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Genetic distinctness of isolated populations of an endangered marsupial, the mountain pygmy-possum, *Burramys parvus*

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Abstract

The mountain pygmy-possum, *Burramys parvus*, exists in isolated and fragmented populations in the Australian alps. To examine the degree of interpopulation divergence, mitochondrial cytochrome *b* and NADH dehydrogenase subunit 2 (NADH2) sequences were obtained from samples representing all populations of *B. parvus*. Three divergent mitochondrial DNA (mtDNA) lineages were identified which exhibited strong phylogeographical structure. This indicates the presence of three maternal clades corresponding to populations in the northern, central and southern Australian alps. Molecular clock estimates suggest that the mtDNA lineages diverged from one another 420–680 thousand years ago. On this basis it is argued that *B. parvus* populations have probably been isolated since the mid-Pleistocene, and that management should focus on maintaining viable *B. parvus* populations in each of the three regional localities.

Keywords: *Burramys parvus*, conservation genetics, cytochrome *b*, fragmentation, mtDNA, NADH2

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Introduction

The mountain pygmy-possum (*Burramys parvus*) is the only marsupial restricted to the subalpine–alpine regions of mainland southeastern Australia (Fig. 1). It is physiologically intolerant to temperatures exceeding 28 °C (Fleming 1985) and is found only above 1400 m in boulder fields associated with vegetation such as mountain plum-pine (*Podocarpus lawrencei*) and alpine pepper (*Tasmania xerophylla*). The specialized nature of its habitat results in a patchy distribution for *B. parvus*, with a total area of available critical habitat of only 10 km^2 (Smith & Broome 1992). Developments associated with alpine ski resorts have resulted in degradation and increased fragmentation of much of this habitat. Combined with small population size, this threatens the survival of the species.

The total number of adult *B. parvus* is currently estimated at around 2600 individuals (Mansergh & Broome 1994). Approximately half the total population is located in the northern Australian alps, in the Kosciusko National Park, with the remainder distributed in the central (Mount Bogong, Mount Hotham and the Bogong High Plains) and southern (Mount Buller) Australian alps. The Mount Buller population of *B. parvus* was only discovered in 1996 and is estimated to comprise 300 adults (D. Heinze, personal communication).

The aim of the present study was to determine the relationships and degree of isolation of the extant *B. parvus* populations using sequence data from the mitochondrial NADH dehydrogenase subunit 2 gene (NADH2) and part of the cytochrome *b* gene. This information is to form part of the development of a conservation management strategy for the species.

Methods

Hair samples were used from 21 wild caught individuals from five sampling localities across the species' range. Tissue samples were also obtained from single specimens from Mount Hotham and Bogong High Plains in the central alps. The localities examined and sample sizes are given in Fig. 1.

DNA was extracted following the method of Gemmel & Akiyama (1996). A mitochondrial DNA (mtDNA)-enriched fraction was extracted from one of the tissue samples using the method of Tamura & Aotsuka (1988)

