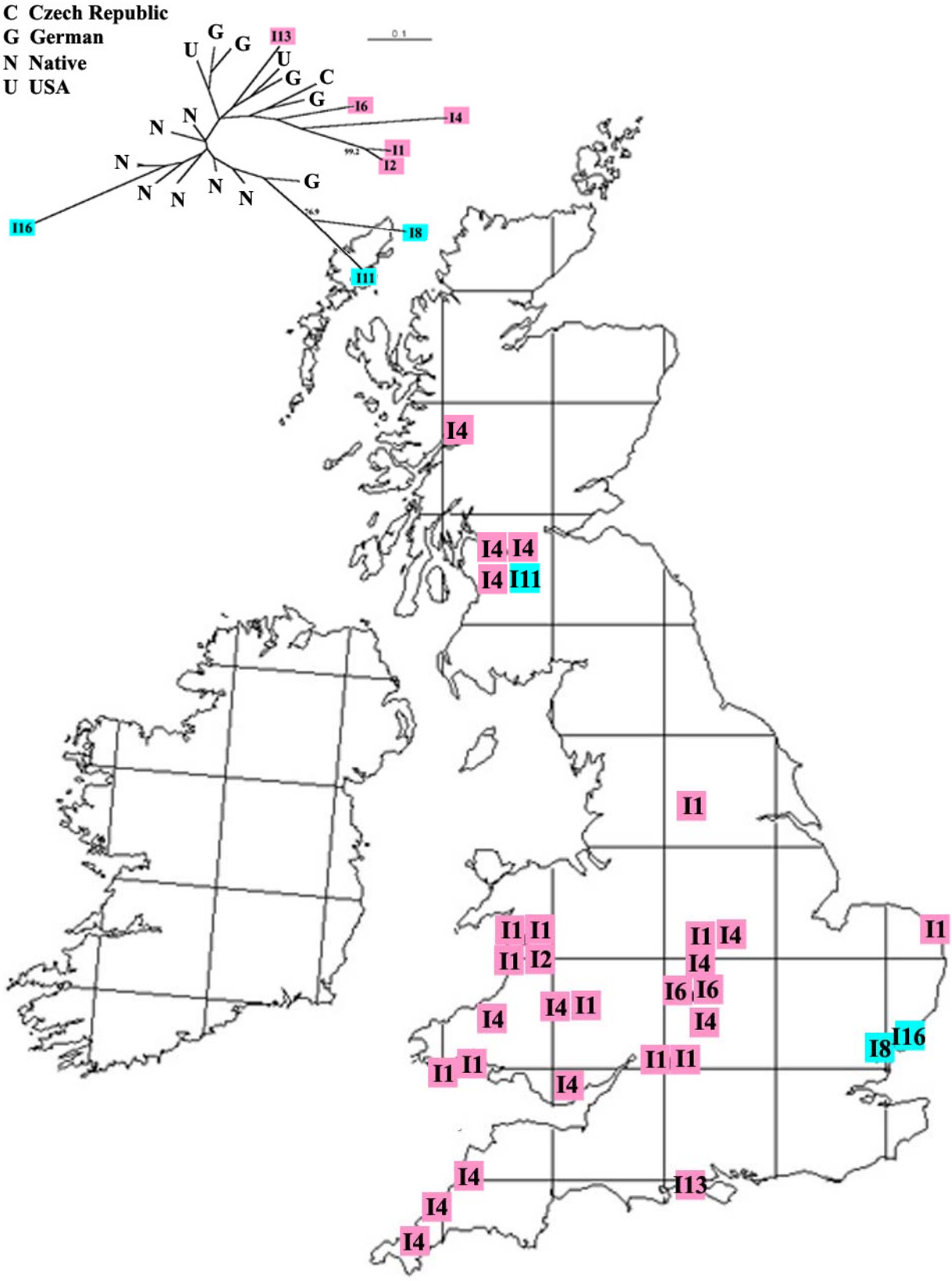



**Figure 8.5** Localities of British *F. sachalinensis* genotypes. The map was produced using Dr. A. J. Morton's DMAP program. The position of some genotypes have been moved slightly, to allow all samples to be seen, due to multiple records in single recording 10 km squares.

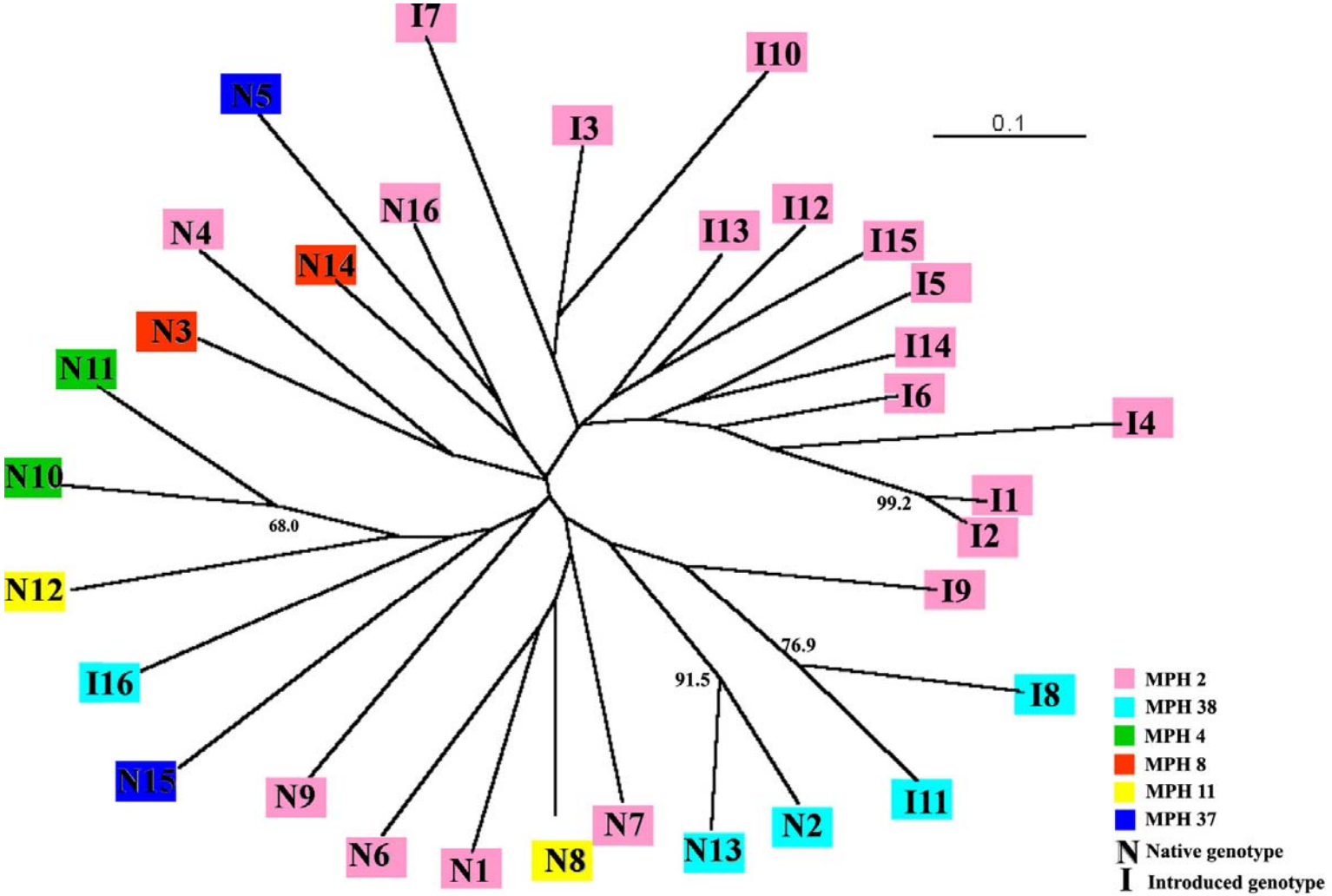



**Figure 8.6** Neighbour-joining tree depicting relationships between the various genotypes, based on the Jaccard's similarity coefficient. Numbers below branching points are confidence values based on bootstrapping 10,000 times. Only bootstrap values greater than 50 are shown. The coloured box superimposed over the genotype represents the corresponding chloroplast haplotype.

