CSIRO PUBLISHING

Wildlife Research, 2010, **37**, 156–165 www.publish.csiro.au/journals/wr

Defining spatial genetic structure and management units for vulnerable koala (*Phascolarctos cinereus*) populations in the Sydney region, Australia

Tristan Lee[A,D], *Kyall R. Zenger*[B], *Robert L. Close*[C], *Marilyn Jones*[A] *and David N. Phalen*[A]

[A]Wildlife Health and Conservation Centre, Faculty of Veterinary Science, The University of Sydney, NSW 2570, Australia.

[B]School of Marine & Tropical Biology, James Cook University, Townsville, Qld 4811, Australia.

[C]School of Biomedical and Health Sciences, University of Western Sydney, NSW 2560, Australia.

[D]Corresponding author. Email: t.lee@usyd.edu.au

Abstract

Context. Mammal populations around the world are increasingly threatened with population fragmentation because of loss of habitat or barriers to gene flow. The investigation of koala populations in the Sydney region not only provides valuable information about this vulnerable species, but also serves as a model for other species that have suffered major rapid declines in population size, and are now recovering in fragmented habitat. The peri-urban study region allows investigation of the impact of landscape features such as major roads and housing developments on koala gene flow.

Aims. Animals originating from four geographic sampling areas around Sydney, New South Wales, Australia, were examined to determine population structure and gene flow and to identify barriers to gene flow and management units.

Methods. The present study examined 12 microsatellite loci and used Bayesian assignment methods and genic frequency analysis methods to identify demographically separate populations and barriers to gene flow between those populations.

Key results. Three discrete populations were resolved, with all displaying moderate to high levels of genetic differentiation among them ($\theta = 0.141$–0.224). The allelic richness and heterozygosity of the Blue Mountains population ($A = 6.46$, $H_O = 0.66$) is comparable to the highest diversity found in any koala population previously investigated. However, considerably lower genetic diversity was found in the Campbelltown population ($A = 3.17$, $H_O = 0.49$), which also displayed evidence of a recent population bottleneck (effective population size estimated at 16–21).

Conclusions. Animals separated by a military reserve were identified as one population, suggesting that the reserve maintains gene flow within this population. By contrast, strong differentiation of two geographically close populations separated by several potential barriers to gene flow suggested these land-use features pose barriers to gene flow.

Implications. Implications of these findings for management of koala populations in the Greater Sydney region are discussed. In particular, the need to carefully consider the future of a military reserve is highlighted, along with possible solutions to allow gene flow across the proposed barrier regions. Because these are demographically separate populations, specific management plans tailored to the needs of each population will need to be formulated.

Additional keywords: gene flow, genetic diversity, koala, microsatellites, population structure.

Introduction

Mammal populations around the world are increasingly threatened with population fragmentation. Populations can become fragmented because of habitat loss or the presence of anthropogenic barriers to gene flow, such as major roads (Balkenhol and Waits 2009). If barriers are present in an important migration or dispersal corridor, they could significantly impede gene flow across that region, effectively isolating adjacent populations (Pérez-Espona *et al*. 2008). Fragmentation and consequent isolation can reduce the genetic diversity and therefore the species ability to respond to environmental change (Frankham *et al*. 2002). In the context of species conservation, *management units* are generally recognised as demographically independent populations (Palsboll *et al*. 2007). The identification of management units is a crucial step in the management and conservation of natural populations (Palsboll *et al*. 2007).

The koala (*Phascolarctos cinereus*) is a valuable iconic Australian marsupial. It is internationally recognisable and plays a significant role in promoting Australian fauna awareness. The koala is also fundamentally important in its biological uniqueness. Koalas are unique in that they are the only extant member of the Family Phascolarctidae and one of the few mammals that feed almost exclusively on eucalypts. Despite this, koala populations have significantly declined over the years through hunting for the koala fur trade and habitat

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10.1071/WR09134 1035-3712/10/020156

destruction from human activities (Hume 1990; Reed and Lunney 1990). Along with many other mammal species, increasing urban sprawl has caused koalas to be increasingly threatened by factors such as motor vehicles, loss of habitat and dog attacks (Dique *et al*. 2003; Lunney *et al*. 2004). The current geographical distribution of the koala ranges across much of the eastern coast of Australia (Fig. 1*A*).

Koalas were believed to have been lost from large areas surrounding Sydney (Reed *et al*. 1991); however, recent sightings indicate that some populations are gradually expanding back into the remnants of their original range (Ward and Close 2004). The Sydney koalas may not have experienced the same degree of genetic bottleneck that occurred in Victorian koalas and were not subjected to translocation programs as seen in Victorian koalas (Houlden *et al*. 1996*b*). Consequently, the Sydney koalas may retain genetic characteristics not found in other koala populations. Additionally, there have been no studies into koala population genetic structure within the Sydney basin. Management of these populations may therefore not be optimal because knowledge of population structure is necessary when developing management plans for scattered remnant populations (e.g. Manel *et al*. 2003; Storfer *et al*. 2007). Koalas also occupy some of the remaining undeveloped land in Sydney's south-west, and therefore careful development planning will be needed to maintain healthy koala populations while allowing for Sydney's growing population, which is projected to increase by ~63% over the next 50 years (ABS 2006). This paper aims to investigate the current population structure of koala populations surrounding the Sydney region, to provide information to better manage this species. In addition, the present study may also offer insights into factors that may affect other animal species in similar urban environments that have also suffered major rapid declines in population size in the past, but are now recovering.

