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Conservation genetics of the koala (*Phascolarctos cinereus*): low mitochondrial DNA variation amongst southern Australian populations

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Summary

Koala (*Phascolarctos cinereus*) populations in southern Australia have a history of bottlenecks – earlier this century the species became extinct in South Australia, and almost so in Victoria. Subsequently large numbers of animals from island populations (founded from very few animals) have been translocated back to mainland sites and to other islands in the region. As part of a larger study of the genetic structure of koala populations in southern Australia, we have undertaken a survey of mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP) variability. Genomic DNA from 91 koalas from five populations was examined using 23 restriction enzymes, and mtDNA fragments were detected using a domestic cat full-length mtDNA clone. Only one of the enzymes, *Taq*I, revealed polymorphism – a relatively low amount of variation compared with other mammals, although low mtDNA-RFLP variation has also been reported in Queensland koalas. French Island and populations established predominantly from French Island immigrant koalas, either directly or via other island populations, were indistinguishable by haplotype frequencies. The mtDNA data are thus consistent with the interpretation that the koala translocation programme has homogenized gene frequencies amongst those populations involved. South Gippsland is not recorded as having received translocated koalas directly, and has significantly different mtDNA-RFLP haplotype frequencies from all other populations examined. The fact that this distinction was not previously observed in nuclear gene frequencies may reflect predominantly male-mediated dispersal in koalas.

1. Introduction

The distribution of the koala (*Phascolarctos cinereus*) spans eastern South Australia in the south, to central Queensland in the north (Fig. 1). Although the species is not widely considered to be endangered, southern populations are likely to be genetically impoverished as a result of their recent history. The documented history of the koala reveals near-extinction in Victoria by the 1930s, at which time the species disappeared from South Australia, largely due to a flourishing fur-trade (Lewis, 1934). Mainland and certain island habitats were subsequently restocked with large numbers of koalas from the rapidly growing French Island population (either directly or via other islands; Fig. 1), with the dual aims of relieving over-browsing on the island and of re-establishing the koala in its former range (Martin & Handasyde, 1990). The French Island population itself has a very narrow genetic base since it is believed to have been founded by as few as two individuals late last century (Warneke, 1978). This raises the possibility of regional genetic uniformity due to the frequent and substantial relocations. The Brisbane Ranges and Stony Rises regions may have retained small numbers of individuals, while koalas had not previously inhabited Kangaroo and Phillip Islands (Fig. 1). The South Gippsland area is thought to have maintained a viable population, apparently without the aid of directly relocated individuals (Martin & Handasyde, 1990). In contrast, Queensland koala populations have been subjected to a much lower level of disturbance resulting from European settlement, and thus might be expected to retain higher levels of genetic diversity than southern populations.

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There exists a high level of concern about loss of genetic diversity, and its effects on extinction probabilities of populations (Frankham, 1995). The role of genetic diversity in fertility, response to pathogenic infection and general fitness is of interest for both conservation planning and evolutionary theory. Data on the fertility of Victorian koalas suggest that both individuals and populations show differential response to infection by the pathogen *Chlamydia psittaci* (Handasyde *et al*., 1988). These populations have also been reported to differ significantly in ejaculate characteristics such as sperm concentration and motility ratings (Wildt *et al*., 1991). Genetic characterisation of populations may indicate the extent to which reproductive and disease resistance parameters are associated with genetic diversity, as well as identifying populations which may have retained remnant gene pools existing prior to the bottleneck.

Our group has examined the genetic diversity and structure of Victorian and South Australian koala populations using several methods. Analysis of DNA minisatellites, microsatellites and allozymes has revealed a low degree of polymorphism and a highly uniform distribution of allele frequencies across populations in this southern region (Taylor *et al*., 1991; Houlden *et al*., 1996; S. Ramus *et al*., unpublished data). This is in contrast to observations of Queensland koala populations, where minisatellite loci show sufficient within-population variability to allow individual identification and parentage determination (Cocciolone & Timms, 1992; Timms *et al*., 1993). In addition, a survey of microsatellite variation amongst koala populations from throughout the species' range revealed significantly higher levels of heterozygosity and allelic diversity amongst northern than amongst southern populations of the koala (Houlden *et al*., 1996).