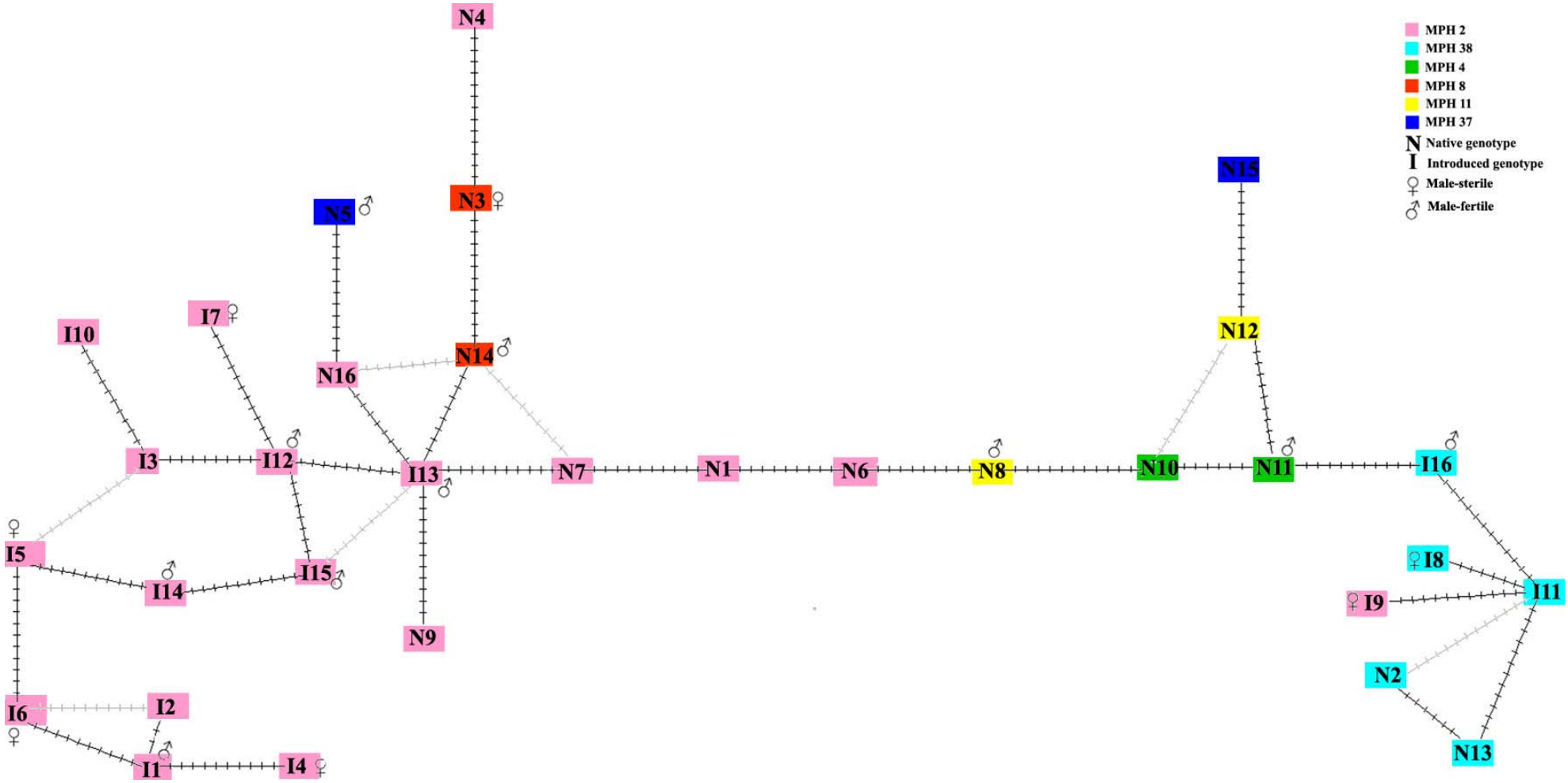



**Figure 8.7** Minimum spanning network showing the number of differences between *F. sachalinensis* genotypes. The cross-links show the number of differences. The grey links are equally likely alternatives between genotypes. The coloured box superimposed over the genotype represents the chloroplast haplotype that corresponds with the genotype. Sex of the genotype is indicated where known.

**Table 8.1** Location details of *F. sachalinensis* included in this study, and sex of the plant where known.

| **P number** | *Location* | **Grid reference** | **Sex** |
|---|---|---|---|
| P055 | Nant Y Frith | SO 2654 | male-fertile |
| P057 | Howey village, Wales | SO 0558 | male-sterile |
| P310 | Amroth Beach | SN 1608 | male-fertile |
| P311 | Amroth Toilet | SN 1608 | male-fertile |
| P326 | Caerynwich, Water gardens | SH 7518 | male-fertile |
| P327 | Caerynwich | SH 7518 | male-fertile |
| P405 | River Kelvin, Botanic gardens bridge, | NS 5865 | |
| P408 | River Kelvin, footbridge, | NS 5865 | |
| P618 | Cwrt Newydd, | SN 4947 | male-sterile |
| P624 | Brithdir, waterside | SH 7217 | male-fertile |
| P626 | Bridge between Congerstone and Bilstone | SK 3605 | male-fertile |
| P706 | River Kelvin, Glasgow | NS 5865 | male-sterile |
| P716 | Glasgow | NS 5865 | |
| P824 | Hempstead Old Rectory | TG 4028 | male-fertile |
| P851 | Colchester, Bunting's nursery | TM 0025 | male-sterile |
| P852 | Colchester, Bunting's nursery | TM 0025 | male-fertile |
| P871 | Cirencester Abbey grounds | SP 0201 | male-fertile |
| P877 | Cirencester Roundabout | SP 0201 | male-fertile |
| P896 | Brithdir | SH 7518 | |
| P1117 | Kirkstall Abbey, Leeds | SE 2635 | male-fertile |
| P1198 | Guys Cliff, Warwick | SP 2865 | male-sterile |
| P1199 | Guys Cliff, Warwick | SP 2865 | male-sterile |
| P1202 | Nuneaton | SP 3592 | |
| P1203 | Scarrier, Cornwall | SW 7244 | male-sterile |
| P1204 | Polzeath, Cornwall | SW 9378 | male-sterile |
| P1205 | Tiscott, Cornwall | SS 2309 | male-sterile |
| P1207 | Epwell, Warwicks | SP 3540 | |
| P1208 | Wanlip, Leicestershire | SK 5919 | |
| P1209 | Coed-y-felin Woods, | ST 1882 | male-sterile |
| P1210 | Christchurch road, South Hampshire | SZ 2392 | male-fertile |
| P1227 | Fort William, Scotland | NN 1074 | male-sterile |

| P number | | Location | Latitude & longitude | Sex |
|---|---|---|---|---|
| P204 | | *R.* x *vivax,* Aachen, Germany | | male-fertile |
| P521 | | Aachen,Stadtwald, Germany | | male-fertile |
| P522 | | Aachen,Stadtwald, Germany | | male-sterile |
| P527 | | Leverkusen, Germany | | |
| P532 | | Umminger, Germany | | |
| P572 | | Benesov nr Komopiste, Czech Republic | | male-sterile |
| P573 | | Kolin, Kostelec, Czech Republic | | male-sterile |
| P834 | | Aberdeen, Washington, USA | | male-sterile |
| P1176 | | Lake City, Seattle, USA | | male-fertile |
| P794a | (38) | Joetsu, Niigata, Honshu, Japan | N 37 06, E 138 15 | |
| P795a | (38) | Joetsu, Niigata, Honshu, Japan | N 37 06, E 138 15 | |
| P918 | (11) | Eai River Valley, Miyagi, Honshu, Japan | N 38 44, E 140 46 | |
| P931 | (11) | Mt. Aoba-yama, Miyagi, Honshu, Japan | N 38 15, E 140 53 | male-fertile |
| P942 | (8) | Hirosaki, Aomori, Honshu, Japan | N 40 36, E 140 26 | male-sterile |
| P943 | (8) | Hirosaki, Aomori, Honshu, Japan | N 40 36, E 140 26 | male-fertile |
| P1055 | (4) | Yonezawa Rd, Fukushima, Honshu, Japan | N 37 50, E 140 22 | |
| P1056 | (4) | Yonezawa Rd, Fukushima, Honshu, Japan | N 37 50, E 140 22 | male-fertile |
| P1062 | (37) | Zao Quasi National Park, Yamagata, Japan | N 38 07, E140 26 | |
| P1076 | (37) | Yamagata, Japan | N 38 38, E 140 10 | male-fertile |
| P1160 | (2) | Ishikari, Hokkaido, Japan | N 43 12, E 141 23 | |
| P1161 | (2) | Ishikari, Hokkaido, Japan | N 43 12, E 141 23 | |
| P1162 | (2) | Ishikari, Hokkaido, Japan | N 43 12, E 141 23 | |
| P1167 | (2) | Memuro, Hokkaido, Japan | N 42 50, E 143 00 | |
| P1169 | (2) | Memuro, Hokkaido, Japan | N 42 50, E 143 00 | |
| P1170 | (2) | Memuro, Hokkaido, Japan | N 42 50, E 143 00 | |

**Table 8.2** Chloroplast haplotypes for the regions *trn*C-*trn*D and *trn*F-*trn*V, and the combined multi-primer-haplotype (MPH).

