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Genetic population structure of the Greater Bilby *Macrotis lagotis*, a marsupial in decline

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Abstract

The Greater Bilby has shown a rapid decline in range during this century and now occupies only a small isolated area in south-western Queensland (QLD) and a larger, but mostly low-density area in the north-western deserts of the Northern Territory (NT) and Western Australia (WA). We have examined variation in the control region of mitochondrial DNA (mtDNA) and at nine microsatellite loci in order to investigate the extent of current and historical subdivision across the species range, and to provide a preliminary assessment of genetic structuring and mating system on a finer scale within the QLD population. Both mtDNA and microsatellite loci had substantial variation within and among populations, with mtDNA divergence being greater between QLD and NT than between NT and WA. The QLD population had two unique and divergent mtDNA lineages, but there was no evidence for strong phylogeographical structure across the range. The available evidence suggests that the bilby should be considered as a single Evolutionarily Significant Unit consisting of multiple Management Units. Augmentation of the remnant QLD population from the NT does not appear necessary at this stage, at least not on genetic grounds. Finer-scale analysis of microsatellite variation for two QLD colonies revealed a deficiency of heterozygotes and significantly greater relatedness within than between colonies. However, structuring was observed only for males; relatedness values for females did not depart from those expected under panmixia. Parentage exclusion analysis for one colony allowed the construction of a partial pedigree which indicated strong polygyny, with one male fathering all but one of the eight offspring assigned. The extent to which fine-scale genetic structuring and differences between sexes is due to sex-biased dispersal vs. effects of mating system remain to be determined.

Keywords: mitochondrial DNA, microsatellites, ESU, conservation genetics, parentage

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Introduction

The Greater Bilby *Macrotis lagotis* is regarded as vulnerable to extinction because of the substantial reduction (≈ 80%) in its range since the late 1800s (Kennedy 1992; Southgate 1994). At the time of European settlement, the species was distributed over 70% of arid and semiarid Australia, but has now contracted to two isolated areas (Southgate 1990); a small region of south-west Queensland (QLD) and a broader, but low density distribution in the western deserts (WA–NT, Fig. 1). The QLD population is estimated as ≈ 700–1000 individuals. Local population reductions have been recorded and are possibly continuing (Gordon *et al*. 1990; P. McRae, unpublished data). The cause of this decline has not been established, although competition with introduced species (cattle, sheep and rabbits) and predation by foxes and cats have been suggested (Watts 1969; Gordon *et al*. 1990; Southgate 1990). An attempt to reintroduce the species to part of its former range (in the NT) failed, although the reasons for this failure are not clear (Southgate 1994; Southgate & Possingham 1995).

As one of the surviving 'critical weight range' mammals (Burbidge & McKenzie 1989) and the only remaining

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species of desert bandicoot, the Greater Bilby is a flagship for arid-zone conservation, yet there are major gaps in our knowledge of the species. One such gap is whether bilbies exist as spatially structured metapopulations and whether local population declines reflect extinction or emigration in response to disturbance or fluctuations in availability of insects. The animals exist in scattered, low-density colonies which may occupy areas of 5–20 ha (reviewed by Johnson 1989). In a reintroduced population, individual males moved between burrows up to 2 km apart over 2 days and a new colony was established 10.5 km from the release site within 2 years (Southgate & Possingham 1995). Detailed information on movements within, and dynamics of, remnant populations is lacking, although Watts (1969) also reports tracking of individuals for 0.8 and 1.2 km and QLD individuals have been radio-tracked over distances of 2–3 km in one night (P. McRae, unpublished data).

Another area of limited knowledge is the extent of genetic differentiation within and among populations across the range. Bilbies are currently managed on a state-by-state basis, but the extent of population continuity and genetic divergence among areas is poorly understood. Southgate & Adams (1993) found no evidence for differentiation at allozyme loci among small samples ($n = 6$–9) from WA, NT and QLD. However, as for other marsupials (Sherwin & Murray 1990), the analysis was restricted by the limited diversity at allozyme loci. Only six of the 42 loci were polymorphic and average heterozygosity was < 3%.

Mitochondrial DNA (mtDNA) and nuclear microsatellites have proved powerful as tools for the analysis of genetic population structure in marsupials (Taylor *et al.* 1994; Robinson 1995; Houlden *et al.* 1996), particularly when used in combination (Moritz *et al.* 1996; Pope *et al.* 1996). The combined data should also permit sensitive definition of conservation units; either Evolutionarily Significant Units (Ryder 1986) or Management Units (*sensu* Moritz 1994). Analyses of molecular population structure would be of particular value for the bilby in that it should shed light on long-term population processes and thereby provide guidance for the translocations proposed for this species (Southgate 1994, 1995). The microsatellites also have the potential to provide information on mating system and individual productivity (e.g. Craighead *et al.* 1995; Altmann *et al.* 1996), especially if it is possible to pedigree individuals from the same and different colonies.

The aims of the current study therefore were:

1 to examine the distribution of molecular genetic diversity among populations in WA, NT and QLD and thereby define conservation units; and

2 to provide an preliminary perspective on local population processes in one area (QLD) through detailed analysis of fine-scale genetic structure and estimation of relationships among individuals.