Mitochondrial DNA (mtDNA), being clonally inherited through matrilines, is often a sensitive indicator of demographic contractions, as maternal lineages can quickly become fixed by founder effects during population bottlenecks (Avise *et al*., 1987). In addition, sex-biased dispersal or hybridization is expected to lead to differential structuring of nuclear and mtDNA markers, so the use of both types of marker should lead to a more complete understanding of the factors structuring phylogeographic variation (Moritz *et al*., 1987; Degnan, 1993). A study using mtDNA to characterize genetic variability from two Queensland koala populations reported only one restriction fragment length polymorphism (RFLP), almost perfectly partitioned between the two populations (Worthington Wilmer *et al*., 1993), and concluded that koala populations are generally low in genetic variation. Here we present the results of a survey of mtDNA-RFLPs in 91 koalas from five southern Australian populations, which also resulted in the discovery of only one polymorphic restriction enzyme site. The distribution of this polymorphism, how it relates to the relocation history of koalas in the region, and the evidence to date regarding genetic diversity in koalas from the north and south of their range, are discussed.

2. Materials and methods

(i) *Samples*

Koala samples used in this study came from populations in the Brisbane Ranges ($n = 21$), Stony Rises ($n = 21$), South Gippsland ($n = 20$) and French Island ($n = 19$) in Victoria, as well as Kangaroo Island ($n = 10$) in South Australia. Fig. 1 shows the location and known translocation histories of these populations.

(ii) *DNA extraction and Southern blot analysis with domestic cat mitochondrial DNA*

Total genomic DNA was extracted from frozen koala whole blood or leucocytes as described in Taylor *et al*., (1991). Restriction digests of 1–2 μg of genomic DNA were performed in a 30 μl reaction containing 1X appropriate restriction enyzme buffer (BRL), 4 mM spermidine trichloride and 10–20 units of restriction enzyme (BRL) for 3 h at the appropriate temperature. Enzymes used were *Acc*I, *Ava*I, *Ava*II, *Bcl*I, *Bgl*II, *Bst*EII, *Dra*I, *Eco*RI, *Hha*I, *Hin*cII, *Hin*dIII, *Hin*fI, *Hpa*I, *Hpa*II, *Nci*I, *Nco*I, *Nde*I, *Pvu*II, *Sst*II, *Stu*I, *Sty*I, *Taq*I and *Xba*I. The reactions were then stopped and loaded onto 20 × 23 cm 1% or 1·3% agarose gels (Seakem GTG) and electophoresed in 1 × TAE (Sambrook *et al*., 1989) for 15–18 h at 60–70 V, with buffer recirculation. After ethidium bromide staining and photography the gels were denatured for half an hour in 0·4 M NaOH, 0·8 M NaCl and neutralized in 1·5 M NaCl, 0·5 M Tris/HCl for half an hour. Southern transfers (from upturned gels) were performed in 10 × SSC overnight to Biotrace nylon membranes (Gelman). Following transfer, the membranes were rinsed briefly in 2 × SSC and vacuum-baked at 80 °C for 2 h.

The full-length domestic cat mtDNA clone Fcamt3-2 (Lopez *et al*., 1995) was labelled using random primer labelling (Boehringer Mannheim kit) and [α-^{32}P]dCT. Prehybridization (3 h) and hybridization (15–20 h) were performed in plastic bags at 37 °C in a solution consisting of 50% formamide, 200 μg/ml salmon sperm DNA, 1 M NaCl, 10 mM EDTA, 50 mM PIPES, 1% SDS and 5 × Denhardt's solution. Stringency washes were performed as follows: two rinses in 2 × SSC/1% SDS at room temperature and a half hour wash at each of 37 °C and 50 °C in 1 × SSC/1% SDS. Autoradiography was carried out at −70 °C for 16 h to 1 week.