| **P number** | trn*C*-trn*D* | ***trn*F-*trn*V** | **MPH** | *P number* | ***trn*C-*trn*D** | ***trn*F-*trn*V** | **MPH** |
|---|---|---|---|---|---|---|---|
| P055 | E | D | 2 | P204 | E | D | 2 |
| P057 | E | D | 2 | P521 | E | D | 2 |
| P310 | E | D | 2 | P522 | E | D | 2 |
| P311 | E | D | 2 | P527 | E | D | 2 |
| P326 | E | D | 2 | P532 | E | D | 2 |
| P327 | E | D | 2 | P572 | E | D | 2 |
| P405 | E | D | 2 | P573 | E | D | 2 |
| P408 | E | D | 2 | P834 | E | D | 2 |
| P618 | E | D | 2 | P1176 | E | D | 2 |
| P624 | E | D | 2 | P794a | D | E | 38 |
| P626 | E | D | 2 | P795a | D | E | 38 |
| P706 | E | D | 2 | P918 | D | F | 11 |
| P716 | D | E | 38 | P931 | D | F | 11 |
| P824 | E | D | 2 | P942 | E | A | 8 |
| P851 | D | E | 38 | P943 | E | A | 8 |
| P852 | D | E | 38 | P1055 | B | A | 4 |
| P871 | E | D | 2 | P1056 | B | A | 4 |
| P877 | E | D | 2 | P1062 | D | G | 37 |
| P896 | E | D | 2 | P1076 | D | G | 37 |
| P1117 | E | D | 2 | P1160 | E | D | 2 |
| P1198 | E | D | 2 | P1161 | E | D | 2 |
| P1199 | E | D | 2 | P1162 | E | D | 2 |
| P1202 | E | D | 2 | P1167 | E | D | 2 |
| P1203 | E | D | 2 | P1169 | E | D | 2 |
| P1204 | E | D | 2 | P1170 | E | D | 2 |
| P1205 | E | D | 2 | | | | |
| P1207 | E | D | 2 | | | | |
| P1208 | E | D | 2 | | | | |
| P1209 | E | D | 2 | | | | |
| P1210 | E | D | 2 | | | | |
| P1227 | E | D | 2 | | | | |

**Table 8.3** Phenotypes generated with each primer. Each band is referred to by the name of the primer that amplified it, and its approximate size in base pairs (bp). Each combination of bands is referred to as a phenotype and is represented by a letter.

| | Primer 881 | | | | | | | Primer AG-4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1772 | 1713 | 1618 | 1152 | 1117 | 1053 | 978 | 2278 | 1620 | 1531 | 1362 | 1328 | 1285 | 1004 | 915 |
| A | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| B | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| C | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| D | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| E | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| F | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 |
| G | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| H | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| I | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| J | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| K | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| L | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| M | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| N | | | | | | | | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |

| | Primer AAG-1 | | | | | Primer CAC-1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2150 | 2060 | 1791 | 1573 | 1321 | 1653 | 1556 | 1493 | 1425 | 1375 | 1336 | 1156 | 1112 | 1095 |
| A | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| B | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| C | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| D | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| E | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| F | | | | | | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| G | | | | | | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| H | | | | | | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| I | | | | | | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| J | | | | | | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| K | | | | | | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| L | | | | | | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| M | | | | | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |

| | Primer 855 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2124 | 2034 | 1940 | 1736 | 1675 | 1504 | 1005 | 754 | 717 |
| A | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| B | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| C | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| D | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| E | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| F | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| G | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| H | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| I | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| J | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| K | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| L | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| M | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| N | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| O | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| P | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| Q | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 |
| R | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| S | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |

| | Primer 840 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1360 | 1275 | 1255 | 1168 | 1011 | 962 | 602 | 574 |
| A | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| B | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| C | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 |
| D | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 |
| E | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| F | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| G | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |
| H | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 |
| I | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| J | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| K | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| L | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| M | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| N | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| O | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| P | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 |
| Q | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| R | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| S | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 |

| | Primer 864 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2442 | 2162 | 2020 | 1858 | 1776 | 1582 | 1502 | 1060 | 1023 | 953 | 924 | 897 | 844 |
| A | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| B | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| C | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| D | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 |
| E | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| F | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| G | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| H | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| I | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| J | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| K | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| L | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| M | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| N | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| O | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| P | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| Q | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| R | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| S | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| T | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| U | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |
| V | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| W | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| X | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| Y | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| Z | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| AA | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| AB | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |

**Table 8.4** The combination of phenotypes that make up each genotype. The table is ordered alphabetically starting with the phenotypes generated by the primer listed on the furthest left of the table. Sex of the plants found with each genotype is listed where known, as is the chloroplast multi-primer haplotype.

| | 881 | CAC-1 | 855 | 864 | 840 | AAG-1 | AG-4 | Plants with genotype | Sex | MPH |
|---|---|---|---|---|---|---|---|---|---|---|
| I1 | A | A | A | A | A | A | A | P055, P310, P311, P326, P327, P624, P626, P824, P871, P877, P1117 | male-fertile | 2 |
| I2 | A | A | A | A | K | A | A | P896 | | 2 |
| I3 | B | A | H | E | E | A | E | P527 | | 2 |
| I4 | B | B | B | B | B | A | B | P057, P405, P408, P573, P618, P706, P1202, P1203, P1204, P1205, P1207, P1208, P1209, P1227 | male-sterile | 2 |
| I5 | C | B | I | G | G | C | I | P572 | male-sterile | 2 |
| I6 | C | B | N | M | C | A | A | P1198, P1199 | male-sterile | 2 |
| I7 | D | G | K | I | J | A | D | P834 | male-sterile | 2 |
| I8 | E | H | L | J | I | B | C | P851 | male-sterile | 38 |
| I9 | F | D | D | O | H | B | H | P522 | male-sterile | 2 |
| I10 | G | E | G | F | F | A | D | P532 | | 2 |
| I11 | H | F | F | H | H | D | C | P716 | | 38 |
| I12 | K | A | M | L | J | A | E | P1176 | male-fertile | 2 |
| I13 | L | B | J | N | J | A | C | P1210 | male-fertile | 2 |
| I14 | L | D | C | D | L | A | G | P521 | male-fertile | 2 |
| I15 | M | C | E | C | D | A | F | P204 | male-fertile | 2 |
| I16 | M | I | J | K | F | B | C | P852 | male-fertile | 38 |
| N1 | C | B | R | V | M | A | K | P1167 | | 2 |
| N2 | C | F | R | R | O | D | E | P794a | | 38 |
| N3 | C | L | S | Y | E | A | E | P942 | male-sterile | 8 |
| N4 | I | K | S | S | F | A | J | P1160 | | 2 |
| N5 | J | B | P | Q | A | B | N | P1076 | male-fertile | 37 |
| N6 | K | B | R | X | R | A | L | P1170 | | 2 |
| N7 | L | D | R | W | Q | A | C | P1169 | | 2 |
| N8 | L | D | R | Z | S | C | K | P931 | male-fertile | 11 |
| N9 | L | H | O | T | J | A | C | P1161 | | 2 |
| N10 | L | H | R | AA | M | B | M | P1055 | | 4 |
| N11 | L | I | R | AB | M | B | A | P1056 | male-fertile | 4 |
| N12 | L | I | R | Y | A | B | C | P918 | | 11 |
| N13 | L | J | R | R | P | D | E | P795a | | 38 |
| N14 | L | L | R | N | N | A | E | P943 | male-fertile | 8 |
| N15 | L | M | R | P | G | E | C | P1062 | | 37 |
| N16 | M | B | Q | U | G | A | E | P1162 | | 2 |

# Chapter 9 General Discussion

Alien species are now a common component of the floras of most countries. Only a small subset of these are believed to become invasive (1993), but when they do they can have severe economic impacts as well as reducing biodiversity (Sakai *et al.*, 2001). In recent years, genetic methods have been used to augment traditional morphological approaches in the study of invasive species (Roderick & Howarth, 1997), but as yet the genetics and evolution of these taxa have received far less attention than their ecology (Sakai *et al.*, 2001). Understanding the genetic diversity and population structure within invasive plants may help to develop programs to both control the current situation, and prevent it from getting worse. As well as this, lessons can hopefully be learnt with regard to the impact that introducing plants that would not normally be sympatric can have on the evolution of these taxa, and to prevent similar situations occurring in the future with different taxa.

## 9.1 Effectiveness of molecular techniques and methods of analysis employed

Deciding on which molecular markers to use to analyse the study taxa is a very important stage in the investigation. Both the technique to produce the raw data, and the methods used to analyse the data have to be suitable to answer the questions posed. For this thesis three different molecular approaches have been utilised.

### 9.1.1 *ISSR PCR*

This technique was used to analyse genetic diversity within populations at the genotypic level. When the study taxa are capable of both sexual and clonal reproduction, a means of detecting the individual clones is required. Although it is theoretically possible that two plants assigned the same genotype with this technique may be different, we can only underestimate the amount of variation. The high numbers of *F.* x *bohemica* genotypes detected, and the fact that all seedlings included in the studies were found to have unique genotypes is testament to the sensitivity of the assays. Whilst it is possible that the large number of genotypes detected is in part attributable to PCR artefacts, *F. japonica* var. *japonica* being shown to be a single clone is one of the aspects of the study that gives confidence that this is not the case. The other being the unexpected detection of the widespread clones of male-fertile and male-sterile *F. sachalinensis*, following the earlier study by Hollingsworth (1998). Whilst it could be argued that the technique is more sensitive to genetic diversity in *F.* x *bohemica* than in *F. sachalinensis*, the high levels of genetic diversity within native and non-British introduced *F. sachalinensis* suggest this is not a factor.

9.1.2 *Chloroplast PCR-RFLP*

Chloroplast PCR-RFLPs were used for two different aspects of this study. In the first instance the level of diversity within the introduced populations was low, so that only one or two chloroplast haplotypes were found within each taxon. This meant that this was an ideal method to use to determine the direction of hybridisation in the octoploid and tetraploid *F*. x *bohemica* in chapters 4 and 5 respectively. The different taxa could be distinguished by this technique and the level of diversity was sufficient to detect enough variation in native taxa to undertake the second aspect of the work, a molecular biogeographical study with the additional aim of identifying the origins of the introduced plants.

