# Investigating the Sex-Selectivity of a Middle Ontario Iroquoian Atlantic Salmon (*Salmo salar*) and Lake Trout (*Salvelinus namaycush*) Fishery through Ancient DNA Analysis

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## Abstract

Prior to European settlement, Indigenous peoples sustainably harvested Atlantic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush)* from Lake Ontario for centuries. Previous studies have suggested Indigenous peoples were able to maintain the productivity of Atlantic salmon and lake trout fisheries in the Great Lakes region through the use of resource management strategies. Since males tend to be the surplus sex among salmonids, one way in which Indigenous peoples could have managed Atlantic salmon and lake trout stocks was through the preferential harvesting of males. Here, we sought to investigate whether Indigenous peoples traditionally used sex-selective fishing to manage Lake Ontario Atlantic salmon and lake trout stocks. To address this question, we modified a DNA-based sex identification method developed for ancient Pacific salmonid (*Oncorhynchus* spp.) remains to make it applicable to archaeological Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) remains. This method assigns sex identities to samples through two PCR assays that co-amplify a fragment of the Y-specific salmonid master sex-determining gene (sexually dimorphic on the Y-chromosome gene) and an internal positive control, consisting of a fragment of the mitochondrial D-loop or nuclear Clock1b gene. We applied this method to 61 Atlantic salmon and lake trout remains from the Antrex site (AjGv-38), a Middle Ontario Iroquoian (ca. AD 1250 to 1300) village located in the Lake Ontario watershed. Using this method, we successfully assigned sex identities to 51 of these remains (83.61% success rate), highlighting our method's sensitivity and efficacy. Statistical analyses indicate neither the aggregate sex ratio nor the sex ratios obtained for the individual species were male-biased. This suggests Antrex's Middle Ontario Iroquoian inhabitants probably did not practice male-selective fishing for Atlantic salmon or lake trout.

**Keywords:** Ancient DNA; Sex identification; Great Lakes; Atlantic salmon (*Salmo salar*); Lake trout (*Salvelinus namaycush*); Ontario Iroquoian archaeology; Zooarchaeology

## 1.0 Introduction

Lake Ontario, in northeastern North America, was historically renowned for its substantial populations of lake trout and potamodromous Atlantic salmon (Dymond et al., 2019; Guiry et al., 2016; Parson, 1973; Smith, 1995). During the 19th century, these populations supported large-scale Euro–North American commercial fisheries, as well as subsistence and recreational fisheries (Bogue, 2000; Elrod et al., 1995; Tiro, 2016). However, by the mid-nineteenth century, Euro–North American–driven overfishing, habitat alteration, pollution, and species introductions, had caused Atlantic salmon and lake trout stocks in Lake Ontario to collapse (Dymond et al., 2019; Elrod et al., 1995; Ketola et al., 2004; Parson, 1973; Smith, 1995). As a result, Atlantic salmon were extirpated from Lake Ontario by 1900, with the last sighting occurring in 1899 (Dymond et al., 2019; Parson, 1973). Although lake trout continued to be commercially harvested into the 20th century, this taxon, too, became locally extinct, by the end of the 1950s (Elrod et al., 1995).

Prior to their extirpation, Atlantic salmon and lake trout were harvested from Lake Ontario by Indigenous peoples for centuries (Hawkins et al., 2019). It has been hypothesized that Indigenous peoples maintained the productivity of salmonid fisheries in the Great Lakes through the use of resource management strategies (Recht, 1997; Thoms, 2004; Tiro, 2016). Ethnohistoric and ethnographic data indicate these resource management strategies included restricted fishing seasons (Tiro, 2016), tenure systems that regulated access to fisheries (Thoms,

2004), and the use of selective fishing technologies, such as weirs (Recht, 1997). These fisheries management strategies were underpinned by what the Wendat historian Georges E. Sioui (1999) terms a fishing theology. This fishing theology consisted of a series of rituals and beliefs that cultivated a reciprocal and respectful relationship between humans and fish (Recht, 1997; Sioui, 1999; Thoms, 2004; but see Tiro, 2016). Understanding the repertoire of strategies that Indigenous peoples traditionally used to manage Lake Ontario's Atlantic salmon and lake trout stocks can inform present-day restoration efforts focused on these taxa (Morales et al., 2017).

One way in which Atlantic salmon and lake trout stocks can be managed is through sex-selective fishing. As males tend to be the surplus sex among salmonids, preferentially harvesting males can enhance the sustainability of salmonid fisheries (Fleming and Einum, 2011; Mathisen, 1962; Reed, 1982). In another salmonid-bearing region of the Americas, northwestern North America, such preferential harvesting of male salmonids, specifically Pacific salmonids (*Oncorhynchus* spp.), was widely practiced by a variety of Indigenous peoples, including the Ahtna (Simeone and Valentine, 2007), Cowichan (Dale and Natcher, 2014), Shasta (Curtis, 1924), Sts′ailes (Ritchie and Springer, 2010), Tla'amin (Barnett, 1975) ,and Tlingit (Langdon, 2006; Ratner et al., 2006). Within this region, male-selective Pacific salmonid fishing was commonly achieved through the use of weirs and traps, which enabled the release of female fish (Dale and Natcher, 2014; Langdon, 2006; Ratner et al., 2006; Ritchie and Springer, 2010). Alternatively, in river pools with clear water and light-coloured substrates it was possible to visually discern male Pacific salmonids and preferentially harvest them with spears or gaffs (Curtis, 1924; Langdon, 2006; Ratner et al., 2006). Ethnographic accounts indicate that in many instances male-selective fishing was purposefully done by Indigenous peoples in order to maintain the productivity of local Pacific salmonid stocks (Barnett, 1975; Dale and Natcher,

2014; Langdon, 2006; Ritchie and Springer, 2010). However, it is important to note that male Pacific salmonids were also preferentially harvested for reasons unrelated to management. For instance, males were targeted by some individuals on account of their larger size or were incidentally harvested in higher numbers due to fluctuations in the relative abundance of the sexes (Langdon, 2006; Ritchie and Springer, 2010; Simeone and Valentine, 2007). As similar fishing technologies were also used by Indigenous peoples in the Great Lakes region (Cleland, 1982; Recht, 1997), it is feasible that they also managed Lake Ontario Atlantic salmon and lake trout stocks through similar male-selective fishing strategies.

Here, we sought to investigate whether male-selective fishing was one of the strategies Indigenous peoples used to manage Lake Ontario's Atlantic salmon and lake trout stocks. To address this question, we developed a DNA-based sex identification method for archaeological Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) remains by adapting a method developed for ancient Pacific salmonid remains (Royle et al., 2018). Following Royle et al. (2018), this method uses two PCR assays that co-amplify a fragment of the Y-specific salmonid master sex-determining gene (sexually dimorphic on the Y-chromosome gene) (Yano et al., 2013, 2012), and an internal positive control consisting of a fragment of the mitochondrial D-loop or nuclear Clock1b gene. Royle et al. (2018) have demonstrated that this DNA-based approach is an efficient sex identification method for archaeological Pacific salmonid remains, but it has yet to be applied to remains from other salmonids. To investigate the sex-selectivity of Indigenous Atlantic salmon and lake trout fisheries in the Lake Ontario basin, we applied our modified DNA-based sex identification method to 28 Atlantic salmon and lake trout remains from the Middle Ontario Iroquoian (ca. 1250–1300 CE) Antrex site (AjGv-38). Our results indicate that our modified method is an efficient sex identification method for archaeological

Atlantic salmonid and char remains and suggest Antrex's inhabitants likely did not practice male-selective fishing for these species.

## 2.0 Archaeological Context

Antrex is an Ontario Iroquoian village located near the north shore of Lake Ontario, in present-day Mississauga, Ontario, Canada (Figure 1). The site is bounded by a tributary of Cooksville Creek and is also situated near the Credit River (Archaeological Services Inc., 2010). During the 19$^{th}$ century, the Credit River supported a substantial Atlantic salmon run harvested by Anishinaabeg and Euro–Canadians (Parson, 1973; Thoms, 2004; Tiro, 2016). The combined results of excavations and surveys conducted by Archaeological Services Inc. (Archaeological Services Inc., 2010, 1991); the Erindale College (now University of Toronto Mississauga) Field School (Smith, 1993); and Mayer, Poulton, and Associates Inc. (Mayer Heritage Consultants Inc., 1998; Mayer Poulton and Associates Inc., 1991) indicate Antrex was a partially palisaded, 0.65 ha village composed of 8 longhouses, some of which were contemporaneous. Analyses of the site's ceramic assemblage indicate it was inhabited during the Middle Ontario Iroquoian period, with multiple radiocarbon dates suggesting a ca. 1250 to 1300 CE occupation (Archaeological Services Inc., 2010; Mayer Heritage Consultants Inc., 1998; Mayer Poulton and Associates Inc., 1991). Within this timeframe, Antrex, like other Middle Ontario Iroquoian villages (Warrick, 1988), was likely only occupied for approximately 20 years before being abandoned (Robertson and Williamson, 2002).

