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Population genetic analysis reveals a long-term decline of a threatened endemic Australian marsupial

BIRGITA D. HANSEN,* DANIEL K. P. HARLEY,† DAVID B. LINDENMAYER‡ and ANDREA C. TAYLOR*

*Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Clayton, Vic. 3800, Australia, †Wildlife Ecology Research Group, School of Biological Sciences, Monash University, Clayton, Vic. 3800, Australia, ‡Fenner School of Environment and Society, The Australian National University, Canberra, ACT 0200, Australia

Abstract

Since European colonization, Leadbeater's possum (*Gymnobelideus leadbeateri*) has declined across its range to the point where it is now only patchily distributed within the montane ash forests of the Central Highlands of Victoria. The loss of large hollow-bearing trees coupled with inadequate recruitment of mature ash forest has been predicted to result in a reduction in population size of up to 90% by 2020. Furthermore, bioclimatic analyses have suggested additional reductions in the species' distribution under a variety of climate change scenarios. Using a panel of 15 highly resolving microsatellite markers and mitochondrial control region sequence data, we infer past and present gene flow. Populations in the northern part of the core range were highly admixed, and showed no signs of either current or historical barriers to gene flow. A marginal, isolated and inbred population at Yellingbo was highly genetically differentiated, both in terms of current and historic genetic structure. Sequence data confirmed the conclusions from earlier genetic simulation studies that the Yellingbo population has been isolated from the rest of the species range since before European-induced changes to the montane landscape, and formed part of a larger genetic unit that is now otherwise extinct. Historic loss of maternal lineages in the Central Highlands of Victoria was detected despite signals of immigration, indicating population declines that most probably coincided with changes in climate at the end of the Pleistocene. Given ongoing habitat loss and the recent (February 2009) wildfire in the Central Highlands, we forecast (potentially extensive) demographic declines, in line with predicted range reductions under climate change scenarios.

Keywords: climate change, conserved sequence blocks, control region, wildfire

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Introduction

The most significant and widespread cause of species decline is the loss of habitat (Gallant *et al.* 2007; Eigenbrod *et al.* 2008). Such loss is exacerbated by fragmentation of larger areas of habitat into small patches, and the subsequent degradation of these remnants (Rankmore & Price 2004; Lindenmayer & Fischer 2006; Banks *et al.* 2007). The situation is predicted to worsen with imminent changes to climate resulting from global warming (Lindenmayer 2000; Mitrovski *et al.* 2007). Furthermore, many forest-dwelling species have experienced dramatic declines across their range as a result of human disturbance (Kerr & Burkey 2002; Rankmore & Price 2004; Goossens *et al.* 2006). One of these is Leadbeater's possum, *Gymnobelideus leadbeateri*, a small (120 g) arboreal petaurid marsupial endemic and restricted to Victoria, Australia, and patchily distributed within an 80×60 km region of montane forest within the Central Highlands (Fig. 1). Leadbeater's possum is listed as endangered under both international (IUCN 2009) and Australian Federal Government legislation (*Environment*

Correspondence: Birgita D. Hansen, Fax: +61 3 99055613; E-mail: birgita.hansen@sci.monash.edu.au

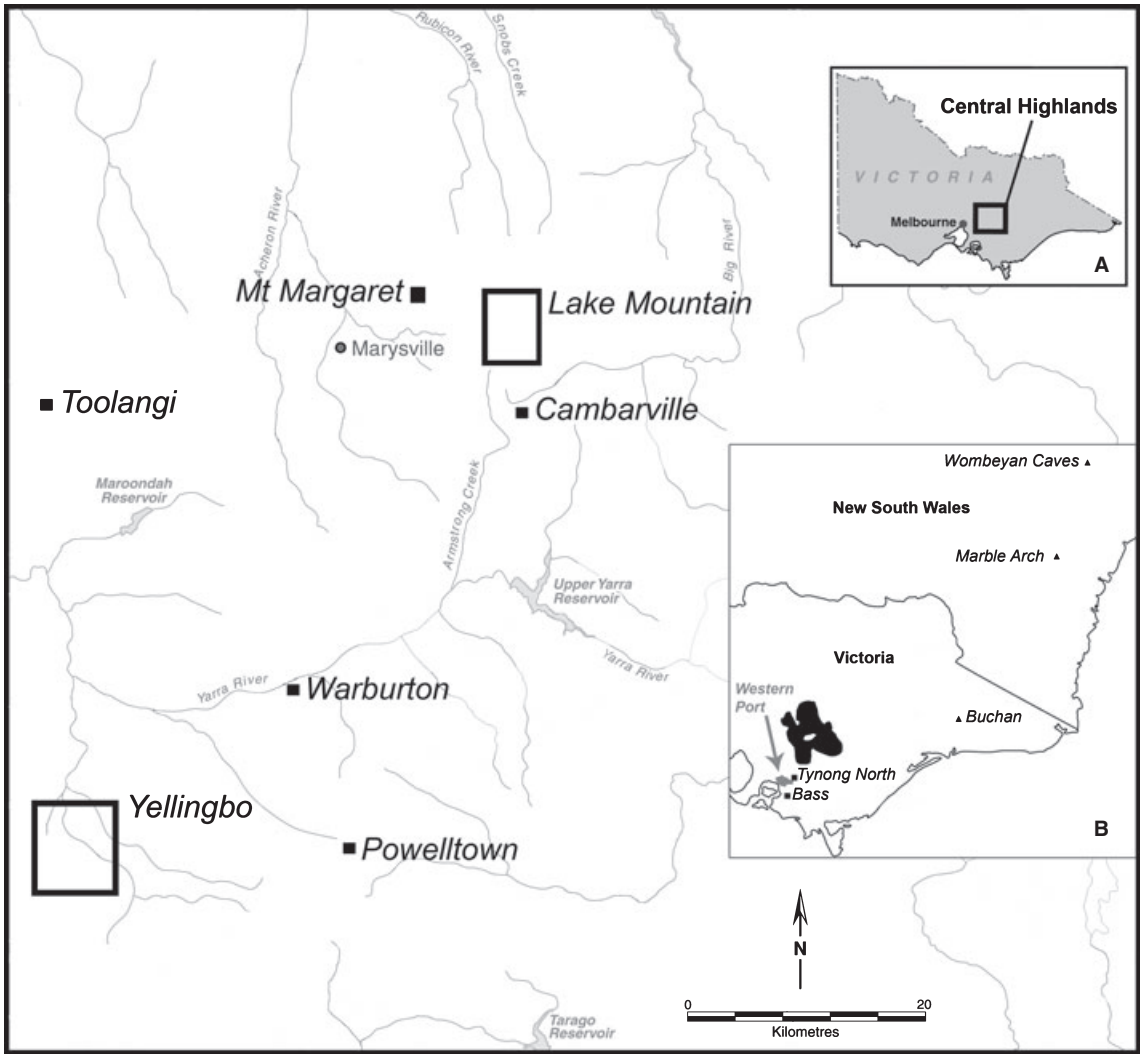

Fig. 1 Map of the Central Highlands of Victoria showing sampling locations. Filled squares depict small populations and open boxes depict large populations. Inset A shows the location (within Victoria) of the study area in the Central Highlands. Inset B shows southeast Australia, the location of the core range of Leadbeater's possum (black shading) and the locations of extinct populations (filled squares) and subfossil remains (filled triangles). The approximate location of the Western Port swamp is indicated with grey shading. Map modified from Clive Hilliker (A.N.U.) with permission.

Protection and Biodiversity Conservation Act 2009: Department of the Environment, Water, Heritage and the Arts).

Leadbeater's possum was first discovered near Bass River in the Western Port region of Victoria in 1867 (Fig. 1). Four specimens were collected from the area around the turn of the century (Fleay 1933; Brazenor 1946, 1962). After 50 years without a sighting, the species was declared 'certainly, or almost certainly extinct' (Calaby 1960). It was re-discovered in 1961 near Marysville in the Central Highlands of Victoria (Wilkinson 1961). Since that time virtually all records of its presence have been made within this region (Smith *et al.* 1985; Lindenmayer *et al.* 1989).

