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Phylogeography of the iconic Australian pink cockatoo, *Lophochroa leadbeateri*

Running title: Phylogeography of the pink cockatoo

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Abstract

The pink cockatoo (*Lophochroa leadbeateri*; or Major Mitchell's cockatoo) is one of Australia's most iconic bird species. Two subspecies based on morphology are separated by a biogeographical divide, the Eyrean Barrier. Testing the genetic basis for this subspecies delineation, clarifying barriers to gene flow and identifying any cryptic genetic diversity will likely have important implications for conservation and management. Here, we used genome-wide SNPs and mitochondrial DNA data to conduct the first range-wide genetic assessment of the species. The aims were to investigate the pink cockatoo's phylogeography, characterise conservation units and reassess subspecies boundaries. We found consistent but weak genetic structure between the two subspecies based on nuclear SNPs. However, phylogenetic analysis of nuclear SNPs and mitochondrial DNA sequence data did not recover reciprocally monophyletic groups, indicating that the subspecies are not evolutionarily distinct. Consequently, we have proposed that the two currently recognized subspecies be treated as separate management units rather than evolutionarily significant units. Because poaching is suspected to be a threat to this species, we assessed the utility of our data for wildlife forensic applications. We demonstrated that a subspecies identification test could be designed using as few as twenty SNPs.

Key words

conservation genetics - *Lophochroa leadbeateri* - phylogeography - population genomics - wildlife forensics - wildlife trade

24 # Introduction

25 The pink cockatoo (also known as the Major Mitchell's cockatoo), *Lophochroa leadbeateri*
26 (Vigors, 1831), is an iconic bird species endemic to Australia. It is considered by many to be
27 the most beautiful and spectacular of the cockatoos (Cacatuidae; Rowley & Chapman, 1991;
28 Schodde, 1994), having pink-white plumage and an impressive bright red, yellow and white
29 crest. The pink cockatoo is a hardy species that occurs in low densities throughout Australia's
30 harsh arid and semi-arid regions.

31

32 Within the pink cockatoo's wide yet patchy distribution, four core breeding regions are
33 apparent (Blakers *et al*., 1984; Fig. 1a). Although previous authors have recognised a variable
34 number (0-4) of subspecies (ssp; e.g. Mathews, 1912 – 3 ssp; Peters, 1937 – 4 ssp; Condon,
35 1975 - 0 ssp; Hall, 1974 – 3 ssp; Schodde, 1997 – 2 ssp), two subspecies*, L. l. leadbeateri* and
36 *L. l. mollis* (*cf* Forshaw & Cooper, 1981) have been generally accepted since the publication
37 of Schodde's 1994 study (Fig. 1a) on the basis of body size and colour and pattern of the
38 crest. These subspecies are separated by the Eyrean Barrier (Fig. 1a): a well-documented
39 biogeographic barrier in southern Australia for a range of bird species (Ford, 1974; Schodde,
40 1982; Kearns *et al.*, 2009; Dolman & Joseph, 2012). *Lophochroa leadbeateri leadbeateri* is
41 east of the Eyrean Barrier and has a more prominent yellow band in its crest and is larger in
42 body size, while *L. l. mollis* is west and north of the Eyrean Barrier (Schodde 1994, 1997;
43 Forshaw & Cooper, 2002).

44

45 Despite its wide distribution, the pink cockatoo is of conservation concern. In the eastern part
46 of its distribution it is listed as Vulnerable (New South Wales and Queensland - Biodiversity
47 Conservation Act, 2016; Nature Conservation Wildlife Regulation, 2006), or Threatened
48 (Victoria - Flora and Fauna Guarantee Act, 1988; Walker *et al.*, 1999) (see Fig. 1a for State

49 localities). The species' abundance and range in north-western Victoria and western New
50 South Wales have been greatly reduced through the removal of habitat, in particular the loss
51 of hollow-bearing trees (Garnett *et al.*, 2011). Like other cockatoo species, the pink cockatoo
52 is unable to excavate its own hollows for nesting and so requires naturally occurring tree
53 hollows (Mackowski, 1984; Cameron, 2007). Further, increased agriculture and clearing of
54 feeding habitat have impacted the species, particularly in the southwest of its range in the
55 Western Australian wheat belt region (Rowley & Chapman, 1991). Another threat to this
56 species is poaching (Forshaw & Cooper, 1981; Higgins, 1999), which Rowley & Chapman
57 (1991) found to impact the most critical stage of the species' life cycle: recruitment of young.
58 Poaching is directly linked to demand for the species in the illegal pet trade. Together, these
59 factors indicate a need for improved understanding of phylogeographic patterns within the
60 species to aid in the conservation management of the species.
61
62 Genomic tools allow researchers to investigate how genetic diversity is distributed among
63 populations. They may help to identify and manage at-risk populations. Characterising
64 discrete units of genetic variation, termed conservation units (Ryder, 1986), and clarifying
65 barriers to gene flow within the pink cockatoo will facilitate conservation strategies that
66 maximize the evolutionary potential of the species (Frankham *et al*., 2010). The putative
67 subspecies barrier, the Eyrean Barrier, comprises the Flinders Ranges and Lake Eyre Basin
68 (Schodde, 1982). It is thought to have limited dispersal during the Plio-Pleistocene due to the
69 presence of vast lakes associated with the Lake Eyre Basin, and then in the Pleistocene due to
70 extreme aridity (Ford & Parker, 1973; Ford, 1974; Schodde, 1982; Joseph *et al.*, 2006).
71 However, the timing and strength with which the Eyrean Barrier has separated populations
72 within species is known to vary between avian taxa (Schodde, 1982; Dolman & Joseph,
73 2012; McElroy *et al*., 2018). Whether the morphological differences between pink cockatoo

74 subspecies at this barrier reflect underlying genetic divergence and potential conservation
75 units is unknown. Schodde (1994) suggested that there is currently no dispersal between
76 subspecies over this barrier, and that the two may even warrant recognition at species rank.
77 Further, it is unknown whether cryptic genetic structure exists across other well-characterised
78 southern Australian arid-zone biogeographic barriers within the pink cockatoo distribution,
79 such as the Nullarbor and Murravian Barriers (see Schodde & Mason, 1999). The impact of
80 these biogeographic barriers varies considerably between species (Neaves *et al*., 2012).
81
82 Clarifying the species' evolutionary history and intraspecific taxonomy have been
83 problematic due to a combination of poor sampling, relatively weak morphological
84 divergence across the species (e.g. see Forshaw, 2011), and the need to disentangle patterns
85 of geographical, sexual and age-related variation. Genomic analyses have the potential to
86 help to characterize conservation units, investigate connectivity among core breeding
87 populations, and resolve lingering taxonomic uncertainties about subspecies boundaries
88 (Baumsteiger *et al*., 2017; Marie *et al*., 2019; Tonzo *et al*., 2019; Ewart *et al*., 2020).
89 Furthermore, genetic data could facilitate the development of wildlife forensic tools, such as
90 geographical provenance and progeny testing, to increase the capacity for detection and
91 prosecute trafficking crimes involving this species (Walker *et al.*, 1999; Huffman & Wallace,
92 2011). The pink cockatoo is listed under CITES Appendix II, and trade in the species is
93 strictly regulated under Australian legislation.
94
95 Here we perform the first comprehensive phylogeographic assessment of the pink cockatoo
96 to address the topics we have raised above. This builds on two earlier genetic studies
97 involving this species based on allozymes (Adams *et al.*, 1984), and a multilocus nuclear and
98 mitochondrial DNA (mtDNA) data set (White *et al.*, 2011); both used only a few individuals