During the Middle Ontario Iroquoian period, subsistence patterns were characterized by an increased dependence on cultigens, most notably maize (*Zea mays* ssp. *mays*) (Dodd et al., 1990). Although maize and other crops were important foodstuffs, stable isotope analyses of Middle Ontario Iroquoian human remains indicate that fish, particularly large piscivorous

species, were significant sources of protein (Feranec and Hart, 2019; Pfeiffer et al., 2016, 2014; van der Merwe et al., 2003). The abundance of fish remains at many archaeological sites dating to this period further reflects the dietary importance of fish at this time (Hawkins et al., 2019; Pfeiffer et al., 2014). The results of a preliminary zooarchaeological analysis, namely an assignment of vertebrate remains to taxonomic class, indicates that fish comprise 33.78% (NISP=4,724) of Antrex's inventoried faunal assemblage (NISP=13,986) (Balmer, 2010). This suggest that, proportionately, fish were a similarly important subsistence item at Antrex. As a more detailed, below-class, analysis of the Antrex faunal assemblage has yet to be completed, the relative abundance of Atlantic salmon, lake trout, and other individual fish species at the site is unknown.

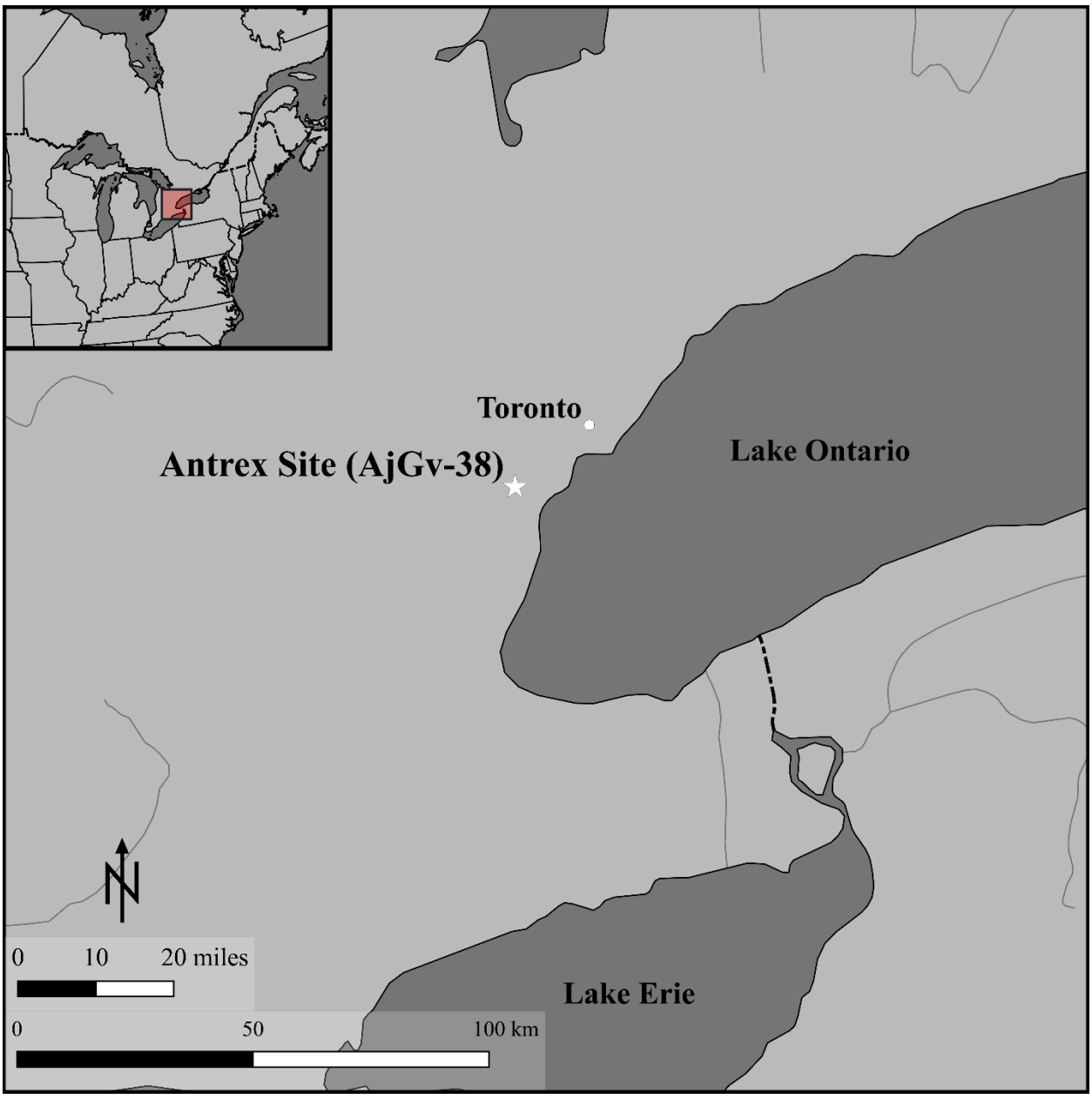


**Figure 1.** Location of the Antrex (AjGv-38) site.

## 3.0 Materials and Methods

### *3.1 Sampling and Zooarchaeological Analysis*

A total of 61 salmonid vertebral elements recovered from Antrex were selected for ancient DNA (aDNA) analysis. We sampled vertebrae rather than unique cranial elements in order to maximize our sample size. In contrast to salmonid vertebrae, which are often archaeologically abundant, salmonid cranial elements are typically rare due to their low bone density relative to vertebrae, which increases their susceptibility to destructive taphonomic processes (Butler and Chatters, 1994; Hawkins et al., 2019; Lubinski, 1996). However, since vertebral elements other than the atlas, penultimate, and ultimate vertebra, are repetitive elements, sampling vertebrae can potentially result in sampling an individual fish multiple times, which would bias our results. Following Cannon and Yang (2006), we sought to mitigate the potential for repeated sampling of individual fish by selecting vertebral elements recovered from different units, features, and layers. Detailed provenience information for each of the analyzed samples is provided in Table S1. As Antrex was likely only occupied for about 20 years, all of the samples, despite coming from different contexts, are roughly contemporaneous.

Taxonomic identifications were assigned to the selected samples by Orchard through comparisons with reference specimens held in the Deborah J. Berg Faunal Collection at the Department of Anthropology, University of Toronto Mississauga (Mississauga, ON, Canada). Uncertain taxonomic identifications were double-checked and confirmed by Needs-Howarth using reference specimens from the Howard G. Savage Faunal Archaeo-Osteology Collection at the Department of Anthropology, University of Toronto (Toronto, ON, Canada). Of the 61 salmonid vertebrae selected for analysis, 35 (Samples LOS1–LOS35) were identified as Atlantic

176 salmon and 26 (Samples LON1–LON26) were identified as lake trout or likely lake trout
177 (*Salvelinus* cf. *namaycush*) (Table S1).

178 Prior to aDNA analysis, a portion of some of the samples (Samples LOS1–LOS17 and
179 LON1–LON11) was removed and subjected to stable carbon and nitrogen isotope analysis
180 (Table S1) and, in some instances (Samples LOS4, LOS14, LON7, LON9, LON10, and LON11),
181 zooarchaeology by mass spectrometry (ZooMS) (Table S1) (Guiry et al., in press). ZooMS
182 confirmed the zooarchaeological taxonomic identifications assigned to four of the six analyzed
183 samples (LOS4, LOS14, LON10, and LON11) (Guiry et al., in press). The remaining two
184 samples (LON7 and LON9) could not be assigned a species identification through ZooMS
185 (Guiry et al., in press).

186 *3.2 Decontamination and DNA Extraction*

187 Decontamination, DNA extractions, and PCR setups were all conducted in a dedicated
188 aDNA laboratory in the Department of Archaeology, Simon Fraser University (Burnaby, BC,
189 Canada) and followed strict contamination controls (Yang and Watt, 2005). In instances where
190 samples were sufficiently large, only a portion of the individual bone was used for DNA
191 extraction. All of the samples were decontaminated prior to DNA extraction using a previously
192 published protocol (Speller et al., 2012). To decontaminate the samples, each sample was, in
193 brief, immersed in a 100% commercial bleach solution (~5% w/v NaOCl) for ≈6–8 mins; rinsed
194 in distilled water for 30 sec–1 min; rinsed again in distilled water for ≈6–11 mins; and UV
195 irradiated for 15–30 mins on two sides. Subsequently, the decontaminated samples were
196 incubated overnight at 50 ˚C in 2.8–5 mL of lysis buffer (0.5 M EDTA [pH 8.0], 0.125–0.25%
197 SDS, and 0.5 mg/mL proteinase K) in a rotating hybridization oven. Following incubation, DNA
198 was extracted from the digested samples using a modified silica-spin column method (Yang et

199 al., 2008, 1998). DNA extraction was repeated for three of the Atlantic salmon samples (LOS7,
200 LOS9, and LOS15) using the remaining bone. Repeat DNA extractions were conducted by an
201 independent analyst within the same laboratory as the initial extractions. To monitor for
202 contamination, blank extraction controls were included in each DNA extraction procedure and
203 subjected to amplification with each combination of primers.