The koala's ability to recover from threatening processes and population declines has varied over its geographical range. Koalas in many areas of Victoria and on Kangaroo Island, South Australia, are now considered to be overabundant (Masters *et al*. 2004). However, in New South Wales (NSW) and Queensland, some koala populations have continued to decline or have become extinct altogether (Reed *et al*. 1991; Phillips 2000). With overpopulation in some areas and declining populations in other areas, koala management in Australia is a complex and controversial topic (e.g. Phillips 2000) that requires specific management plans at a local scale, rather than a single uniform national approach.

Despite the complex issues surrounding koala management and conservation, there have been few genetic studies into koala population structure in Australia (Houlden *et al*. 1996*b*, 1999; Taylor *et al*. 1997; Fowler *et al*. 2000; Sherwin *et al*. 2000). When comparing microsatellite data from 10 populations around Australia, Houlden *et al*. (1996*b*) found significantly lower levels of intra-specific population differentiation among Victorian koala populations than among northern NSW and Queensland populations. They concluded that this reflects the extensive human perturbation in most Victorian populations. Taylor *et al*. (1997), using mitochondrial DNA data, supported these findings. Fowler *et al*. (2000) used mitochondrial DNA in a study on Queensland koala populations and concluded that female-mediated gene flow is likely to be limited, and

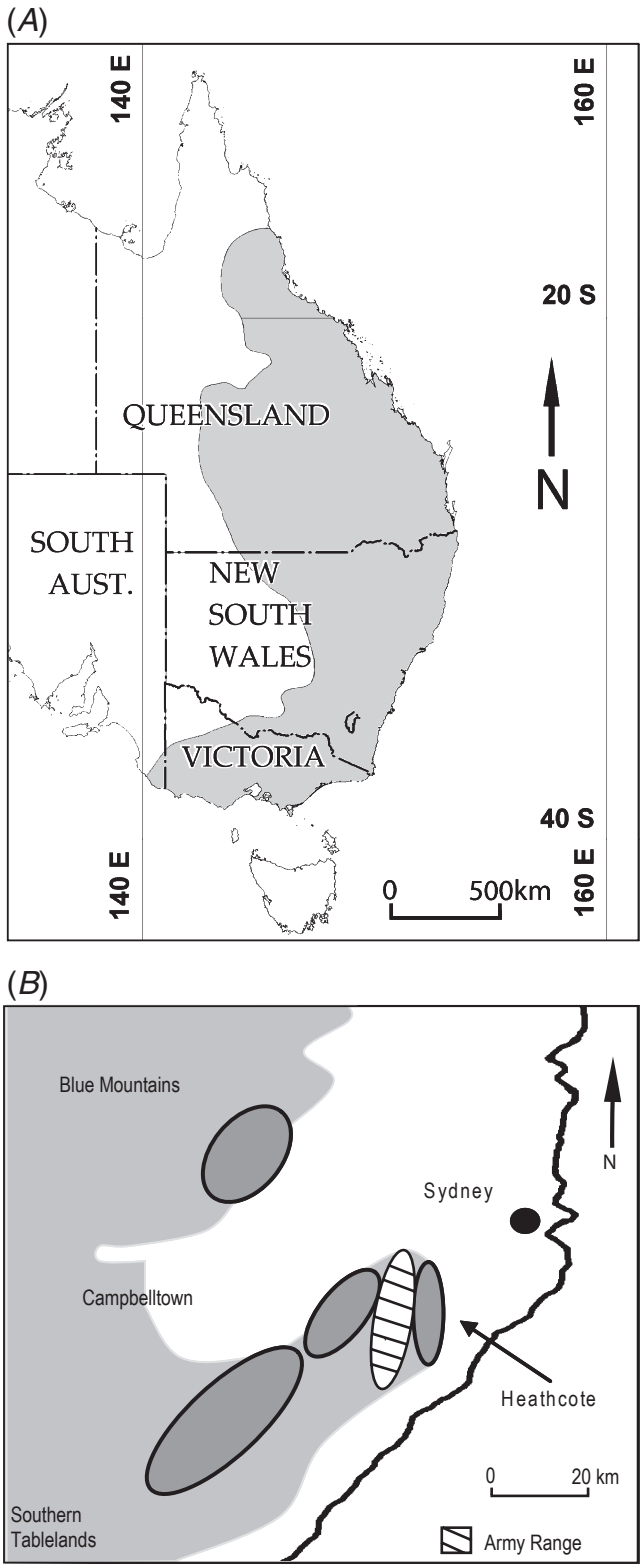

Fig. 1. (*A*) East coast map of Australia, with shading indicating the broad geographical range of koalas. Koala populations in this range are highly fragmented. Reproduced with permission from the Australian Koala Foundation. (*B*) The greater Sydney region showing sampling locations (circled) and potential koala habitat (shaded).

noted the need for further work to investigate variation in nuclear DNA.