(iii) *Statistical analyses*

The maximum likelihood estimate of the number of sequence substitutions between mtDNA restriction

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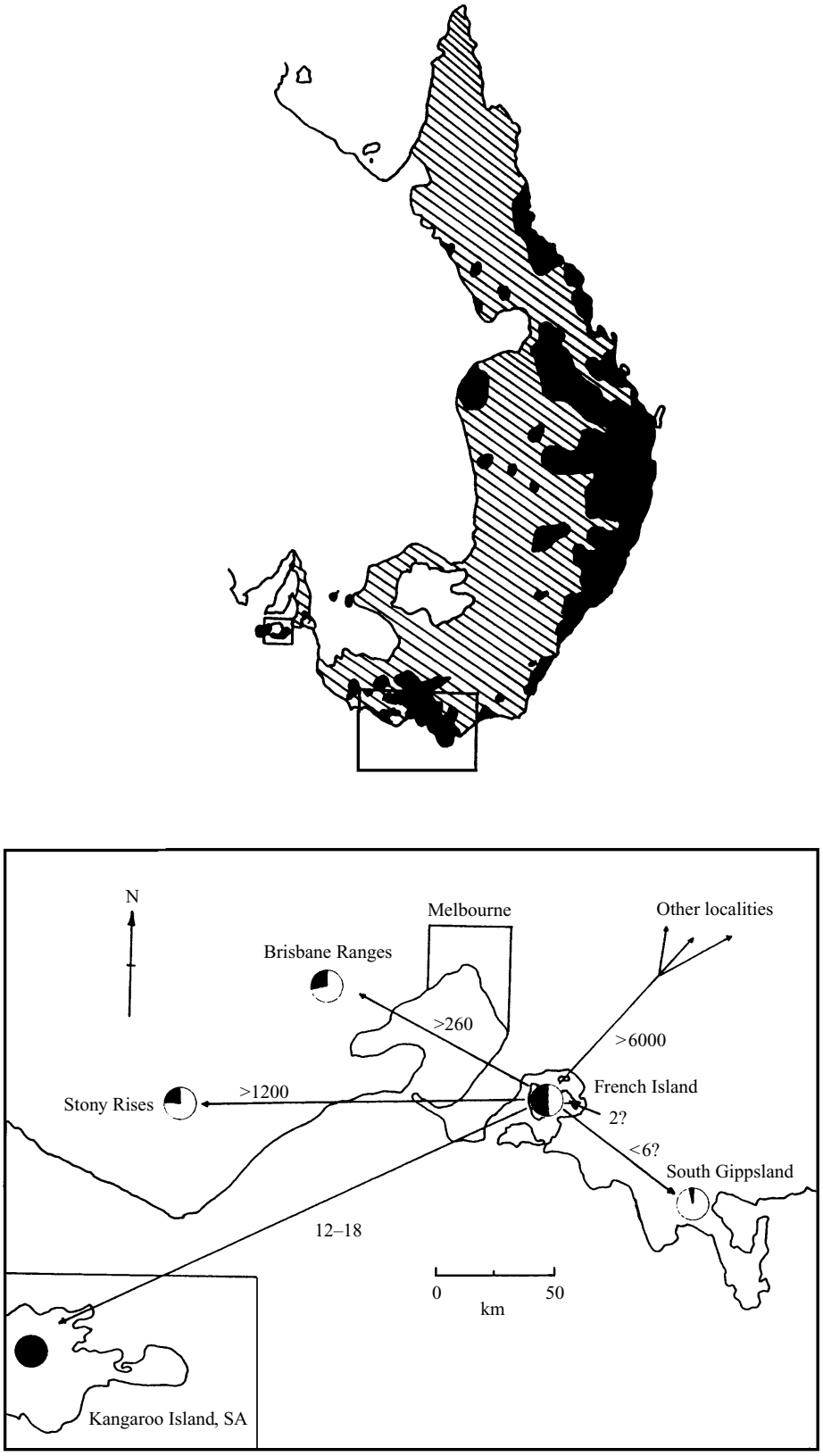