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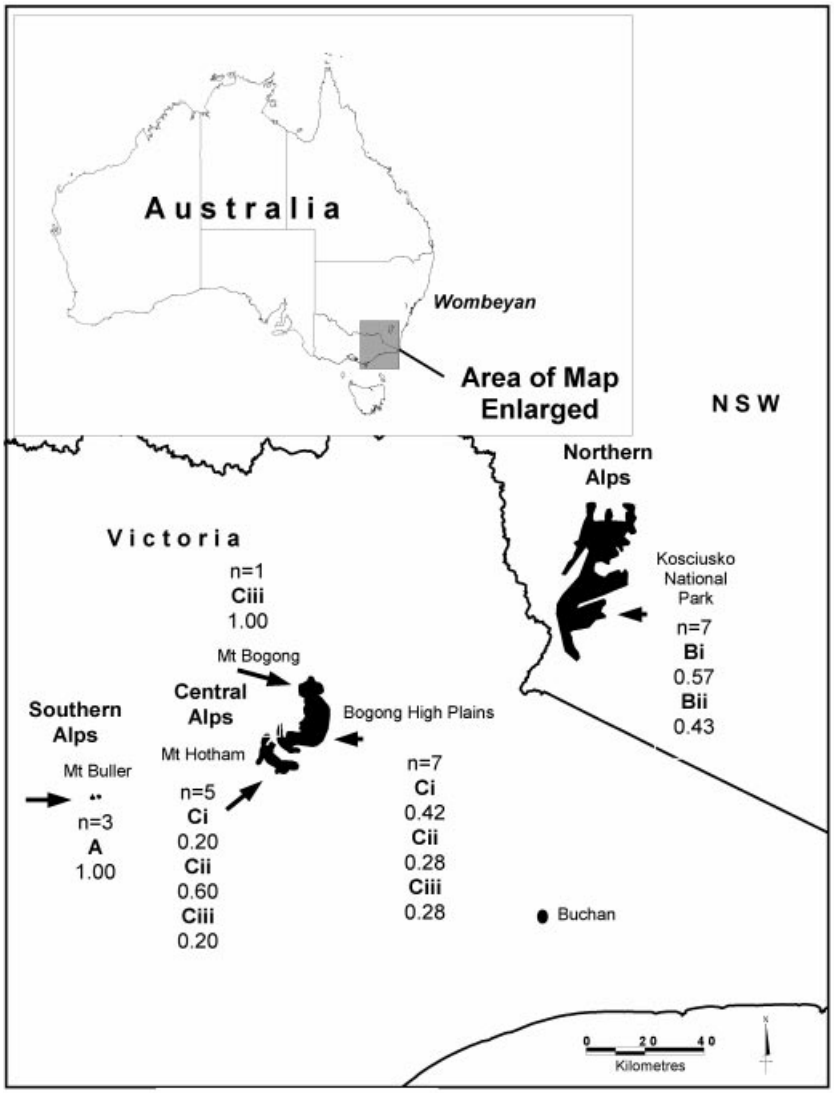

Fig. 1 Distribution map of *Burramys parvus* showing regions above 1400 m where populations occur. Sample sizes (*n*) and haplotype frequencies are given.

and purified using the Qiagen DNA plasmid kit to confirm the mitochondrial origin of sequences. The NADH2 gene was amplified and sequenced using the methods and primers described in Osborne & Norman (submitted). An additional double-stranded sequence was obtained using the primers ND2.2 h (5′-actcaaaagtggaatggtgc), and ND2b (5′-gttgggtcattgaagcttaccg). A portion of the cytochrome *b* gene was initially amplified and sequenced using L15424 and H15767 (Edwards *et al*. 1991). From this sequence, *Burramys parvus*-specific primers were designed: BpcytbL (5′-cttctagccttattctccccagac) and BpcytbH (5′-cccccaattcatgtaagggtaaata) which span a region of 264 bp. The cycling conditions were essentially the same as for NADH2 but with an annealing temperature of 56 °C and an extension time of 20 s. Polymerase chain reaction (PCR) products were purified using the Qiagen QIAquick PCR purification kit. Cycle sequencing was carried out using the Promega *fmol*™ thermal cycle sequencing kit.

Combined NADH2 and cytochrome *b* sequences were analysed using the published opossum (*Didelphis virginiana*) sequence (Janke *et al*. 1994) as an outgroup. Pairwise distances were estimated by Kimura's (1980) 2-parameter model. Sequences were aligned and a distance matrix was created using the DNADIST option of PHYLIP 3.572 (Felsenstein 1996). From this distance matrix, neighbour-joining trees were generated using the NEIGHBOR option of this program. PAUP 3.1.1 (Swofford 1993) was used to find the most parsimonious tree. The robustness of the branching patterns of the trees was assessed using bootstrapping (1000 replicates).

