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Assessment of genetic diversity in the critically endangered Australian corroboree frogs, *Pseudophryne corroboree* and *Pseudophryne pengilleyi*, identifies four evolutionarily significant units for conservation

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Abstract

The iconic and brightly coloured Australian northern corroboree frog, *Pseudophryne pengilleyi*, and the southern corroboree frog, *Pseudophryne corroboree* are critically endangered and may be extinct in the wild within 3 years. We have assembled samples that cover the current range of both species and applied hypervariable microsatellite markers and mitochondrial DNA sequences to assess the levels and patterns of genetic variation. The four loci used in the study were highly variable, the total number of alleles observed ranged from 13 to 30 and the average number of alleles per locus was 19. Expected heterozygosity of the four microsatellite loci across all populations was high and varied between 0.830 and 0.935. Bayesian clustering analyses in STRUCTURE strongly supported four genetically distinct populations, which correspond exactly to the four main allopatric geographical regions in which the frogs are currently found. Individual analyses performed on the separate regions showed that breeding sites within these four regions could not be separated into distinct populations. Twelve mtND2 haplotypes were identified from 66 individuals from throughout the four geographical regions. A statistical parsimony network of mtDNA haplotypes shows two distinct groups, which correspond to the two species of corroboree frog, but with most of the haplotype diversity distributed in *P. pengilleyi*. These results demonstrate an unexpectedly high level of genetic diversity in both species. Our data have important implications for how the genetic diversity is managed in the future. The four evolutionarily significant units must be protected and maintained in captive breeding programmes for as long as it is possible to do.

Keywords: Anuran, Australia, conservation genetics, frog, microsatellite, mitochondrial DNA, Myobatrachidae

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Introduction

The recent decline of frogs worldwide is now well established but the causes of this decline remain unclear (Alford & Richards 1999; Stuart *et al*. 2004; Beebee & Griffiths 2005). Australia has a highly diverse and species-rich frog fauna and a number of species have declined significantly over the last 30 years. Two iconic species in particular have been the subject of considerable concern because their decline has been so rapid and extreme. The northern corroboree frog, *Pseudophryne pengilleyi*, and the southern corroboree frog, *Pseudophryne corroboree* (Family Myobatrachidae), are small, pond-breeding and very brightly coloured terrestrial frogs restricted parts of the alpine and subalpine regions of the

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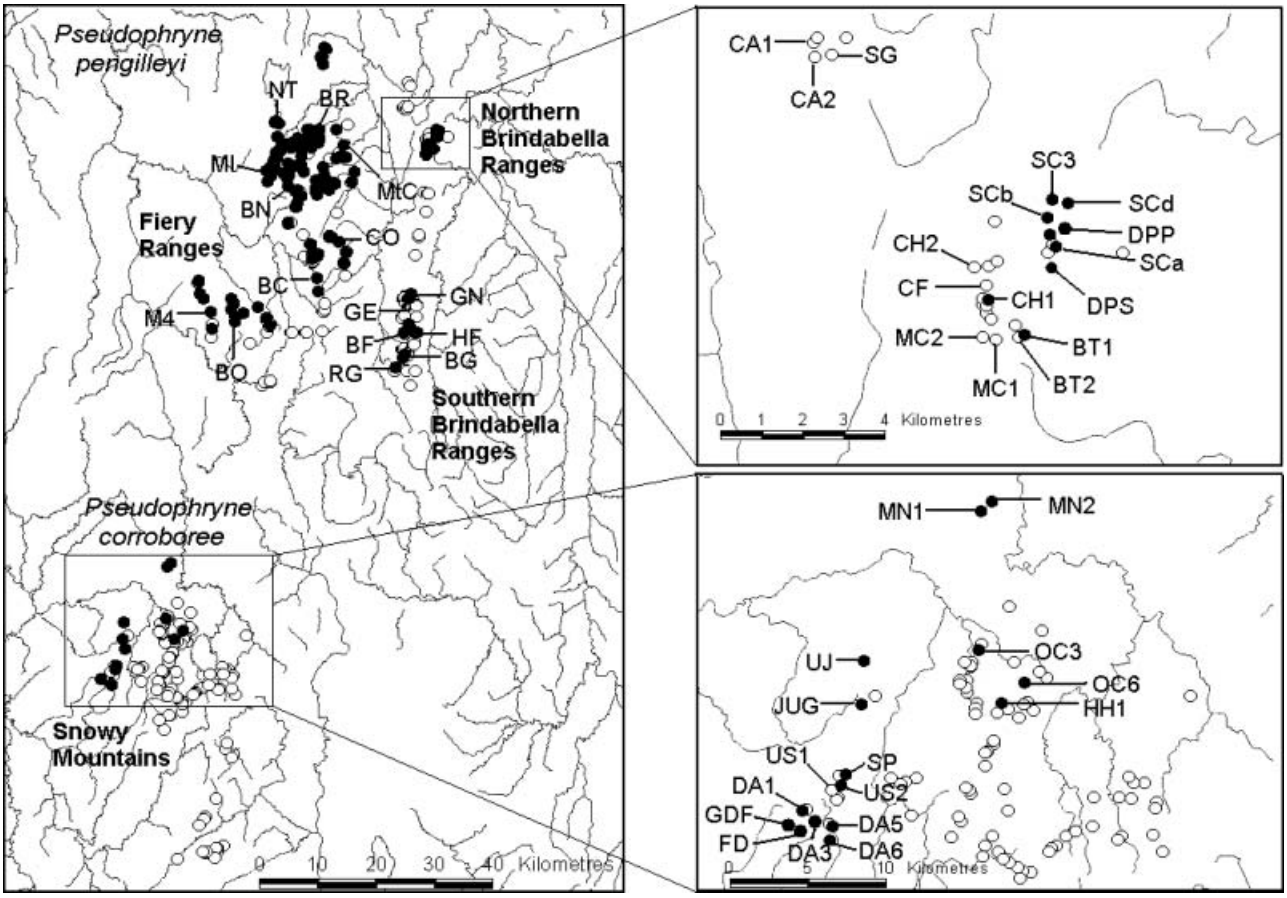

Fig. 1 Map showing disjunct distributions of *P. pengilleyi* and *P. corroboree* and the locations of historical records of now extinct populations (white dots) and the 41 sample sites used in the genetic analyses (black dots). Site names and haplotypes correspond to the codes in Table 1.

southern highlands of New South Wales and the Australian Capital Territory in Australia (Woodruff 1975; Osborne 1989) (Fig. 1). *Pseudophryne pengilleyi* is known from sites within three disjunct geographical regions; the Fiery Ranges, the Northern Brindabella Ranges and Southern Brindabella Ranges, whereas *P. corroboree* is known only from sites within the Snowy Mountains (Fig. 1). Once considered a single species, populations now considered *P. pengilleyi* have been taxonomically distinguished from *P. corroboree* based on hybridization experiments (Osborne & Norman 1991), morphology (Pengilley 1966; Woodruff 1975; Osborne *et al*. 1996), call structure (Osborne *et al*. 1996) and genetic data including allozymes and immunological distance (Roberts & Maxson 1989; Daly *et al*. 1990; Osborne & Norman 1991).

The rapid decline of both species of corroboree frog is noteworthy because it has been so well documented. Observations until 1966 indicated that both species were abundant and occurred in large numbers during the breeding season (Colefax 1956; Pengilley 1966), but numbers of *P. corroboree* in the Snowy Mountains and *P. pengilleyi* in the Brindabella Ranges had declined markedly by the 1980s (Osborne 1989; Osborne *et al*. 1999). Population monitoring as part of a recovery programme has documented further declines in both species. A recent census, taken in the summer of 2006, found the number of calling male *P. corroboree* to have fallen from 450 frogs at 79 sites in 1999 to just 39 frogs at 14 sites in 2006 (Hunter *et al*. 2006), making this species possibly the most critically endangered vertebrate in Australia. Declines in *P. pengilleyi* numbers have been equally severe in several parts of this species' range with the most recent census data finding fewer than 450 calling males across this species' known distribution (Hunter *et al*. 2006). The disease chytridomycosis, caused by infection with the Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*), has been identified as the primary causal factor in the decline of both corroboree frog species (Hunter 2007). The initial decline of these species coincided with the first appearance of this disease in populations (Hunter 2007), and also with the decline of other frog species in the Australian Alps (Osborne *et al*. 1999) and elsewhere along the eastern ranges of Australia which have been attributed to chytridomycosis (Berger *et al*. 1998).

