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RESEARCH ARTICLE

Genetic variation and structuring in the threatened koala populations of Southeast Queensland

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Abstract Habitat fragmentation can act to cause reproductive isolation between conspecifics and undermine species' persistence, though most studies have reported the genetic condition of populations that have already declined to a very small size. We examined genetic diversity within the vulnerable, declining koala (*Phascolarctos cinereus*) population in Southeast Queensland, Australia to determine the genetic impact of ongoing threatening processes. Five hundred and twelve koalas from ten Southeast Queensland Local Government Areas on the mainland and one island were genotyped at six polymorphic microsatellite loci. Based on Bayesian cluster analysis incorporating spatial data, the regional koala population was subdivided into six clusters, with location of major roads and rivers appearing to be consistent with being barriers to gene flow. The distribution of mtDNA control region haplotypes identified distinct coastal and inland clades suggesting that historically there was gene flow between koalas along the coast (though little interchange between coastal and inland animals). In contrast, koalas from the Koala Coast (Brisbane City, Logan City and Redland Shire) were shown by microsatellite analysis to be genetically distinct from adjacent areas. It is likely, therefore, that more recent reductions in population size and restricted gene flow through urbanisation have contributed to the genetic differentiation of koalas in the Koala Coast region.

Keywords Koalas · Habitat fragmentation · Mitochondrial DNA · Microsatellites · Genetic isolation · Island population

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Introduction

The negative impacts of habitat fragmentation on species' persistence are well known (Noss and Copperrider 1994; Tilman et al. 1994). However, the mechanistic links between extinction and fragmentation are sometimes poorly understood and so simply ascribed to demographic stochasticity; that is, population variability due to random differences in reproduction and survival among individuals (Simberloff 1994). Isolation can reduce the plasticity of population responses to habitat change (Segelbacher et al. 2003), so identifying the scale at which populations respond to fragmentation is an important step toward

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understanding mechanisms by which fragmentation affects population viability.

Southeast Queensland, Australia, is undergoing rapid, urban-mediated development causing fragmentation of habitat; thus understanding the effects of this process on natural populations is a key to conserving the species in the region. The koala (*Phascolarctos cinereus*) is a large, arboreal marsupial for which breeding dynamics are well described (Ellis et al. 2002) and preliminary phylogeographic studies have been undertaken (Houlden et al. 1999; Fowler et al. 2000); it thus presents a suitable model for evaluation of the molecular evolutionary consequences of habitat fragmentation on a declining species. Historical impacts on the koala populations in Queensland have arisen from broad-scale habitat clearing, disease epizootics and the export of 1–2 million pelts by the end of the last koala open hunting season in 1927, which all contributed to a decline to the present day estimate of less than 35,000 koalas in Southeast Queensland (Phillips 1990; de Villiers unpublished data). A study on the mitochondrial DNA (mtDNA) variation of koalas from four Southeast Queensland locations suggested that there has been female-mediated gene flow historically (on the basis that identical haplotypes were detected in adjacent populations), but creation of recent barriers and hence reduced gene flow have resulted in limited distribution of haplotypes (Fowler et al. 2000).

Koalas are widely distributed in Queensland, but they are most abundant in the south-east region (Phillips 1990). Under the Regulations of *The Nature Conservation Act* (Qld) 1992, koalas were classified as "common protected wildlife" in Queensland. In March 2004, koalas were reclassified as "vulnerable wildlife" in the Southeast Queensland Bioregion (from the New South Wales border north to Gladstone and west to Toowoomba). This reassessment of conservation status was based on the estimate of the current population size, the rate of habitat loss and calculated rates of koala mortality for the region. The *Nature Conservation [Koala] Conservation Plan 2006 and Management Program 2006–2016* (Queensland Government 2006) was implemented to address the issue of the declining koala population in the State's south-east where dramatic declines and some local extinctions have resulted from habitat loss, fragmentation and anthropogenic mortality (Queensland Government 2006; Preece 2007). Clearing of koala habitat continues in the south-east region to accommodate a rapidly increasing human population and the threat to the viability of these koalas is intensified by motor vehicles, dogs and disease, especially around urban centres (Queensland Government 2006).

Although the impact of habitat fragmentation on the genetic integrity of a koala population has not previously been investigated, in another marsupial species in Queensland (*Petrogale penicillata*) habitat fragmentation has been shown to disrupt natural population dynamics, including effects on reproduction, survival and movement of animals (Hazlitt et al. 2006). In general, threats to species from habitat fragmentation occur through decreased gene flow between populations, leading to genetic isolation of populations, a loss of heterozygosity through genetic drift and increased inbreeding (and inbreeding depression) as populations become small (Lacy 1988). More particularly in mammals, it is thought that decreased genetic variation can result in reduced reproductive success, reduced disease resistance and decreased ability to adapt to changing environmental pressures (O'Brien et al. 1985; O'Brien and Evermann 1988; Sherwin et al. 2000; Aguilar et al. 2008). As populations become small, stochastic events can have significant impacts on their persistence. Infections with Chlamydiacae are prevalent in most koala populations and resulting chlamydial diseases are widespread in Southeast Queensland; these can present as blindness, pneumonia, cystitis/nephritis and infertility (Girjes et al. 1988; Weigler et al. 1988; Carrick 1996), thereby reducing individual and thus population capacity for long-term survival. Stress and habitat disturbance can precipitate the expression of clinical signs from chlamydial infections (Ellis et al. 1993).

Parts of Brisbane (south of the Brisbane River), Logan (east of the M1 Motorway) and Redland Local Government Areas (LGAs) make up a conservation region known as the Koala Coast, comprised of 375 sq km of land that has been identified as one of the most significant natural koala populations in Australia (Carrick 2004; Queensland Government 2007). The 2008 survey estimated a population of 2,279 koalas, which represents a 51% decline in 3 years and a 64% decline since the original 1996–1999 survey (Dique et al. 2004; Queensland Government 2007, 2009). Thompson (2006) described genetic isolation on a very fine scale in the Koala Coast (1–5 km) and so at the outset of this study genetic isolation over larger distances across Southeast Queensland was expected.

We evaluated microsatellite allelic diversity and mitochondrial haplotypic diversity in koala populations in the Southeast Queensland region, focusing on an area incorporating ten LGAs on the mainland and one island, to estimate within-population genetic variation and investigate the effect of habitat fragmentation as a result of urban expansion on the level of genetic differentiation among populations.