Materials and methods

Sampling

The samples available for analysis included those from the previous study of allozyme variation (details in Southgate & Adams 1993) and material from freshly caught bilbies. The former were frozen blood lysates and the latter

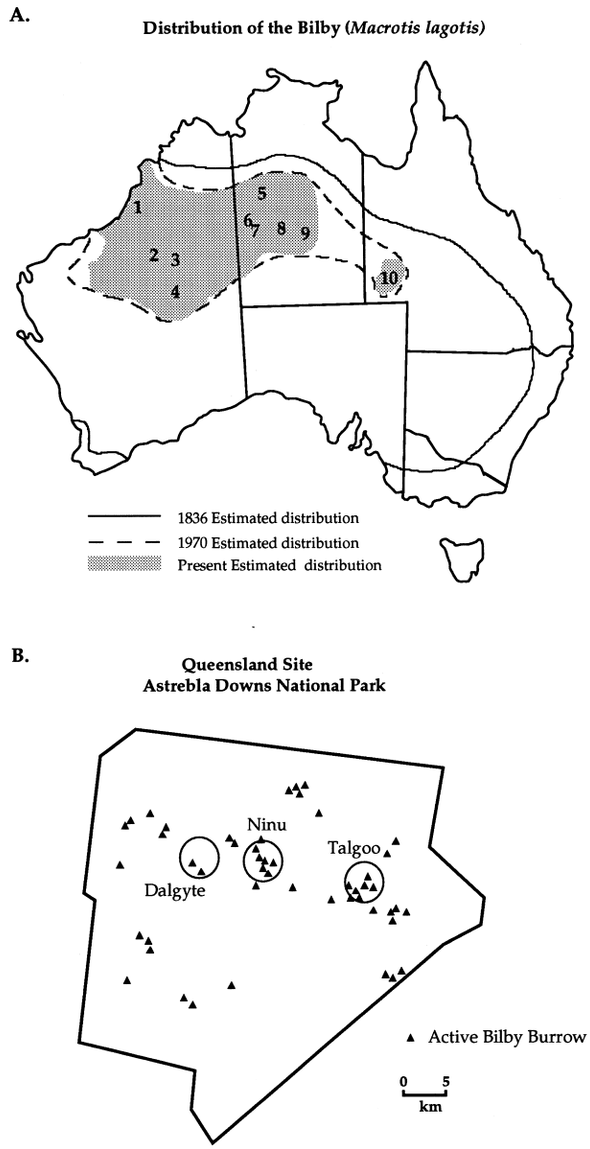

Fig. 1 (A) Map of Australia showing the approximate locations sampled (numbers as in Table 1), the limit of distribution of the Greater Bilby at 1836, at 1970 and currently (modified from Southgate 1990). (B) Detailed distribution of colonies sampled within the QLD population (Astrebla Downs National Park; corresponding to location no. 10 in A). The locations of main colonies sampled, Dalgyte, Ninu and Talgoo, are indicated. Triangles indicate the locations of active burrow complexes. The numbers refer to localities listed in Table 1

Table 1 List of localities sampled and sample sizes for analysis of mtDNA and microsatellites.

Location	Lat × Long.	Sample size	
		mtDNA	microsatellite
Western Australia (WA)			
1. La Grange	18°42′ × 121°52′	1	NA
2. Purnmu	22°04′ × 123°07′	3	NA
3. Well 33	22°19 × 124°41′	1	NA
4. Patjarr	24°45′ × 124°41	1	NA
Northern Territory (NT)			
5. Tanami Desert	various (see Fig. 1)	6	13
6. Rabbit Flat	20°20′ × 130°02	2	2
7. The Granites	20°51′ × 130°05′	2	2
8. Yinapuka	20°23′ × 131°55′	1	1
9. Ali Kurung	20°58′ × 123°12′	1	1
Queensland (QLD)			
Astrebla Downs National Park			
10a. Dalgyte	24°10′ × 140°33′	1	5
10b. Ninu	24°10 × 140°36′	6	10*
10c. Talgoo	24°11 × 140°42′	5	17*

NA, not analysed.
* For analysis of *F*-statistics and relatedness, pouch young were excluded from Ninu ($n = 2$) and Talgoo ($n = 3$).

2–3 mm² ear biopsies stored in 20% DMSO and saturated NaCl. The samples for QLD ($n = 27$, excluding pouch young) were obtained from three colonies distributed over ≈ 20 km within Astrebla Downs National Park (Fig. 1B; Table 1). Those from the NT ($n = 19$) came from more widely separated locations in the Tanami desert and the WA samples ($n = 6$) also came from widely dispersed sites (Fig. 1; Table 1).

Amplification and sequencing of mitochondrial DNA

The 5′ segment of the mtDNA control region, located between the $tRNA^{pro}$ gene and the conserved central domain, was targeted for analysis as this has proved highly variable in other mammals, including marsupials (e.g. Pope *et al*. 1996). Amplification of the entire control region was achieved using primers designed against conserved portions of the flanking $tRNA^{pro}$ and $tRNA^{phe}$ sequences (primers mt15996L and mt00607; M. Elphinstone, personal communication). This produced fragments of ≈ 1.8 kb, but some individuals had two amplification products differing in length by up to ≈ 200 bp. Sequencing of these products from both 5′ and 3′ ends revealed two distinct sequences, both of which had long arrays of short tandem repeats (i.e. a mtDNA microsatellite) at the 3′ end of the control region. This observation raised the possibility that one copy was mitochondrial, and the other nuclear in origin, as has been reported in other taxa (Zhang & Hewitt 1996). To resolve this, we purified mtDNA by ultracentrifugation (Dowling *et al*., 1995) from tissues from a single, previously killed individual and determined that one copy was mitochondrial in origin. We then designed an mtDNA specific primer near the 3′ end of the control region (‘3BilbyH’) and, from sequences obtained from a mt15996 L–3BilbyH amplimer, designed an internal primer (2BilbyH: 5′ GGGCTTGCTCTGAAGGATGTTG 3′) to amplify, in combination with mt15996 L (CTCCACCATCAGCACCCA), a 630-bp segment of the 5′ end of the control region. Conditions for the mt15966 L and 2bilbyH amplifications were: denaturation, 94 °C, 40 s; annealing, 50 °C, 40 s; extension 72 °C, 1 min, for 30 cycles. All amplifications included negative controls to test for cross-contamination. Manual cycle sequencing was performed using γ^{33}P end-labelled primers as described in Pope *et al*. (1996).