In response to the continued decline and critically low population size, a captive husbandry programme has

commenced. This recovery action currently includes (i) collection of eggs from the wild each summer with captive rearing of tadpoles through to metamorphosis and subsequent re-introduction of frogs to the wild, and (ii) establishment of a captive breeding colony for producing frogs for re-introduction. Saving species from extinction through captive breeding and re-introduction is a highly controversial conservation tool (Fischer & Lindenmayer 2000; Seigel & Dodd 2002), although the introduction of captive-bred individuals has been successful in the Mallorcan midwife toad, *Alytes muletensis* (Bloxan & Tonge 1995; Kraaijeveld-Smit *et al*. 2005, 2006).

An understanding of the magnitude and patterns of genetic diversity among populations is critical for conservation efforts attempting to maintain evolutionarily viable species (Avise 2004). Pond-breeding amphibians such as corroboree frogs often employ distinct environments for breeding, development and adult survival. Breeding habitat is typically distributed patchily throughout a species range, and landscape features that affect the genetic connectivity of populations, as well as life-history characteristics of the individual species, can have a major effect on population structure. Recent studies of genetic structure in pond-breeding amphibians have shown that some species may show significant genetic structure across small geographical distances whereas others show little structure across similar scales (Rowe *et al*. 2000; Newman & Squire 2001; Burns *et al*. 2004; Palo *et al*. 2004; Shaffer *et al*. 2004; Funk *et al*. 2005; Spear *et al*. 2005; Pabijan & Babik 2006; Zamudio & Wieczorek 2007). Both corroboree frog species are slow-moving and exhibit high breeding site fidelity (W. Osborne, D. Hunter, University of Canberra, unpublished data). The distances travelled from breeding sites and dispersal capabilities through nonbreeding habitat are not known, although local extirpation and population declines have resulted in many breeding sites being separated by more than 5 km. Such distances are potentially beyond the dispersal capability of adult anurans (Newton & Squire 2001). These factors (low vagility, high site fidelity and effective fragmentation) in addition to the severe population declines, promote the possibility that breeding populations become genetically diverse, differentiated from each other and maintain their own genetic identity (Palo *et al*. 2004; Shaffer *et al*. 2004; Zamudio & Wieczorek 2007). Understanding the geographical scale over which genetic processes, such as gene flow, operate is important for the effective management of potentially important genetic diversity. Thus, understanding the extant level of genetic diversity and the genetic structure of wild and captive frog populations is of vital importance to the long-term persistence of both these species.

In addition, maintaining captive populations can potentially generate adverse genetic effects that jeopardize the ability of individuals to reproduce and survive in the wild (Woodworth *et al*. 2002; Gilligan & Frankham 2003). In particular, the maintenance of high genetic diversity in captivity is important to the long-term viability of populations because it may enable populations to adapt in response to environmental changes, and low heterozygosity is directly linked to reduced population fitness in many species, including frogs (Reed & Frankham 2003; Halverson *et al*. 2006; Kraaijeveld-Smit *et al*. 2006).

A previous allozyme study of seven wild corroboree frog populations (Osborne & Norman 1991) found: (i) no private alleles, but significant differences in allele frequencies between frogs in the Snowy Mountains region and those in the northern regions at five of seven loci; (ii) some evidence of genetic differentiation between populations in allopatric geographical regions, that is, those the Fiery Ranges and those in the Brindabella Ranges; (iii) little genetic differentiation between populations within geographical regions; and (iv) levels of genetic diversity (number of polymorphic loci, heterozygosity, average number of alleles per locus) in *P. corroboree* within the Snowy Mountains region to be lower than *P. pengilleyi* in the Fiery Ranges and Brindabella Ranges.

Osborne & Norman's (1991) study encompassed only seven populations: two in the Fiery Ranges (Cromwell Hill and Broken Cart), two in the Brindabellas (Coree Flats and Ginini Flats), and three in the Snowy Mountains (Maragale Range, Ogilive's Creek and Mount Jagungal). Here we re-address the findings of the previous investigation using recently acquired samples from 49 locations, which greatly improves coverage of the current range of both species. We employ hypervariable microsatellite markers and mitochondrial DNA sequences to provide baseline genetic diversity estimates, identify genetic populations within each species, and identify isolated populations of potential conservation importance as well as to inform captive breeding strategies. Specifically, we address four key questions: (i) should *P. corroboree* and *P. pengilleyi* be managed as separate species? (ii) Does significant genetic structure exist between geographical regions that should be taken into account in any conservation actions? (iii) Does significant genetic structure exist within geographical regions that should be taken into account in any conservation actions, for example, are individual breeding sites a suitable unit for conservation? (iv) In light of the dramatic recent declines in both species, do patterns of genetic diversity and genetic structure reflect potentially important losses of genetic variation in either species, and how can this be managed?

Materials and methods

Sampling locations and collection

Genetic samples were obtained from adult male frogs across the majority of the known extant distribution of

Pseudophryne pengilleyi. For *P. pengilleyi*, sampling occurred during the breeding season in early 2000 (northern and southern Brindabella Range sites) and again in 2006 (Fiery Range sites). Toe clips were taken from adult frogs and the tissue stored individually in 1.5-mL tubes containing 95% ethanol. In total, tissue samples were taken from 483 individuals from 35 locations with sample sizes ranging from 1 to 59 male frogs per site (Table 1).

Pseudophryne corroboree samples were obtained from tadpoles in the captive breeding programme at The Amphibian Research Centre, Melbourne, and from one wild population at an undisclosed location. This site was discovered in 2004 and census data indicate the presence of a large population at the site (approximately 140 calling males, Hunter *et al*. 2006). The large number of individuals in combination with the geographical isolation of the site makes this population of great potential conservation value, and as such the site location data have been withheld. The captive tadpoles were reared from eggs sourced from wild populations in 2005 for captive breeding or population augmentation programmes (e.g. Hunter *et al*. 1999), and provide a sample of the extant genetic diversity of wild populations. Tadpole tail tips were removed using sterile techniques and stored in 1.5-mL tubes containing 95% ethanol. Toe clips from the wild population were taken as described above. In total, tissue samples were taken from 89 individuals from 17 locations with sample sizes ranging from one to 17 animals per site (Table 1).

DNA extraction, genotyping and sequencing

DNA was extracted from samples using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Sambrook *et al*. 1989). Seven microsatellite loci were used initially to genotype individuals as described elsewhere (Morgan *et al*. in press). Three loci (Pc110, Pc116 and Pc166a2) amplified in less than 95% of individuals of one or both species and were discarded from subsequent analyses. Additionally, 66 individuals across the range of both species were sequenced for ~450-bp fragment at the 3′ end of the mitochondrial ND2 gene (Table 1). Polymerase chain reaction (PCR) and sequencing were performed using the forward primer L4882 and reverse primer tRNA_Trp under conditions described elsewhere (Morgan *et al*. 2007). Sequences were edited in SEQUENCHER 3.0 (Gene Codes Corporation) and aligned using CLUSTAL_X (Thompson *et al*. 1997).

Defining populations

The recent population declines and associated extreme rarity of both species means that many breeding pools have fewer than 10 calling males (e.g. Osborne *et al*. 1999). To facilitate genetic analyses samples obtained from discrete locations that comprised multiple breeding ponds were pooled if sample sizes from individual ponds were less than 10. For *P. pengilleyi*, samples were only pooled if the ponds were geographically close (within 2 km) and obviously connected by potential breeding habitat. Pooled sites were as follows: CA comprised CA1, CA2 and SG; CH comprised CH1 and CH2; BT comprised BT1 and BT2; SC1 comprised SCa and SCb; SC2 comprised SCc and SCd; GF comprised GN and GE (Fig. 1). Breeding sites containing fewer than 10 individuals that could not be justifiably pooled were discarded from subsequent population genetic analyses (excluded sites were MtC, M4 and HF). For *P. corroboree*, samples were pooled on the basis of common drainage systems as this represents the pooling system employed in the captive rearing programme (G. Marantelli, Amphibian Research Centre, personal communication): Drainage 1 (D1) comprised MN1 and MN2. Drainage 2 (D2) comprised UJ and JUG. Drainage 3 (D3) comprised OC3, UO6 and HH1. Drainage 4 (D4) comprised DA1, DA3, FD and GFD. Drainage 5 (D5) comprised DA5 and DA6. Drainage 6 (D6) comprised US1 and SP (Fig. 1). After this pooling stage, D1 and D5 contained fewer than 10 frogs so were not included in the microsatellite analysis, but were included in the mitochondrial DNA analysis.

