

Isolated remnant or recent introduction? Estimating the provenance of Yellingbo Leadbeater's possums by genetic analysis and bottleneck simulation

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Abstract

Effective conservation management requires that genetically divergent populations potentially harbouring important local adaptations be identified and maintained as separate management units. In the case of the endangered Australian Leadbeater's possum (*Gymnobelideus leadbeateri*), an arboreal marsupial endemic to Victoria, uncertainty over the evolutionary origin of a potentially important extant wild population recently discovered in atypical habitat (lowland swamp) at Yellingbo is hampering such efforts. The population is rumoured to be a recent introduction. Microsatellite allele frequencies at Yellingbo differed substantially from those in sampled populations in montane ash forest (F_{ST} between 0.23 and 0.36), and Bayesian clustering analyses of genotypes strongly separated them ($K = 2$). We conducted a suite of bottlenecking tests which all indicated that Yellingbo had undergone a recent reduction in size. The extent to which the distinctiveness of Yellingbo animals might be expected solely through bottlenecking associated with a recent introduction, was tested by simulating population–history scenarios seeded with genotypes from candidate wild and captive sources. No bottleneck scenario reproduced anything approaching the genetic distinction of the Yellingbo population, with all STRUCTURE analyses placing Yellingbo in a separate cluster to simulated populations ($K = 2$, minimum $F_{ST} = 0.13$). These results suggest that Yellingbo does not share recent ancestry with other extant populations and instead may be a remnant of an otherwise extinct gene pool. Importantly, this may include genes involved in adaptation to a lowland swamp environment, substantially adding to the conservation importance of this population, and suggesting that separate management may be prudent until evidence suggests otherwise.

Keywords: conservation biology, microsatellites, population, rare alleles

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Introduction

The decline of native species is almost always attributable to degradation and fragmentation of habitat, introduction of exotic competitors and predators, and/or to disease. If a threatened species suffers through loss of habitat or the introduction of non-native competitors, information on the evolutionary history of populations of that species may be important in guiding management programmes. In the case of some threatened Australian fauna introduced to regions outside their native range, information on the source of introduced animals is important for guiding

re-introduction programmes (Taylor & Cooper 1999; Eldridge *et al.* 2001). With the refinement and application of population genetic techniques to conservation, it is possible to investigate these scenarios using molecular markers such as microsatellites. Here, we apply this approach to Leadbeater's possum, a threatened Australian endemic, in order to elucidate the ancestry of a population with a questionable genetic and demographic history.

Leadbeater's possum (*Gymnobelideus leadbeateri*) is most notable for its status as Victoria's faunal emblem, but this status has not prevented a widespread decline in its range since European colonization. It was first described in the late 19th century from two specimens collected near Bass in the Western Port region along Victoria's coast (Brazener 1962) (Fig. 1). Only four records from Western Port were

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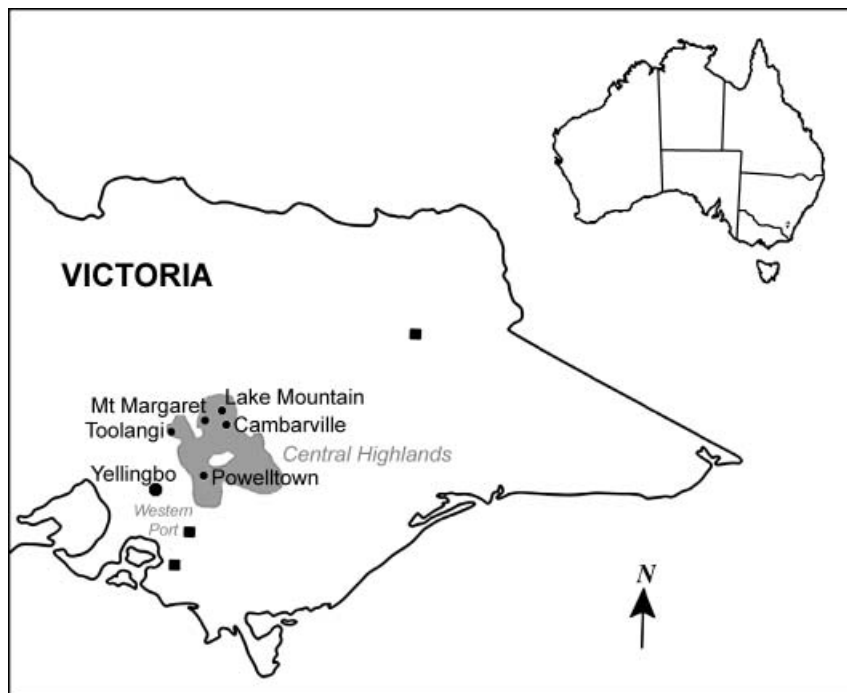


Fig. 1 Map of Australia showing Victoria in detail. The core range (shaded region) of *Gymnobelideus leadbeateri* and the locations of extinct populations (represented by squares) are shown. Black dots represent extant populations. Source of Australia map outline: www.ga.gov.au. Map modified from Harley (2005) (with permission).

ever made, and by the mid-20th century it was presumed extinct. The species was subsequently re-discovered in 1961 in the montane habitats of Victoria's Central Highlands (Wilkinson 1961) (Fig. 1). Since that time, the species has been found in a variety of locations throughout the highlands, although patchily distributed and at low densities. The habitat at these locations is characterized by montane ash forest with large, hollow-bearing trees and a complex understory of *Acacia* (Lindenmayer *et al.* 1989, 1991; Lindenmayer & Possingham 1995). The distribution of *G. leadbeateri* in the central highlands is restricted by the availability of trees of suitable age and size for provision of nesting hollows (Smith & Lindenmayer 1988). This is seen as a major conservation issue, as suitable nesting trees are either being felled for timber, succumbing to postlogging fire operations or collapsing due to wildfire-induced decay (Macfarlane & Seebeck 1991; Lindenmayer 2000).

In 1986, a small, geographically isolated population of *G. leadbeateri* was discovered in lowland swamp near Yellingbo, some 50 km east of Melbourne (Smales 1994), 16 km from the nearest known Central Highlands population (Harley 2004) and approximately 70 km north of Bass Valley in Western Port. The population is restricted to a narrow, linear reserve located within an agricultural landscape. The high rate of occupancy of most available artificial denning sites (85% in suitable habitat; Harley 2005) suggests the population is near carrying capacity (annual population estimates vary between 80 and 100 animals; Harley 2005). The local rarity of suitable unoccupied habitat, the absence of corridors for movement between Yellingbo and the highlands (Harley 2005), and the low average dis-

persal distance of individuals – between 450 and 500 m at this site, with the maximum being 1.5 km (Harley 2002) – suggest it is highly unlikely that Yellingbo exchanges migrants with highlands populations.

The unexpected discovery of a successfully breeding population in lowland swamp changed the perspective on the conservation priorities of this rare species. At any given time, approximately 64% of the population is comprised of adults, with the remainder being largely juveniles (less than 12 months old; 26%) and some subadults (12–16 months; 10%) (Harley 2005). Maximum litter size in this species is two, with breeding occurring all year round (Harley 2005). Average juvenile survivorship is relatively high (55%), and breeding vacancies are rare (Harley 2005). As a result, approximately 65% of adults hold a breeding position (Harley & Lill 2007), the sex ratio of these being at parity. Habitat at Yellingbo is clearly deficient in large hollow-bearing ash trees and the possum's major food source in ash-type forest, *Acacia* spp. (Smith 1984). Furthermore, animals readily occupy nest boxes (Harley 2002, 2005), which attests to the lack of natural denning sites upon which they are so reliant in the highlands. These floristic and vegetation structure differences between Yellingbo and montane ash habitats have led to speculation that the population was not a natural remnant but had been introduced in Yellingbo. Such speculation was fuelled by rumours that some animals from a private captive population, most of which were ultimately housed at Melbourne Zoo, had been released at Yellingbo (Harley 2006).