In the core of its range, Leadbeater's possum inhabits montane ash forest with large hollow-bearing Mountain Ash *Eucalyptus regnans* (typically over 200 years old) and a thick middlestorey of *Acacia*, an important food resource (Smith 1984; Lindenmayer *et al.* 1991a; Lindenmayer 2000). The majority of suitable and/or occupied habitat coincides with timber and pulpwood production activities, as the bioclimatic conditions that are suitable for Leadbeater's possum also promote good growth rates for eucalypts (Lindenmayer 2000). Clearfelling operations have a rotation time of 50–80 years, which is insufficient time for the development of hollows in Mountain Ash trees (Ambrose 1982; Lindenmayer *et al.* 1993). Hence, current conservation efforts for Leadbeater's

possum involve protection of remaining old-growth stands, and maintenance of younger stands that are allowed to attain hollow-bearing age (Macfarlane *et al.* 1998). Multi-aged and old growth stands are comparatively rare within the Central Highlands of Victoria, and hence, the species faces a habitat crisis as mature trees are lost and not replaced (Lindenmayer *et al.* 1997).

During the past 30 years, two populations have been discovered that do not occur in this typical ash-type habitat. The first was discovered in 1986 at Yellingbo Nature Conservation Reserve (Smales 1994), only 17 km from the nearest record of the species in montane ash (Harley 2004) (see Fig. 1). The habitat utilized by the species at Yellingbo is lowland swamp dominated by Mountain Swamp Gum *Eucalyptus camphora* with *Melaleuca* and *Leptospermum* species in the middlestorey (Harley *et al.* 2005). Previous work on this population has identified it as being highly genetically distinct from the rest of the extant species, and having undergone a bottleneck from an unsampled source (Hansen & Taylor 2008). While microsatellite analysis was able to determine that Yellingbo is not a recent remnant from the Central Highlands, coalescent analyses using mitochondrial markers will be more informative in determining the timing and nature of its differentiation. The long-term viability of this population is questionable due to its isolation and signal of inbreeding as a function of the population's small size (<100 individuals) (Hansen 2008). Furthermore, the stochastic extinction risk posed by wildfire is high due to the small and isolated nature of the reserve.

The second atypical population was discovered in 1993 in subalpine woodland at the popular cross-country ski resort of Lake Mountain, 80 km northeast of Melbourne (Jelinek *et al.* 1995) (see Fig. 1) and ~10 km north of Cambarville. Colonies occur across a plateau supporting subalpine woodland dominated by Snow Gum *Eucalyptus pauciflora*, with *Leptospermum grandifolium* and *Nothofagus cunninghamii* thickets occurring along drainage lines (Harley 2007). The habitat utilized by Leadbeater's possum at both sites differs to montane ash forest in lacking large hollow-bearing ash trees and dense stands of *Acacia*.

For species that are threatened and/or declining, extinction risk is expected to increase with loss of genetic diversity (Frankham 2005). For highly range-restricted species such as Leadbeater's possum, which are subject to ongoing threatening processes like habitat destruction, loss of genetic diversity may severely compromise population resilience. Population viability analyses (Lindenmayer & Lacy 1995; Lindenmayer & Possingham 1996) have identified two key knowledge gaps that currently restrict our ability to assess accurately extinction risk for Leadbeater's possum: (i) its ability to recolonize logged and regenerated forest and (ii) its dispersal capability. Although radio-tracking and recapture data have recently been collected to investigate dispersal of the species in lowland swamp forest (Harley 2005), dispersal information from the Central Highlands of Victoria is still lacking. Thus, at a broader spatial scale, this study aims to quantify population genetic variation using a panel of highly resolving microsatellite markers to infer gene flow between extant populations. We also use mitochondrial control region sequence data to infer historic gene flow and past population structure. Using this information, we identify populations of concern or requiring special management, and past population dynamics that may influence future extinction risk.

Methods

Collection and preparation of genetic material

Leadbeater's possum blood and ear biopsy samples were collected from six extant wild populations (Cambarville $N = 7$, Lake Mountain $N = 162$, Mt Margaret $N = 3$, Toolangi $N = 2$, Powelltown $N = 6$ and Yellingbo $N = 198$) as detailed in Hansen & Taylor (2008). Juveniles were distinguished from adults by the presence of long fluffy rump fur and by weight, with juveniles typically weighing up to 100 g, subadults 100–120 g and adults over 120 g (Harley and Lill 2007). This species is extremely difficult to capture in the wild and animals do not readily enter traps (Lindenmayer 1996). Therefore, while highly desirable, additional samples from under-represented localities (for example, Powelltown) and unsampled populations that are known to lie between Cambarville and Powelltown were not obtainable.

In addition to the field sampling, hair and/or tissue samples were obtained from seven Leadbeater's possum specimens held in the collection of the Museum of Victoria. These include the two Bass Valley type specimens C4380 and C4379 (Wilkinson 1961; Brazenor 1962) (Fig. 1), which were provided as plucked hair samples, and two other historic specimens C4378 (Tynong North, north of Bass) and C1965 (Bass Valley) (Wilkinson 1961), for which hair and a tissue sample from the footpad were obtained. The other three museum specimens, which were provided as plucked hair samples, were collected from extant populations after 1961 (Warburton C4321, Marysville/Cambarville C8175 and Yellingbo C28009). Samples were extracted and polymerase chain reaction (PCR) amplified in a UV-irradiated laminar flow hood (cleaned after each use with sodium hypochlorite) to reduce the risk of DNA contamination.

Whole genomic DNA was extracted from tissue samples following the salting out protocol in Sunnucks & Hales (1996), and from blood samples using a DNeasy™ Tissue Kit (Qiagen) according to the manufacturer's protocol. Extractions from multiple (usually a small tuft) plucked hair samples followed the protocol of Larwill *et al.* (2003).

Microsatellite genotyping

All field-collected samples were genotyped for 15 polymorphic petaurid microsatellite markers, as detailed in Hansen & Taylor (2008). PCR amplification of museum footpad DNA was trialled using two methods. The first was the multiplex method of Piggott *et al.* (2004), with the addition of 1 M betaine in each PCR reaction mixture. The second method was single 20 μL PCR reactions using 8 μL template DNA and 75 mM Tris–HCl, 20 mM ($(NH_4)_2SO_4$), 0.01% Tween 20, 2.5 mM $MgCl_2$, 0.2 mM each of dTTP, dCTP and dGTP, and 0.02 mM of dATP, 1 M betaine, 0.5% bovine serum albumin (BSA; Panvera), 0.5 U *Taq* DNA polymerase (MBI Fermentas) and 0.05 μL [α^{33}P]-dATP (10 mCi/mL; PerkinElmer). Cycling conditions followed those outlined in Piggott *et al.* (2004), with the annealing temperature the same for each locus.

Extracts from all museum specimens amplified sporadically at only seven loci (GL7, GL13, GL24, GL26, GL28, 5A and GL44). Locus GL5A was re-designed to produce a smaller fragment size. The new primers (5′–3′ forward: TGT ATC CTC TTC CCC CAG TAA C; reverse: AGA GTT CTC TCA TCC ACA AGA GG) produced a 158–178 bp fragment, which proved more suitable for amplification of museum extracts, suggesting the DNA was degraded. Genotyping of museum specimens was performed using a minimum of eight replicate PCRs per sample per locus.