99 to address the species' systematic position with respect to other cockatoos. Pink cockatoo
100 specimens from across the species' range have been collected over many decades and are
101 stored in museums throughout Australia and elsewhere. Owing to developments in museum
102 genomics, genome-wide data of use in population-level studies can be generated from old
103 museum specimens (Rowe *et al.*, 2011; Ewart *et al.*, 2019). We generated thousands of
104 genome-wide single nucleotide polymorphism markers (SNPs) and sequence data at three
105 mtDNA markers from pink cockatoo frozen tissue and toe pad samples across their entire
106 distribution. We performed comprehensive population genomic analyses to investigate
107 potential barriers to gene flow for the purposes of clarifying taxonomy and informing
108 conservation management. These data can be interpreted in light of the biogeography and
109 palaeoenvironmental history of Australia's arid and semi-arid zones, and compared to the
110 steadily increasing body of phylogeographic analyses of species having broadly similar
111 distributions across southern Australia (Neaves *et al.*, 2009; Dolman & Joseph, 2012, 2015;
112 Engelhard *et al.*, 2015; Ansari *et al.*, 2019).
113

114 Methods

115 *Sample acquisition and DNA extractions*

116 We acquired pink cockatoo frozen tissue (frozen liver/muscle) (*n*=45) and toe pad (*n*=51)
117 samples from across their distribution (Fig. 1a & Table S1). Samples were obtained from: the
118 Australian National Wildlife Collection, Canberra (ANWC); the Australian Museum, Sydney
119 (AM); Museum Victoria, Melbourne (MV); and the Western Australian Museum, Perth
120 (WAM). Collection dates for these samples ranged from 1883 to 2011 (Table S1).
121

122 Thinly sliced toe pads (~2 mm thick) were sampled from traditional museum specimens, and
123 DNA was extracted following Ewart *et al.* (2019). These DNA extractions were performed in
124 a clean room facility dedicated to historical museum samples likely to have degraded DNA.
125 Genomic DNA was extracted from frozen tissue samples following the manufacturer's
126 protocols for the 'Bioline Isolate II Genomic DNA kit' Bioline (Australia). DNA
127 concentration was measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).
128

129 *SNP genotyping*

130 SNP data was generated using DArTseq™, a reduced representation sequencing method
131 (methods described in Kilian *et al.*, 2012; Cruz *et al.*, 2013). This was performed by
132 Diversity Arrays Technology (DArT) in Canberra, Australia. DArTseq has previously been
133 used to generate SNP data for a range of phylogeographic, phylogenetic, and population
134 genetic studies on vertebrate species (Melville *et al*., 2017). Briefly, different combinations
135 of restriction enzymes were tested, and the *PstI*-*SphI* enzymes were selected for digestion of
136 cockatoo DNA. DNA was then processed as per Kilian *et al.* (2012), using two different
137 adaptors that correspond with the restriction site overhangs, both containing an Illumina flow
138 cell attachment sequence, and one (the *PstI*-compatible adapter) also containing a sequencing
139 primer sequence and varying length barcode region. The library was subject to PCR (using
140 REDTaq DNA Polymerase, Sigma-Aldrich) as follows: initial denaturation at 94°C for 1
141 min, then 30 cycles of 94°C for 20 sec, 58°C for 30 sec and 72°C for 45 sec, and a final
142 extension step at 72°C for 7 min. The library was then normalized and sequenced by first
143 performing a c-Bot (Illumina) bridge PCR, followed by single end sequencing for 77 cycles
144 on an Illumina Hiseq2500.
145

146 The resultant short-read sequences were processed using the DArT Pty Ltd analytical
147 pipelines. First, poor quality sequences were removed (using a Phred score ≥10), and
148 sequences were demultiplexed (using a barcode Phred score ≥30). Second, sequences were
149 trimmed to 69 bp and clustered with a Hamming distance threshold of 3. Low-quality regions
150 from singleton tags were corrected where possible. Third, SNPs were called using the
151 proprietary DArTsoft14 SNP calling pipeline. Real alleles were discriminated from
152 paralogous sequences by assessing a range of parameters including sequence depth, allele
153 count and call rate.
154

155 *SNP filtering*

156 We applied numerous SNP filtering criteria depending on the analysis (following Ewart *et*
157 *al.*, 2019). First, we removed the duplicate/triplicate samples with the highest amount of
158 missing data. Second, we removed potentially erroneous SNPs, and SNPs with a high level of
159 missing data, based on reproducibility (100%) and call rate (>80%), using the R package
160 dartR version 1.0.5 (Gruber *et al.*, 2018). Third, to meet the population genetic assumptions
161 of some analyses, we removed linked SNPs, outlier SNPs that potentially represented loci
162 under selection, and SNPs out of Hardy-Weinberg equilibrium (HWE). To remove linked
163 SNPs to meet the assumption of linkage disequilibrium for some of the analyses, we retained
164 only one SNP per DArTseq locus using the R package dartR. To identify and remove outlier
165 SNPs that are potentially under directional or balancing selection to meet the assumption of
166 neutrality for some analyses, we used LOSITAN (Beaumont & Nichols, 1996; Antao *et al.*,
167 2008). For this analysis, samples were divided into subspecies, then performed 100,000
168 simulations, applying the 'infinite alleles' mutation model, a 0.95 confidence interval and a
169 0.1 false discovery rate. To identify departure from HWE, we used ARLEQUIN version 3.5
170 (Excoffier & Lischer, 2010), implementing 1,000,000 Markov Chain steps and a burnin of

100,000. We removed loci with a p-value <0.01 that potentially deviate from HWE. For this analysis, we considered all samples as one population, which is likely a conservative approach, as we would expect some false positives due to the Wahlund effect.

To investigate whether remnant poor-quality SNPs were skewing results, additional filters were applied to represent a 'stringently filtered' data set, and analyses were repeated. Here, we filtered SNPs for average locus coverage (>20 X) using the R package dartR, and minor allele frequency (MAF) (>0.05) using the R package poppr version 2.6.1 (Kamvar *et al.*, 2014, 2015).

Additionally, to ensure that the inclusion of toe pad samples from old museum specimens did not skew results, SNPs were re-called using only the more contemporary tissue samples (using the SNP calling methods outlined in the previous section). SNPs were subsequently re-filtered. Additional details on SNP filtering methods and variants are provided in the Supplementary Material (Appendix I).

