A species in decline: genetic diversity and conservation of the Victorian eastern barred bandicoot, *Perameles gunnii*

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ABSTRACT

The eastern barred bandicoot, *Perameles gunnii*, has undergone a dramatic decline in distribution and abundance on the mainland of Australia during the 20th century. In 1988 a captive breeding program was initiated to reduce the chance of extinction. With the extinction of the last wild mainland population in the early 1990's, reintroductions from captive-bred *P. gunnii* have met limited success, and currently only two extant populations persist in predator proof enclosures in the State of Victoria. With ~20 years of breeding, there are concerns that the genetic diversity within the breeding program has declined and may inhibit current and future success of the program. We have used ten nuclear microsatellite loci and sequencing of two partial mitochondrial genes (*cytochrome oxidase I* and *ATPase 6*) to determine genetic diversity within current Victorian *P. gunnii*. These diversity estimates are compared with historic samples from the captive breeding program dating back to 1995, historic samples from the last wild mainland population found at Hamilton in 1992 and contemporary Tasmanian wild populations. Results indicate that the captive *P. gunnii* population in the State of Victoria has lost significant genetic diversity through time. Genetic diversity is also reduced in populations at Hamilton Community Parklands and Mount Rothwell. Samples from the last wild population at Hamilton collected in 1992, along with samples from Tasmanian *P. gunnii*, had significantly greater genetic diversity than contemporary mainland populations. The results are discussed with reference to management options for maintaining genetic diversity within Victorian *P. gunnii*, including crossing Victorian and Tasmanian *P. gunnii* to increase genetic diversity, adaptability and evolutionary potential.

KEYWORDS: CONSERVATION GENETICS, MICROSATELLITE, EFFECTIVE POPULATION SIZE, CAPTIVE BREEDING PROGRAM.

INTRODUCTION

The resilience of populations to environmental change, parasites and resource competition depends largely on the genetic variation present in quantitative traits, recruitment and gene flow (Hoffmann and Parsons, 1997). Genetic studies are increasingly part of natural resource management because they can either directly or indirectly estimate these important parameters. Genetic tools can provide information on the evolutionary history, population size, current levels of genetic diversity and gene flow, and other key factors contributing to the fitness of populations (Hedrick, 1999). Importantly, they can also be used as tools in captive breeding programs to manage genetic diversity, determine levels of inbreeding, assess parentage and integrate genotypic data into pedigree charts. Understanding the impacts of captive rearing on genetic diversity is integral to the viability of re-introduction programs, where it is assumed that losses of neutral diversity (commonly assessed by neutral markers) will also indicate a loss of genetic diversity in quantitative traits that are important for fitness, particularly in changing environments (Markert et al. 2010).

The eastern barred bandicoot, *Perameles gunnii*, is a small terrestrial marsupial found in south-eastern Australia. The species is highly fecund with up to five litters per year with an average of 2-3 young per litter, gestation period of 12 days, and sexual maturity reached at 3 months (females) or 5 months (males) (Winnard and Coulson 2008). This species once had a distribution on mainland Australia that extended from Melbourne, through the western part of the State of Victoria to the south-eastern corner of South Australia (DSE Victoria 2009). Since European settlement, however, the distribution and abundance of *P. gunnii* on the mainland has gone through a dramatic decline due largely to the invasive predatory fox (*Vulpes vulpes*) and is now considered extinct in the wild (DSE Victoria 2009). Wild populations of *P. gunnii* are still found in Tasmania (which is largely fox free), with its distribution restricted to the north and south-eastern parts of the state. Populations in Tasmania are also thought to be declining, and the species is listed as *near threatened* nationally by the IUCN (Menkhorst and Richards 2008).

The last known population of mainland *P. gunnii* was found at Hamilton, in western Victoria. It was recognised as being on the verge of extinction in 1991, and this is

supported by a lack of confirmed sightings in the wild since 1993 (DSE Victoria 2009). A captive colony was initiated in 1988, first managed by the Department of Natural Resources and Environment at Woodlands Historic Parklands and in 1992 taken over by Zoos Victoria. The colony started with a base of 40 wild individuals from the Hamilton area, however, due to unequal reproductive contributions it is thought that the effective population size may have been reduced to 19 individuals early on in the breeding program (Myroniuk 1993). Over the last 20 years, individuals from the captive colony have been released at various sites in the State of Victoria. These sites contain either predator proof enclosures (Hamilton Community Parklands and Mount Rothwell), or a combination of semi-predator proof fencing and/or fox control programs (Mooramong, Woodlands, Floating Islands Nature Reserve, Lake Goldsmith Wildlife Reserve, Lanark, Cobra Killuc Wildlife Reserve). Currently, only Hamilton Community Parklands and Mount Rothwell are known to support *P. gunnii* populations. The Mount Rothwell population was established in 2004 and founded by 22 released individuals, with a current population size estimate of >200 individuals (Winnard and Coulson 2008; R. Hill, pers. comm.). Releases at Hamilton Community Parklands began in 1989 and continued until 2003 (Winnard and Coulson 2008). In 2005, the population was presumed extinct due to a lack of captures and sightings over the six previous years. The demise of the population was attributed to several breaches of the fencing by foxes, and therefore significant upgrades to the predator proof fencing were undertaken. In 2007, 30 captive-bred individuals were released at Hamilton Community Parklands and current trapping efforts estimate the population size to be approximately 70 individuals (R. Hill, pers. comm.). Fox-free habitat appears critical to maintaining successful *P. gunnii* populations.

Despite the *P. gunnii* captive breeding program producing and releasing over 700 individuals in a 20 year period, there has been no assessment of genetic diversity within this program. Given that the initial underlying relationships between the founders of this program were not known, and given the low number of individuals that contributed to offspring (between 19 and 40 wild founders; Myronuik 1993), there are concerns that genetic diversity may be low within the captive breeding program and this may be reducing the fitness and adaptability of released individuals (e.g. inbreeding depression). This has been compounded by the recent finding of a morphological deformity (undercut jaw) in offspring from a breeding pair in the

captive breeding program at Zoos Victoria (Peter Courtney, pers. comm.). These individuals were released into the Hamilton Community Parklands population, and the same undercut jaw phenotype has since been found in wild-bred young (Amy Winnard, pers. comm.). It is not known whether this phenotype is a spontaneous mutation or heritable, nor if it causes any fitness effects.

Previous genetic studies (Robinson et al. 1993; Robinson 1995) have indicated that Victorian *P. gunnii* have greater genetic diversity and are genetically differentiated from Tasmanian *P. gunnii*, with Robinson (1995) suggesting that they be called different sub-species. Here we use ten microsatellite markers to determine levels of genetic variation in *P. gunnii* from Hamilton Community Parklands and Mount Rothwell using hair samples collected in 2008/09. We also assess genetic variation in broodstock individuals currently held at Zoos Victoria, historic samples taken from the captive breeding program broodstock dating back to 1995 and samples taken from the now extinct wild population at Hamilton in 1992. Levels of genetic variation are compared to determine the impacts of the breeding program through time. Also estimates of genetic variation using microsatellites and DNA sequence data from two mitochondrial genes were compared with contemporary Tasmanian samples to provide insight into historical relationships between Tasmanian and mainland populations.

METHODS

Sample Collection

Perameles gunnii hair samples were collected from two locations in the State of Victoria [Hamilton Community Parklands (37° 43’ 22” S, 142° 1’ 22” E) and Mount Rothwell Sanctuary (37° 53’ 46” S, 144° 26’ 25” E)] between February 2008 and June 2009. A total of 25 individuals were collected from Hamilton Community Parklands, while 18 individuals were collected from Mount Rothwell. Hair samples were also collected from *P. gunnii* wild individuals in the north (n = 18) and south (n = 51) of Tasmania between March and December 2008. Collection sites of contemporary *P. gunnii* in Tasmania and Victoria are shown in Figure 1. All hair samples were placed in 100% ethanol in 2 ml microcentrifuge tubes and stored at -20 °C until DNA extraction.