9.1.3 *Chloroplast sequencing*

Sequencing is a better method than PCR-RFLP analysis for understanding the types of mutations that cause variation in chloroplast haplotypes, and the relationship between the haplotypes. For identifying variation between plants and general clustering of related haplotypes the two methods were found to be congruent, with PCR-RFLP being the quicker and cheaper method to collect the data and less time consuming to analyse the raw data. Some of the mutations that grouped some of the taxa were found to be of questionable phylogenetic use, a fact that would not have been obvious from PCR-RFLP analysis.

The method used to analyse the data did have some effect on the phylogenetic structure of the ingroup. The removal of microsatellites caused by mono and di-nucleotide repeats had no effect on the main relationships determined by the study, but did have an effect on those that were less strongly associated leading, at the extreme, to an unresolved polytomy at the base of the ingroup. However, the main clades predicted by the current study were consistent regardless of which of the three ways of analysing the combined data was used. Those three ways being a parsimony analysis of the whole data set, a parsimony analysis excluding variation caused by microsatellite repeats, and the maximum likelihood analysis.

## 9.2 Hybridisation and introgression in Japanese Knotweed *s.l.* in Britain

9.2.1 *Genetic diversity in the introduced taxa*

*F. japonica* var. *japonica*, the most common of the plants that comprise Japanese Knotweed *s.l.* and currently regarded as the most problematic, was shown to be represented in Britain by a single male-sterile octoploid clone (Hollingsworth & Bailey, 2000a). Seven accessions of *F. japonica* var. *japonica* were analysed from plants found in Dolgellau in chapter 4; two accessions from Cirencester, three from Preston and one from Leeds in chapter 5; and one accession from each of South Woodchester and Hempstead (Pashley, unpublished). These

data confirm the presence of a single clone in Britain as found by Hollingsworth & Bailey (2000a). This same clone was also found in an *F. japonica* var. *japonica* from Seattle, U.S.A, and six accessions from Leiden, Holland (Pashley, unpublished). The presence of the clone in Leiden is of particular interest. These plants were found near to the site where Phillipe von Siebold was believed to have had his Garden of Acclimatisation in the late 1840s. This finding supports the belief that all material of this type arose from a single introduction from Japan to this garden in Leiden, and from there was sold to gardens throughout Europe (Bailey & Conolly, 2000; Conolly, 1977).

The clonal nature of *F. japonica* var. *japonica* was one of the main factors that made this taxon a prime target for a biological control program (Shaw, 1999). The other two introduced taxa, *F. sachalinensis* and *F. japonica* var. *compacta*, are not as invasive as *F. japonica* var. *japonica*, but they do contribute to the Japanese Knotweed *s.l.* problem through their role as a pollen source for the male-sterile *F. japonica* var. *japonica*, producing the hybrid *F.* x *bohemica* and the intra-specific *F. japonica* hybrid respectively; and as the parental taxa for the tetraploid *F.* x *bohemica*. Given the clonal nature of *F. japonica* var. *japonica*, the genetic composition of the other parental taxa is an important factor for potentially introducing novel variation into the Japanese Knotweed *s.l.* complex.

In chapter 5, five accessions of *F. japonica* var. *compacta* from locations in England, Scotland and Ireland were analysed. Three of the accessions were found to share a chloroplast haplotype; these consisted of two accessions with one genotype and one that possessed a unique genotype. The other two accessions shared a different chloroplast haplotype, and also possessed unique genotypes. This would indicate that, unlike the clonal *F. japonica* var. *japonica*, there is a high level of genetic diversity among British *F. japonica* var. *compacta* plants.

In chapter 8, thirty-one *F. sachalinensis* plants from Britain were analysed for both chloroplast haplotype and genotype. Two chloroplast haplotypes were found in British *F. sachalinensis*, a rare haplotype found in only three accessions, and a common haplotype found in the remaining 29 British accessions, and also in a further nine accessions from non-British countries to which *F. sachalinensis* has been introduced. The three accessions with the rare chloroplast haplotype were found to each possess a unique genotype. Of those with the common chloroplast haplotype, the majority, 24 of the 28 accessions, were found to be one of two genotypes, either a widespread male-fertile, or a widespread male-sterile clone. The remaining four accessions were found at three localities, each locality having a unique

genotype. There is clearly a higher level of genetic diversity in *F. sachalinensis* than there is in *F. japonica* var. *japonica*. However, there is strong evidence that a lot of the current British distribution has resulted from the spread of two clones rather than via sexual reproduction.

The nine non-British *F. sachalinensis* accessions from the introduced range, five from Germany, two from the Czech republic and two from the USA, were all found to have unique genotypes with the exception of a male-sterile individual from the Czech republic that was found to share the common British male-sterile genotype. This would indicate a higher level of genetic diversity in the rest of the introduced countries, compared to Britain, potentially indicative of sexual reproduction. The shared genotype between Britain and the Czech republic would indicate a common source for at least some of the introduced *F. sachalinensis*.

These results have important implications for both current control and management programs, and long-term biological control plans. Due to the expense of control and management programs aimed at Japanese Knotweed *s.l.*, only certain populations are targeted. *F. sachalinensis* and *F. japonica* var. *compacta* are not considered to be as much of an invasive threat as *F. japonica* var. *japonica*. However, whilst *F. japonica* var. *japonica* can only spread by vegetative means, the pollen from the other two can lead to sexual reproduction within the plant complex, and often the seeds can travel further distances without the aid of man. Understanding the genetic composition of Japanese Knotweed *s.l.* populations can mean that potential pollen sources such as male-fertile *F. sachalinensis* and male-fertile *F. japonica* var. *compacta* can be targeted as a pre-emptive strike against hybridisation and the introduction of greater variation into the more invasive components of Japanese Knotweed *s.l.*

One of the aims of biological control is to introduce an organism that will specifically target the problem species without affecting other closely related crops or endangered species. The clonal nature of *F. japonica* var. *japonica* means that there should be little chance of some plants having greater resistance to the control organism than others. If the control agent is too specific there is the possibility that it will not be effective against the hybrid *F.* x *bohemica*, which is already showing signs of being as invasive if not more so than its parental taxa, (Bímová *et al.*, 2001; Brabec & Pyšek, 2000). In the extreme case *F. japonica* var. *japonica* may be reduced or eradicated at some locations, leaving a niche for *F.* x *bohemica* to invade. Alternatively if the agent is too general it may have a detrimental effect on the non-invasive *F. japonica* var. *compacta* that is often found as a garden plant. To fully assess the impact of the biological control agent an understanding of the genetic population structure of all

members of Japanese Knotweed *s.l.* should be considered. This study contributes towards understanding the genetic diversity in the other parental taxa.

### 9.2.1 *Genetic diversity in* Fallopia *x* bohemica

As already suggested, *F* x *bohemica* is showing signs of becoming a serious invasive weed in terms of ability to invade, and difficulty of eradication or control. Clonal growth is given as the main way in which it spreads (Ellstrand & Schierenbeck, 2000), but sexual reproduction also plays a role (Hollingsworth & Bailey, 2000b; Hollingsworth *et al.*, 1998). A major aim of this work was to investigate the levels of genetic diversity in a Japanese Knotweed *s.l.* population in North Wales, renowned for having a large number of both sexes of hexaploid hybrids, and in three other English locations where both sexes of tetraploid *F*. x *bohemica* occur. The production of a successful invader, capable of sexual reproduction, is of great concern to the many people who are struggling to control the highly invasive *F. japonica* var. *japonica* that invades so well by clonal means alone. By analysing the levels of genetic diversity within hybrid populations, predictions can be made to the relative contribution clonal spread versus sexual reproduction has on the invasive potential of these plants. *F*. x *bohemica* is found at three ploidy levels, tetraploid, hexaploid and octoploid. All three have the potential to play an important role in the further evolution of this plant complex and have been investigated to some extent in this thesis.

#### 9.2.1.1 Tetraploid *Fallopia* x *bohemica*

The tetraploid *F*. x *bohemica,* which arises from a cross between *F. sachalinensis* and the dwarf *F. japonica* var. *compacta,* has been given very little attention in comparison to the more common hexaploid hybrid, but is thought to be fully fertile, and in particular in Leeds and Preston is showing signs of being more invasive than either of its parental taxa.

In chapter 5, three locations in Britain where both male-fertile and male-sterile tetraploid hybrids are found were analysed for both chloroplast haplotype and genotype. Hybridisation was shown to occur in both directions, with the plants at Preston having *F. sachalinensis* as the maternal parent, and those at Cirencester and Leeds having *F. japonica* var. *compacta*.

At Cirencester four genotypes were detected from thirteen tetraploid accessions; at Preston there were two genotypes from ten accessions; and at Leeds four genotypes were detected from six stands. The distribution of tetraploid hybrids at Cirencester and Preston appeared to be more a product of the clonal spread of a few genotypes rather than a result of sexual reproduction, whilst at Leeds there was little evidence of clonal spread but a high level of

genetic variation between the different genotypes. The high level of genetic diversity was unsurprising as the site at Leeds is associated with the Wood's Hardy Plant Club, who were known for having sold *F. sachalinensis*, *F. japonica* var. *japonica* and *F. japonica* var. *compacta* (Bailey, J.P., pers. comm.). There was no common genotype found between the different sites. At Cirencester there was at least one case of long distance dispersal of a tetraploid genotype; a male-sterile genotype that was found in multiple stands within the Abbey grounds was also found at a single stand some distance away along a main road. This was probably a case of secondary spread through human interference as opposed to planting.