SNP quality control

To quantify genotyping error, we included 18 replicate and 4 triplicate samples among the 96 pink cockatoo samples analysed (indicated in Table S1). We used various replicate/triplicate types to investigate the factors that may influence error, including: frozen tissue replicates (from the same and different DArTseq plates), toe pad replicates (from the same and different DArTseq plates), frozen tissue / toe pad replicates (i.e. a frozen tissue and toe pad from the same individual), and tissue DNA replicates (from the same and different DArTseq plates).

195 We calculated SNP error rates (i.e. the number of SNP mismatches between replicate pairs
196 over the total number of SNPs that were not missing in both replicates) using R functions
197 from Mastretta-Yanes *et al*. (2015). Error rates were calculated pre- and post- SNP-filtering.
198

199 *Generation of mitochondrial DNA sequence data*

200 To generate mitochondrial reference genomes, we performed low-coverage whole genomic
201 sequencing for four pink cockatoo samples (indicated in Table S1), following the NEBNext
202 DNA library preparation protocol, with a pre-treatment of 500 bp shearing using Covaris
203 M220. The libraries were then sequenced on an Illumina MiSeq using paired-end 251 bp
204 sequencing. Library preparation and sequencing were performed at the Monash University
205 Malaysia Genomics Facility (Selangor, Malaysia). The resultant paired sequence reads were
206 trimmed using the BBDuk plugin in Geneious version 10.2.4 (Kearse *et al*., 2012), then
207 assembled using Geneious and NOVOPlasty (Dierckxsens *et al.*, 2017). We then designed
208 primers for the *ND4* and *ND5* genes and d-loop (for *ND2*, we used primers from Sorenson,
209 2003), and amplified and sequenced 15 samples from across the pink cockatoo range
210 (indicated in Table S1). Thus, the mtDNA analyses were carried out using 19 samples (4
211 using low-coverage whole genomic sequencing, and 15 using Sanger sequencing). The d-
212 loop marker was subsequently excluded as it was unable to be reliably sequenced (possibly
213 due to the presence of control region duplications, which are often found in parrot species;
214 Schirtzinger *et al*., 2012; Eberhard & Wright, 2016). Additional details on mitochondrial
215 genome assemblies, primers, PCR conditions and sequencing can be found in the
216 Supplementary Material (Appendix II).
217
218
219

220 *Identifying population structure*

221 We used five methods to investigate population structure present in the SNP genotype data.
222 Details of the different SNP filtering strategies and samples used in the different analyses are
223 provided in the Supplementary Material (Appendix I; Table S1). First, genetic variation was
224 summarized and visualized using a principal coordinates analysis (PCoA). This was
225 performed using the R packages dartR and ade4 version 1.7 (Chessel *et al.*, 2004). Second,
226 STRUCTURE version 2.3 (Pritchard *et al.*, 2000) was used to investigate genetic structure and
227 admixture. For this analysis, we modelled up to five ancestral populations (K=1-5),
228 implementing 10 replicates for each K, assuming admixture and correlated allele frequencies
229 (Porras-Hurtado *et al*., 2013). We ran the analysis for 2 million iterations with a burn-in of 1
230 million. This analysis was parallelized and automated using StrAuto version 1.0 (Chhatre &
231 Emerson, 2017). We considered six different estimators to determine the optimal value of K,
232 generated using StructureSelector (Li & Liu, 2018). Replicate runs were merged and bar plots
233 were generated using CLUMPAK (Kopelman *et al.*, 2015), implemented through
234 StructureSelector. We took a hierarchical approach, whereby the population clusters
235 identified using the full dataset were separated, re-filtered, then run independently. Third, to
236 investigate whether patterns of genetic differentiation derived from continuous (i.e. isolation
237 by distance; IBD) or discrete (e.g. biogeographic barriers) phylogeographic processes, we
238 performed a conStruct analysis (Bradburd *et al*., 2018), implementing the spatial model. A
239 conStruct (i.e. 'continuous structure') analysis is similar to the STRUCTURE analysis, but
240 controls for geographic distance between samples. Based on initial optimization, we ran two
241 independent conStruct analyses, with the 'adapt delta' parameter (the target average proposal
242 acceptance probability) set at 0.85, implementing two chains with 100,000 MCMC iterations
243 for each run. We checked for consistency between chains and independent runs, and visually
244 checked for convergence using the trace plots generated by conStruct. To determine an

245 appropriate level of parameterization, we ran five replicates of a cross-validation analysis
246 comparing the spatial and non-spatial models for $K = 1$-5 for each replicate. We used a
247 random 90% subsample as the training partition, and ran the analysis for 10,000 MCMC
248 iterations.

249

250 Fourth, to measure genetic divergence between subspecies, we calculated pairwise F_{ST} values
251 (Weir & Cockerham, 1984) using the R package hierfstat version 0.4.22 (Goudet & Jombart,
252 2015). F_{ST} values were considered significant if their associated confidence intervals (based
253 on 0.025 and 0.975 quantiles, implementing 1000 bootstraps) did not encompass 0. To
254 investigate differentiation within and between subspecies we performed an AMOVA using
255 the R package poppr, and checked for significance using 10,000 permutations implemented
256 in the R package ade4. To investigate whether any genetic structure patterns were driven by
257 closely related individuals (e.g. cousins), we performed an inter-individual kinship analysis
258 using the R package SNPRelate version 1.14 (Zheng *et al.*, 2012).

259

260 We performed a haplotype network analysis to investigate population structure within the
261 mtDNA sequence dataset. We performed this analysis using PopART (Leigh & Bryant,
262 2015), based on concatenated *ND2*, *ND4* and *ND5* sequences (a total of 2037 bp) and 19
263 samples, implementing the statistical parsimony TCS method (Clement *et al.*, 2000).
264 Additionally, we calculated net nucleotide divergence (*Da*) between the two subspecies based
265 on the mtDNA sequence dataset using the R package strataG version 2.4.905 (Archer *et al.*,
266 2017).