We used STRUCTURE 2.1 (Pritchard *et al*. 2000) to test whether frogs from the four geographical regions (Fiery Range, Northern Brindabella Range, Southern Brindabella Range, and Snowy Mountains) are indeed separate populations. For breeding sites within these regions, we then tested whether they form separate populations or should be treated as one population. For all analyses, ln posterior probabilities were estimated using a correlated frequency model for $1 \leq K \leq 15$ from 500 000 generations after 200 000 generations were discarded as burn-in. Stationarity was evaluated by examination of F_{ST} and likelihood value plots, and by performing each run twice and evaluating the consistency of the results. The optimal value of K was calculated from the resulting $-\ln P(X \mid K)$ using Bayes' rule (Pritchard *et al*. 2000).

Genetic diversity analysis

Genetic diversity analyses were performed at two hierarchical levels: for each geographical region and at the level of the individual populations.

Evidence of linkage disequilibrium between microsatellite loci was assessed in GENEPOP (Raymond & Rousset 1995) using Markov chain approximations (dememorization 1000, batches 100, iterations per batch 1000). The inbreeding coefficient, F_{IS}, which describes how heterozygote frequencies deviate from Hardy–Weinberg proportions, was estimated for each locus and globally using FSTAT 2.9.3 (Goudet 1995). Significant deviations were assessed at the 5% level employing a sequential Bonferroni correction for multiple

Table 1 Geographical coordinates of sampled locations in Australian Map Grid units (zone 55 Easting and Northing). Number of individuals per site (*N*) and mitochondrial DNA haplotypes (number of individuals sequenced in parentheses) is also reported

Monitoring site name	Code	*N*	mtDNA	UTM Easting	UTM Northing
Pseudophryne pengilleyi					
Fiery Range					
Barnett's Road	BN	15	A(2)	642074	6083154
Broken Cart Fire Trail	BC	15	A(2)	645045	6074317
Bogong Peaks	BO	19	A,E(2)	632000	6065000
Brindabella Road	BR	15	A,H(2)	647922	6091320
Cooleman Fire Trail	CO	15	A(2)	648017	6078012
Michalong Swamp	MI	21	A(2)	637127	6089336
Nottingham Road	NT	14	A(2)	641219	6093284
Mount Cromwell	MtC	6	E,H,G(3)	650619	6091744
Kosciusko site M4	M4	7	A,I,K(5)	627500	6065200
Wee Jasper	WJ	14	A,E,F(6)	649000	6113000
Yankee Ned	YN	21	A,E,H,J(6)	638000	6096000
Northern Brindabella Range					
California Flats	CA1	4	B(1)	660600	6100300
California Flats South	CA2	2		660550	6099850
Silvery Gully	SG	4		660950	6099900
Coree Flats	CF	59	A,C(5)	664700	6093950
Coree Hut East	CH1	10		664950	6093800
Coree Hut West	CH2	2		664400	6094750
Coree Flats South	CS	14		664700	6093650
Broken Track East	BT1	6		665650	6093100
Broken Tack West	BT2	7		665500	6093050
Middle Creek	MC1	6		664600	6093050
Middle Creek Lower	MC2	3		664250	6093500
Devils Peak Patch	DP1	19		666400	6095850
Devils Peak Seep	DP2	11		666200	6095100
Swamp Creek 1	SCa	7	A(1)	666250	6095550
Swamp Creek 2	SCb	19	A(1)	666200	6095950
Swamp Creek 3	SCc	5	A(3)	666300	6096400
Swamp Creek 4	SCd	30	A(2)	666700	6096300
Swamp Creek 3 (2006)	SC3	11	A,B(2)	666300	6096400
Southern Brindabella Range					
Ginini Flats North	GN	32	A(2)	661700	6067750
Ginini Flats East	GE	1		661300	6067500
Brumby Flats	BF	14		660700	6061500
Hanging Flat	HF	5	D(1)	662500	6061650
Blackfellows Gap	BG	14		660300	6058850
Rolling Ground Gap	RG	33	A(1)	660400	6057250
Pseudophyrne corroborre					
Snowy Mountains					
Far Dargals	FD	12	L(1)	608400	6001400
Grassy Far Dargals	GFD	4		608300	6001500
Dargals 1	DA1	1		608700	6002400
Dargals 3	DA3	6		609000	6001800
Dargals 5	DA5	4		610600	6001400
Dargals 6	DA6	2	L(1)	610600	6001800
Snakey Plain	SP	15	L(1)	611500	6004700
Upper Snakey 1	US1	2		611000	6004200
Upper Snakey 2	US2	2		611200	6004000
Jugumba Fire Trail	JUG	4		613600	6010000
Upper Jugumba	UJ	7	L(2)	612600	6012100
Ogilives Creek 3	OC3	3		620100	6013100
Upper Ogilives 6	UO6	2	L(2)	622500	6008400
Hell Hole 1	HH1	5		620000	6005000
Manjar Fire Trail 1	MN1	2	L(2)	620100	6021600
Manjar Fire Trail 2	MN2	1		620800	6022200
Undisclosed site	XX	19	L(2)		

tests. Genetic diversity was measured as mean number of alleles, observed (H_O) and expected heterozygosity (H_E) and allelic richness (R_S) using FSTAT. Allelic richness is the mean number of alleles expected within a sample of standard size and is therefore appropriate to compare between populations of varied sample size.

Significant levels of differences in allelic richness, H_E and F_{IS} between regions and populations were tested in FSTAT using 1000 permutations, and separately using a nonparametric Kruskal–Wallis test. The level of genetic differentiation among populations was quantified using pairwise F_{ST} calculated in FSTAT and the significance of pairwise comparisons tested using 1000 iterations without assuming Hardy–Weinberg equilibrium. In addition, pairwise Φ_{ST} values were estimated via AMOVA in GENALEX 6 (Peakall & Smouse 2005) and significance assessed by 1000 permutations. Patterns of genetic differentiation were examined by performing a principle components analysis (PCA) using pairwise Φ_{ST} estimates in GENALEX. The relationship between genetic and geographical distance was tested using $F_{ST}/(1 - F_{ST})$ (Rousset 1997), with the significance of the association tested using a Mantel test in GENALEX with 1000 permutations.

Bottleneck events

To detect recent or long-term genetic bottlenecks reducing effective population size, we used BOTTLENECK (Piry *et al*. 1999). This program tests whether populations exhibit 'heterozygosity excess' due to the loss of rare alleles during genetic bottleneck events (Luikart & Cornuet 1998). Analyses were performed using the mixed model option with the assumption that the loci follow a 90% stepwise-mutation model with a variance of 30%. A complication of this type of analysis is that the results may be sensitive to the mutation model employed (Luikart & Cornuet 1998). Therefore these analyses also were performed using the infinite allele model (IAM). Following the recommendations of Luikart & Cornuet (1998), populations were concluded to have been recently bottlenecked if significant results were obtained under both models, or if one was significant and the other very close to being significant, which was defined as $P < 0.080$ (Luikart & Cornuet 1998). Heterozygosity excess at a significant number of loci was tested using the one-tailed Wilcoxon signed rank test. The Bonferroni correction for multiple testing was not applied, as this correction reduces the risk of type I error but increases risk of type II (i.e. falsely rejecting that a genetic bottleneck has occurred in favour of the null hypothesis). Given the dire conservation status of the species, the potential effects of type II errors are more severe than the effects of type I errors, and that highlighting populations under potential threat is highly desirable. The mode-shift indicator (Luikart *et al*. 1998) also was used to detect any evidence of population bottlenecks, although it should be noted that this method is qualitative in that no statistical test is performed, and the majority of the sample sizes used in this study were below the recommended minimum of 30 individuals (Luikart *et al*. 1998).