Results and Discussion

Haplotype diversity

A complete NADH2 sequence (1044 bp) was obtained

Table 1 Pairwise comparisons of percentage sequence divergences, below diagonal, and transition, transversion differences, above diagonal

Haplotype	A	Bi	Bii	Ci	Cii	Ciii
A	****	14, 1	15, 1	16, 0	20, 0	17, 0
Bi	1.16	****	1, 0	8, 1	12, 1	9, 1
Bii	1.22	0.08	****	9, 1	13, 1	10, 1
Ci	1.22	0.7	0.80	****	4, 0	1, 0
Cii	1.53	1.01	1.10	0.31	****	5, 0
Ciii	1.30	0.80	0.84	0.08	0.38	****

from 10 individuals representing the five sampling localities. Twenty variable sites were observed: four at the first position and 16 at the third position. These identified five haplotypes (Table 1) referred to as: A, Bi, Bii, Ci and Cii. Differences between haplotypes involved transitions except for one transversional change separating the B haplotypes (Bi, Bii) from the rest. All sites were verified by obtaining a double-stranded sequence for a single representative of each haplotype. Partial NADH2 sequence data comprising 685 bp was obtained from an additional 13 individuals. This region contained 14 of the 20 variable sites and was sufficient to identify the five haplotypes detected using the complete NADH2 sequences. A 264 bp segment from the 3′ end of cytochrome *b* was also sequenced from the same 23 individuals. This identified an additional haplotype (Ciii). GenBank Accession nos for the NADH2 and cytochrome *b* sequences are: AF206308 and AF206307. The combined NADH2 and cytochrome *b* sequences (1308 bp) identified six haplotypes (A, Bi, Bii, Ci, Cii, Ciii) with percentage divergences ranging from 0.08 to 1.53% (Table 1).

Phylogeographical patterns

Neighbour-joining and PAUP analyses (Fig. 2b) recognized three distinct lineages among the six haplotypes. These corresponded to the three broad geographical regions of the species range — the southern (A), central (Ci, Cii, Cii) and northern (Bi, Bii) alps. Bootstrap values for the central and northern clades were high in the neighbour-joining tree (95% and 100%, respectively) and moderate in the PAUP tree (73% and 80%, respectively). Both neighbour-joining and PAUP trees (Fig. 2b) identified the northern (haplotypes Bi, Bii) and central (haplotypes Ci, Cii, Ciii) clades as sister lineages relative to the southern clade (haplotype A).

The hierarchical pattern of sequence divergences (Table 1) also reflected the presence of three genetically distinct groups corresponding to the northern, central and southern alps. The levels of sequence divergence recorded between regions (0.70–1.53%) were higher than between populations within regions (0.08–0.38%). Despite the small sample sizes used, multiple haplotypes were detected at three of the five sampling localities; Mount Hotham and Bogong High Plains in the central alps, and Kosciusko National Park in the northern alps. Further sampling of individuals is required to determine more precisely the distribution of haplotypes within the central and northern alpine populations. This may reveal an additional level of population structuring not apparent in this preliminary study.

The above results indicate that genetic variation in *Burramys parvus* occurs at three levels; between regional groups (northern, central and southern alps), within a regional group, and within populations. Three factors are likely to have been important in determining the observed pattern of mtDNA differentiation: (i) the impact of Pleistocene glacial cycles on the availability and distribution of suitable contemporary habitat; (ii) the physiological intolerance of the species to high temperatures; and (iii) the effects of biased genetic sampling (e.g. stochastic lineage sorting, founder events and genetic drift).

The Pleistocene period was characterized by short-term climatic oscillations which resulted in repeated glacial and interglacial cycles (Galloway & Kemp 1981). The impact of these phases on a species is dependent on its habitat requirements. Ride & Davies (1997) suggested that populations dependent on alpine habitats (such as *B. parvus*) would have contracted during the warmer interglacials and expanded during the colder glacial cycles. They further postulated that populations of these species would have been reconnected during subsequent glacial cycles when the alpine zone extended 1000 m below its present level (Twidale & Campbell 1993). Ride & Davies's (1997) proposition is supported by the presence of *B. parvus* fossils in cave deposits at Wombeyan (north of Kosciusko; Fig. 1) and Buchan (southeast of Mount Buller; Fig. 1) (Broom 1896; Ride 1956) indicating a more extensive distribution for *B. parvus* during the most recent glacial phase (30 000–18 000 years ago). These low-altitude populations probably became extinct as conditions became warmer (Morris *et al*. 1997).

