



Original Article

Koalas (*Phascolarctos cinereus*) From Queensland Are Genetically Distinct From 2 Populations in Victoria

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Abstract

The koala (*Phascolarctos cinereus*) suffered population declines and local extirpation due to hunting in the early 20th century, especially in southern Australia. Koalas were subsequently reintroduced to the Brisbane Ranges (BR) and Stony Rises (SR) by translocating individuals from a population on French Island descended from a small number of founders. To examine genetic diversity and north-south differentiation, we genotyped 13 microsatellite markers in 46 wild koalas from the BR and SR, and 27 Queensland koalas kept at the US zoos. The Queensland koalas displayed much higher heterozygosity ($H_o = 0.73$) than the 2 southern Australian koala populations examined: $H_o = 0.49$ in the BR, whereas $H_o = 0.41$ in the SR. This is consistent with the historical accounts of bottlenecks and founder events affecting the southern populations and contrasts with reports of high genetic diversity in some southern populations. The 2 southern Australian koala populations were genetically similar ($F_{ST} = 0.018$, $P = 0.052$). By contrast, northern and southern Australian koalas were highly differentiated ($F_{ST} = 0.27$, $P < 0.001$), thereby suggesting that geographic structuring should be considered in the conservation management of koalas. Sequencing of 648 bp of the mtDNA control region in Queensland koalas found 8 distinct haplotypes, one of which had not been previously detected among koalas. Queensland koalas displayed high mitochondrial haplotype diversity ($H = 0.753$) and nucleotide diversity ($\pi = 0.0072$), indicating along with the microsatellite data that North American zoos have maintained high levels of genetic diversity among their Queensland koalas.

Subject areas: Population structure and phylogeography, conservation genetics, and biodiversity

Key words: bottleneck, genetic diversity, microsatellites, mitochondrial DNA, population structure

Introduction

Koalas in Australia have experienced periods of population decline, local extirpation, and over hunting throughout their history. In the early 20th century, koala fur gained popularity among consumers and millions were hunted for their pelts (Martin et al. 1999). By the 1930s, the species was on the brink of extinction in southern Australia, with populations nearly extirpated in Victoria and completely extirpated in mainland South Australia (Jackson 2007), although offshore island populations were established by the translocation of koalas. However, many island populations were founded by only a few individuals; for example, French Island was stocked using only 2–3 koalas (Jackson 2007). The severe population bottlenecks led to inbreeding and low genetic diversity on the islands. Consequently, when animals from French Island were later translocated to mainland Victoria, this resulted in the establishment of populations with low genetic diversity (Houlden et al. 1996b).

Currently, koala populations are found across eastern Australia, specifically within Queensland, New South Wales, Victoria, and South Australia (Martin et al. 1999; Jackson 2007). The koala is listed as vulnerable by the Australian government, and as threatened under the US Endangered Species Act (Department of the Environment 2013). Koalas in North American zoos that originated in Queensland (nominally *Phascolarctos cinereus adustus*) have been managed as a separate stock from southern Australian koalas, which include koalas from New South Wales (nominally *P. c. cinereus*) and Victoria (nominally *P. c. victor*). For convenience, we will refer to the North American zoo population as Queensland koalas, because Queensland was the source of all but one individual.

Studies that have characterized nuclear genetic markers in koalas reported higher levels of heterozygosity and allelic diversity in koalas from northeastern Australia (Houlden et al. 1996b; Lee et al. 2010a, 2010b), than in southeastern populations (Taylor et al. 1991; Houlden et al. 1996b). Cristescu et al. (2009) found low genetic diversity among koalas in Kangaroo and French Island. Although studies have found that koala populations in South Australia (Seymour et al. 2001) and Victoria (Taylor et al. 1991) carry low levels of nuclear genetic diversity, koalas from the South Gippsland region in Victoria have higher genetic diversity and are genetically distinctive from other Victorian koala populations (Lee et al. 2012). Recently, a study using genome-wide single nucleotide polymorphisms concluded that isolation by distance was present among koalas in Australia, with geographically distant populations showing higher levels of differentiation (Kjeldsen et al. 2016). In contrast to the previous reports, nuclear genetic diversity was reported as high in both northern and southern populations (Kjeldsen et al. 2016).

Other studies have examined koala populations in Australia for their mtDNA diversity, revealing substantial geographic structuring for the mtDNA (Fowler et al. 2000; Lee et al. 2010a), and also reporting lower diversity across southern Australian populations (Taylor et al. 1997) than across northern Australian populations (Houlden et al. 1999). Houlden et al. (1999) sequenced 860 bp of the mitochondrial DNA control region in 16 koala populations distributed along the eastern coast of Australia and revealed low-average nucleotide diversity ($\pi = 0.16$) and haplotype diversity ($H = 0.18$) within the populations (despite the higher diversity across the northern Australian populations). Mitochondrial genetic differentiation across koala populations in Queensland and New South Wales was found to be high (Fowler et al. 2000; Lee et al. 2010a), while koala populations in Victoria were little differentiated (Houlden et al. 1999), possibly due to their recent descent from

translocated individuals from French and Philip Islands. In a recent study, Tsangaras et al. (2012) analyzed mitochondrial DNA haplotypes of historical museum koala samples and found that the haplotypes were identical to those found in modern koala populations. This study suggested that mtDNA diversity among Queensland koalas was similar before and after the populations decline caused by hunting (Tsangaras et al. 2012).