Hair samples were also taken from individuals that are held at Zoos Victoria (n = 47) and currently forming the captive breeding population of *P. gunnii* (individuals that have bred or have been kept for future breeding). Zoos Victoria also took hair and tissue samples from *P. gunnii* individuals in the captive breeding program dating back to 1995, with samples stored in 100% ethanol at -20 °C. We extracted DNA from historic *P. gunnii* sampled from the captive breeding program in 1995 (tissue samples, n = 23) and 2002 (tissue samples, n = 33). Finally, we obtained DNA samples from the now extinct wild population at Hamilton, collected in 1992 (n = 32) and used by Robinson (1995).

DNA Extraction, Genotyping and Sequencing

Extraction of genomic DNA from hair samples was performed with Chelex (Bio-Rad™) following the protocol outlined in Mitrovski et al. (2005). Briefly, ten hairs with follicles were transferred to a 0.5 ml microcentrifuge tube that was placed in liquid nitrogen (1 min) and then centrifuged at 20,800 g for 1 min to move the hairs to the bottom of the tube. 200 µl of a 5% Chelex solution was added, samples were mixed, incubated at 90 °C for 10 min and stored at -20 °C. Immediately prior to PCR, samples were centrifuged at 20,800 g for 2 min.

Genomic DNA was extracted from tissue samples (ear punch) using a phenol-chloroform extraction method (Sambrook and Russell 2001). Approximately 5 mg of tissue from an ear clipping was placed in a 2 ml microcentrifuge tube and 500 µl of lysis buffer [20 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 400 mM NaCl, 1% (w/v) SDS, 400 µg/ml proteinase K] was added to each tube and incubated overnight at 55 °C. After incubation, 500 µl of phenol: chloroform:isoamyl alcohol (25:24:1) was added to each tube and inversion mixed for 1 min, followed by centrifugation at 20,800 g for 5 min. The upper aqueous phase was then transferred to a new 1.5 ml microcentrifuge tube and 1.5 volumes of cold isopropanol added to precipitate the DNA. The samples were left for 1 h in the -20 °C freezer and then centrifuged at 20,800 g for 15 min at 4 °C. The isopropanol was removed and 1 ml of 70% ethanol added, mixed by inversion and then centrifuged at 20,800 g for 5 min. The ethanol

was then removed, the DNA pellet dried and 100 µl of sterile water added to dissolve the pellet. Extractions were stored at -20 °C until required.

All individuals were genotyped for six microsatellite loci (B3-2, B7-2, B15-1, B20-5, B34-1, B34-2) that were originally characterized for the southern brown bandicoot (Zenger and Johnston 2001). Additionally, we developed four new microsatellite markers using the 454-sequencing platform. For this, approximately 10 µg of genomic DNA was extracted from tissue of one specimen of *P. gunnii* using a QIAGEN DNA Easy kit (Qiagen). The prepared DNA was sent to the Australian Genome Research Facility for 454 sequence analysis (AGRF, Queensland, Australia). DNA was nebulised, ligated with 454 adapters and subjected to high throughput DNA sequencing on 1/16 of a 70 x 75 mm PicoTiterPlate using the Roche GS FLX (454) system (Margulies et al. 2005). The software GDD (Meglécz et al. 2010) and PRIMER 3 (Rozen and Skaletsky 2000) were used to select unique sequence contigs (1,008) containing repeat units and design 20 primer sets. We assessed polymorphisms of loci by screening eight *P. gunnii* individuals sampled in 2009 from the captive breeding program at Zoos Victoria. Primers were pooled into groups of four where they were co-amplified by multiplex PCR using a Qiagen multiplex kit. Four new polymorphic loci were identified using the following primer combinations: (i) PG20 (F: 5'-GCCAAGAATACCTGGGTTCA-3'; R: 5'-CACAAAACTATGCTTACTGTGCG-3'), (ii) PG36 (F: 5'-CAAAAGTTCCAGAAAAGTGTGG-3'; R: 5'-TCTTCTTGTCCAGTCACTCTTG-3'), (iii) PG38 (F: 5'-ATGGCTCCCTTCACATCATC-3'; R: 5'-TGGGTTCAAATGAGGTCATACA-3'), and (iv) PG39 (F: 5'-AGGACAGATGACTAACAGGGAGA-3'; R: 5'-GGAGCTAATTATCCATGAGCTTTC-3'). Corresponding size repeat motifs and size ranges were; $(AC)_{10}$ (279-283 bp), $(AT)_9$ (167-171 bp), $(AAT)_{15}$ (118-157 bp) and $(AT)_{10}$ (118-193 bp), respectively.

In order to distinguish PCR products upon capillary separation, primers for the ten microsatellite markers were tagged with a unique fluorescent label during PCR using the method outlined in Blacket et al. (2012). Reactions matrices for PCR amplification consisted of 5 µL Qiagen multiplex mix (Qiagen, Chadstone, Victoria,

Australia), 4 µL of primer mix (0.2 µM of each primer) and 2 µL of template DNA. PCR conditions consisted of an initial 15 min denaturing step at 94 °C, followed by 40 cycles of 94 °C for 30 s, 59 °C for 1:30 min, and 72 °C for 1:00 min, with a final extension step of 60 °C for 30 min. Genotyping was subsequently performed using an Applied Biosystems 3730 capillary analyzer and product lengths were determined relative to a GS500LIZ_3730 size standard. Fragment analyses were conducted using an ABI3730 XL DNA analyzer. Microsatellite profiles were examined and scored manually and assessed for polymorphisms using GeneMapper version 4.0 (Applied Biosystems).

A 728 base pair fragment of the mitochondrial *cytochrome oxidase* subunit I gene (*COI*) was amplified using *P. gunnii* specific primers (forward primer 5' - ATGCCTTCGTAATAATTTTCT - 3'; reverse primer 5' - GGRTARTCTGAGTATCGTCG - 3'). DNA from 33 Victorian *P. gunnii* (15 Hamilton wild 1992, 8 Mt Rothwell, 10 Zoo 2009) and 48 Tasmanian *P. gunnii* (15 north Tasmania and 33 south Tasmania), was amplified in a 30 µl reaction containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 0.6 units *Taq* polymerase (New England Biolabs) and 4 µL template DNA. PCR cycling conditions were 5 min at 94 °C, followed by 35 cycles of 94 °C (30 sec), primer annealing at 51 °C (1 min) and extension at 72 °C (1 min), with a final extension at 72 °C for 7 min, using an Eppendorf EP Gradient Mastercycler. We also amplified a fragment of the *ATPase 6* mitochondrial gene using primers developed from sequence data representing *P. gunnii* and the brown bandicoot, *Isoodon obesulus*, on GenBank. These primers (forward primer 5' - CCCCATTCATCACCCCTAC - 3'; reverse primer 5' - TATGGCGACGGCAAATTCTA - 3') produced a 605 bp fragment using PCR mixes and reaction conditions identical to those specified for *COI*. The partial *ATPase 6* gene was amplified from 30 samples in total (10 Hamilton wild 1992, 10 Zoo 2009 samples and 10 samples from Deviot, Tasmania). The *COI* and *ATPase 6* PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) in both directions on an ABI 3730 sequencer, and a consensus sequence was generated from forward and reverse sequences in SEQUENCHER (Gene Codes, Ann Arbor, USA).

Microsatellite Data Analysis

Genetic Data Analysis version 1.0 (Lewis & Zaykin 2001) was used for estimating Weir & Cockerham's (1984) inbreeding coefficient (F_{IS}), as well as testing for deviations from Hardy-Weinberg equilibrium within populations and loci. Diversity estimates (observed and expected heterozygosities, average number of alleles, allelic richness using rarefaction) were calculated using FSTAT (Goudet 2001) and GENALEX (Peakall & Smouse 2006). Pairs of loci were tested for linkage disequilibrium using a log-likelihood ratio test in FSTAT (Goudet 2001). Evidence for null alleles and large allele dropout for each locus within each population were assessed using MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004).