Haplotype diversity of mitochondrial DNA sequences

The aligned mtND2 sequences were used to estimate genealogical relationships among haplotypes using statistical parsimony (Templeton *et al*. 1992) as implemented in TCS 1.21 (Clement *et al*. 2000). Cladograms were constructed using the 95% confidence criterion (Templeton *et al*. 1992).

Results

Microsatellite marker diversity

All four microsatellite loci used in the study were highly variable – the total number of alleles observed ranged from 13 (Pc109) to 30 (Pc72) and the average number of alleles per locus was 19. Expected heterozygosity of individual loci across all sampled populations was high and varied between 0.830 (Pc109) and 0.935 (Pc72). After correcting for multiple comparisons, significant departures from Hardy–Weinberg proportions were only found for one locus (Pc129). A significant heterozygote deficiency was observed at this locus in both southern Brindabella Range populations. However, since the locus did not deviate significantly from Hardy–Weinberg equilibrium in all other populations, it was retained for subsequent analyses.

At the scale of geographical regions, Fiery Range locations had 13 private alleles at the four common loci, the Northern Brindabellas had five and the southern corroboree frogs had three. Animals from the Southern Brindabella region did not exhibit any private alleles. The frequency of private alleles was generally low (< 0.029), although two private alleles occurred at relatively high frequency (0.392, Pc109; Fiery Ranges; 0.118, Pc72, Snowy Mountains). No significant linkage disequilibrium was discovered between any pair of loci. For four sites (WJ, YN, BG and BF) less than 10 frogs could be reliably genotyped for all four loci, so these populations were discarded from further microsatellite analysis.

Defining populations

Bayesian clustering analyses in STRUCTURE strongly supported the presence of four genetically distinct populations (Table 2; $\Pr(K = 4) = 0.97$). These genetic subdivisions corresponded exactly to the four main allopatric geographical regions in which the frogs are currently found. Individual analyses performed on the separate regions showed that

Table 2 The proportion of individuals assigned to populations according to STRUCTURE assignments ($K = 4$). Rows represent populations of origin. Columns show assigned populations

	P. corroboree		*P. pengilleyi*	
	Snowy Mountains	Fiery Ranges	North Brinds	South Brinds
Snowy Mountains	**0.957**	0.013	0.013	0.018
Fiery Ranges	0.022	**0.834**	0.068	0.076
North Brinds	0.033	0.074	**0.559**	0.334
South Brinds	0.080	0.104	0.161	**0.655**

breeding sites within these four regions could not be separated into distinct populations [for all regions $Pr(K = 1) > 0.99$].

The majority of animals were assigned to their region of origin (55.9% for Northern Brindabellas to 95.7% for Snowy Mountains), although fewer frogs from the Northern and Southern Brindabella Ranges could be assigned to separate populations than Snowy Mountains or Fiery Ranges frogs (Table 2). The majority of frogs from Snowy Mountain and Fiery range sites showed strong membership to a single population ($Q = 0.90$ for 89/90 Snowy Mountain frogs and 73/121 Fiery Range frogs), whereas comparatively few frogs from both Brindabella regions showed strong or moderate membership to a single population ($Q = 0.80$ for 70/200 Northern Brindabella sites and 28/56 Southern Brindabella sites). For the majority of individuals from the Brindabella Range, the next most probable population membership was the other Brindabella population. Combining these probabilities resulted in at least moderate assignment of the majority of animals to a 'Brindabella Range' population ($Q = 0.80$ for 168/200 Northern Brindabella sites and 44/56 Southern Brindabella sites). However, the relatively low posterior probability for $K = 3$ ($P < 0.0001$), and the result that the majority of individuals could be assigned to different genetic populations (Table 2) indicates that the Northern and Southern Brindabellas constitute distinct genetic populations.

Genetic variability

Mean expected heterozygosity (H_E) at individual breeding sites was high and ranged from 0.557 in D2 in the Snowy Mountains to 0.861 in BR in the Fiery Range (Table 3). Significant differences in H_E among the four geographical regions were detected (two-sided test $P = 0.001$; Kruskal–Wallis $P = 0.0023$), with the lowest levels detected in the Snowy Mountains populations. The mean number of alleles per locus (N_A) varied from 4.5 at the undisclosed southern corroboree frog site (XX) to 11.5 at Coree Flats (CF). Relative allelic richness corrected for differences in sample size ranged from 4.1 at XX in the Snowy Mountains to 7.9 in BR in the Fiery Range. Allelic richness did not vary significantly between individual breeding sites within geographical regions, but the allelic richness at breeding sites within the Snowy Mountains was significantly lower than at *Pseudophryne pengilleyi* breeding sites (two-sided test $P = 0.001$; Kruskal–Wallis $P = 0.0004$). The coefficient of inbreeding, F_{IS}, indicated significant heterozygote deficiency in the Fiery Ranges and Southern Brindabella geographical regions (Table 3). A two-tailed test performed in FSTAT showed F_{IS} for the Southern Brindabellas was significantly higher than for other regions. Within populations, significant heterozygote deficiency was only observed at both Southern Brindabella sites. Inspection of separate loci showed that only one locus (Pc129) had a significant heterozygote deficit at these sites.

Population differentiation

Significant genetic structure across all four geographical regions was detected using multilocus F_{ST} values ($P < 0.001$). Between geographical regions, F_{ST} values were significant and largest between the *P. pengilleyi* and *P. corroboree* populations. Values were lower between *P. pengilleyi* regions but still significantly different from zero (Table 4). Within regions F_{ST} values were low (0.007–0.038) but significant in the Fiery Ranges and the Snowy Mountains ($P < 0.001$; Table 4, along diagonal). Levels of population genetic differentiation within each geographical region were not significantly different to each other ($P = 0.972$). In pairwise population analysis, most F_{ST} comparisons between regions were highly significant and most within regions were nonsignificant (Table 5).

Global Φ_{ST} showed that 18.7% of molecular variation was partitioned among populations. Between geographical regions, Φ_{ST} values were highly significant ($P < 0.001$) and very large (Table 4). Within regions, Φ_{ST} values were low (0.008–0.066) but significant in the Fiery Ranges, Northern Brindabellas and the Snowy Mountains ($P < 0.001$; Table 4). Pairwise population comparisons showed a similar trend with highly significant ($P < 0.001$) and very large values ($\Phi_{ST} \geq 0.134$) for all pairwise comparisons between populations in different regions, but considerably smaller values between populations within regions ($\Phi_{ST} \leq 0.118$). Most

Table 3 Summary of genetic diversity within geographical regions and individual populations within regions. Only populations where 10 or more individuals were sampled and successfully genoptyped for the four loci are shown. Populations sampled, the number of individuals per population (N), average observed (H_O) and expected (H_E) heterozygosity, average number of alleles (N_A), average allelic richness, number of private alleles, average F_{IS} and indication of deviation from Hardy–Weinberg proportions (NS, nonsignificant; *significant at $\alpha = 0.05$ with sequential Bonferroni correction $P < 0.00052$). Results of bottleneck analysis are indicated (BN; a, significant genetic signature of bottleneck under Wilcoxon signed ranks test; b, evidence of mode-shift)