Methods

Samples

Ear tissue samples and the addresses of capture locations were obtained for 512 sick, injured, orphaned or dead

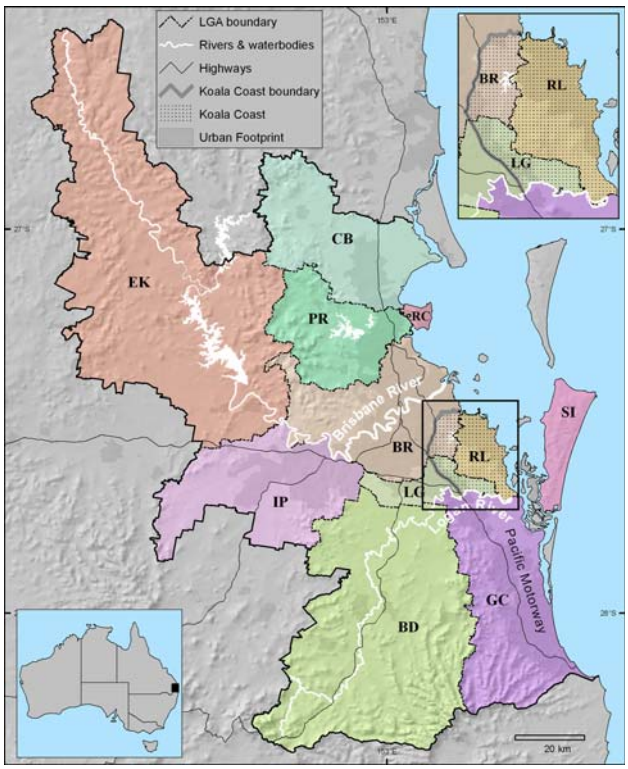

Fig. 1 Local Government Areas in Southeast Queensland from which samples were obtained. *BD* Beaudesert, *BR* Brisbane, *CB* Caboolture, *EK* Esk, *GC* Gold Coast, *IP* Ipswich, *LG* Logan, *PR* Pine Rivers, *RC* Redcliffe, *RL* Redland, *SI* North Stradbroke Island. Koala Coast shown in *inset*

koalas presented to Moggill Koala Hospital or the Australian Wildlife Hospital, from various Southeast Queensland locations indicated in Fig. 1 (Beaudesert Shire $n = 18$, Brisbane City $n = 60$, Caboolture Shire $n = 46$, Esk Shire $n = 50$ Gold Coast City $n = 48$, Ipswich City $n = 50$, Logan City $n = 78$, Pine Rivers Shire $n = 62$, Redcliffe City $n = 18$, Redland Shire $n = 62$ and North Stradbroke Island $n = 20$). North Stradbroke Island is part of the Redland LGA, but for the purposes of this study, it was treated as a separate population because there is no land connection to the mainland part of the Redland LGA. Since the commencement of this study, most of the LGAs in Southeast Queensland have undergone amalgamations but for present purposes, the original LGA designations have been retained. Although LGA boundaries are not barriers to koala movement, we used the term 'population' when referring to koalas located in individual LGAs for convenience of description. Ear tissue was stored in 70% ethanol at room temperature until extraction. Total genomic DNA was extracted by standard phenol:chloroform and ethanol precipitation procedures following overnight incubation at 55°C in lysis buffer (40 mM Tris hydrochloride, 0.1 M NaCl, 0.5% sodium dodecyl sulphate, 20 mM EDTA) and 0.3 mg/mL Proteinase K. The pellet was resuspended in 50 µL water and stored at −20°C.

Microsatellite screening

Koalas were genotyped using six polymorphic, dinucleotide microsatellite loci previously isolated by Houlden et al. (1996a). The PCR amplification protocols have been previously described by Ellis et al. (2002). Genotypes were resolved on an Applied Biosystems/Hitachi 3130xl Genetic Analyser and analysed in Genemapper v 3.7 (Applied Biosystems).

Genetic diversity

The mean number of alleles per locus, an unbiased estimate of heterozygosity (H_e; Nei 1987) and observed heterozygosity were calculated using GenAlEx6 (Peakall and Smouse 2006). Genotype distributions were tested for conformity to Hardy–Weinberg equilibrium using Markov chain analysis and linkage disequilibrium in GENEPOP on the Web (http://genepop.curtin.edu.au/; Raymond and Rousset 1995). A test for null alleles was performed in Micro-Checker (van Oosterhout et al. 2003). Allelic richness (mean number of alleles corrected for sample size) was calculated in FSTAT (Goudet 2001).

Population structure

While LGA boundaries are generally arbitrary with respect to biology, they represent the basis on which day-to-day management of koala habitat occurs and thus were chosen for the initial grouping for analysis. Pair-wise population F_{ST} (based on LGAs) was calculated in Genetix v.4.03 (Belkhir et al. 2004) and significance assessed after Bonferroni correction. To visualize the data and gain a basic understanding of the partition of the total genetic variation across individuals, a Factorial Component Analysis (FCA) was performed in Genetix v.4.03 (Belkhir et al. 2004). The detailed spatial information available for each sample was utilised in Geneland (Guillot et al. 2005) to infer the number of clusters based on both genetic and geographic data. Under the Dirichlet model, five replicates of $K = 2$–9 were tested with 100,000 iterations and 100 thinning. Pair-wise population F_{ST} based on Geneland clusters was calculated in Genetix v.4.03 (Belkhir et al. 2004) and significance assessed after Bonferroni correction. Assignment of koalas to a cluster or population based on their genotype alone rather than an LGA or geographic location, was implemented in STRUCTURE 2.2 (Pritchard et al. 2000). To infer the number of genetic clusters (K), twenty independent runs of models $K = 1$–7 were used, deduced by posterior probabilities [Ln $P(D)$] using 100,000 iterations

after a 100,000 iteration burn-in period. The average Ln $P(D)$ for each K was plotted to determine the highest likelihood and the number of population clusters was determined by calculating ΔK (Evanno et al. 2005). Koalas were assigned to a particular cluster if they had a probability of membership to that cluster (q-value) ≥ 0.8. Koalas with a q-value $= 0.19$–0.79 were regarded as mixed or hybrid animals. Each koala record was colour-coded according to the cluster to which it belonged and plotted in ArcGIS 9 (ESRI) using the co-ordinates of its capture location. To determine if any sub-structure existed within any of the inferred clusters, each cluster was tested individually in STRUCTURE 2.2 in the manner described above. Observed and expected heterozygosity, allelic richness and pair-wise population F_{ST} were re-calculated based on the Geneland and STRUCTURE 2.2 cluster populations. Mantel tests (Mantel 1967) were performed in GenAlEx6 (Peakall and Smouse 2006) with 999 permutations to test for isolation by distance within and between both Geneland and STRUCTURE 2.2 clusters. Analysis of molecular variance (AMOVA) was performed in GenAlEx6 (Peakall and Smouse 2006) by partitioning the data into five different groups of pooled populations, to determine the group design that explained the highest percentage of the total variation.