Samples obtained from the captive breeding program in 1995, 2002 and 2009 were compared with the historic samples from the last wild population at Hamilton collected in 1992, contemporary samples from Hamilton Community Parklands and Mount Rothwell, and samples from northern and southern Tasmania. A Wilcoxon signed-rank test (Sokal & Rohlf 1995) was used to determine significant differences between diversity estimates (allelic richness and expected heterozygosity) for all population samples. Similarly, a Wilcoxon signed-rank test was used to determine significant differences between all Victorian and all Tasmanian samples for the number of alleles at each locus. The software package BOTTLENECK was also run to test for evidence of recent reductions in the effective population size based on a comparison of allele numbers and gene diversity at polymorphic loci (Cornuet and Luikart 1996) for all Victorian samples separately. BOTTLENECK tests were performed using the infinite allele model (IAM), stepwise mutation model (SMM) and the two-phased model of mutation (TPM), with the intermediate TPM considered most suitable for microsatellite loci (Cornuet and Luikart 1996). The variance for TPM was set to 30 and the proportion of SMM in TPM set to 70%. Due to the relatively small number of loci, the Wilcoxon's signed-rank test was applied to determine significance (Cornuet and Luikart 1996) based on 1000 iterations.

Pairwise F_{ST} for sample groups were estimated with FSTAT and their significance determined through permutation (1000 replicates). An additional estimate of population differentiation, D_{est} was obtained using SMOGD: software for the measurement of genetic diversity (Crawford, 2010). This estimate is suggested to be more accurate for identifying population differentiation by overcoming the limitations

of F_{ST} (Jost, 2008). Significance of pairwise estimates of D_{est} were determined from 1,000 bootstrap replicates using the program DEMEtics (Gerlach et al. 2010). To further explore differences between contemporary *P. gunnii* samples and historic samples from the last wild population, we undertook an analysis of molecular variation (AMOVA) in GENALEX using pairwise F_{ST} as the distance measure, with 10,000 permutations to test for significance. The model for analysis partitioned variation among temporal samples (contemporary samples from Mt Rothwell, Hamilton Community Parklands and the Zoo captive breeding program and the historic wild sample from Hamilton collected in 1992), between populations within regions (the three contemporary populations) and within populations. A principal coordinate analysis, implemented in GENALEX (Peakall and Smouse 2006) was undertaken to visualise the genetic differentiation between historic and contemporary Victorian *P. gunnii* and also samples from Tasmania.

All tests involving multiple comparisons were corrected at the table-wide $\alpha' = 0.05$ level with the Dunn-Šidák method (Sokal & Rohlf 1995) or, in the case of the Wilcoxon signed-rank tests where the number of loci limit the significance level, we used the False Discovery Rate graphically sharpened method (Benjamini & Hochberg 2000).

Effective Population Size

The *P. gunnii* studbook held by Zoos Victoria suggests that since the captive breeding program was initiated in 1988 there have been approximately four, seven and ten breeding generations (M. Parrott, pers. comm.) when individuals were sampled for this study in 1995, 2002 and 2009, respectively . We determined the average N_e through time between each sampling period that has resulted in the genetic diversity estimates using the following formula:

$$H_t = H_0[1-(1/2N_e)]^t$$

where H_t = expected heterozygosity in generation *t*, H_0 = initial heterozygosity, and N_e = the effective population size (Hedrick 1999). The estimate from the Hamilton wild population sample from 1992 was used as a surrogate for the initial captive breeding program genetic diversity estimate. The effective population size is

approximately the harmonic mean of effective population sizes in individual generations and therefore will be downwardly biased when the effective population size differs greatly between generations (Hedrick 1999).

Using the microsatellite genotypic data generated for the now extinct wild population at Hamilton in 1992, we undertook simulations to predict the change in heterozygosity and average allele number over 25 generations based on the average N_e estimated from the formula above. Simulations were based on 10,000 replicates, assume random mating, non-overlapping generations and a constant effective population size over the 25 generations. All simulations were undertaken in the Microsoft Excel add in PopTools (Hood 2002). These simulations also provide insight into the expected continued loss of genetic diversity through time.

Mitochondrial DNA analysis

DNA sequences for the mitochondrial *COI* and *ATPase 6* partial gene fragments were aligned, edited and translated in MEGA version 4 (Tamura et al. 2007). Genetic distances between *COI* haplotypes were calculated using the Kimura 2-parameter model (Kimura 1980) in MEGA version 4. Genealogical relationships between mitochondrial haplotypes were inferred from a haplotype network (Templeton *et al.* 1992). Unrooted networks were generated with TCS version 1.21 (Clement *et al.* 2000), using maximum parsimony to connect haplotypes with a 95% confidence interval.

RESULTS

Microsatellite statistics

MICRO-CHECKER found no evidence for large allele dropouts for any loci in each population, while the incidence of null alleles for each locus was generally low and only significant for three loci in the Zoo 2009 samples (loci B20-5, B34-2 and PG39), and three loci in the south Tasmanian samples (loci B7-2, B20-5 and PG38). No evidence of linkage disequilibrium was found between any loci across all population samples from 360 pairwise tests after corrections for multiple comparisons.

The Zoo 2009 samples had a significantly positive inbreeding coefficient, displaying an overall excess of homozygotes at the microsatellite loci (Table 1). This sample was also out of Hardy-Weinberg equilibrium. All other Victorian samples (Zoo 1995, Zoo 2002, Hamilton Community Parklands, Mount Rothwell and the extinct wild population collected in 1992) did not have significant inbreeding coefficients and all were in Hardy-Weinberg equilibrium. After correction for multiple comparisons, the south Tasmanian samples showed evidence for significant deviations from Hardy-Weinberg equilibrium with a corresponding significant F_{IS} estimate (Table 1).

Genetic Diversity and Population Differentiation

A total of 34 alleles were found across the ten microsatellite loci for the Victorian contemporary samples (from 90 individuals). Significantly more alleles were found at each locus in the Tasmanian samples (Wilcoxon signed-rank test, $P < 0.01$), with a total of 80 alleles detected from 69 individuals. When historic Victorian samples are included (23 individuals from Zoo 1995, 33 from Zoo 2002 and 32 individuals from the wild Hamilton population collected in 1992), the total allele numbers are higher for Victoria, with 51 alleles detected (17 alleles more than in contemporary Victorian samples).

Current diversity estimates are low within Victorian *P. gunnii* (Zoo 2009) when compared to samples from the now extinct wild population at Hamilton collected in 1992 (Table 1). This extinct Victorian wild population had similar levels of heterozygosity as to that found in current Tasmanian populations, although allelic diversity estimates were lower. The lowest diversity estimates were found for the contemporary Victorian samples [Hamilton Community Parklands, Mount Rothwell and the current captive breeding program broodstock (Zoo 2009); Table 2, Supplementary Material 1].

Overall, there has been a reduction of 30% in allelic richness, 39% in observed heterozygosity and 25% in expected heterozygosity over a 17 year period of captive breeding. Figure 2 shows the differences in average allele numbers, allelic richness, observed and expected heterozygosities between samples from the last wild population (collected in 1992), samples from the captive breeding program (Zoo 2009, Zoo 2002 and Zoo 1995), contemporary samples from Hamilton Community

Parklands and Mount Rothwell in Victoria, and contemporary samples from north and south Tasmania.

The Wilcoxon signed-rank population pairwise comparisons for allelic richness and expected heterozygosity indicate differences are greater for allelic richness (Table 2). All contemporary Victorian population samples have significantly lower allelic diversity than the last wild population sample from Hamilton in 1992, and generally significantly lower expected heterozygosities (except Mount Rothwell, which is approaching significance). All Victorian samples (contemporary and historic) are significantly different from the north Tasmanian samples for allelic richness, although only Hamilton Community Parklands and the current captive breeding broodstock (Zoo 2009) are significantly different from south Tasmanian samples. The expected heterozygosity estimate from the last wild population sample from Hamilton in 1992 is not significantly different from the Tasmanian samples (north or south), whereas estimates from Hamilton Community Parklands and Zoo 2009 are significantly lower than the north Tasmanian samples. Only the expected heterozygosity from Hamilton Community Parklands is significantly lower than the south Tasmanian samples. A significant difference between allelic richness and expected heterozygosity was also found between north and south Tasmanian samples.