Population	N	H_O	H_E	N_A	Allele richness average	F_{IS}	BN
Geographical regions							
Fiery Ranges	121	0.809	0.879	17.25	14.7	0.076*	
North Brindabellas	200	0.823	0.835	14.50	11.5	0.016 NS	
South Brindabellas	56	0.602	0.802	9.75	9.5	0.234*	
Snowy Mountains	90	0.568	0.621	7.25	6.8	0.082 NS	
Individual populations							
Fiery Ranges							
BN	13	0.831	0.847	9.3	7.4	0.063 NS	
BC	15	0.773	0.775	8.8	6.7	0.039 NS	
BO	18	0.742	0.816	10.5	7.3	0.121 NS	
BR	15	0.861	0.861	10.5	7.9	0.037 NS	
CO	15	0.878	0.851	10.3	7.7	0.004 NS	
MI	21	0.849	0.848	11.0	7.5	0.024 NS	
NT	14	0.857	0.832	8.8	6.8	0.076 NS	
North Brindabellas							
CA	10	0.863	0.795	7.3	6.5	–0.026 NS	
CF	53	0.788	0.822	11.5	6.6	0.051 NS	
CH	10	0.822	0.792	7.5	6.7	0.017 NS	a
CS	11	0.793	0.792	8.0	6.8	0.052 NS	a
DP1	20	0.839	0.830	9.0	6.7	0.017 NS	
DP2	10	0.850	0.803	7.8	6.9	0.003 NS	
BT	12	0.936	0.799	8.0	6.5	–0.127 NS	
SC1	22	0.909	0.834	10.0	7.0	–0.066 NS	
SC2	32	0.764	0.790	9.5	6.4	0.050 NS	
SC3	11	0.864	0.810	7.5	6.5	–0.019 NS	
South Brindabellas							
GF	19	0.625	0.780	8.3	6.2	0.228*	a
RG	22	0.619	0.785	8.8	6.2	0.237*	
Snowy Mountains							
D2	10	0.629	0.557	5.3	4.5	–0.069 NS	
D3	10	0.600	0.559	4.8	4.3	–0.018 NS	b
D4	23	0.567	0.589	5.3	4.3	0.063 NS	ab
D6	19	0.630	0.607	5.8	4.7	–0.006 NS	a
XX	19	0.489	0.581	4.5	4.1	0.188 NS	a

Table 4 Estimates of genetic differentiation based on Φ_{ST} within (along diagonal in bold) and between (above diagonal) geographical regions. Estimates based on F_{ST} are shown below the diagonal. Values in italics are *not* significantly different from zero ($P < 0.05$)

	Fiery Ranges	North Brinds	South Brinds	Snowy Mountains
Fiery Ranges	0.029/**0.049**	0.136	0.138	0.301
North Brinds	0.076	*0.007*/**0.013**	0.108	0.323
South Brinds	0.083	0.063	*0.009*/***0.008***	0.200
Snowy Mountains	0.191	0.197	0.108	0.038/**0.066**

Φ_{ST} values were significantly different from zero. Non-significant comparisons were also recorded within regions, most notably between populations within the Northern Brindabella Ranges and within the Southern Brindabella Ranges (Table 5).

Patterns of genetic differentiation

PCA was performed using pairwise Φ_{ST} to visualize the patterns of pairwise relationship (Fig. 3). The first two dimensions of the PCA account for a total of 84% of the

Table 5 Estimates of pairwise population differentiation F_{ST} (below diagonal) and Φ_{ST} (above diagonal). Figures in italics indicate comparisons are significant at 5% level. Figures in bold italics indicate comparsions are significant at 1% level. Shading is used to distinguish populations for comparsions

	BN	BC	BO	BR	CO	MI	NT	CA	CF	CH	CS	DP1	DP2	BT	SC1	SC2	SC3	GF	RG	D2	D3	D4	D6	XX
BN		***0.088***	*0.056*	*0.029*	*0.050*	*0.041*	*0.058*	***0.134***	***0.136***	***0.124***	***0.123***	***0.137***	***0.119***	***0.135***	***0.126***	***0.178***	***0.106***	***0.138***	***0.142***	***0.350***	***0.340***	***0.322***	***0.320***	***0.315***
BC	0.047		0.019	*0.053*	***0.076***	0.024	***0.117***	***0.219***	***0.210***	***0.221***	***0.213***	***0.198***	***0.204***	***0.244***	***0.220***	***0.235***	***0.230***	***0.194***	***0.208***	***0.396***	***0.389***	***0.398***	***0.388***	***0.372***
BO	0.031	0.010		*0.047*	*0.051*	*0.035*	***0.118***	***0.142***	***0.179***	***0.147***	***0.150***	***0.162***	***0.175***	***0.191***	***0.161***	***0.208***	***0.176***	***0.173***	***0.159***	***0.349***	***0.354***	***0.345***	***0.343***	***0.351***
BR	0.015	*0.028*	0.026		0.027	*0.026*	0.052	***0.137***	***0.144***	***0.133***	***0.129***	***0.094***	***0.121***	***0.130***	***0.144***	***0.157***	***0.110***	***0.133***	***0.156***	***0.320***	***0.315***	***0.339***	***0.318***	***0.306***
CO	0.026	0.039	0.027	0.014		*0.041*	***0.093***	***0.149***	***0.154***	***0.156***	***0.136***	***0.118***	***0.145***	***0.178***	***0.140***	***0.184***	***0.149***	***0.146***	***0.154***	***0.354***	***0.347***	***0.365***	***0.336***	***0.340***
MI	0.021	*0.012*	0.018	0.013	*0.021*		***0.081***	***0.136***	***0.134***	***0.137***	***0.125***	***0.114***	***0.128***	***0.137***	***0.132***	***0.167***	***0.139***	***0.133***	***0.134***	***0.333***	***0.335***	***0.342***	***0.325***	***0.316***
NT	0.031	*0.064*	0.068	0.028	*0.050*	*0.043*		***0.191***	***0.171***	***0.180***	***0.175***	***0.136***	***0.149***	***0.195***	***0.171***	***0.204***	***0.181***	***0.184***	***0.197***	***0.382***	***0.371***	***0.398***	***0.356***	***0.354***
CA	*0.072*	0.124	0.081	*0.074*	0.079	*0.073*	0.108		0.012	0.000	0.000	0.033	0.003	0.032	0.000	0.030	0.019	***0.101***	*0.070*	***0.369***	***0.365***	***0.358***	***0.338***	***0.351***
CF	*0.076*	*0.122*	*0.104*	*0.081*	*0.086*	*0.074*	*0.097*	0.005		0.000	0.001	0.007	0.000	*0.037*	0.007	0.008	0.008	***0.130***	***0.104***	***0.338***	***0.343***	***0.342***	***0.325***	***0.331***
CH	*0.069*	*0.127*	*0.085*	*0.073*	*0.085*	*0.074*	*0.103*	0.005	0.005		0.000	0.033	0.000	0.021	0.015	0.000	0.005	***0.129***	***0.090***	***0.377***	***0.380***	***0.359***	***0.355***	***0.350***
CS	0.068	*0.124*	0.088	0.071	*0.074*	*0.068*	0.100	0.011	0.000	0.009		0.022	0.004	0.020	0.000	*0.036*	0.000	***0.131***	*0.056*	***0.387***	***0.392***	***0.387***	***0.374***	***0.360***
DP1	*0.076*	*0.112*	*0.094*	*0.050*	*0.063*	*0.061*	*0.075*	0.016	0.003	0.017	0.011		0.022	*0.039*	0.023	*0.023*	0.010	***0.103***	***0.096***	***0.339***	***0.346***	***0.352***	***0.331***	***0.325***
DP2	*0.065*	*0.115*	*0.102*	*0.065*	*0.078*	*0.069*	*0.083*	0.001	0.007	0.018	0.001	0.011		0.038	0.001	0.011	0.000	***0.148***	***0.115***	***0.394***	***0.383***	***0.388***	***0.373***	***0.375***
BT	*0.071*	*0.134*	*0.108*	*0.067*	*0.092*	*0.072*	*0.106*	0.014	0.019	0.010	0.010	0.019	0.018		*0.053*	*0.052*	0.000	***0.098***	***0.066***	***0.336***	***0.328***	***0.315***	***0.311***	***0.286***
SC1	*0.065*	*0.121*	*0.089*	*0.075*	*0.072*	*0.069*	*0.091*	0.006	0.003	0.007	0.008	0.011	0.000	0.025		*0.041*	0.011	***0.111***	***0.074***	***0.344***	***0.345***	***0.350***	***0.327***	***0.336***
SC2	*0.102*	*0.138*	*0.123*	*0.088*	*0.104*	*0.094*	*0.119*	0.015	0.004	0.004	0.019	0.011	0.005	0.027	0.020		0.029	***0.159***	***0.147***	***0.351***	***0.353***	***0.360***	***0.338***	***0.346***
SC3	*0.057*	*0.131*	*0.102*	*0.059*	*0.079*	*0.075*	*0.102*	0.009	0.004	0.002	0.008	0.005	0.003	0.002	0.005	0.015		***0.128***	***0.106***	***0.393***	***0.386***	***0.383***	***0.375***	***0.356***
GF	*0.083*	*0.121*	*0.109*	*0.079*	*0.087*	*0.078*	*0.113*	0.059	*0.075*	0.078	0.080	0.059	*0.089*	*0.056*	*0.062*	*0.095*	*0.076*		0.014	***0.159***	***0.155***	***0.175***	***0.142***	***0.138***
RG	*0.086*	*0.133*	*0.101*	*0.095*	*0.093*	*0.080*	*0.124*	0.041	*0.060*	0.055	0.033	0.056	*0.069*	*0.038*	*0.042*	*0.089*	0.064	0.008		***0.193***	***0.212***	***0.195***	***0.195***	***0.200***
D2	*0.214*	*0.247*	*0.221*	*0.189*	*0.210*	*0.199*	*0.239*	0.220	*0.209*	*0.227*	0.244	*0.203*	*0.238*	*0.185*	*0.196*	*0.217*	*0.236*	0.099	*0.125*		*0.076*	*0.080*	*0.044*	*0.096*
D3	*0.210*	*0.245*	*0.229*	*0.190*	*0.209*	*0.203*	*0.234*	*0.222*	*0.214*	*0.235*	*0.253*	*0.212*	*0.235*	*0.183*	*0.200*	*0.221*	*0.235*	*0.098*	*0.141*	0.037		***0.097***	0.037	*0.093*
D4	*0.201*	*0.257*	*0.223*	*0.212*	*0.228*	*0.212*	*0.261*	*0.226*	*0.215*	*0.225*	*0.254*	*0.221*	*0.247*	*0.185*	*0.210*	*0.229*	*0.241*	*0.110*	*0.128*	0.041	0.052		*0.038*	*0.077*
D6	*0.194*	*0.244*	*0.216*	*0.190*	*0.200*	*0.195*	*0.221*	*0.203*	*0.200*	*0.215*	*0.237*	*0.200*	*0.227*	*0.176*	*0.189*	*0.209*	*0.227*	*0.087*	*0.126*	0.021	0.018	0.019		***0.085***
XX	*0.205*	*0.247*	*0.237*	*0.195*	*0.218*	*0.200*	*0.236*	*0.231*	*0.211*	*0.230*	*0.245*	*0.208*	*0.248*	*0.173*	*0.207*	*0.224*	*0.232*	*0.090*	*0.137*	0.054	0.054	*0.044*	*0.048*	