Mitochondrial sequencing

A stratified random subset of 77 individuals encompassing all LGAs was selected for mitochondrial analysis. The mitochondrial control region was amplified by PCR in 10 μL reaction volumes containing 0.5 U of Qiagen HotStar DNA polymerase, 1× Qiagen PCR buffer, 1.25 mM $MgCl_2$, 0.15 mM dNTPs, 0.25 μM each of KmtL2 and KmtH2 primers (Fowler et al. 2000) and 80–280 ng of DNA. The PCR cycling conditions were as described in Fowler et al. (2000). Reactions were purified using 2.5 U Exonuclease I (Fermentas) and 0.25 U Shrimp Alkaline Phosphotase (Fermentas). Sequencing was carried out using the BigDye® Terminator v3.1 (Applied Biosystems) sequencing kit. Haplotypes were edited and aligned by eye and aligned with previously published Southeast Queensland haplotypes Q1–Q8 (Fowler et al. 2000). A haplotype network based on statistical parsimony was constructed using TCS (Clement et al. 2000).

Results

Genetic variability at microsatellite loci

Genotyping of 512 koalas from Southeast Queensland revealed that all six loci were polymorphic with 2–17 alleles per locus. There were only two significant deviations from Hardy–Weinberg expectations calculated for each locus and LGA population (Locus 2 at Esk and Locus 4 at Redcliffe). Tests for linkage disequilibrium revealed significant linkage between Loci 1 and 6 (Phc-1 and Phc-25), which may be a result of non-random mating, recent admixture or genetic drift (Frankham et al. 2002). Physical linkage of loci is unlikely because linkage at Loci 1 and 6 was not significant in every population (LGA). There was no evidence of null alleles among loci.

Populations from each LGA displayed high microsatellite variation with average expected heterozygosity ranging between 69 and 80%; the only exception was North Stradbroke Island, where variability was significantly lower (56%, $P < 0.05$, Tukey's multiple comparison; Table 1). The North Stradbroke Island population also had lower allelic richness (3.5 alleles per locus) compared to the mainland LGAs (5.2–8.1 alleles per locus; Table 1). The island koalas had a subset of the alleles present in the mainland koalas, with a small number of alleles at high frequencies. However, three koalas on North Stradbroke

Table 1 Genetic variation detected at six microsatellite loci in Southeast Queensland koala populations defined by Local Government Areas for convenience of description

Population	n	Mean no of alleles	Mean allelic richness	Average observer heterozygosity	Average expected heterozygosity
Beaudesert	18	7.8	7.8	0.81	0.78
Brisbane	60	7.8	6.3	0.72	0.74
Caboolture	46	7.0	6.3	0.74	0.76
Esk	50	10.2	8.1	0.79	0.80
Gold Coast	48	9.7	7.9	0.73	0.79
Ipswich	50	9.3	7.8	0.73	0.76
Logan	78	6.5	5.5	0.72	0.72
Pine Rivers	62	7.3	6.1	0.73	0.77
Redcliffe	18	6.0	6.0	0.68	0.71
Redland (mainland)	62	6.2	5.2	0.72	0.69
North Stradbroke Island	20	3.5	3.5	0.53	0.56

Island possessed an allele at Locus 2 that was not identified from any koalas on the mainland.

Population genetic structure

The level of differentiation between populations from each LGA was measured using pair-wise F_{ST} (Table 2). Out of 55 pairwise comparisons, there were four pairs that were not significantly different from each other after Bonferroni correction (Brisbane–Logan, Brisbane–Redland, Caboolture–Pine Rivers and Pine Rivers–Redcliffe); these pairings represent adjacent LGAs. Population structure was visualised in an FCA (Fig. 2), which indicated three regions that diverged from the rest of the samples: the Koala Coast, North Stradbroke Island and the coastal region north of the Brisbane River encompassing Caboolture, Pine Rivers and Redcliffe LGAs.

Adding spatial information but not pre-assigned populations to the genetic data, the clustering program Geneland identified six clusters (Fig. 3), extending the initial structure seen on the FCA plot. These six clusters were 1) Beaudesert and Ipswich ($n = 68$), 2) Caboolture, Pine Rivers and Redcliffe ($n = 128$), 3) Koala Coast (Brisbane, Logan, Redlands; $n = 196$), 4) North Stradbroke Island ($n = 20$), 5) Gold Coast ($n = 48$) and 6) Esk ($n = 52$). Each of the clusters was from a geographically restricted region. Cluster 4 was limited to North Stradbroke Island. On the coastal plain, Cluster 2 was found north of the Brisbane River, and to the south of the river, Cluster 3 was in the region termed the Koala Coast and Cluster 5 further south in the Gold Coast. Inland, Cluster 6 was found to the north, separated from Cluster 1 by the Warrego Highway. All Geneland-defined clusters were significantly differentiated from each other (Table 3). However, North Stradbroke Island was the most divergent population with greatest differentiation from the Koala Coast cluster and least from the Gold Coast cluster. Expected heterozygosity in the Geneland clusters ranged from 0.56 (Cluster 4, North Stradbroke Island) to 0.81 (Cluster 6, Esk). Cluster 3 (Koala Coast) had the lowest heterozygosity of any mainland cluster (0.72). Allelic richness ranged from 3.5 alleles per locus in Cluster 4 (North Stradbroke Island) to 8.5

Table 2 Pair-wise population differentiation (F_{ST}) between Southeast Queensland koala populations based on Local Government Areas estimated from six polymorphic microsatellite loci

	Brisbane	Caboolture	Esk	Gold Coast	Ipswich	Logan	Pine Rivers	Redcliffe	Redland	North Stradbroke Island
Beaudesert	0.07	0.06	0.04	0.04	0.03	0.1	0.07	0.1	0.11	0.19
Brisbane		0.09	0.09	0.04	0.06	**0.01**	0.09	0.11	**0.01**	0.22
Caboolture			0.03	0.06	0.08	0.11	**0**	0.04	0.12	0.23
Esk				0.04	0.06	0.1	0.04	0.06	0.12	0.18
Gold Coast					0.05	0.06	0.07	0.09	0.07	0.14
Ipswich						0.09	0.08	0.09	0.09	0.19
Logan							0.1	0.12	0.01	0.24
Pine Rivers								**0.02**	0.12	0.23
Redcliffe									0.15	0.25
Redland										0.26