Landscape features, both natural (e.g. rivers, mountains) and artificial (e.g. roads, housing) can be barriers to gene flow for certain species, depending on their methods of migration. Several putative barriers exist between the sampling regions in the present study, and are investigated to determine whether they have any effect on gene flow between the two sampling regions. One hypothesised barrier region is transected by a major arterial road that may impede koala dispersal. A 2-km stretch of this road has previously been identified as a koala fatality blackspot (Ward 2002). In addition, this area is surrounded by an urban area, a colliery, steep river tributaries and contains habitat not considered prime koala habitat. These factors might all combine to create effective barriers to dispersal.

Two sampling regions in the present paper are separated by the 18 000-ha Holsworthy Army Range, which lies ~30 km from the Sydney central business district, and has been in active use since ~1914. The future of the Holsworthy Army Range is currently uncertain and therefore it is necessary to examine whether the range provides connectivity between the two sampling regions.

There is a mix of habitat within the area, some of which would be expected to support koalas. However, koala presence within the area is difficult to confirm because of restricted access. One ear-tagged young male koala originally observed and tagged within the Campbelltown sampling region has been sighted near the north-eastern border of the Army Range (Ward 2002), indicating that males at least are able to move through some of the range. Although there are no specific reasons to assume koalas do not inhabit the range, the uncertainties regarding koala status, combined with the potential importance of the land and the uncertain future, deem the subject to be worthy of further investigation.

The objectives of the present study were to help guide management decisions aimed at conserving koala populations inhabiting the rapidly changing environment in the Sydney region. The paper seeks to use genetic information to define management units for koala conservation. Potential natural and anthropogenic barriers are also investigated with an aim to determine whether they impede gene flow. The paper will also aim to determine the importance of the Holsworthy Army Range to local koala populations and, consequently, to inform future land-use decisions within the Army Range. To achieve these objectives, the present paper uses 12 microsatellite loci to investigate population structure, genetic diversity and barriers to gene flow in remnant Sydney koala populations.

Materials and methods

Study area

Samples were obtained from the following four geographic regions: Campbelltown ($n = 101$), Heathcote ($n = 9$), Southern Tablelands ($n = 16$) and Blue Mountains ($n = 18$), for a total of 144 samples (Fig. 1*B*). These four regions were selected because they represent all known koala-inhabited areas of the Sydney basin (Ward 2002). The habitat is eucalyptus forests, covering terrain varying from steep gorges dispersed throughout a plateau (Campbelltown and Heathcote sites), a rugged, steep, mountainous area (Blue Mountains populations) and a tablelands region (Southern Tablelands). The areas vary from peri-urban (Campbelltown and Heathcote) to agricultural (Southern Tablelands) to well forested (Blue Mountains). Although the Campbelltown and Southern Tablelands sampling areas are in close proximity, they are considered separate geographical regions for the purposes of the present study because of their differing land uses and because the intervening landscape has several possible barriers to gene flow. The land to the north of Campbelltown and the Southern Tablelands and to the east of the Blue Mountains is the heavily urbanised Sydney region.

Sample collection and genotyping

Samples were collected during the period of 1998–2008. The koalas sampled were part of an ongoing study monitoring koala populations in the Greater Sydney region, conducted by The University of Western Sydney and The University of Sydney. Samples were obtained opportunistically from deceased animals along roadways or from local field-survey programs. Consequently, samples were more abundant from urbanised areas than from non-populated regions. A small number of samples was also collected as a result of deliberate expeditions aimed at sampling koalas to fill in spatial gaps. At the time of sampling, Universal Transverse Mercator (UTM) coordinates were recorded with a handheld global positioning device. The samples were collected from both male and female koalas that ranged from 1 to ~14 years of age. Each tissue sample consisted of a 2-mm-diameter plug of tissue created when the animals were ear tagged. Animals were captured by 'flagging' down from trees, and then restrained for a brief period by experienced koala handlers while ear tags were applied and tissue samples obtained. All work was authorised under licence (Licence No. S10293) from New South Wales National Parks and Wildlife Service and ethics approval by the relevant University bodies. A similarly sized piece of ear tissue was collected by sharp dissection from deceased animals. The tissue samples were stored in 70% ethanol at room temperature before DNA extraction. Tissues were processed with DNeasy tissue kits (QIAGEN, Melbourne) to extract genomic DNA following the DNeasy tissue kit protocol.

Repeatability and polymorphism levels of 17 microsatellite primers that had been previously identified (Houlden *et al*. 1996*a*; Cristescu *et al*. 2009) were tested in replicate PCR reactions with 12 randomly selected DNA samples from across the sample sites. On the basis of the ease of genotype scoring, error rates, reliability of PCR amplification, and level of polymorphism, 12 primer pairs were selected (results not shown). The 12 primer pairs used in analysis were Phc 2, Phc 4, Phc 13 (Houlden *et al*. 1996*a*) and Pcv 2, Pcv 6.3, Pcv 24.2, Pcv 25.2, Pcv 26, Pcv 30, Pcv 31, K 2.1, K 10.1 (Cristescu *et al*. 2009).