204 *3.3 Development of DNA-based Sex Identification Method*

205 Across the family Salmonidae, sex is principally determined through an XY genotypic
206 sex-determination system wherein males are the heterogametic sex (Davidson et al., 2009).
207 Among most salmonids, including Atlantic salmonids and char, the master sex-determining gene
208 responsible for sex differentiation is hypothesized to be *sdY (*sexually dimorphic on the Y-
209 chromosome gene), a male-specific gene located on the Y-chromosome (Yano et al., 2013). The
210 results of recent studies suggest the expression of *sdY* in developing gonads triggers male
211 differentiation by preventing estrogen synthesis, which promotes testis development (Bertho et
212 al., 2018; Yano et al., 2013, 2012). Recently, Royle et al. (2018) have demonstrated that
213 archaeological Pacific salmonid remains can be assigned accurate sex identities using two PCR
214 assays that screen for the presence of *sdY* and an internal positive control (IPC). However, not all
215 the primers in these assays are conserved in Atlantic salmonids and chars, necessitating the
216 modification of this method to make it applicable to our samples.

217 Atlantic salmonid and char *sdY* sequences obtained from GenBank (Sayers et al., 2019)
218 were aligned with ClustalW (Thompson et al., 1994) through BioEdit v7.2.5 (Hall, 1999).
219 Through a visual examination of this alignment in BioEdit, we designed several primer pairs that
220 targeted small fragments (<100 bp) of *sdY*. NetPrimer
221 (http://www.premierbiosoft.com/netprimer) and Primer-BLAST (Ye et al., 2012) were used to

assess the potential efficiency and specificity of these potential primer pairs. We subsequently included these primers in various potential PCR sex identification assays that, following Royle et al. (2018), co-amplify *sdY* alongside an IPC consisting of a fragment of mitochondrial or nuclear DNA. We evaluated the efficiency of these potential assays by testing them on modern Atlantic salmon (3 males, 1 female) and Arctic char (*Salvelinus alpinus*) (1 male) samples whose genotypic sex was known and a subset of our archaeological samples. The genotypic sex of the modern samples was determined using the 18S rRNA gene/*sdY* co-amplification PCR sex identification assay described by Yano et al. (2013). Reaction conditions for the assays were optimized by applying them with varying PCR conditions to subsets of our modern and ancient samples.

Based on the results of these tests, we selected two PCR sex identification assays to apply to the entire set of Atlantic salmon and lake trout samples from Antrex. Following Royle et al. (2018), the first assay co-amplifies a 98 bp fragment of *sdY* alongside an IPC consisting of a 255 bp fragment of the mitochondrial D-loop. The *sdY* fragment targeted in this assay is amplified with primers *sdY*-F100 and *sdY*-R101, whilst the D-loop fragment was amplified with previously published primers Smc7 and Smc8 (Yang et al., 2004) (

Table ***1***). In the second assay, primers *sdY*-F102 and *sdY*-103 were used to amplify a 98 bp fragment of *sdY*, which was amplified in tandem with a 116 bp fragment of the nuclear Clock1b (*Clk1b*) gene (

Table ***1***). This *Clock1b* fragment serves as the IPC in this assay and was amplified with primers *Clk1b*-F106 and *Clk1b*-R107 (

Table ***1***). Since the X-chromosome is not conserved between or within salmonid species due to *sdY* being a transposable element (Eisbrenner et al., 2014; Faber-Hammond et al., 2015; Lubieniecki et al., 2015), primers directly targeting it were not included in either assay. In both assays, the co-amplification of the IPC functions as a surrogate for the presence of the X-chromosome. Application of these assays to our small sample of genotypically-sexed modern Atlantic salmon (3 males, 1 female) and Arctic char (*Salvelinus alpinus*) (1 male) produced sex identification results concordant with their known genotypic sex (Table S2). All pre-PCR laboratory work involving the modern samples was conducted in a laboratory in the Centre for Forensic Research, Simon Fraser University (Burnaby, BC), that is dedicated to the analysis of modern DNA samples and physically separated from the aDNA laboratory.

**Table 1**. Primer pairs used in this study.

| **Locus** | **Primer**[1] | **Sequence (5'-3')** | **Amplicon Size**[2] | **Source** |
|---|---|---|---|---|
| Cytochrome *b* | CytB5 (F) | AAAATCGCTAATGACGCACTAGTCGA | 168 bp | Yang et al. (2004) |
| | CytB6 (R) | GCAGACAGAGGAAAAAGCTGTTGA | | Yang et al. (2004) |
| *Clock1b* | *Clk1b*-F106 (F) | CTGGTGCAGATGTTCCTCCAAC | 116 bp | This study |
| | *Clk1b*-R107 (R) | ACCACCTGGCCCTGCATGTTGAGAGC | | This study |
| D-loop | Smc7 (F) | AACCCCTAAACCAGGAAGTCTCAA | 255 bp | Yang et al. (2004) |
| | Smc8 (R) | CGTCTTAACAGCTTCAGTGTTATGCT | | Yang et al. (2004) |
| *sdY* | *sdY*-F100 (F) | ATCTCTCTCCCAAAGCCCCC | 98 bp | This study |
| | *sdY*-R101 (R) | CTTAAAACCACTCCACCCTCCAT | | This study |
| *sdY* | *sdY*-F102 (F) | GGGGAGTGATGTCAGAATTGC | 98 bp | This study |
| | *sdY*-R103 (R) | AGATGGGAATGGTGTCGGG | | This study |

[1]F denotes a forward primer and R denotes a reverse primer.
[2]Predicted size of mitochondrial DNA, *Clock1b*, and *sdY* amplicons is based on the position of their corresponding primer pair within Atlantic salmon mitochondrion genome (Genbank accession number: NC001960) (Hurst et al., 1999), *Clock1b* (Genbank accession number: GU228525) (Paibomesai et al., 2010), *sdY* (Genbank accession number: KP898412) (Lubieniecki et al., 2015) reference sequences, respectively.

*3.4 PCR Amplification and Sex Identification*

PCR amplifications and post-PCR procedures were conducted in a dedicated post-PCR laboratory physically separated from the aDNA laboratory. PCR amplifications for the sex

identification assays were performed on a Mastercycler Personal or Gradient thermal cycler (Eppendorf, Mississauga, ON, Canada) in a 30 μL reaction volume that contained 1.5× PCR Gold Buffer (Applied Biosystems, Carlsbad, CA, USA), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 μM of each *sdY* primer, 0.1 μM of each D-loop (D-loop/*sdY* assay) or *Clock1b* (*Clock1b*/*sdY* assay) primer, BSA (1 mg/mL), 1–4 μL of DNA solution, and 0.75–1.25 U/μL AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA). The thermal program for the PCRs consisted of an initial denaturation step at 95 °C for 12 min followed by 60 cycles at 95 °C for 30 s (denaturation), 58 °C (D-loop/*sdY* assay) or 56 °C (*Clock1b*/*sdY* assay) for 30 s (annealing), and 70 °C for 40 s (extension), and a final extension step at 72 °C for 7 min. To identify instances of allelic drop-out, a multi-tube procedure was used for both sex identification assays (Taberlet et al., 1996). Both sex identification assays were applied to each of the samples between two and five times (Sugimoto et al., 2006). Negative PCR controls were included in each PCR run.

Five microlitres of PCR product was pre-stained with SYBR Green I (Life Technologies, Carlsbad, CA, USA), electrophoresed on a 2% (D-loop/*sdY* assay) or 3% (*Clock1b*/*sdY* assay) agarose gel, and visualized with a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO, USA). Sex identities were assigned to the samples with each of the assays through a visual analysis of the electrophoresis gels of the generated PCR products using a modified version of the criteria outlined by Sugimoto et al. (2006). For both assays, a sample was identified as male if *sdY* or both *sdY* and the IPC were amplified at least twice (Sugimoto et al., 2006). Samples were identified as female if the IPC was amplified at least three times with an individual assay (Sugimoto et al., 2006) and *sdY* was not amplified by any of the five PCR replicates carried out for potential females (Janečka et al., 2008). A sex identity was not assigned to a sample with an individual assay if neither of these criteria were met. Following Royle et al.

(2018), a final consensus sex identity was assigned to the samples based on the sex identities assigned with the individual assays. A final consensus sex identity was assigned to a sample if it was successfully identified as the same sex by both the D-loop/*sdY* and *Clock1b/sdY* assay. A sample was not assigned a sex identity if the assays yielded inconsistent results or if a sex identity could not be assigned to the sample with one or both of the assays.