We used molecular clock estimates to determine the impact of Pleistocene glacial cycles on population structuring in *B. parvus*. A rate of 2% sequence divergence per million years was assumed (Brown *et al*. 1979) and this rate has been used in other studies of marsupials (Taylor *et al*. 1994; Krajewski *et al*. 1997). Accordingly, individual haplotypes within the central (Ci, Cii, Ciii) and northern (Bi, Bii) regions are estimated to have diverged 40 000–190 000 years ago. The levels of genetic differentiation among regional haplotypes (northern, central, southern) suggest that they diverged much earlier, between 420 000 and 680 000 years ago. We emphasize that such estimates relate to divergence times for the haplotypes, and may

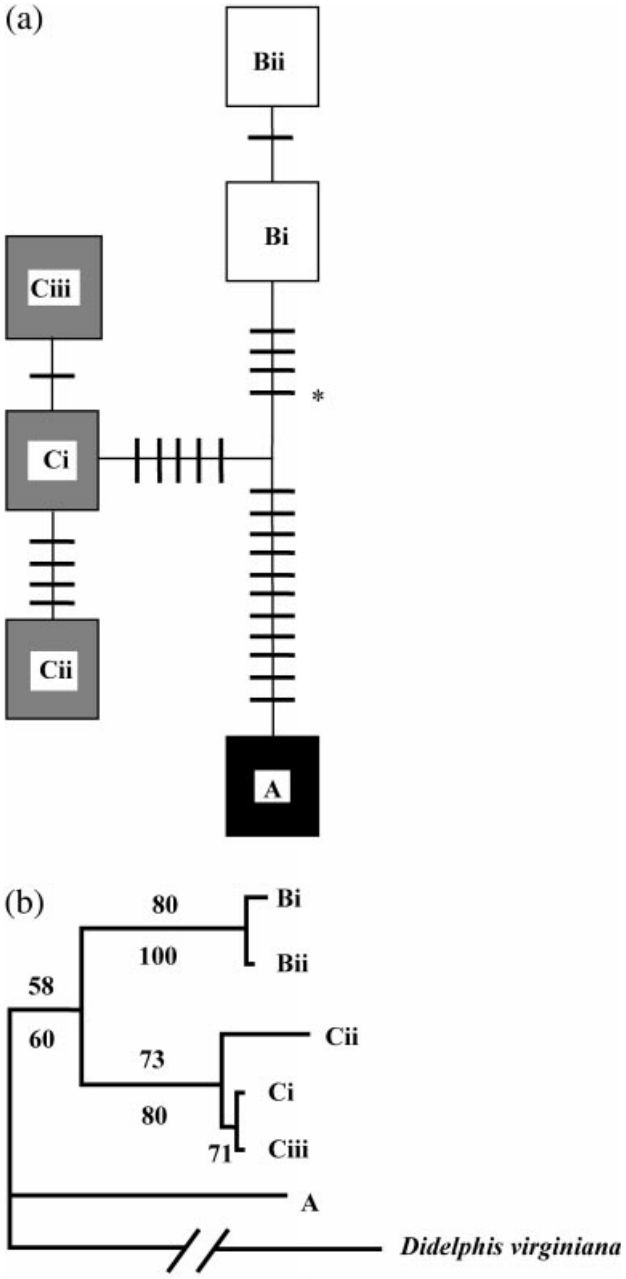

Fig. 2 (a) Unrooted haplotype network, all changes are transitions except one transversion indicated by an asterisk; (b) neighbour-joining tree based on Kimura (1980) 2-parameter distance (bootstrap values below branches) and single shortest PAUP tree (29 steps; retention index of 0.71; consistency index of 0.76; bootstrap values above branches). Haplotypes Ci, Cii, Ciii form an unresolved trichotomy in the PAUP tree.

