# This document provides supplementary material for the article titled:

# Genomic population structure aligns with vocal dialects in palm cockatoos (*Probosciger aterrimus*); evidence for refugial late-Quaternary distribution?

**Authors:** Miles V. Keighley\*, Robert Heinsohn, Naomi E. Langmore, Stephen A. Murphy, and Joshua V. Peñalba.

*\* Corresponding author. E-mail: miles.keighley@anu.edu.au*

# Supplementary methods sections

## DNA sequencing

We used a modified version of the hyRAD protocol to screen the nuclear genome for anonymous SNPs (Suchan *et al.* 2016). The hyRAD protocol uses double-digest restriction digest (ddRAD) sequencing libraries as probes for a sequence capture to screen for thousands of SNPs (Peterson *et al.* 2012; Suchan *et al.* 2016). As the hyRAD protocol has been described in detail by Suchan et al. (2016), we focus on the modifications. The hyRAD protocol converts a ddRAD library into hybridization probes to capture anonymous loci across the genome (Figure S1). This “hybrid” protocol uses the SNP discovery capability of ddRAD but bypasses the limitation of ddRAD being unable work on highly-degraded samples since it requires in-tact fragments between cut sites. Sequence capture methods have proven to be more effective for highly-degraded samples, therefore, the combination of the two methods allows for SNP discovery and genotyping many samples into one efficient procedure. Unfortunately, without an available reference genome it is difficult to predict the number of loci which comprises the probe set a priori.

We used the Iron Range samples for probe design as the DNA was extracted from blood and had the highest quality. A standard ddRAD library preparation protocol was carried out using the restriction enzymes *PstI* and *EcoRI* (Peterson *et al.* 2012). For each sample, two parallel reactions containing 500ng of DNA were digested with *PstI* and *EcoRI*. Standard ddRAD adapters, described in Peterson *et al*. (2012), were ligated onto the digested DNA. After the adapter ligation step, all five samples were pooled together and we used a LabChip XT to select for a size range of 345-407 bp. Half of the output from the LabChip XT proceeded to a standard ddRAD library while the other half was converted into probes for hyRAD-like capture.

The half that were converted into ddRAD libraries were amplified and indexed with external Illumina barcodes for sequencing. For the subsample to be turned into probes, we amplified the libraries using internal IS7 (5’ ACACTCTTTCCCTACACGAC 3’) and IS8 (5’ GTGACTGGAGTTCAGACGTGT 3’) primers to generate enough probe DNA. The probes were deadapterized with another restriction digest of *PstI* and *EcoRI*. Finally, we attached short biotinylated adapters, specifically designed to bind only to the restriction sites, using NEB Quick Ligase. The probes were stored in -20°C until the capture was ready.

To prepare shotgun genomic libraries from the feather and toe-pad samples, we used the protocol outlined by Meyer and Kircher (2010). All samples except for those from the Iron Range were prepared as genomic libraries. The raw DNA quality was checked using an agarose gel. From the agarose results, only the feather samples needed additional shearing. The feather samples were sheared using a Diagenode BioRuptor on high for 6 cycles of 15 seconds on and 90 seconds off prior to library preparation. All samples underwent a double-ended bead size selection before and after the library preparation to reduce DNA fragments < 200bp and > 500bp. Samples from the different tissue type (toe-pads, and feathers) were pooled separately into three 2ug pools. Within each pool, the samples (5 toe-pad, 4 toe-pad, and 18 feather samples) were pooled equimolarly.