In a previous study (Ruiz-Rodriguez et al. 2014), we developed and evaluated novel microsatellite markers among Queensland koalas. Given that previous studies using nuclear genetic markers have reported somewhat different results, and that the genetic diversity of Queensland koalas in North American zoos has not been previously assessed, we here examined and compared genetic diversity and genetic structure in samples of koalas from Queensland (kept in zoos), and in wild koalas from Victoria, Australia. We also compared mtDNA diversity in the zoo koalas with those previously reported for wild koalas.

Materials and Methods

Samples

Samples of northern Australian koalas from Queensland were from unrelated individuals sampled from zoos in the United States. Whole blood samples were collected during routine veterinary care from koalas in the Cleveland Zoo, Columbus Zoo, Dallas Zoo, Riverbanks Zoo, San Diego Zoo, and San Francisco Zoo. DNA was extracted from the blood samples using the DNeasy Blood and Tissue Kit (QIAGEN), following the recommended protocol. Southern koala samples had been collected from wild koalas from Victoria, Australia, and used in a previous study (Taylor et al. 1997), including 22 samples from the Brisbane Ranges (BR) and 24 samples from the Stony Rises (SR).

Microsatellite Genotyping

Thirteen primer pairs were used to genotype 73 koala samples. We used 4 novel microsatellite markers, as well as 9 of 14 that had been developed recently (Ruiz-Rodriguez et al. 2014). Another 5 recently developed microsatellite markers (*Phci10*, *Phci12*, *Phci16*, *Phci28*, and *Phci31*) were not used by this study for several reasons: *Phci12* and *Phci16* had not been recommended for use (Ruiz-Rodriguez et al. 2014), while among our samples there was substantial missing data when *Phci28* and *Phci31* were used. Some koalas had a 2 bp indel in the 3'-end of the flanking region of *Phci10*. The indel was verified by Sanger sequencing and precluded our inclusion of that marker. Instead of these problematic markers, 4 novel microsatellite markers (*Phci21*, *Phci23*, *Phci24*, and *Phci30*) were developed and are reported in this study for the first time. The development of the new markers followed the same procedures reported in Ruiz-Rodriguez et al. (2014).

The PCR setup and algorithm were the same as used by Ishida et al. (2012). All forward primers included a “primer tail” consisting of the M13 forward sequence (5' TGTAACGACGCCAGT), which enabled labeling with a fluorescent tag (Boutin-Ganache et al. 2001). Forward and reverse primer sequences are listed in Supplementary Table S1. PCR consisted of 15 μ L reaction mixture that consisted of a final concentration of 200 μ M of each dNTP, 1x PCR buffer II, 2 mM MgCl₂, 0.04 units/ μ L of AmpliTaq Gold Polymerase along with 1.2 μ L of primer mix (Ishida et al. 2012) and 0.5 μ L of template DNA (of variable concentrations). In some reactions, 0.8 μ g/ μ L final concentration of bovine serum albumin (New

England BioLabs, Ipswich, MA) was included. Touchdown PCR consisted of the following algorithm: initial 95 °C for 10 min; with cycles of 15 s at 95 °C; followed by 30 s at 60 °C, 58 °C, 56 °C, 54 °C, 52 °C (2 cycles each), or 50 °C (last 30 cycles); and 45 s at 72 °C; with a final extension of 30 min at 72 °C. An aliquot of each PCR product was examined on 1.5–2% agarose gels stained with ethidium bromide. Amplicons were then diluted depending on the intensity of the image in the gel (a 5x dilution for dimmer bands and a 10x dilution for brighter bands) and electrophoresed on an ABI 3730XL capillary sequencer at the UIUC Core Sequencing Facility. Microsatellite fragments were scored and binned with GENEMAPPER v. 3.7 (Applied Biosystems).

Microsatellite Analyses

Allelic diversity, observed heterozygosity, expected heterozygosity, and F_{ST} were calculated using ARLEQUIN v. 3.5 (Excoffier and Lischer 2010). F_{ST} values were estimated using the method of Weir and Cockerham (1984) for pairs of populations. Linkage disequilibrium and deviations from Hardy–Weinberg equilibrium were examined using ARLEQUIN. The modified Garza–Williamson (GW) index ($M = k/r + 1$) was calculated using ARLEQUIN. The GW index (M) measures the ratio of the number of alleles k to the range in allele size r across microsatellite loci. When a population undergoes a bottleneck, rare alleles tend to be lost through genetic drift. The loss of any allele will contribute to a reduction in k , but only the loss of the largest or smallest allele will contribute to a reduction in r , so that k would be reduced more quickly than r . Thus, the value of M may be reduced following a bottleneck (Garza and Williamson 2001).