Fig. 1. Past (hatched area) and present (black area) distribution of koalas in eastern Australia (redrawn from Lee & Martin, 1988), and summarized relocation history of koalas in southern Australia (magnified area). The magnitude of koala relocations from French Island to other island and mainland habitats is shown (from translocation records held by the Victorian Department of Conservation and Environment), and pie charts indicate the frequencies of mitochondrial haplotypes 1 (white) and 2 (black) in each population. Unknown numbers of koalas may have existed in the Brisbane Ranges and Stony Rises prior to the translocations. Koalas have persisted at South Gippsland throughout recorded history, but none inhabited Kangaroo Island or French Island prior to translocations.

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site haplotypes was calculated according to Nei & Tajima (1983). This estimate was then used in equation 22 of Nei & Li (1979) to calculate nucleotide diversity within populations.

Chi-squared contingency analyses (Sokal & Rohlf, 1981) were used to assess haplotype frequency differences between populations. Geographic distribution of haplotypes was examined using the G_{ST} method of Takahata & Palumbi (1985) and significance was assessed by randomization tests performed using the program of Palumbi & Wilson (1989).

3. Results

(i) *Mitochondrial DNA haplotypes and genetic variation*

Using 23 restriction enzymes, a total of 98 restriction sites were scored in this survey, representing 424 bp of mtDNA. This is approximately 2·4% of the koala mitochondrial genome as sized by Worthington Wilmer *et al*., (1993). Only *Taq*I showed variation, at a single site, amongst the 91 koalas examined; the remaining 22 restriction enzymes gave monomorphic patterns. Fig. 2*a* shows the *Taq*I polymorphism which distinguishes two haplotypes: 1 and 2. The expected smaller molecular weight fragment in the haplotype 1 pattern was not resolved. This may have been due to relatively low homology of the fragment to the probe, as the polymorphic *Taq*I restriction site was shown to reside in the D-loop [data not shown; see Section 3(ii) below].

The estimated sequence divergence between haplotypes 1 and 2 is 0·12% and the within-population nucleotide diversity (π) ranged from zero (Kangaroo Island) to 0·03% (French Island) (Table 1).

Haplotype proportions amongst the study populations are shown in Fig. 1. Only one population sample, that from Kangaroo Island, was fixed for mtDNA haplotype (2); all others possessed both haplotypes. Overall, the five populations examined