To prepare for the capture, the library pools were dried down completely and we added a hybridization mix similar to Peñalba *et al.* (2014). We added 25uL Agilent Hybridization Buffer, 5uL 10X Agilent blocking agent, 5uL Hybloc Chicken, 3uL of a blocking oligo mix, and 12uL containing 500ng of the biotinylated ddRAD probes. The reaction was incubated in 95°C for 10 minutes to denature the DNA and probes and incubated at 65°C for 48 hours to perform the hybridization. We cleaned 20uL of Streptavidin beads using 1X TEN buffer according the hyRAD protocol. We resuspended the cleaned Strepatividin beads with the 50uL hybridization mix and incubated for 30 min at 23°C to attach the biotin to the Streptavidin. We removed the supernatant and cleaned the beads using the SSC/SDS buffers according to the hyRAD specifications. To melt the libraries off the beads, we added 30uL of water to the cleaned beads and incubated the reaction in 95°C for 5 minutes. We used 15uL of this to amplify using IS5 (5’ AATGATACGGCGACCACCGA 3’) and IS6 (5’ CAAGCAGAAGACGGCATACGA 3’) for sequencing. Finally, we sequenced the hyRAD and ddRAD libraries using 47% and 6%, respectively, of a high-throughput NextSeq500 lane for 150bp, paired-end in the ACRF Biomolecular Resource Facility.

The whole mitochondrial genome was recovered as by-catch from the sequence capture, we then sequenced an additional fragment of the mitochondrial ND2 (NADH dehydrogenase subunit 2) gene for the Iron Range samples, which were not included in the capture pools. We amplified ND2 using the primers L5204 (5’ TAACTAAGCTATCGGGCGCAT 3’) and H6312 (5’ CTTATTTAAGGCTTTGAAGGCC 3’)(Sorenson *et al.* 1999). We used this gene as it is proposed to be fast-evolving (Pacheco *et al.* 2011) and to use a different mitochondrial gene from previous studies (Murphy *et al.* 2007). Lastly, we sequenced the amplified fragments using an ABI 3100.

## Data processing

The hyRAD and ddRAD-derived data sets were filtered differently. Since the hyRAD data is from a sequence capture, we utilized the first two scripts from the existing pipeline (https://github.com/MVZSEQ/SCPP) to filter the raw reads. The scripts trim adapters using cutadapt, remove low quality bases using Trimmomatic, merge reads using FLASH, and filter contaminants using Bowtie2. For details on the scripts see Peñalba *et al.* (2014). For the ddRAD-derived data set, we used a custom python script to filter out low complexity reads (reads comprising >50% of the same base and with trailing Guanine bases) common in NextSeq500 data. Finally, we utilized Trimmomatic to trim off low quality bases and the first 9 bp which contains the individual barcodes and restriction cut sites (Bolger *et al.* 2014). The resulting cleaned reads were used for remaining analyses.

The cleaned Iron Range ddRAD data were used for marker discovery and to assemble the nuclear reference sequence set. For each sample, we used the assembler Rainbow (default settings) which is specifically designed to assemble paired-end ddRAD data sets (Chong *et al.* 2012). To finalize the reference contig set we used vsearch to cluster homologous contigs between the individual Rainbow assemblies (Rognes *et al.* 2016). The same reference contig set was used to map all the individuals. For the mitochondrial sequences, we performed a de novo assembly of the samples AMNH781401, AMNH619297, AMNH619295, AMNH425703, and AMNH425700 using SOAPdenovo (Luo *et al.* 2012) and the final assemblies script in (https://github.com/MVZSEQ/SCPP). We used BLAST in the assembled contig to find the mitochondrial genome and used the contig from AMNH781401 as a reference (Altschul *et al.* 1990). Finally, Bowtie2 was used to map the cleaned reads to the reference contig set (Langmead and Salzberg 2012).

For low-coverage data-sets, using genotype likelihoods performs better for calculating population genetic statistics compared to direct genotyping (Nielsen *et al.* 2011; Nielsen *et al.* 2012). ANGSD was used for SNP filtering and genotype likelihood calculations (Korneliussen *et al.* 2014). We employed multiple filters to obtain high quality SNPs for analyses. We only used contigs with a minimum coverage of 2x. For the population filter, at least 3 (out of 5) and 10 (out of 27) individuals within the Iron Range and Cape York Peninsula + Papua New Guinea populations should pass the coverage filter, respectively. We used tools within ngsTools to find SNPs that overlapped between the two populations so the genetic distances are not biased to SNPs that are were only genotyped within a single population (Fumagalli *et al.* 2014). Lastly, we filtered against contigs with >5 SNPs which may be putative repeat or paralogous region (< 1% of the contigs). Only unlinked SNPs (one SNP per locus) were carried through to the population structure and all SNPs within all loci were carried through for the other population genetic statistics.