Mitochondrial single-stranded conformation polymorphism and sequencing

Mitochondrial DNA amplification was undertaken using the universal marsupial control region (D-Loop) primers L16517M and H605M (Fumagalli *et al.* 1997). For the purpose of distinguishing haplotypes via single-stranded conformation polymorphism (SSCP), 10 pmol of each primer was used in a 10 μL PCR reaction containing 75 mM Tris–HCl, 20 mM ($(NH_4)_2SO_4$), 0.01% Tween 20, 2.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.1% BSA, 0.5 U *Taq* DNA polymerase and 0.05 μL [α^{33}P]-dATP (10 mCi/mL). Cycling conditions follow those of Fumagalli *et al.* (1997). PCR products were combined with 10 μL of formamide loading buffer and analysed using SSCP gel electrophoresis (Sunnucks *et al.* 2000). Haplotypes were scored using a combination of SSCP and commercial sequencing on an ABI sequencer (Macrogen). For uncommon haplotypes, all samples representing each SSCP banding pattern were sequenced.

The extracts from museum specimens failed to amplify using the standard marsupial mitochondrial primers. PCR amplification and sequencing were achieved by designing five new primers (two pairs and one for use in conjunction with H605M) from previously obtained Leadbeater's possum sequence to amplify the same region in smaller fragments (Appendix I). The PCR volume was increased to 20 μL containing 10 pmol of each primer, 0.5% BSA and 1 U *Taq* polymerase. All other reagent concentrations remained unchanged.

Microsatellite DNA analysis

The four largest samples (Yellingbo, Lake Mountain, Cambarville and Powelltown) were used in population genetic analyses. In addition to a suite of standard diversity indices (observed and expected heterozygosity and allelic diversity; Hansen & Taylor 2008), allelic richness, which effectively standardizes allelic diversity by the smallest sample size, was calculated by rarefaction in FSTAT 2.9.3 (Goudet 1995). FSTAT provides statistical tests for comparisons of gene diversity and allelic richness among groups of populations (requiring at least two groups per population or sample). To quantify the differences between Yellingbo and Lake Mountain, 10 and four groupings, respectively (chosen arbitrarily to approximately represent the numbers of clusters identified in Structure; see below), were tested for significance against 10 000 random permutations. For comparisons with/between Cambarville and Powelltown, a Wilcoxon test for matched pairs was used.

Pairwise population F_{ST}-values were calculated in Arlequin 3.11 (Excoffier *et al.* 2005) and tested against a null distribution obtained by 50 000 permutations of genotypes between populations. A principle coordinates analysis (PCA) using a standardized covariance distance matrix was performed in GENALEX 6 (Peakall & Smouse 2006). GENALEX was also used to perform Mantel tests on pairwise F_{ST} and geographic distance matrices for tests of isolation by distance. Genotypes from all extant populations were analysed in Structure 2.0 (Pritchard *et al.* 2000) as described in Hansen & Taylor (2008).

Mitochondrial DNA analysis

DNA sequences were aligned in BIOEDIT (Hall 1999) and screened for variable and parsimoniously informative sites in MEGA4 (Tamura *et al.* 2007). Identification of the

mammalian conserved sequence blocks (CSB) II and III (Janke *et al.* 1994) in the possum sequences aided alignment and provided assurance that sequences represented mitochondrial control region and not pseudogenes (nuclear homologues). Haplotype networks using all sequences were constructed in Network 4.5.0.0 (Bandelt *et al.* 1999). MEGA4 was also used to construct a phylogenetic neighbour-joining tree with 1000 bootstrap replicates to test for support of any clades identified in the network. Pairwise differences between sequences were used to compute molecular diversity, analysis of molecular variance and population pairwise F_{ST}-values in Arlequin. Congruence in patterns of population differentiation inferred separately from mitochondrial and microsatellite data was assessed by Mantel testing (in GENALEX) of pairwise population F_{ST}-matrices derived from each. We also performed a PCA on variable sites only in GENALEX using a standardized covariance distance matrix.

Equilibrium and neutrality tests of sudden population expansion were performed in Arlequin for the two largest populations, Lake Mountain and Yellingbo. The observed mismatch distribution provides an estimate of θ_0 and θ_1 (initial population size N_0 and population size after expansion N_1, scaled by mutation rate), and τ, the time since population expansion. This was tested against the expected mismatch distribution using the sums of squared deviations (SSD) between the observed and expected, divided by the number of parametric bootstrap replicate simulations (in this case 10 000). Tajima's *D*-statistic and Fu's F_S-test the null hypothesis of selective neutrality and population equilibrium, which is rejected at $P < 0.05$. Fu and Li's *D**- and *F**-test statistics were computed in DnaSP version 4 (Rozas *et al.* 2003) and were undertaken as additional (and complementary) tests to Fu's F_S and Tajima's *D*, to distinguish between population dynamic effects and potential selection.

We then used a maximum-likelihood estimate of the parameters of population growth (g) and population size scaled by mutation rate (θ) in FLUCTUATE (Kuhner *et al.* 1995) to infer past population dynamics. Six replicate runs of 10 short chains and five long chains (10 000 and 100 000 steps respectively) were done for Lake Mountain. Too few variable sites and a star-like phylogeny (see Network results) precluded estimation of θ and g for Yellingbo or Bass, even when they were combined into a single population or when only the growth parameter g was allowed to vary as recommended when haplotypes represent a star-like phylogeny (Kuhner *et al.* 1995).

FLUCTUATE is intended to test for population growth or decline in the absence of selection, migration and recombination. The last was not relevant here as only mitochondrial control region sequence data were used for tests. The first was covered by the previously described tests of neutrality. However, it is possible that signals of population changes may be a result of ongoing directional migration, and this was tested for separately using LAMARC (Kuhner 2006). The maximum-likelihood method in LAMARC version 2.0 was used to estimate theta and relative rates of migration between Lake Mountain and its nearest neighbour Cambarville, and between pooled (on the basis of microsatellite patterns—see Results) northern Central Highlands samples from Lake Mountain/Cambarville/Mt Margaret and the southern population of Powelltown. Five replicate runs of 10 short chains and two long chains (10 000 and 200 000 steps respectively) were performed for the northern and southern Central Highlands groupings. Simultaneous estimation of growth and migration was not possible due to the small sample size of comparative groupings (Cambarville and the southern Central Highlands grouping).

Effective population size estimation

Effective population sizes (N_e) were estimated using both marker sets. A point estimation method based on linkage disequilibrium in microsatellite data was computed in NEESTIMATOR 1.3 (Peel *et al.* 2004) using genotypes from all individuals at Lake Mountain and Yellingbo. Relative N_e was estimated from mitochondrial DNA (mtDNA) sequence data using the mean number of pairwise differences method to compute theta (pi) in Arlequin. The relative differences in N_e between each population using the two data sets were used in combination with estimates of θ and g to assess recent changes in population size in the context of current predicted species-wide declines.

Results

Microsatellite DNA analyses

Allelic diversity, observed and expected heterozygosity and allelic richness were significantly lower at Yellingbo than at Lake Mountain (all $P < 0.002$) (Table 1). Yellingbo also had significantly lower gene diversity and allelic richness than Cambarville ($P < 0.02$). Gene diversity and allelic richness were also lower at Yellingbo than at Powelltown, but the difference was not significant, possibly due to the small sample from the latter.

The number of genetic clusters inferred by Structure was interpretable in several different ways. The first is after the method of Evanno *et al.* (2005), who use the second-order rate of change of the ln $P(X \mid K)$, given by

Table 1 Measures of genetic diversity for the four largest extant populations of Leadbeater's possum *Gymnobelideus leadbeateri*, based upon analyses of 15 microsatellite loci

Population	n	H_E	H_O	A	AR
Powelltown	4	0.65	0.83	2.9	2.39
Cambarville	7	0.71	0.71	4.9	2.69
Lake Mountain	162	0.79	0.74	11.2	3.00
Yellingbo	198	0.55	0.53	3.4	2.12

n is total number of individuals sampled. H_E and H_O are expected and observed heterozygosity, A is average number of alleles per locus and AR is allelic richness.

