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The identity of the Depuch Island rock-wallaby revealed through ancient DNA

D. Haouchar[A], *J. Haile*[A], *P. B. S. Spencer*[B] *and M. Bunce*[A,C]

[A]Ancient DNA Laboratory, School of Biological Sciences, Murdoch University, Perth, WA 6150, Australia.
[B]Wildlife Identification Laboratory, School of Biological Sciences, Murdoch University, Perth, WA 6150, Australia.
[C]Corresponding author. Email: m.bunce@murdoch.edu.au

Abstract. Ancient DNA is becoming increasingly recognised as a tool in conservation biology to audit past biodiversity. The widespread loss of Australian biodiversity, especially endemic mammal populations, is of critical concern. An extreme example occurred on Depuch Island, situated off the north-west coast of Western Australia, where an unidentified species of rock-wallaby (*Petrogale* sp.) became extinct as a result of predation by red foxes. Two potential candidate species, *Petrogale lateralis* and *P. rothschildi*, both have ranges adjacent to Depuch Island, making identification based on geography difficult. A museum bone (one of the only surviving Depuch Island specimens) was subjected to standard ancient DNA analyses and procedures. Mitochondrial DNA cytochrome *b* and hypervariable control region were targeted for species identification. Ancient DNA was successfully recovered from the bone: 200 base pairs (bp) of control region and 975 bp of the cytochrome *b* gene. Bayesian phylogenetic analyses were employed to model the Depuch Island rock-wallaby DNA sequences together with sequences of other rock-wallaby taxa from GenBank. Evidence suggests that of the two *Petrogale lateralis* subspecies proposed to have inhabited Depuch Island, *Petrogale lateralis lateralis* was identified as the most likely. The identification of the Depuch Island rock-wallaby population may assist in the reintroduction of an insurance population of *Petrogale lateralis lateralis*, which is becoming increasingly threatened on mainland Australia.

Additional keywords: Past biodiversity, Conservation.

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Introduction

The Australian mammal fauna has undergone several extinctions and population declines since European settlement (Burbidge and Manly 2002; Johnson 2006). Among those species most severely affected have been the medium-sized species, more specifically marsupials that are in the 'critical weight range' (between 35 and 5500 g) (Johnson and Isaac 2009). As a result, combinations of environmental pressures such as introduced feral predators, and demographic and genetic stochasticity have contributed to putting isolated populations of critical-weight marsupials, such as those that exist on islands (Frankham 1997; Eldridge *et al*. 1999), under extreme pressure.

Located off the north-west coast of Western Australia (Fig. 1*a*), Depuch Island (DI) was previously separated from the mainland by ~3 km (2 miles) of shallow water and mud flats (Ride 1964). Whilst neighbouring islands in the Forestier's Archipelago are low and sandy, DI has undulating outcrops of dolerite and granite (Ride 1964; McCarthy 1961), which provide ideal refuge sites and habitat for rock-wallabies. DI, known as Womalantha by Aboriginal people, was named in 1801 by Nicolas Baudin on board the *Le Géographe* and revisited 40 years later by Captain J. C. Wickham, commander of the *H.M.S. Beagle*, who conducted the first biological survey of the island (Ride 1964). DI is well known to anthropologists as an important site of Aboriginal rock art and engravings (Ride 1964) and was declared a sanctuary in 1958 for the protection of engravings and fauna (McCarthy 1961). The island was previously inhabited by several bird, reptile and mammal species including a rock-wallaby (recorded as a small kangaroo by Péron 1807, and as *Petrogale lateralis* by Stokes 1846), which became extinct as a consequence of predation by the introduced red fox (*Vulpes vulpes*) (Kinnear *et al*. 1988, 1998). Rock-wallaby colonies were reported on DI at the mouth of the Balla Balla River and the animals were known to be present in 1964. Judging by the vast quantities of their droppings around the island, the rock-wallabies seemed plentiful at the time (Ride 1964). However, 20 years later they were considered locally extinct (Hall and Kinnear 1991) before any secure species identification could be made. Prior to any reintroduction attempts, a sound species and genetic characterisation of the extinct wallaby should be undertaken.