To recover the corresponding ND2 sequence from the mitochondrial genomes, we extracted a fasta file per mitochondrial genome alignment. Any bases that were suspected to be heterozygotes or had coverage lower than 10X were converted to ambiguous Ns. We used a local BLAST alignment to find the sequence fragment that corresponded to the Sanger sequenced ND2 sequence (Altschul *et al.* 1990). Finally, we aligned the samples using MAFFT (Katoh *et al.* 2002; Katoh and Standley 2013).

## Population structure and statistics

We recovered the nuclear genome population structure using ngsDist in the ngsTools kit (Fumagalli *et al.* 2014; Vieira *et al.* 2016). We used the genotype likelihood output of ANGSD as input for ngsDist to incorporate the uncertainty in the distance measures. Finally we used the distance matrix produced by ngsDist to create a network in SplitsTree (Dress *et al.* 1996). Lastly, we used the getMDS.R in ngsTools to summarize the distance information using multidimensional scaling (MDS). For the ND2 population structure, we visualized the haplotype network using a minimum spanning network in PopArt (Leigh and Bryant 2015).

We then used ngsAdmix to try to detect additional population structure and estimate admixture between populations (Skotte *et al.* 2013). We ran ngsAdmix from K = 2 to K = 5 with 10 replicates for each K. We then used the standard deviation of the replicates within each K to select the best number of clusters. Finally, we used CLUMPP to combine the different replicates within K = 2 and K = 3 (Jakobsson and Rosenberg 2007).

We used the allele frequencies from ANGSD to calculate population genetics summary statistics. We used FST (Reynolds *et al.* 1983) to estimate population differentiation between the Iron Range population and the Cape York Peninsula + Papua New Guinea population with ANGSD’s realSFS. To calculate population divergence (dxy) we used calcDxy.R in ngsTools. The calculation uses the allele frequencies from the genotype likelihoods and the following equation

dxy = ∑(fA1 \* (1 - fA2) + (fA2 \* (1 - fA1)) / n

where fA1 is the allele frequency in one population, fA2 is the allele frequency in the other population and n is the sequence length. Lastly, we calculated per site heterozygosity (θ) and per site nucleotide diversity (π) for each population using ngsTools and ANGSD. Since we didn’t have an ancestral reference sequence, we used a folded site frequency spectrum to obtain θ. Lastly, we calculated divergence after population split (DA) using the equation

DA = Dxy – (π1 + π2) / 2.

## A note on formal analysis of gene flow

Unfortunately, estimating gene flow parameters with the data available in this study will yield inaccurate estimates for multiple reasons. Firstly, migration (and other demographic) parameter estimates from RAD data is highly sensitive to preprocessing (see Shafer *et al.* 2017), and although all the processing may be appropriate to the data the parameter estimates remain elusive. The sampling of the Iron Range population is too low and would not contain enough information for formal SFS-based migration estimates (Excoffier *et al.* 2013). Although the methods described above would yield an estimate, preliminary ABC analysis suggest that the data is not informative enough for parameter estimation. Although formal analysis of gene flow would greatly improve the inferential power of this study, the current state of both the sampling and available methods are likely to lead to inaccurate and unreliable estimates. Estimates of gene flow were therefore not included to maintain the conservative nature of claims made.