Isolation and screening of microsatellite loci

Microsatellite loci were isolated from a partial plasmid library of bilby DNA (from an NT individual) following the protocol described by FitzSimmons *et al*. (1995). Thirty of 205 positive clones were sequenced and 15 of these contained uninterrupted CA repeats of > 12 copies. Primers were designed to 11 of these (Appendix 1) and amplification with these primers revealed reliably scorable polymorphism at all loci except B61. Amplifications were achieved with the following conditions: denaturation, 94 °C, 40 s; annealing, 55–57 °C, 40 s; extension 72 °C, 50 s for 28 cycles using one primer end-labelled with γ^{33}P-ATP.

Products were separated on acrylamide gels as described in Pope *et al*. (1996). Each gel included multiple sequence ladders to allow scoring of exact allele sizes. Detailed statistical analysis excluded one further locus (B08) because preliminary analyses revealed atypically strong deficiencies of heterozygotes in both the NT (F_{IS} = 0.56, $P < 0.001$) and QLD (F_{IS} = 0.29, $P < 0.014$) samples, suggesting the presence of either null alleles or scoring errors.

Statistical analyses

For mtDNA, sequences were aligned using CLUSTAL V (Higgins *et al*. 1992) and adjusted manually to minimize gaps. Sequence divergence was estimated using the Kimura (1980) 2-parameter model in MEGA (Kumar *et al*. 1993) and nucleotide diversity and divergence was estimated using REAP 4.0 (McElroy *et al*. 1992). The phylogeny of the sequence was estimated using a variety of methods including Neighbour-Joining (in MEGA) and Quartet Puzzling (an analogue of maximum likelihood; Strimmer & von Haeseler 1996). The distribution of sequence variation among range states (QLD, WA and NT, see Fig. 1) was determined using AMOVA (Excoffier *et al*. 1992). For microsatellites, estimates of observed and (unbiased) expected heterozygosity were obtained from BIOSYS vs. 1.7 (Swofford & Selander 1989). Exact tests for fit of genotype proportions to Hardy–Weinberg equilibrium (Guo & Thompson 1992) were carried out using GENEPOP (Raymond & Rousset 1995). The Weir & Cockerham (1984) analogues of Wright's *F*-statistics were estimated, and their significance tested by appropriate permutations in FSTAT (Goudet 1995).

For the QLD colonies, we used two approaches to determine relatedness among individuals – an index of relatedness (*R*, Queller & Goodnight 1989) and parentage exclusion. Pairwise estimates of *R* and their standard errors (obtained by jack-knifing across loci) were obtained using a program written by E. Geffen. We estimated the number of loci needed to provide consistent estimates of relatedness by rarefaction analysis in which loci are added sequentially up to the total number assayed (see Altmann *et al*. 1996). To test whether individuals within colonies are more closely related to each other than to randomly selected individuals, we used Monte-Carlo simulations which compared observed *R*-values for dyads within and between colonies with those calculated from random assignment of individuals to colonies, keeping colony sizes and sex ratios constant. These simulations were also carried out separately on all, male–male, and female–female pairwise comparisons.

To estimate parentage within a colony we used exclusions based on comparison of multilocus allele profiles. Using a computer program, again written by E. Geffen, we assigned all possible parental combinations to each offspring. The number of exclusions with each parent and with each pair of parents allowed us to locate the most probable parents, these being combinations that had zero exclusions. Additional behavioural and morphological information (e.g. estimated age, presence in the study site, etc.), as well as matching of mtDNA sequences, were used to refine and verify estimates of parentage further.

Results

Variation in mitochondrial DNA

A sequence of ≈ 600 bp (21 bp from $tRNA^{pro}$ and ≈ 580 bp from a 5′ segment of the control region) was obtained from 30 individuals; 12 from each of the NT and QLD populations and all six individuals available from WA. The samples from QLD were all from nearby localities within Astrebla Downs NP, whereas those from both NT and WA were from geographically dispersed populations (Fig. 1, Table 1). Homologous sequence was also obtained from

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QLD1          CTATATAAAT  TTTTAAAAGA AATAACCTAA ACTTATCAAT CTCA
QLD3(5)       .........G. .......... .......... .......... ....
QLD4          .........G. ......G... .......... .......... ....
QLD5          .........G. .......... .......... .......... T...
QLD2(4)       .C...C.... ......G.T. .....TT... ..A..C...C TC.G
NT1           .A...C.... C........A. .....TT... ..A......C TC..
NT2           .....C.... .........A. .....TT... ..A......C TCTG
NT3(2)        T....C.... CACCTG.G.. ..C.GTT... ..AC.....C TC..
NT4           .A...C.G.. .........A. .....TT... ..A......C TC..
NT5           .....C.... .......... .....TT... ..A......C T.T.
NT6(3)        T....C.... C.CCTG.G.. ..C.GTT... ..AC.....C TC..
NT7           .....C.... .......... .....TT... ..A......C TCTG
NT8           .....C.... .......... .....TT.G. ..A...T..C TCTG
NT9           .CTAGC..G. .C......AG G..T.TT... ..A......C TC.G
WA1           .C...CG..C .......... .....TT..G .TA....G.C TC.G
WA2           .C...C..G. .....G.T.. .....TT... ..A.....GC TC..
WA3           .C........ .......... .....T.... G.A......C T..G
WA4           .C...C..G. C....G..A. .G...TT... ..A.G..... TC..
WA5           .C...CG..C .......... .....TT... ..A....G.C TC..
WA6           .C...C.... .......... G....TTC.. ..A......C TC..
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Fig. 2 Sequence variation within the 5′ segment of the mtDNA control region for 20 alleles detected among 30 bilbies. Only variable positions are shown. The full sequence alignment can be obtained from the European Bioinformatics Institute site (ftp // ftp. ebi. ac. uk/pub/data bases/embl/align/) with accession number DS30659. Alleles are designated according to their state of origin (QLD = Queensland; NT = Northern Territory, WA = Western Australia) and numbers in parentheses are the number of individuals with that allele where $n > 1$