*3.5 Statistical Analyses of Sex Identification Results*

Statistical analyses of the sex identification results were performed in R v3.5.1 (R Core Team, 2018) through RStudio v1.1.456 (RStudio Team, 2015). Two-tailed exact binomial tests were used to assess whether the aggregate sex ratio or the sex ratios obtained for each of the species was significantly male or female biased (McDonald, 2014). The significance of inter-specific sex ratio differences was evaluated through a two-tailed Fisher's exact test of independence (McDonald, 2014). P-values less than or equal to 0.05 were considered significant.

*3.6 Species Identification*

To confirm the samples' species identities, we sequenced and analysed the D-loop fragment co-amplified by the D-loop/*sdY* assay (Royle et al., 2018). In instances where this D-loop fragment was only weakly amplified by this assay or failed to amplify, we attempted to amplify this fragment with the same D-loop primers in a singleplex PCR. Following Yang et al. (2004), we sought to confirm the D-loop–based species identifications though the analysis of a 168 bp fragment of cytochrome *b* amplified in a singleplex PCR with primers CytB5 and CytB6 (

Table ***1***). The conditions for the singleplex PCRs targeting these D-loop and cytochrome z*b* fragments were the same as above, with the exception of their primer concentrations,

polymerase concentrations, and annealing temperatures which were as follows: 0.3 μM of each D-loop or cytochrome *b* primers, 1–1.5 U AmpliTaq Gold, and 54 °C, respectively. Negative PCR controls were included in each of the singleplex PCR runs. The PCR products generated by the singleplex PCRs were separated on a 2% agarose gel and visualized in the same manner as described above. Unpurified D-loop and cytochrome *b* amplicons were directly sequenced in the reverse and/or forward direction with their respective amplification primers at Eurofins Genomics (Toronto, ON, Canada).

The sequences obtained from the Antrex samples were visually edited, truncated to remove the primer sequences, and assembled using ChromasPro v2.1.8 (http://www.technelysium.com.au). To determine their closest taxonomic match, the edited sequences were compared against reference sequences accessioned in GenBank through a BLASTn search (Altschul et al., 1990). Multiple alignments of the edited sequences, reference sequences from all Atlantic salmonid and char species (Atlantic salmon, brook trout [*Salvelinus fontinalis*], and lake trout) native to southern Ontario (Holm et al., 2009), and a huchen (*Hucho hucho*) reference sequence to serve as an outgroup in the phylogenetic analyses, were performed for each marker using ClustalW (Thompson et al., 1994) through BioEdit v7.2.5 (Hall, 1999). Maximum-likelihood phylogenetic trees were constructed for each of the aligned datasets using PhyML v3.1 (Guindon et al., 2010) with 1000 bootstrap replicates. Each phylogenetic analysis was performed with the best–fit substitution model determined by PhyML's automated Smart Model Selection (SMS) method (Lefort et al., 2017) using the Akaike Information Criterion. SMS selected HKY85 as the best-fit substitution model for the D-loop sequences and HKY85+G as the best-fit substitution model for the cytochrome *b* sequences. Both of the resulting phylogenetic trees were visualized and annotated with iTOL v4.4.1 (Letunic and Bork, 2019).

Species-level identifications were assigned to samples if all the sequences obtained from a given sample matched or closely resembled sequences from a single species and differed from other, closely related species.

## 4.0 Results

### *4.1 Sex Identification*

DNA was successfully amplified with the sex identification assays from 60 of the 61 samples (Table S3; See Figures and 3 for exemplar electrophoresis gels). The results of the individual PCR replicates carried out for each sample with both sex identification assays are provided in Table S3, whilst Table 2 presents a summary of the sex identification results for each of the samples. Of the 60 samples that yielded amplicons, the D-loop/*sdY* and *Clock1b*/*sdY* assays generated concordant sex identities for 51 samples, enabling a sex identification to be assigned to these samples (83.6% success rate) (Table 2; See Figures 2 and 3 for exemplar electrophoresis gels). The sex identification results obtained for the repeat DNA extractions of samples LOS7, LOS9, and LOS15 matched the sex identities generated from the initial extractions (Table S3). Of the 51 remains that were successfully sexed, 29 were Atlantic salmon and 22 were lake trout. The remaining ten samples could not be assigned a sex identity using the outlined criteria (Table 2). Likely owing to DNA degradation, one of these samples (LOS16) could not be assigned a sex identity as a result of the failure to amplify DNA with either assay (Table S3). Stable isotope analyses of LOS16 indicate it has poorly preserved collagen (Table S1) (Guiry et al., in press), suggesting that overall biomolecular preservation in this sample was poor. DNA was amplified at least once with both assays from the remaining nine samples, but these could not be assigned to a sex due to the replicates of one or both assays yielding inconsistent results (Table 2; Table S3). The failure to obtain consistent results for these samples potentially reflects allelic drop-out due

to degradation, inhibition, amplification competition with the IPC in the case of males, or a combination thereof. No DNA was amplified from any of the blank extraction or negative PCR controls with either the sex identification assays or singleplex PCRs.

When all 51 of the sexed samples are considered as a whole, irrespective of species, no sex bias is evident. Although females were more abundant than males (Table 3), no significant difference from a 1:1 sex ratio was observed (Exact binomial test, two-tailed, $p=0.2624$). Amongst the Atlantic salmon samples assigned a sex, females were more than twice as abundant as males (Table 3). However, the sex ratio obtained for the Atlantic salmon samples is not significantly sex-biased (Exact binomial test, two-tailed, $p=0.06143$). No sex bias was observed in the sample of sexed lake trout (Exact binomial test, two-tailed, $p=0.8318$), with male and female lake trout being roughly equally abundant (Table 3). The sex ratios obtained for each species did not significantly differ from each other (Fisher's exact test, two-tailed, $p=0.1504$).

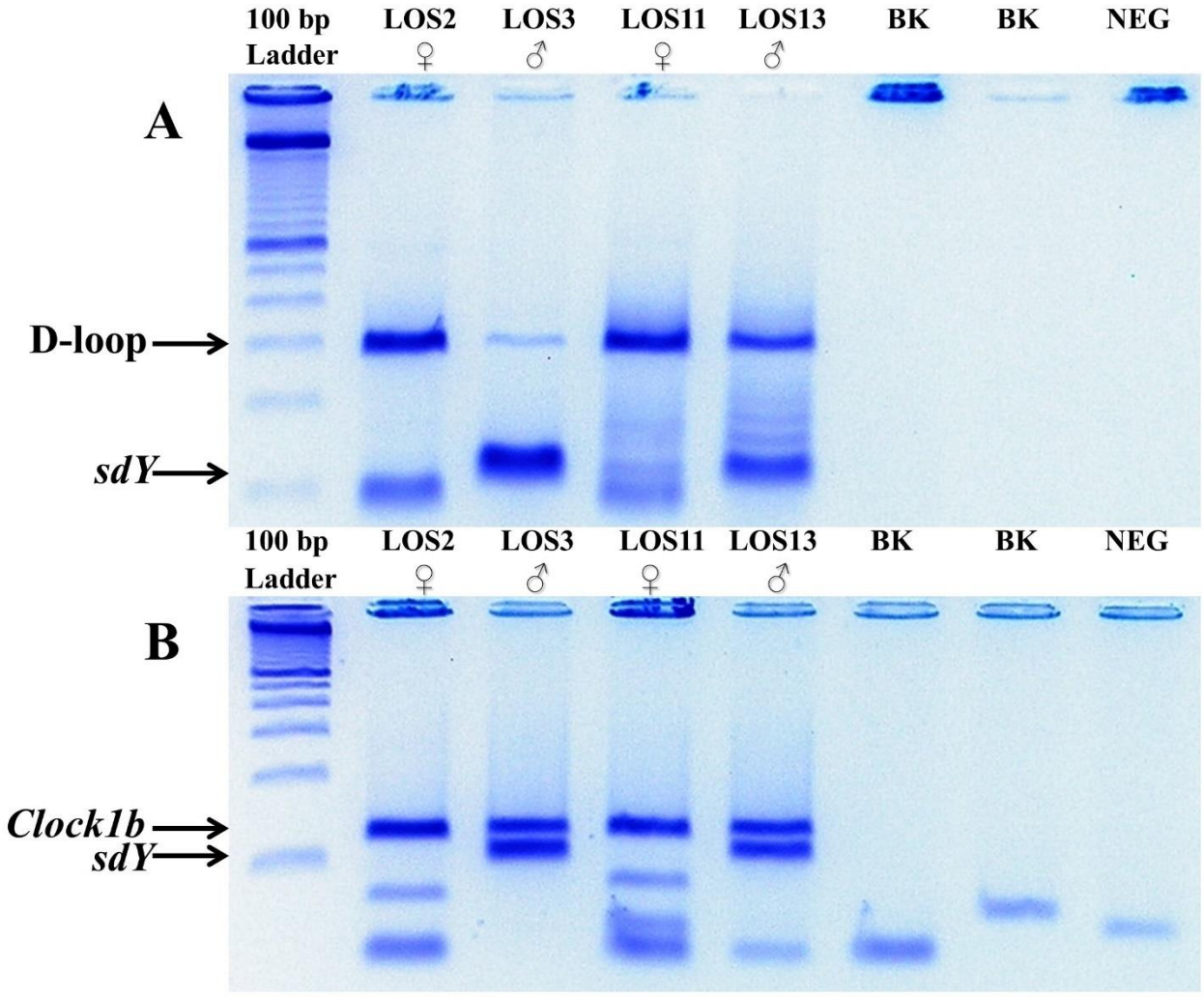


**Figure 2.** Negative images of electrophoresis gels showing the (A) D-loop/*sdY* PCR and (B) Clock1b/*sdY* assay results for four of the analyzed Atlantic salmon (*Salmo salar*) samples (LOS#). The Mars (♂) and Venus (♀) symbols beneath the sample names denote samples identified as male and female, respectively. The approximate positions of the internal positive control (D-loop and *Clock1b*) and *sdY* amplicons generated by the assays are indicated by the labelled arrows. BK denotes the blank extraction controls processed alongside the samples. NEG indicates negative PCR controls. The 100 bp ladder is from Invitrogen (Waltham, MA, USA).