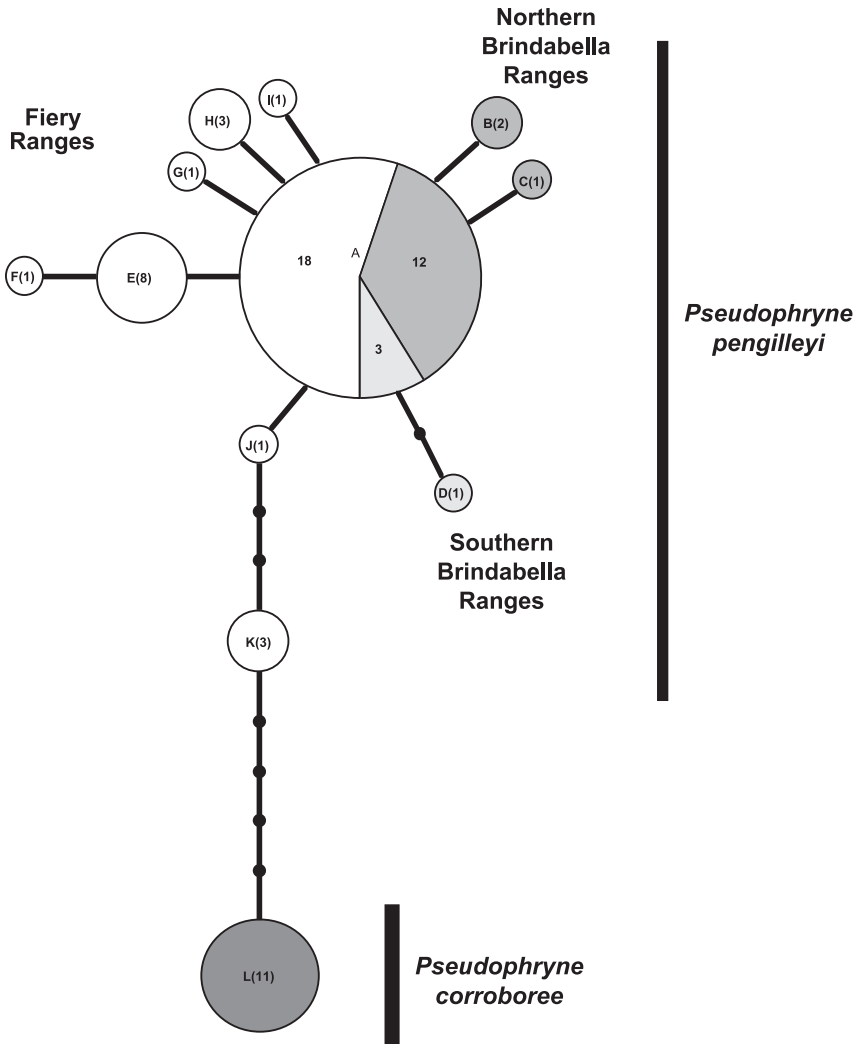

Fig. 2 Statistical parsimony network of 12 corroboree frog mtND2 haplotypes. Circles represent observed haplotypes and black dots represent mutational steps between haplotypes. Shading indicates proportion of frogs from individual geographical regions displaying each haplotype. The number of individuals sharing each haplotype is displayed in each circle.

variance, and the first three dimensions account for 89%. The four well-differentiated groups observed corresponded to the four main geographical regions.

Under drift–mutation equilibrium, a relationship between genetic differentiation and geographical distance is predicted (Rousset 1997). A highly significant relationship was found between $F_{ST}/(1 - F_{ST})$ and geographical distance among all sampled populations (Fig. 4; Mantel's $P = 0.001$) suggesting a strong isolation by distance at the level of all populations. However, investigation of isolation by distance on a finer scale within individual regions found no significant linear correlation between $F_{ST}/(1 - F_{ST})$ and either raw geographical distance or the natural logarithm of geographical distance.

Population bottlenecks

Signatures of genetic bottlenecks were detected at seven of the 24 breeding sites depending on the test used (Table 3). The Wilcoxon signed-rank test indicated bottlenecks in six populations under both the mixed model and IAM (Table 3). A further eight populations showed a significant heterozygote excess under the IAM but not under the mixed model. There was evidence of a mode-shift in two populations. Both tests were significant for one population only (D4, Snowy Mountains).

Phylogeography of mitochondrial DNA sequences

All mtND2 sequences displayed open reading frames and alignment did not require gap placement. The final alignment comprised 443 bp for 66 individual frogs from 28 breeding sites. Twelve mtND2 haplotypes were identified throughout the four geographical regions (Haplotypes A–L, Fig. 2; Table 1). Uncorrected *P* distances between sequences ranged from 0% to 2.3%. The statistical parsimony network of mtDNA haplotypes shows two distinct groups which correspond to the two species of

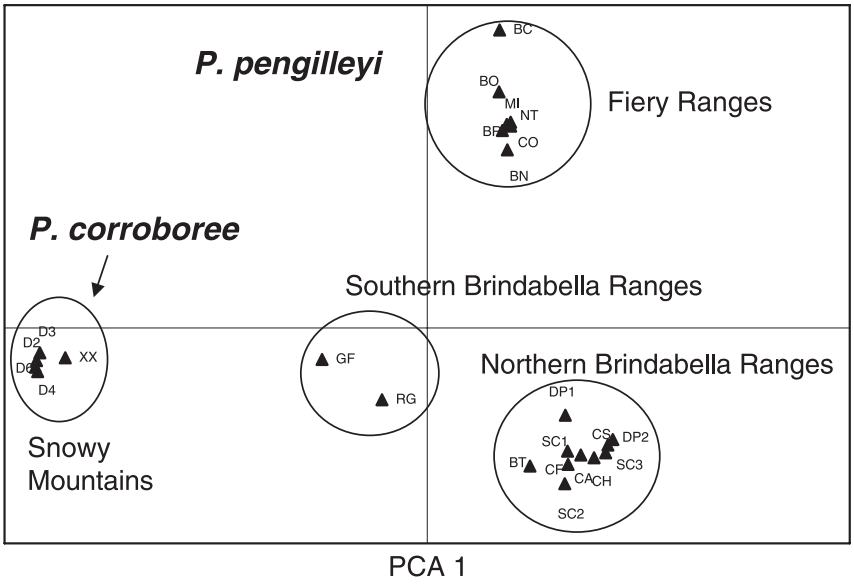

Fig. 3 Principle components analysis from pairwise Φ_{ST} esimates for four loci and 24 sampling locations. The clustering of sites into the four main geographical regions is indicated.