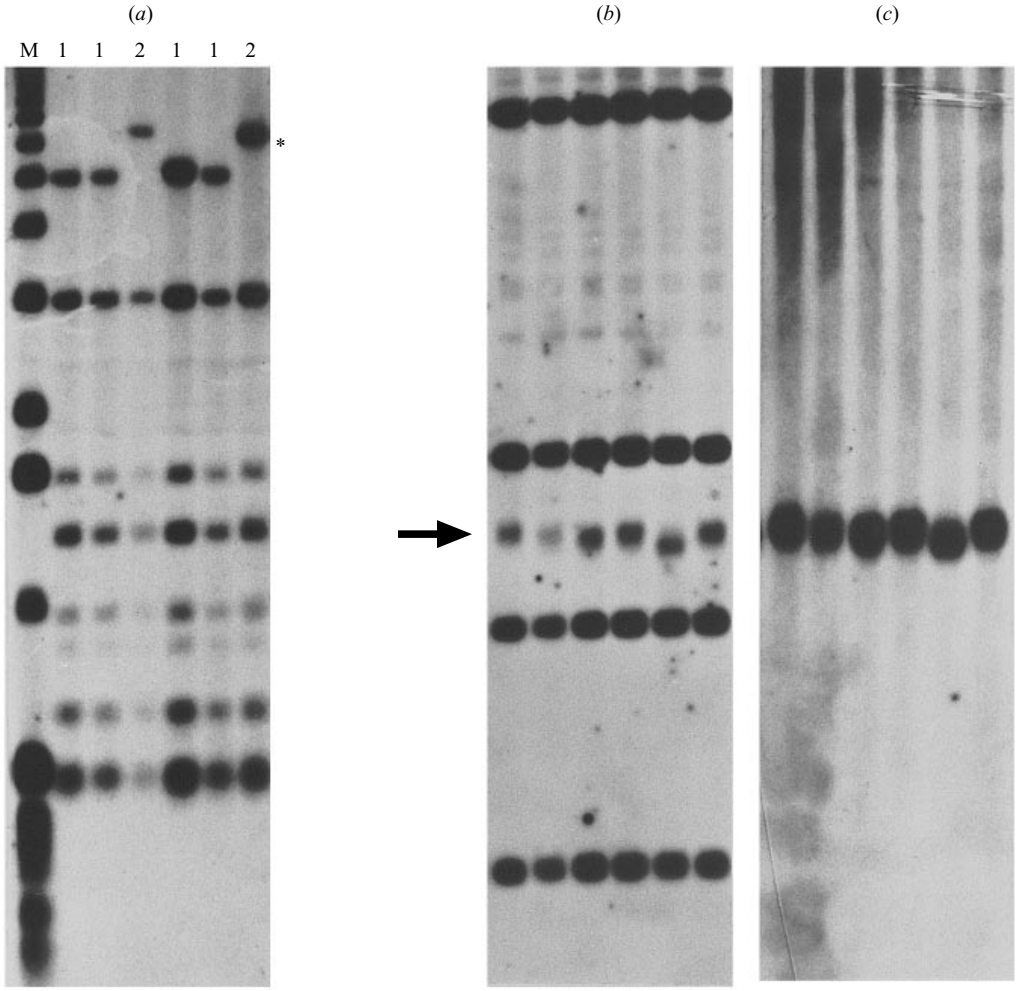

Fig. 2. Autoradiographs showing restriction fragment length polymorphisms observed in southern Australian koala populations. (*a*) The *Taq*I polymorphism defining two haplotypes (1 and 2). Lane M contains the molecular weight marker Ad2-*Bam* HI/*Eco* RI. (*b*) *Xba*I fragments probed with full-length mtDNA clone, with 'fuzzy' bands referred to in the text (representing putative length heteroplasmy) shown with an arrow. (*c*) *Xba*I fragments probed with D-loop polymerase chain reaction product.

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Table 1. *Levels of microsatellite and mtDNA restriction fragment length polymorphism in koalas, other marsupials and eutherians* (*averaged over subspecies, lineages or geographic region*)

	mtDNA							Microsatellites						
Species/locality	Ind	Bp	Hap	δ	π	G_{ST}	Reference	Ind	Loci	P	H	A	D	Reference
Marsupials														
Koala – southern	—	—	—	—	—	0·12		—	—	—	—	—	0·072	
French Island, VIC	19	424	2·0	0·12	0·03			43	6	67	37	3·0		
Brisbane Ranges, VIC	21	424	2·0	0·12	0·02			24	6	100	41	3·3		
Stony Rises, VIC	21	424	2·0	0·12	0·02		This study	17	6	83	39	3·0		Houlden *et al.* (1996)
South Gippsland, VIC	20	424	2·0	0·12	0·01			47	6	100	48	4·3		
Kangaroo Island, SA	10	424	1·0	0·00	0·00			12	6	67	33	1·7		
Koala – Queensland	11	326	1·5	0·06	0·01	0·80	Worthington Wilmer *et al.* (1993)	19	6	100	76	6·3	0·411	Houlden *et al.* (1996)
Southern hairy-nosed wombat														
Murraylands, SA	4	456	2·0	1·60	0·36	na	Taylor (1995)	23	16	94	66	5·0	na	Taylor *et al.* (1994)
Yellow-footed rock wallaby														
South Australia	10	265	2·0	0·24	0·05	na	Eldridge (in press)	na	na	na	na	na	na	Pope *et al.* (1996)
Queensland	8	265	1·0	0·00	0·00	na		23	4	100	69	5·5	na	
Eastern barred bandicoot	—	—	—	—	—	0·15								
Victoria	16	275	3·0	0·70	na		Robinson (1995)	na	na	na	na	na	na	
Tasmania	35	275	2·0	0·26	na			na	na	na	na	na	na	
Eutherians														
Tassel-eared squirrel	10	335	3·8	0·48	na	na	Wettstein *et al.* (1994)	na	na	na	na	na	na	
Woodrat	23	na	5·2	0·48	na	na	Hayes & Harrison (1992)	na	na	na	na	na	na	
European field vole	72	530	41·5	0·73	0·79	na	Jaarola & Tegelstrom (1995)	na	na	na	na	na	na	
Humpback whale	21	468	3·5	na	0·10	na	Baker *et al.* (1990)	na	na	na	na	na	na	