Populations that are not significantly differentiated from each other are indicated in bold, all other populations are significantly differentiated after Bonferroni correction. Pair-wise population differentiation is based on Weir and Cockerham (1984)

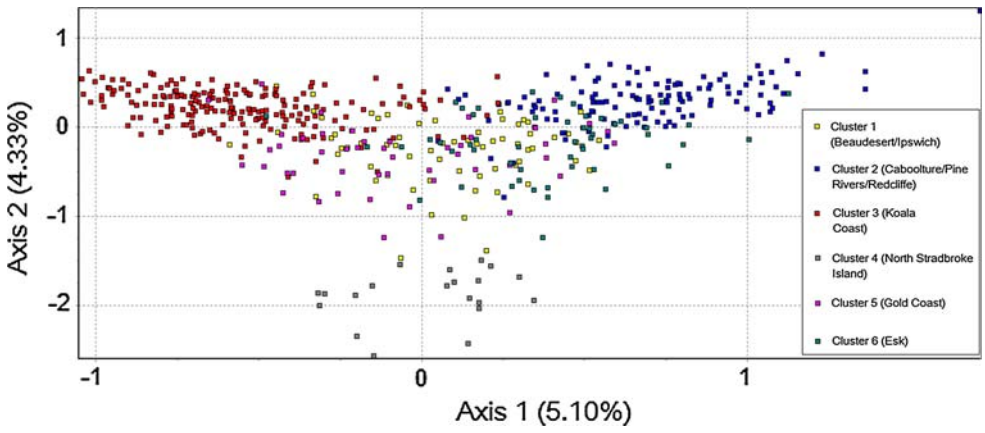

Fig. 2 Factorial Component Analysis (*FCA*) of koala samples coloured by Geneland cluster

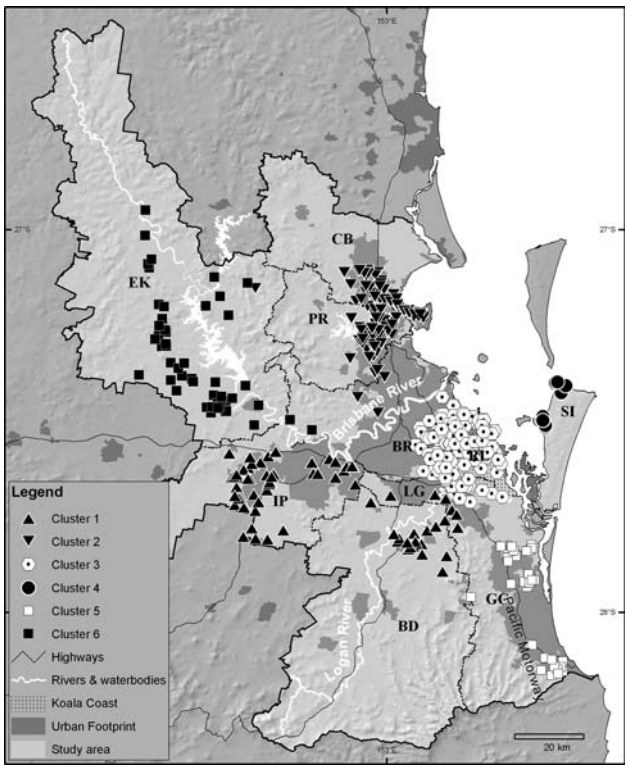

Fig. 3 Clusters as determined by Geneland. Cluster 1▲: Beaudesert, Ipswich; Cluster 2▼: Pine Rivers, Caboolture, Redcliffe; Cluster 3⬡: Koala Coast (Brisbane, Logan, Redland); Cluster 4●: North Stradbroke Island; Cluster 5□: Gold Coast; Cluster 6■: Esk

Table 3 Pair-wise population differentiation (F_{ST}) between South-east Queensland koala populations based on six Geneland clusters estimated from six polymorphic microsatellite loci: Symbols correspond to symbols in Fig. 3

	Cluster 2▼	Cluster 3⬡	Cluster 4●	Cluster 5□	Cluster 6■
Cluster 1▲	0.07	0.08	0.18	0.04	0.05
Cluster 2▼		0.10	0.22	0.07	0.04
Cluster 3⬡			0.24	0.06	0.11
Cluster 4●				0.14	0.18
Cluster 5□					0.04

All clusters are significantly differentiated after Bonferroni correction. Pair-wise population differentiation is based on Weir and Cockerham (1984)

alleles per locus in Cluster 1 (Beaudesert and Ipswich). Cluster 3 (Koala Coast) had the lowest allelic richness of any mainland cluster (5.6 alleles per locus). Mantel tests did not detect any evidence of isolation by distance within the Geneland-defined clusters (Cluster 1 $R^2 = 0.043$, Cluster 2 $R^2 = 0.05$, Cluster 3 $R^2 = 0.093$, Cluster 4 $R^2 = 0.169$, Cluster 5 $R^2 = 0.051$, Cluster 6 $R^2 = 0.028$, $P < 0.001$ for Clusters 1–5, $P < 0.005$ for Cluster 6).

Without spatial data, Bayesian clustering analysis of the six microsatellite loci in STRUCTURE 2.2 identified two clusters as the highest level of structuring. The number of clusters (K) was determined by ΔK, when a value of K could not be accurately determined from the Ln $P(D)$ plot (Fig. 4a, b). 193 koalas (38%) belonged to Cluster 1, 260 koalas (50.5%) belonged to Cluster 2 and 59 koalas (11.5%) were considered to be mixed or hybrids ($q = 0.19$–0.79; Fig. 5). Mantel tests failed to detect any evidence of isolation by distance within or between the clusters (Cluster 1 $R^2 = 0.029$, Cluster 2 $R^2 = 0.0034$, Cluster 1 and Cluster 2 $R^2 = 0.12$, $P < 0.001$). No koalas from north of the Brisbane River or from North Stradbroke Island belonged to Cluster 1. The vast majority of koalas in Cluster 1 were restricted to the Koala Coast and the coastal area immediately south. Koalas in Cluster 1 had a total of 47 alleles across all loci. The heterozygosity levels in Cluster 1 were high in comparison to those reported from southern Australia ($H_e = 0.72$), as was allelic richness (6.6 alleles per locus). This cluster had three unique alleles at two loci. There was no additional substructure within Cluster 1 (Koala Coast) when tested. This cluster corresponded with the Geneland-defined Cluster 3.