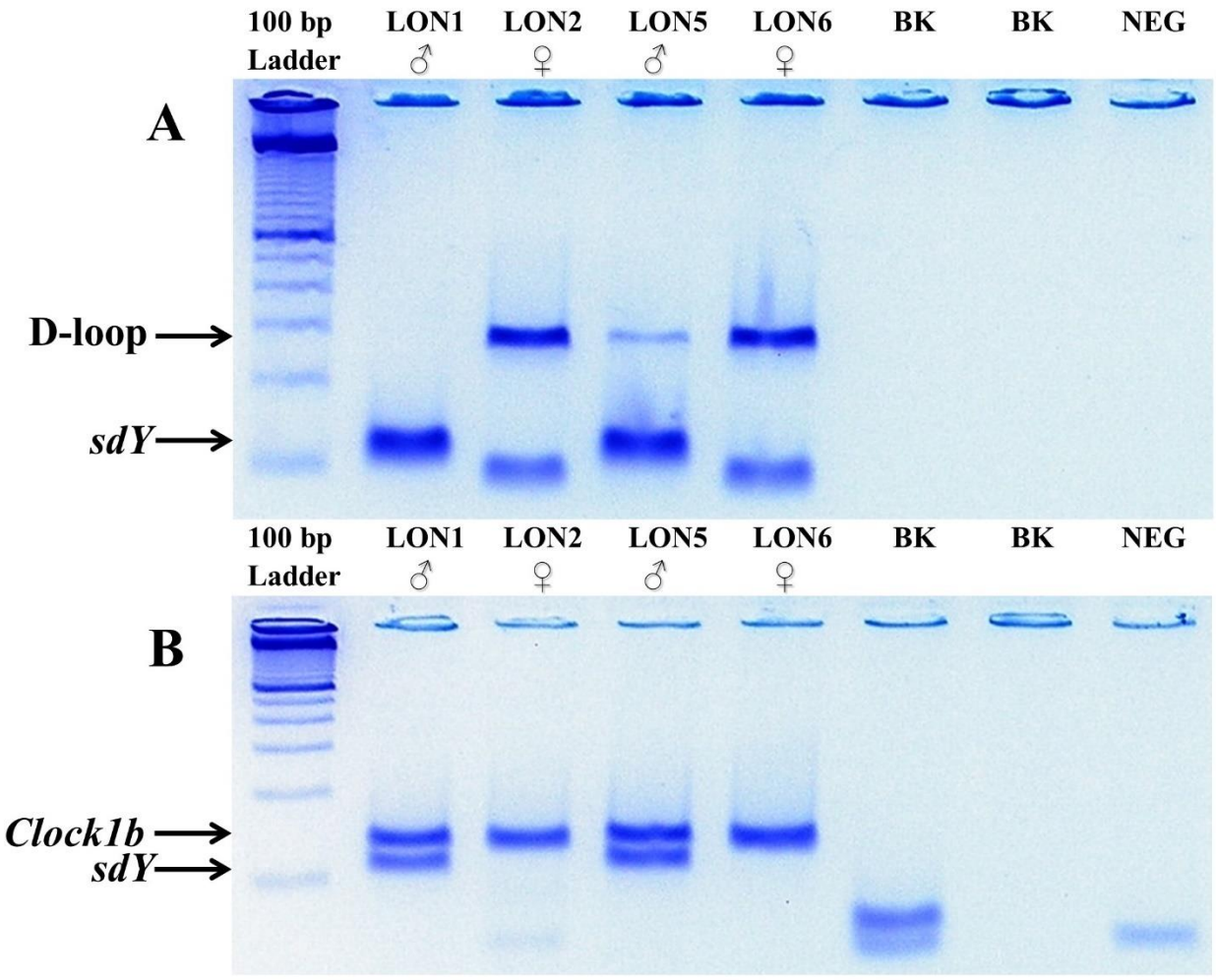


**Figure 3.** Negative images of electrophoresis gels showing the (A) D-loop/*sdY* PCR and (B) Clock1b/*sdY* assay results for four of the analyzed lake trout (*Salvelinus namaycush*) samples (LON#). The Mars (♂) and Venus (♀) symbols beneath the sample names denote samples identified as male and female, respectively. The approximate positions of the internal positive control (D-loop and Clock1b) and *sdY* amplicons generated by the assays are indicated by the labelled arrows. BK denotes the blank extraction controls processed alongside the samples. NEG indicates negative PCR controls. The 100 bp ladder is from Invitrogen (Waltham, MA, USA).

386

387 **Table 2**. Sex and species identification results for the analyzed samples. ZooMS species identifications are from Guiry et al. (in press).

| Sample | Zooarchaeological Species ID | ZooMS Species ID | D-loop Species ID | *Cytb* Species ID | Consensus Genetic Species ID | D-loop/*sdY* Sex ID | Clock1a/*sdY* Sex ID | Consensus Sex ID |
|---|---|---|---|---|---|---|---|---|
| LOS1 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS2 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS3 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS4 | Atlantic salmon | Atlantic salmon | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS5 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS6 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Indeterminate | Indeterminate | Indeterminate |
| LOS7 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS8 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS9 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS10 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS11 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS12 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS13 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS14 | Atlantic salmon | Atlantic salmon | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS15 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS16 | Atlantic salmon | - | Indeterminate | Indeterminate | Indeterminate | Indeterminate | Indeterminate | Indeterminate |
| LOS17 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS18 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Indeterminate | Indeterminate |
| LOS19 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS20 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Indeterminate | Indeterminate | Indeterminate |
| LOS21 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS22 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Indeterminate | Indeterminate |
| LOS23 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS24 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS25 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS26 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Indeterminate | Indeterminate |
| LOS27 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS28 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS29 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS30 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS31 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS32 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| LOS33 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Male | Male | Male |
| LOS34 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LOS35 | Atlantic salmon | - | Atlantic salmon | Atlantic salmon | Atlantic salmon | Female | Female | Female |
| LON1 | Lake trout | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON2 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON3 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON4 | Lake trout | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON5 | Lake trout | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON6 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON7 | Lake trout | Indeterminate | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON8 | Lake trout | | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON9 | Lake trout cf. | Indeterminate | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON10 | Lake trout cf. | Lake trout | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON11 | Lake trout cf. | Lake trout | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON12 | Lake trout cf. | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON13 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Indeterminate | Indeterminate |
| LON14 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON15 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON16 | Lake trout | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON17 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON18 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON19 | Lake trout cf. | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON20 | Lake trout cf. | - | Lake trout | Lake trout | Lake trout | Male | Indeterminate | Indeterminate |
| LON21 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON22 | Lake trout | - | Lake trout | Lake trout | Lake trout | Male | Male | Male |
| LON23 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Indeterminate | Indeterminate |
| LON24 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON25 | Lake trout | - | Lake trout | Lake trout | Lake trout | Female | Female | Female |
| LON26 | Lake trout | - | Lake trout | Lake trout | Lake trout | Male | Indeterminate | Indeterminate |

| Species | Females | Males | Sex Indeterminate | Total Analyzed |
|---|---|---|---|---|
| Atlantic salmon | 20 | 9 | 6 | 35 |
| Lake trout | 10 | 12 | 4 | 26 |
| Aggregate | 30 | 21 | 10 | 61 |

**Table 3**. Number of identified females and males by species.

*4.2 Species Identification*

Both D-loop and cytochrome *b* were successfully amplified from 60 of the 61 samples, with LOS16 being the only sample to not yield any mitochondrial DNA. The D-loop and cytochrome *b* sequences obtained from the repeat DNA extractions of samples LOS7, LOS9, and LOS15 matched those obtained from the initial extractions. The results of the BLASTn searches indicate the D-loop and cytochrome *b* sequences obtained from each sample matched or closely resembled Atlantic salmon or lake trout reference sequences. Each sample's D-loop and cytochrome *b* sequences matched reference sequences from the species to which it was identified using zooarchaeological methods and differed from closely related taxa. The phylogenetic analyses yielded similar results (Figures 4 and 5). All the sequences obtained from samples zooarchaeologically identified as lake trout formed a group with lake trout reference sequences, whilst those from samples zooarchaeologically identified as Atlantic salmon clustered with references sequences from that species (Figure 4 and 5). Based on these data, species-level identifications could be confidently assigned to each of the 60 samples that yielded mitochondrial DNA (Table 2). The DNA-based species identities assigned to the samples agreed with the species identities assigned to them through zooarchaeological methods and, in the case of four samples, ZooMS (Table 2).