Cornuet and Luikart's (1996) test for evidence of reduction in the effective population size support the differences found above in allelic richness and heterozygosity. All contemporary and historic Victorian samples showed evidence for severe reduction in size, with significant heterozygote excess detected under all three mutation models (Supplementary Material 2). The sample from the last wild population at Hamilton (1992) also showed evidence for a severe reduction in population size (Supplementary Material 2).

The differences between Victorian and Tasmanian *P. gunnii* are large with an average F_{ST} of 0.359 (95% CI, 0.279, 0.453) and D_{est} of 0.602 (95% CI, 0.574, 0.639). The principal coordinate analysis (Figure 3) highlights these differences, with complete separation of all Tasmanian *P. gunnii* samples from Victorian historic and contemporary samples. While F_{ST} and D_{est} differed for most comparisons (Table 3), the overall pattern of significance was the same. Pairwise comparisons of F_{ST} and D_{est}

for the contemporary Victorian samples are not significantly differentiated. The contemporary Mount Rothwell samples also did not differ significantly from the historic Zoo 2002 samples. All other pairwise comparisons for the two estimates were highly significant after corrections for multiple comparisons, including all comparisons between samples from the captive breeding program. Finally, the AMOVA analysis indicates highly significant variation between the contemporary Victorian samples and samples from the extinct wild population at Hamilton (17%, $P < 0.001$), significant differences between contemporary Victorian samples (2%, $P < 0.001$), and the majority of variation in microsatellites found within populations (81%).

Effective Population Size

Solving the formula of Hedrick (1999) and using the average heterozygosity from contemporary captive breeding program samples (Zoo 2009), the average N_e in the captive breeding program through the ten generations is 17.57, while N_e estimates from 1988 to 1995 (four generations), 1995 to 2002 (three generations) and 2002 to 2009 (three generations) are 16.91, 20.29 and 16.22, respectively. Figure 4 depicts the simulation of the changes through time (generations) in (a) average allele numbers over the ten microsatellite loci, and (b) expected heterozygosity over the ten microsatellite loci, using an average N_e of 18. The observed diversity estimates for contemporary Victorian *P. gunnii* fit well within the 99% CI's for these simulations (Figure 4). Importantly, these simulations show the continued decline in genetic variation if the effective population size remains at its current size, with a 57% reduction in average allele numbers and 50% reduction in heterozygosity compared with the extinct wild population, to occur in the next 15 generations.

Mitochondrial diversity

At the *ATPase 6* mtDNA locus, only two haplotypes (differentiated by a single nucleotide) were identified, with one haplotype representing samples from Victoria and Tasmania and the other representing samples only from Tasmanian (Table 1). At the *COI* mtDNA locus, eight haplotypes were identified (Table 1), with less than 0.5% sequence divergence between haplotypes (mean 0.4%, range 0.1% - 0.8%; see Supplementary Material 3). Two haplotypes were present in Victorian samples and six present in Tasmanian samples (Figure 5). Two fixed base pair differences were

identified between all Victorian and Tasmanian *P. gunnii*, and a single haplotype dominated in each state (H1 in Tasmania, H2 in Victoria). Four haplotypes were identified in north Tasmania (H1, H4, H7, H8), and three in south Tasmania (H1, H5, H6). The second Victorian haplotype (H3) was identified in only one individual that was derived from Mount Rothwell.

DISCUSSION

The results indicate that the genetic health of Victorian *P. gunni* is in a progressive state of erosion. The current breeding program was initiated ~ 20 years ago, with a likely founder population of between 19 and 40 individuals contributing to the initial genetic diversity (Myroniuk 1993). The results here suggest that the effective founder size is more likely to have been less than 19 individuals and the effective population size has remained below 20 individuals per generation for the duration of the captive breeding program. This is an inadequate number of individuals to prevent losses of genetic diversity (Hedrick 1999; Hedrick and Fredrickson 2010). The simulation in Figure 4 suggests continued declines may occur in the captive breeding program unless the management of this population is changed.

Currently it is unknown what impacts the observed losses in genetic diversity have had on the fitness of *P. gunnii*. Losses in genetic diversity are likely to significantly increase the threat of extinction (Markert et al. 2010) and affect the adaptive potential of the species (Willi et al. 2006; Weeks et al. 2011). Of particular concern is the recent 'undercut jaw' phenotype found in both captive breeding broodstock (P. Courtney, pers. comm.) and in individuals derived from this broodstock at Hamilton Community Parklands conservation park (A. Winnard, pers. comm.). This suggests the phenotype may be heritable, although it is not known whether this causes any fitness effects or results from the expression of deleterious alleles. Small populations are, however, known to be vulnerable to the accumulation of deleterious alleles (genetic load) that can become fixed through inbreeding and random genetic drift.

While there were no significant differences between contemporary Victorian samples for genetic diversity estimates, the Mount Rothwell population had slightly higher diversity estimates and was the only contemporary sample that did not differ

487 significantly from the historic Zoo 2002 samples. The Mount Rothwell population
488 was founded from individuals from the captive breeding program around this time
489 (2004). This may suggest the Mount Rothwell population of *P. gunnii* has been able
490 to attain a population size that has limited further losses of genetic diversity since the
491 population was founded by ~20 individuals.
492
493 Tasmanian samples of *P. gunnii* are more genetically diverse and highly differentiated
494 from Victorian samples at nuclear microsatellite loci, including the extinct wild
495 population samples from Hamilton collected 1992. However, there were relatively
496 low levels of haplotype diversity and divergence in mitochondrial sequences for the
497 *ATPase 6* and *COI* genes. These results are in contrast to those of Robinson et al.
498 (1993) and Robinson (1995) who found higher levels of genetic diversity in the same
499 wild Hamilton samples collected in 1992 (identical samples to those used in this
500 study), compared with samples from Tasmania for both nuclear VNTRs and
501 mitochondrial RFLPs. Robinson (1995) also reported much greater mitochondrial
502 DNA divergence between Victorian and Tasmanian *P. gunnii*, and suggested these
503 should be considered separate sub-species. It is difficult to reconcile the differences
504 between studies, although the methods of assessing genetic diversity used by
505 Robinson et al. (1993) and Robinson (1995) were different to those used here,
506 targeting different genes. Robinson (1995) also undertook RFLPs on the whole
507 mitochondrial genome, rather than the single genes used here. Some differences may
508 also be attributed to a greater sample size of Tasmanian *P. gunnii* in our study, and
509 those samples being collected over a broader area than in Robinson et al. (1993) and
510 Robinson (1995).
511
512 Genetics differences were also found between *P. gunnii* collected in the north and
513 south of Tasmania, with samples collected from the south having lower levels of
514 genetic diversity than samples from the north. These results must be treated with
515 caution, however, as samples were combined in the north and south for comparisons
516 with Victorian samples, and it is likely that samples are derived from multiple
517 populations in each region. An assessment of a larger number of contemporary *P.*
518 *gunnii* samples from Tasmania is currently underway to determine patterns of genetic
519 variation and gene flow throughout their current distribution (Weeks, unpubl. data).
520

Victorian *P. gunnii* are losing genetic diversity due to a small effective population size in the captive breeding program. Unless this decline is mediated it will have consequences for the success of future captive breeding, reintroduction translocations, and the extinction threat for Victorian *P. gunnii*. Therefore, from a genetic perspective, there are several options that need to be considered (Weeks et al. 2011). To stop the decline in genetic diversity, the effective population size needs to be increased substantially within the next few generations. The captive breeding program at Zoos Victoria only has space for 15 breeding pairs (P. Courtney, pers. comm.). Consideration should therefore be given to managing all populations of Victorian *P. gunnii* as a metapopulation, with the transfer of individuals between Mount Rothwell, Hamilton Community Parklands and *P. gunnii* held at Zoos Victoria. This will elevate the census size in excess of 300, which should greatly increase the effective population size beyond current levels and slow the decline of genetic diversity. The actual migration rate between populations need only be between 1 and 10 individuals, with at least one effective migrant contributing in successive generations (Mills and Allendorf 1996). This will provide adequate gene flow to connect populations, slow the loss of genetic diversity, decrease genetic load, and yet still allow differences in allele frequencies between populations to persist (Hedrick 1995; Hedrick 1999; Mills and Allendorf 1996).