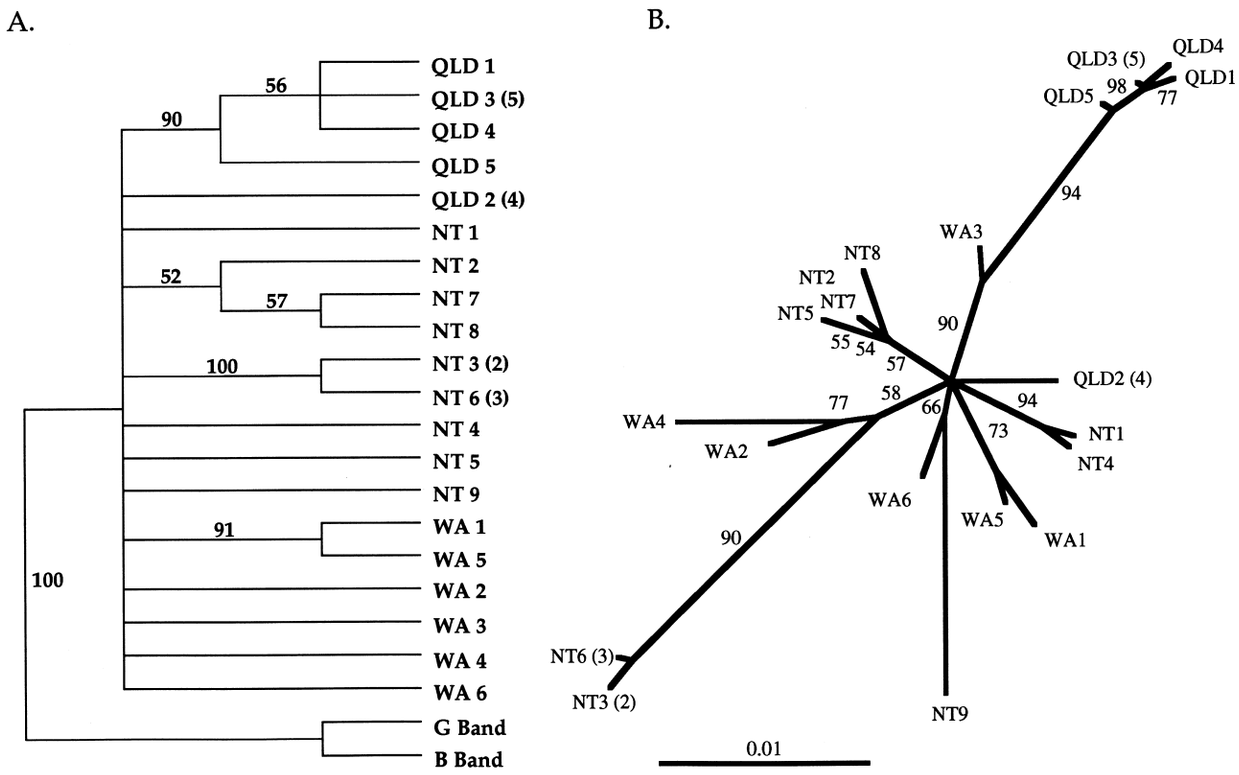

Fig. 3 Estimates of phylogeny of mtDNA alleles from the bilby obtained from (A) 500 bootstrapped Neighbour Joining (NJ) trees and (B) Quartet Puzzling. The NJ tree (A) was outgroup rooted using the golden and brown bandicoots; whereas (B) is shown as an unrooted phylogram with branch lengths scaled according to molecular distance. Allelic designations follow Fig. 2. Numbers above branches refer to the proportions of bootstraps (in A) and Quartet Puzzling replicates (in B) in which the clade to the right appears

two outgroups (the golden and northern brown bandicoots; *Isoodon auratus* and *I. macrourus*), although in these species the corresponding segment of the control region was ≈ 100 bp shorter with indels distributed throughout the sequence (data not shown). Excluding the outgroups, there were 44 variable nucleotide positions, of which 28 were phylogenetically informative (bold in Fig. 2). This sequence variation defined 20 different haplotypes; five in QLD, nine in NT and six from WA. Levels of sequence divergence between haplotypes ranged up to 3.6% (Kimura 2 parameter estimate between NT3 and NT9). However, most of the 20 mtDNA haplotypes were similar, differing by ≈ 2% or less.

Attempts to reconstruct the phylogeny of the haplotypes were limited by the low level of variation relative to the large number of haplotypes present and strong biases in substitution processes. The region analysed is highly AT rich (80% A + T) with a strong bias towards transitions [maximum likelihood (ML) estimate; ti/tv = 8.2] and marked heterogeneity in rates of substitution among sites (ML estimate; gamma parameter, $\alpha = 0.106$). An outgroup rooted neighbour-joining tree produced with 500 bootstrap resamplings is shown in Fig. 3(A). This identified just three reasonably supported (> 90%) clades, each of which was restricted to a single geographical area. Another way of viewing these relationships is as an unrooted consensus phylogram, derived by Quartet Puzzling, in which branch lengths are scaled according to estimated sequence divergence (Fig. 3B). Details vary according to the method of analysis, but three features are consistent: (i) both the QLD and NT samples include multiple, highly divergent clusters of mtDNA haplotypes; (ii) there is an unresolved

Table 2 Statistical analysis of sequence variation among samples of bilbies from different states. Figures below the diagonal are nucleotide divergence, those on the diagonal (in bold) are within-population nucleotide diversity, and those above the diagonal are the proportion of variation distributed between states (i.e. ϕ_{ST}). The significance of ϕ_{ST} values is indicated as ***, $P < 0.001$ or *, $0.01 < P < 0.05$

	n	QLD	NT	WA
QLD	12	**0.011**	0.485***	0.352*
NT	12	0.0085	**0.016**	0.197*
WA	6	0.0048	0.0034	**0.013**

Table 3 Allele frequencies at nine microsatellite loci among bilbies from the Northern Territory (NT) and Queensland (QLD). Exact allele sizes can be obtained from the senior author on request