Population structure was examined using the software STRUCTURE 2.3.2 (Pritchard et al. 2000). STRUCTURE was run 10 independent times for each of the 4 models combining assumptions on genetic ancestry of individuals and genetic relatedness among populations; admixture model with correlated or uncorrelated allele frequencies, and no admixture model with correlated or uncorrelated frequencies. Each run used a burn-in of 10 000 iterations, followed by 100 000 Markov chain Monte Carlo iterations testing values of K between 1 and 10. Population structure was examined using 22 samples from BR, 24 samples from SR, and 27 Queensland koala samples from zoos, using genotypes for 13 microsatellite markers in each population. The uppermost hierarchical level of population structure was examined using the ad hoc statistics ΔK based on the rate of change in $\ln P(D)$ between successive K values (Evanno et al. 2005). The web-based program STRUCTURE HARVESTER (Earl and Vonholdt 2012) was used to assess likelihood values and determine the K -value that best fit the data based on the method of Evanno et al. (2005). To further examine the genetic relationships among populations, principal coordinate analyses (PCoA) were conducted using GenAIEx (Peakall and Smouse 2006, 2012). One dataset included 13 microsatellite markers, the 2 southern populations, and the Queensland population. Another dataset used the same number of markers but included only the southern populations.

Mitochondrial DNA Amplification, Sequencing, and Analyses

A 648 bp fragment of the mitochondrial control region was amplified via PCR and Sanger sequenced for 26 Queensland koalas using the primers PCI_CR_1F (5'-AAATAACCAACCAACACTCACATCC-3') and PCI_CR_NR (5'-TTCTAGGTACGTCCGCAATCT-3').

Published control region mtDNA sequences (Houlden et al. 1999; Fowler et al. 2000; Lee et al. 2010a; Seddon et al. 2014) were downloaded from Genbank: AJ005846-AJ005863, AJ012057-AJ012064, GQ851933-GQ851940, and KF745869-KF745875. Sequences were aligned in MEGA6 (Tamura et al. 2013) and conserved regions were visually identified. Primers were designed to target the control region of mtDNA using Primer3 (Untergasser et al. 2012). PCR reactions for mtDNA control region (25 µL), consisted of 0.5 µL of DNA template, 0.4 µM final concentration of each oligonucleotide primer, 2 mM MgCl₂, 200 µM of each of the dNTPs (Applied Biosystems Inc. [ABI], Foster City, CA), and 1X PCR Buffer II (ABI) with 0.04 units/µL final concentration of AmpliTaq Gold DNA Polymerase (ABI). PCR consisted of an initial 95 °C for 9.45 min; with cycles of 20 s denaturing at 94 °C, followed by 30 s annealing at 60 °C (3 cycles); 58 °C, 56 °C, 54 °C, 52 °C (5 cycles each temperature); or 50 °C (last 22 cycles), followed by 1.5 min extension at 72 °C, with a final extension after the last cycle of 7 min at 72 °C. After PCR, primers and dNTPs were removed from the PCR products with Exonuclease I (USB Corporation, Santa Clara, CA) and shrimp alkaline phosphate (USB Corporation) (Hanke and Wink 1994). Sequences in both directions were generated using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) with 2.5 µL of purified PCR products and 2 µM primer as previously described (Ishida et al. 2011) and resolved on an ABI 3730XL capillary sequencer.

The software SEQUENCER 5.1 (Gene Codes Corp., Ann Arbor, MI) was used to edit chromatograms, assemble contigs for each amplicon, and concatenate the mtDNA sequences for each individual. Shared and unique haplotypes were identified using DNAsP v. 5 (Librado and Rozas 2009). F_{ST} , nucleotide diversity, and haplotype diversity were estimated for each population using ARLEQUIN (Excoffier and Lischer 2010). Our southern Australian samples overlapped those previously sequenced for their mtDNA (Houlden et al. 1999), hence we excluded those samples. Previously published mtDNA haplotypes from Houlden et al. (1999) were aligned together with our novel control region sequences using MUSCLE implemented MEGA6. A neighbor joining tree was constructed in MEGA6 using 648 bp of the aligned mtDNA sequences and rooted using the mtDNA control region sequences of the closest sequenced relatives of the koala the common wombat (*Vombatus ursinus*) and the northern hairy-nosed wombat (*Lasiorhinus krefftii*). NETWORK v. 4.6.1 (Bandelt et al. 1999) was used to create a median-joining network using 18 previously published koala haplotypes from Houlden et al. (1999) and mtDNA control region sequences of Queensland koalas.