The captive population was originally sourced from multiple locations within the highlands, including around

Cambarville and Marysville in the north, Powelltown and Noojee in the south and Toolangi in the west. A breeding program was initiated at Melbourne Zoo in 1970, and animals were subsequently transferred to establish breeding colonies at other zoos/sanctuaries. Captive populations have since declined and have not been replenished. The future outlook for *G. leadbeateri* in the wild is grim, with a 90% extinction probability predicted by 2025 (Smith *et al.* 1985). Thus, the re-establishment of captive populations and a translocation program are high priority short-term conservation priorities. The population at Yellingbo is a potential source of animals for both conservation options due to its successful breeding and apparent saturation of the available habitat. However, if the population at Yellingbo is a long-term and locally adapted isolate from those in the highlands, then it may be inappropriate to mix them. Such a course of action may result in loss of local adaptation (Frankham 2005) and/or outbreeding depression (by mixing of genomes adapted to different environments; Marshall & Spalton 2000).

Here, we use microsatellite genetic variation to address the hypothesis that the Yellingbo population is the result of a recent introduction rather than being a natural remnant. Whether the population is introduced or remnant, it may harbour reduced genetic diversity because of a recent founder effect or long-term isolation and small size, respectively. Thus, genetic diversity per se will not allow us to distinguish between these two scenarios. However, analysis of experimentally bottlenecked populations of *Drosophila melanogaster* (England 1998; England *et al.* 2003) showed that intense (small number of animals for brief periods) and diffuse (larger numbers for longer periods) bottlenecks produced measurable differences in a variety of alternative genetic signatures. We here apply these tests to microsatellite data from Yellingbo, to assess whether they indicate an intense or diffuse bottleneck, which would mimic introduction and remnant scenarios, respectively. In addition, we utilize computer simulations to produce artificial gene pools resulting from a variety of bottleneck scenarios applied to potential source populations (wild Central Highlands populations or captive colonies). We then assess whether simulated bottlenecks produce populations with genetic characteristics (genotypic structure and allele frequencies) resembling those observed at Yellingbo by using statistics that describe differences in allelic frequencies.

Materials and methods

Sample collection and microsatellite genotyping

Leadbeater's possum blood and ear-biopsy samples were collected from animals captured during previous studies at five locations within Victoria's Central Highlands: Cambarville ($n = 7$), Lake Mountain ($n = 3$), Mount (Mt) Margaret ($n = 3$),

Powelltown ($n = 4$) and Toolangi ($n = 2$), and at a sixth location, Yellingbo ($n = 11$) (Lindenmayer & Meggs 1996; D. Lindenmayer, personal communication). In addition to these samples collected in earlier studies, ear-clip samples were also taken from adults and immature animals residing in nest boxes at Lake Mountain ($n = 159$). Ear-clip samples were taken from all animals representing multiple cohorts of adults and offspring captured at Yellingbo ($n = 187$) as part of an intensive ecological study between 1996 and 2001 (Harley 2005). Blood samples were also collected from captive animals held at Melbourne Zoo ($n = 17$), Taronga Zoo (Sydney) ($n = 7$) and Healesville Sanctuary (Victoria) ($n = 16$) (D. Lindenmayer, personal communication). The original colonies were established in Melbourne and subsequent transfers of descendant individuals gave rise to new colonies at other locations. Therefore, for the purpose of genetic analyses, all sampled captive animals were treated as having come from a single captive population.

Whole genomic DNA was extracted from tissue samples using the salting-out protocol in Sunnucks & Hales (1996), and from blood samples using a DNeasy™ Tissue Kit (Qiagen) according to the manufacturer's protocol.

All samples were genotyped using 14 polymorphic microsatellite markers developed for *Gymnobelideus leadbeateri* (GL4, GL5A, GL6, GL7, GL13, GL19B, GL24, GL28, GL33, GL35, GL38, GL39, GL42, GL44; Hansen *et al.* 2005) and one from another possum, the striped possum, *Dactylopsila trivirgata* (DT1; Hansen *et al.* 2003). Primer sequences and polymerase chain reaction (PCR) conditions for amplification of microsatellite markers are described in Hansen *et al.* (2005) except for DT1, which was amplified using touchdown cycling from 62 to 55 °C.

Statistical analysis of genetic diversity and structure

Standard measures of genetic diversity were obtained for the four largest population samples (Cambarville, Lake Mountain, Yellingbo and the captive colony). Conformance to Hardy–Weinberg expectations was tested using exact probability tests with 10 000 permutations in GENEPOP version 3.4 (Raymond & Rousset 1995). Linkage disequilibrium between every pair of loci in every population was tested in ARLEQUIN 3.11 (Excoffier *et al.* 2005) at α/c (0.05/no.loci*pops) (Quinn & Keough 2002). Pairwise population differentiation F_{ST} were calculated in ARLEQUIN and tested against a null distribution obtained by 10 000 permutations of genotypes between populations. Expected heterozygosity and allelic diversity were compared statistically using a Wilcoxon test for matched pairs.

Genotypes were analysed in STRUCTURE 2.0 (Pritchard *et al.* 2003), a model-based Bayesian clustering method that identifies genetic groups and probabilistically assigns individuals to them. The simulation was run with an initial

burn-in and thinning interval of 200 000 followed by 500 000 iterations, for five replicates of each K from 1–10.

To determine if our wild (excluding Yellingbo) plus captive samples adequately represent the total potential genetic diversity across the unsampled range of Leadbeater's possum, the cumulative number of alleles (for all alleles having a frequency of 0.05 or greater) was plotted as each individual was added. Five percent was chosen to represent the threshold for rare alleles (Sjögren & Wyöni 1993; Taylor & Cooper 1999). This was done twice; (i) by predefining rare alleles (which in this case was 89 out of a total of 200 sampled from the highlands) and plotting them as they appeared in the sample; and (ii) using alleles present at greater than 5% after the addition of each individual. All individuals were plotted in order of population, starting with those from Lake Mountain and finishing with those from the captive colony.

Analysis of bottlenecking patterns

A variety of genetic diversity measures was examined from the largest wild populations ($n > 25$ samples) of Lake Mountain and Yellingbo to reveal the presence (if any) of genetic bottleneck signatures in those populations. Three methods were used to test for bottleneck signatures in microsatellite data. The first was the M ratio of number of alleles k divided by the allelic size range r , averaged across all loci in each sample (Garza & Williamson 2001). This ratio is intended to quantify gaps in the allele size frequency distribution resulting from loss of alleles through bottlenecking. The critical value M_c for a bottlenecked population was computed separately for Lake Mountain and Yellingbo using the program CRITICAL_M (Garza & Williamson 2001). Starting values of theta, p_s (proportion of one-step mutations) and Δ_g (average size of non one-step mutations) for Lake Mountain were theta = 2 and 10, $p_s = 0.8$ and $\Delta_g = 3.3$, and for Yellingbo were theta = 1.5 and 4, $p_s = 0.4$ and $\Delta_g = 4.4$. Values lower than M_c tend to represent populations that have experienced a recent reduction in size. Loci with alleles that do not represent multiples of a recognized repeat unit violate the mutation models upon which this method hinges (Garza & Williamson 2001) and were not included in the simulation modelling process. On that basis, GL38 and GL44 were removed from calculations of the M ratio.