Allele no.	Bil02		Bil16		Bil17		Bil22		Bil41		Bil55		Bil56		Bil63		Bil66	
	NT	Qld	NT	Qld	NT	Qld	NT	Qld	NT	Qld	NT	Qld	NT	Qld	NT	Qld	NT	Qld
1	0.053	0	0.632	0.204	0	0.037	0.026	0	0.105	0	0.026	0	0.158	0.074	0.132	0	0.026	0
2	0.026	0	0.079	0	0.026	0	0.053	0	0.105	0.5	0.026	0	0.263	0.019	0.079	0.093	0.026	0
3	0.132	0.148	0.132	0.093	0.026	0.056	0.105	0.315	0.263	0.019	0	0.278	0.105	0.019	0.026	0.056	0.053	0.037
4	0.184	0.056	0.079	0	0.289	0.074	0.184	0.185	0	0.037	0	0.019	0.053	0	0.184	0.426	0	0.296
5	0.316	0.426	0.026	0.093	0.132	0.056	0.342	0.5	0.447	0.222	0.053	0.259	0.158	0.056	0.079	0.037	0.079	0.333
6	0.079	0.241	0	0.111	0	0.019	0.263	0	0	0.056	0.158	0.056	0.026	0.444	0.026	0.148	0.237	0.241
7	0.079	0	0	0.352	0.105	0	0.026	0	0	0.056	0.105	0.019	0.026	0	0.263	0	0.237	0.019
8	0	0.019	0.026	0.074	0.079	0			0	0.093	0.132	0.333	0.026	0.167	0	0.037	0.158	0
9	0	0.019	0.026	0.056	0.026	0.056			0	0.019	0.263	0	0.079	0.222	0	0.056	0.079	0
10	0.026	0	0	0.019	0.026	0			0.079	0	0.237	0.037	0.079	0	0	0.13	0.053	0.056
11	0.105	0.074			0.079	0.333							0.026	0	0.026	0	0.053	0
12	0	0.019			0.105	0.204									0.105	0.019	0	0.019
13					0.053	0.037									0.053	0		
14					0.053	0									0.026	0		
15					0	0.13												

polytomy linking samples from all three states; and (iii) there is no obvious geographical component to the phylogenetic structure. The QLD samples include two divergent clusters that are also divergent from NT and WA samples, but there is no support for monophyly of the QLD haplotypes relative to those from the rest of the geographical range. Haplotypes from both clades were present in each of the two major colonies from QLD.

The amount of sequence variation within and among populations was summarized as haplotype diversity, nucleotide diversity and divergence values, respectively (see Table 2 for the latter two measures). For the species as a whole, both nucleotide and haplotype diversity are substantial (π = 1.8%, H = 0.95). Values within states are broadly similar, although the QLD population tended to have less sequence variation (π = 1.1%) and lower haplotype diversity (H = 0.76) than the samples from NT (π = 1.6%, H = 0.94) and WA (π = 1.4%, H = 1.00). These patterns should be interpreted with caution because of differences in sampling scale between states. It is possible that the slightly lower diversity of the QLD population is due to the smaller geographical scale of sampling compared with NT and WA (Fig. 1).

Despite the lack of obvious phylogeographical structure, over 30% of the sequence variation was distributed among states (AMOVA: ϕ_{ST} = 0.317, $P < 0.001$). Pairwise comparisons (Table 2) revealed substantial nucleotide divergence and a significant proportion of sequence diversity distributed between populations. The divergence was greatest between QLD and NT or WA and less, but still significant, between NT and WA.

Table 4 Measures of within population variation at microsatellite loci Values are observed/expected heterozygosity, and the number of alleles (A)

	NT (n = 19)			QLD (n = 27)		
	H_O	H_E	A	H_O	H_E	A
B2	0.789	0.844	10	0.778	0.743	8
B16	0.526	0.585	7	0.778	0.811	8
B17	0.895	0.879	12	0.593	0.828*	10
B22	0.789	0.785	7	0.407	0.628*	3
B41	0.789	0.721	5	0.704	0.697	8
B55	0.947	0.839	8	0.667	0.753	7
B56	0.947	0.875	11	0.815	0.730	8
B63	0.895	0.873	11	0.556	0.776**	9
B66	0.737	0.863	10	0.593	0.752	7
Mean	0.813	0.807	8.9	0.654	0.746**	7.7
+ SE	0.044	0.033	0.77	0.044	0.020	1.0

* Deviations between observed and expected levels of heterozygosity at these loci were found to be marginally significant in exact tests with $0.01 < P < 0.05$, but the deviations are insignificant when α levels are adjusted for multiple tests.
** significant deficiency of heterozygotes ($P < 0.005$).

Microsatellite variation

The analysis of microsatellite variation was restricted to the NT and QLD populations for which sample sizes were 19 and 27, respectively. Known relatives (i.e. pouch young) were excluded. The small number ($n = 6$) of samples available for the WA population precluded statistical analysis and these results are not included here. The numbers of alleles detected overall at the nine loci analysed varied from seven (B22) to 15 (B17) and the number of alleles detected per population varied from three (B22 in QLD) to 12 (B17, NT), indicating that these loci should have considerable power for paternity analysis as well as fine-scale analysis of genetic structure. Allele frequencies for each of the nine loci across the two population samples are given in Table 3.