DNA was amplified by using PCR methodology based on M13 tailed forward primers (Neilan *et al*. 1997). Optimised conditions for PCR consisted of ~100 ng of total genomic DNA, 10× PCR buffer, 0.5 mM each dNTP, 1.5 units *Taq* DNA polymerase (QIAGEN), $MgCl_2$ concentration of 1.5 mM, 0.25 µM of forward primer, 1 µM reverse primer, 0.5 µM of either NED, VIC, FAM or PET fluorescently labelled M13 primer (Applied Biosystems, Melbourne), and sterile water to bring to a total volume of 10 µL. Loci were amplified using a

touchdown PCR protocol as follows: initial 94°C denaturation for 3 min, followed by six touchdown cycles of 94°C for 30 s, annealing temperature at 60°C (and decreasing by 2°C each cycle) for 45 s, and extension step of 72°C for 60 s. On completion of the final touchdown cycle, a further 30 cycles were performed at the 50°C annealing temperature, followed by a final extension of 72°C for 10 min.

Amplification products for each sample were genotyped using an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Genotypes were scored using GeneMapper 4.0 (Applied Biosystems) and verified manually. All allele scorings were independently checked by eye by at least two people. All genotypes with low signal intensity or patterns that were difficult to interpret on GeneMapper 4.0 were re-electrophoresed and/or re-amplified. As a quality control measure, 30 random individuals were amplified a second time across all loci and re-scored blindly, to assess error rates for each locus.

Loci and population statistics

Deviations from Hardy–Weinberg (HW) equilibrium and presence of null alleles and/or substructuring were assessed by measuring F_{IS} and its statistical significance (10 000 permutations) for all loci within all sampling regions with FSTAT 2.9.3 (Goudet 1995). In addition, Mendelian inheritance was confirmed where mother–offspring relationships were known. Genotypic linkage disequilibrium for each pair of loci was calculated in FSTAT 2.9.3 (Goudet 1995). Number of alleles for each locus and average number of alleles across loci and populations were calculated in FSTAT 2.9.3 (Goudet 1995). The false discovery rate (FDR) control (Benjamini and Yekutieli 2001) for multiple testing was used where applicable for the *P*-values throughout the paper.

Population structure

Genotypic population structure was analysed with two programs, GENELAND 3.1.4 (Guillot *et al*. 2005*a*, 2005*b*, 2008; Guillot 2008) and STRUCTURE 2.1 (Pritchard *et al*. 2000; Falush *et al*. 2003). Both programs are based on Bayesian approaches that allow populations to be defined by the genetic data, rather than requiring an *a priori* estimation of population definition. In addition, GENELAND incorporates spatial information (the location in which each individual was sampled), and assigns greater probability to genetic clusters that are continuous within the spatial landscape (Guillot *et al*. 2005*a*).

GENELAND analysis was performed with an initial series of runs (12 runs at 500 000 Markov chain Monte-Carlo (MCMC) iterations each) to determine the most probable number of genetically distinct clusters (K). The uncertainty associated with the spatial coordinates was set to 1000 m, to allow variation within the koala's home range (Ward 2002). Minimum K was fixed at one and maximum K at 12. The Dirichlet model was used as a model for allelic frequencies, and the option to account for the presence of null alleles was selected (Guillot *et al*. 2008). Default values were used for remaining parameters. The number of populations was inferred on the basis of the mode of these 12 runs. To assign individuals to the inferred number of populations, the MCMC was run 10 times, with K set to the inferred number of populations (Guillot *et al*. 2005*a*). Other parameters remained the same as used in the runs with variable K. GENELAND was used to produce a Voronoi tessellation (Dupanloup *et al*. 2002) map of posterior probabilities of population membership for each of these 10 population assignment runs. To assess potential barriers to gene flow, a thematic map (1 : 200 000) including the potential barriers (e.g. roads, waterways, land use) was compared visually with the tessellation map.

STRUCTURE analysis was performed with five runs at each value of K, with values of K set from 1 to 10. Maximum number of populations was set at 10 to give a large margin of error in our estimates of maximum number of populations, and to allow for possible genetic structuring within each site. Each run was performed with a burn-in of 50 000 MCMC iterations, followed by 1 000 000 MCMC iterations. The correlated allele-frequency model and the admixture model were used, because each sampling region may have some contact. All other values were set to their default values. The mean log-likelihood of each K and the ΔK method described by Evanno *et al*. (2005) was used to estimate K. To assign individuals to populations, we performed five final runs at the estimated K.

To visualise the genetic similarity among individuals and populations, a neighbour-joining (NJ) distance tree was constructed, based on 1 – proportion of shared alleles between all individuals (Goldstein *et al*. 1999). The distance matrices were generated with the program MICROSAT (Minch *et al*. 1995) and the NJ tree was built with the program MEGA version 2.1 (Kumar *et al*. 2001).

Population pairwise F_{ST} values were used to measure the level of genetic differentiation between the populations inferred by GENELAND and STRUCTURE. F_{ST} values and their significance (10 000 permutations) were estimated in FSTAT 2.9.3 (Goudet 1995). For comparison, the same tests were then performed with each of our four original sampling regions as separate populations. To gain some indication of the timescale of the population splits and to assess whether the populations have been historically separated over the very long term, we compared observed R_{ST} values to R_{ST} values generated by random permutation of allele-size information in the program SPAGEDI 1.3 (Hardy and Vekemans 2002). Significantly smaller permuted (pR_{ST}) than observed R_{ST} values suggested that mutation may have contributed to the observed population differentiation, rather than genetic drift alone.