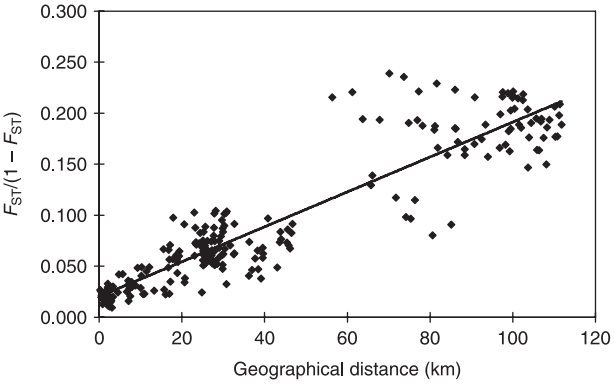

Fig. 4 Isolation by distance between populations across the four disjunct geographical regions. The solid line represents the best-fit linear regression ($P = 0.001$). $R^2 = 0.8489$.

corroboree frog; the Snowy Mountain region for *P. corroboree* and the combined northern regions for *P. pengilleyi*. The Snowy Mountain region is fixed for a single private haplotype (haplotype L), whereas the Fiery Ranges, Northern Brindabella and Southern Brindabella populations contained multiple mtND2 haplotypes. All three northern populations share a common high frequency mtND2 haplotype (A). The network shows the remaining 10 alleles are genetically similar to the common allele (separated by between one and four steps), but none of these are shared across geographical regions (Fig. 2).

Discussion

We have assembled mitochondrial and nuclear microsatellite data for the endangered corroboree frogs to complement and compare to allozyme and morphological data (Osborne & Norman 1991). Regrettably, the continued decline of these species through the 1990s has resulted in the total or imminent local extinction of frogs at four of the seven populations sampled by Osborne and Norman (Cromwell Hill, Maragale Range, Ogilive's Creek and Mount Jagungal, 1991), making direct population genetic comparisons difficult. Therefore, we assess the results of our study in the context of the overall conclusions of Osborne & Norman (1991). Our results demonstrate a high level of genetic diversity in both species. Significant genetic structuring was evident on a large scale (10 s of kilometres) and much of this structure is associated with four geographically disjunct populations. Within sampled sites and geographical regions, allelic richness and heterozygosity were similar to, or greater than, those reported for a wide variety of amphibian species. This is inconsistent with the small extant population size, and instead reflects the recent and rapid population decline with insufficient time for genetic drift to erase the signature of a large population size. Potential fragmentation and population isolation associated with the dramatic population declines observed in both species have not yet had a significant negative genetic impact, which may not be surprising given the decline in these species began so recently (Osborne *et al*. 1999).

We recognize that our inferences from the nuclear genome are based on data from relatively few microsatellite markers. Recent population genetics studies in other amphibians have been based on between 4 and 11 microsatellite loci, and/or a single mitochondrial locus (Newman & Squire 2001; Monsen & Blouin 2003; Burns *et al*. 2004; Palo *et al*. 2004; Funk *et al*. 2005; Kraaijeveld-Smit *et al*. 2005; Spear *et al*. 2005; Johansson *et al*. 2006; Pabijan & Babik 2006; Johansson *et al*. 2007; Zamudio & Wieczorek 2007).

While our data set is at the lower end of this scale, the concordance between our nuclear and mitochondrial data, and the broad agreement of those data with published allozyme data (Osborne & Norman 1991), justify our biologically reasonable conclusions and the inferences concerning the patterns of genetic diversity and conservation recommendations arising from them.

Here, we discuss how both the mtDNA and microsatellite data contribute to our understanding of overall genetic structure in corroboree frogs, genetic diversity within major groups, and the implications of these data for future conservation efforts in these two iconic Australian frogs.

Should P. corroboree *and* P. pengilleyi *be managed as separate species?*

These data support the continued recognition of *P. pengilleyi* as a distinct species to *P. corroboree*. It is worth noting that the sequence divergence between these species is considerably lower than that found at the mtND2 locus between closely related Myobatrachid species (e.g. Read *et al*. 2001; Morgan *et al*. 2007) or even within Myobatrachid species with wider distributions (Edwards 2007; Edwards *et al*. 2007). However, our data support the widely held view that species determination should not solely be based on evidence from a single locus (Avise 2000). While the isolation-by-distance result supports the hypothesis of Woodruff (1975) that the northern and southern corroboree frogs represent variation at extreme ends of a latitudinal cline, high levels of genetic differentiation and the presence of a divergent fixed diagnostic mitochondrial haplotype in *P. corroboree* suggest long-term genetic isolation of these populations. Additionally, in contrast to the findings of Osborne & Norman (1991), a high-frequency private allele was observed in *P. corroboree* at one locus (Pc72). In combination with hybridization experiments (Osborne & Norman 1991), morphology (Pengilley 1966; Woodruff 1975; Osborne *et al*. 1996), call structure (Osborne *et al*. 1996) and genetic data including allozymes and immunological distance (Roberts & Maxson 1989; Daly *et al*. 1990; Osborne & Norman 1991), this provides strong evidence for the recognition of two species.

Does significant genetic structure exist between geographical regions that should be taken into account in any conservation actions?

Multiple lines of evidence suggest that the four disjunct geographical regions making up the distribution of these frogs comprise distinct genetic populations (Figs 2–4, Tables 2–5). In particular, there was high concordance between the clustering of individual populations and geographical location. Levels of population differentiation between geographical regions were highly significant ($P < 0.001$) further implying low gene flow between these regions. Our genetic data clearly identified four evolutionarily significant units for conservation (Moritz 1994, 1995; Moritz & Faith 1998) that should be maintained as unique entities if possible.

The three disjunct *P. pengilleyi* regions correspond to genetic populations, although STRUCTURE was unable to distinguish strongly between the northern and southern Brindabella Range populations. No frogs were found between these geographical regions during the sampling period, although historical records show the existence of corroboree frogs at sites in this area. This implies that gene flow may have been possible in the recent past and could explain the STRUCTURE result. Osborne & Norman (1991) considered these a single geographical region connected by frogs in breeding habitat despite finding relatively high genetic distances between them. Osborne & Norman (1991) observed significant differences in allele frequencies at a single allozyme locus between the northern and southern Brindabella Range populations, and excluded the locus on those grounds. Both the mitochondrial and microsatellite data suggest that these regions are indeed genetically differentiated and this action was possibly not necessary. The existence of the common mitochondrial haplotype (haplotype A) in all three regions suggests a recent common origin for frog populations in these regions, and the observation that the remaining 10 haplotypes are unique to geographical regions while some are shared by populations within regions, implies highly restricted gene flow between regions.

Are individual breeding sites a suitable unit for conservation?

Levels of within-region genetic differentiation were not significantly different between geographical regions ($P = 0.972$) implying similar population structure and levels of gene flow within each region. In common with Osborne & Norman (1991), we found levels of differentiation within regions were generally low, suggesting that management of wild populations need not focus on individual breeding sites as discrete populations, although some pairwise comparisons were found to be significant. The sharing of five mitochondrial haplotypes A, B, E, H, and L across populations within geographical regions (Table 1) implies that current or historical gene flow within regions was common. Given the recency of the rapid declines experienced by these species, it is unclear whether the apparently low differentiation of populations within geographical regions over relatively large scales represents high levels of current gene flow or is a remnant of historical gene flow. The low densities of frogs at occupied sites and the current absence of frogs from potential breeding habitat

throughout the historical range of both species may be contributing to increased fragmentation and isolation of populations. However there may be a substantial time lag between the onset of population isolation and resulting negative genetic and fitness effects (Hitchings & Beebee 1998). The inability of STRUCTURE to find substructure within any geographical region when F_{ST} and Φ_{ST} were able to detect structure suggests that allele frequency differences between predefined populations are subtle (Pritchard *et al*. 2000). This is consistent with the theory that recent population declines and local extinctions have severely restricted current gene flow in this species. The absence of significant isolation by distance between sites within geographical regions suggests either that gene flow has been sufficiently large to confound genetic differentiation or that dispersal does not predominantly occur between geographically proximate breeding assemblages (Burns *et al*. 2004). Ecological observations suggest that a high occurrence of long-range dispersal in these species is unlikely. It is possible that other landscape effects such as elevation or connectivity through breeding or nonbreeding habitat may be a better predictor of gene flow than raw or ln-transformed geographical distances in these species (e.g. Funk *et al*. 2005; Spear *et al*. 2005), but these hypotheses remain to be tested.