Variation within populations, as measured by either numbers of alleles or heterozygosity, was substantial (Table 4). Mean observed heterozygosity was higher in the NT population, although the difference in expected heterozygosity is slight and nonsignificant ($P = 0.11$) in a Wilcoxon signed rank test. The average numbers of alleles per locus were similar between the NT and QLD populations (Table 4) although, when adjusted for equal sample sizes (Ewens 1972), the QLD sample had significantly less alleles (Wilcoxon signed rank test, $P = 0.03$). Exact tests confirmed that genotype frequencies were in Hardy–Weinberg proportions in the NT population. However, for the QLD population, three loci showed deficiencies of heterozygotes (Table 4). For two loci, B17 and B22, the deviations are marginal ($0.01 < P < 0.05$) and nonsignificant when type 1 error levels are corrected for multiple tests (Rice 1989); the heterozygote deficiency at the third locus (B63, $P < 0.005$) remains significant and, likewise, the deviation across all loci is significant ($P < 0.005$).

Comparisons of allele frequencies between the QLD and NT population revealed highly significant heterogeneity ($P < 0.001$) at 8 of the 9 loci, and there was also significant heterogeneity at the 9th locus (B02, $P = 0.023$). Analysis of *F*-statistics revealed significant divergence among populations (jack-knife $F_{st} = \theta = 0.11 \pm 0.017$; permutation test, $P < 0.0005$).

The *F*-statistics analysis also revealed low, but significant deviation from random mating within populations ($F_{IS} = f = 0.069 \pm 0.036$; permutation test, $P = 0.004$). Subsequent analysis of individual populations indicated that this was due to the significant deficiency of heterozygotes within the QLD sample ($F_{is} = 0.134 \pm 0.055$, $P < 0.0005$), whereas the NT population showed no such deviation ($F_{IS} = 0.001 + 0.032$, $P = 0.632$).

The QLD sample was comprised mostly of individuals captured from two major clusters of burrows in 1995 and 1996 (the 'Ninu' and 'Talgoo' colonies; Table 1; Fig. 1B). These colonies are each distributed over ≈ 5 km² and are separated by ≈ 10 km. Estimated colony sizes are ≈ 10 and 25 bilbies, respectively. To explore further the nature of the nonrandom mating within the QLD sample, we analysed the distribution of pairwise estimates of relatedness (*R*) within and among colonies. Sample sizes for this were $n = 10$ for Ninu (four males and six females) and $n = 17$ for Talgoo (nine males and eight females). Resampling analysis indicated that estimates of *R* asymptote relative to the value for nine loci after seven loci have been combined, so that the *R*-values for nine loci are expected to be reasonably precise (analysis not shown).

For all individuals, the observed mean *R* within colonies was positive and significant relative to values observed in randomizations ($R = 0.072$, $P < 0.001$), whereas *R* between colonies was negative and also significant ($R = -0.103$, $P < 0.001$). This pattern holds for comparisons among males (within $R = 0.104$, $P < 0.001$; among $R = -0.163$, $P < 0.001$) but not for comparisons among females (within $R = 0.005$, $P = 0.164$; among $R = -0.052$, $P = 0.245$). The results, although limited by small sample size, suggest that there is greater relatedness within areas and lower relatedness among areas than expected if the QLD population was panmictic, but also that this pattern differs between sexes.

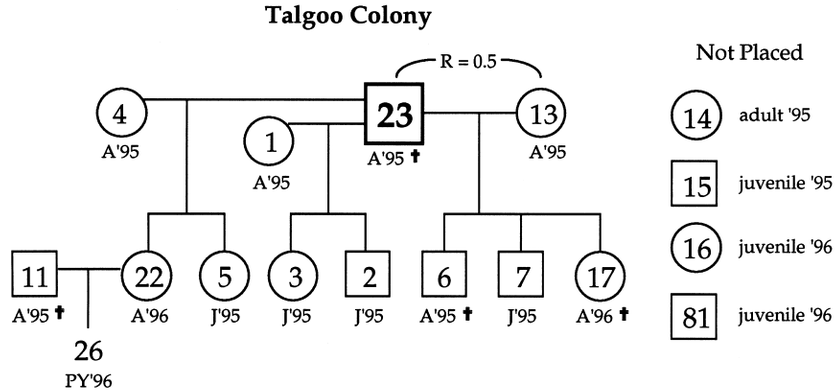

Fig. 4 Partial pedigree for the Talgoo colony inferred from exclusions based on multilocus microsatellite profiles. Three juveniles and two adults could not be placed within the pedigree. The dotted line connecting male no. 23 and female no. 13 indicated probably first-order relatedness inferred from the high *R*-value (see text). The individuals marked with crosses were known to be dead in early 1997

An insight into the cause of this pattern was provided by assignment of parentage within the larger Talgoo colony. Comparison of multilocus genotypes of individuals and all possible combinations of parents from the sampled bilbies unambiguously identified both parents for five juveniles and three adults. Four individuals from Talgoo could not be assigned to the pedigree; parents were not identified for three juveniles and one adult female (no. 14) present in 1995 was excluded as a mother of any of the captured offspring. The partial pedigree assembled from this information (Fig. 4) indicates strong polygyny with one male (no. 23) having mated three females to father seven of the eight offspring for which parentage was determined. Two other males present in 1995 (no. 11 and no. 6) did not contribute to the sampled offspring in that year, but one (no. 11) mated a daughter of the dominant male to produce a single offspring in 1996. Male no. 6 was a small adult in 1995. The dominant male (no. 23), two other males (nos 6 and 11) and one female (no. 17) were known to be dead by August 1996.

Estimates of R among parents in the pedigree were mostly low (< 0) with the exception of the dominant male (no. 23) and female no. 13, for which $R = 0.50$. To assess whether this high value of R indicates first-order relationship, we compared observed distributions of R for individuals of known relatedness (from the pedigree; Fig. 4) and compared these with unrelated individuals. The latter were defined as pairs of individuals, one from Ninu and the other from Talgoo, having mtDNAs from the two different major lineages (i.e. QLD2 vs. other alleles). The observed value for unrelated individuals was $R = -0.02 \pm 0.043$ and for second-order relatives was $R = 0.28 \pm 0.076$. Mean values for the first-order relatives were between 0.46 and 0.57 (Fig. 5). The estimate of $R = 0.50$ between male no. 23 and its mate, female no. 13, suggests therefore that they are likely to be first-order relatives.