The second method compares gene diversity excess relative to that expected if a population were at mutation–drift equilibrium. That is, recently bottlenecked populations lose relatively more allelic diversity than heterozygosity, which results in a testable signal when more than 10 microsatellite loci are screened (using the software BOTTLENECK, Cornuet & Luikart 1996). The third method uses a Wilcoxon signed-rank test to evaluate estimators of bottleneck-induced distortion under a two-phase mutation model (TPM). TPM settings were 90% one-step changes

(stepwise mutation model) and 10% multistep changes (after the infinite allele model), with estimates based on 10 000 replications. BOTTLENECK also performs a qualitative graphical assessment ('mode-shift indicator') describing bottleneck-induced changes in allele frequency distributions (Luikart *et al.* 1998). We concluded that a bottleneck had occurred if all three methods were in agreement.

Bottleneck simulations

Thirty-four (out of a total of 53) microsatellite alleles present at frequencies of greater than 10% in the pooled highlands sample were absent from the large sample taken from Yellingbo, giving a first indication that sampled highlands populations were unlikely to have been the source of a recent Yellingbo introduction. To investigate this more quantitatively, two simulated populations established with the allele frequencies observed in the pooled Central Highlands sample (all five highlands populations) and the captive colony, respectively, were bottlenecked under various scenarios.

Bottleneck simulations were performed using modelling software, GENELOSS (England & Osler 2001), which randomly re-samples alleles in each of a given number of generations from replicate simulated populations created from starting allele frequencies. GENELOSS reports, per locus, the proportion of (in this case 1000) replicate bottlenecks in which a given allele is retained, as well as mean observed heterozygosity and the mean number of alleles per locus retained (allelic diversity).

Two levels of bottleneck intensity were simulated for each of the two source populations: intense (two breeding pairs, that is, eight randomly chosen alleles – each generation) and diffuse (50 breeding pairs each generation) (after England 1998). One generation was defined as two years, which is equivalent to the age at first breeding in this species (Lindenmayer & Lacy 1995; Harley 2005). Duration of simulated bottlenecks were 1, 5, 10 and 50 generations. One generation was intended to represent a single founder event (introduction) of brief duration. Five generations (10 years) was intended to represent the maximum bottleneck duration (1986 was chosen as a starting point –15 years after the first wild animal was acquired for captive breeding) that could still enable post-bottleneck reproduction to produce 12 breeding pairs (assuming that all offspring go on to breed). This number approximated the average annual number of breeding adults present in the population during the sampling period (Harley 2005). A bottleneck of 10 generations was chosen arbitrarily as an intermediate figure to the other simulations, and 50 generations was intended to represent the approximate amount of time that had elapsed between first European discovery of the species and the sampling period.

Allelic retention rates were used to determine the probability that source-population alleles could be absent in the

Table 1 Sample size (n), observed (H_O) and expected (H_E) heterozygosity, allelic diversity (A), percent unique alleles and loci deviating from Hardy–Weinberg expectations (HW disequilibrium) at 15 microsatellite loci for the five largest sampled populations of *Gymnobelideus leadbeateri* samples. NA means test not performed

Population	n	H_E	H_O	A	% unique	Loci with unique alleles	HW disequilibrium
Toolangi	2	0.72	0.77	2.4	2.8	GL4	NA
Mt Margaret	3	0.73	0.76	3.3	4.0	GL38 GL7	NA
Powelltown	4	0.65	0.83	2.9	4.5	GL38 GL5A	–
Cambarville	7	0.71	0.71	4.9	0	–	–
Lake Mountain	162	0.79	0.74	11.2	23.2	all except GL4 & GL5A	GL35 GL44
Yellingbo	198	0.55	0.53	3.4	5.9	GL38 GL35 GL33	GL4 GL19B
Captive colony	42	0.74	0.69	6.8	1.0	GL24	–

Yellingbo sample given a particular bottleneck scenario by multiplying rates across all retained alleles matching those in the Yellingbo sample (as in Taylor & Cooper 1999). Retention rates were also used to calculate allele frequencies representative of each bottleneck scenario, and the similarity of these to the observed Yellingbo gene pool was examined by calculation of pairwise F_{ST} . Although F_{ST} may not be strictly valid in this situation (where there is no obvious ancestral population) it nevertheless gives an indication of population genetic similarity between samples. Real and simulated population pairwise F_{ST} values were calculated from allele frequencies using modified Wright's F -statistics according to the following equation

$$F_{ST} = \frac{H_T - H_e}{H_T}$$

after Peakall & Smouse (2006). For consistency, F_{ST} values for the real population pairs were re-computed from genotypes using the same method (in GENALEX 6; Peakall & Smouse 2006). Pairwise F_{ST} values were plotted in a neighbour-joining tree using MEGA version 2.1 (Kumar *et al.* 2001) for convenient visualization of allele frequency similarities and dissimilarities (Supporting Information, Fig. S1).

Simulated allele frequencies were used to construct 100 genotypes (this figure represents the average annual population census size at Yellingbo; Harley 2005) in GEMINI (Valière *et al.* 2002). These genotypes were included in STRUCTURE analyses to determine if bottlenecks of any intensity could produce a population as strongly differentiated from other populations as Yellingbo.

Results

Genetic diversity and structure of real populations

Yellingbo exhibited significantly lower expected heterozygosity (all $P < 0.05$) than any other sampled population, and significantly lower allelic richness (all $P < 0.05$) than all

except Powelltown ($0.05 < P < 0.1$). By contrast, the captive colony showed negligible reduction in diversity compared to Lake Mountain and Cambarville (Table 1). Initially, genotypes were obtained from a single PCR, with unique alleles being verified in replicate PCRs. Genotype frequencies at both Lake Mountain (GL35 and GL44) and Yellingbo (GL4 and GL19B) deviated significantly from Hardy–Weinberg expectations (heterozygote deficit) (Table 1). Heterozygote deficits at one or a small number of loci may indicate the presence of null alleles. However, the trend for heterozygous deficits across most loci, the fact that the loci involved were not the same in the two populations, and the lack of evidence for null alleles in parentage analyses (Hansen & Taylor, unpublished) all suggest that null alleles were not the cause here. Rather, a Wahlund effect due to strong demic structure is a more likely explanation, given that our sampling included all animals encountered in nest boxes, which typically consist of family groups (Harley 2005). Furthermore, significant linkage disequilibria were detected for 57% and 27% of locus pairs (after Bonferroni correction) at Yellingbo and Lake Mountain, respectively. Disequilibria were not consistently caused by the same locus pairs in each population so are unlikely to be reflecting physical linkage. Strong linkage disequilibrium is also consistent with the presence of demic substructure in our sample. Such effects have been previously observed when families of related species showing similar social organization are sampled from nest-boxes (for example, sugar gliders *Petaurus breviceps*; Kendal 2008). This possibility will be further explored in future research.