STRUCTURE 2.2 Cluster 2 occupied a broad geographical area and included koalas from ten LGAs, extending north of the Brisbane River, west of Brisbane and the region south-west of the Koala Coast area. The

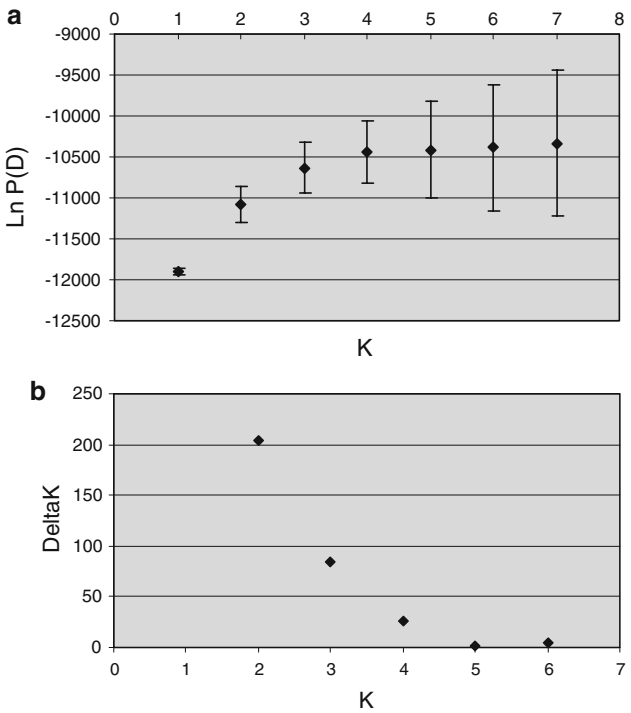

Fig. 4 Determination of number of clusters in STRUCTURE 2.2 analysis. **a** Mean Ln $P(D)$ (±mean variance) over 20 runs for each K, showing a gradual increase in log-likelihood values. **b** ΔK, showing $K = 2$ as the modal value of the distribution and therefore, the true number of population clusters

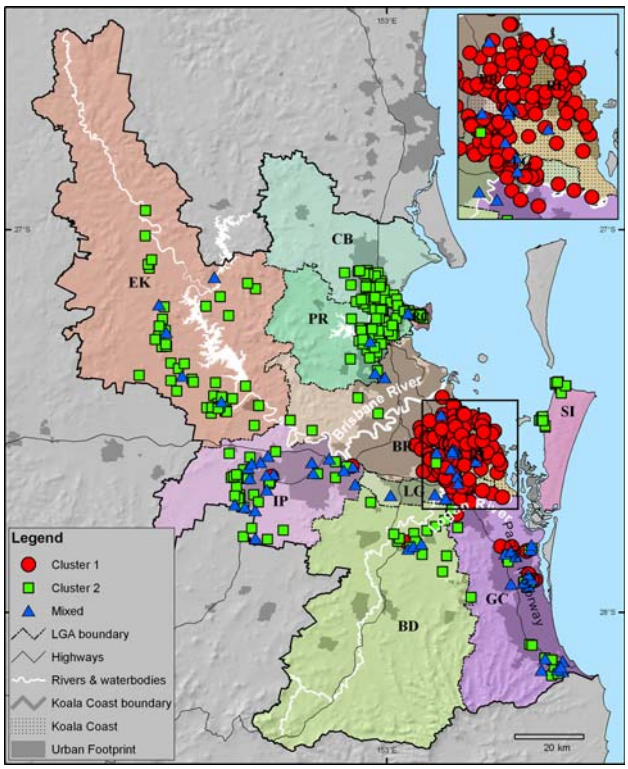

Fig. 5 Clusters as determined by STRUCTURE 2.2. Map also shows major highways, river systems and the Southeast Queensland urban footprint. LGA abbreviations as in Fig. 1. Koala Coast shown in *inset*

majority of koalas in Beaudesert, Caboolture, Pine Rivers, Esk, Ipswich, Redcliffe and all animals from North Stradbroke Island belonged to this cluster. This population cluster had significantly higher heterozygosity than Cluster 1 ($H_e = 0.81$, $P < 0.05$, Tukey's multiple comparison) and higher allelic richness (10.4 alleles per locus). Cluster 2 had a total of 73 alleles across all loci, with 14 unique alleles across five of the six loci. Koalas in Cluster 1 had a subset of the alleles present in koalas from Cluster 2 and had greater frequencies of approximately one-third of the alleles it shared with Cluster 2. Pair-wise F_{ST} comparison of Clusters 1 and 2 showed the two populations are significantly differentiated from each other ($F_{ST} = 0.09$, $P < 0.05$). The koalas that were considered to be of mixed genotype ($q = 0.19$–0.79) on initial STRUCTURE 2.2 analysis were scattered throughout the geographical areas studied, but proportionately more tended to be found in the coastal region south of the Logan River, extending to the Gold Coast and also in the area to the west of the Koala Coast (Cluster 1) into the Cluster 2 region around Ipswich. These koalas also had high heterozygosity ($H_e = 0.80$) and allelic richness (10.2 alleles per locus).

As there were differences identified between the STRUCTURE 2.2 and Geneland clustering pattern, a hierarchical approach was adopted, in which each STRUCTURE 2.2 cluster was tested for sub-clustering using STRUCTURE 2.2. Additional sub-structuring was identified in Cluster 2, with three sub-clusters detected, Cluster 2A was comprised of koalas from Caboolture, Pine Rivers and Redcliffe (north of the Brisbane River)—corresponding to Geneland Cluster 2—and Cluster 2B separated the population of North Stradbroke Island (Geneland Cluster 4). The third sub-cluster, 2C, consisted of koalas in Beaudesert, Esk, Gold Coast and Ipswich (west and south of the Brisbane River), although further subdivision within Cluster 2C separated clusters consistent with the Geneland Clusters 1, 5 and 6 (Beaudesert and Ipswich, Gold Coast, Esk, respectively). Hence, there was a general correspondence of the six regional groupings of koalas between STRUCTURE 2.2 and Geneland analyses.

An AMOVA was undertaken using regional groupings identified from clustering analysis of microsatellite data and from mtDNA analysis. Although each tested grouping explained a significant level of variation at the among-location level, the Geneland grouping explained the highest percentage of variation (15%, $P < 0.001$) among populations in the study area (Table 4). However, similar levels of variation among locations was explained by the two clusters identified at the highest level of structuring in STRUCTURE 2.2 with North Stradbroke Island as a separate group (14%, $P < 0.001$).