In total, ten different genotypes of established tetraploid *F.* x *bohemica* were detected by this study and there are other known tetraploids in Britain that have not yet been analysed. This implies that the hybridisation event is not a rare occurrence. The limiting factor to the number of such hybrids is more likely a result of the scarcity of the *F. japonica* var. *compacta* parent, and the careful management of the plants found in maintained gardens such as those in Cirencester and Leeds.

9.2.1.2 Hexaploid *Fallopia* x *bohemica*

Hexaploid *F.* x *bohemica* is by far the most common of the Japanese Knotweed hybrid taxa, being recorded as present in 190 of the 3859 10km recording squares (Preston *et al.*, 2002) in the New Atlas of The British & Irish Flora. This is in contrast to the tetraploid hybrid that is known from seven locations in Britain, and the octoploid hybrid that has only been recorded from one area of Wales (Bailey J.P. pers. comm.). Although capable of producing F2 and backcrossed seed, it is not capable of reproducing at the hexaploid level because the gametes produced, and subsequent plants, are mostly aneuploid. This does not detract from the significance of the hexaploid as a potential invader. *F. japonica* var. *japonica* is as successful as it is through purely vegetative spread. Hexaploid *F.* x *bohemica* is as capable as *F. japonica* var. *japonica* at spreading through clonal means, with the added advantage of new genotypes being constantly produced when *F. sachalinensis* and *F. japonica* var. *japonica* hybridise. Hexaploid *F.* x *bohemica* is also capable of producing balanced octoploid backcrosses with *F. sachalinensis* via the production of unreduced gametes.

The main investigation into hexaploid *F.* x *bohemica* plants concentrated on a study site in Merioneth, North Wales. As detailed in chapter 4, forty-eight established *F.* x *bohemica* stands were analysed, of which 46 were hexaploid. Twenty-six genotypes were detected among these accessions, 64% of which were found as a single stand. Only ten of the genotypes were found at two or more stands, the most dispersed being found at seven stands.

This is clear evidence that both clonal spread and sexual reproduction play a role in the distribution of these plants at this site. Two important dispersal-linked findings were discovered in this study. The first was the genotype that was found at seven stands covering an overall distance of approximately 4.5 km. This genotype was found the furthest upstream of all the genotypes, the most likely explanation for its distribution being that it arose at Caerynwch hall and was dispersed to its downstream locations by the water. The second was a shared genotype between plants at the cattle market in Dolgellau with Caerynwch, which gives some indication of secondary spread. The implication being that the primary dispersal was from Caerynwch via the river to Dolgellau, followed by subsequent movement by man. A further nine genotypes of hexaploid *F*. x *bohemica* were revealed from the three sites studied in chapter 5, with no genotypes shared between the sites and no genotypes shared with those found in plants from Dolgellau (chapter 4). At Cirencester there were eighteen hexaploid stands represented by five different genotypes, at Preston there were three stands represented by two different genotypes, and in Leeds only a single hexaploid accession was found.

Additionally, ISSR genotypes have been determined for a number of other hexaploid *F*. x *bohemica* in Britain. Two genotypes were detected in *F*. x *bohemica* from South Woodchester; three in accessions from Hempstead; two in accessions from Freshford; four from accessions in Colchester; one in an *F*. x *bohemica* from Warwick; and one from an accession from Cornwall (Pashley, unpublished). Each of the genotypes listed here were unique to the sites in which they were found. These results give further evidence of the high level of genetic diversity in hexaploid *F*. x *bohemica*. However it should be noted that the plants from the first four locations listed were analysed due to the fact that the locations were potential "hot spots" in terms of Japanese Knotweed *s.l.* evolution, due to the presence of more than one of the taxa that comprise Japanese Knotweed *s.l.* The plants from the other two locations were sent as *F. sachalinensis* to the University of Leicester, Department of Biology, and subsequently identified as being of hybrid origin. There is therefore a high chance that they were found in sites that could potentially be "hot-spots". These results do not imply that all sites where hexaploid *F*. x *bohemica* is found in Britain will contain unique genotypes.

9.2.1.3 Octoploid *Fallopia* x *bohemica*

Octoploid *F*. x *bohemica* is only known from one region of Wales, although octoploid *F*. x *bohemica* plants have also been recorded from Germany, France and the Czech republic (Bailey, 1997). Two different genotypes of octoploid *F*. x *bohemica* were recorded from

Dolgellau in chapter 4, representing male-fertile and male-sterile genotypes. The fact that the production of octoploid *F*. x *bohemica* has occurred at least twice in Dolgellau, as indicated by the two different genotypes found in locations where they are unlikely to have been planted, is evidence that it is not a rare incident. The discovery of both sexes of octoploid *F*. x *bohemica* means that there are three key ways these octoploid plants can affect the Japanese Knotweed *s.l*. population. Firstly a male-sterile octoploid *F*. x *bohemica* can be pollinated by a male-fertile *F. sachalinensis*. The resultant backcrossed hybrids will be hexaploid, with a larger proportion of their genome being attributable to *F. sachalinensis* than the F1 hexaploid *F.* x *bohemica* plants. As mentioned in chapter 4, seed of this constitution has already been found at Dolgellau (Bailey, pers. comm.). Secondly, the octoploid *F*. x *bohemica* plants could reproduce with each other, and thirdly a fully fertile hermaphrodite octoploid *F*. x *bohemica* could potentially replace the missing male fertile *F. japonica* var. *japonica* within the British flora, leading to introgression and thereby giving sexual reproduction as an additional means of spread to *F. japonica* var. *japonica*, a plant that has invaded so well by clonal means alone.

9.2.1.4 Backcrossed, and F2 *Fallopia* x *bohemica*

Hybridisation between two tetraploid hybrids, or backcrossing to either of the parental taxa, produces hybrids that cannot be determined through chromosome counts as they will also be tetraploid. Tetraploid hybrids appear to be fully capable of hybridisation and seed production, and as mentioned in chapter 5, seedlings presumably of F2 constitution were found growing in Preston showing that germination in the British climate is possible. Whether these seedlings would establish is not known, but is indeed plausible.

The situation involving hexaploid *F*. x *bohemica* is more complicated than with the tetraploids. From a cytological perspective none of the established hybrids studied in chapters 4 and 5 were thought to be F2 plants derived from hexaploid F1 individuals, as these tend to be aneuploid (Bailey, pers. comm.). In chapter 4, the seedlings that were aneuploid were found growing under *F. japonica* var. *japonica* stands in Dolgellau and were much more likely to be backcrosses. As mentioned previously, the hybrids that result from the octoploid *F*. x *bohemica* backcrossing with *F. sachalinensis* are hexaploid. In chapter 4, some larger leaved hybrids were hypothesised to be of this constitution, but there was insufficient evidence to prove or refute the hypothesis.

## 9.3 Native *Fallopia* section *Reynoutria* taxa

The introduction of species into regions of the world where they have become invasive has altered natural environments and severely threatened native diversity, as well as having other detrimental impacts as discussed in chapter 1. In many cases the species introduced is not invasive in its native land. The ability to control an invasive plant may rely on understanding its origin and genetic structure, especially as the scenarios of invasions constructed from morphological analysis can be misleading (Schaal *et al.*, 2003).

Whilst the morphological and cytological variation in British *Fallopia* section *Reynoutria* taxa has received considerable attention, the variability in the native region remained largely unexplored (Hollingsworth, 1998). Several of the aims of this thesis were designed to address this imbalance. On the basis of morphological and cytological analysis, and historical documentation it is strongly believed that the *F. japonica* var. *japonica* and *F. japonica* var. *compacta*, found naturalised in Britain and Europe originated from Japan, whilst *F. sachalinensis* may have arisen from Japan, or the Sakhalin Islands (Bailey & Conolly, 2000). As a result of this Japan was selected as the main focus of the investigations into native *Fallopia* section *Reynoutria* taxa.

Before completing any genetic analyses the plants needed to be identified, and one of the aims of this project was to see if the leaf characters that distinguish *F. japonica* and *F. sachalinensis* in the introduced range can be of taxonomic use when studying native material. The combined use of cuticular ornamentation and trichome type did appear to be a useful tool in differentiating between the taxa in their native range, as detailed in chapter 6, whilst leaf shape was less useful. To fully utilize these characteristics though a study of the interspecific and intraspecific native hybrids is required.

### 9.3.1 *Molecular Biogeography of Japanese* Fallopia sachalinensis *and* Fallopia japonica *taxa*

In general the multi-primer-haplotypes detected by the PCR-RFLP analysis of chloroplast regions in chapter 6 were good indicators of geographical distribution. Four major clades (A-D) were detected, and a further group of OTUs formed an unresolved polytomy at the base of the majority rule consensus tree.