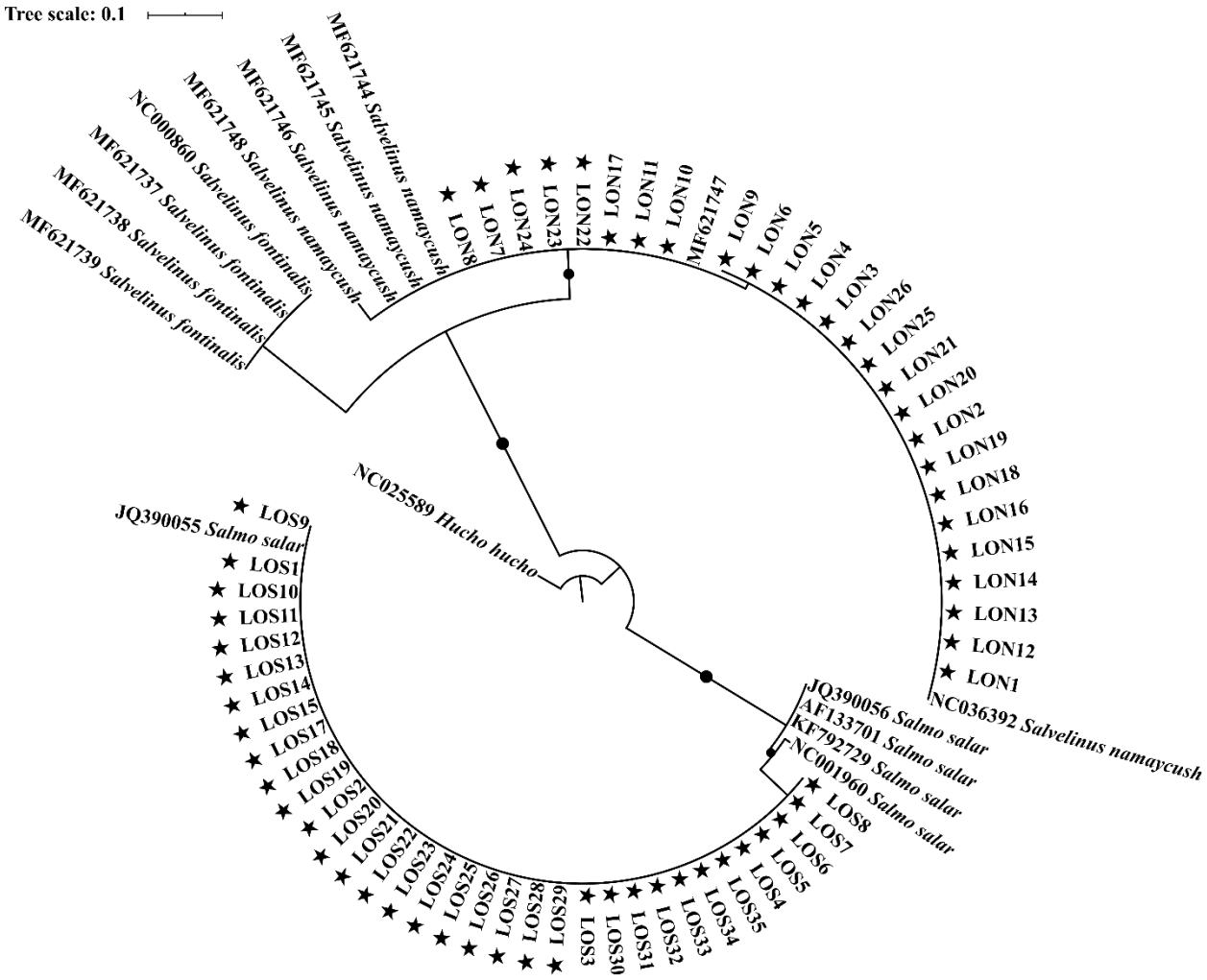


**Figure 4.** Maximum-likelihood phylogenetic trees displaying the relationship between the D-loop sequences obtained from the Antrex samples (denoted with stars) and references sequences (GenBank accession number shown) from all Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) species native to southern Ontario. The tree was rooted using a huchen (*Hucho hucho*) reference sequence as an outgroup. LOS# indicates samples from Antrex zooarchaeologically identified as Atlantic salmon (*Salmo salar*), whereas LON# denotes samples zooarchaeologically identified as lake trout (*Salvelinus namaycush*). The circles indicate nodes with bootstrap support values greater than 50% after 1,000 replications. The scale bar represents the number of nucleotide substitutions per site.

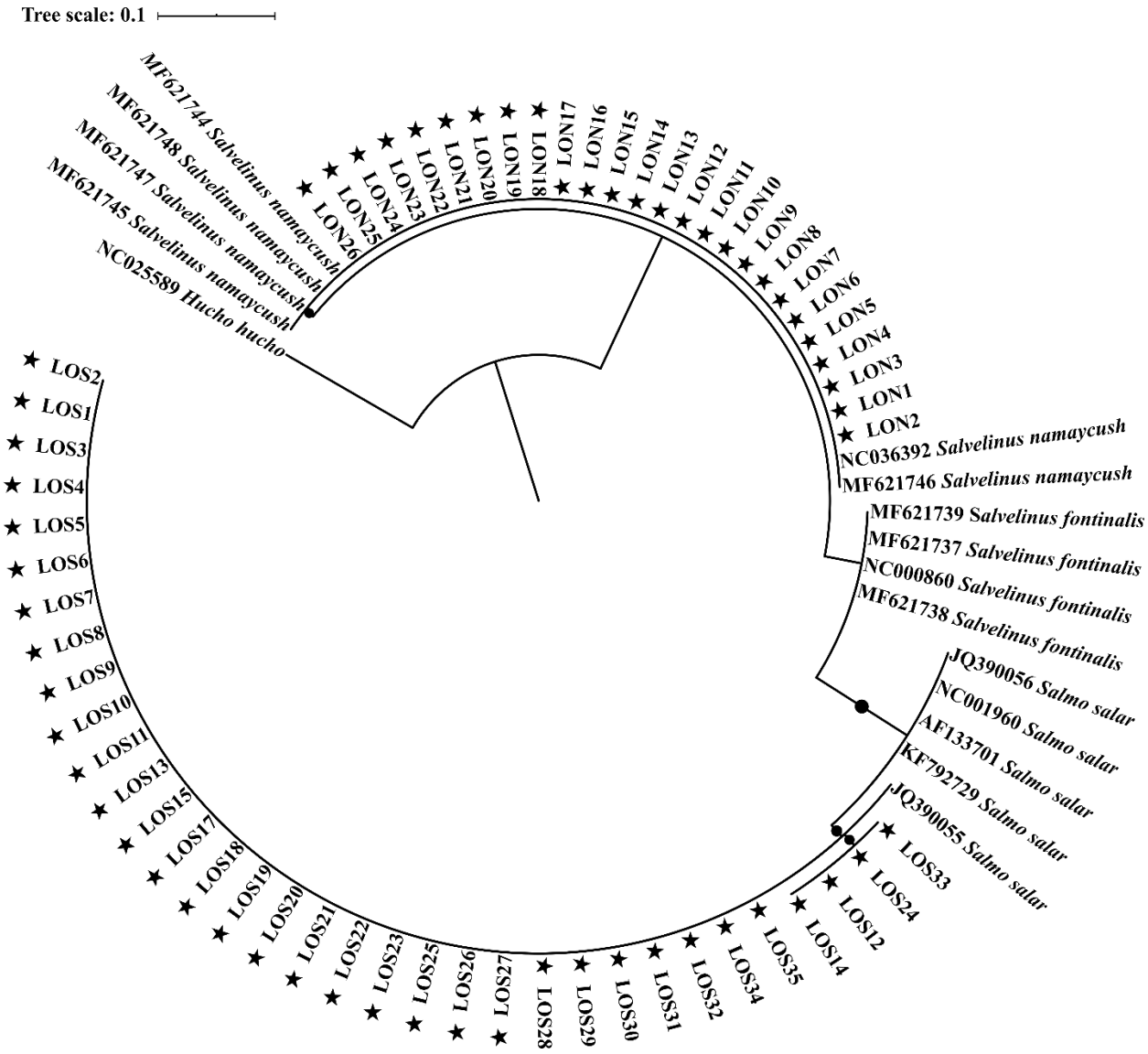


**Figure 5.** Maximum-likelihood phylogenetic trees displaying the relationship between the cytochrome *b* sequences obtained from the Antrex samples (denoted with stars) and references sequences (GenBank accession number shown) from all Atlantic salmonid (*Salmo* spp.) and char (*Salvelinus* spp.) species native to southern Ontario. The tree was rooted using a huchen (*Hucho hucho*) reference sequence as an outgroup. LOS# indicates samples from Antrex zooarchaeologically identified as Atlantic salmon (*Salmo salar*), whereas LON# denotes samples zooarchaeologically identified as lake trout (*Salvelinus namaycush*). The circles indicate nodes with bootstrap support values greater than 50% after 1,000 replications. The scale bar represents the number of nucleotide substitutions per site.