Genetic differentiation for all sample pairs was highly significant (all F_{ST} values had $P \leq 0.00001$: Table 2), with the exception of that for Lake Mountain and Cambarville ($P \leq 0.01$), consistent with their close geographical proximity (Fig. 1). Yellingbo was most differentiated from all other populations, with all pairwise F_{ST} values being greater than 0.23 (Table 2) and highly significant. The lowest pairwise F_{ST} values for the captive colony were with the Lake Mountain and Cambarville populations, in agreement

	Cambarville	Lake Mountain	Powelltown	Yellingbo
F_{ST}				
Lake Mountain	0.084**			
Powelltown	0.203***	0.136***		
Yellingbo	0.302***	0.235***	0.358***	
Captive colony	0.131***	0.085***	0.172***	0.309***

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

Table 3 Results of the STRUCTURE analysis of all real populations. Bold indicates the best number of clusters K using the method of Pritchard *et al.* (2003) ($K = 7$) and Evanno *et al.* (2005) ($K = 2$)

K	$\ln P(X K)$	Var $\ln P(X K)$	ΔK
1	-10713.1	82.4	—
2	-8562.5	149.3	908.9
3	-8634.9	273.8	1.3
4	-8160.4	343.3	1.5
5	-8108.7	464.7	0.1
6	-8063.6	571.9	10.6
7	-7866.5	650.5	1.5
8	-8007.5	799.8	0.7
9	-7899.7	830.7	0.1
10	-7869.9	952.2	—

with the fact that many of these animals are descendents of individuals sourced from sites local to these populations.

Two methods were used to infer number of genetic clusters from STRUCTURE results for the combined wild and captive sample. The first is based on the recommendations of Pritchard *et al.* (2003) and involves computing the posterior probability of K ($P(K | X)$) from multiple replicates of each different value of K . The estimated number of clusters was seven (Table 3). The second method of interpretation after the method of Evanno *et al.* (2005), who use the second-order rate of change of the $\ln P(X | K)$ given by the value ΔK . ΔK is computed from the mean and standard deviation of the $\ln P(X | K)$; the maximum value provides the best estimate of the number of clusters, which for this data set was two (Table 3). Either way, meta-population subdivision was most strongly defined by the exclusion of Yellingbo from all other populations.

The accumulation of allelic diversity asymptotes at approximately 20 individuals, all from a Lake Mountain (Fig. 2). The subsequent addition of more individuals from the remainder of the highlands sample (and with it, the addition of new rare alleles) does not increase the accumulation of common allelic diversity. The first plot effectively represents the mean of the second plot (Fig. 2). The second plot fluctuates with the addition of every 10 individuals, the greatest fluctuation occurring between 107 alleles at $n = 10$ individuals to 64 alleles at $n = 11$ individuals. These

Table 2 Genetic differentiation (pairwise F_{ST}) computed in ARLEQUIN, between the five largest sampled populations of *G. leadbeateri*

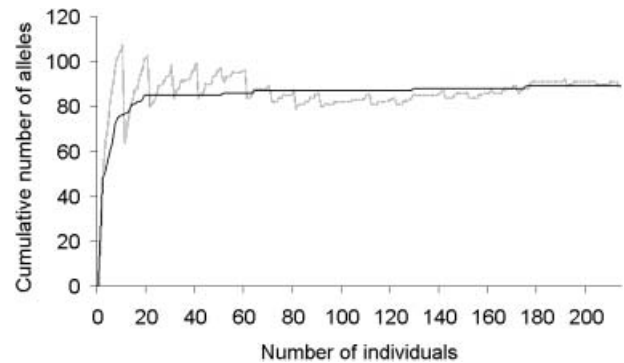


Fig. 2 Cumulative number of alleles (with frequency > 5% in pooled highlands and captive samples) plotted for each individual sample from all populations of *G. leadbeateri*, excluding Yellingbo. The solid line indicates the accumulation of new alleles present at 5% or higher in the total sample, and the dashed line indicates the accumulation of alleles at 5% or higher each time an individual is added.

fluctuations occur because (i) the addition of more alleles changes the total sample allele frequency distribution with each new individual; and (ii) common alleles become diluted by the accumulation of new alleles as individuals are added. The plateau in accumulation of allelic diversity indicates that our sampling has likely detected all except rare alleles and that the pooled sample captures the majority of meta-population genetic diversity within the central highlands.

Yellingbo alleles were extremely skewed in their relative size and frequency. In per-locus allelic size frequency plots, there was a bimodal distribution of allele size classes in at least seven loci (GL35, GL4, GL13, GL39, GL33, GL28 and DT1), with the pooled and captive source populations tending to contribute to one mode and Yellingbo to the other (see Appendix I). This further suggests that alleles at Yellingbo are not simply a subsample of those in potential source populations, as might be expected if Yellingbo were a bottlenecked founding population from the latter.

Bottlenecking patterns

The M ratio for the Lake Mountain sample was 0.729 and for Yellingbo was 0.606, confirming that numerous alleles

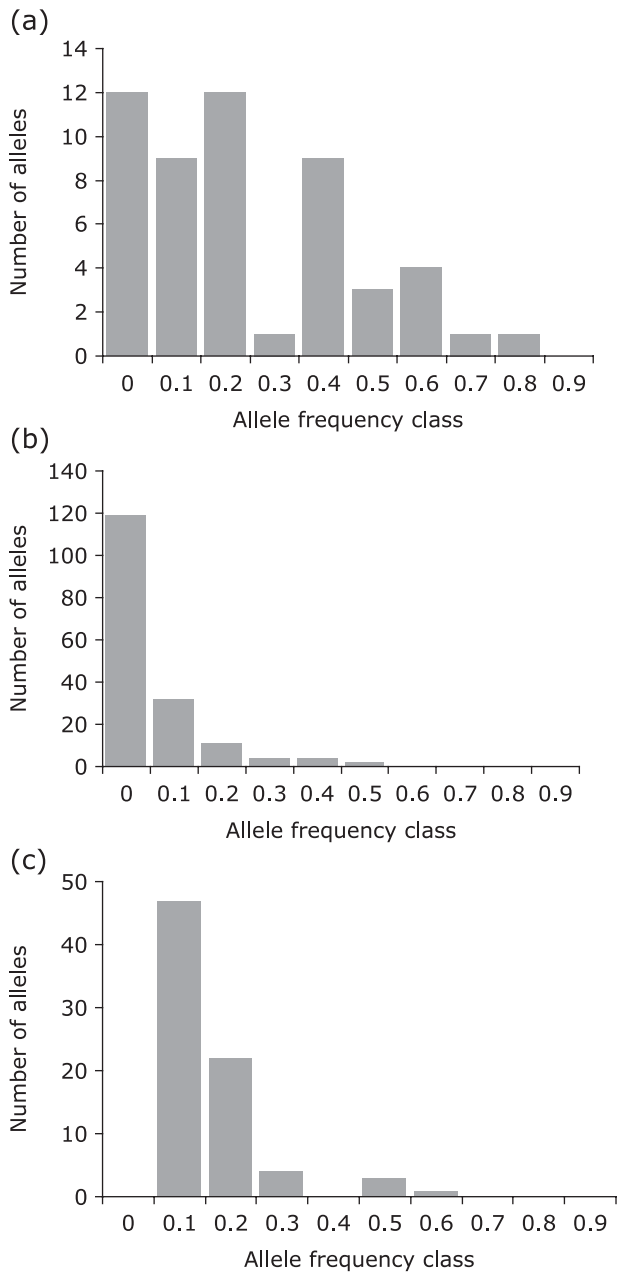


Fig. 3 Microsatellite allele frequency distributions for (a) Yellingbo (b) Lake Mountain and (c) one representative simulated bottlenecked population (CH int-1).

are absent from the Yellingbo sample relative to the total number we would expect to be present under the TPM. The critical value of M (M_c) generated for the Lake Mountain data set was 0.712 at $\theta = 2$, and 0.713 at $\theta = 10$, and for Yellingbo was 0.462 at $\theta = 1.5$ and 0.504 at $\theta = 4$. In the latter case, 88.4% of 10 000 simulated replicates would be below M for a population with $N_e = 750$ ($\theta = 1.5$) at equilibrium, indicating that there has probably been a reduction in population size (as inferred by the other methods; see below) but it may not be recent.