Relationship of control-region haplotypes

Fifteen haplotypes were detected from the mitochondrial control region of 77 Southeast Queensland koalas; the haplotypes were identified by letters A–N in this study. A previous study (Fowler et al. 2000) detected eight haplotypes identified as Q1–Q8, all but one of which (their Q5) were detected in the present study. A further eight haplotypes were detected in this study that were not identified in the previous mtDNA study and these sequences have been deposited in GenBank/EMBL (accession nos: GQ851933–40). Haplotypes differed from each other by 1–11 bp in the 626 bp sequence.

Evolutionary relationships among the 15 haplotypes derived by using a statistical parsimony network are shown in Fig. 6. Three divergent clades were identified, separated by at least four mutational steps. The first clade containing Haplotypes D (Q8), H and K and the second clade containing Haplotypes I and O (Q7) were both restricted to the inland northwest region (west Ipswich and Esk; Fig. 7). The third clade was found in coastal regions both north and south of the Brisbane River, extending to meet the other clades in the central west (Ipswich and Esk area). Of the haplotypes in this coastal clade, Haplotype B (Q1) was the most widely distributed, being detected in coastal regions both north and south of the Brisbane River and on North Stradbroke Island

Table 4 Results from Analysis of Molecular Variance (AMOVA) showing the amount of variation explained among regions based on different groups of pooled populations

Region	% of variation explained	PhiPT	*P* value
Mitochondrial pattern: Inland LGAs (Esk and Ipswich) *vs. Coastal LGAs* (Beaudesert, Brisbane, Caboolture, Logan, Pine Rivers, Redcliffe, Redland, North Stradbroke Island)	3	0.101	0.001
Mitochondrial pattern plus island: Inland LGAs (Esk and Ipswich) *vs. Coastal LGAs* (Beaudesert, Brisbane, Caboolture, Logan, Pine Rivers, Redcliffe, Redland) *vs. North Stradbroke Island*	7	0.186	0.001
Geneland clusters	15	0.171	0.001
Initial STRUCTURE pattern: Koala Coast LGAs (Brisbane, Logan, Redland) *vs. rest of Southeast Queensland LGAs* (Beaudesert, Caboolture, Esk, Gold Coast, Ipswich, Pine Rivers, Redcliffe, Redland, North Stradbroke Island)	10	0.189	0.001
Initial STRUCTURE pattern plus island: Koala Coast LGAs (Brisbane, Logan, Redland) *vs. rest of Southeast Queensland LGAs* (Beaudesert, Caboolture, Esk, Gold Coast, Ipswich, Pine Rivers, Redcliffe, Redland) *vs. North Stradbroke Island*	14	0.198	0.001

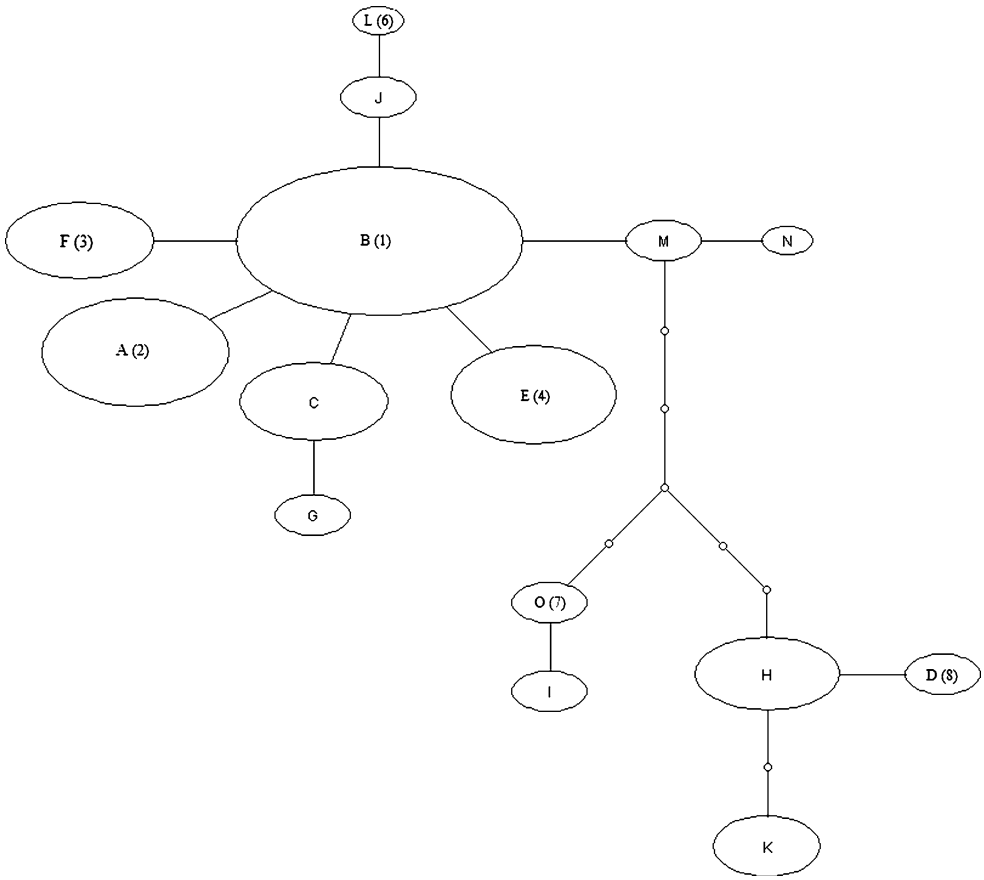

Fig. 6 Statistical parsimony network showing mutational steps between clades. Haplotypes are identified by *letters* A–N (this study) and *numbers* 1–8 (Fowler et al. 2000)

(Fig. 7). Haplotype A (Q2) was also found at several coastal locations but at only one location north of the Brisbane River. Haplotype C was only found at coastal locations south of the Brisbane River (Beaudesert and Gold Coast). Haplotypes J, L, M, N and O were each unique to a particular location (J in Redland; L in Brisbane; M, N and O in Esk).