Data Availability

In accordance with the *Journal of Heredity* data archiving policy (Baker 2013), we have deposited the data underlying these analyses as follows: 1) Sampling locations and microsatellite genotypes: Dryad. 2) Mitochondrial DNA haplotype: Genbank accession KX646398.

Results

Nuclear Genetic Diversity

Wild koalas from the BR and SR of Victoria were genotyped, as well as zoo koalas derived from the Queensland population. Of the 13 microsatellite markers used for analyses, 3 (*Phci21*, *Phci23*, and *Phci27*) were monomorphic across 22, 19, and 21 individuals from BR, whereas only 1 locus *Phci23* was monomorphic across

19 koalas from SR (Table 1). Linkage disequilibrium was detected between loci *Phci5* and *Phci27*. No locus deviated from Hardy-Weinberg equilibrium after Bonferroni correction ($P > 0.0006$). All 13 loci were highly polymorphic in koalas from the Queensland zoo population (Table 1). The BR and SR koala populations had a lower number of alleles at each locus compared with the Queensland koalas. The BR koala population had a total of 31 alleles across all loci with an average of 2.3 alleles per locus, whereas SR had a total of 35 alleles with an average of 2.6 alleles per locus. The Queensland koalas had a total of 85 alleles across all loci with an average of 6.5 alleles per locus. The Queensland koala population exhibited the broadest range of allele sizes with frequency patterns distinct from the 2 southern populations, whereas similar allele size distributions and allele frequency patterns were seen between the BR and SR populations (Supplementary Figure S1). The average allelic size range was 3.4, 2.7, and 7.1 for BR, SR, and Queensland, respectively. Overall mean observed and expected heterozygosity for polymorphic loci were $H_o = 0.49$ and $H_e = 0.47$ for BR, $H_o = 0.41$ and $H_e = 0.43$ for SR, and $H_o = 0.73$ and $H_e = 0.72$ for Queensland.

Population Structure

Low genetic differentiation was observed between the 2 southern Australian koala populations in Victoria, with $F_{ST} = 0.018$ between BR and SR ($P = 0.052$). High levels of genetic differentiation were observed between the Queensland population and either southern koala population. Between Queensland and BR, $F_{ST} = 0.283$ ($P < 0.001$); whereas between Queensland and SR, $F_{ST} = 0.281$ ($P < 0.001$). When the 2 southern populations were combined, $F_{ST} = 0.27$ ($P < 0.001$) between northern and southern Australian koalas. When STRUCTURE was run at $K = 2$, the 2 partitions corresponded almost completely to 1) Queensland koalas from northern Australia and 2) the southern Australian koalas including both BR and SR (Figure 1). The ad hoc ΔK method implemented in STRUCTURE HARVESTER estimated a total of 2 clusters ($K = 2$) for all models (Evanno et al. 2005). These STRUCTURE results indicate that the Queensland koala population is genetically distinctive from those of the southern Australian populations, regardless of model assumptions (Figure 1).

Principal coordinate analyses implemented using the software GenAIEx also found distinctiveness between northern and southern koala populations. When koalas from all 3 localities were included in the analysis, the first principal component separated the Queensland koalas from the southern Australian populations (BR and SR) (Figure 2). In this principal coordinate analysis, no distinctiveness was evident between BR and SR group on any axis (Figure 2). An additional PCoA using only the BR and SR koala samples also failed to separate these 2 populations along any axis (Supplementary Figure S2), indicating that these 2 populations are not genetically distinguishable. The modified Garza-Williamson index was low for the southern population ($M = 0.37 \pm 0.18$) but much higher for the Queensland population ($M = 0.80 \pm 0.16$). Garza and Williamson (2001) suggested that data sets with 7 or more loci that show an $M < 0.68$ can be assumed to have experienced a bottleneck, as proved to be the case for the southern Australian koalas.

Mitochondrial DNA Diversity

Among the 26 Queensland zoo koalas, 18 variable sites defined a total of 8 distinct control region haplotypes (Table 2). One of these haplotype (KH1) had not been reported previously among koalas and was carried by 5 of the Queensland koalas. All other sequences matched previously published koala mtDNA haplotypes (Houlden et al. 1999; Fowler et al. 2000; Lee et al. 2010a; Seddon et al. 2014). Haplotype and nucleotide diversity for Queensland koalas were $H = 0.753$ and $\pi = 0.0072$ (Table 2), respectively, and genetic differentiation was high when compared with the southern koalas reported by Houlden et al. (1999) (Supplementary Figure S3). A median joining-network was generated (Figure 3A) to show the genetic relationships among our Queensland haplotypes and haplotypes from Houlden et al. (1999). Within the network, haplotypes formed 6 groups when separated by at least 3 mutations between groups.