While the loss of genetic diversity can be slowed by increasing the effective population size quickly, additional management actions will be required to increase current levels of genetic diversity. Substantial genetic diversity (25-35%) has already been lost from Victorian *P. gunnii* populations and this is likely to reduce the ability of *P. gunnii* to adapt to environmental change and predators/pathogens (Willi et al. 2006; Weeks et al. 2011), and greatly increase the risk of extinction (Markert et al. 2010). To reinvigorate genetic diversity, consideration should be given to crossing Victorian and Tasmanian *P. gunnii*. An obvious concern to crossing Victorian and Tasmanian *P. gunnii* is the phenomenon known as outbreeding depression (Frankham 1999). However this concern is overstated in the literature (Frankham et al. 2011) and is often not based on an assessment of risk (Weeks et al. 2011). Similarly, local adaptation (which is an extrinsic cause of outbreeding depression) is likely to be of minimal concern in populations with very low effective population size and should be of low concern for conservation in such situations (Lopez et al. 2009). The ability to

perform controlled crosses of *P. gunnii* in captivity provides an ideal way of assessing outbreeding depression in subsequent generations, and could be a first step in determining whether this course of action will have any viability and/or fitness consequences. If no outbreeding depression is found in the F_2 or F_3 generations, then there would be a strong case for implementing strictly controlled gene flow from Tasmanian *P. gunnii* populations (source) into Victorian *P. gunnii* populations (recipient) to increase genetic variation. This strategy, termed 'genetic rescue/restoration' has been undertaken several times in wild animal populations previously (Hedrick & Fredrickson 2010; Weeks et al. 2011). The genetic rescue/restoration would have to be managed carefully so that adaptations unique to Victorian *P. gunnii* are maintained.

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Table 1. Sample size (*n*), average number of alleles (N_a), allelic richness (R_a), observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}) and Hardy Weinberg Equilibrium (HWE) *P* value and haplotypes for *ATPase 6* and *COI* for the different samples.

Population	*n*	N_a	R_a	H_O	H_E	F_{IS}	HWE *P* value	*ATPase 6* Haplotype (*n*)	*COI* haplotype (*n*)
Hamilton Community Parklands	25	3.1	2.949	0.394	0.417	0.056	0.360	-	-
Mount Rothwell	18	3.0	2.961	0.447	0.488	0.087	0.106	-	H2 (7), H3 (1)
Zoo 2009	47	3.1	2.902	0.369	0.457	0.194*	<0.001*	H1 (10)	H2 (10)
Zoo 2002	33	3.3	3.114	0.503	0.502	-0.002	0.323	-	
Zoo 1995	23	3.9	3.637	0.557	0.541	-0.03	0.709	-	
Wild (1992)	32	4.5	4.142	0.600	0.610	0.008	0.039	H1 (10)	H2 (15)
North Tasmania	18	7.0	6.640	0.609	0.685	0.113	0.009	H1 (4), H2 (6)	H1 (5), H4 (7), H7 (2), H8 (1)
South Tasmania	51	6.8	5.427	0.542	0.625	0.134*	<0.001*	-	H1 (26), H5 (3), H6 (3)

* denotes significance at the α' = 0.05 level after corrections for multiple comparisons.

717 Table 2. Wilcoxon signed-rank *P* values for population pairwise comparisons of loci
718 for allelic richness (above diagonal) and expected heterozygosity (below diagonal).
719 Bold indicates significance after correcting for multiple comparisons using the False
720 Discovery Rate (Benjamini & Hochberg 2000). HCP = Hamilton Community
721 Parklands, MTR = Mount Rothwell.
722

	HCP	MTR	Zoo 2009	Zoo 2002	Zoo 1995	Wild (1992)	North Tasmania	South Tasmania
HCP	-	0.953	0.594	0.263	0.086	**0.021**	**0.013**	**0.028**
MTR	0.126	-	0.284	0.515	0.093	**0.017**	**0.007**	0.051
Zoo 2009	0.185	0.333	-	0.11	0.037	**0.011**	**0.005**	**0.028**
Zoo 2002	0.074	0.541	0.169	-	0.11	**0.008**	**0.007**	0.051
Zoo 1995	**0.009**	0.407	**0.047**	0.721	-	0.069	**0.017**	0.066
Wild (1992)	**0.022**	0.059	**0.022**	**0.047**	0.139	-	**0.028**	0.139
North Tasmania	**0.037**	0.059	**0.037**	**0.047**	0.074	0.203	-	**0.007**
South Tasmania	**0.047**	0.139	0.059	0.203	0.153	0.575	**0.009**	-

723

Table 3. Estimates of differentiation between *P. gunnii* samples. Above diagonal D_{est}, below diagonal F_{ST}. Bold indicates non-significant pairwise comparisons after corrections for multiple comparisons. Significance determined through bootstrapping (D_{est}) and permutation (F_{ST}). HCP = Hamilton Community Parklands, MTR = Mount Rothwell.

	HCP	MTR	Zoo 2009	Zoo 2002	Zoo 1995	Wild (1992)	North Tasmania	South Tasmania
HCP	-	**0.0054**	**0.0032**	0.0137	0.0272	0.1689	0.6041	0.6439
MTR	**0.0260**	-	**0.004**	**0.005**	0.0341	0.1346	0.5577	0.575
Zoo 2009	**0.012**	**0.0182**	-	0.0126	0.0361	0.1706	0.6037	0.6257
Zoo 2002	0.0373	**0.0283**	0.0469	-	0.0338	0.1592	0.5435	0.5523
Zoo 1995	0.0623	0.0671	0.0672	0.0557	-	0.0641	0.5167	0.5521
Wild (1992)	0.2094	0.1393	0.1904	0.1592	0.0963	-	0.4355	0.5051
North Tasmania	0.3722	0.314	0.3643	0.3169	0.299	0.2479	-	0.0517
South Tasmania	0.3875	0.3398	0.375	0.3338	0.3326	0.2967	0.0515	-