267

268

269

270 *Gene flow patterns*

271 To investigate the influence of geographic distance in our genetic structure results, we
272 investigated the correlation between genetic and geographic distance (i.e. IBD). As there are
273 no discrete sampling sites (reflecting the pink cockatoo's continuous distribution; Fig. 1a),
274 we analysed inter-individual distances. Individual-based genetic distances were based on
275 PCA-based Euclidean distance, following Shirk *et al.* (2017), calculated using 45 principal
276 components (35 when using only fresh tissue samples), and performed using the R package
277 adegenet version 2.1.0 (Jombart, 2008). We then performed a Mantel test using these
278 Euclidean genetic distances and geographic distance (in kilometres) using the R packages
279 adegenet and dartR.
280
281 Due to the ongoing debate surrounding the use of Mantel tests to infer IBD patterns (e.g.
282 Diniz-Filho *et al*., 2013), especially when considering inter-individual distances, we analysed
283 interpopulation gene flow along a transect following methods in Ogden & Thorpe (2002).
284 Indirect gene flow inferences were based on pairwise F_{ST} measurements (calculated as above,
285 but scaled by pairwise geographic distance) between five 'sample clusters' (three individuals
286 per cluster) across Australia, focusing on the putative subspecies barrier (Fig. 3b; Table S1).
287 Willing *et al*. (2012) demonstrated that F_{ST} values can be estimated with relatively small
288 sample sizes when using thousands of SNPs. To complement this analysis of gene flow
289 across the Eyrean Barrier, we ran a conStruct analysis using the same 15 samples in the
290 transect above. We used the same settings as the previous conStruct analysis, except the
291 'adapt delta' parameter was set to 0.7.
292
293
294

295 *Genetic diversity*

296 To measure the genetic diversity within each subspecies, we calculated allelic richness,
297 heterozygosity and private allele counts for each SNP marker. Allelic richness was calculated
298 using the R package PopGenReport version 3.0.4 (Adamack & Gruber, 2014), implementing
299 rarefaction to account for differences in sample size. Observed and expected heterozygosity
300 were calculated using GenAlEx (Peakall & Smouse, 2006, 2012). A count of private alleles
301 per population was calculated using the R package poppr. Mitochondrial DNA diversity was
302 measured in terms of nucleotide diversity, proportion of polymorphic sites, and number of
303 haplotypes using Geneious and the R package pegas version 0.1 (Paradis, 2010).

304

305 *Population growth*

306 To investigate factors that may have caused discordant mtDNA and nuclear DNA clustering
307 patterns (see the '*Results*' section) and to test for population growth, we computed Tajima's
308 D (Tajima, 1989), Fu's Fs (Fu, 1997) and Ramos-Onsin's R2 (Ramos-Onsins & Rozas, 2002)
309 statistics using DnaSP 6.12.03 (Rozas *et al*., 2017), based on mtDNA sequence data (2037 bp
310 of concatenated *ND2*, *ND4* and *ND5* sequences). The significance of the statistics was
311 inferred using coalescent simulations with 1000 replicates. Additionally, a mismatch
312 distribution plot was generated using the R package pegas.

313

314 *Phylogenetic methods*

315 We performed phylogenetic analyses to investigate whether genetic units identified in the
316 population genetic analyses were evolutionarily distinct within a phylogenetic framework.
317 Phylogenetic analyses based on SNPs were performed using SNAPP (Bryant *et al*., 2012),
318 implemented in BEAST version 2.4 (Bouckaert *et al*., 2014), to compare 'species'
319 hypotheses (ESU hypotheses in this case). We used SNAPP to compare the relative support

320 for two models: one enforcing monophyly of each of the two subspecies (which corresponds
321 to two genetic units in population genetic analyses; see Results section), and one without
322 enforcing monophyly. As SNAPP is computationally intensive, we included four individuals
323 per subspecies and 1000 randomly selected SNPs with no missing data from the putatively
324 neutral SNP data set (see Supplementary Material, Appendix I, for more details) to improve
325 computational tractability. We ran SNAPP for 4 million Markov chain Monte Carlo (MCMC)
326 steps, sampling every 1000 steps after a burn-in of 400,000 steps. We used allele frequencies
327 for the forward and backward mutation rates, and the default settings for priors. Model
328 support was subsequently estimated using the AICM (Akaike information criterion through
329 MCMC) method in Tracer version 1.6 (Rambaut *et al*., 2014). AICM was chosen over the
330 preferred stepping-stone and path sampling analyses to improve computational tractability.
331 As AICM has been shown to suffer from poor repeatability (Baele *et al*., 2012), we ran three
332 replicate SNAPP analyses for each model (i.e. three enforcing monophyly of subspecies, and
333 three not enforcing monophyly) and subsequently estimated AICM for each of the six runs.
334
335 To complement the SNAPP analysis, we performed a maximum likelihood phylogenetic
336 analysis using RAxML (Stamatakis, 2014) based on concatenated SNP data (see
337 Supplementary Material, Appendix I). We implemented the GTR substitution model with
338 gamma-distributed rates among sites and the Lewis-type ascertainment bias correction to
339 account for the exclusion of invariant sites, and performed 1000 bootstrap replicates to
340 estimate node support. Trees were rooted using the midpoint method and visualised using
341 Figtree 1.4.2 (Rambaut, 2009).
342
343 We performed a Bayesian phylogenetic analysis of mtDNA data (2,037 bp of concatenated
344 *ND2*, *ND4* and *ND5*) using MrBayes 3.2 (Ronquist *et al*., 2012). This analysis was performed

345 using four independent Markov chains, each run for 100 million steps with a 25% burn-in,
346 and sampled every 100 steps with convergence diagnostics calculated every 100 steps. We
347 implemented the HKY substitution model with gamma-distributed rates among sites.
348 Convergence diagnostics were assessed using Tracer (ESS values <200 were considered
349 inadequate). This analysis was performed with and without an outgroup (*Cacatua pastinator*;
350 GenBank accession: JF414240). Trees were rooted using either the midpoint method or an
351 outgroup, and visualized using Figtree.
352
353 *Testing SNPs for wildlife forensic applications*
354 We filtered a subset of SNPs based on their utility in a geographic provenance assignment
355 test by investigating SNP contributions in a discriminant analysis of principal components
356 (DAPC). DAPC minimizes variation within groups, and maximizes variation between
357 groups. First, we performed DAPC on the entire SNP dataset with no missing data (see extra
358 filtering details in Supplementary data, Appendix I) using the R package adegenet. We
359 considered two populations (K=2), corresponding to separation of the two subspecies, then
360 repeated the analysis considering three populations (K=3) to investigate whether more fine-
361 scale geographic assignment was possible. Second, SNPs were ranked based on their
362 contribution to the clustering analysis. Third, we iterated through decreasing numbers of
363 SNPs (increments of five SNPs) to investigate the minimum number of SNPs required to
364 separate the two subspecies clusters. Finally, we tested the utility of a refined set of SNPs for
365 geographic/subspecies assignment by assigning six randomly selected individuals (three
366 individuals per subspecies) in separate tests using GeneClass2 (Piry *et al.*, 2004). For this
367 analysis, we implemented the frequency-based assignment method (Paetkau *et al.*, 1995) and
368 a 0.05 assignment threshold. The individual being tested was removed from the 'reference'