Table 4 Comparisons of genetic diversity between real and simulated *G. leadbeateri* populations. A refers to allelic diversity for real populations and its equivalent, average allelic retention, for simulations. H is expected heterozygosity for real populations and observed heterozygosity for simulated populations (output from GENELOSS). The probability (P) that common alleles in the source population could be absent from Yellingbo following each bottleneck scenario is given

Population	H	A	P
<i>Non-bottlenecked</i>			
Lake Mountain	0.74	11.2	—
Captive colony	0.74	6.8	—
<i>Bottlenecked</i>			
Yellingbo	0.53	3.4	—
<i>Simulated introduction</i>			
CH int-1	0.68	4.6	1.6×10^{-17}
CH int-5	0.41	2.3	2.1×10^{-32}
CC int-1	0.60	3.7	0.0
CC int-5	0.35	2.1	2.8×10^{-14}
<i>Simulated remnant</i>			
CH dif-10	0.62	4.3	5.5×10^{-19}
CH dif-50	0.22	1.7	3.9×10^{-30}
CC dif-10	0.64	5.7	0.0
CC dif-50	0.52	3.6	0.0

Source populations are CH (Central Highlands), and CC (captive colony).

Bottleneck severity is represented by: int (intense = two breeding pairs) and dif (diffuse = 50 breeding pairs) (adapted from England 1998).

Number of generations in bottleneck is shown as the suffix.

The Wilcoxon test (in BOTTLENECK) revealed significantly higher gene diversity than expected for the observed allelic diversity at Yellingbo ($P = 0.0007$, indicative of a recent bottleneck) but not Lake Mountain ($P = 0.7727$). Analyses of bottleneck-induced distortion of allele frequencies indicated an allele frequency distribution mode-shift (Fig. 3a) at Yellingbo, but a normal L-shaped mode for Lake Mountain, as expected for non-bottlenecked population at mutation-drift equilibrium (Fig. 3b). The strength of bottleneck patterns at Yellingbo confirms that this population has undergone a recent reduction in size; conversely, the absence of these patterns at Lake Mountain suggests otherwise.

Bottleneck simulations

Simulated bottlenecks were highly sensitive to starting allele frequency. This is not surprising as virtually all bottleneck detection methods acknowledge that changes in relative allele frequencies are a signal of a genetic bottleneck (Nei *et al.* 1975; Frankham *et al.* 2004). As expected, all bottleneck simulations resulted in a loss of allelic diversity and heterozygosity relative to the original source (Table 4). Initially, allele frequencies from 10 randomly chosen

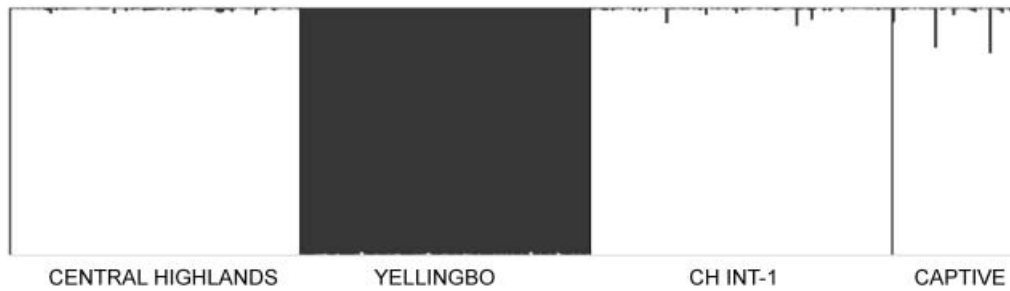


Fig. 4 STRUCTURE plot of genotypic patterns in all real populations and a representative simulated bottlenecked population CH int-1. Shaded bars indicate the proportion of an individual's membership to a given genetic cluster K (where $K = 2$). The abbreviation CAPTIVE refers to the captive colony.

replicate bottlenecks (per scenario) were examined and compared using the probability of allelic retention and F_{ST} comparisons to the Yellingbo sample. The average allelic retention rate was subsequently used to summarize the bottlenecking patterns (probability of retention and F_{ST}) per scenario. In no single replicate, nor in the average, were alleles retained at frequencies similar to those observed in the Yellingbo sample. This was also true when bottlenecking the captive colony source population. Allele frequencies in all simulated populations regardless of source or bottleneck type (intense or diffuse) were substantially more similar to those at Lake Mountain ($F_{ST} = 0.006$ – 0.053) and the captive colony ($F_{ST} = 0.001$ – 0.061) than to Yellingbo ($F_{ST} = 0.133$ – 0.191) (Supporting Information, Figs S1 and S2).

The probability that a founding population could have lost the alleles that are found to be absent from the Yellingbo sample was effectively zero, depending on the bottleneck scenario (Table 4). In particular, the probability that alleles could have been lost from a population founded with captive colony animals in such a short time was effectively zero. The allele frequency distribution of one representative simulated bottleneck replicate, which clearly demonstrates the mode-shift, is shown in Fig. 3(c).

Consistent with the allele frequency patterns produced by artificial bottlenecking, replicate simulated bottlenecked populations did not cluster with the Yellingbo population in any STRUCTURE analysis. Population subdivision in all bottleneck scenarios was best described by $K = 2$. This pattern was strong and consistent over different bottleneck intensities and durations. Each replicate simulation produced very similar results in STRUCTURE, so only the most conservative outcome, that is, a randomly chosen replicate from the simulation scenario with average allele frequencies most closely resembling those at Yellingbo (measured by F_{ST}), is presented (Fig. 4). It is clear that, using either candidate source population, be it the sampled highlands populations or the captive colony, the Yellingbo bottleneck could not be replicated.

Discussion

Yellingbo is the only known *Gymnobelideus leadbeateri* population surviving in the lowland swamp habitat type from which the species was first described (Smales 1994; Macfarlane *et al.* 1998, 2003; Harley 2004; Harley *et al.* 2004). Individuals residing there are geographically and demographically separated from conspecifics in the mountain ash forest of the Central Highlands of Victoria (Smales 1994; Harley 2005). Genetic analyses support the hypothesis that Yellingbo is an isolated population losing genetic diversity as a function of its small size and isolation. Its allele frequency patterns are consistent with bottleneck-induced distortion resulting from a loss of rare alleles, and there is low allelic diversity and heterozygosity, as commonly seen in other bottlenecked populations (see for example Taylor *et al.* 1994; Garza & Williamson 2001; Hellborg *et al.* 2002; England *et al.* 2003; Eldridge *et al.* 2004; Kraaijeveld-Smit *et al.* 2005).