Plants found within clade A were predominantly *F. japonica* var. *uzenensis* or hybrid taxa found in northern Honshu in the Tōhoku and Horuriku districts, but not found as far north as Aomori. The accessions found within clade B had the widest overall distribution, being found from the furthest south prefecture of Honshu, Yamaguchi, to the furthest north Aomori, and

also on the island of Hokkaido. They were not found on either Shikoku or Kyushu. The individual MPHs within this clade did show some geographical clustering. This was a very mixed group of taxa comprising primarily of both *F. sachalinensis* and *F. japonica* var. '*japonica*', but some of the clade B MPHs were also found in *F. japonica* var. *uzenensis* and hybrid taxa. Clade C was only found in Toyama and Niigata, and contained both *F. sachalinensis* and tetraploid *F. japonica* var. '*japonica*'. The MPHs within clade D were found on three of the main islands, Shikoku, Kyushu and the southern part of Honshu. Clade D contained both tetraploid and octoploid *F. japonica* var. '*japonica*'. The unresolved group was comprised of Japanese *F. japonica* var. '*japonica'* with a very similar distribution to those from clade D. Also included within this unresolved section were the Chinese and Korean taxa.

A number of hypotheses were given in chapter 6 that could explain the current distribution of the clades and the taxa within them. These included:
1) *F. sachalinensis* entering Japan from the northern land bridge, and *F. japonica* from the southern land bridge during the Pleistocene.
2) The plants that formed clade B, which consists primarily of tetraploid *F. japonica* var. '*japonica'* and *F. sachalinensis,* had a common ancestor that originated in the region to the north of Japan. The taxa arrived in Japan during several different glaciation cycles, the tetraploid *F. japonica* var. '*japonica'* plants arriving during one of the earlier climatic warming then cooling periods.
3) During the glacial periods the volcanoes became refugia for the early progenitors of the present taxa.

The three hypotheses are not necessarily mutually exclusive. The first and the second hypotheses were both based on explanations for geographical distribution in other Japanese species; however, the results from this study would suggest that the third hypothesis was the most parsimonious explanation of the data, especially given their affinity with volcanoes, although a combination of factors is highly probable.

9.3.2 *Relationship between and within introduced and native* Fallopia *section* Reynoutria *taxa*
In chapter 6, six regions of the chloroplast genome were analysed by PCR-RFLP analysis to investigate the relationship between and within introduced and native *Fallopia* section *Reynoutria* taxa. In chapter 7, sequence analysis of three fragments derived from sequencing three of the regions analysed in chapter 6 from one direction only were used to clarify the

relationship between a subset of the taxa, using three closely related species from different sections within the genus *Fallopia* as outgroup taxa.

The taxa within *Fallopia* section *Reynoutria* were found to form a monophyletic group. Within the outgroup taxa, *F. baldschuanica* and *F. convolvulus* formed a strong well supported group, with *F. multiflora* being found neither closer to the ingroup or the other outgroup taxa. This challenges the current taxonomic segregation that puts *F. multiflora* and *F. baldschuanica* together in section *Sarmentosae*, with *F. convolvulus* being found in section *Fallopia*. *F. multiflora* is a rhizomatous herbaceous plant with the same base number as the ingroup (n = 11). It is believed to be either a diploid progenitor to the ingroup, or at least a close relative of the progenitor (J. P. Bailey, pers. comm.). The other two outgroup species are climbers, unlike both the ingroup and *F. multiflora*, and have a base number of n = 10.

*F. sachalinensis* and *F. japonica* were found interspersed within the tree derived from the sequence data, and were likewise mixed together in the trees derived from the PCR-RFLP data. This implies that they are not phylogenetically distinct species, at least according to the chloroplast genome. From a morphological perspective these plants do appear to be distinctive taxa, however the high instance of hybridisation between these two taxa in both the native (chapters 6 and 7) and introduced (chapters 4 and 5) ranges, which results at the tetraploid level in what appear to be fully fertile hybrids (chapter 5), raises questions as to the relationship between these plants. Although only recently recognised in the Japanese Flora, hybridisation is clearly occurring between these native taxa, particularly in prefectures such as Aomori where the different taxa are being planted in disturbed habitats. In chapter 7, the sequence data showed the British tetraploids *F. japonica* var. *compacta* and *F. sachalinensis* to have chloroplast haplotypes that were genetically closer to each other than to that of the British octoploid *F. japonica* var. *japonica*. In chapter 6 there were five cases of chloroplast haplotypes being shared by more than one taxon. In one of these cases the MPH was shared by two different varieties of *F. japonica*, var. '*japonica'* and var. *uzenensis*. In the other four cases the MPHs were shared between the species, *F. sachalinensis* and *F. japonica*.

There is always the possibility that what is regarded as two closely related but different species could in fact be different ecological races of the same species, such as is the case with some of the members of the *Achillea millefolium* complex. For example, the dwarf hexaploid race *A. millefolium* var. *borealis* (Bong) Farwell hybridises readily with the giant hexaploid race *A. millefolium* var. *gigantia* (Pollard) Nobs with highly segregating F2 progeny. Likewise, the dwarf subalpine tetraploid *Achillea millefolium* var. *alpicola*, and the tall

tetraploid *Achillea millefolium* var. *puberla*, hybridise readily and the F1 hybrids are highly fertile (Hiesey & Nobs, 1970).

The PCR-RFLP data revealed four clades of equal evolutionary distance to each other, with the remaining plants forming a polytomy at the base of the group implying that they were no closer to each other than they were to any of the four clades. The sequence data also revealed four clades and an unresolved polytomy, but in this case the four clades paired up into two major clades comprised of two sister clades. The plants that made up the clades were the same for both studies, with one major difference. In chapter 6 some of the plants that made up the unresolved group formed clade C in chapter 7, whilst those from clade D in chapter 6 became part of the unresolved group in chapter 7. Three plants were consistently in the unresolved group, P113, P476 and P134. P134 is *F. japonica* var. *terminalis*. In chapters 6 and 7, *F. japonica* var. *terminalis* was found to merit taxonomic recognition. P476 is the Korean *F. sachalinensis*. The combination of cytological, chloroplast and morphological evidence would suggest that some level of taxonomic recognition be required for these plants from Ullung. The chloroplast DNA sequence data showed the Korean *F. sachalinensis* to be genetically closer to the other non-Japanese native plants compared to the other *F. sachalinensis* plants from this study. P113 is the Chinese octoploid *F. japonica* var. '*japonica*'. In general the plants that comprise the unresolved group and clade D in chapter 6, and those that comprise the unresolved group and clade C in chapter 7 consist of all of the non-Japanese native plants from China and Korea, all of the Japanese plants found from the south-western islands Shikoku and Kyushu, *F. japonica* var. *terminalis* from the southern Hachijo Islands and the vast majority of the accessions from the south-western most prefectures of Honshu. The Korean *F. elliptica* was part of this group, with very few unique mutations separating it from the other Chinese and Japanese *F. japonica* var. '*japonica'* accessions supporting the argument that *F. elliptica* does not deserve taxonomic recognition at the species level.

These studies show a high degree of similarity between the Korean, Chinese, and the Japanese plants from the southwestern region of Japan. This may indicate that Japan is the centre of diversity for *F. japonica* and that the plants found in Korea and China may prove to be of limited genetic variation.

An unexpected find in chapter 7 was a 27 base pair inversion shared between the British *F. japonica* var. *japonica*, the anomalous accession of Japanese octoploid *F. japonica* var. '*japonica',* the accessions of *F. japonica* var. *uzenensis,* the Chinese decaploid and Korean *F.*

*elliptica* and two tall tetraploid Japanese *F. japonica* var. '*japonica'* from the southwestern part of Japan. In phylogenetic analyses single base changes (transitions and transversions) were given a weighting of one, as were all indels and the inversion. The inversion, together with a single base transition, are the two mutations that support the major clade that includes the sister clades C and D that comprise the above taxa. The bootstrap support for this major clade is 75, 76 or 79% for different parsimony analyses and 61% with maximum likelihood. The chance of this inversion being the result of homoplasy in the data is much lower than, for example, a single base mutation. Given that a further two mutations within this inverted region affect only a subset of the taxa in only one of the orientations. This would justify increasing the weighting of the mutation in the analyses, which would lead to higher bootstrap support for this major clade. These data indicate that the British *F. japonica* var. *japonica* is genetically closer to the Chinese and Korean *F. japonica* var. '*japonica'* than was originally hypothesised from earlier cytological and morphological analysis and historical documentation.

*F. japonica* var. *uzenensis* appears to be a distinct taxon, albeit one that readily hybridises with other sympatric taxa. In contrast, no genetic difference was found between high altitude dwarf plants, and the tall lowland taxon, leading to the conclusion that the dwarf high altitude plants do not deserve formal recognition, for example the use of the name *F. japonica* var. *compacta* at least when considering native material.

Clearly the relationship between the taxa in this study is confusing, and whilst this study represents an increase in the understanding of the relationships between them, many questions remain to be answered.

### 9.3.3 *Geographical origin of the introduced plants*

One of the main aims of this study was to identify probable native regions that the introduced taxa may have originated from. In chapter 6, using PCR-RFLP analysis of regions of the chloroplast, exact matches to the chloroplast MPHs discovered in plants from the introduced range were found in plants from the native range. All the matches were found in Japan, with no matches between the introduced material and China or Korea, and no exact matches between China, Korea and Japan. Figure 9.1 shows the most likely Japanese regions that the introduced material came from.

Two different chloroplast haplotypes were found in British *F. japonica* var. *compacta*. On the basis of cytological, morphological and chloroplast PCR-RFLP data in chapter 6, the most

likely origin of *F. japonica* var. *compacta* was Mt. Fuji or the immediately surrounding region, region C on Figure 9.1. Chloroplast sequence analysis in chapter 7 supported this finding. Given how Mt. Fuji is a popular destination for visitors to Japan, this is a highly plausible conclusion.