## 5.0 Discussion

*5.1 Authenticity of Ancient DNA Data*

Although archaeological fish remains often exhibit exceptional DNA preservation (Oosting et al., in press), they, like all ancient skeletal remains, are highly susceptible to contamination from exogenous sources of modern DNA (Yang and Watt, 2005). However, various lines of evidence suggest our aDNA data are authentic rather than the result of systematic contamination. First, all pre-PCR laboratory work was conducted in a dedicated aDNA laboratory that is physically separated from modern DNA and post-PCR laboratories (Cooper and Poinar, 2000). Second, prior to DNA extraction, the samples were decontaminated using both bleach and UV irradiation (Yang and Watt, 2005). Third, no DNA was amplified from any of the blank extraction or negative PCR controls, indicating a lack of systematic contamination (Cooper and Poinar, 2000). Fourth, the sex identities assigned to samples were successfully reproduced with two independent PCR assays and two to five replicates of each assay (Cooper and Poinar, 2000). Fifth, analysis of the amplified D-loop and cytochrome *b* fragments yielded identical species identifications for each of the 60 samples that yielded mitochondrial DNA (Yang et al., 2004). Sixth, the DNA-based species identities assigned matched those assigned to them through conventional zooarchaeological methods and ZooMS in the case of four samples, providing independent support for the aDNA data (Yang et al., 2004). Seventh, all repeat DNA extractions produced sex identities as well as D-loop and cytochrome *b* sequences that matched those obtained from the initial extractions (Cooper and Poinar, 2000). Eighth, the successful amplification of DNA from associated passenger pigeon (*Ectopistes migratorius*) remains from Antrex (Guiry et al., 2020), provides supporting evidence for the preservation of DNA in fish remains from the site (Cooper and Poinar, 2000). Finally, with the exception of LOS16, all of the samples (n=27) that underwent stable isotope analysis had well-

454 preserved collagen (Table S1; Guiry et al, in press), indicating that the samples exhibit good
455 overall biomolecular preservation (Cooper and Poinar, 2000).

456 *5.2 Efficacy of Sex Identification Method*

457 In order to be an efficient sex identification method for archaeological or
458 palaeontological remains, PCR-based sex identification methods must be both sensitive and
459 accurate. The high proportion of samples to which we successfully assigned sex identities
460 (83.61%) with our method indicates it is highly sensitive. In this study, we did not assess our
461 method's accuracy by applying it to a large sample of Atlantic salmonids and char of known
462 phenotypic sex. However, the congruence between the sex identifications we assigned to a small
463 sample of modern Atlantic salmon and Arctic char with our method and the validated method
464 described by Yano et al. (2013), suggests our method is reliable. The results of previous studies
465 provide further support for the reliability of our method. Previous studies have demonstrated a
466 strong relationship between Atlantic salmonids' *sdY* genotype and their phenotypic sex
467 (Eisbrenner et al., 2014; King and Stevens, 2019; Quéméré et al., 2014; Yano et al., 2013). For
468 instance, amongst the Atlantic salmon analyzed by Eisbrenner et al. (2014), *sdY* was present in
469 97.66% of analyzed males (n=555) and absent in 98.96% of analyzed females (n=384).
470 Although *sdY* in char has not been as extensively studied, Yano et al. (2013) found a similar
471 strong relationship between *sdY* genotype and phenotypic sex amongst char species, including
472 lake trout. This correspondence between *sdY* genotype and phenotypic sex observed among
473 Atlantic salmonids and char indicates *sdY* is an accurate sex identification marker for these taxa,
474 suggesting our method is reliable. However, males lacking *sdY* and females possessing *sdY* have
475 been documented among char and Atlantic salmonids (e.g., Eisbrenner et al., 2014; Yano et al.,
476 2013), indicating our method is not foolproof.

Several other design aspects of our sex identification method also contribute to its reliability. Critical to our method's reliability is the use of two PCR assays to assign sex identities to samples. By facilitating the detection of Y-chromosome dropout due to degradation (Quéméré et al., 2014; Royle et al., 2018; Taberlet et al., 1996), a common issue in aDNA studies (Kim et al., 2013), the use of two assays reduces false female identifications. False female identifications are further reduced in our method through the co-amplification of an IPC in both assays. The co-amplification of these IPCs provides for ascertaining whether the failure to amplify *sdY* is indeed due to the sample being female or due to a lack of amplifiable DNA as result of inhibition or degradation. However, the co-amplification of the IPCs in the assays can, by outcompeting *sdY*, lead to *sdY* drop-out, resulting in the erroneous identification of males as females (Sinding et al., 2016). Following Royle et al. (2018) and Speller and Yang (2016), our method reduces the probability of the IPCs outcompeting *sdY* by designing the assays to preferentially amplify *sdY*. Both assays promote the preferential amplification of *sdY* by targeting *sdY* fragments shorter than the IPCs fragment and by using a higher concentration of *sdY* primers relative to the IPC primers (Royle et al., 2018; Speller and Yang, 2016). Although these measures promoted the preferential amplification of *sdY*, our data indicates that the amplification of the IPC, but not *sdY*, from male samples did still occur. For example, one of the D-loop/*sdY* and two of the Clock1a/*sdY* PCR replicates performed for LON7, which was identified as male, failed to amplify *sdY* but amplified the IPC (Table S3). However, the performance of PCR replicates for both assays enabled the identification of instances of *sdY*, and in the case of females, IPC dropout, that could influence the sex identification results, minimizing their effect. In addition to the above factors, the drop-out of *sdY* and subsequent misclassification of male salmonid samples as females can also occur as a result of primer–

template mismatches (King and Stevens, in press). To an extent, by using different *sdY* primers in each assay, our method mitigates the potential for such misidentification related to primer–template mismatches (Royle et al., 2018; Szpak et al., in press).

On top of being an efficient sex identification method for Atlantic salmonid and chars remains the method described in this study is also useful for species identification. Through the sequence analysis of the D-loop fragment co-amplified as an IPC in the D-loop/*sdY* assay, we were able to assign species-level identifications to 60 of the 61 samples. As this fragment exhibits intra-specific variation amongst both Atlantic salmon and lake trout, analysis of this fragment may also shed light on the historic genetic diversity of these taxa. Here, we confirmed the D-loop species identities assigned to these 60 samples through the amplification and analysis of a fragment of cytochrome *b.* Although not needed for species identification, the amplification and analysis of cytochrome *b* functions as an internal reproducibility test useful for detecting contamination (Yang et al., 2004). Any discrepancies between the species identities indicated by these D-loop and cytochrome *b* fragments might be indicative of contamination (Yang et al., 2004).

*5.3 Sex-Selectivity of Antrex's Atlantic Salmon and Lake Trout Fisheries*

At Antrex, neither the aggregate sex ratio nor the sex ratio obtained for the individual species were significantly male-biased. In the case of lake trout, the Antrex fishery appears to have targeted males and females relatively equally, whilst female fish appear to have been to some extent preferentially harvested by the site's Atlantic salmon fishery. This suggests the site's Middle Ontario Iroquoian inhabitants did not preferentially target male Atlantic salmon and lake trout, and, by inference, did not manage these salmonids through male-selective fishing. The lack of evidence at Antrex for the management of Atlantic salmon and lake trout through male-

selective fishing is potentially the product of a myriad of factors. These include the fishing technologies used by the site's inhabitants, a lack of pronounced sexual dimorphism amongst lake trout, natural biases in Atlantic salmon sex ratios, and the local abundance of both species.