Although a standard suite of tests identified Yellingbo as a bottlenecked population they could not indicate whether this was due to it being a recent introduction or a remnant from a more widespread distribution. Simulations were employed to distinguish these two scenarios. All simulated bottlenecked populations clustered closely with the real populations from the Central Highlands, to the exclusion of Yellingbo. Over a range of scenarios, simulated bottlenecks for the known potential source populations did not approximate genetic patterns at Yellingbo. This effect was consistently strong, indicating that this population is not recently descended from one like Lake Mountain or any other in its immediate vicinity and unlikely to be descended from any other sampled extant Central Highlands population. Moreover, it certainly did not arise from an introduction event in the last 100 years (50 generations). Thus, we conclude that the Yellingbo population has as its source an ancestral gene pool either too poorly represented in highlands populations to have been sampled, or from a location separate from the highlands (and its derivative captive colony).

The sample used in this study encompasses populations from the southern (Powelltown) and northern (Lake Mountain/Cambarville) parts of the core range, and presumably a significant proportion of the genetic diversity as well. This was demonstrated in the allele frequency accumulation curve where, at a sample size of 20 individuals (from a single population), virtually all alleles occurring at a frequency of 5% or higher had been detected. Thus, we are confident that the addition of more sampling sites to the highlands source gene pool would not alter the sample allele frequency distribution, such that a bottleneck of that source could replicate the Yellingbo scenario. Therefore, we consider it implausible that Yellingbo was founded by any un-sampled extant highlands population(s).

Our evidence indicates that Yellingbo has not recently descended from populations like Lake Mountain or Cambarville, and that it is therefore unlikely to be a recent remnant from the Central Highlands. Instead, we propose that the Yellingbo gene pool is a recent remnant from a broader, now extinct lowland/swamp population. This assertion is buoyed by speculation in the literature that Yellingbo may be a surviving representative of now extinct coastal populations of the Western Port region (Smales 1994; see also Harley 2004 for an overview). This speculation is largely based upon habitat similarities between the two locations. Part of the Western Port region was lowland swamp, drained to make way for agriculture in the early 20th century (Brazenor 1946). Anecdotal historical evidence suggests that prior to its reclamation, this swamp may have been *Melaleuca* and *Eucalyptus ovata* dominated, similar to that occurring at Yellingbo (Smales 1994). Swamp habitat of this type is largely absent from the Central Highlands.

If Yellingbo is indeed a remnant from an extinct population or meta-population, its degree and nature of genetic distinctiveness may indicate long-term separation of *G. leadbeateri* populations according to habitat type. This postulation has significant conservation implications, because it suggests that *G. leadbeateri* populations from the two habitat types not only constitute separate management units but may also fit the criteria of Evolutionary Significant Units (Moritz 1995). Mitochondrial genetic data are necessary to assess this proposition in more detail. Nevertheless, geographical isolation and habitat specialization of the kind proposed here may be sufficient evidence to justify this separation (Moritz 1995; Frankham *et al.* 2004). We would therefore advise caution in mixing animals sourced from different habitat types for breeding or translocation programmes, until it can be ascertained whether they represent entities that differ in important adaptive characteristics. Our mitochondrial DNA sequence analyses in progress, including samples from the recently extinct Western Port populations, may help pinpoint the timing and nature of the Yellingbo population divergence. Experiments on ecological exchangeability of the form suggested

by Rader *et al.* (2005) would be warranted if they can be conducted without deleterious impacts on populations.

Conclusion

We have shown that simulating various population genetic scenarios using high resolution genetic data is a powerful approach to inferring population ancestry. Standard genetic and genotypic differentiation analyses are insufficient for distinguishing between alternative historical scenarios in the absence of precise information on both separation times and population sizes. By contrast, simulations as employed here allow exploration of the possible genetic outcomes of scenarios incorporating a range of possible values for these parameters. Such a rigorous, quantitative approach has left little doubt that the Yellingbo population of *Gymnobelideus leadbeateri* is an important remnant of a previously more widespread, genetically divergent lowland swamp form of the species whose persistence and separate management may be important for maintaining historical genetic diversity and adaptation, particularly in the face of ongoing loss of habitat. There are clear management implications associated with this finding, and these are currently being incorporated into the updated Leadbeater's Possum Action Statement (Macfarlane *et al.* 2003). Our work demonstrates a useful approach for analysis of other threatened species with restricted ranges and uncertain divergence history, and also those that have been translocated outside their native range, given the often poor historical records associated with such programmes.

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References

- Brazenor CW (1946) Last chapter to come. A history of Victoria's rarest possum. *Wild Life*, **8**, 382–384.
- Brazenor CW (1962) Rediscovery of a rare Australian possum. *Proceedings of the Zoological Society of London*, **139**, 429–431.
- Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001–2014.
- Eldridge MDB, Browning TL, Close RL (2001) Provenance of a New Zealand brush-tailed rock-wallaby (*Petrogale penicillata*)