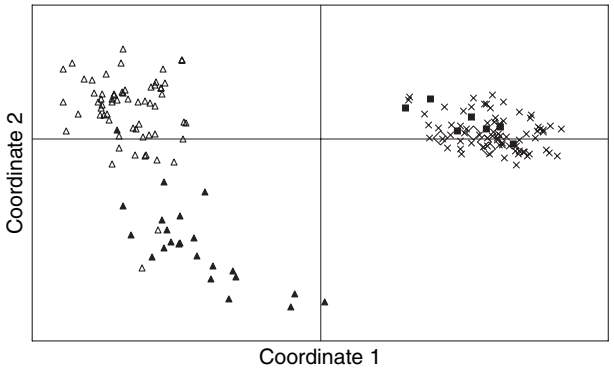

Fig. 2 Principle coordinates analysis based on 15 microsatellite locus genotypes from the four largest populations of Leadbeater's possum, Powelltown (open diamonds), Cambarville (filled squares), Lake Mountain (crosses), Yellingbo north subgroup (open triangles) and Yellingbo south subgroup (closed triangles). Coordinate 1 explains 49% of the variation and coordinate 2 explains 20%.

the value ΔK. ΔK is computed from the mean and standard deviation of the ln $P(X \mid K)$ and the maximum value provides the best estimate of the number of clusters, which for this data set was two. This method best described the split between Yellingbo and other populations.

Based on the recommendations of Pritchard *et al.* (2000), the 'correct' estimate of K [that is, when the $P(K)$ 'more-or-less plateaus'] is the smallest value that gives the largest ln $P(X \mid K)$, in this case seven (Fig. S1). Pritchard *et al.* (2000) also suggest computing the posterior probability of K, [$P(K \mid X)$], from multiple replicates of each different value of K. Using this methodology, the number of clusters was 13.

For all three methods, meta-population subdivision was most strongly defined by the exclusion of Yellingbo from all other populations. For the second and third methods, Yellingbo was subdivided into two exclusive groups that contributed two of the 7/13 clusters respectively. Lake Mountain was also subdivided in much the same manner. This substructure was best represented by $K = 4$ and 11, although population membership to some clusters was not inclusive, and was partially shared with individuals from Mt Margaret, Cambarville and Toolangi (Table 2).

Using either $K = 7$ or $K = 13$, Powelltown clustered separately from other populations. This clustering of Powelltown is consistent with its geographic separation in the southern part of the sampled highlands. The four northern-highlands populations of Toolangi, Mt Margaret, Lake Mountain and Cambarville showed considerable levels of both admixture and shared cluster membership (Fig. S1). The distribution of clusters in this region was not congruent with the geographic locations of sampling sites.

Genetic variation was best described by differences between individuals. Molecular variance among individuals (77%) was much higher than among populations (23%). This was reflected in a global fixation index (0.23) differing little to population-specific fixation indices (between 0.22 and 0.24 for all extant populations). Despite this, population differentiation was still significant: pairwise F_{ST}-values varied from 0.08 between Cambarville and Lake Mountain, to 0.36 between Yellingbo and Powelltown (all $P \leq 0.003$). Yellingbo was most strongly differentiated from all other populations (Fig. 2), with all pairwise F_{ST}-values being >0.30 for all but the Yellingbo/Lake Mountain comparison (Table 3).

Analyses of molecular variance and population differentiation produced incongruent results, so we recomputed pairwise F_{ST}-values to factor in population substructure, and to allow for the possibility that the individual (or groups of individuals) is/are the unit of spatial genetic variance rather than the population. The Yellingbo and Lake Mountain samples were split into multiple groupings and pairwise population F_{ST}-values calculated treating the groupings as separate populations. Groupings were arbitrarily chosen on the basis of geographic location of nest boxes and were two at Yellingbo: a northern and southern, and four at Lake Mountain: a northwestern, far-eastern, southern and central grouping. Not only was population differentiation between Powelltown, Cambarville and the Yellingbo/Lake Mountain groupings large, but there also was significant intrapopulation differentiation at both Yellingbo and Lake Mountain. The northern and southern demes at Yellingbo were significantly differentiated ($F_{ST} = 0.23$, $P < 0.00001$). In addition, the far-eastern (LME) and northwestern (LMNW) demes at Lake Mountain were significantly differentiated from neighbouring group/s, despite indications of potential dispersal events between demes (on the basis of the Structure analyses).

Table 2 Population cluster membership where $K = 7$, inferred from microsatellite genotypes in STRUCTURE

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
Mt Margaret	31	18	4	0	18	0	35
Powelltown	0	99	0	0	0	0	0
Toolangi	5	7	2	0	2	0	84
Cambarville	1	2	3	0	0	0	93
Lake Mountain	44	1	19	0	9	0	26
Yellingbo	0	0	0	73	0	26	0

Numbers give the percentage cluster membership in each population. Values <1% are expressed as zero.

Table 3 Pairwise population differentiation F_{ST} between the four largest extant populations of Leadbeater's possum based on 15 microsatellite loci, and between four extant and one extinct location based on mitochondrial control region sequence data

	Powelltown	Cambarville	Lake Mountain	Yellingbo
Microsatellite F_{ST}				
Cambarville	0.203**			
Lake Mountain	0.136***	0.084***		
Yellingbo	0.359***	0.302***	0.235***	
Mitochondrial F_{ST}				
Cambarville	0.226*			
Lake Mountain	0.226**	0.116*		
Yellingbo	0.972***	0.908***	0.631***	
Bass region	0.766*	0.418**	0.364**	0.795***

Significance codes *$P < 0.05$, **$P < 0.01$, ***$P < 0.00001$.

Coupled with this differentiation was a significant isolation-by-distance effect ($r^2 = 0.52$, $P = 0.001$). Pairwise F_{ST}-values for most comparisons with Powelltown were outliers, as were those for both Yellingbo demes, and LME and LMNW. The Powelltown comparisons tended to show lesser differentiation over a larger distance, whereas the Yellingbo/Lake Mountain groupings showed the opposite.

No reliable or consistent genotypes (that is, matching genotypes in more than three replicate PCRs, at more than two loci for every sample) were obtained from museum specimens despite repeat extractions from fresh material. Therefore, no comparative microsatellite analyses involving museum specimens could be undertaken.

Mitochondrial DNA analyses

A total sequence length of 653 bp was aligned for all samples from extant populations and from four museum specimens (C4380, C4379, C1965 and C8175; 192 samples in total). However because three museum specimens (C4378, C4321 and C28009 the first two representing otherwise unsampled localities) did not sequence successfully beyond 559 bp, all sequences were truncated to 559 bp for phylogenetic analysis. This truncated sequence contained a total of 37 variable sites, which included 26 parsimoniously informative sites and three single base-pair indels. The CSB II and III were identified in all sequences and aligned with the equivalent CSBs in *Didelphis virginiana* (GenBank Accession no. Z29573.1). Unlike CSB III, CSB II in *G. leadbeateri* only shared 74% of the sequence with *D. virginiana*. All unique sequences were lodged with GenBank (Table S1).

In total, 24 unique haplotypes were identified among all samples analysed: 22 from the seven extant localities and two from the (now-extinct) type locality (Table 4). The largest number of haplotypes was found at Lake Mountain ($N = 12$), identified from 71 sequences. In contrast, among 97 individuals from the Yellingbo sample there were only two haplotypes. The two Yellingbo haplotypes were separated by only a single base indel (verified by replicate sequencing of every sample representing the rarer haplotype). Only three haplotypes were shared between populations: one between Yellingbo and Bass (Bass specimens C4380 and C28009 and 92 Yellingbo samples), one between Lake Mountain and Mt Margaret, and one between Lake Mountain and Cambarville. All other haplotypes were unique to their sampling locality (Fig. 3). With the exception of Yellingbo, the number of haplotypes observed in a population generally increased with sample size.