in fact predate population separation (Avise *et al*. 1984). Under conditions that favour short periods of stochastic lineage sorting (e.g. small population size), divergence times can be significantly overestimated (Hoelzer *et al*. 1998). This could also occur under conditions promoting high levels of mtDNA diversity within populations, such that a recently isolated group could already be very distinct from the original population as a result of sampling bias (Hoelzer & Melnick 1994). Nevertheless, we consider that the geographical distance between the three alpine regions, the absence of intervening habitat patches (Galloway & Kemp 1981; McDougall 1982; O'Brien & Gowans 1985) and movement corridors (Mansergh & Scotts 1989) probably severely limited dispersal opportunities in *B. parvus*. Consequently, the observed genetic structure in *B. parvus* more likely reflects the effects of the climatic phases of the Pleistocene than founder events or lineage sorting. Various climatic phases may correspond to different levels of genetic variation in *B. parvus*; with the higher levels of divergence (between regions) a result of earlier Pleistocene events, and the lower levels (haplotype frequency differences, within regions) a consequence of more recent factors. The high level of differentiation between regional groups conflicts with conclusions reached by Ride & Davies (1997), that the extant populations of *B. parvus* are remnants of a species that had a more contiguous distribution as recently as 18 000 years ago.

Taxonomic and conservation implications

On the basis of behaviour and morphology, Mansergh & Broome (1994) suggested that populations from the northern and central alps may be subspecifically distinct from one another. This is unlikely, given that molecular data link these populations to the exclusion of the recently discovered southern alpine population. Instead, the molecular data demonstrate that three regional genetic groups of *B. parvus* should be recognized. These have been isolated for a significant length of time and thus have had separate evolutionary histories.

The results can be used to make some broad recommendations for the conservation of the species. Management decisions should consider as a priority that there are three genetically distinct regional populations of *B. parvus* from the northern, central and southern alps. Management of *B. parvus* should focus on maintaining viable representative populations from each of these regions. As stated previously, additional samples need to be considered to determine, more precisely, the distribution of the haplotypes identified in this preliminary study. The most immediate threat to *B. parvus* is the encroachment of tourist developments into areas of critical habitat. Conservation of *B. parvus* may be achieved best by maintaining and enhancing existing habitat areas and maximizing connectivity between populations within each of the regions.

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References

Avise JC, Neigel JE, Arnold J (1984) Demographic influences on mitochondrial DNA lineage survivorship in animal populations. *Journal of Molecular Evolution*, **20**, 99–105.

Broom R (1896) On a small fossil marsupial with large grooved premolars. *Proceedings of the Linnean Society of New South Wales (Ser., 2)*, **10**, 563–567.

Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Science USA*, **76**, 1967–1971.

Edwards SV, Archtander P, Wilson AC (1991) Mitochondrial resolution of a deep branch in the genealogical tree for perching birds. *Proceeding of the Royal Society of London*, **243**, 99–107.

Felsenstein J (1996) *Phylogenetic Inference Programs (PHYLIP)*. University of Washington, Seattle; University Herbarium, University of California, Berkeley.

Fleming MR (1985) The thermal physiology of the Mountain Pygmy-possum *Burramys parvus* (Marsupialia: Burramyididae). *Australian Mammalogy*, **8**, 79–90.

Galloway RM, Kemp EM (1981) Late Cainozoic environments in Australia. In: (ed. Keast A) *Ecological Biogeography of Australia*, pp. 53–80. Dr W Junk BV, London.

Gemmel NJ, Akiyama S (1996) An efficient method for the extraction of DNA from vertebrate tissues. *Trends in Genetics*, **12** (9), 338–339.