Quantities given are: ind, number of individuals examined; bp, number of base pairs surveyed; hap, number of haplotypes observed; δ, per cent nucleotide divergence between mtDNA haplotypes within populations (Nei & Tajima 1983); π, per cent nucleotide diversity within populations (Nei & Li 1979); G_{ST}, proportion of variation distributed among populations (Takahata & Palumbi 1985); loci, number of loci examined; P, per cent polymorphic loci; H, per cent Hardy–Weinberg expected heterozygosity; A, allelic diversity; D, average Nei's unbiased genetic distance between pairs of populations (Nei, 1978). na, data not available.

were not homogeneous in haplotype frequency ($\chi^2 = 30{\cdot}53$; d.f. = 4; $P < 0{\cdot}001$). However, French Island and those populations to which it has contributed large numbers of immigrants (Brisbane Ranges and Stony Rises) form a homogenous group with respect to mtDNA haplotype frequency ($\chi^2 = 4{\cdot}83$; d.f. = 2; $P > 0{\cdot}05$). By contrast, this group of populations shows significantly different haplotype frequencies to both the South Gippsland ($\chi^2 = 5{\cdot}12$; d.f. = 1; $P < 0{\cdot}025$) and Kangaroo Island ($\chi^2 = 12{\cdot}30$; d.f. = 1; $P < 0{\cdot}005$) populations.

G_{ST} was estimated to be 0·12, indicating that 12% of the genetic variation in the southern region could be explained by between-population variation. Results from 100 bootstrap resamplings of the data suggested the observed G_{ST} was significantly greater than would be expected at random ($P = 0{\cdot}02$).

(ii) *Apparent length heteroplasmy in the D-loop*

Many of the restriction enzymes produced weakly hybridizing fragments which appeared to vary in mobility and thickness, raising the possibility of length variation in less-conserved regions of the mitochondrial genome (Fig. 2*b*). This putative variation could not be resolved under the conditions used in the present survey. However, hybridization with a polymerase chain reaction (PCR)-amplified koala D-loop fragment confirms that these faint bands represent D-loop sequence (Fig. 2*c*). It therefore appears likely that southern Australian koalas have: (1) length variation in the D-loop and (2) frequent heteroplasmy, with individuals having more than one mtDNA haplotype. Heteroplasmy for length variants in this region was also observed in Queensland koala populations (Worthington Wilmer *et al.*, 1993).

4. Discussion

(i) *Low haplotype diversity in koalas*

Mitochondrial DNA haplotype diversity in southern Australian koalas was found to be of similar magnitude to that observed by Worthington Wilmer *et al.*, (1993) in Queensland koalas (Table 1). In both studies only two haplotypes were seen, defined by a single restriction site change (a different site in each case), and differing at only 0·12% of nucleotide positions. By contrast, a survey of another member of the superfamily Vombatoidea, the southern hairy-nosed wombat (*Lasiorhinus latifrons*), revealed two highly divergent haplotypes (1·6%) in a small sample of individuals (Taylor, 1995). MtDNA nucleotide diversity within populations also appears to be generally low in koalas: estimates for other mammals, including at least one marsupial, are more than an order of magnitude greater (Table 1). Low mtDNA diversity was predicted for koalas in southern Australia because of the near-extinction and subsequent relocations from islands with a narrow genetic base, but the same was not expected of the relatively undisturbed northern populations.