The geographic distributions of koala mtDNA haplotypes were plotted on a map (Figure 3B), which also shows the locations of the BR and SR. Northern Australian koalas have a higher number of haplotypes (11 in Queensland, including US zoo haplotypes) compared to the southern Australian (6 in New South Wales and 5 in Victoria) koalas (Figure 3B). The southern Australian koalas carried a very

Table 1. Characteristics of koala microsatellite loci

Locus	Brisbane ranges				Stony rises				Queensland			
	N	H_o	H_e	A	N	H_o	H_e	A	N	H_o	H_e	A
<i>Phci2</i> ^a	22	0.27	0.30	2	24	0.25	0.26		27	0.77	0.83	#
<i>Phci5</i> ^a	21	0.76	0.60	3	24	0.62	0.59		27	0.88	0.82	6
<i>Phci9</i> ^a	22	0.45	0.44	2	24	0.29	0.40		27	0.59	0.66	5
<i>Phci15</i> ^a	19	0.52	0.44	2	22	0.63	0.49		27	0.74	0.71	5
<i>Phci17</i> ^a	22	0.45	0.50	4	24	0.37	0.43		27	0.88	0.81	7
<i>Phci18</i> ^a	22	0.22	0.21	3	24	0.50	0.49		27	0.66	0.65	6
<i>Phci19</i> ^a	21	0.47	0.52	3	23	0.52	0.65		27	0.40	0.38	4
<i>Phci21</i> ^b	22	Monomorphic		1	23	0.04	0.04		27	0.88	0.83	7
<i>Phci22</i> ^a	22	0.86	0.8	5	24	0.79	0.70		27	0.77	0.78	#
<i>Phci23</i> ^b	19	Monomorphic		1	19	Monomorphic			27	0.74	0.73	6
<i>Phci24</i> ^b	20	0.45	0.40	2	24	0.41	0.50		26	0.65	0.79	7
<i>Phci27</i> ^a	21	Monomorphic		1	19	0.05	0.05		27	0.92	0.84	8
<i>Phci30</i> ^b	20	0.50	0.46	2	22	0.45	0.52		27	0.62	0.51	3

N is the number of individuals successfully genotyped, H_o is observed heterozygosity, H_e is expected heterozygosity. A is the number of alleles per locus.

^aLoci from Ruiz-Rodriguez et al. (2014).

^bLoci developed for this study.

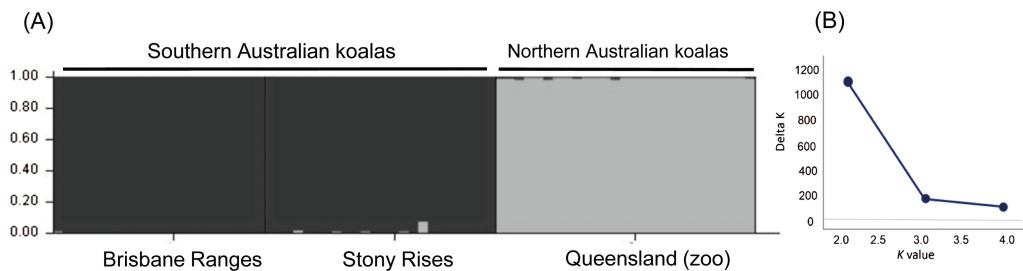


Figure 1. (A) Genetic partitioning of koala populations using the program STRUCTURE 2.3.2 (Pritchard et al. 2000). The 2 partitions almost completely correspond to the southern koala populations of BR and SR (left) and the koala population from Queensland (right). The analysis shown assumed admixture between populations and correlated allele frequencies, with $K = 2$. (B) The method of Evanno et al. 2005 found that the most likely number of clusters was $K = 2$ (Evanno et al. 2005).