369 data before each analysis. Likelihood ratios were calculated from the assignment likelihood
370 results, considering different prosecution and defence hypotheses.
371
372 Results
373 *SNP genotyping*
374 Seventy-eight samples were successfully genotyped using DArTseq (Table S1). DNA
375 extracts from one frozen tissue sample (out of 45) and 20 toe pad samples (out of 51) were
376 unsuitable for successful DArTseq library preparation. The oldest sample successfully
377 genotyped was collected in 1912; all samples collected before this date failed. The
378 DArTsoft14 pipeline called 20,324 SNPs from the successfully genotyped 78 samples (with
379 36.32% missing data). This SNP data set was reduced to 4,135 SNPs (with 12.26% missing
380 data) after filtering for quality and missing data, 2,131 SNPs (with 11.78% missing data) after
381 filtering for neutrality and linkage, and 1,279 SNPs (with 10.35% missing data) after
382 applying more stringent filtering (see Supplementary Material, Appendix I for data filtering
383 details, and Table S1 to view which individuals were used in each analyses). When using
384 only the more contemporary tissue samples for SNP calling, the DArTsoft14 pipeline called
385 16,472 SNPs (with 16.79% missing data), which was reduced to 6,466 SNPs (with 3.07%
386 missing data) after filtering for quality and missing data, and to 4,891 SNPs (with 1.95%
387 missing data) after filtering for neutrality and linkage.
388
389 *SNP quality control*
390 Of the 18 replicate and 4 triplicate samples examined, some failed. We found two additional
391 replicate samples based on their genetic signature (i.e. they had different sample numbers and
392 were held in different Museums but they were parts from the same individual in two

393 collections). This was subsequently confirmed with the relevant Museums. Overall, a total of
394 13 replicates and 4 triplicates were used to quantify genotyping error (Table S2).
395
396 Filtering reduced the allele error rate in all samples except one (ANWC B38557; this sample
397 also had a very high proportion of missing data) (Table S1). After filtering, SNP error rates
398 for frozen tissue and DNA replicates/triplicates were all <3%. The SNP error rate and/or
399 shared missing data (missing in both replicates) was particularly high in eight 'toe pad/toe
400 pad' and 'tissue/toe pad' replicates (ranging from 12.10-23.08% and 0.63-97.17%
401 respectively after filtering). Although several problematic samples were removed from many
402 of the population genetic analyses (see Supplementary Material, Appendix I), error in toe pad
403 samples was variable, ranging from 2.87-23.08% in 'toe pad/toe pad' replicates after
404 filtering, hence toe pad samples with relatively high error rates are likely present in some
405 analyses.
406
407 *Genetic structure*
408 The PCoA revealed three distinct clusters: one *L. l. mollis* cluster and two *L. l. leadbeateri*
409 clusters (Fig. 1b). Within *L. l. leadbeateri*, five individuals from central Queensland formed a
410 cluster that was distant from the other samples. Kinship between these individuals was
411 relatively high (0.045-0.144; Table S3) compared to the average kinship of the entire dataset
412 (0.008; excluding self-kinship values), which may distort the level of genetic structure in this
413 region. When removing four out of the five central Queensland samples in a PCoA, the
414 remaining sample clusters with the other *L. l. leadbeateri* individuals (this result is consistent
415 when different central Queensland individuals are used; Figure S1). The only other
416 Queensland individual in the data set, from southern Queensland (see Fig. 1a), clustered with
417 the other *L. l. leadbeateri* samples. There were five other outlier samples. The four outliers

418 near the origin of the PCoA plot (Fig. 1b) are likely explained by their high level of missing
419 data (>70%) (missing data are replaced by the mean allele frequency in the PCoA analysis).
420 The origin of the outlier from the Northern Territory (MV Z50083) is unclear. It may have
421 been either a migrant, an escaped aviary bird from the *L. l. leadbeateri* range, or the result of
422 a processing error (e.g. mislabelling, DNA contamination etc.).
423
424 Genetic variability in the STRUCTURE analysis was best explained using *K*=2-5, depending on
425 the estimator considered (Fig. S2). We present the major modes generated by CLUMPAK for
426 *K*=2 and *K*=3 (Figs 1c, d). The STRUCTURE analysis revealed a clear genetic break between
427 the two subspecies, with the exception of one outlier sample from the Northern Territory
428 (identified in the PCoA; Fig. 1). Individuals from central Queensland were distinct when
429 using *K*=3 (Fig. 1d) and in the analysis based on *L. l. leadbeateri* samples only (Fig. S3a).
430 Similar to the case for the PCoA, this result is likely driven by the relatively high relatedness
431 between these central Queensland individuals. In the STRUCTURE analysis based on *L. l.*
432 *mollis* samples only, subtle population differentiation, although not robustly supported,
433 coincided with samples from the south-western wheatbelt region (Fig. S3b-c).
434
435 Genetic variability in the conStruct analysis was best explained using *K*=2-3 (Fig. S4). Some
436 isolation by distance is evident as the spatial model is preferred over the non-spatial model.
437 In the conStruct analysis using *K*=2, there is clear population differentiation between the two
438 subspecies (excepting the Northern Territory outlier sample identified above; this sample was
439 removed from subsequent analyses; Fig. S5a-b), corroborating the STRUCTURE analysis (Fig.
440 1c-d). There was slight variability in the admixture plots between different chains and
441 independent analyses, however the main patterns were consistent (we present one chain from
442 each independent analysis; Fig. S5a-b). Inadequate convergence and consistency between

443 chains/analyses when using K=3 indicated that the results were unreliable at this level of
444 parameterization.
445
446 Relatively low but significant genetic differentiation was evident between the two subspecies
447 (F_{ST} = 0.039; confidence interval: 0.035, 0.042). In the AMOVA based on the full dataset
448 (i.e. 56 individuals and 2131 SNPs), the proportion of genetic variation within individuals
449 was 69.8%. This is significantly lower than expected based on random permutations ($p <$
450 0.001). The proportion of genetic variation within and between subspecies (25.8% and 4.4%,
451 respectively) were, however, both greater than expected ($p < 0.001$) (Table S4 & Fig. S6).
452 These patterns are indicative of population structure, and not a single panmictic population.
453 In the PCoA, STRUCTURE, F_{ST} and AMOVA analyses, use of different SNP datasets (i.e.
454 SNPs based on only tissues, and SNPs that underwent more stringent filtering) exhibited very
455 similar results (Figs S6, S7, S8 & Tables S4, S5).
456
457 Ten haplotypes were observed from the 19 mtDNA samples that were sequenced (i.e. 2,037
458 bp of concatenated *ND2*, *ND4* and *ND5* genes; Table S7). The haplotype network analysis
459 based on mtDNA exhibited a star-like pattern (Fig. 2a). A central haplotype predominated,
460 while other haplotypes were connected by the common haplotype. The common central
461 haplotype comprises individuals from both subspecies from across the species range. The
462 mtDNA *Da* between subspecies was 0.004%. Overall, mtDNA structure did not reflect
463 patterns found in SNP clustering analyses.
464

465 *Gene flow patterns*

466 The inter-individual Mantel tests revealed significant IBD when analysing the full dataset and
467 when analysing only more contemporary frozen tissue samples (all $p < 0.001$) (Fig. S9).