At present, the taxonomic identity of the Depuch Island rock-wallaby is unresolved, with two candidate species inhabiting north-west Australia: the black-footed (black-flanked) rock-wallaby (*Petrogale lateralis*) and Rothschild's rock-wallaby (*Petrogale rothschildi*). The location of DI within the range of both species makes it difficult to predict which taxon might have formerly occupied this island (see Fig. 1*a*). Ride (1964) set out to confirm Stokes' (Stokes 1846) observation of an unknown

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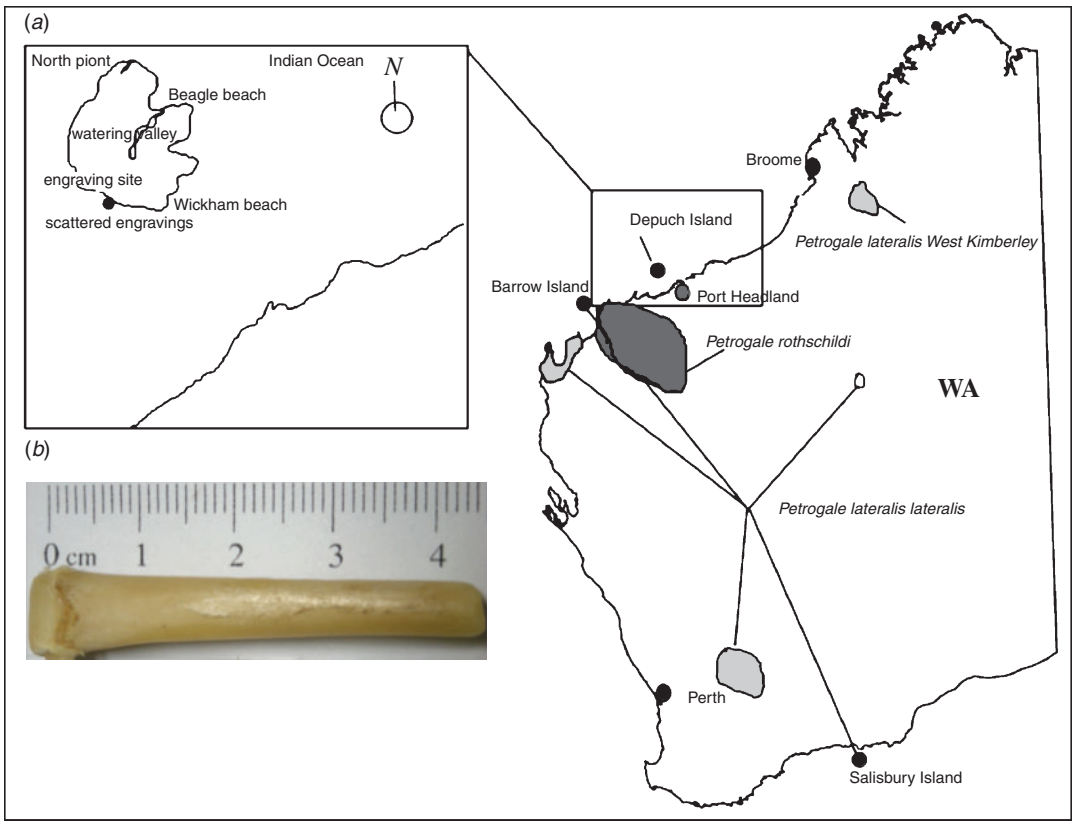

Fig. 1. (*a*) Current species distribution of *Petrogale rothschildi* (dark grey), *Petrogale lateralis lateralis* (light grey) and *Petrogale lateralis* West Kimberley race (medium grey) in relation to Depuch Island. Inset shows an enlargement of Depuch Island, showing sites mentioned in the text. (*b*) Western Australian Museum fossil bone of fourth metatarsal (proximal end missing) (M5233; Depuch No. 48) from Depuch Island (collected on 15 June 1962) used in this study. Map (*a*) based on Mason *et al*. (2011) and (Eldridge *et al*. 1994).

species of rock-wallaby on DI, and stressed the importance of identifying the species for zoogeographical and taxonomic reasons. Ride (1964) noted that two species occurred in the vicinity of the island, *P. rothschildi* inhabiting islands of the Dampier Archipelago and *P. lateralis* being dispersed throughout the Western Australian mainland and also Barrow Island (Ride 1964).