To test for spatial genetic structure at the level of individuals, a spatial autocorrelation test was performed in GENALEX 6 (Peakall and Smouse 2006), using the method of Smouse and Peakall (1999). The spatial autocorrelation coefficient was calculated for all genotypes and represented as a correlogram. Geographical distance was measured as linear Euclidean distance. To test for no spatial genetic structure in the combined dataset, 95% confidence intervals were estimated using 999 permutations, whereas 999 bootstraps were used to estimated confidence intervals for *r*, for given geographical distance classes.

Population genetic diversity

Genetic diversity within each of the three inferred populations was evaluated by calculating the mean number of alleles per locus

(A), observed heterozygosity (H_O), expected heterozygosity (H_E), the number of rare (frequency of less than 5%) alleles (A_{RARE}) and the number of unique alleles (A_U) with GENALEX 6.1 (Peakall and Smouse 2006). Allelic Richness (A_R), a measure that adjusts the alleles per locus to account for variation in the sample size, was calculated in FSTAT 2.9.3 (Goudet 1995). Private allelic richness (A_{UR}), a measure that uses rarefaction to adjust the number of unique alleles per locus to account for variation in sample size was calculated in HPRARE 1.1 (Kalinowski 2004, 2005).

Testing for a recent population bottleneck

A combination of methods was used to test for evidence of a population bottleneck. The program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996) was used with the two-phase mutation (TPM) (Di Rienzo *et al.* 1994; Luikart *et al.* 1998) model, with 90% single-step mutations (12% variance). Significance was assessed using Wilcoxon's sign-rank tests. In addition, the effective population size was estimated with ONESAMP 1.1 (Tallmon *et al.* 2008), which uses a Bayesian framework, and NEESTIMATOR 1.3 (Peel *et al.* 2004), which incorporates a linkage disequilibrium analysis. For these bottleneck and effective population size analyses, only the inferred Campbelltown population (i.e. Campbelltown and Heathcote sampling areas combined) was tested because of insufficient sample sizes in the other populations.

Results

Loci and population statistics

When evaluated across all populations, all loci were in Hardy–Weinberg equilibrium in the pooled sampling regions test. F_{IS} values for each population were not significantly different from zero. There was no significant linkage disequilibrium detected between any of the loci. All loci were polymorphic, and the number of alleles per locus ranged from four (Pcv 2) to 13 (Pcv 6.3), with a mean of 7.33 alleles per locus. Blind re-scoring of genotypes of 30 animals did not result in any contradictions.

Population structure

Both GENELAND and STRUCTURE inferred that the most likely number of populations was three. The population boundaries found by GENELAND were well defined and geographically distinct (Fig. 2) and corresponded to the sampling regions, except the Heathcote and Campbelltown animals that were consistently assigned to the same population. Similarly, assignments by STRUCTURE corresponded to the sampling regions and also grouped the Campbelltown and Heathcote animals together as one population, and all assignments were very strong. For all subsequent population analyses, the Campbelltown and Heathcote animals are to be considered a single population. The location of environmental barriers corresponded to the border found by GENELAND between the Campbelltown region and the Southern Tablelands population. The existence of three discrete populations was also clearly shown in the NJ distance tree (Fig. 3) where three clear groups were identified, and these groups are virtually identical to the population assignment given by GENELAND and STRUCTURE. Spatial autocorrelation tests revealed no significant genetic spatial structure (i.e. isolation by distance).