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**Figure Captions**

**Figure S1.** Methodology pipeline.

**Tables**

Table S1. List of samples and their corresponding tissue types and geographic source.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | CATALOGUENUMBER | COLLEC-TION | | TISSUE TYPE | | YEAR | | REGION | | SPECIFIC LOCALITY | | LATITUDE | | LONGITUDE | |
| ANU17 | | Field | Blood | | 1999 | | CYP, Australia | | Iron Range | | -12.67632 | | 143.34909 | |
| ANU19 | | Field | Blood | | 1999 | | CYP, Australia | | Iron Range | | -12.66113 | | 143.32266 | |
| ANU21 | | Field | Blood | | 1999 | | CYP, Australia | | Iron Range | | -12.67703 | | 143.34976 | |
| ANU24 | | Field | Blood | | 2000 | | CYP, Australia | | Iron Range | | -12.67584 | | 143.35144 | |
| ANU25 | | Field | Blood | | 2000 | | CYP, Australia | | Iron Range | | -12.80072 | | 143.33482 | |
| AMNH781401 | | AMNH | Toe pad | | 1948 | | Papua New Guinea | | Aroa | | -8.890675\* | | 146.963524 | |
| AMNH619297 | | AMNH | Toe pad | | 1898 | | Papua New Guinea | | Aroa | | -8.890675\* | | 146.963524 | |
| AMNH619295 | | AMNH | Toe pad | | 1903 | | Papua New Guinea | | Aroa | | -8.890675\* | | 146.963524 | |
| AMNH425703 | | AMNH | Toe pad | | 1936 | | Papua New Guinea | | Fly R, Upper | | -6.981407\* | | 141.024615\* | |
| AMNH425700 | | AMNH | Toe pad | | 1936 | | Papua New Guinea | | Fly R, Lower | | -8.715992\* | | 142.943354\* | |
| AMNH619319 | | AMNH | Toe pad | | 1912 | | CYP, Australia | | Bamaga | | -10.8928\* | | 142.3848\* | |
| AMNH619315 | | AMNH | Toe pad | | 1912 | | CYP, Australia | | Bamaga | | -10.8928\* | | 142.3848\* | |
| AMNH619316 | | AMNH | Toe pad | | 1912 | | CYP, Australia | | Bamaga | | -10.8928\* | | 142.3848\* | |
| AMNH619318 | | AMNH | Toe pad | | 1912 | | CYP, Australia | | Bamaga | | -10.8928\* | | 142.3848\* | |
| ANU201 | | Field | Feather | | 2013 | | CYP, Australia | | Wenlock R, West | | -12.38074 | | 142.18012 | |
| ANU203 | | Field | Feather | | 2014 | | CYP, Australia | | Weipa | | -12.623796 | | 141.887573 | |
| ANU205 | | Field | Feather | | 2013 | | CYP, Australia | | Archer R, Central | | -13.4058 | | 142.60457 | |
| ANU207 | | Field | Feather | | 2014 | | CYP, Australia | | Archer R, Central | | -13.42912 | | 142.69478 | |
| ANU208 | | Field | Feather | | 2014 | | CYP, Australia | | Archer R, Central | | -13.40582 | | 142.53998 | |
| ANU210 | | Field | Feather | | 2014 | | CYP, Australia | | Wenlock R, Central | | -12.45514 | | 142.64272 | |
| ANU211 | | Field | Feather | | 2014 | | CYP, Australia | | Archer R, Central | | -13.20916 | | 142.61003 | |
| ANU216 | | Field | Feather | | 2012 | | CYP, Australia | | Piccaninny Creek | | -13.19264 | | 142.69425 | |
| ANU217 | | Field | Feather | | 2014 | | CYP, Australia | | Mapoon | | -12.02647 | | 141.8863 | |
| ANU218 | | Field | Feather | | 2014 | | CYP, Australia | | Mapoon | | -12.02132 | | 141.9044 | |
| ANU221 | | Field | Feather | | 2013 | | CYP, Australia | | Wenlock R, West | | -12.38074\* | | 142.18012\* | |
| ANU223 | | Field | Feather | | 2015 | | CYP, Australia | | Wenlock R, West | | -12.38074\* | | 142.18012\* | |
| ANU224 | | Field | Feather | | 2015 | | CYP, Australia | | Wenlock R, West | | -12.38074\* | | 142.18012\* | |

Abbreviations used: ANU Australian National University; AMNH American Museum of Natural History; CYP Cape York Peninsula.