The phylogenetic network indicated the presence of one distinct clade (with 87% bootstrap support) containing all Yellingbo samples and the Bass Valley specimens (C4390, C4379 and C1965). This phylogeny was star-like indicating nonequilibrium past population histories. There was 99% bootstrap support for a single Lake Mountain haplotype representing a distinct lineage from the group containing all other haplotypes. Contrary to our expectations (based on collection site information accompanying museum specimens), the Tynong specimen C4378 did not cluster with the Yellingbo/Bass clade but rather grouped with Central Highlands samples. No other population-specific patterns

Table 4 Mitochondrial haplotype and nucleotide diversity for all locations sampled, including one extinct locality (Bass region)

Population	*n* Samples	*n* Haps	Prop. haps unique	Mean no. pairwise diff.	Nucleotide diversity
Cambarville	8 (1)	4	0.75	6.04 ± 3.22	0.0108
Lake Mountain	71	12	0.75	6.76 ± 3.22	0.0121
Mt Margaret	3	2	0.33	1.33 ± 1.10	0.0024
Toolangi	2	1	1.0	0.00 ± 0.00	0.0000
Powelltown	6	2	1.0	2.33 ± 1.48	0.0042
Yellingbo	97 (1)	2	0.5	0.12 ± 0.19	0.0002
Warburton	(1)	1	1.0	na	na
Bass region	(4)	4	0.75	3.5 ± 2.2	0.0063
Total	192	24			

n samples is the number of individual sequences obtained for that population/locality. Numerals in parentheses are the number of museum specimens contributing to a sample.

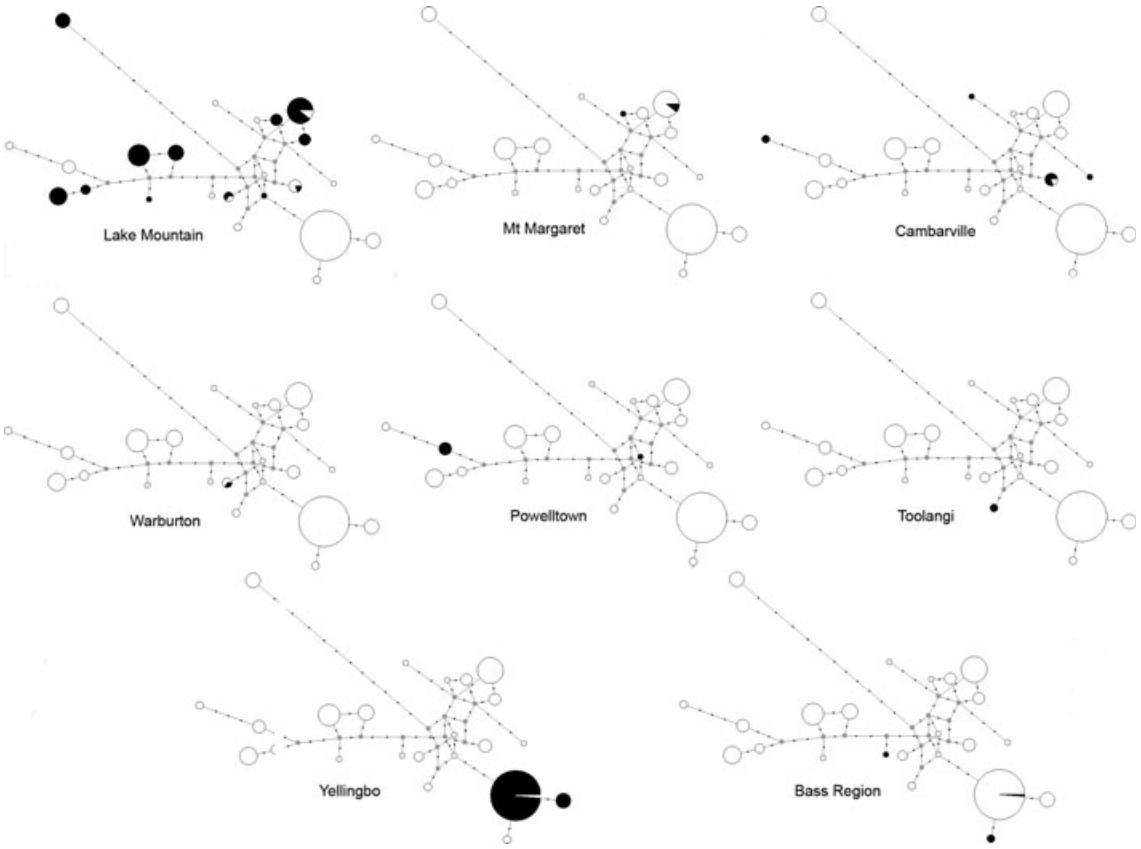

Fig. 3 Mitochondrial haplotype networks for eight sampling localities based upon a 559-bp region of control region sequence from 180 individuals. Circle size is proportional to number of individuals sharing a haplotype. Grey nodes represent haplotypes missing from any locality, and small black nodes represent mutations. Circle size corresponds to the number of samples sharing a haplotype and shading is used to indicate which haplotypes were present in a given population.

were detected in the network. Closely related and missing haplotypes were interspersed among Central Highlands populations rather than being confined exclusively to one or the other (Fig. 3). No suitable outgroup was found to be available to root the network.

Mitochondrial molecular variance among populations was 60%, with the remaining 40% assigned to within-population differences, based on pairwise differences between sequences. The global fixation index was 0.60 ($P \sim 0.0$), indicating significant population

differentiation. Given (i) the distribution of haplotypes (not necessarily concordant with sampling locality); (ii) the significant contribution of within-population variance in the microsatellite data; and (iii) the clustering of Yellingbo and Bass haplotypes (Fig. 3), we re-analysed molecular variance in the mitochondrial data as follows. Haplotypes from Yellingbo and the Bass region were combined into one grouping, and all other Central Highlands samples into another. This resulted in a re-partitioning of the variance, with the among-groups component being the highest at 47%, and the remainder being partitioned largely within populations (37%) and less so among populations (16%). All variance components were highly significant when tested against 50 000 random permutations. The global fixation index tested by permuting populations among groups (F_{CT}) was 0.47 ($P = 0.099$), indicating that the two groupings explained only marginally more of the genetic variance than that explained within populations. Removal of the Tynong specimen from the Bass sample altered neither the variance nor the global fixation index. Regardless, it was removed from subsequent analyses of pairwise population F_{ST} and tests for sudden population expansion owing to its clear phylogenetic exclusion from the Yellingbo/Bass clade.

Pairwise population microsatellite F_{ST} was highest between Yellingbo and each of the other populations, including Bass (>0.60), whereas much lower values resulted for comparisons between Lake Mountain and other populations (Table 3; Fig. 4). Mantel testing of microsatellite and mitochondrial pairwise F_{ST}-matrices produced a strong correlation ($r^2 = 0.90$, $P = 0.044$, based upon 999 permutations). A single outlier was present, between Cambarville and Powelltown, indicating higher microsatellite population differentiation than expected given mtDNA differentiation.

Consistent with the pattern of microsatellite diversity, the two largest Central Highlands samples from Lake Mountain and Cambarville had the highest mtDNA nucleotide diversity and largest mean number of pairwise sequence differences (Table 5). Conversely, Yellingbo had the lowest nucleotide diversity and smallest mean number of pairwise differences (excluding Toolangi, where the two individuals sampled had the same haplotype) despite the large sample size, and these values were nearly two orders of magnitude lower than in the Lake Mountain sample.