However, *Petrogale lateralis* is polytypic, consisting of five distinct subspecies/races – *P. l. lateralis*, *P. l. hacketti*, *P. l. pearsoni*, *P. l.* 'MacDonnell Ranges' and *P. l.* 'West Kimberley' (Sharman *et al*. 1990; Eldridge *et al*. 1994, 2001; Eldridge and Close 1997; Pearson and Kinnear 1997; Potter *et al*. 2012) – some of which are readily distinguished by traditional morphology (e.g. skull/body morphology/size, coat colour and markings). However, such features are highly variable within rock-wallaby species and are therefore an unreliable means of classification (Eldridge and Close 1992, 1997; Eldridge and Spencer 1997). Prior to DNA studies one of the most convincing genetic species determiners had been chromosome and allozyme similarities (Sharman *et al*. 1989; Eldridge *et al*. 1991*a*, 1991*b*, 1994). However, the use of chromosomes to provide definitive species identification is limited by the technique's requirement for living tissue from which rapidly dividing cells can be cultured for analyses (Eldridge and Spencer 1997; Loupis and Eldridge 2001). Now with DNA studies becoming a diagnostic tool for species identification, it is mandatory that this research be used in identifying which subspecies/races of *P. lateralis* likely occurred on DI. Given the location of *P. l. lateralis* and *P. l.* 'West Kimberley' near the island, either of these could be potential candidates. Therefore, the use of mitochondrial DNA (mtDNA) targeting cytochrome *b* and control region can be useful in telling these taxa apart.

Previously, chromosome analyses categorised *Petrogale* species into three distinct groups: the *xanthopus*, *brachyotis* and *lateralis–penicillata* groups (Eldridge and Close 1992, 1997). However, recent molecular analyses shows that the *xanthopus* group is not monophyletic and comprises independent lineages for *P. xanthopus*, *P. persephone* and *P. rothschildi*, despite their shared ancestral karyotypes (Potter *et al*. 2012). Further analyses show that four distinct lineages were identified on the basis of mitochondrial and nuclear DNA: (1) the *brachyotis* group, (2) *Petrogale persephone*, (3) *Petrogale xanthopus* and (4) the *lateralis–penicillata* group (Potter *et al*. 2012).

The aim of this study was to isolate ancient DNA from a museum specimen for species identification, namely targeting mtDNA. A marginally degraded subfossil museum bone (Fig. 1*b*) was subjected to mitochondrial DNA (mtDNA) analysis of the cytochrome *b* gene and hypervariable control region. Until now

no attempts had been made to clearly identify the endemic species of rock-wallaby that previously inhabited the island, which is important as it will allow informed decisions to be made in any biodiversity restoration projects onto the now fox-free DI. Since this species is completely extinct on the island and there are only a few trace rock-wallaby museum specimens left, ancient DNA analysis is the most suitable technique and is highly recommended in order to handle and retrieve relevant data for species identification useful in this study.

Materials and methods

Sample collection and DNA extraction

Due to sampling restrictions, a single DI rock-wallaby subfossil bone (fourth metatarsal) was accessed from the Western Australian Museum (M5233; collected on 15 June 1962) and sampled for DNA (Fig. 1*b*). DNA extraction procedures were carried out in a dedicated ancient DNA laboratory, minimising contamination from PCR amplicons and modern DNA. A sample of ~100 mg of bone powder was obtained using a Dremel tool (part no. 114; Germany) at high rotational speeds. Bone powder was collected in a 1.5-mL Eppendorf tube, weighed and stored for later digestion.

The digestion step included an overnight incubation at 55°C with rotation in a 1.5-mL digestion buffer containing 20 mM Tris pH 8.0 (Sigma, MO, USA), 10 mM dithiothreitol (Thermo Fisher Scientific, MA, USA), 1 mg mL^{-1} proteinase K powder (Amresco, OH, USA), 0.48 M EDTA (EDTA) (Invitrogen) and 1% Triton X-100 (Invitrogen). After digestion, centrifugation at 13 000*g* was initiated for 1 min to pellet undigested material. The supernatant containing the DNA was concentrated to ~100 µL in a Vivaspin 500 column (MWCO 30000; Sartorius Stedim Biotech, Germany) at 13 000*g*, and then combined with five volumes of PBi buffer (Qiagen, CA, USA). DNA was immobilised on silica spin columns (Qiagen) and washed with 700 µL of AW1 and AW2 wash buffers (Qiagen). Finally, the DNA was eluted from the silica in 50 µL of 10 mM Tris pH 8.0 (Sigma, MO, USA).

Primer design

Primers were designed in order to amplify small segments of DNA spanning the targeted mtDNA regions, ranging in size from 150 to 420 base pairs (bp) (see Table 1 for primer combinations). PCR amplification of 975 bp of cytochrome *b* gene and 200 bp of the hypervariable control region was targeted by designing specific primers for rock-wallabies using GENEIOUS 4.8 (Biomatters, New Zealand).