468 However, inter-individual genetic distances were found to be relatively invariable (note the
469 near-horizontal relationship between genetic and physical distance in Fig. S9a). Relatively
470 low genetic distances across Australia indicate that differentiation among geographic
471 locations is weak. Further, in some cases, spatial patterns inferred from Mantel tests are
472 problematic (Legendre & Fortin, 2010; Legendre *et al.*, 2015). We did not consider mtDNA
473 in this analysis, as mtDNA is known to produce unreliable IBD results (Teske *et al.*, 2018).
474
475 There was a reduction in gene flow between the 'sample clusters' spanning the putative
476 subspecies along the transect (Fig. 3). Although the level of differentiation was relatively
477 low, all pairwise F_{ST} estimates along the transect were significant except for one (between
478 'cluster 1' and '2'; see Fig. 3b). The conStruct analysis based on these 15 transect samples
479 corroborated the other population structure analyses. Clear genetic differentiation was evident
480 between the two subspecies (Fig. S5c-d). Although there was slight variability between the
481 independent analysis and separate chains, the main population structure patterns were
482 consistent.
483

484 *Genetic diversity*

485 *Lophochroa leadbeateri mollis* had the highest genetic diversity for all metrics, although not
486 considerably higher than *L. l. leadbeateri* (Table 1). Genetic diversity measurements varied
487 when using different SNP datasets, but were qualitatively consistent (Table S6). As expected,
488 when applying more stringent filtering (including a MAF filter), the number of private alleles
489 and allelic richness decreased. Without subspecies divisions, mtDNA nucleotide diversity
490 was 0.0012 (Table S7); *ND2* was considerably more diverse than *ND4* and *ND5*.
491
492

493 *Population growth*

494 Analyses of 'randomness', 'neutrality', Tajima's D (-1.851), Fu's Fs (-4.865) and Ramos-
495 Onsin's R2 (0.052), were all significant ($p < 0.05$ in each case). The unimodal mismatch
496 distribution (with a high value at zero mismatches) of the mtDNA data also indicates the
497 occurrence of an expansion event (Figure S10; Rogers & Harpending, 1992). These results
498 are consistent with a scenario of rapid growth in population size.

499

500 *Phylogenetics*

501 The SNAPP model for which monophyly was not enforced received the highest support
502 (Table S8). AICM was relatively consistent between replicates, ranging from 16838.7 to
503 16846.6 for model one (monophyly not enforced), and from 16874.8 to 16879.1 for model 2
504 (monophyly enforced). The two subspecies each exhibited monophyly in the RAxML
505 analysis (excepting the one aforementioned outlier sample from Northern Territory), although
506 bootstrap support was relatively low (i.e. 73%; Figure S11). These results indicate that the
507 existence of two ESUs corresponding each of the two subspecies are not unambiguously
508 supported.

509

510 Similar to the haplotype network analysis, phylogenetic analysis of mtDNA did not
511 correspond to the SNP population structure results and did not exhibit any discernible
512 geographic patterns (Fig. 2b, S12).

513

514 *Wildlife forensics*

515 The initial DAPC used for SNP selection clearly separated the two subspecies (Fig. 4a), in
516 line with the other genetic structure analyses. We retained 35 principal components for this

517 analysis. The minimum number of SNPs required to separate the subspecies via DAPC was
518 twenty (Fig. 4b). We considered adequate separation when all samples were correctly sorted
519 into their corresponding subspecies clusters. We retained five principal components when
520 performing the DAPC using twenty SNPs. When considering three populations (K=3), the
521 central Queensland individuals formed a separate cluster having no overlap but only when
522 ≥75 SNPs were utilized (Fig. S13). It should be noted, however, that this clustering is likely
523 driven by the high relatedness between these central Queensland samples.
524
525 The GeneClass2 analyses correctly assigned all six individuals with high support. When
526 assigning an individual to the correct subspecies (e.g. claiming a *L. l. leadbeateri* individual
527 was *L. l. leadbeateri*), all likelihood ratios were >28.71, and averaged 1.81×10^{7} (Table S9).
528 The likelihood ratios were higher when assigning *L. l. mollis* than when assigning *L. l.*
529 *leadbeateri*, averaging 3.54×10^{8} and 8.23×10^{6} respectively. When assigning an individual to
530 the incorrect subspecies (e.g. claiming an *L. l. leadbeateri* individual was an *L. l. mollis*
531 individual), all likelihood ratios were $<3.48 \times 10^{-2}$, and averaged 5.81×10^{-3}.
532

533 # Discussion

534 We have performed the first comprehensive phylogeographic study of one of Australia's
535 most charismatic but relatively understudied parrots, the pink cockatoo. Our extensive data
536 set revealed two major genetic clusters corresponding to the currently recognized subspecies,
537 and an additional, divergent cluster comprising closely related Central Queensland members
538 of *L. l. leadbeateri* (importantly, this cluster disappeared when only one representative was
539 used). We use these results to reassess the species' conservation priorities and taxonomy,
540 which are currently based on morphology.
541

542 *Population structure*

543 *Lophochroa leadbeateri* is a widespread species that does not have defined geographically
544 disjunct population isolates. Our SNP data show consistent but relatively weak levels of
545 genetic structure between the two currently recognized subspecies at the Eyrean Barrier. It is
546 important to determine whether this result is derived from historical biogeography (i.e. the
547 Eyrean Barrier) or sampling gaps (i.e. IBD) as has been highlighted by several authors (Latch
548 *et al*., 2014; Bradburd *et al*., 2018; Chambers & Hillis, 2020). We found that genetic structure
549 between the two subspecies based on SNPs was apparent even when accounting for
550 geographic distance (Figs. 3 & S5). Contrastingly, distinct subspecies clusters were not
551 apparent in the mtDNA analyses. This is possibly due to incomplete lineage sorting and/or
552 higher female dispersal, and is consistent with the weak and/or recent phylogeographic
553 structure across the continent inferred by the SNP analyses. Large effective population sizes
554 retaining ancestral variation even after long periods of isolation and/or recent divergence
555 times could potentially preclude signals of population divergence in mtDNA (Hartl *et al.*,
556 1997; Maddison, 1997).
557
558 The significant population expansion result, further evidenced by the star-like haplotype
559 network (Fig. 2a), may have proliferated the frequency of a common haplotype and explain
560 the absence of distinct geographically disjunct haplotype clusters. The common haplotype
561 (see Fig. 2a) comprised individuals from across the species' range, including an individual
562 from central Queensland (B28102) and individuals from south-west Western Australia
563 (A35378, Z23813 and B53847), indicating that the species has the capacity to disperse over
564 long-distances. However, the weak differentiation detected by SNPs indicates that the Eyrean
565 barrier may have limited dispersal, similar to other vertebrate species found in this region
566 (Neaves *et al*., 2012; McElroy *et al*., 2018).

567

568 Overall, these data suggest that the Eyrean barrier has been either a somewhat effective,
569 although relatively recent biogeographic barrier to gene flow in this species, or a more long-
570 term but porous barrier. The subtle morphological divergence between subspecies reported
571 by Schodde (1994) is consistent with a relatively recent divergence time. Morphological
572 differences can accumulate rapidly in bird taxa, often before mtDNA genetic divergence
573 (Zink & Barrowclough, 2008; Safran *et al.*, 2016).