Estimates of the parameters τ, θ_0 and θ_1 (Table 5) indicated a significant difference between the mismatch distributions *expected* under the null hypothesis of sudden population expansion, and those *observed* at Lake Mountain, Cambarville and Powelltown, but not at Yellingbo. Neither Fu's F_S nor Tajima's D rejected the null hypothesis of selective neutrality in any population

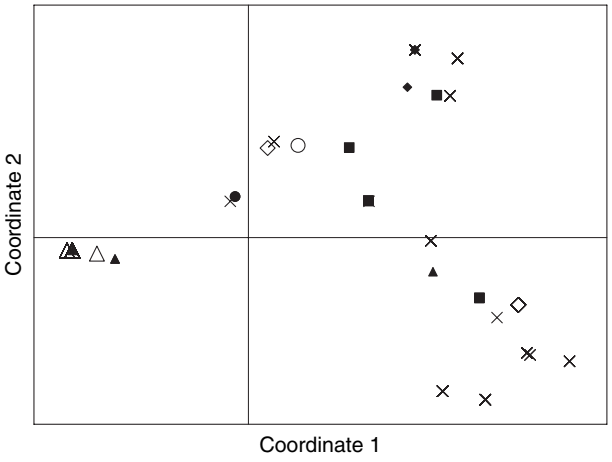

Fig. 4 Principle coordinates analysis of haplotype sequences from all sampling localities of Leadbeater's possum. Labels as follows: Powelltown (open diamonds), Cambarville (filled squares), Lake Mountain (crosses), Yellingbo (open triangles), Mt Margaret (filled diamonds), Toolangi (filled circle), Bass region (filled triangles) and Warburton (open circle). Coordinate 1 explains 42% of the variation and coordinate 2 explains 26%.

when tested against 10 000 simulated samples. These computations were repeated using three groupings of samples, (i) Yellingbo/Bass (based on clustering of these haplotypes), (ii) Central Highlands north (Mt Margaret/Lake Mountain/Cambarville, based on patterns of microsatellite variation), and (iii) Central Highlands south (Powelltown, based on its geographic separation from the northern Central Highlands populations), to test for 'regional' past population dynamics. The Central Highlands north and south groups had significantly different observed mismatch distributions, but not Fu's F_S nor Tajima's D (Table 5). Estimates of Fu and Li's D*- and F*-test statistics were not significant for the samples from Lake Mountain, Central Highlands north, nor Powelltown (Table 5). Estimates of Fu and Li's D*- and F*-test statistics could not be obtained for the Yellingbo sample in DnaSP. These tests combined indicate that background selection was not responsible for the mtDNA patterns observed in any population. Yellingbo was the only population that fitted the pattern of having undergone expansion.

Tests of past population dynamics in FLUCTUATE identified Lake Mountain as a declining population. All replicates produced a maximum-likelihood growth rate of around −3. The average maximum-likelihood estimates of the parameters g and theta were -77.73 ± 23.43 and 0.0075 ± 0.0008, respectively, which corresponded to an approximate change in population size over 2000 generations of one order of magnitude at a mutation rate of 1×10^{-05}. Lessa *et al.* (2003) use $g > 3SD(g)$ as a conservative test for population growth. For Lake Mountain,

Table 5 Relative measures of sudden population expansion (τ and its 95% confidence intervals, and θ_1), selective neutrality (Tajima's D and Fu's F_S) and effective population sizes estimated from mitochondrial haplotypes [theta (pi)] and from microsatellite genotypes (N_e)

Population/grouping	τ	τ (95% CI)	θ_1	SSD	D	F_S	Fu & Li's D*	Fu & Li's F*	Theta (pi)
Lake Mountain	9.4	3.9–13.8	12.23	0.05*	1.14	3.19	1.44	1.45	6.73
Yellingbo	3.0	0.3–3.0	0.12	<0.01	0.00	−0.43	—	—	0.10
Yellingbo/Bass	3.0	0.5–3.0	0.16	<0.01	0.00	−1.78	—	—	0.14
CH north	8.7	4.2–12.1	14.03	0.04*	0.85	1.63	0.34	0.31	6.72
CH south (Powelltown)	3.0	0.4–3.0	0.29	0.16*	−1.37	3.36	−1.40	−1.49	2.33

The sum of squared deviations (SSD) between the observed and expected mismatch distribution is the test statistic of parameters τ, θ_0 and θ_1. CH north groups Lake Mountain, Cambarville and Mt Margaret into one unit. θ_0 was 0.0 in all cases and is therefore not listed. All values are not significant unless otherwise marked.
*$P < 0.05$.

$g << 3SD(g) = 70.29$ indicating no population growth. Sunnucks *et al.* (2006) used the criterion of g plus $3SD < 0$ to differentiate significant population decline from stability. For Lake Mountain, this equated to −7.44 provides strong support for a population decline there.

These analyses were repeated on the Central Highlands north grouping and revealed average maximum-likelihood estimates of the parameters g and theta of −13.42 ± 5.35 and 0.0197 ± 0.0028 respectively. The mean growth parameter g was $<3SD(g) = 16.05$, also indicating no population growth for the region represented by samples from Lake Mountain, Cambarville and Mt Margaret. Using g plus $3SD < 0$ criterion (which equalled 2.63) indicated either population stability or a signal of decline that is masked by the sample sizes from Cambarville and Mt Margaret.

Five replicate runs in LAMARC produced a maximum-likelihood estimate of theta = 0.0072 (95% CI: 0.0039–0.0124) for Lake Mountain and 0.0053 (95% CI: 0.0014–0.0206) for its nearest neighbour, Cambarville. Migration from Cambarville to Lake Mountain was estimated as 378.55 (95% CI: 81.3–1124.3) compared with 151.83 (95% CI: 32.91–416.79) in the other direction. Five replicate runs using the northern and southern highlands groupings produced a maximum-likelihood estimate of theta = 0.0107 (95% CI: 0.0069–0.0176) for the Central Highlands north group and 0.0014 (95% CI: 0.0001–0.0056) for Powelltown (Central Highlands south). Relative inferred migration from Powelltown to the north grouping was approximately seven times higher than in the other direction (south to north: 705.13, 95% CI: 105.02–4596.61 cf. north to south: 98.33, 95% CI: 12.29–291.41).

Effective population size

Effective population sizes were between six and nine times larger at Lake Mountain (56.8, 95% CI: 52.7–61.4) than at Yellingbo (7.4, 95% CI: 6.7–8.2) when estimated from microsatellite data in NEESTIMATOR. However, the relative difference estimated from haplotype sequence data using theta (pi) in Arlequin was between one and two orders of magnitude larger at Lake Mountain than Yellingbo (Table 5). These differences in estimates suggest that substantial negative population size changes have occurred at Lake Mountain relative to Yellingbo over the timescale represented by the two markers (long-term past vs. recent past).

Discussion

Using genetic analysis of individual animals captured in nest boxes and from trapping, we gathered a substantial sample from animals in wild populations of Leadbeater's possum. This provided us with a unique opportunity to examine some genetic attributes of the species. We detected a signal of population decline and a signal of immigration in the largest sampled population in the northern part of the current species' range. We have also explored past population processes in detail for the population at Yellingbo and found that, consistent with earlier findings from population genetic simulations, it is not recently isolated from populations from the Central Highlands (Hansen & Taylor 2008). Rather, our analyses suggest that the Yellingbo population is a remnant of a larger, now extinct meta-population that centred on lowland swampy habitats.

Based on extensive long-term monitoring data (Lindenmayer *et al.* 2003), the current population size of Leadbeater's possum may be as low as 2500. Recent wildfire across much of the species' range is likely to have substantially reduced population sizes to well below this figure. The sample obtained for this study thus represents ≥14% of the estimated population. While our genetic samples are skewed in geographic distribution and population representation, they are nevertheless a significant portion of the extant meta-population of the species and thus also presumably a significant portion of the genetic diversity.