Definitive statements on normal levels of genetic variation in koala populations have been problematic in the past due to differences in population history and lack of concordance between the markers and/or methods used. However, largely as a result of an examination of koala microsatellite variation from 10 populations from across the species' range (Houlden *et al.* 1996), it is now clear that low genetic variation is not a general feature of koala populations. Microsatellite heterozygosity and allelic diversity in Queensland and New South Wales koala populations are similar to those reported for other marsupials surveyed with these markers (Table 1).

In light of ample nuclear variation in at least some koala populations, the apparent paucity of mtDNA variation in all populations so far examined requires some explanation. Only a limited comparison between fragment sizes estimated in this study and those for Queensland populations (J. M. Worthington Wilmer, unpublished data) is possible. Data on the number and size of fragments produced by restriction enzymes common to both studies suggest that haplotype A of Worthington Wilmer *et al.* (1993) equates to haplotype 2 of the present study. Although definitive conclusions must await a more direct comparison of northern and southern mtDNA haplotypic variation, there is apparently minimal divergence between haplotypes in Queensland and Victorian koala populations. There are several possible non-exclusive explanations: (1) low evolutionary rate in koala mtDNA, (2) a recent 'selective sweep' affecting mtDNA diversity, and (3) a global historic bottleneck reflected disproportionately in mtDNA diversity. The first explanation has some merit, since variation in mtDNA evolutionary rate amongst mammalian lineages has been documented, and low mtDNA evolutionary rate is strongly correlated with low metabolic rate, which is characteristic of koalas (Rand, 1994; Degabriele & Dawson, 1979). However hairy-nosed wombats (close relatives of koalas), with amongst the lowest metabolic rate of any mammal (Wells, 1978), do not show restricted mtDNA diversity (Table 1).

A selective sweep may occur when a single mtDNA type gains a selective advantage over other types, resulting in fixation of the favourable mtDNA type amongst interbreeding populations. Selective sweeps have been documented for both mtDNA and low-recombining nuclear regions in *Drosophila* spp. (Berry *et al.*, 1991; Begun & Aquadro, 1992; Rand *et al.*, 1994), but no examples are known in other animals. Such an explanation for the low mtDNA diversity in koalas would require historically high levels of gene flow over thousands of kilometres. Although microsatellite data show a strong disjunction between northern and southern populations which would tend

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to argue against such gene flow, this genetic discontinuity may be due to an isolation by distance effect (Houlden *et al*., 1996). Analysis of koala populations at intermediate geographic distances would be required to assess gene flow accurately. This would require methods capable of estimating historic levels of gene flow, such as phylogenetic relationships between alleles of known spatial distribution (Moritz & Lavery, 1996).

The present data may also be consistent with the third explanation hypothesizing a historic bottleneck causing loss of mtDNA but not nuclear variability. This disparity can arise because of the lower effective population size of mtDNA relative to nuclear genes (Birky *et al*., 1983). Microsatellite diversity may have been retained through such a bottleneck or had sufficient time to regenerate, given the high mutation rate for microsatellite loci (average $1{\cdot}2 \times 10^{-3}$ in humans; Weber & Wong, 1993). To explain the similarity of haplotypes in southern and northern populations, however, the bottleneck would need to have been followed by recent expansion of the species, again over thousands of kilometres, from a single refugium. An extensive analysis of D-loop sequence variation in koala populations throughout their range is currently being undertaken (Bronwyn Houlden, University of New South Wales, personal communication) and should provide the data required to address these hypotheses more adequately.