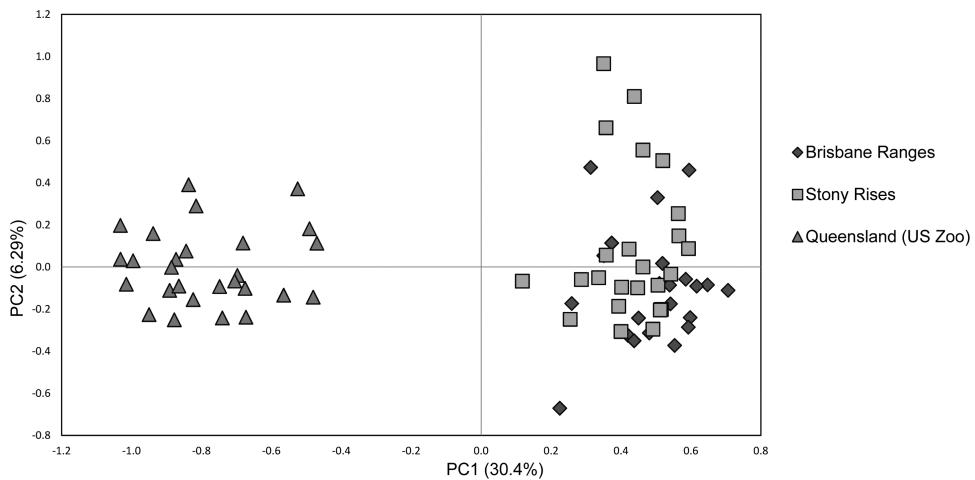


Figure 2. PCoA of 3 koala populations using 13 microsatellite loci. The diamonds represent koalas from BR, squares represent SR, and triangles represent the Queensland population (in the US zoos). The variation explained by coordinates 1, 2, and 3 was 30.4%, 6.29%, and 5.66%, respectively. Coordinate 3 is not shown as it was not informative for distinguishing populations. The PCoA shows that southern Australian koala populations are genetically distinct from Queensland koalas along coordinate 1. The BR and SR populations could not be distinguished along any coordinate.

common haplotype (KH2) that is found across the region including: BR, French Island, Kangaroo Island, Phillip Island, South Gippsland, SR, and Strzelecki Ranges. A rooted neighbor-joining tree was created using koala haplotypes to illustrate the genetic relationship of previously published haplotypes (Houlden et al. 1999) and the 8 haplotypes found in this study (Supplementary Figure S4). The phylogenetic relationship of 44 koala mtDNA control region sequences (Supplementary Figure S4) showed relationships consistent with the 6 groups defined in the median-joining network (Figure 3A). Previous analyses of relationships among mtDNA haplotypes had not rooted the tree (Houlden et al. 1999; Seddon et al. 2014). Use of an outgroup suggested that the most basal divergence among koala mtDNA haplotypes separates haplotype KH12 (Haplotype group 6 in the network) from all other haplotypes (Supplementary Figure S4). Haplotype KH12 is only found in western New South Wales (Figure 3B).

Discussion

The findings of this study have contributed to our understanding of the population structure and genetic diversity of koalas both ex situ and in situ. The koalas in the US zoos showed high levels of heterozygosity, consistent with those of mainland Queensland populations reported by previous studies (Houlden et al. 1996b; Lee et al. 2010a; 2010b; Seddon et al. 2014). We found higher levels of nuclear genetic diversity

in the Queensland zoo koalas compared with the koalas from Victoria, consistent with the previous reports (Houlden et al. 1996b). The North American zoo koalas also carried the same haplotypes as wild Queensland koalas (Houlden et al. 1999; Fowler et al. 2000; Seddon et al. 2014). Both the BR and SR populations had low levels of heterozygosity, consistent with the previous reports (Houlden et al. 1996b). Genetic differentiation was high between the Queensland zoo koalas and the 2 populations in Victoria, but low between BR and SR koalas.

The origin of the zoo animals in Queensland was initially based on their studbook records, but was verified by our mtDNA results. The Queensland zoo koalas showed levels of haplotype diversity ($H = 0.753$) and nucleotide diversity ($\pi = 0.0072$) that were higher than those of wild Queensland koalas ($H = 0.735$; $\pi = 0.0076$), which we calculated using the data reported by Houlden et al. (1999) (Supplementary Table S2 and S3). The founder koalas in zoos likely come from multiple geographic localities in Queensland, and thus likely represent greater diversity than may be found among wild koalas at a single geographic locality (Seddon et al. 2014).

Oddly, 1 haplotype (KH2) found in just 1 of the zoo koalas was commonly found throughout populations of koalas in Victoria, but not among koalas in Queensland or New South Wales. Further investigation revealed that this koala (Simba, studbook no. 526) is unique among the North American zoo koalas in having potential admixture from southern Australia (Pye G, personal communication). All other haplotypes found in our Queensland zoo koalas

Table 2. Sequence variation across 8 mtDNA control region haplotypes

Haplotype	Nucleotide position																			
	0	0	0	0	1	1	1	2	2	3	3	3	3	3	3	3	3	5	5	N
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	2	7	9	4	4	5	7	7	0	0	1	6	7	9	9	2	2	3	3	3
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	2	4	9	4	5	3	0	9	6	9	1	6	9	2	4	7	9	—	—	—
KH1	C	T	T	T	A	T	C	A	G	A	T	T	T	A	T	5	5	5	5	5
KH2	T	•	•	•	G	C	T	G	•	A	•	•	•	•	•	•	•	•	•	1
KH3	T	•	•	•	•	C	•	T	•	G	A	•	C	•	•	•	•	•	•	1
KH4	T	•	•	•	•	C	•	T	•	G	A	•	C	•	•	•	•	•	•	1
KH5	T	•	•	•	•	C	•	T	•	•	A	•	C	•	•	•	•	•	•	12
KH6	•	C	C	C	•	C	•	T	•	•	A	•	•	•	•	•	•	•	•	2
KH7	•	•	•	•	•	•	•	T	•	•	A	G	•	•	•	•	•	•	•	3
KH8	T	•	•	•	•	C	•	T	•	•	G	A	•	C	•	•	•	•	•	1

Only variable positions are shown. Dots indicate identity with reference sequence KH1, and letters designate base substitutions. Haplotype diversity (H) = 0.753, nucleotide diversity (π) = 0.0072 as calculated in ARLEQUIN. N indicates number of koalas.