Discussion

This study, the most comprehensive of its kind on koalas to date, found high genetic diversity in Southeast Queensland koala populations, which was substantially higher than that reported for koala populations in southern Australia (Houlden et al. 1996b) and comparable to or higher than

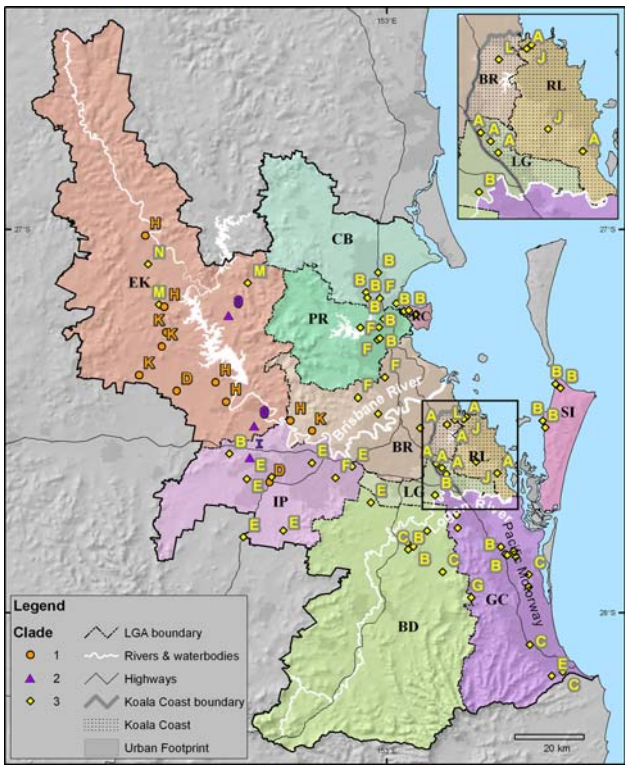

Fig. 7 Spatial distribution of mtDNA haplotype clades. LGA abbreviations as in Fig. 1. Koala Coast shown in *inset*

other Australian marsupials (e.g. *Macrotis lagotis* [Moritz et al. 1997]; *Petrogale lateralis* [Eldridge et al. 1999]). Consistent with the expectation that island populations show reduced genetic diversity compared to mainland populations due to isolation and the susceptibility of relatively small populations to the effects of genetic drift (Frankham 1997), the genetic diversity on North Stradbroke Island was significantly lower than in the mainland Southeast Queensland koalas. However, it was substantially higher than for koalas on Kangaroo Island, South Australia (33%) and Victorian mainland populations (40–44%); populations that have been affected by extreme founder events and translocations (Houlden et al. 1996b). The koala population on North Stradbroke Island is the only known naturally occurring island population of koalas in Australia. There are no records of koala translocation from the mainland to the island and it is likely that current island koalas are descended from a population isolated following the last sea level rise ~8,000 years ago (Sloss et al. 2006). This is supported by the similarities with the mainland koalas, sharing a mtDNA haplotype found in adjacent coastal regions and clustering of the microsatellite genotypes in the first stage STRUCTURE 2.2 analysis. It is likely that subsequent genetic drift is responsible for the significant differentiation of the island population from all other LGAs in pair-wise population F_{ST} analysis, Geneland and FCA analyses.

Although LGA boundaries are arbitrary with respect to animal ecology, they often form the basis for management of species at the local level, with differing priorities and resources depending on the LGA. The majority of populations defined by LGAs were significantly differentiated according to pair-wise F_{ST}. Those populations that were not significantly different from each other were always neighbouring LGAs (e.g. Caboolture, Pine Rivers and Redcliffe; Brisbane, Logan and Redland), indicating that there is almost certainly gene flow across these arbitrary LGA boundaries and, therefore, illustrating the importance of co-ordinated conservation and management efforts between LGAs.

The separation of the 512 koalas from such a broad geographical area into a maximum of only six genetic clusters was unexpected. Because of a recent finding of genetic differentiation on a very fine-scale (1–5 km) within the Koala Coast (Thompson 2006), we expected many more clusters to be spread over the region. Thompson's (2006) results suggested that habitat fragmentation affected the gene flow potential of koalas within the Koala Coast. Hence, it was expected that the effects of urbanization and distance would have an important impact on the gene flow potential and, therefore, genetic structuring in our study area, because it covered a much broader region than Thompson's (2006) study. Alternatively, "corridors" of habitat enabling effective gene flow might still be expected to result in some level of isolation by distance, either between the northernmost and southernmost locations, or within localities, such as within Geneland clusters. However, it is likely that the level of habitat fragmentation, particularly around the City of Brisbane, or the recent and so non-equilibrium nature of the fragmentation processes, have resulted in the observed lack of isolation by distance. According to Schwartz and McKelvey (2009), clustering programs such as STRUCTURE 2.2 and Geneland will only work efficiently when isolation by distance is absent; thus we have confidence that the clustering analysis is reliable.

Several of the boundaries between clusters seemingly correspond to rivers and roads. For example, the Koala Coast animals are separated from the adjoining cluster to the north by the Brisbane River, from the cluster to the south by the Logan River and interchange is limited to the west by the M1 Motorway (previously known as the Pacific Motorway or Highway). Shared mtDNA haplotypes occurring north and south of the Brisbane River provide evidence that coastal koala populations were connected in the past and that the river was not a significant barrier to gene flow. However, it is likely that loss of habitat through high density urban development along the river and

transport corridors has contributed to the isolation and subsequent differentiation of the koalas in the Koala Coast region. Roads impact on populations not only through reducing numbers (road kills), but also by acting as partial or complete barriers to dispersal (Dixon et al. 2007). Evidence for population fragmentation and reduced gene flow caused by roads has been reported for several species [e.g. moor frog *Rana arvalis* (Vos et al. 2001), eastern red-backed salamander *Plethodon cinereus* (Noël et al. 2007) and Florida black bear *Ursus americanus floridanus* (Dixon et al. 2007)]. The M1, first constructed as the Pacific Highway in the 1920s, is the major highway connecting Brisbane and the Gold Coast and is now up to 10 traffic lanes in width. It is probable that the loss of koalas through hunting early last century and the construction and upgrading of what is now the M1 could have maintained or reinforced a pre-existing differentiation through more effective genetic isolation of the Koala Coast population.

The distribution of mtDNA haplotypes reflects longer term gene flow patterns, since mtDNA has a slower mutational rate than microsatellite DNA. Hence, the divergent clades found in the north-west inland areas indicate a long-term division of these koala populations from those nearer the coast. It may be relevant that the D'Aguilar Range runs roughly north–south, separating Brisbane, Pine Rivers and Caboolture to the east and Esk and Ipswich to the west. This range coincides with a change in vegetation from wet coastal to dry inland eucalypt forest and may be causally related to a physical separation of koala populations. The observation of a morphological difference in koalas inhabiting coastal Southeast Queensland compared with those further inland (Lawson and Carrick 1998) may also be informative. This east–west division north of the Brisbane River is also found in the microsatellite data with the Geneland and STRUCTURE 2.2 analyses, as is the extension of the coastal clade inland along the Brisbane River valley, indicating an historical patterning of koala distribution.