Two chloroplast haplotypes were also found in British *F. sachalinensis*. The two chloroplast haplotypes of *F. japonica* var. *compacta* originate from a single geographical region, which could support a single or multiple introduction. *F. japonica* var. *japonica* as a clone must only have been introduced once. However, the molecular analyses in this thesis indicate there have probably been two separate introductions of *F. sachalinensis*. There is documented evidence for multiple introductions of *F. sachalinensis* (Bailey & Conolly, 2000).

The results found within this thesis predict that the rare British *F. sachalinensis* chloroplast haplotype originated from Niigata, on Honshu, Japan, region S2 on Figure 9.1. This result was supported by the chloroplast PCR-RFLP analysis in chapter 6, the sequencing study in chapter 7 and the ISSR-PCR study in chapter 8.

In chapter 6, the more common *F. sachalinensis* haplotype was the only chloroplast type found in the *F. sachalinensis* plants analysed from two distinct areas of Hokkaido, and was also found in Aomori along with other chloroplast types. These are shown as region S1 on Figure 9.1. Given that it was the only chloroplast haplotype found in the plants from Hokkaido, whereas in Aomori there was a mixture of both chloroplast haplotypes and taxa, it was argued that of the areas analysed in this study, it was more likely that it came from Hokkaido. In chapter 7, accessions of *F. sachalinensis* with the matching chloroplast haplotype to the common British *F. sachalinensis* from Aomori and Hokkaido were sequenced along with a British accession. Unfortunately the study was unable to distinguish Aomori from Hokkaido as the probable source of the British invasive *F. sachalinensis*. In chapter 8, the neighbour joining tree based on ISSR-PCR variation implied that the introduced *F. sachalinensis* were more closely related to the samples from the East of Hokkaido than the west of Hokkaido or from Honshu, however this was a very tentative suggestion that was not supported by the minimum spanning network, but neither was it refuted.

In terms of biological control, *F. japonica* var. *japonica* is clearly the most important taxon. Unfortunately these results were the least conclusive of the three introduced taxa. The chloroplast haplotype determined in chapter 6 from the invasive clone of *F. japonica* var. *japonica* was found within clade A, and was present in plants from six different sites in Japan.

Five of these plants were *F. japonica* var. *uzenensis* found in the region to the north of central Japan in the prefectures Niigata, Yamagata and Miyagi; region J(a) on Figure 9.1. The exception was an accession of octoploid *F. japonica* var. '*japonica'* found in Osaka; region J(b). It would be tempting to say that Osaka was where it arose; however this may not be the case. Firstly this specimen was found growing along the roadside in Osaka, and we have been informed that both *F. japonica* and *F. sachalinensis* were frequently used to protect roadside cutting from erosion in Japan (Yonekura K. pers. comm.). Secondly this individual was found to be at a different ploidy level, and to be genetically distinct from the other plants from this region.

In overall leaf shape and colour some of the *F. japoninca* var. *uzenensis* plants were strongly reminiscent of the British *F. japonica* var. *japonica* clone (personal observation), with the obvious exception of possessing dense uniseriate trichomes of between 3 and 5 cells in length. Many morphological character differences, particularly those of presence versus absence, are frequently governed by one or two genes. Pubescence is often thought to be one of these presence versus absence characters. Dependant on the genetic basis of pubescence, the clone that we are familiar with in Britain may have arisen from *F. japonica* var. *uzenensis* found in the Tōhoku and Horuriku districts of Japan.

An alternative theory is that chloroplast capture between *F. japonica* var. *uzenensis* and *F. japonica* var. '*japonica'* has occurred. If this were the case one would expect the plants to come from a similar geographical region which would still point towards the Tōhoku and Horuriku districts of Japan, unless the event occurred at, for example, a nursery garden.

The chloroplast sequence data produced in chapter 7 supported the findings of chapter 6 for *F. japonica* var. *japonica*, but was unable to further clarify the relationship between British *F. japonica* var. *japonica*, the anomalous accession of Japanese octoploid *F. japonica* var. '*japonica'* from Osaka, and the accessions of *F. japonica* var. *uzenensis*.

One other factor has become clear through these studies, and that is that octoploid *F. japonica* var. '*japonica'* is relatively rare in Japan and has limited chloroplast diversity, although there does appear to have been at least two separate chromosome-doubling events that have led to the production of octoploid *F. japonica* var. '*japonica'*.

### 9.4 Future projects

As with most studies, a number of questions and areas for further studies were raised.

The production of tetraploid hybrids was shown not to be a rare occurrence. To fully establish the risk factor caused by these tetraploid *F.* x *bohemica* plants, better estimates of the fertility of these hybrids should be conducted. It would also be interesting to compare the invasive ability of the hybrids that have *F. sachalinensis* as the maternal parent with those that have *F. japonica* var. *compacta*. The analysis of the other known British tetraploid hybrids not included in this study would also be of interest. They were not included because there is only known to be a single sex present at the various locations and therefore no immediate risk of further evolution through hybridisation. However if the analyses showed these other hybrids to be of the same genotype as each other or some of the genotypes in this study, it may help to determine where these hybrids arose. If they are different it gives further strength to the concern that their production is not an isolated event, and that these hybrids are cause for concern from an invasive point of view.

The native regions of origin of the introduced *F. sachalinensis* and *F. japonica* var. *compacta* have most likely been elucidated in chapter 6. The origin of *F. japonica* var. *japonica* was less clear. Two aspects created the confusion, the first was the fact that the only match to *F. japonica* var. '*japonica'* was found in an accession that did not appear to be in a "natural" location or match the ploidy and chloroplast types of the other local accessions. A wider study into plants from that region may help to either eliminate the region from the investigation, or give stronger support to the match. The second factor was the strong similarity between the chloroplast types found in the British *F. japonica* var. *japonica* and Japanese *F. japonica* var. *uzenensis*. A study to understand the genetic component behind pubescence may shed some light on the situation. Likewise, the analysis of the plants with nuclear markers may eliminate the concern that introgression or chloroplast capture has made the plants appear to be more similar than they truly are.

The clades predicted by the sequence analysis in chapter 7 show a high degree of geographical clustering, ideal for predicting where the introduced taxa came from. However, for understanding the phylogenetic relationship between the taxa there is a risk of confusion caused by hybridisation, and in the extreme scenario the risk that chloroplast capture has occurred. To clarify the relationships within *Fallopia* section *Reynoutria* further studies concentrating on non-chloroplast sequences such as ribosomal DNA or single copy nuclear genes would help.

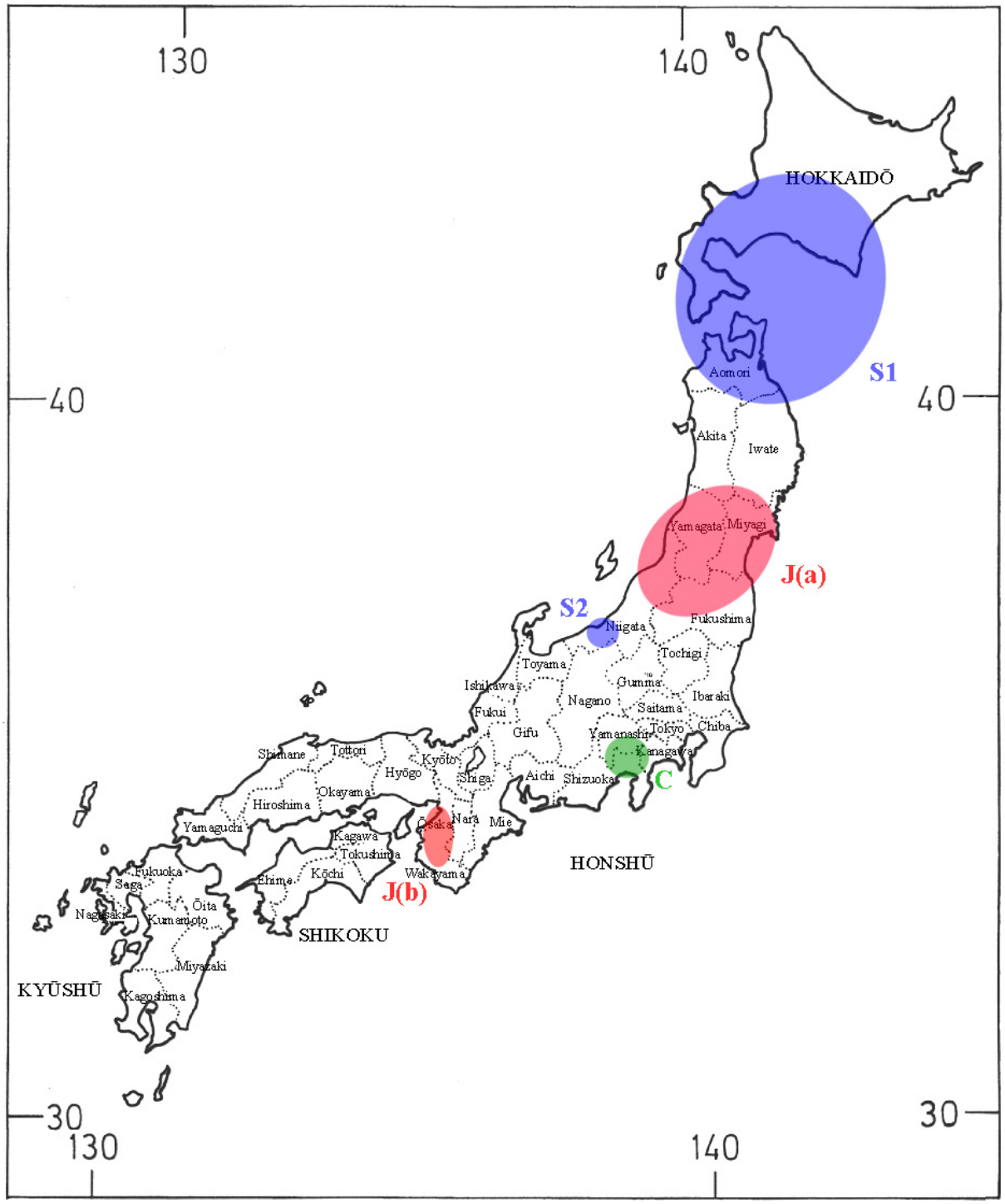



**Figure 9.1** Chloroplast haplotype evidence for the putative origins of the British members of Japanese Knotweed *s.l.*