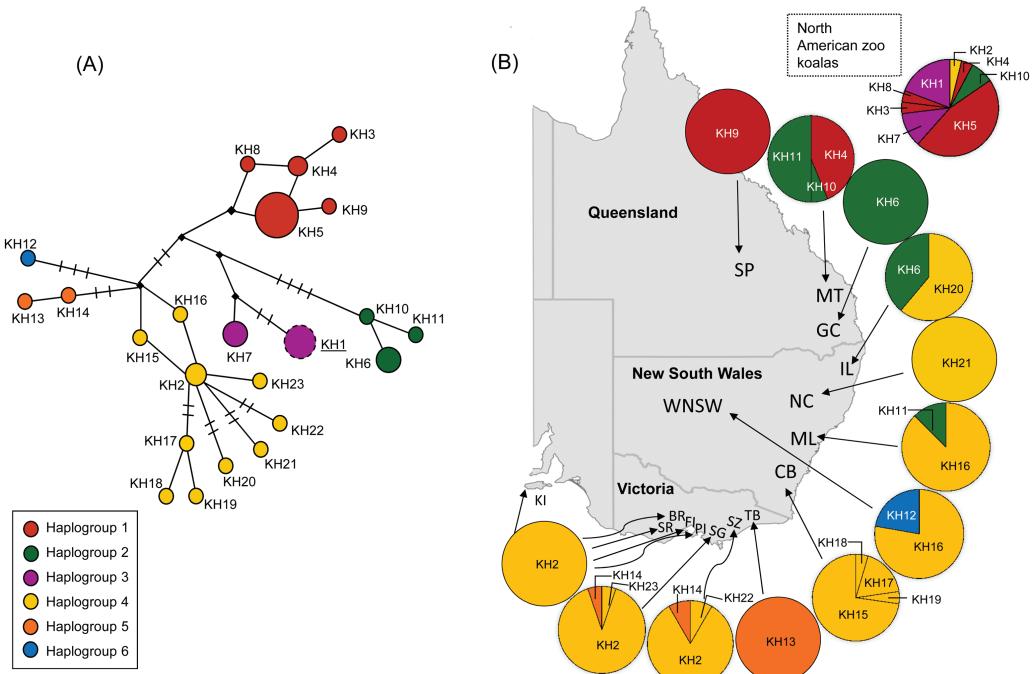


Figure 3. (A) Median-joining network of distinct mitochondrial control region haplotypes (648 bp in length). The network includes sequences of Queensland koalas in North American zoos, and previously published haplotypes from Queensland, New South Wales, and Victoria for which haplotype frequency information was known (Houlden et al. 1999). Circle size is proportional to number of koala samples. The novel haplotype (KH1) is indicated by a dashed circle. Haplogroups are color coded and defined by a separation of at least 3 mutational steps between each haplogroup. Nucleotide differences are indicated (if >1) by hatch marks. (B) Distribution of koala mtDNA control region haplotypes from this study and Houlden et al. (1999). The colors correspond to the haplogroups show in the network in panel A. Pie charts show haplotype frequencies within localities, although circle sizes are not proportionate to sample size by locality. Queensland zoo koalas were grouped in a separate pie chart from wild Queensland koalas. The zoo koala (studbook no. 526) that carried haplotype KH2 is unique among the North American zoo koalas in having potential admixture from southern Australia. The locations from north to south are as follows: Springsure (SP), Mutdapilly (MT), Gold Coast (GC), Iluka (IL), Nowendoc (NC), Coonabarabran (WNSW), Maitland (ML), Campbelltown (CB), Tubut (TB), Strzelecki Ranges (SZ), South Gippsland (SG), French Island (FI), Phillip Island (PI), Stony Rises (SR), Brisbane Ranges (BR), and Kangaroo Island (KI).

corresponded to those found among wild populations in Queensland (Houlden et al. 1999) (Figure 3). One novel haplotype sequence was detected among the zoo koalas (Figure 3A), which was similar in sequence to previously reported haplotypes carried by wild Queensland koalas. For koalas maintained ex situ in Australia, both high (Worthington-Wilmer et al. 1993) and low (Seddon et al. 2014) levels of inbreeding have been reported. The high genetic diversity

reported here among both mitochondrial sequences and microsatellite alleles is an indication that North American zoos have successfully managed their koalas to minimize inbreeding or genetic drift.