Hoelzer GA, Melnick DJ (1994) Patterns of speciation and limits to phylogenetic resolution. *Trends in Ecology and Evolution*, **9**, 104–107.

Hoelzer GA, Williams J, Melnick DJ (1998) The effects of social structure, geographical structure, population size on the evolution of mitochondrial DNA: II. Molecular clocks and the lineage sorting period. *Journal of Molecular Evolution*, **47**, 21–31.

Janke A, Feldmaier-Fuchs G, Thomas WK, von Haeseler Paabo S (1994) The marsupial genome and the evolution of placental mammals. *Genetics*, **137**, 243–256.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequence. *Journal of Molecular Evolution*, **116**, 111–120.

Krajewski C, Blacket M, Buckley L, Westerman M (1997) A multigene assessment of phylogenetic relationships within the marsupial subfamily Sminthopsinae. *Molecular Phylogenetics and Evolution*, **8** (2), 236–248.

Mansergh I, Broome L (1994) *The Mountain Pygmy-possum of the Australian Alps, Australia*. New South Wales University Press, NSW, Australia.

Mansergh I, Scotts D (1989) Habitat continuity and social organisation of the Mountain Pygmy-possum restored by tunnel. *Journal of Wildlife Management*, **53** (3), 701–707.

McDougall K (1982) *The Alpine Vegetation of the Bogong High Plains*. Environmental Studies Publication no. 357. Ministry for Conservation, Victoria.

Morris DA, Augee ML, Gillieson D, Head J (1997) Analysis of a late Quaternary deposit and small mammal fauna from Nettle Cave, Jenolan, New South Wales. *Proceedings of the Linnean Society of New South Wales*, **117**, 135–162.

O'Brien M, Gowans RM (1985) *The Distribution of Boulder Streams and Podocarpus Heath, Components of the Preferred Habitat of* Burramys parvus, *in Alpine and Subalpine Areas of Eastern Victoria*. Arthur Rylah Institute for Environmental Research, Technical Report Series 8. Department of Conservation Forests and Lands, Australia.

Osborne MJ, Norman JA (submitted) Versatile primers for the amplification of the mitochondrial NADH dehydrogenase subunit 2 gene in marsupials. *Molecular Ecology*.

Ride WDL (1956) The affinities of *Burramys parvus* Broome — a fossil Phalangeroid marsupial. *Proceedings of the Zoological Society, London*, **127** (3), 413–429.

Ride WDL, Davies AC (1997) Origins and settings: mammal Quaternary palaeontology in the Eastern Highlands of NSW. *Proceedings of the Linnean Society NSW*, **117**, 197–222.

Smith A, Broome L (1992) The effects of season, sex and habitat on the diet of the Mountain Pygmy-possum (*Burramys parvus*). *Wildlife Research*, **19**, 755–768.

Swofford DL (1993) *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1*. Illinois Natural History Survey, Champaign.

Tamura K, Aotsuka T (1988) Rapid isolation of animal mitochondrial DNA by the alkaline lysis procedure. *Biochemical Genetics*, **26**, 815–819.

Taylor AC, Sherwin WB, Wayne RK (1994) Genetic variation of microsatellite loci in a bottlenecked species: the Northern Hairy-nosed wombat *Lasiorhinus krefftii*. *Molecular Ecology*, **3**, 277–290.

Twidale CR, Campbell EM (1993) *Australian Landforms: Structure, Process and Time*, pp. 367–374. Gleneagles, Adelaide.

Megan Osborne's research is currently focused on the population genetics of *Burramys parvus*. This is part of her PhD research on the molecular evolution of possums. Dr Leslie Christidis is Director of the Science Program at the Museum of Victoria and Dr Janette Norman is head of its Molecular Evolutionary Genetics Unit. Both undertake collaborative research on the evolutionary biology and phylogeny of Australasian birds, and the population genetics of diverse Australian faunas. Dr Neil Murray is a senior lecturer at La Trobe University, whose research interests include the conservation genetics of Australian endangered species.