Current genetic structure of wild populations

Leadbeater's possum is remarkably genetically diverse in the core of its range. This is despite extensive alterations to montane ash forest at a landscape scale from logging and wildfire, both of which have been identified as having significant negative impacts on the persistence of populations of Leadbeater's possum (Lindenmayer & Possingham 1996; Lindenmayer 2000). Both expected heterozygosity and allelic richness (diversity) (based on microsatellite data) were higher in all highlands populations sampled than at Yellingbo, which supports the only extant lowland population (Hansen & Taylor 2008). Microsatellites provide the most practical and economical tool for threatened species conservation at this time and we therefore draw conclusions regarding relative population genetic structure using the data obtained from them, despite the possibility that they may not accurately reflect genome-wide diversity (Väli *et al.* 2008). Given this caveat, we suggest that genetic patterns detected here potentially under-estimate rather than over-estimate true population differentiation in this species.

Lake Mountain, Cambarville and Mt Margaret in the northern part of the species range appear to form a continuous genetic unit whose subunits have experienced regular genetic exchange. Gene flow is probably still occurring across a relatively large area ($\sim$20 km^2) reflecting relatively continuous habitat in the region. The population at Powelltown, on the other hand, appears to be genetically distinct from other sampled highlands populations, suggesting either isolation by distance or relatively recent disruption to gene flow between these regions. While it is possible the small size of the Powelltown sample inadequately represents true genetic diversity for the region, we consider this a less plausible explanation for its distinction given that a more poorly sampled Mount Margaret site (N = 3) clustered with other northern populations in this analysis.

There was generally a high correlation between population genetic differentiation using both molecular markers, with the exception of Powelltown, which showed lower mitochondrial divergence (based on sequence similarity rather than haplotype frequency) than microsatellite divergence compared with other populations. This suggested that the genetic differentiation of Powelltown from northern populations may be a recent phenomenon, and that habitat fragmentation is having an additive affect to patterns of isolation by distance. Powelltown is disconnected from the northern distribution of the species by mixed species forest and extensive cleared valley floors that are unsuitable habitat for Leadbeater's possum. Therefore, we suggest that recent disruptions to gene flow are the cause of Powelltown's differentiation.

Keyghobadi (2007) has commented that time lags may exist between fragmentation and its subsequent effect on gene flow. It is therefore plausible that changes due to timber production, land clearance and wildfire are ongoing contributors to the genetic signal of fragmentation between regions within the highlands. The flow-on effects of fragmentation may therefore continue to accrue even if there is immediate cessation of timber production. Furthermore, the extended periods required for recruitment of new trees to hollow-bearing maturity (Ambrose 1982; Lindenmayer *et al.* 1993) will inflate fragmentation effects due to punctuation of suitable habitat with the loss of den sites. Additional sampling in between the northern and southern units, and sampling in the eastern part of the highlands is necessary to assess the extent of fragmentation across the entire species range.

Historical population genetic structure

We found many similar haplotypes shared among Central Highlands populations of Leadbeater's possum, but few were unique to their sampling locality. This indicated that populations have not been separated for any substantial time period in the past, and probably formed part of a larger panmictic group. This group may have been centred on the current core range, or may have extended north and east to other regions in Victoria and southern New South Wales where subfossils have been found (Wakefield 1967; Hall 1974; Menkhorst 1995; Harley 2004).

There were many 'missing' haplotypes identified in the network, indicating either that they have not been sampled or have been lost due to extinction of maternal lineages. We consider the former explanation unlikely, given that even for the well-sampled Lake Mountain population there was evidence of missing haplotypes, yet the detection of only 12 haplotypes in 71 individuals would tend to suggest sampling was sufficient to detect most extant haplotypes. We have explored the accumulation of microsatellite allelic diversity previously (Hansen & Taylor 2008) and found it adequately represents the majority of diversity across the species' range. Moreover, tests of sudden population expansion failed to find any positive changes to population size, from which we infer a potential negative change in past population dynamics. Coalescent analysis at Lake Mountain produced a significantly negative value for the growth parameter g indicating that the population has experienced declines.

In addition to the population decline detected at Lake Mountain, coalescent analysis of the northern part of the Central Highlands of Victoria grouping (that encompasses Lake Mountain, Cambarville and Mt Margaret)

also produced negative values of population growth, rejecting the hypothesis of population growth for the region. We used LAMARC to distinguish true signals of decline at Lake Mountain from potentially confounding effects of emigration. Substantially higher estimates of immigration than emigration were detected for Lake Mountain compared with its nearest neighbour Cambarville, and for the northern complex of Lake Mountain/Cambarville/Mt Margaret when compared with Powelltown. While the known presence of unsampled populations between the northern and southern populations (Lindenmayer *et al.* 1991b) potentially confounds the validity of this finding (Beerli 2004), it does not supersede the signal of decline (which would be otherwise invalid if there was substantial emigration from Lake Mountain). Given that Lake Mountain appears to have never represented a discrete genetic unit and clearly exchanges migrants with nearby populations, it is plausible that the decline detected there is neither restricted to that site, nor a peculiarity of that location. Given the apparent reduction in the species' range since the last glaciation (Hope 1974; Lindenmayer 1989) and bioclimatic modelling of range reductions under global warming scenarios (Lindenmayer *et al.* 1991b), the population decline detected here may represent a general trend across at least the northern half of the species' core, Central Highlands range.

The evolutionary history of lowland swamp populations of Leadbeater's possum

Previous work on the lowland swamp population at Yellingbo has revealed that it is not a recent remnant from the Central Highlands of Victoria. Instead, it may be the sole representative of a broader swamp population that extended to the Bass Valley region in Western Port (Hansen & Taylor 2008). The clustering of Bass and Yellingbo haplotypes to the exclusion of populations from the Central Highlands supports the argument that these two lowland areas were previously connected. Leadbeater's possum disappeared from Western Port at the start of the 20th century, and well before any serious attempt to study the species was made (Brazenor 1946; Menkhorst 1995; Harley 2004). Anecdotal observations of habitat in the Bass Valley (Nicholls 1911) suggest that the region where Leadbeater's possum was first discovered may have floristically and structurally resembled Yellingbo (Harley 2004). Swampy habitats that covered a substantial portion of Western Port were drained and much of the region cleared of scrub cover, almost certainly contributing to the local extinction of Leadbeater's possum (Nicholls 1911; Spencer 1921; Wilkinson 1961; Menkhorst 1995). Cockatoo Creek (along which the population at Yellingbo occurs) may have been connected to Western Port prior to urban and agricultural development of the area. Our research findings support this hypothesis by concluding that populations from the Bass and Yellingbo were historically connected, and that Leadbeater's possum either used corridors of suitable habitat to move between the two districts or else was continuously distributed throughout the intervening area.

Nonequilibrium past population dynamics was inferred for Yellingbo and Bass Valley from the results of the test for sudden population expansion. Our results suggest that this region has either experienced recent population growth or some other non-negative population size change. We have previously identified the patterns of a genetic bottleneck at Yellingbo, probably occurring prior to European-induced changes to the montane landscape (Hansen & Taylor 2008). The two Yellingbo haplotypes detected in this study were separated only by a single base-pair deletion, suggesting a severe bottleneck down to a single haplotype and the appearance of a second haplotype by mutation. Therefore, while this bottleneck could have occurred anytime between the last Pleistocene glacial maximum [up to 25 000 years before present (ybp)] and the arrival of Europeans, it is more likely to have occurred during or nearer the Pleistocene.

The mitochondrial control region evolves rapidly and at a highly variable rate. As a result, caution is advised in attempting to use a mitochondrial molecular clock approach for dating in the absence of fewer than four independent time points (Moritz *et al.* 1987). Attempts to do so may result in divergence time estimates that are uninformative, due to the large confidence intervals surrounding those estimates (for example, the Mountain Pygmy Possum *Burramys parvus*—Mitrovski *et al.* 2007). Virtually no sequence similarities were found in the control region of Leadbeater's possum and its nearest relative, the striped possum *Dactylopsila trivirgata* (GenBank Accession no. NC_008134), and only CSB II and III were shared with the American Opossum *Didelphis virginiana* (and only partly so). Thus we were unable to obtain a suitable calibration to date divergence times for Leadbeater's possum sequence, which would have allowed us to test if the bottleneck signature at Yellingbo is a result of an early split between highlands and swamp populations.