The Koala Coast population is a focus of concern because of its current high exposure to anthropogenically generated threatening processes. The koalas in the Koala Coast had reduced allelic variation and a significantly lower level of heterozygosity compared to other mainland regions in Southeast Queensland, although both measures can be considered high when compared to other koala populations in south-eastern Australia. The Koala Coast cluster contained few alleles that were not also present in the rest of the mainland populations and the koalas on North Stradbroke Island; however, the remainder of the mainland koalas had many alleles that were not present in the Koala Coast animals. The mtDNA analysis suggests that, prior to European occupation, koalas within the coastal plains of the Southeast Queensland region were connected and gene flow was relatively uninhibited. It is possible that the koalas that remained in or recolonised the Koala Coast after hunting ceased in the late 1920s possessed a subset of the microsatellite alleles that were present prior to the severe reduction in the population's size; subsequent fragmentation and isolation might, therefore, have resulted in the continued differentiation of this population. We consider that the high level of differentiation of the Koala Coast population is responsible for the results obtained from the initial STRUCTURE 2.2 analysis which clustered the remaining koalas on the mainland and North Stradbroke Island together. This is supported by a similar percentage of variation explained by a separation of the Koala Coast from the remainder of the mainland koalas and from North Stradbroke Island (14%) as for the increased grouping into six Geneland clusters (15%).

Our results demonstrating that the Koala Coast population is effectively isolated and differentiated from other koalas in Southeast Queensland, probably at least partly due to ongoing anthropogenic disturbance, provide clear evidence for the Koala Coast population to be recognised as a distinct management unit (*sensu* Houlden et al. 1999). For an already significantly declining population, genetic isolation increases the risk of stochastically-mediated population extinction. Factors such as habitat loss and fragmentation, attack by dogs, injury from road trauma and disease are key threatening processes in the decline of koalas in this region, requiring urgent implementation of more effective actions to manage these threats. Due to the observed rapid decline (Queensland Government 2009) in what we have now shown is a recognisably distinct Koala Coast population, this population meets the criteria for classification as "endangered wildlife" under *The Nature Conservation Act* (Qld) 1992.

The *Nature Conservation [Koala] Conservation Plan 2006 and Management Program 2006–2016* requires rehabilitated or rescued animals to be released in the immediate vicinity of their point of capture and proscribes the translocation of koalas displaced by habitat destruction associated with development. Although we do not know to what extent the genetic differences between the Southeast Queensland populations are important for the species' evolutionary potential, they indicate differentiation to which a precautionary approach should be taken. In particular, longer-term historical differentiation indicated by mtDNA coincides with a change in vegetation and potentially may correspond to adaptive differences. Introduction of homogenising translocations within Southeast Queensland has the potential to result in the loss of some adaptive traits or introduction of deleterious alleles (Cristescu et al. 2009) as has occurred in the southern Australian populations. While directly applicable to koalas, there are more general lessons to be derived which are applicable

whenever translocations of animals are proposed for purposes not specifically contemplated by the International Union for the Conservation of Nature's (1987) position statement (such as the "rescuing" of animals displaced by development—urban, agricultural or infrastructure).

The initial translocations of koalas in southern Australia (see Houlden et al. 1996b) in the 1920s and 1930s were prompted by the perceived threats of imminent extinction of the species on mainland Australia (with the actual extinction of the original South Australian koala population occurring around this time). Whilst this kind of intervention is not specially addressed in the IUCN's position statement *Translocation of Living Organisms* (IUCN 1987), it can probably be viewed as being consistent with a valid purpose for "Intentional Introduction". The later extensive translocations of koalas that were undertaken in Victoria during the mid Twentieth Century appear to have been designed to alleviate a management problem due to overabundance of koalas on the Victorian islands onto which earlier introductions had taken place. More recently, this activity has continued in Victoria to address overabundance problems on islands and habitat isolates on the mainland, as well as from the problematic introduced population on Kangaroo Island, South Australia. As indicated by Houlden et al. (1996b), these translocations have had the unintended consequence of contributing to loss of heterozygosity in mainland Victoria due to the genetically impoverished nature of the main source population (French Island).

In Queensland, there are currently no overabundance issues or demonstrated need to reintroduce koalas to a former part of their range, nor is there a demonstrated need for restocking of wild populations. However, Southeast Queensland is experiencing Australia's highest growth in its human population and consequent development pressure which continues to result in loss and fragmentation of koala habitat. The direct relocation of koalas by wildlife management authorities in response to such habitat alienation is not infrequently proposed by interest groups and simplistically this may seem reasonable. However, it is not an effective conservation strategy since it has been shown that it can actually result in an increase in morbidity and mortality (Nattrass and Fiedler 1996). Also proponents generally ignore the vital requirements for determination of the genetic and other characteristics of the animals proposed for relocation, as well as the potential impacts on recipient populations and habitats, prior to undertaking such a scheme (IUCN 1987). In order to maintain current genetic diversity, gene traffic may need to be assisted in some circumstances, but any translocation must follow IUCN guidelines and to date in Queensland, none have. Hence, the outcomes are unpredictable, but if they hasten local extinction through artificially exceeding local carrying capacity or some other factor, then the impact of the translocation will not be to increase gene traffic, but rather to extinguish or replace local genotypes. It should be noted that although more genetically diverse than French Island koalas, the most likely source populations for development driven translocations in Southeast Queensland would be those with the lowest diversity on the coastal mainland—producing the same (though less drastic) reduction in heterozygosity of recipient populations.

The Queensland Department of Environment and Resource Management (formerly the Environmental Protection Agency) runs a comprehensive koala rescue and rehabilitation system, the conservation purpose of which is to support the source populations, especially those most threatened by development pressure (Queensland Government 2006). Typically, about 300 sick, injured or orphaned koalas per year from the Koala Coast have been rehabilitated and returned to the wild (i.e. the equivalent of about half the number of animals required to be produced each year to maintain a stable population by natural increase). This has helped overcome losses due to premature mortality caused by motor vehicles, domestic dogs and disease. If successfully rehabilitated koalas are not returned to their source population and/or those displaced by development are deliberately removed, such actions hasten the decline of these threatened populations towards local extinction.

Our study has demonstrated significant differentiation of Southeast Queensland's koala populations and thus proponents for translocation of koalas within the region are obligated to address the genetic constraints and other requirements as established by the IUCN position statement. This differentiation might reflect a feature of koala dispersal, so *ad hoc* movement or translocation of koalas in this region is to be avoided. The findings of the study also have implications more generally for wildlife management approaches that involve translocation of animals (particularly mammals) as a response to habitat destruction. However, the most widespread implications lie in the example provided of how habitat fragmentation due to residential and infrastructure development can produce quite rapid reproductive isolation and genetic differentiation in populations of relatively mobile and long lived species.

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