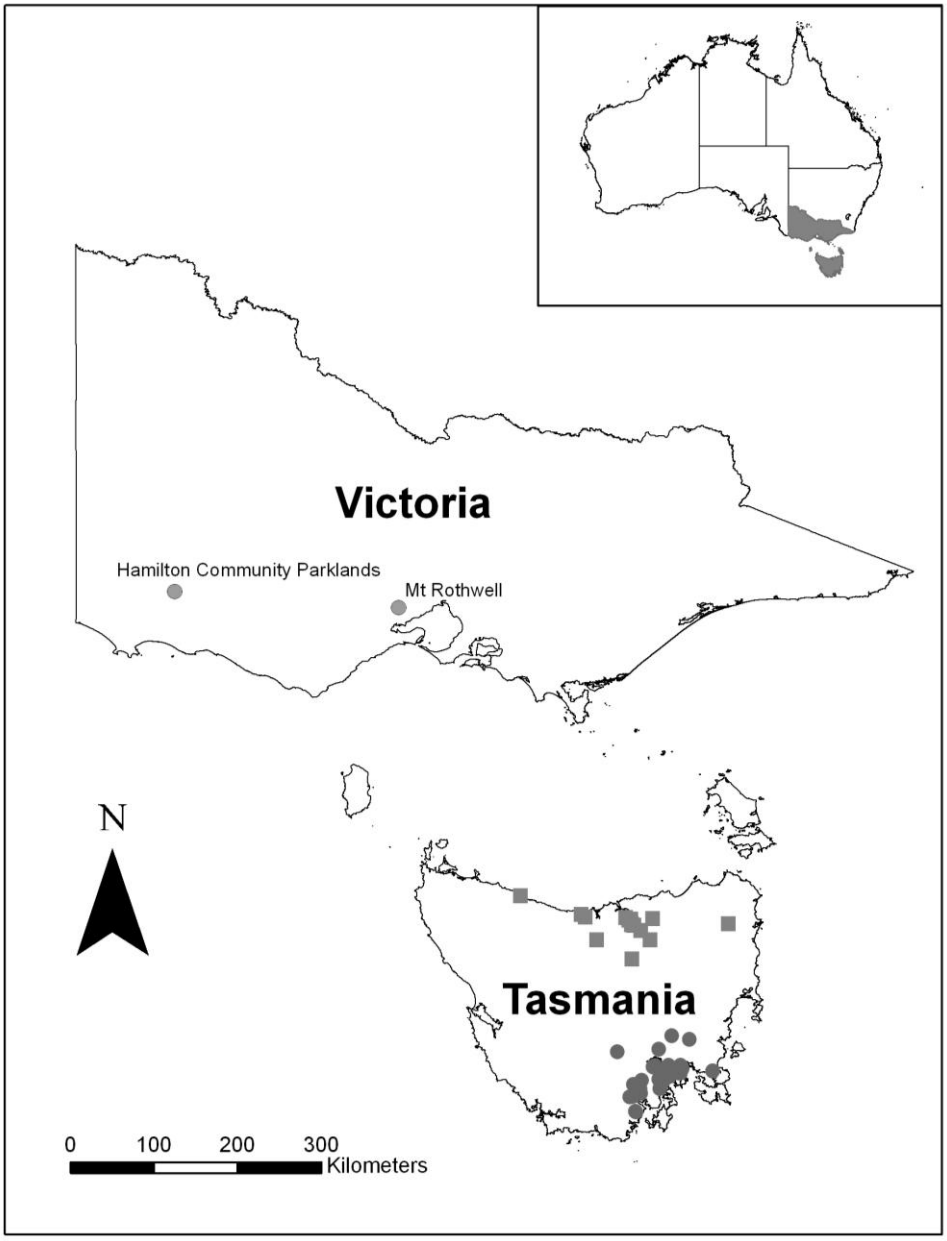
Figure 1. Sites where contemporary *P. gunnii* individuals were sampled in 2008/2009. Tasmanian samples are grouped as north (closed squares) and south (closed circles) for analyses.

Figure 2. Average allele number (Na), allelic richness (Ra), observed (Ho) and expected (He) heterozygosity in *P. gunnii* samples from north (Nth Tas) and south (Sth Tas) Tasmania, the last wild population at Hamilton (Vic 1992), Hamilton Community Parklands (HCP), Mount Rothwell (MTR) and historic (Zoo 1995, Zoo 2002) and contemporary (Zoo 2009) samples from the captive breeding program.

Figure 3. Two-dimensional plot showing the relationship among samples of *P. gunnii* collected in Victoria (MTR = Mount Rothwell, Zoo = Zoo 2009, Wild = Hamilton wild 1992, Zoo_95 = Zoo 1995, HCP = Hamilton Community Parklands, Zoo_02 = Zoo 2002), northern (Nth Tas) Tasmania and southern (Sth Tas) Tasmania based on a principal coordinate analysis of genotypes at 10 microsatellite loci in each individual.

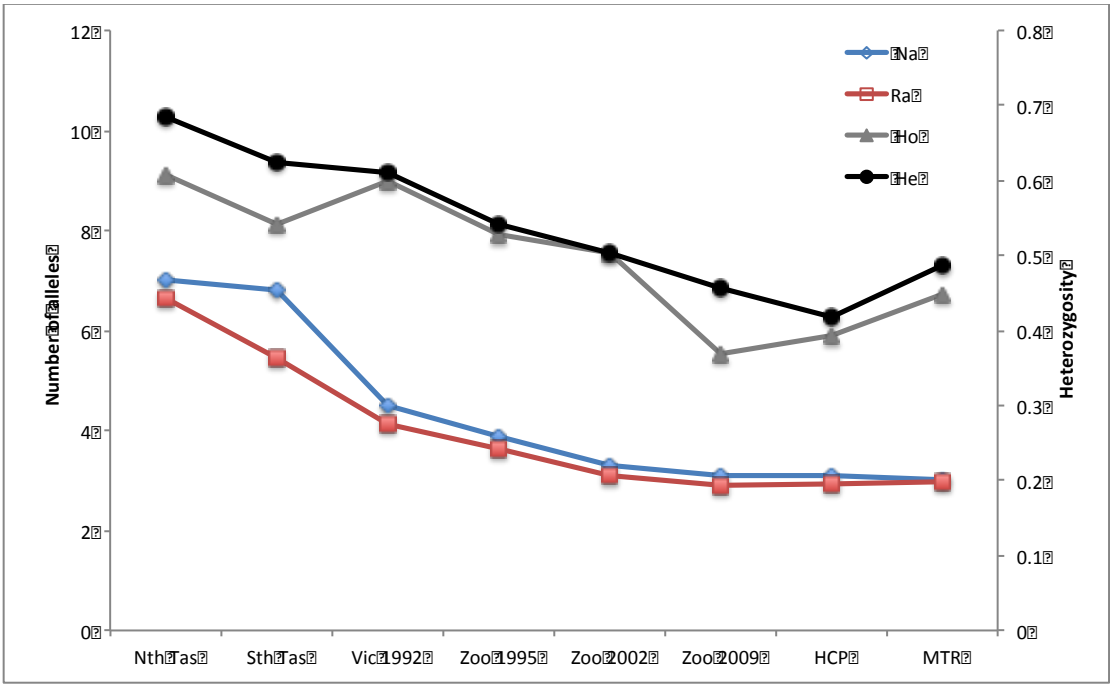
Figure 4. Simulations of *P. gunnii* microsatellite data from the wild population at Hamilton showing predicted change through time in average allele numbers (top) and expected heterozygosity (bottom), across ten microsatellite loci. The data is based on 1000 simulations assuming random mating within the population and non-overlapping generations. Dotted lines are 99% confidence intervals. Black enclosed circles represent actual observed data for contemporary Victorian *P. gunnii* from the captive breeding program.

Figure 5. Network analysis (as implemented in TCS) showing haplotypes at the *CO1* mtDNA gene, relationships between haplotypes, and their relative frequencies in *P. gunnii* samples from Victoria and Tasmania. Numbers for each haplotype are: H1 = 33; H2 = 32; H3, H8 = 1; H5-7 = 2; H4 = 8. Numbers on branches are position of base changes in the partial *COI* mtDNA gene. Tasmanian and Victorian haplotypes are indicated by blue and red coloration, respectively.