Leadbeater's possum is represented in the fossil record by Pleistocene subfossils from southern New South Wales, and later ones from the Buchan district in eastern Victoria (dated between the late Pleistocene and 2500 ybp) (Wakefield 1967; Lindenmayer 1989). Two-hundred and seventy-seven individual fossils, dated at 15 000 ybp, were detected in the Buchan sample but only a single fossil was found that dated at 2500 ybp (Wakefield 1967). This change in fossil representation

indicates a possible range contraction since the last Pleistocene glacial (most likely climate-induced), which could have lead to the species' current restricted range in southeastern Australia. Thus, on the basis of a climate-induced range contraction (Wakefield 1967), we suggest that the divergence time of populations from Yellingbo and Bass Valley may be sometime around or after the appearance of subfossils in the Buchan area.

The historic specimen recorded as being from Tynong North did not cluster with Yellingbo and Bass, as would be expected on the basis of its geographic proximity. This specimen was collected only 30 km south of the nearest contemporary Central Highlands record (Loyn & McNabb 1982), both within the Western Port catchment. Its genetic affinity with other Central Highlands specimens suggests a past connection between the two. An ecological reconstruction of the Western Port swamps has demonstrated the presence of an 'inner' permanently inundated reed and rush swamp and an 'outer' paperbark scrub swamp subject to regular flooding (Yugovic & Mitchell 2006). The inner swamp lies directly between Tynong and the region where the more southerly Bass specimens were collected, and was probably impassable for Leadbeater's possum. The lack of clustering may therefore represent long-term physical separation of the two localities. Alternatively, this specimen may represent a retained ancestral haplotype prior to the divergence of Yellingbo/Bass, although the possibility that the specimen may be mislabelled cannot be definitively excluded.

Implications of the genetic data for future species conservation

The pattern of the genetic data collected in this study, coupled with the results of bioclimatic analyses that simulated a considerable range contraction associated with global warming (Lindenmayer *et al.* 1991b), suggest that Leadbeater's possum has experienced past climate-induced declines in parts of its range (including the intensely studied area around Cambarville). Current and ongoing monitoring programmes have also indicated a recent decline in abundance across much of the core range (Lindenmayer D.B., unpublished data). Thus, recent declines (due to the loss of hollow-bearing trees; Lindenmayer *et al.* 1997) are occurring in addition to historic declines (due to shifts in climate).

Population viability analyses conducted over a decade ago predicted a high risk of a meta-population crash within 50 years (Lindenmayer & Lacy 1995; Lindenmayer 2000). At a finer scale, simulations revealed that only populations over a threshold size (200 individuals, which is approximately the number of animals on the Lake Mountain plateau) had a 90% chance or higher of persistence over a 100-year period.

A high level of genetic diversity and signs of successful breeding [80 offspring, both weaned ($N = 71$) and pouch young ($N = 9$) encountered in colonies over three visits in a 12-month period; B. Hansen and D. Harley, unpublished data] were detected at the Lake Mountain population. On this basis, we expect that the Lake Mountain population should be relatively stable. Instead it showed a signal of past decline despite high inferred immigration rates, suggesting that meta-population extinction risk might be higher than currently predicted. State government plans to establish a montane reserve system will be important for reducing this risk (Macfarlane & Seebeck 1991; Macfarlane *et al.* 1998), but management actions that incorporate conservation of existing genetic diversity will also be necessary to safeguard against further loss of maternal lineages. This includes targeted management of areas identified as distinct genetic units (in this case, Powelltown and the Lake Mountain/Cambarville complex) (Moritz 1995). Maintenance of habitat connectivity between populations or subpopulations is clearly an important factor in facilitating gene flow.

The population at Yellingbo should be managed separately, as it fits the criteria of an evolutionarily significant unit (ESU) (Moritz 1995; Fraser & Bernatchez 2001) in being a historically isolated, and sole representative of an otherwise extinct genetic unit. To definitively demonstrate reciprocal monophyly (with the Central Highlands), a criterion proposed to define ESUs (Moritz 1994), we would need either an outgroup (which is not available at this time) or alternative mitochondrial sequence data (for example, cytochrome *b*). The accumulation of novel mutations (adaptive or otherwise) through long-term isolation means that Yellingbo qualifies as an ESU or distinct genetic lineage (after Fraser & Bernatchez 2001). We have previously suggested that experiments on ecological exchangeability would be warranted (Hansen & Taylor 2008), which would also fulfil the criteria of defining *ESU*s according to Crandall *et al.* (2000) (reviewed in Fraser & Bernatchez 2001). As the population at Yellingbo harbours a unique subset of genetic diversity that is absent from the rest of the species' range (Hansen & Taylor 2008), its protection (along with Central Highlands complexes like Lake Mountain/Cambarville/Mt Margaret) should be viewed as a high conservation priority.

What is the long-term prognosis for the survival of Leadbeater's possum?

We note that potential lags in accumulation of genetic signals (Keyghobadi 2007) mean that the effects of

ongoing alteration to montane ash forests may be leading to further genetic fragmentation of populations of Leadbeater's possum than the current field data suggest. There may be continued isolation of populations within the Central Highlands, especially those towards the edge of the core range. Not only is it important to take action immediately to conserve genetic diversity in this species, but it will be equally as important to monitor changes in this diversity over time, especially if a severe bottleneck occurs due to loss of nest trees (Lindenmayer *et al*. 1990).

In early February 2009, severe wildfires damaged extensive areas of montane ash forests in the Central Highlands of Victoria. Large trees that have been damaged or destroyed by fire may be removed through salvage logging operations, which is a common practice in wet forests subject to stand-replacing fires around the world (Lindenmayer *et al*. 2008). If salvage logging proceeds without appropriate prescriptions to conserve habitat for Leadbeater's possum, local populations of the species may be substantially reduced, thereby increasing the risk of extinction. Our data provide an important baseline for postfire monitoring of both population genetic structure and genetic diversity, and will allow realistic estimates of effective population sizes for incorporation into future PVA models and short-term survival estimates.

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This study forms part of Birgita Hansen's PhD research on conservation genetics of Leadbeater's possum. Dan Harley has undertaken a comprehensive ecological study on the population of Leadbeater's possum at Yellingbo as part of his PhD research, and is an active member of the Leadbeater's Possum Recovery team where he specialises in population monitoring. David Lindenmayer specialises on large-scale long-term empirical and natural experiments, particularly those associated with the impacts of natural resource use on biodiversity. Andrea Taylor has applied molecular ecological tools and analyses to a variety of situations involving both conservation management and population ecology of many Australian native species, primarily marsupials.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 This supporting information contains Table S1 and Fig. S1 referred to in the text.

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Appendix I

Details of *Gymnobelideus leadbeateri* control region mitochondrial primers. The majority of sequences obtained occur between $tRNA^{phe}$ and the site of initiation of heavy strand replication (O_H) (Taberlet 1996)

Primer name	Sequence (5′–3′)	Complementary primer	Fragment size (bp)*	Location†
L16216GL	ATTCGTAGAGGCATATGTGATG	Forward	465	16184
H196GL	GCTTTTTGGGGTGGGAAAG	Reverse		16704
L16204GL	CCTAAACATGCTATTCGTAGAGGC	Forward	406	16175
H120GL	AATCATTTAATCAAGGGGGAAAG	Reverse		16634
L16613GL	TTGTTGCTCACGCTAAAC	H651B‡	388	16617

*This is the approximate size of fragment calculated from alignments of *Gymnobelideus leadbeateri* sequences. The fragment size of L16613GL is from PCR with H651B.
†Location refers to the approximate location on the light strand of the control region of *Didelphis virginiana*. Primers L16204GL and L16613GL partially overlap with CSB II.
‡For details of H651B, refer to Fumagalli *et al.* (1997).