- population determined by mitochondrial DNA sequence analysis. *Molecular Ecology*, **10**, 2561–2567.
- Eldridge MDB, Rummery C, Bray C *et al.* (2004) Genetic analysis of a population crash in brush-tailed rock-wallabies (*Petrogale penicillata*), from Jenolan Caves, south-eastern Australia. *Wildlife Research*, **31**, 229–240.
- England PR (1998) *Conservation genetics of population bottlenecks*, PhD Thesis, Macquarie University.
- England PR, Osler GHR (2001) GENELOSS: a computer program for simulating the effects of population bottlenecks on genetic diversity. *Molecular Ecology Notes*, **1**, 111–113.
- England PR, Osler GHR, Woodworth LM *et al.* (2003) Effect of intense versus diffuse population bottlenecks on microsatellite genetic diversity and evolutionary potential. *Conservation Genetics*, **4**, 595–604.
- Evanno G, Regnaut S, Goudel J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.
- Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.
- Frankham R (2005) Genetics and extinction. *Biological Conservation*, **126**, 131–140.
- Frankham R, Ballou JD, Briscoe DA (2004) *A Primer of Conservation Genetics*. Cambridge University Press, Cambridge.
- Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology*, **10**, 305–318.
- Hansen BD, French J, Handasyde KA *et al.* (2003) A set of microsatellite primers for the striped possum, *Dactylopsila trivirgata* (Petauridae: Marsupialia). *Molecular Ecology Notes*, **3**, 212–214.
- Hansen BD, Sunnucks P, Blacket M, Taylor AC (2005) A set of microsatellite markers for an endangered arboreal marsupial, Leadbeater's possum. *Molecular Ecology Notes*, **5**, 796–799.
- Harley D (2002) The discovery of Leadbeater's Possum *Gymnobelideus leadbeateri* along the Woori Yallock Creek, Yellingbo. *Victorian Naturalist*, **119**, 233–235.
- Harley DKP (2004) A Review of Recent Records of Leadbeater's Possum (*Gymnobelideus leadbeateri*). In: *The Biology of Australian Possums and Gliders* (eds Goldingay RL, Jackson SM), pp. 330–338. Surrey Beatty & Sons, Chipping Norton.
- Harley DKP (2005) *The life history and conservation of Leadbeater's Possum (Gymnobelideus leadbeateri) in lowland swamp forest*. PhD Thesis, Monash University.
- Harley D (2006) The Yellingbo population of Leadbeater's Possum – remnant or introduced? *Victorian Naturalist*, **123**, 170–173.
- Harley DKP, Lill A (2007) Reproduction in a population of the endangered Leadbeater's possum inhabiting lowland swamp forest. *Journal of Zoology*, **272**, 451–457.
- Harley DKP, Worley MA, Harley TK (2004) The distribution and abundance of Leadbeater's possum *Gymnobelideus leadbeateri* in lowland swamp forest at Yellingbo Nature Conservation Reserve. *Australian Mammalogy*, **27**, 7–15.
- Hellborg L, Walker CW, Rueness EK *et al.* (2002) Differentiation and levels of genetic variation in northern European lynx (*Lynx lynx*) populations revealed by microsatellites and mitochondrial DNA analysis. *Conservation Genetics*, **3**, 97–111.
- Kendall P (2008) *Molecular Population Ecology of the Sugar Glider (Petaurus breviceps) in Fragmented and Unfragmented Habitat*. PhD Thesis, Monash University.
- Kraaijeveld-Smit FJL, Beebe TJC, Griffiths RA, Moore RD, Schley L (2005) Low gene flow but high genetic diversity in the threatened Mallorcan midwife toad *Alytes muletensis*. *Molecular Ecology*, **14**, 3307–3315.
- Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**, 1244–1245.
- Lindenmayer DB (2000) Factors at multiple scales affecting distribution patterns and their implication for animal conservation – Leadbeater's Possum as a case study. *Biodiversity and Conservation*, **9**, 15–35.
- Lindenmayer DB, Lacy RC (1995) Metapopulation viability of Leadbeater's possum, *Gymnobelideus leadbeateri*, in fragmented old-growth forests. *Ecological Applications*, **5**, 164–182.
- Lindenmayer DB, Meggs RA (1996) Use of Den Trees by Leadbeater's Possum (*Gymnobelideus leadbeateri*). *Australian Journal of Zoology*, **44**, 625–638.
- Lindenmayer DB, Possingham HP (1995) Modelling the viability of metapopulations of the endangered Leadbeater's possum in south-eastern Australia. *Biodiversity and Conservation*, **4**, 984–1018.
- Lindenmayer DB, Smith AP, Craig SA, Lumsden LF (1989) A survey of the distribution of Leadbeater's Possum, *Gymnobelideus leadbeateri* McCoy in the Central Highlands of Victoria. *Victorian Naturalist*, **106**, 174–178.
- Lindenmayer DB, Nix HA, McMahon JP *et al.* (1991) The conservation of Leadbeater's possum, *Gymnobelideus leadbeateri* (McCoy): a case study of the use of bioclimatic modelling. *Journal of Biogeography*, **18**, 371–383.
- Luikart G, Allendorf FW, Cornuet JM, Sherwin WB (1998) Distortion of Allele Frequency Distributions Provides a Test for Recent Population Bottlenecks. *Journal of Heredity*, **89**, 238–247.
- Macfarlane MA, Seebeck JH (1991) Draft management strategies for the conservation of Leadbeater's Possum, *Gymnobelideus leadbeateri*, in Victoria. *Arthur Rylah Institute Technical Report Series no. 111*. Department of Conservation and Environment, Melbourne.
- Macfarlane MA, Smith J, Lowe K (1998) *Leadbeater's Possum Recovery Plan 1998–2002*. Department of Natural Resources and Environment, Melbourne.
- Macfarlane MA, Lowe K, Smith J (2003) *Flora and Fauna Guarantee Action Statement: Leadbeater's Possum Gymnobelideus Leadbeateri*. Department of Sustainability and Environment, Victoria. Available at <http://www.dse.vic.gov.au>.
- Marshall TC, Spalton JA (2000) Simultaneous inbreeding and outbreeding depression in reintroduced Arabian oryx. *Animal Conservation*, **3**, 241–248.
- Moritz C (1995) Uses of molecular phylogenies for conservation. *Philosophical Transactions of the Royal Society of London Series B*, **349**, 113–118.
- Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1–10.
- Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.
- Pritchard JK, Stephens M, Donnelly P (2003) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 845–859.
- Quinn GP, Keough MJ (2002) *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, Port Melbourne.
- Rader RB, Belk MC, Shiozawa DK, Crandall KA (2005) Empirical test for ecological exchangeability. *Animal Conservation*, **8**, 239–247.
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

- Sjögren P, Wyöni P (1993) Conservation genetics and detection of rare alleles in Finite Populations. *Conservation Biology*, **8**, 267–270.
- Smales IJ (1994) The discovery of Leadbeater's Possum, *Gymnobelideus leadbeateri* McCoy, Resident in a Lowland Swamp Woodland. *Victorian Naturalist*, **111**, 178–182.
- Smith A (1984) Diet of Leadbeaters Possum, *Gymnobelideus leadbeateri* (Marsupialia). *Australian Wildlife Research*, **11**, 265–273.
- Smith AP, Lindenmayer D (1988) Tree Hollow Requirements of Leadbeater's Possum and Other Possums and Gliders in Timber Production Ash Forests of the Victorian Central Highlands. *Australian Wildlife Research*, **15**, 347–362.
- Smith A, Lindenmayer D, Suckling G (1985) *The Ecology and Management of Leadbeater's Possum*. Research Report to the World Wildlife Fund Australia. University of New England.
- Sunnucks P, Hales DF (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biology and Evolution*, **13**, 510–524.
- Taylor AC, Cooper DW (1999) Microsatellites identify introduced New Zealand tamar wallabies (*Macropus eugenii*) as an 'extinct' taxon. *Animal Conservation*, **2**, 41–49.
- Taylor AC, Sherwin WB, Wayne RK (1994) Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiornhinus krefftii*. *Molecular Ecology*, **3**, 277–290.
- Valière N, Berthier P, Mouchiroud D, Pontier D (2002) GEMINI: software for testing the effects of genotyping errors and multi-tubes approach for individual identification. *Molecular Ecology Notes*, **2**, 83–86.
- Wilkinson HE (1961) The Rediscovery of Leadbeater's possum, *Gymnobelideus leadbeateri* McCoy. *Victorian Naturalist*, **78**, 97–102.

This study forms part of Birgita Hansen's PhD research on conservation genetics of Leadbeater's possum. 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.