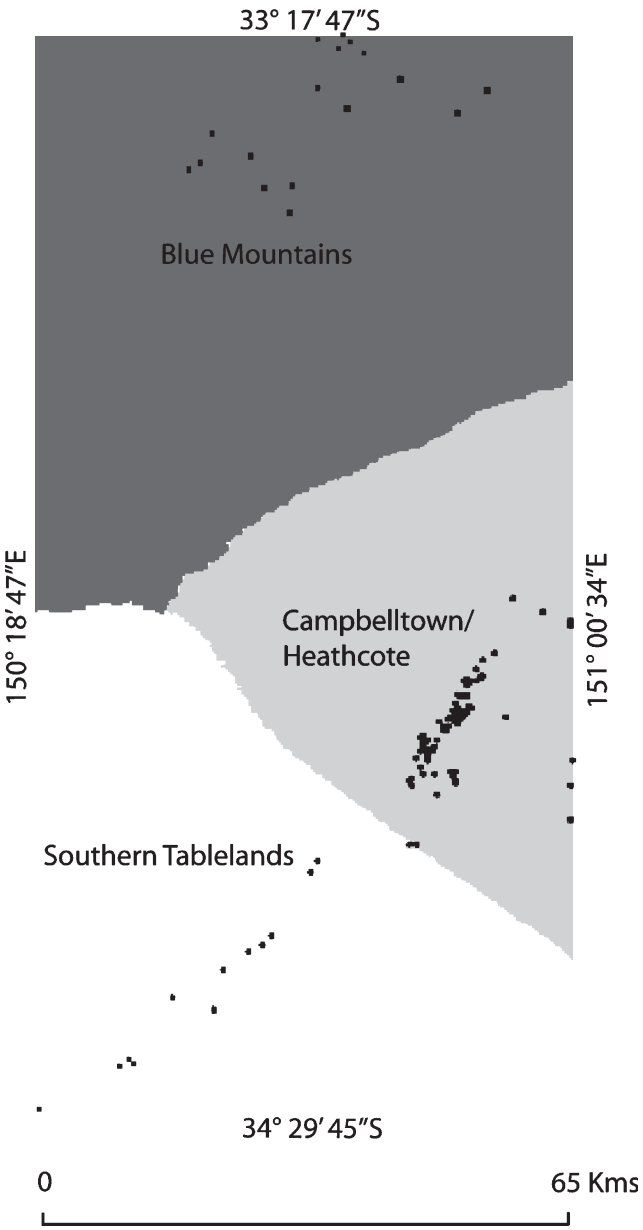

Fig. 2. Individual assignments from GENELAND. Each sample is represented by a black dot and placed according to sample site spatial coordinates. Samples are grouped into one of three populations.

The R_{ST}/pR_{ST} comparison test was not significant, indicating it is not possible to say that the barriers are based on evolutionary timescales, and therefore it is possible that any observed differentiation is due to a contemporary barrier to gene flow.

Genetic differentiation of inferred populations and of sampling regions

Pairwise F_{ST} of the inferred populations showed considerable differentiation, and all F_{ST} values were significant ($P < 0.001$). Pairwise F_{ST} values were as follows: $\theta = 0.224$ (Campbelltown + Heathcote – Southern Tablelands), $\theta = 0.220$ (Campbelltown + Heathcote – Blue Mountains) and $\theta = 0.141$

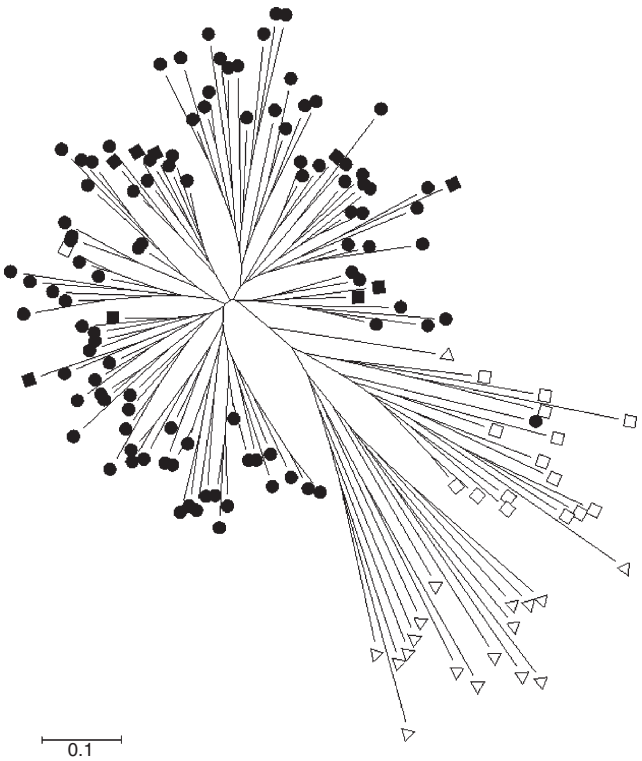

Fig. 3. Unrooted neighbour-joining tree displaying 1 – proportion of shared alleles genetic distance between sampled individuals. ● = Campbelltown, ■ = Heathcote, □ = Southern Tablelands, △ = Blue Mountains.

(Southern Tablelands – Blue Mountains). F_{ST} analysis was also performed on the basis of sampling regions, and the results were similar, except that the pairwise F_{ST} between Campbelltown and Heathcote was $\theta = 0.006$ ($P = 0.297$).

Within-population genetic diversity

The Blue Mountains region had the highest genetic diversity and greatest proportion of unique alleles, whereas the Campbelltown + Heathcote population had the lowest genetic diversity and the fewest unique alleles. Observed (H_O) and expected (H_E) heterozygosity, average number of alleles per locus (A), allelic richness (A_R) and private allelic richness (A_{UR}) are provided in Table 1.

Testing for a recent population bottleneck

Analysis of the inferred Campbelltown + Heathcote population with BOTTLENECK detected significant evidence of a recent population bottleneck by using the heterozygote excess test ($P = 0.02$). Effective population size estimates for this population with ONESAMP and NEESTIMATOR were very low and ranged from 16.3 (95% CI: 15.4, 17.3) to 21.2 (95% CI: 16.1, 29.7) respectively. The mode-shift test in BOTTLENECK did not detect evidence of a bottleneck. However, this may simply mean that the bottleneck was of short duration, the bottleneck occurred sufficiently long ago so that the population size has since recovered, or even that there has been a low level of migration, because any of these factors could prevent a mode-shift signal (e.g. Keller *et al*. 2001; Eldridge *et al*. 2004; Busch *et al*. 2007).

Discussion

The impacts of external threats and the viability of koala populations vary across their geographic range. Therefore, koala management plans in Australia need to be developed and implemented on a local scale. Although the NSW Koala Recovery Plan has identified the Campbelltown population as a priority population for study, there are no management plans specific to koalas in the Sydney region (DECC 2008). The objectives of this research were to formulate management recommendations that will maximise the long-term conservation of koala populations in the Sydney region by resolving their genetic structure.