Victoria
Hamilton Community Parklands
Mt Rothwell
N
Tasmania
0
100
200
300
Kilometers

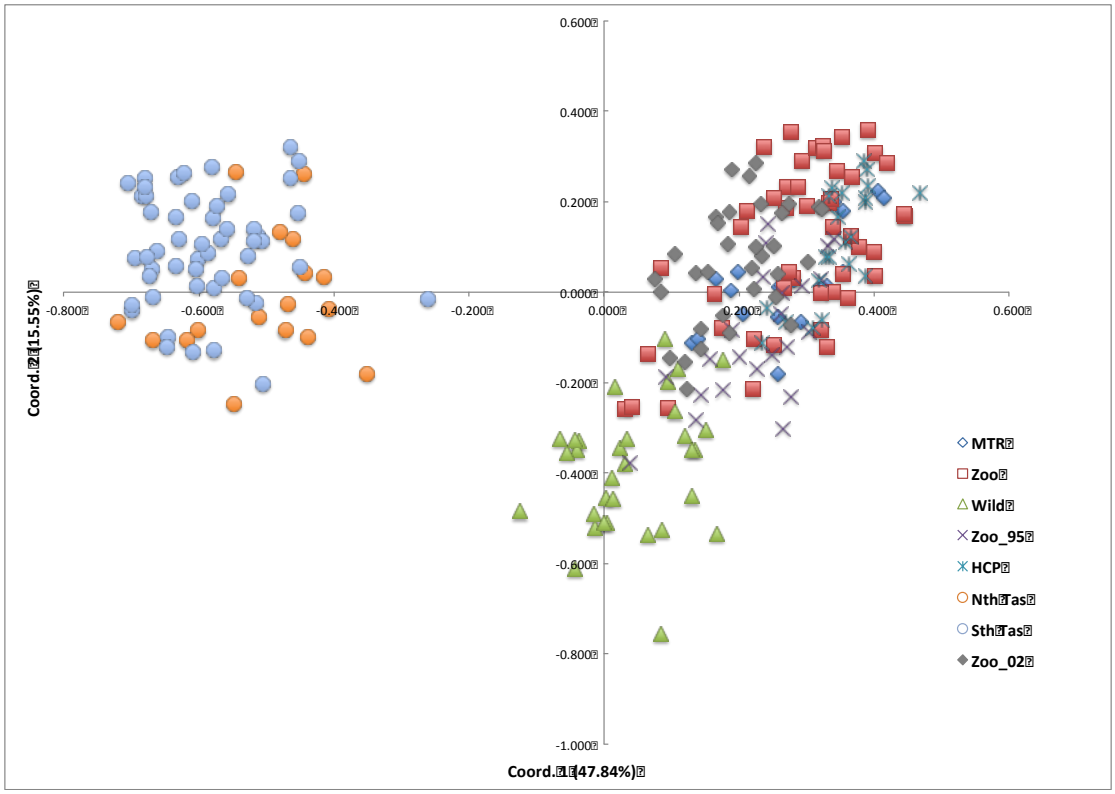
762

763 Fig 1.


12
10
8
6
4
2
0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Na
Ra
Ho
He
Heterozygosity
Nth Tas
Sth Tas
Vic 1992
Zoo 1995
Zoo 2002
Zoo 2009
HCP
MTR

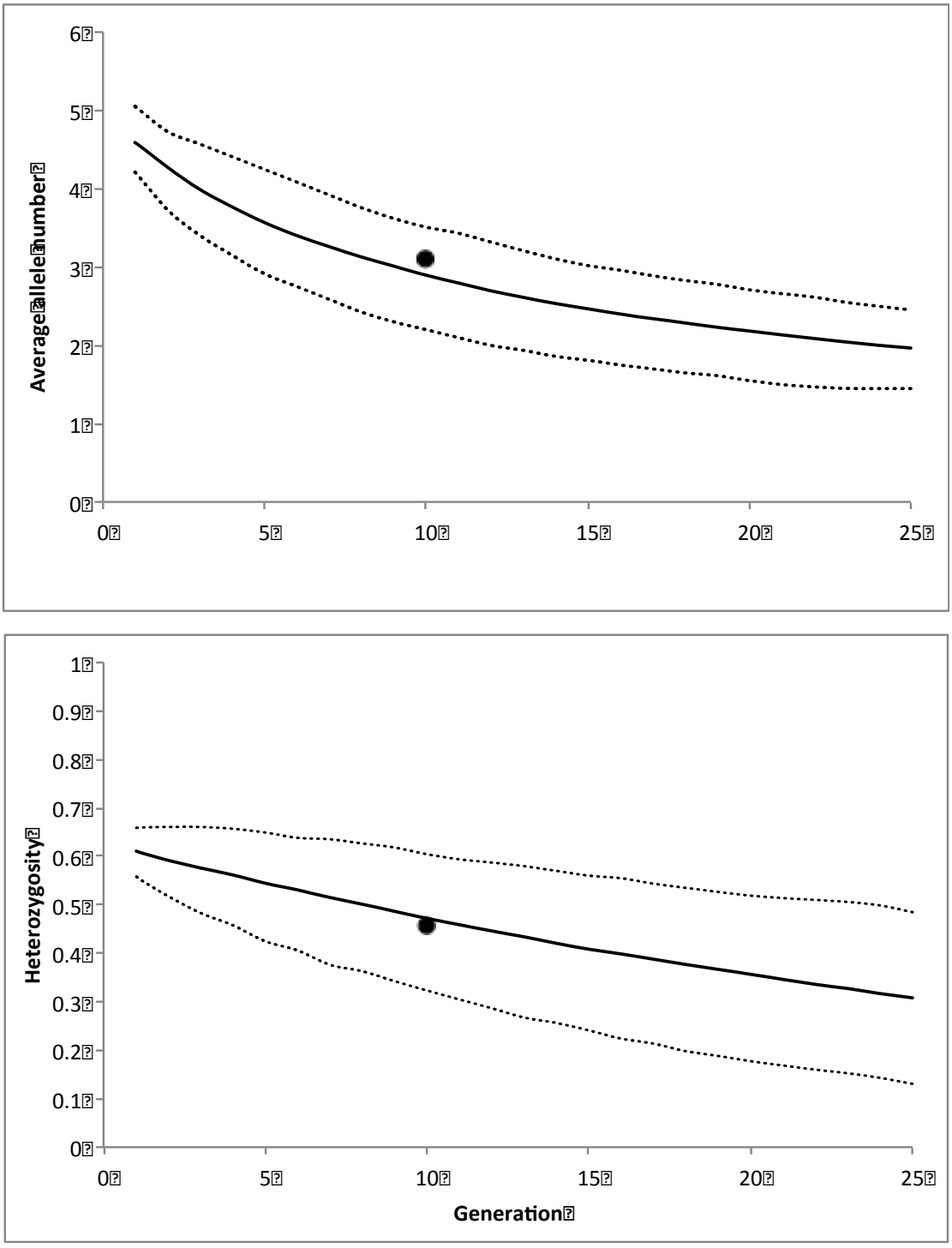
764

765 Fig 2.


0.600
0.400
0.200
0.000
-0.200
-0.400
-0.600
-0.800
-1.000
-0.800
-0.600
-0.400
-0.200
0.000
0.200
0.400
0.600
Coord. 2 (15.55%)
Coord. 1 (47.84%)
MTR
Zoo
Wild
Zoo_95
HCP
Nth Tas
Sth Tas
Zoo_02

766

767 Fig 3.


Average allele number
6
5
4
3
2
1
0
0
5
10
15
20
25
Heterozygosity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
Generation

768

769 Fig 4.

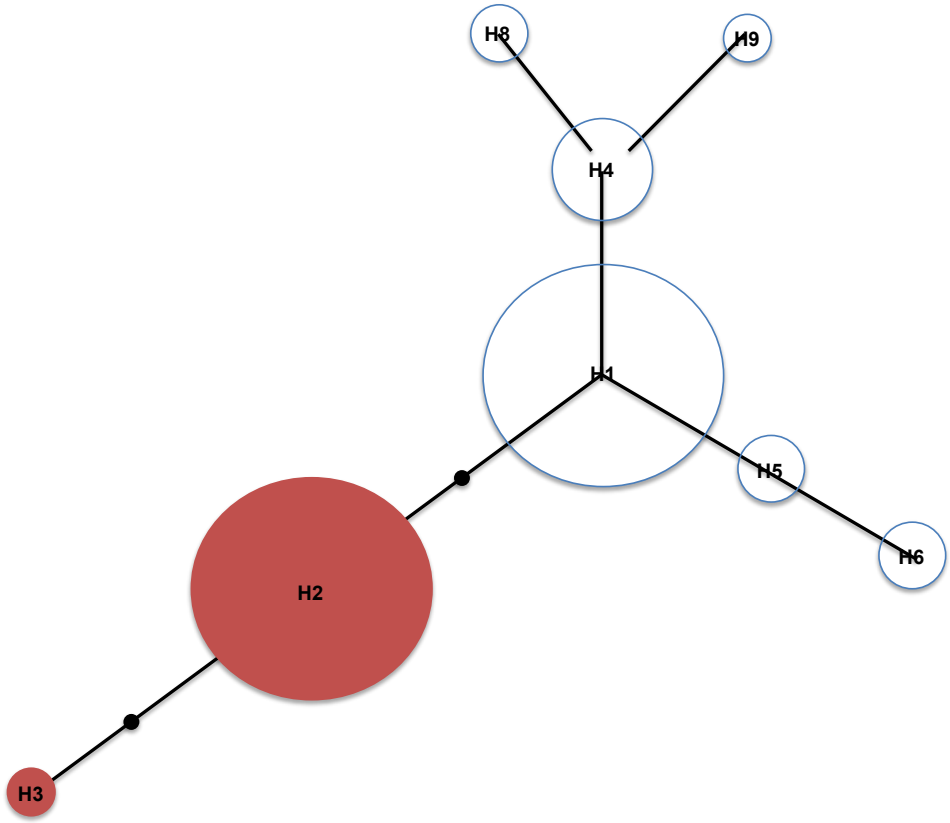


Fig 5.