574

575 The weak substructure evident within each of the two subspecies is consistent with relatively
576 regular gene flow between members of the four core breeding populations (Fig. 1a). In *L. l.*
577 *leadbeateri*, the genetic differentiation we identified between individuals from central
578 Queensland individuals and all other populations is likely an artefact of analysing related
579 individuals. Although the relatively high relatedness between these individuals may be due to
580 actual genetic structure in this region (i.e. higher levels of inbreeding in a genetically isolated
581 population), it is more likely that individuals from a family unit were sampled. All five
582 central Queensland individuals were collected in the same region, four of which were
583 collected three days apart (while the other was collected ~3 years later), and the kinship
584 analysis suggests these individuals could be second- and/or third-order relatives (Table S3).
585 In *L. l. mollis*, there is limited genetic differentiation between the population in the south-
586 westernmost 'wheatbelt' area and other populations (Fig. S3b). This population inhabits
587 mulga shrubland, and was previously considered a separate subspecies (Peters, 1937).
588 However, the genetic structure in this region is subtle and inconsistent; notably, some of the
589 associated samples do have high levels of missing data. Analysing additional geographically
590 intermediate samples may help clarify the presence of potential cryptic genetic diversity

591 within the two subspecies, and hence elucidate management strategies to conserve their
592 genetic variation.
593
594 The pink cockatoo's shallow phylogeographic structure across its range corresponds to that
595 seen in some other Australian arid zone bird species (Joseph & Wilke, 2006; Dolman &
596 Joseph, 2015). Engelhard *et al.* (2015), for example, found mtDNA genetic structure, albeit
597 weak, in another cockatoo in the same subfamily (Cacatuinae), the galah (*Eolophus*
598 *roseicapilla*). However, there are numerous examples of similarly distributed bird species
599 that do exhibit more marked genetic differentiation across much the same range, such as the
600 copper-backed and chestnut quail-thrush (*Cinclosoma clarum* and *C. castanotum*,
601 respectively), the white-eared honeyeater (*Nesoptilotis leucotis*), the splendid fairy-wren
602 (*Malurus splendens*), and the Australian ringneck (*Barnardius zonarius*) (Joseph & Wilke,
603 2006; Kearns *et al.*, 2009; Dolman & Joseph, 2015, 2016). We recently found evolutionarily
604 distinct isolates within arid zone populations of another inland cockatoo species, the red-
605 tailed black-cockatoo *Calyptorhynchus banksii*. In that case the southwestern 'wheatbelt'
606 population was found to be genetically and taxonomically distinct (Ewart *et al.*, 2020).
607 Varying responses to biogeographical barriers among the pink cockatoo and these other arid
608 bird taxa are likely due to differences in habitat specificity and vagility (Toon *et al.*, 2007).
609

610 *Conservation implications*

611 Robust delineation of conservation units is vital for effective conservation prioritization.
612 Conservation units can be apportioned as either management units (i.e. a demographically
613 independent unit of genetic variation; Moritz, 1994; Palsbøll *et al.*, 2007) or evolutionarily
614 significant units (i.e. independently evolving units of genetic variation; Ryder, 1986; Moritz,
615 1994). Based on the genetic structure results presented above, the two subspecies should be

616 considered separate management units. Given the lack of support for two evolutionarily
617 distinct clades (i.e. they do not exhibit reciprocal monophyly) in the phylogenetic analysis
618 based on nuclear SNPs, the low F_{ST} values, and the lack of mtDNA support, these
619 conservation units do not appear to constitute separate ESUs.
620
621 Assessing population fragmentation within each of the two subspecies is critical, as small
622 isolated populations often suffer from genetic erosion (Frankham *et al*., 2017). The additional
623 substructure we identified in central Queensland could indicate that this population is at risk
624 of genetic isolation, although it is likely that the genetic differentiation detected in this region
625 is likely driven by high relatedness among the samples examined (see above). Denser
626 sampling of unrelated individuals, and geographically wider sampling to fill gaps in this
627 study should be implemented to clarify the genetic structure in this region and determine
628 whether or not it should be regarded as separate management unit.
629

630 *Taxonomic reassessment*

631 Incorrect delineation of subspecies can misguide subsequent studies and conservation
632 strategies (Zink, 2004; Braby *et al.*, 2012; Huang & Knowles, 2016). Typically, different
633 subspecies exhibit at least some mtDNA phylogenetic resolution (e.g. Kearns *et al.*, 2015,
634 2016). Net divergence, *Da*, at the mtDNA *ND2* gene between the two nominal pink cockatoo
635 subspecies was only 0.009%. In several other avian species that exhibit ND2 differentiation
636 at the Eyrean Barrier, the value is much higher. Examples include the white-eared honeyeater
637 (2.23%; Dolman & Joseph, 2015), the mulga parrot subspecies (1.92%; McElroy *et al*.,
638 2018), and the Australian ringneck (1.72%; Joseph & Wilke, 2006). Accordingly, the
639 minimal mtDNA differentiation may be taken to suggest that the species is monotypic (i.e.,
640 no subspecies). Conversely, a lack of mtDNA-based subspecies divergence does not

641 necessarily justify/dictate taxonomic modifications (Ball & Avise, 1992; Funk & Omland,
642 2003; Omland *et al.*, 2006). Traits other than genetics and morphology, including
643 vocalizations, ecological characteristics, and frequency of subspecies hybrids, can be taken
644 into account (Remsen, 2005; also see Ford & Parker, 1973). Therefore, although they may
645 not be evolutionary distinct genetically (i.e. they may not represent separate ESUs), we
646 advocate continued recognition of two subspecies within the pink cockatoo.
647

648 *Wildlife forensics implications*

649 The generation of SNP data and the population genetic inferences presented in this study
650 could facilitate the development of wildlife forensic techniques for the pink cockatoo (Ogden,
651 2011). Typically, a species or subspecies identification test is based on analysis of mtDNA
652 due its high mutation rate, lack of recombination, availability of homologous reference data,
653 and the ease with which it is amplified and sequenced (Linacre & Tobe, 2011; Johnson *et al.*,
654 2014). However, the lack of reciprocal monophyly of subspecies/populations in our analyses
655 of mtDNA loci means they may not be suitable for performing a subspecies identification or
656 geographic provenance tests. Any forensic testing of pink cockatoo subspecies should
657 therefore rely on nuclear DNA markers. We have provided proof of concept that reliable
658 population identification testing can be performed in this species using as few as 20 SNPs (all
659 likelihood ratios were >28 when the prosecution hypothesis was correct). Including more
660 SNPs and samples would intuitively yield greater assignment power and confidence.
661 Furthermore, different SNPs could be selected that are more informative to identify
662 individuals in certain subregions. Being able to identify source populations will help direct
663 enforcement and compliance resources to areas most vulnerable to illegal collection and
664 allow repatriation of seized animals to their subspecies/population of origin (Alacs &
665 Georges, 2008). This study serves as an example on how to construct subspecies/population