Do patterns of genetic diversity and genetic structure reflect potentially important losses of genetic variation in either species, and how can this be managed?

Our microsatellite data show that the level of genetic diversity observed over all populations is high (mean $NA = 19$, $H_E = 0.885$) for the four polymorphic loci amplified in both *P. pengilleyi* and *P. corroboree*. The level of genetic diversity observed in *P. pengilleyi* is significantly higher than in *P. corroboree* (allelic richness = 6.9 and 4.4, respectively, $H_E = 0.840–0.607$, respectively) but it is important to note that the genetic diversity in both species was equivalent to or greater than those reported for other amphibian species, including those under significant threat of extinction (e.g. Scribner *et al*. 1994; Rowe *et al*. 1998; Shaffer *et al*. 2000; Newman & Squire 2001; Burns *et al*. 2004; Kraaijeveld-Smit *et al*. 2005). Within each species, estimates of genetic diversity were not significantly different among sites, indicating that the effects of fragmentation and population declines are not associated with local or regional causes, but have occurred across the geographical ranges of these frogs with similar magnitude.

In common with Osborne & Norman (1991), we found that *P. corroboree* contains significantly lower genetic diversity than the *P. pengilleyi* populations. Census data collected over the last three decades show that population declines have been severe in *P. corroboree* (Hunter *et al*. 2006), and the signature of a genetic bottleneck was observed in four of the five populations analysed (Table 3). The geographical isolation of undisclosed site XX has been proposed as a reason for the high density of frogs occupying the site. However, the observation of very low genetic diversity, high inbreeding coefficient ($F_{IS} = 0.188$), and evidence of a recent genetic bottleneck suggests that this population may be inbreeding. In light of these results, the low genetic diversity is consistent with a recent history of decline, fragmentation and isolation coupled with genetic drift (e.g. Luikart & Cornuet 1998; Luikart *et al*. 1998). While the low genetic diversity in *P. corroboree* could be a result of ascertainment bias in the microsatellite loci used here (Ellegren *et al*. 1995; Hutter *et al*. 1998; Chambers & MacAvoy 2000), this seems unlikely because reduced genetic diversity also was observed in mitochondrial sequence, and our results are in agreement with the previous allozyme study (Osborne & Norman 1991).

Although low genetic diversity in peripheral populations of amphibians may be due to historical factors (e.g. Hoffman & Blouin 2004), the well-documented declines, evidence of genetic bottlenecks and high inbreeding coefficients observed in some populations imply that low genetic diversity is due to current population fragmentation and isolation. In such circumstances it is usual to recommend the preservation of habitat to allow connections between populations (e.g. Burns *et al*. 2004). However in this case, the range of *P. corroboree* is contained within the Jagungal Wilderness Area within Kosciusko National Park, and habitat connectivity between populations has not been identified as a major cause of concern (Osborne *et al*. 1999; Hunter *et al*. 2006). It is possible that fragmentation of these populations has been established through local extinctions between currently occupied sites leading to distances that frogs are unable to disperse across. This seems likely since recent surveys suggest that *P. corroboree* has become locally extinct from over 85% of areas where it was historically known to occur (Hunter 2007).

An obvious potential remedy to this situation would be the translocation of individuals between populations; however, this is a controversial strategy (e.g. Dodd & Seigel 1991; Rowe *et al*. 1998; Seigel & Dodd 2002) and the genetic and ecological consequences are difficult to predict (e.g. Ficetola & De Bernardi 2005). A similar but lower-risk strategy would be to establish the captive breeding strategy in a way that crosses frogs from across the range of *P. corroboree* in order to mitigate the effects of inbreeding and assess the effects on fitness traits such as larval mortality or metamorph success (Kraaijeveld-Smit *et al*. 2006). A possible criticism of this strategy is that this will effectively homogenize the genome (Woodworth *et al*. 2002), potentially destroying co-adapted complexes and reducing the frogs' ability to adapt to novel environmental challenges (e.g. Reed & Frankham 2003). A possible remedy would be to group captive animals into two (or more) populations

and maintain them as separate 'fragmented' populations. Occasional genetic exchange between the two could then be used as another mechanism to avoid inbreeding (Woodworth *et al*. 2002). The current prognosis for *P. corroboree* is so dire (Hunter *et al*. 2006) that this may provide the only strategy to keep this species from extinction.

Of the *P. pengilleyi* populations, the Southern Brindabella population shows the lowest heterozygosity and allele richness and appears to be most at risk from negative genetic effects. Most alarmingly, both Southern Brindabella sites also showed highly significant levels of heterozygote deficiency and one site (GF) showed evidence of a genetic bottleneck signature (Table 3). As with other populations in this study, GF and RG represent composite populations of frogs pooled across continuous breeding habitat, and hence significant F_{IS} values could be explained by population substructure at these sites not investigated in this study due to low sample sizes. Another cause of departure from Hardy–Weinberg equilibrium is the presence of null alleles (Chambers & MacAvoy 2000). Null alleles in this population would be expected to cause individual loci to deviate from Hardy–Weinberg equilibrium but not affect others, whereas a loss of genetic diversity due to inbreeding might be expected to affect all loci to the same extent. Data inspection revealed that only one locus (Pc129) deviated significantly from Hardy–Weinberg equilibrium, although two further loci (Pc109 and Pc72) showed evidence of homozygote excess. Although the presence of null alleles at this locus may be a confounding factor, the low genetic diversity at other loci, lack of private alleles and evidence of a genetic bottleneck in one population provide evidence that the Southern Brindabellas comprise a genetically depauperate population. Under this interpretation, low genetic diversity could be explained either by restricted gene flow, isolation and inbreeding similar to a genetic bottleneck, or it could be the result of a relatively recent founder event. The finding of a unique ND2 mitochondrial haplotype in addition to the common *P. pengilleyi* haplotype implies that frogs within the Southern Brindabellas have been isolated for some period of time, and this interpretation is strengthened by the existence of an isolation-by-distance pattern across the two species (Fig. 4). In combination with one of the two sites showing evidence of a genetic bottleneck, we therefore favour the bottleneck hypothesis, partly in the face of the genetic evidence and partly due to the conservative nature of this interpretation. Given this interpretation, supplementation, where conspecific animals from another population are added to an existing population, is a possible management action to increase genetic diversity at these sites (Storfer 1999; Ficetola & De Bernardi 2005). However, this can have negative genetic effects because it leads to a reduction in the genetic differences between populations. Furthermore, outbreeding depression could result in reduced fitness of these populations: frogs in the Southern Brindabellas breed at higher altitude than other northern corroboree frogs and could represent adaptive genetic diversity that would be compromised by outbreeding. A captive breeding strategy incorporating outcrossing for South Brindabella frogs might be the only way to maintain genetic diversity and prevent these populations from extinction, but careful monitoring of fitness effects is vital.

Conclusion

We have assembled mitochondrial and nuclear microsatellite data for the endangered corroboree frogs to complement and compare to allozyme and morphological data (Osborne & Norman 1991). Our results are in broad agreement with the major findings of the previous study, although we find significant levels of differentiation within the Brindabella Ranges and clearer genetic differences between the Northern and Southern corroboree frogs. We found a high level of genetic diversity in both species and significant genetic structure is associated with four geographically disjunct populations. These geographical regions represent four evolutionarily significant units for conservation (Moritz 1994, 1995; Moritz & Faith 1998) that where possible should be maintained as unique entities in further conservation efforts.

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