### *5.3.1 Fishing Technology*

Traditionally, gillnets were commonly used by the Wendat and other Indigenous peoples in the Great Lakes region to harvest salmonids, particularly chars and whitefish (*Coregonus* spp.) (Cleland, 1982; Tooker, 1964). Gillnets are nets suspended in the water column that passively ensnare fish that fall within a narrow size range, with the size range of ensnared fish being determined by the net's mesh gauge (Hubert et al., 2012). Fish that are substantially larger than a gillnet's mesh gauge are unable to breach the net, whilst small fish are able to slip through the net without being ensnared (Hubert et al., 2012). However, as male and female Atlantic salmon and lake trout often overlap considerably in size (e.g., Halttunen et al., 2013; Miller and Kennedy, 1948), it is potentially difficult to preferentially target individuals from these taxa belonging to a specific sex with gillnets. Nonetheless, through regular monitoring of gillnets and the release of salmonids belonging to an undesired sex, gillnets could potentially be operated in a sex-selective manner. Historic Wendat fishers, however, often left gillnets in place for extended periods of time (Tooker, 1964), which reduces the potential for the release of undesired individuals by increasing mortality among ensnared fish (Buchanan et al., 2004). The unbiased lake trout sex ratio at Antrex might reflect its inhabitants' reliance on similar gillnetting strategies with limited sex-selective capabilities to harvest this species. However, there is scant direct evidence for the use of gillnets by Antrex's inhabitants. Nonetheless, the presence of bone netting needles at the site indicates fishing nets—potentially gillnets—were manufactured and/or mended, and hence used, by its inhabitants (Cooper, 2010).

546 *5.3.2 Degree of Sexual Dimorphism*

547 Due to their greater accessibility and predictability during their spring to fall spawning
548 run, Ontario Iroquoians likely harvested Atlantic salmon as they migrated upstream from Lake
549 Ontario (Hawkins et al., 2019; Holm et al., 2009). Similarly, lake trout were likely harvested
550 during the fall, when they aggregate on shoals in Lake Ontario in order to spawn (Holm et al.,
551 2009; Martin and Olver, 1980; Needs-Howarth and Thomas, 1998). During this spawning period,
552 lake trout, unlike many other salmonids, exhibit relatively muted sexual dimorphism (Martin and
553 Olver, 1980). Notably, spawning male lake trout do not typically develop the prominent kype
554 seen among spawning males belonging to other salmonid species (Royce, 1951). Male lake trout
555 do develop dark bands during the spawning season that set them apart from females, but only for
556 a very brief period (Martin and Olver, 1980; Royce, 1951). In addition, whilst breeding tubercles
557 are a male-specific trait in some lake trout populations, this trait is not universally male-specific,
558 with females also developing breeding tubercles in some populations (Martin and Olver, 1980).
559 By impeding the ready sex identification of individual lake trout, this lack of pronounced,
560 sustained, and consistent, sexual dimorphism may have hampered Middle Ontario Iroquoians'
561 ability to fish sex-selectively for this species. As spawning Atlantic salmon exhibit pronounced
562 sexual dimorphism (Fleming and Einum, 2011), this hypothesis likely does not account for the
563 lack of a male-selective Atlantic salmon fishery at Antrex.

564 *5.3.3 Naturally Biased Sex Ratios*

565 Amongst some modern Atlantic salmon populations, the sex ratio of spawning runs has
566 been observed to temporally vary (Harvey et al., 2017; Pérez et al., 2005). Reflecting females'
567 earlier migration timing, Atlantic salmon spawning runs in some populations have been observed
568 to be female dominated during the early portion of the spawning season (Dahl et al., 2005;

Harvey et al., 2017; Pérez et al., 2005; Sparholt et al., 2018). As the spawning season progresses, and males begin to migrate in larger numbers, spawning runs become less female-biased (Harvey et al., 2017; Pérez et al., 2005). During the peak of the spawning run, the sex ratio may be relatively unbiased, yet it may become male-biased following this peak, with some males persisting in spawning creeks throughout the winter (Harvey et al., 2017; Holm et al., 2009).

In modern recreational fisheries targeting Atlantic salmon spawning runs, the sex demographics of harvested salmon often mirror those of the spawning run at their time of harvest (Harvey et al., 2017; Pérez et al., 2005). Consequently, assuming they were harvested during their spawning runs in a non-sex-selective manner, the sex ratios of archaeological Atlantic salmon assemblages may provide insights into when they were harvested. Although not statistically more abundant, the predominance of female rather than male Atlantic salmon at Antrex might reflect the harvesting of salmon early in their spring to fall spawning run when females may have been more prevalent. Support for such targeting of early-run Credit River salmon by Indigenous fisheries can be found in Euro-Canadian historic records. In a diary entry dated June 16$^{th}$, 1796, Elizabeth Simcoe (Robertson, 1911:328) reported that Indigenous people congregated along the Credit River "at this season to fish for salmon." While likely referring to Mississauga rather than Iroquoians related to Antrex's inhabitants, Simcoe's statement does indicate Indigenous peoples did harvest the early-run salmon that migrated up the Credit River during the spring. Alternatively, the predominance of female Atlantic salmon at Antrex could also reflect females being incidentally harvested in larger numbers due to the Credit River run, similar to some modern populations (Fleming, 1998), having a female-biased sex ratio, regardless of season.

Historically, the condition of Atlantic salmon running up the Credit River and other nearby Lake Ontario tributaries appear to have seasonally varied, which may have influenced the timing of Antrex's Atlantic salmon fishery. During the early nineteenth-century, Thomas W. Magrath (1833:299) described salmon taken from the Credit River during the spring as being in "fine" condition and "firm and full of curd". Similarly, Samuel Wilmot (1872:79) later in the century remarked that spring running salmon in the nearby Humber River were "rich and fat in flesh, in prime condition" while fall running salmon were "lean and lank, out of condition." The early-run timing potentially suggested by Antrex's female-dominated Atlantic salmon sex ratio, might reflect a strategy to maximize access to prime condition salmon. However, we stress additional samples from Antrex need to be analyzed in order to confirm the female-bias of the site's Atlantic salmon fishery.

*5.3.4 Local Abundance*

Historical records suggest Atlantic salmon and lake trout were potentially abundant in the vicinity of the Antrex site during its occupation. For instance, the nearby Credit River was historically described as supporting one of the largest Atlantic salmon runs on the north shore of Lake Ontario (Dymond et al., 2019; Parson, 1973). Although lake trout were likely less abundant than Atlantic salmon, they are hypothesized to have been quite abundant in the lake (Elrod et al., 1995; Smith, 1995). As Antrex was likely only occupied by approximately 400 people for roughly 20 years (Robertson and Williamson, 2002), the fishing pressure exerted by the site's inhabitants may have been insufficient to depress these locally abundant Atlantic salmon and lake trout stocks. Since only 11,000 Iroquoians are estimated to have occupied south-central Ontario during the early Middle Ontario Iroquoian period, when Antrex was occupied (Warrick, 2008), regional fishing pressures may have also been relatively minimal. Without resource

depression of the locally abundant Atlantic salmon and lake trout stocks, there may have been little incentive for Antrex's inhabitants to manage them through male-selective fishing (Alvard and Kuznar, 2004). Alternatively, other management strategies, such as the ethnographically documented tenure systems (Thoms, 2004), seasonal closures (Tiro, 2016), and a fishing theology (Sioui, 1999), may have also been sufficient to maintain the productivity of local Atlantic salmon and lake trout stocks.

## 6. Conclusion

Here, we reported on a DNA-based sex identification method for archaeological Atlantic salmonid and char remains that is adapted from a method developed for ancient Pacific salmonid remains. This method assigns sex identities to samples through two PCR assays that screen for the presence of the Y-linked *sdY* gene and IPCs consisting of D-loop or *Clock1b* fragments. Reflecting this method's efficiency and sensitivity, we were able to assign sex identities to 51 of the 61 analyzed Atlantic salmon and lake trout remains from the Antrex site. By sequencing the D-loop fragment co-amplified as IPC and additional cytochrome *b* fragment, this method also allowed for species identifications to be assigned to 60 of the remains. Although only applied to remains from a single site in Ontario, this DNA-based sex identification method likely has applicability to Atlantic salmonid and char assemblages from sites across their global range. Moreover, the high proportion of salmonid remains assigned sex identities in this study and that of Royle et al. (2018) highlights the potential for PCR-based sex identification methods for other fish taxa represented in zooarchaeological assemblages. Similar PCR-based sex identification methods can potentially be developed for the remains of other fish taxa, such as Atlantic cod (*Gadus morhua*) (Kirubakaran et al., 2019), and sablefish (*Anoplopoma fimbria*) (Rondeau et al., 2013), whose putative master sex-determining genes have also been identified.

637 The sex identification data generated in this study suggests Antrex's Middle Ontario
638 Iroquoian inhabitants did not manage Atlantic salmon and lake trout fisheries through male-
639 selective fishing. As Ontario Iroquoian fishing strategies geographically varied due to differing
640 local environmental conditions (Hawkins et al., 2019), this lack of male-selective fishing may
641 not have been universal among Middle Ontario Iroquoians. Likewise, mirroring temporal
642 changes in other aspects of Ontario Iroquoian fishing strategies (Hawkins et al., 2019), the sex-
643 selectivity of fisheries may have also temporally varied in response to changing cultural and
644 environmental conditions. Documenting such potential geographic and temporal variation in the
645 sex-selectivity of Ontario Iroquoian fisheries will require the analysis of remains from sites from
646 other regions and time periods. Conducting such studies will provide insights into the factors that
647 influenced the sex-selectivity of Ontario Iroquoian Atlantic salmon and lake trout fisheries.

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