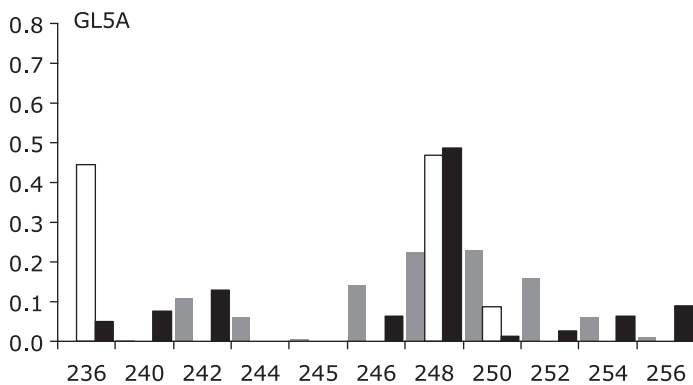
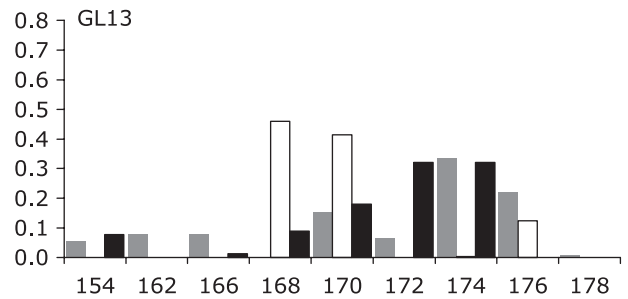
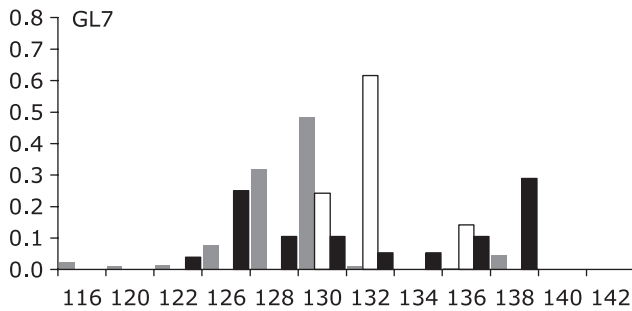
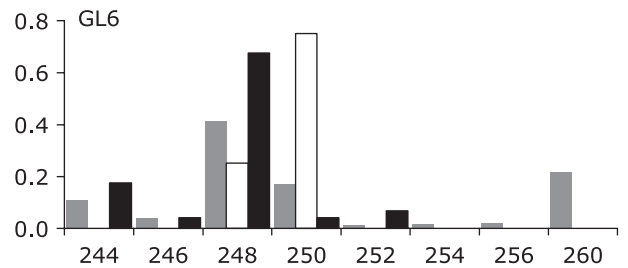
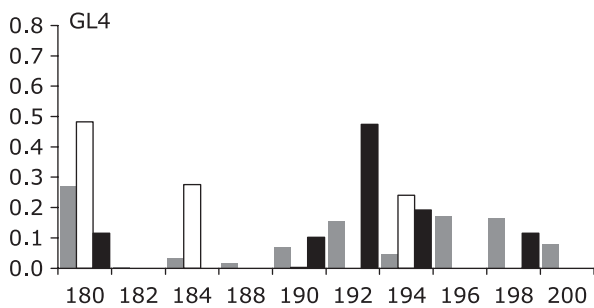
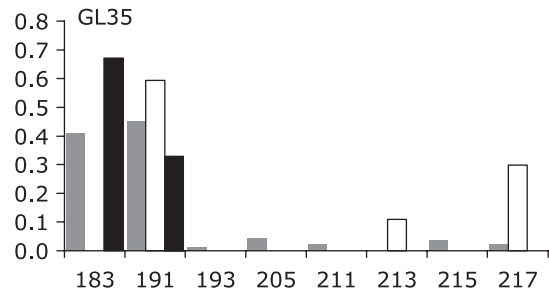
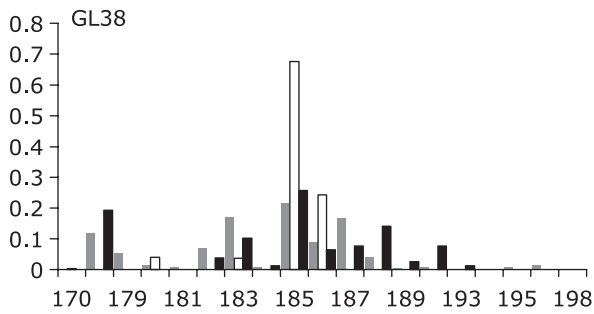
Fig. S1 Neighbour-joining (NJ) tree of pairwise F_{ST} values (calculated using allele frequencies) for real (Yellingbo, Lake Mountain and CAPTIVE) and simulated populations (CH, central highlands source; CC, captive colony source).

Fig. S2 Population pairwise F_{ST} values (computed from allele frequencies) for comparisons of each real and simulated population. LM refers to Lake Mountain, YELL is Yellingbo and CC is the captive colony.

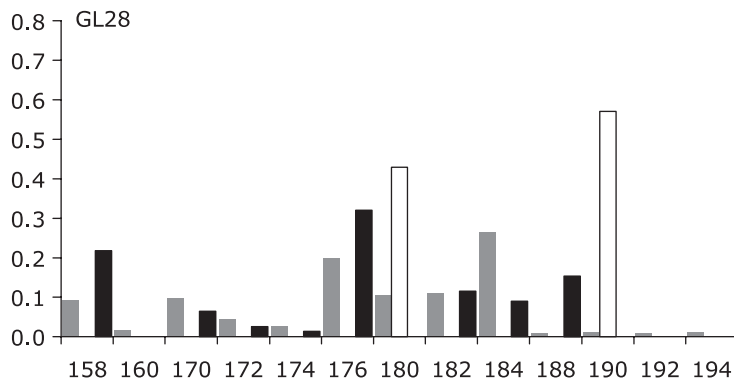
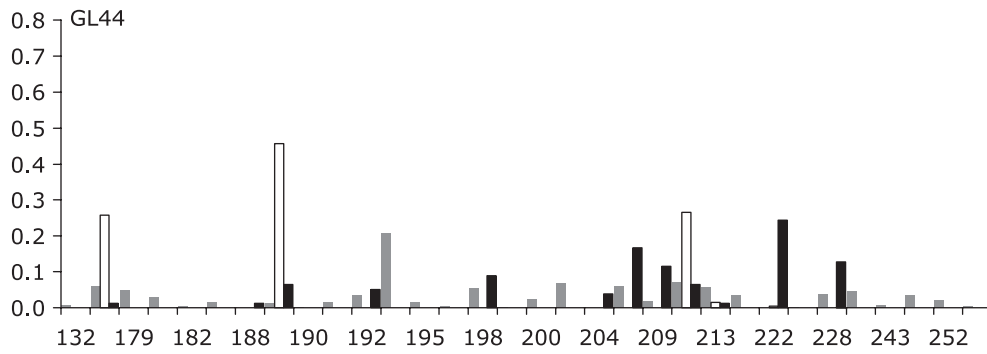
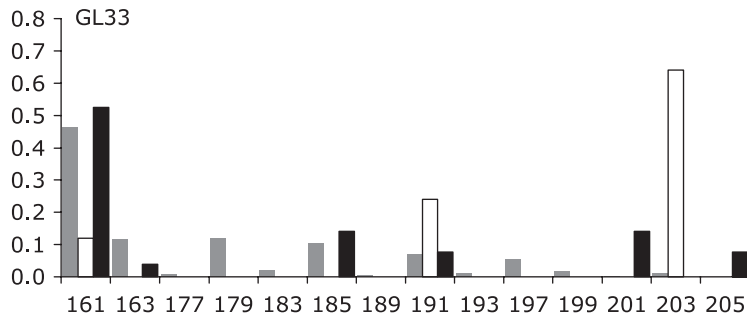
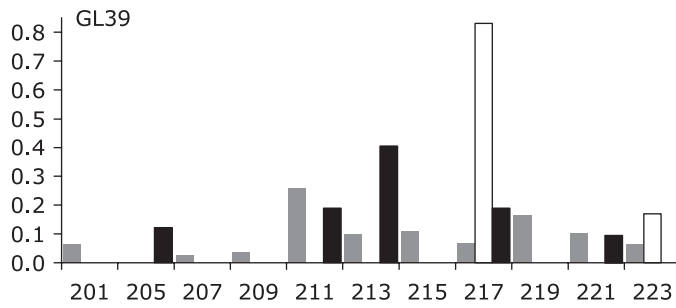
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Appendix I

Frequency of allele sizes (in bp) of 15 microsatellite loci for *Gymnobelideus leadbeateri*. Grey bars are the pooled central highlands sample, black bars are the captive colony and white bars are Yellingbo. The allele frequency is given on the Y axis and allele sizes for each locus are given on the X axis.



Appendix I Continued



Appendix I *Continued*