SUPPLEMENTARY MATERIAL 1.

Pop	Locus	*n*	N_a	R_a	H_O	H_E	F_{IS}
Hamilton Community	B3-2	22	2	1.914	0.136	0.130	-0.05
Parklands	B7-2	22	3	2.091	0.091	0.090	-0.012
	B15-1	22	4	3.598	0.591	0.595	0.007
	B20-5	22	5	4.876	0.636	0.737	0.139
	B34-1	22	3	2.986	0.545	0.606	0.102
	B34-2	22	3	2.994	0.500	0.495	-0.011
	PG20	22	2	1.998	0.227	0.274	0.173
	PG36	22	2	2	0.318	0.384	0.174
	PG38	22	3	2.51	0.227	0.210	-0.082

	PG39	18	4	3.895	0.667	0.649	-0.028
Mount Rothwell	B3-2	18	2	1.667	0.056	0.056	0
	B7-2	18	4	3.864	0.389	0.494	0.217
	B15-1	18	4	3.961	0.611	0.665	0.083
	B20-5	18	5	4.856	0.500	0.716	0.308
	B34-1	18	3	3	0.611	0.667	0.086
	B34-2	18	2	2	0.389	0.437	0.112
	PG20	18	2	2	0.500	0.437	-0.15
	PG36	18	2	2	0.667	0.489	-0.378
	PG38	18	3	2.864	0.278	0.256	-0.09
	PG39	17	3	3	0.471	0.670	0.304
Zoo 2009	B3-2	47	2	1.995	0.298	0.286	-0.044
	B7-2	47	3	2.893	0.340	0.361	0.056
	B15-1	46	5	3.637	0.565	0.606	0.068
	B20-5	46	5	4.739	0.543	0.699	0.225
	B34-1	47	3	2.999	0.574	0.648	0.115
	B34-2	35	3	2.993	0.286	0.554	0.488
	PG20	46	2	1.998	0.261	0.318	0.182
	PG36	44	2	1.999	0.250	0.342	0.272
	PG38	46	3	2.79	0.326	0.289	-0.131
	PG39	36	3	2.559	0.250	0.468	0.469
Zoo 2002	B3-2	32	2	1.949	0.125	0.173	0.279
	B7-2	30	4	3.378	0.533	0.540	0.012
	B15-1	33	5	4.805	0.727	0.709	-0.027
	B20-5	25	5	4.401	0.720	0.672	-0.073
	B34-1	30	4	3.79	0.700	0.686	-0.02
	B34-2	12	3	3	0.750	0.620	-0.222
	PG20	32	2	1.998	0.375	0.310	-0.216
	PG36	32	2	2	0.438	0.490	0.109
	PG38	32	3	2.827	0.313	0.280	-0.117
	PG39	26	3	2.996	0.346	0.539	0.363
Zoo 1995	B3-2	23	2	1.955	0.087	0.162	0.47

	B7-2	23	5	4.274	0.435	0.414	-0.053
	B15-1	23	3	3	0.783	0.665	-0.182
	B20-5	21	6	5.463	0.714	0.748	0.046
	B34-1	23	6	5.563	0.652	0.669	0.025
	B34-2	23	5	3.82	0.696	0.546	-0.282
	PG20	23	2	2	0.522	0.433	-0.211
	PG36	23	2	2	0.522	0.487	-0.073
	PG38	19	4	3.632	0.579	0.664	0.132
	PG39	19	4	3.631	0.579	0.620	0.068
Wild	B3-2	32	2	2	0.438	0.347	-0.265
	B7-2	32	5	4.848	0.750	0.760	0.013
	B15-1	32	7	5.219	0.656	0.749	0.126
	B20-5	31	5	4.737	0.548	0.593	0.076
	B34-1	32	7	6.007	0.750	0.751	0.001
	B34-2	32	6	4.811	0.563	0.591	0.049
	PG20	32	2	2	0.438	0.411	-0.066
	PG36	32	2	2	0.625	0.458	-0.372
	PG38	26	4	3.944	0.462	0.679	0.325
	PG39	23	5	4.949	0.826	0.762	-0.086
North Tasmania	B3-2	18	6	5.56	0.611	0.797	0.238
	B7-2	17	5	4.545	0.412	0.636	0.36
	B15-1	17	9	7.662	0.647	0.800	0.196
	B20-5	14	10	9.634	0.857	0.868	0.013
	B34-1	18	8	6.96	0.833	0.819	-0.018
	B34-2	16	12	11.001	1.000	0.919	-0.091
	PG20	18	3	2.967	0.222	0.379	0.421
	PG36	18	3	2.992	0.611	0.608	-0.005
	PG38	18	11	9.313	0.778	0.849	0.086
	PG39	17	3	2.626	0.118	0.169	0.312
South Tasmania	B3-2	51	6	5.076	0.667	0.724	0.08
	B7-2	51	4	2.982	0.353	0.553	0.364

B15-1	51	9	6.89	0.667	0.692	0.037
B20-5	49	10	7.895	0.694	0.857	0.192
B34-1	50	8	7.06	0.740	0.836	0.115
B34-2	45	13	8.701	0.800	0.874	0.086
PG20	51	2	1.995	0.275	0.294	0.065
PG36	50	2	2	0.480	0.492	0.025
PG38	50	10	7.419	0.640	0.825	0.226
PG39	48	4	2.129	0.104	0.102	-0.026

778

779

SUPPLEMENTARY MATERIAL 2. BOTTLENECK analysis for Victorian samples of *Perameles gunnii*. Probability of heterozygote excess according to the Wilcoxon sign-rank test under the IAM (Infinite Allele Model), TPM (Two-phased model of mutation) and SMM (Stepwise Mutation Model) for each population. All values are significant after corrections for multiple comparisons.

Population	IAM	TPM	SMM	Mode Shift
Hamilton Comm. Parklands	0.00004	0.00060	0.00553	Yes
Mount Rothwell	0.00049	0.00049	0.00049	Yes
Zoo 2009	0.00049	0.00049	0.00146	Yes
Zoo 2002	0.00098	0.00244	0.00342	Yes
Zoo 1995	0.00049	0.00049	0.00146	Yes
Wild (1992)	0.00049	0.00049	0.00098	Yes

SUPPLEMENTARY MATERIAL 3. Genetic distance between *Perameles gunnii* mtDNA *COI* haplotypes based on Kimura's 2-parameter model (Kimura 1980).

	COI haplotypes						
	H1	H2	H3	H4	H5	H6	H7
H1							
H2	0.003						
H3	0.006	0.003					
H4	0.001	0.004	0.007				
H5	0.001	0.004	0.007	0.003			
H6	0.003	0.006	0.008	0.004	0.001		
H7	0.003	0.006	0.008	0.001	0.004	0.006	
H8	0.003	0.006	0.008	0.001	0.004	0.006	0.003