Campbelltown and Heathcote regions

The Campbelltown and Heathcote koalas should be considered a single continuous population. Both genic and genotypic analysis could not discriminate between the two sampling regions.

These sample regions are separated by the Holsworthy Army Range. The present study has shown that animals separated by the Army Range represent a single population. One can therefore assume that koalas use the range as important connective habitat for dispersal between the Campbelltown and Heathcote regions. On the basis of habitat considerations mentioned previously, it appears likely, although cannot be proven from the present data, that koalas also permanently inhabit the Army Range, in addition to using it as an important thoroughfare.

Although these genetically informed conclusions are based on only a relatively small sample size from the Heathcote region, there is also ecological evidence to further support the findings. Previously published estimates of male migration distance in other regions also confirm the potential for migration between the Campbelltown and Heathcote regions (e.g. Mitchell and Martin 1990; Dique *et al*. 2003).

There is evidence that the Campbelltown + Heathcote population has undergone a genetic population bottleneck. The BOTTLENECK analysis detected significant evidence of

Table 1. Summary of genetic diversity parameters

Population	N^A	A	A_R	A_{UR}	H_O	H_E	$F_{IS}{}^B$
Campbelltown + Heathcote	110	3.17	2.93	0.11	0.499	0.542	0.079
South Tablelands	16	5.08	4.88	0.88	0.520	0.586	0.113
Blue Mountains	18	6.83	6.46	2.62	0.655	0.743	0.118
Mean	–	5.027	4.757	15.333	0.558	0.624	–

[A]Total number of koalas genotyped.
[B]All F_{IS} values were not significantly different from zero.

a population bottleneck when using the heterozygosity excess test (Cornuet and Luikart 1996). This is also supported by the small effective population size estimates for this region (16 and 21), especially considering that the population census size is conservatively estimated to be at least 400 (Ward 2002). Finally, the reduced number of rare alleles and the overall lower genetic diversity than for the other populations further support the hypothesis of a sustained reduction in the effective population size (Nei *et al*. 1975; Maruyama and Fuerst 1985). Earlier ecological data suggested a bottleneck was possible, because a severe disease outbreak occurred in the region in the early 1920s (Tilley and Uebel 1990) and koalas were not seen around Campbelltown from ~1920 until 1986 (Ward and Close 2004). The genetic data in this paper now support a bottleneck hypothesis.

Considering the low genetic diversity and strong evidence for a single continuous population, management of the Campbelltown and Heathcote regions as a single population, incorporating the Holsworthy Army Range, is essential and will minimise chances for the additional loss of genetic diversity. Relationships between neutral genetic-marker diversity and functional genetic diversity are not always strong and a more accurate picture of genetic diversity would be obtained by directly measuring quantitative genetic traits (Reed and Frankham 2001). If the land-use changes and koalas are no longer able to move through the Army range, this will cause a major fragmentation of the Campbelltown + Heathcote population and is likely to accelerate genetic drift and potentially lead to inbreeding. The future of the Holsworthy Army Range is currently uncertain. If the Army Range land is modified or developed in any manner, it will be essential that development plans incorporate strict measures to ensure that gene flow is maintained across the range between the Campbelltown and Heathcote regions. Such measures are likely to include substantial migration corridors. On the basis of our evidence of barriers to gene flow in the south of the region, care would need to be taken to ensure that the corridors are well clear of roads or housing and contain preferred koala habitat.

Population differentiation and barriers to gene flow

The Campbelltown + Heathcote, Southern Tablelands and Blue Mountains populations were clearly differentiated by each of the different methods of analysis in the present study and therefore should be considered discrete populations. These three populations have apparently little gene flow between them. Isolation by distance was not detected, so the observed differentiation may be explained by the presence of barriers to gene flow and founder and bottleneck effects (Schwartz and McKelvey 2009).

Significant geographical and artificial barriers are present throughout our study area. On the basis of our genetic analysis and sampling locations, an abrupt demarcation was found between koalas in the Campbelltown + Heathcote population and the Southern Tablelands population. The R_{ST} analysis found no significant difference in observed *v.* permuted R_{ST} values, lending some support to the idea that these are contemporary barriers to migration, rather than a substantially 'pre-European' evolutionary separation. Some samples were obtained from roadkill, which may have lead to non-random sampling in some areas. However, only a small minority of samples were roadkill, and the main bias introduced is likely to be towards young dispersing male koalas. If anything, this bias is likely to increase the ability to detect gene flow and therefore should not underestimate the amount of gene flow existing.

The low genetic diversity and small effective population size of the Campbelltown + Heathcote population could make it more vulnerable to habitat change and survival pressures in the future (Frankham *et al*. 2002). Allowing natural gene flow between the Campbelltown region and the Southern Tablelands population to occur would naturally enrich the genetic diversity in both populations by introducing new alleles, would increase overall N_e and reduce future loss of genetic diversity (e.g. Frankham *et al*. 2002). Natural migration can be encouraged by creating and maintaining habitat corridors that allow the safe navigation through the potential barriers to gene flow mentioned above.