**S1** Predominant *F. sachalinensis* chloroplast haplotype

**S2** Rare British chloroplast haplotype of *F. sachalinensis*

**C** The two haplotypes of *F. japonica* var. *compacta*

**J(a)***F. japonica* var. *uzenensis* plants sharing the British *F. japonica* var. *japonica* haplotype

**J(b)**'Aberrant' accession of *F. japonica* var. '*japonica*' sharing the British *F. japonica* var. *japonica* haplotype

# Appendix. Materials, suppliers and formulation details.

## 1.1 DNA Extractions

CTAB buffer

| | Final concentration | Quantity added per 1000 ml $dH_2O$ |
|---|---|---|
| Sodium chloride (NaCl) (Fisher) | saturated solution | Variable |
| Cetyltrimethylammonium bromide (CTAB) (Fisons) | 3 % | 30 g |

Other Reagents.

Liquid Nitrogen (BOC)

Sand (low in iron, 40 – 100 mesh) (Fisher)

Polyvinyl polypyrrolidone (PVPP) (Sigma)

DNeasy plant mini kits (Qiagen catalogue number 69104)

λ-DNA (EcoR1/HindIII restricted) ladder (MBI Fermentas)

## 1.2 DNA electrophoresis

10 x Tris borate (TBE) buffer.

| | Final concentration | Quantity added per 1000 ml distilled water |
|---|---|---|
| Tris-base (Fisons) | 1.78M | 108 g |
| Boric acid (Fisher) | 1.78M | 55 g |
| Ethylenediaminetetraacetic acid (EDTA) (Sigma) | 4mM | 7.44 g |

pH adjusted to 8.3 using HCl (Fisher)

6% Polyacrilamide gel solution

| | Quantity added per 50 ml distilled water |
|---|---|
| 10 x TBE | 5 ml |
| 40% Accugel 19:1 (National Diagnostics) (19:1) Acrylamide:Bis-Acrylamide solution | 7.5 ml |
| 10% Ammonium persulphate (Fisons) | 250 µl |
| N,N,N'N'-tetramethylethylenediamine (TEMED) (Sigma) | 50 µl |

One dye loading buffer .

| | Final concentration | Quantity added per 1000 ml distilled water |
|---|---|---|
| Glycerol (Fisher) | 50 % | 50 ml |
| Phenol Blue (Sigma) | 0.01 % | 10 mg |
| Ethylenediaminetetraacetic acid (EDTA) (Sigma) | 0.25 M | 25ml |

Other Reagents.

10mg/ml Ethidium bromide (Sigma)

Multi purpose melt agarose (Boehringer Mannheim)

MetaPhor XR agarose (FMC)

λ-DNA *EcoR*I/*Hind*III restricted (FBI Fermentas)

### 1.3 PCR amplifications

1.3 .1 *Chloroplast DNA.*

Reaction mixture 1

| | Final amount/ concentration | Quantity added per 25 μl distilled water |
|---|---|---|
| 10 x $NH_4$ reaction buffer (Bioline) | 1 x | 2.5 μl |
| 2 mM dNTPs (Bioline) | 0.1 mM | 1.25 μl |
| 50 mM $MgCl_2$ (Bioline) | 2 mM | 1 μl |
| 10 μM primer 1 (Bioline) | 0.2 mM | 0.5 μl |
| 10 μM primer 2 (Bioline) | 0.2 mM | 0.5 μl |
| 5u/μl taq polymerase (Bioline) | 1 unit | 0.2 μl |
| 1 ng/μl DNA sample | 2.5 ng | 2.5 μl |

Reaction mixture 2

| | Final amount/ concentration | Quantity added per 25 µl distilled water |
|---|---|---|
| 10 x $NH_4$ reaction buffer (TaKaRa) | 1 x | 2.5 µl |
| 2.5 mM dNTPs (TaKaRa) | 0.1 mM | 1 µl |
| 10 µM primer 1 (Bioline) | 0.2 mM | 0.5 µl |
| 10 µM primer 2 (Bioline) | 0.2 mM | 0.5 µl |
| 5 unit/µl Ex taq polymerase (TaKaRa) | 0.5 unit | 0.1 µl |
| 1 ng/µl DNA sample | 2.5 ng | 2.5 µl |

Other Reagents.

Mineral oil (Sigma)

100 X Bovine Serum Albumin (BioLabs)

1.3.2 *Nuclear ISSR amplification*

| | Final amount/ concentration | Quantity added per 25 µl distilled water |
|---|---|---|
| 10 x $NH_4$ reaction buffer (Bioline) | 1 x | 2.5 µl |
| 2 mM dNTPs (Bioline) | 0.2 mM | 2.5 µl |
| 50 mM $MgCl_2$ (Bioline) | 2.5 mM | 1.25 µl |
| 15 µM primer (Gibco BRL) | 0.2 mM | 0.33 µl |
| 5u/µl taq polymerase (Bioline) | 1 unit | 0.2 µl |
| 1 ng/µl DNA sample | 10 ng | 10 µl |

**1.4 RFLP analysis**

| | Final amount/ concentration | Quantity added per 10 µl distilled water |
|---|---|---|
| CpPCR product | variable | 7.8 µl |
| 10 x Restriction buffer (BioLabs) | 1 x | 1 µl |
| 10 units/µl Restriction enzyme (BioLabs) | 0.2 units | 0.2 µl |

## 1.5 Sequencing of PCR products

Sequencing reaction

| | Final concentration | Quantity added per 10 µl distilled water |
|---|---|---|
| PCR product | ≈ 250 ng | variable |
| 3.2 µM primer (Bioline) | 1.6 mM | 0.5 µl |
| Terminator ready reaction mix (Perkin Elmer–ABI) (contains AmpliTaq® DNA polymerase, magnesium chloride and fluorescently labelled Big Dye terminators) | N/A | 4 µl |

Other Reagents.

Ethanol (Fisher)

2M Sodium acetate (NaOAc) (pH 5.2) (BDH)

QIAquick PCR Purification Kits (Qiagen catalogue number 28104

DyeEx spin Kit (Qiagen catalogue number 63104)

### 1.5.1 *Sequencing by cloning*

DNA ligation

| | Final amount/ concentration | Quantity added per 10 µl distilled water |
|---|---|---|
| Insert (cleaned PCR product) | 50 ng | 1 µl |
| 50 ng/µl PGEM®-T (Promega) | 25 ng | 0.5 µl |
| T4 ligase (3 Weiss units/µl) (Promega) | 1.5 Weiss units | 0.5 µl |
| 2 x ligation buffer (Promega) | 1 x | 5 µl |

Other Reagents.

DH5∝ competent cells (Stratagene) (Produced by A. F. Wardle, Department of Biology, University of Leicester).

Luria Broth (LB)

| | Quantity added per 1000 ml distilled water |
|---|---|
| Sodium chloride (NaCl) (Fisher) | 10 g |
| Tryptone (Difco) | 10 g |
| Yeast extract (Difco) | 5 g |

Luria Agar (LA)

| | Quantity added per 1000 ml distilled water |
|---|---|
| Sodium chloride (NaCl) (Fisher) | 10 g |
| Tryptone (Difco) | 10 g |
| Yeast extract (Difco) | 5 g |
| Bacto agar (Difco) | 15 g |

Selection media

| | Quantity added per 500 ml LA |
|---|---|
| 20 mg/ml X-gal (5-Bromo-4-chloro-3-Indolyl-β-D-galactoside) (Melford) | 1 ml |
| 100 mg/ml ampicillin (Melford) | 500 µl |
| 200 mg/ml IPTG (Isopropyl-β-D-Thiogalactopyranoside) (Melford) | 60 µl |

**1.6 Chromosome counts**

Pre-treatment solution

0.002M 8-Hydroxyquinoline (Fisher) dissolved in $dH_2O$

Fixative

3 parts Ethanol (Fisher)

1 part Glacial acetic acid (Fisher)

Aceto – orcein stain

2g Certified orcein (Sigma) dissolved in 100 ml 45% acetic acid (Fisher)

Other Reagents.

5 N Hydrochloric acid (HCl) (Fisher)

70% Industrial methylated spirits (IMS)

45% Acetic acid (Fisher)

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