\*Approximate coordinates

Locations of data sampling for species distribution models on next page.

Table S2. Locations of data sampling for species distribution models post filtering

|  |  |
| --- | --- |
| Longitude | Latitude |
| 130.16670 | -1.83330 |
| 131.00000 | -1.33330 |
| 131.00000 | -0.33330 |
| 135.53330 | -0.80000 |
| 138.06670 | -5.38333 |
| 141.58500 | -3.38900 |
| 141.88000 | -13.27000 |
| 142.53330 | -10.68330 |
| 142.66670 | -4.50830 |
| 142.85000 | -4.21670 |
| 142.91600 | -11.96600 |
| 142.69480 | -13.42910 |
| 143.13330 | -12.76670 |
| 143.31670 | -12.76670 |
| 143.31670 | -6.39170 |
| 143.46110 | -13.82780 |
| 143.46670 | -13.83330 |
| 145.16670 | -7.76670 |
| 145.38510 | -7.88750 |
| 145.73330 | -5.43333 |
| 146.13330 | -7.80000 |
| 146.14720 | -8.10220 |
| 146.46670 | -6.80000 |
| 149.71250 | -10.02500 |
| 149.85000 | -9.76667 |
| 146.90170 | -8.98384 |
| 141.24390 | -7.07230 |
| 141.71630 | -7.86745 |
| 142.43770 | -10.85070 |
| 142.64270 | -12.45510 |
| 141.85430 | -12.64890 |
| 142.54000 | -13.40580 |
| 142.61000 | -13.20920 |
| 141.88620 | -12.02650 |

Table S3. Summary statistics of sequencing reads and missing data. The coverage is an average among all loci with at least 2x coverage.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| CAT NO. | FILTERED READS | MAPPED READS | PERCENT RECOVERED LOCI | COVERAGE |
| ANU17 | 2153539 | 1475231 | 0.98 | 246 |
| ANU19 | 8251378 | 5748911 | 0.99 | 1607 |
| ANU21 | 1152254 | 844106 | 1.00 | 146 |
| ANU24 | 405772 | 301921 | 0.94 | 58 |
| ANU25 | 668822 | 488312 | 0.98 | 112 |
| AMNH781401 | 310302 | 126341 | 0.53 | 195 |
| AMNH619297 | 4761184 | 1003960 | 0.76 | 497 |
| AMNH619295 | 4868602 | 954323 | 0.79 | 457 |
| AMNH425703 | 9048132 | 2846844 | 0.87 | 1286 |
| AMNH425700 | 5219503 | 1323435 | 0.82 | 652 |
| AMNH619319 | 12813460 | 2736715 | 0.89 | 1150 |
| AMNH619315 | 13322206 | 3866471 | 0.92 | 1551 |
| AMNH619316 | 12960175 | 2787101 | 0.92 | 1193 |
| AMNH619318 | 13673497 | 4894845 | 0.90 | 1989 |
| ANU201 | 6540785 | 1594054 | 0.84 | 749 |
| ANU203 | 8425545 | 2262999 | 0.89 | 1041 |
| ANU205 | 7270660 | 2322985 | 0.86 | 1081 |
| ANU207 | 10143725 | 3163808 | 0.89 | 1465 |
| ANU208 | 2317548 | 414331 | 0.67 | 249 |
| ANU210 | 8446025 | 2320476 | 0.87 | 1027 |
| ANU211 | 8186678 | 3328563 | 0.87 | 1493 |
| ANU216 | 8883537 | 2361250 | 0.87 | 1024 |
| ANU217 | 8682085 | 3267926 | 0.84 | 1502 |
| ANU218 | 9133104 | 3835430 | 0.80 | 1842 |
| ANU221 | 7637002 | 2115553 | 0.85 | 981 |
| ANU223 | 2290255 | 118321 | 0.42 | 114 |
| ANU224 | 6136192 | 1777166 | 0.85 | 855 |