(ii) *Low population differentiation in southern Australian koalas*

The distribution of mtDNA haplotypes in southern koala populations reveals a lower degree of genetic differentiation amongst populations than was observed in the Queensland study (Worthington Wilmer *et al*., 1993). The present study found that only 12% of the variation could be accounted for by between-population variation, compared with 80% in the Queensland sample (based on G_{ST}). Furthermore, the Stony Rises and Brisbane Ranges populations, which received large numbers of animals from French Island during the relocation programme, do not have significantly different haplotype frequencies to the source population (Fig. 1). In contrast, mtDNA haplotypes in Queensland are almost perfectly partitioned between two natural populations, perhaps reflecting the lower level of human disturbance in that region (Worthington Wilmer *et al*., 1993). If such substantial genetic differentiation is characteristic of undisturbed koala populations, the pattern of mtDNA variability we see in southern Australia is consistent with the hypothesis that the relocation programme has contributed to genetic uniformity amongst populations. This supports the conclusions of previous surveys of minisatellite, microsatellite and allozyme variation from a similar sample set (Taylor *et al*., 1991; Houlden *et al*., 1996; Ramus *et al*., unpublished data). In particular, average genetic distance between northern koala populations is substantially larger than that between southern populations, based on microsatellite data (Table 1; Houlden *et al*., 1996).

In the search for relic gene pools in southern Australia, the significant difference in mtDNA haplotype frequencies between South Gippsland and the French Island/Brisbane Ranges/Stony Rises group is of interest. Whilst South Gippsland is thought to have maintained its koala population in the absence of directly translocated individuals, it is possible that natural migration and/or unrecorded translocations have occurred into South Gippsland from surrounding areas (Martin & Handasyde, 1990; records of the Victorian Department of Conservation and Environment). Houlden *et al*. (1996) found no significant differences in microsatellite allele frequencies, and only low genetic distances, between South Gippsland and other mainland populations (Table 1), suggesting sufficient genetic exchange to cause homogenization of gene frequencies. The significant deviation in mtDNA haplotype frequencies may be due simply to the lower effective population size associated with the mtDNA genome (Birky *et al*., 1983) and the small number of haplotypes, with resulting stochastic effects. Alternatively, the disparity between the mtDNA and microsatellite data may indicate that migration into the South Gippsland population has been by males alone. Such a suggestion is consistent with the observed dispersal behaviour of koalas; males (particularly juveniles) are the more nomadic sex (Smith, 1987; Mitchell, 1988).

The apparent absence of one of the two mtDNA haplotypes from Kangaroo Island may be due to founder effect and genetic drift in the population, or simply to the small number of individuals (10) examined. However significant microsatellite allele frequency differences also exist between this population and French Island (from which it was founded) (Houlden *et al*., 1996), suggesting that substantial genetic drift has occurred since founding of the Kangaroo Island population.

(iii) *Size of founder population on French Island*

The detection of two mtDNA haplotypes on French Island, as well as in South Gippsland (the latter thought not to have received any relocated individuals directly), suggests that both haplotypes were endemic in the pre-crash Victorian population, rather than one having arisen by mutation on French Island. Therefore at least three koalas must have founded the French Island population: two females and one male. This conclusion is consistent with the presence of a maximum of six alleles for microsatellite loci in the same population (Houlden *et al*., 1996). Historical records suggest the number of founders was two

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(Warneke, 1978), so it is possible that additional, unrecorded liberations of koalas onto French Island were made.

(iv) *Conclusions*

MtDNA-RFLP analysis of southern Australian koala populations revealed a very low degree of nucleotide diversity and genetic differentiation amongst populations which were largely derived from French Island during large-scale translocations. While these findings are consistent with the recent history of bottlenecks and translocations in the region, this history may not completely explain the observations: similar levels of mtDNA diversity within two relatively undisturbed Queensland koala populations have previously been reported. This raises the possibility that low mtDNA variation is a feature of koalas – an intriguing suggestion which will need to be addressed with more extensive sampling and higher-resolution techniques.

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