666 identification or geographic provenance tests for species with relatively shallow phylogenetic
667 structure.
668
669 Additionally, the SNPs generated in this paper could be utilized in the development of
670 parentage testing. A parentage test of offspring along with their putative mother and/or father
671 could determine in an investigation whether a pink cockatoo is wild or captive-bred. SNPs
672 with a high MAF are particularly useful for parentage analysis (Andrews *et al.*, 2018); even
673 after filtering for missing data, locus quality, outliers, HWE, and linkage, the dataset
674 contained 176 SNPs with a MAF >0.4 (data not shown).
675

676 *Benefits and caveats of this genome-wide SNP data set*

677 This study serves as another example of genome-wide SNP data being able to resolve
678 populations where mtDNA and/or relatively few nuclear markers lacked resolution or were
679 misleading (Leslie & Morin, 2016; Rodríguez-Ezpeleta *et al.*, 2016; Younger *et al.*, 2017).
680 Utilizing many genetic markers alleviates issues faced when basing important conservation
681 decisions and/or taxonomy on a small number of markers. Further, we have harnessed
682 advancements in museum genomics to successfully genotype numerous old museum
683 specimens (toe pads) collected over decades with reasonably high success (61% of toe pads
684 were successfully genotyped), which provided critical representation of the species'
685 distribution (Fig. 1a).
686
687 Utilizing SNPs generated from old museum specimens, however, presents a number of
688 issues. Even after filtering, some toe pad genotypes had high SNP error rates and
689 considerable missing data (Table S2). Although some troublesome genotypes were removed
690 from most analyses (Appendix I), given the variability of the error rates of toe pad samples,

691 some samples with relatively high error rates may not have been excluded during the filtering
692 stages. The error and missing data in these old museum specimens are expected to be biased
693 towards low diversity SNPs, and random errors are expected to homogenize genetic structure
694 (Ewart *et al.*, 2019). Further, even though the pink cockatoo is a very long-lived species
695 (Brouwer *et al.*, 2000), it is possible that the genetic structure may have changed over the
696 sampling period (i.e. genotyped individuals were sampled between 1912 and 2011). To
697 ensure these biases did not lead to false population genetic inferences, we demonstrated that
698 comparable results were obtained when analysing a SNP dataset called exclusively from
699 more contemporary tissue samples. These results corroborate the value of using toe pad
700 samples genotyped with this platform to support spatial conclusions, but may present
701 problems for temporal inferences (Ewart *et al.*, 2019).
702
703 *Conclusion*
704 This is the first species-wide genetic study on the pink cockatoo. The extensive dataset
705 provides a basis for effective conservation management for this species. Well-informed
706 management strategies based on genetics can now be implemented with the aim of
707 maximising the species' genetic diversity and its potential to adapt to changing environments
708 (Frankham, 2005; Huffman & Wallace, 2011). Further, preliminary analyses with these data
709 indicate they could be successfully used in the development of a wildlife forensic toolbox to
710 detect and prosecute trafficking crimes associated with this species.
711
712 The phylogeographic analyses we have performed represent a robust approach for
713 investigating species that are widespread, yet have shallow phylogeographic structure. SNPs
714 revealed subtle patterns of genetic differentiation that were not detected through analysis of
715 mtDNA and morphology.

716

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731

732 Collection of pink cockatoos for research purposes of this project was approved by the
733 Australian Museum Animal Care and Ethics Committee (approval number 16-02). All earlier
734 samples had been collected under all appropriate ethics and scientific collecting permits.
735 Sulphur-crested cockatoo samples were obtained as part of another project, under the ethics
736 permit AEC 151020-0. Transfer of pink cockatoo DNA from Australia to Malaysia was
737 authorized under CITES permit number PWS2018-AU-000019.

738

739 Conflict of interest: none

740

741 # Data accessibility

742 SNP data and mtDNA sequence data will be made available on Dryad Digital Repository and
743 GenBank respectively upon acceptance.

744

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746

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1093

1094 # Figure and table legends

1095

1096 **Figure 1.** (a) The distribution of *Lophochroa leabeateri leadbeateri* (blue) and *L. l. mollis*
1097 (orange) in Australia, adapted from Schodde (1994) and Menkhorst et al. (2017), and
1098 localities of the frozen tissue samples (stars) and toe pads samples (circles) genotyped in this
1099 study. The thick grey line represents the Eyrean Barrier, the darker shading represents core
1100 breeding zones, and the lighter shading and blurred fringes represent areas of potentially
1101 sparser distribution and/or non-breeding based on records from the Atlas of Living Australia
1102 database (https://www.ala.org.au; accessed 4 November 2020). (b) A PCoA plot for 57 pink
1103 cockatoo individuals using 4,135 SNPs. (c) and (d) STRUCTURE plots for 57 pink cockatoo
1104 individuals based on 2,131 SNPs when K=2 and K=3, respectively. The bottom-left photo is
1105 of *L. l. leadbeateri*, Mt. Hope, NSW. Photo: Corey Callaghan.

1106
1107 **Figure 2.** (a) TCS-based haplotype network analysis based on 19 pink cockatoo individuals
1108 using 2,037 bp of concatenated *ND2*, *ND4* and *ND5* genes. (b) Phylogeny of the pink
1109 cockatoo based on mtDNA data (see Table S1 for samples details), isolated from Fig. S12a
1110 (outgroup removed for clarity). Bayesian posterior probabilities are given above relevant
1111 branches. The 'CQ' and 'SW' labels next to the haplotypes (a) and taxon names (b) represent
1112 samples from central Queensland and south-west Western Australia respectively (see Fig. S3
1113 for additional details). NB: the common haplotype in the haplotype network (a) contains
1114 haplotypes from both *L. l. leadbeateri* populations and the south-western Western Australia
1115 *L. l. mollis* population, but not the more north-easterly *L. l. mollis* population.
1116
1117 **Figure 3.** (a) Genetic divergence of populations along a transect based on inter-population
1118 pairwise $F_{ST}/(1 - F_{ST})$ calculated using 2,131 SNPs, divided by pairwise geographic distance,
1119 and plotted against the midpair distance of adjacent localities. (b) For this analysis, 15 pink
1120 cockatoo individuals were divided into five 'sample clusters' (3 individuals per cluster) along
1121 a transect. The vertical dotted red line in (a) indicates the pairwise comparison across the
1122 putative subspecies barrier.
1123
1124 **Table 1.** Genetic diversity measurements based on 2,131 SNPs in 56 pink cockatoo
1125 individuals, and 2,037 bp of concatenated *ND2*, *ND4* and *ND5* genes in 19 pink cockatoo
1126 individuals. Genetic diversity was measured within subspecies. Note, the haplotype common
1127 to both subspecies (see Fig. 2a) was counted twice in the 'number of haplotypes.'
1128
1129 **Figure 4.** DAPC analyses showing separation between *Lophochroa leadbeateri mollis*
1130 (orange) and *L. l. leadbeateri* (blue). The analyses were based on 49 pink cockatoo
1131 individuals using (a) 1,307 SNPs, and (b) 20 informative SNPs.
1132