Discussion

Genetic diversity within populations

As is the case for other marsupials studied to date, allelic diversity in bilbies revealed by sequencing of mtDNA control region and analysis of nuclear microsatellites is far higher than was detected from allozyme electrophoresis. The mean number of alleles per locus at allozyme loci was 1.19 across 42 loci (Southgate & Adams 1993), compared with 11.2 across nine microsatellite loci and 20 for mtDNA. This elevated diversity is expected to allow more sensitive analyses of both geographical structuring and parentage.

Levels of molecular diversity within populations of the bilby are within the range reported for other widespread marsupials in Australia. Mean expected heterozygosity for microsatellites in the bilby ($H = 0.78$) is similar to that seen in QLD populations of the yellow-footed rock wallaby ($H = 0.73$; Pope *et al*. 1996) and the koala ($H = 0.85$; Houlden *et al*. 1996) and greater than observed in the southern hairy-nosed wombat ($H = 0.65$; Taylor *et al*. 1994). Similarly, within-population nucleotide diversity for the bilby mtDNA control region (1.36%) is intermediate between values observed in the yellow-footed rock wallaby (1.03%; Pope *et al*. 1996) and the red kangaroo (2.81%; S. Clegg *et al*. unpublished data).

Microsatellite diversity in the isolated QLD population of the bilby ($H = 0.75$) compares favourably with that in other remnant or size-reduced populations of marsupials (e.g. translocated koalas, $H = 0.44$, Houlden *et al*. 1996; remnant northern hairy-nosed wombat, $H = 0.27$, Taylor *et al*. 1994; bridled nailtail wallaby, $H = 0.83$, Moritz *et al*. 1996). Concern has been expressed about the effects of low genetic diversity on population viability in these koala and wombat populations (refs above), and it has been suggested that the remnant QLD population of bilbies also may have lost genetic diversity (Gordon *et al*. 1990). However, the above evidence for substantial diversity of mtDNA sequences and nuclear microsatellites suggests that the viability of bilby populations is unlikely to be limited by lack of genetic variation *per se*.

Genetic diversity among populations

Significant genetic differentiation was observed between the NT and QLD populations for both mtDNA sequence variation and microsatellite allele frequencies, and between NT and WA for mtDNA. However, the propor-

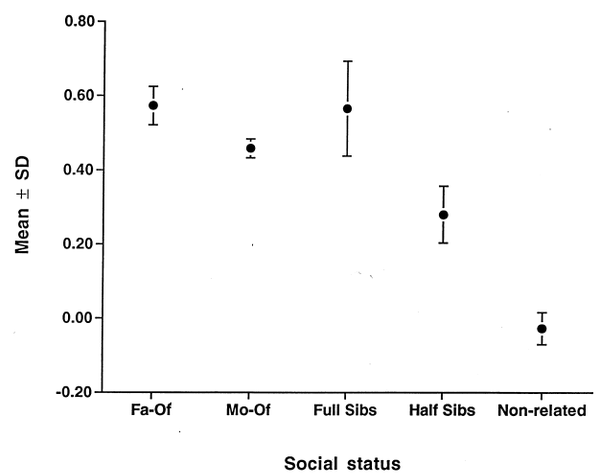

Fig. 5 Observed values of R observed for different classes of relatives as inferred from the Talgoo pedigree (Fig. 4) and for unrelated individuals (see text).

tion of mtDNA variation distributed between NT and QLD (ϕ_{ST} = 0.485) was over twice that between NT and WA (ϕ_{ST} = 0.197). The estimate of divergence between WA and QLD (ϕ_{ST} = 0.352) is lower than that between the geographically closer NT and QLD regions, but this could be due to the lower sample size for WA. The proportion of microsatellite variation distributed between the NT and QLD populations (θ = 0.11) was approximately fourfold less than for mtDNA, as expected if sex ratios of breeding adults are even (but see below) and rates of migration are equal between sexes (Birky *et al*. 1989).

To put these values into context, levels of mtDNA divergence among bilby populations are lower than seen in the yellow-footed rock wallaby, a habitat specialist (ϕ_{ST} = 0.77; Pope *et al*. 1996), but higher than among populations of the highly mobile and generalist red kangaroo (ϕ_{ST} = 0.18; S. Clegg *et al*. unpublished data). The microsatellite differentiation between the NT and QLD bilby populations is similar to that among QLD populations of the yellow-footed rock wallaby (θ = 0.11; Pope *et al*. 1996) and the koala ($0.09 < \theta < 0.16$; Houlden *et al*. 1996).

The range contraction in the bilby has occurred since the late 1800s and by 1936 the species had contracted to the northern populations (Fig. 1). Although records are scant, it appears that the WA and NT regions remain connected by low density populations, whereas the QLD population is now isolated from the rest of the range. The timing of this isolation is not clear, but is likely to date back to some time between 1936 and 1970 (Southgate 1990). These events are mirrored qualitatively by patterns of mtDNA variation which suggest greater connectivity between the WA and NT populations than between NT and QLD.

The observations on changes in range establish, at least roughly, when the QLD population became physically isolated, but do not indicate the pre-existing level of long-term gene flow between it and geographically separated locations. In particular, it is not clear to what extent the QLD population has been isolated historically. The presence of two highly divergent mtDNA lineages and substantial heterozygosity at microsatellite loci suggests either that the QLD population was formerly much larger, or that that it has been connected to others historically, although levels of gene flow to the NT could have been low prior to the population separation. The marked absence of intermediate genotypes in the mtDNA phylogeny is unexpected under equilibrium coalescence theory (Crandall & Templeton 1993) and may reflect losses of geographically and phylogenetically intermediate mtDNA lineages as a result of recent extinction events.