Key strategies for encouraging natural migration should also involve measures allowing koalas to safely cross the road separating the Campbelltown + Heathcote and Southern Tablelands populations. Our genetic results suggest few koalas safely cross this road, resulting in little effective gene flow. Creating road culverts (Taylor and Goldingay 2003) and/or reducing the speed limit (Dique *et al*. 2003) around koala blackspot zones may help reduce koala fatalities and facilitate safe road crossings.

Although there was strong differentiation, it is possible that a low level of individual migration is occurring, although not enough to create effective gene flow among generations. For example, the NJ tree (Fig. 3) shows two potential candidates for migratory exchange between the Campbelltown and Southern Tablelands populations. Of these, the candidate migrant into the Campbelltown region in particular could be a genuine migrant, on the basis of the fact that STRUCTURE also assigned this animal into the Campbelltown population. The potential migrant into the Southern Tablelands as suggested by the NJ tree was not supported by STRUCTURE results.

Comparative genetic diversity

The Blue Mountains population had the highest level of genetic diversity and is comparable to the highest diversity found in Houlden *et al*.'s (1996*b*) study. Three of Houlden's six microsatellite primers were used in the present study, allowing some degree of comparison between the studies. The Southern Tablelands population and the Campbelltown + Heathcote population, however, have much less diversity when compared with the Blue Mountains population. As we have no prior samples we cannot be sure whether the Southern Tablelands population has 'lost' genetic diversity or has always had less diversity than the Blue Mountains population. However, in Campbelltown, the BOTTLENECK program gives us some confidence that there has been a genuine loss of genetic diversity. Habitat in the Campbelltown + Heathcote and Southern Tablelands regions is generally considered high quality for koalas (Tilley and Uebel 1990; Ward 2002), so it is unlikely that the habitat historically supported smaller, less diverse populations. In addition, anecdotal evidence regarding the size of the koala fur trade in the Campbelltown region suggests that the population was historically considerably larger than today. The lower genetic

diversity seen in the Campbelltown + Heathcote and the Southern Tablelands populations is possibly the result of the fur trade, disease (Tilley and Uebel 1990) or habitat loss. Relationships between neutral genetic-marker diversity and functional genetic diversity are not always strong and a more accurate picture of genetic diversity would be obtained by directly measuring quantitative genetic traits (Reed and Frankham 2001). The Blue Mountains koalas may have been spared some of the impact of the fur trade or habitat loss because they inhabit terrain that is more remote and difficult to access. The Blue Mountains population appears to be of high conservation value because it holds a reservoir of genetic diversity not seen in other populations in the Sydney region. Far fewer animals were sampled in the Blue Mountains and the Southern Tablelands populations than in the Campbelltown + Heathcote population and therefore an increased sample size may uncover even more genetic diversity for these populations.

Areas for future research

Few recent sightings of koalas in the forests to the south-east of the Campbelltown region have been recorded, possibly because these areas are part of the Sydney water catchment and access is restricted. Surveys for koalas and analysis of genetic material from known populations on either side of the disputed region may help determine whether animals are present in this area. The major highway between Sydney and Canberra bisects the Southern Tablelands population. Most of our public sightings and capture samples came from animals on the western side of this highway. Additional samples from the eastern side of the highway could be used to test the hypothesis that large roads such as this pose a barrier to gene flow, assuming the roads have created a new barrier and were not built on landscape features that already had a barrier effect. Our analyses of Sydney koalas may provide insights into factors that may affect other mammals in the region, because they have shown that although some mammals can exist in highly fragmented semi-urban areas, certain landscape features are able to further reduce the gene flow. Therefore, research into other species in the outer Sydney region should consider landscape features as potentially causing further fragmentation in populations already fragmented through loss of habitat.

Conclusion

The present paper has identified three discrete koala populations in the Sydney region. Little gene flow among these populations was inferred. Although the Campbelltown + Heathcote and Southern Tablelands populations abut, there appears to be a barrier to gene flow between them that may be the result of geographic features, human alterations of the land or a combination of these. Also revealed is the high level of genetic diversity in the Blue Mountains population, which is comparable to the highest levels previously published for koala populations. However, the Campbelltown + Heathcote population has a relatively low diversity, and there is evidence indicating this population has suffered a recent genetic bottleneck. The fact that these are demographically separate populations has important implications for koala management in the Sydney region. The three confirmed koala populations should be considered separate management units and will need specific management plans tailored to the conservation issues and priorities of those regions. The present paper has also shown that the army land is likely to be critical for the viability of the Campbelltown population because of the connectivity it provides for the Campbelltown and Heathcote regions. The most effective and the simplest solution for conservation of koalas in the Sydney region would appear to be preventing or limiting any further loss of population connectivity.

Acknowledgements

Thanks go to all who assisted in the collection of samples, especially Melissa So, Lynn Bowden, Wendy and Michael Fairs, Steven Ward, Kieran Griffin, Mariette Ennik, Sharon Andronicos, members of the NSW Wildlife Information Rescue and Education Service (WIRES), staff at NSW National Parks and Wildlife Service, the Macarthur Advertiser, NPA Macarthur and the many members of the community who alerted us to koala sightings. We acknowledge funding from the Wildlife Health and Conservation Centre, The University of Sydney and The University of Western Sydney. We also thank the referees for their help in improving the paper.

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Manuscript received 1 October 2009, accepted 10 February 2010