Using microsatellite markers, we examined the nuclear genetic structure and diversity of koalas from 2 southern Australian populations (BR and SR), along with the northern Queensland koalas housed in North American zoos. Our analysis of nuclear genetic diversity

showed that microsatellite variability was quite low in the southern Australian koalas from BR ($H_O = 0.49$) and SR ($H_O = 0.41$) compared with the northern koala population ($H_O = 0.73$). Low levels of genetic diversity would be consistent with the known history of southern Australian koalas, which were hunted to near extinction in the early 1900s, leading to the extirpation of many southern populations. The BR and SR were subsequently repopulated with koalas from an inbred koala population on French Island (Houlden et al. 1996b). With an average of 2 alleles per locus, the 2 southern koala populations show low levels of genetic diversity consistent with this history. The results are also consistent with those of Houlden et al. (1996b), who reported low levels of genetic diversity using 6 microsatellite loci in southern populations ($H_e = 0.436$) compared with northern Australian populations ($H_e = 0.851$). Houlden et al. (1996a) used microsatellite loci developed in southern Australian koalas, so that our own results cannot be attributed to ascertainment bias from the use of markers developed in northern Australian koalas.

Evidence for a population bottleneck affecting the southern Australian populations was also provided using the method of Garza and Williamson (2001). The modified Garza–Williamson value was low in the southern koalas ($M = 0.37$) compared with the northern koalas ($M = 0.80$). The low value in the south is an indication that alleles of intermediate sizes were lost due to a population bottleneck. In addition, genetic differentiation was not detected between the 2 southern populations in the BR and SR by STRUCTURE or by the PCoA (Figures 1 and 2), while F_{ST} was low and not significant between the 2 localities. This would be consistent both with the geographic proximity of the 2 localities and with historical accounts that koalas from French Island were translocated to both localities, given that quantitative evidence for a bottleneck is also present among koalas on French Island (Cristescu et al. 2009).

Our results contrast with a recent report that examined single nucleotide polymorphisms (SNPs) in populations of koalas from across Australia (Kjeldsen et al. 2016). Kjeldsen et al. (2016) reported relatively high nuclear genetic diversity in southern Australian populations, whereas our southern Australian populations show low genetic diversity when compared to northern Australian koalas. The populations from Victoria that they examined were different from ours, which could account at least in part for the different findings. They also attributed differences between northern and southern populations to isolation by distance, rather than to strong genetic substructure between regions (Kjeldsen et al. 2016). Our STRUCTURE analysis found almost complete partitioning between northern and southern koalas (Figure 1), whereas incomplete differentiation among populations, such as that caused by isolation by distance, may have been expected to lead to incomplete separation of populations by STRUCTURE partitions (Brandt et al. 2014). It is possible that our results may have been influenced by the translocations of koalas first to French Island and later to southern Australian locations, since founder effects and bottlenecks can increase genetic distance between populations (Baker and Moeed 1987). However, at locus *Phci19*, the size ranges of alleles do not overlap between northern and southern Australian koalas (Supplementary Figure S1). This may suggest that our results could reflect pre-bottleneck differentiation of southern Australian koalas from those in Queensland.

A high degree of genetic differentiation between the northern and southern Australian koalas was suggested by several analyses (Figures 2 and 3). F_{ST} was high and highly significant between the Queensland koalas and koalas from the BR ($F_{ST} = 0.283$) or the SR ($F_{ST} = 0.281$). This result would not have been due to the use of zoo animals. By combining koalas from different Queensland localities,

within-population Queensland zoo koala genetic diversity would have risen relative to wild populations. This, in turn, would lower the value of F_{ST} in the comparison between Queensland and Victorian koalas, making the differences detected between Queensland zoo koalas and southern populations even more compelling. STRUCTURE partitions ($K = 2$) separated almost completely the Queensland population from the southern koala populations (Figure 1), and a high degree of differentiation could be seen with the PCoA (Figure 2). Finally, the mtDNA sequences also suggested strong phylogeographic subdivisions among koalas. This was especially evident when examining groups of similar haplotypes identified in a network analysis of control region haplotypes (Figure 3A). The haplotypes in the network were color-coded by group when showing their geographic distribution across eastern Australia (Figure 3B). The geographic distribution of mitochondrial haplogroups distinguished Queensland koalas from populations further south. Taken together, our results support the management of Queensland koalas ex situ as a separate stock from southern Australian koalas. Should the differences that we detected be supported using a broader geographic analysis of populations, the distinct conservation management of wild northern and southern Australian koalas would be supported, as would the potential recognition of Queensland koalas as a subspecies (*Phascolarctos cinereus adustus*).

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxford-journals.org/>.

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Data Availability

Data deposited at Dryad: <http://dx.doi.org/10.5061/dryad.6g735>.

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