Identification of conservation units

Moritz (1994; see also Avise 1994) suggested a binary approach to defining conservation units that seeks to identify populations that have been isolated historically (Evolutionarily Significant Units, ESUs) vs. those that are currently isolated, or effectively so, but were connected to others historically (= Management Units, MUs). ESUs can be recognized as having reciprocal monophyly of mtDNA lineages among areas, together with supporting divergence in nuclear allele frequencies, whereas MUs differ in allele frequency but are not phylogeographically distinct.

Application of the above scheme to the bilby is complicated by the presence of unresolved nodes at the base of the mtDNA phylogeny (Fig. 3). No haplotypes are shared between NT and WA, but the phylogenies estimated by a variety of methods do not indicate any reciprocal monophyly and, thus, long-term separation between these areas. The QLD population includes two unique and highly divergent mtDNA lineages, but there is no evidence that these are monophyletic to the exclusion of the NT–WA variants. Our tentative conclusion is therefore that the bilby currently represents a single ESU, with populations from each of the range states representing a separate MU. More detailed analysis within each area may well indicate the presence of additional MUs within states.

This conclusion bears on strategies for translocation to re-establish populations of the bilby (Southgate 1994), in particular whether bilbies should be moved between states or mixed for re-introductions. Elsewhere, it has been suggested that translocations could include mixtures of individuals from different historically connected populations (i.e. MUs), but, where possible, should not be conducted between historically isolated population units (Vrijenhoek *et al*. 1985; Moritz 1995). If the goal of management is to maintain historical population processes (Moritz 1995), it would be appropriate to encourage connectivity between the historically connected NT and WA populations. The situation for QLD populations is less clear. Although it seems unlikely that the QLD population has been historically isolated, there also does not appear to have been *extensive* exchange across the geographical distance now separating the NT and QLD populations. The best option therefore is to restore the QLD population by identifying and addressing local causes of decline (Caughley 1994); augmentation from the NT should be considered only as a last resort and, even then, only if the threatening process(es) have been ameliorated.

Genetic structure among colonies within QLD

The significant deficit of heterozygotes within the QLD sample could, in principle, be due to (i) selection against heterozygotes; (ii) mating among close relatives; (iii) non-random mating among colonies within the area, or (iv) some combination of the three. Although sample sizes were limited, the indication of higher relatedness within areas, and lower relatedness between areas, than expected

under panmixia tends to favour either or both of mechanisms (ii) and (iii), above. An unexpected result of these analyses was that the nonrandom distribution of relatedness was specific to males; by contrast, relatedness among females did not differ significantly from the distribution expected under panmixia.

A possible explanation for this result is provided by the preliminary analysis of parentage within the Talgoo site, wherein a single male appears to have sired seven of the eight assigned offspring. This is the first evidence for polygyny in natural populations of the bilby, but is consistent with behavioural observations on captive colonies, which suggested that males form dominance hierarchies, and also with the presence of strong sexual dimorphism in size and scent marking by males, features that are unusual in bandicoots (Johnson & Johnson 1983). Radiotracking has revealed that the dominant male from Talgoo frequently makes excursions of 2–3 km in one night, covering much of the total area occupied by the colony. Recent theory (Chesser 1991) has emphasized the strong effect of polygyny on genetic correlations within and among populations and, for the bilby, this alone may account for the greater within-population relatedness among males than among females. Further progress towards separating the contributions of limited dispersal and breeding system to the local genetic structure of bilby populations will require more intensive population sampling combined with direct estimates of dispersal distances for both males and females.

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Craig Moritz heads the molecular population genetics group at University of Queensland and is occasionally allowed to indulge in science. Anita Heideman has been the backbone of the lab for several years as Craig's research assistant and did all the work described herein. Eli Geffen works in the field of molecular ecology with a strong interest in social organization and Peter McRae is a field biologist currently studying the ecology of the bilby

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Locus	Primers (5′ –> 3′)	No. CA repeats	
B02	BIL02 A	GCA TGT ACT TAA CCC CCT TTG CC	17
	BIL02B	CCC GAC AAT CCA GCC TGT TAT TC	
B08	BIL08 A	GAA GCC TCA GAC TTT GGT CCC	23
	BIL08B	GCG CAC TCT TTG TAC CAT GTA TA	
B16	BIL16 A	GTT AGA AGA CAG GAT GGG GAT TG	12
	BIL16B	TGA GTA CAC AGT GCA GCA AGA GC	
B17	BIL17 A	GAG AAC TGG AAC TTC GCG TAG GC	16
	BIL17B	CTC CAA TTC ACT TTT CCT GAG AC	
B22	BIL22 A	GGT ATG AGG AAT TAG AAT TAC AGG	16
	BIL22B	CGG TAT TAA ATG GGC TAT GGA GT	
B41	BIL41 A	CAT TGT CAT AGA AGC ATC ATC AAC	14
	BIL41B	GGA AAA GTT TTT AGC CTA ATA GTG G	
B55	BIL55 A	GCA CCA ACC TAT CCT CTT CAT TC	24
	BIL55B	CTA CAA GTC TGA TAA TTC CAG GC	
B56	BIL56 A	GCA TGA ATG AAT CTC CTG GCT CC	23
	BIL56B	CAC TAA CAA ATA TGC TTG GGA AAG G	
B61	BIL61 A	AGA AGA ATT AGG ATT AAG GGA AAG	24
	BIL61B	AGC AGC TAG GAC AAC TGT ATT AG	
B63	BIL63 A	CTT AGG CAA ATA GGG TGA AGT GG	14
	BIL63B	CAG AAC CAT TAG GAA GGA GTT TC	
B66	BIL66 A	GCC TTG GGT ATG CTG TCA TCT GA	45
	BIL66B	CTC CAA GCC AAG AAT CCC ATT CC	

Appendix 1 Primers developed to amplify 11 microsatellite loci from the Greater Bilby and number of CA repeats in the original clones