PCR of cytochrome b and control region

Amplification of DNA product was carried out using real-time PCR to assess the amount of DNA preserved in the sample, a protocol advocated in many ancient DNA procedures (Cooper and Poinar 2000; Pruvost and Geigl 2004). The StepOne real-time PCR system (Applied Biosystems) was used with a final reaction concentration of: 1 × High Fidelity PCR Buffer (Invitrogen), 50 mM $MgSO_4$ (Invitrogen), 0.25 mM of each dNTPs (Austral Scientific), 8 µM of each primer, 0.25 U HIFI Taq polymerase (Invitrogen), 1 µL (10 mg mL^{-1}) bovine serum albumin (Fisher Biotech), 0.6 µL SybrGreen (Invitrogen cat no S7563, 1 : 2000 dilution), ultrapure H_2O and 2 µL of DNA extract in a 25-µL reaction. PCR thermal cycling was initiated with a 5-min denaturation step at 94°C, followed by 50 cycles of 94°C for 45 s, with a variable annealing temperature either 53°C or 57°C according to primer set used (see Table 1), followed by 68°C for 45 s and a final extension at 72°C for 10 min. Quantitative PCR data were analysed using Applied Biosystems StepONE software version 2.00 software. The relative yield of DNA was assessed between each dilution sample according to the C_T values. Samples were visualised on a 2% w/v DNA-grade agarose gel electrophoresis (Bio-Rad) stained with ethidium bromide.

Cloning mtDNA

All DNA products were amplified at least twice, and control region PCR products that contained amplified fragments of the appropriate size were purified using a Qiaquick Purification kit (Qiagen) and cloned using pGEM-T vector system (Promega). Successfully cloned products were chosen after being screened for blue (which do not contain the pGEM-T vector) and white (which contain the pGEM-T vector) colonies on LB/ampicillin/IPTG/X-Gal plates. A random selection of ~10 white colonies and at least one blue colony was selected for size comparison and screened on a 2% agarose w/v gel. Amplicons were checked

Table 1. Mitochondrial control region and Cytochrome b primer sequences and amplification conditions used to genetically characterise the DI rock wallaby

Primer name	Primer sequence 5′ → 3′	Annealing temperature (°C)	Amplicon size (bp)
DL1F	CCACAACACATCAACTYATTTG	53	150
DL1R	ATTCATTTTATGTATTACTAGAATTATGTA		
DL3F	TGTATTAAGACAGATATGTATAAAGT	53	250
DL2R	ATTCATTTTATGTATTACTAGAATTATGTA		
DL3F	TGTATTAAGACAGATATGTATAAAGT	53	280
DL3R	AGTCAGAGATTTGTTAGGTACG		
Cytb_WallabyF1	GACACCCTAACAGCCTTCTCATCAG	57	260
Cytb_WallabyR1	CGGTAGCTCCTCAGAATGATATTT		
Cytb_WallabyF2	AAATATCATTCTGAGGAGCTACCG	57	340
Cytb_WallabyR2	GAGAAGTTGTCTGGGTCGCC		
Cytb_WallabyF3	GGCGACCCAGACAACTTCTC	57	240
Cytb_WallabyR3	GGCTGTAAGGATTCAGAATAGGAT		

to ensure that products were of predicted size, purified and finally prepared for sequencing. This cloning step ensured the production of clean chromatographs facilitating unambiguous base calls, as well as allowing an assessment of DNA damage and polymerase error.

Sequencing of Cytochrome b and control region

Sanger sequencing was carried out at the commercial facility Macrogen (Seoul, South Korea) using BigDye ver. 3.1 (Applied Biosystems) chemistry on a cycling ABI3730XL capillary sequencer (Applied Biosystems). Sequences were analysed using GENEIOUS 5.4.3 (Biomatters, New Zealand) and deposited on GenBank under accession numbers JN898804 and JN898805.

Phylogenetic analysis of Cytochrome b and control region sequence

Alignments of nucleotide sequences were carried out in GENEIOUS 5.4.3, with any ambiguities resolved by eye. Reference species used in this analysis were derived from published sequence data (Potter *et al*. 2012). GenBank accession numbers for cytochrome *b* – *P. lateralis lateralis* (JQ042127-S972, Nangeen Hill, south-west Western Australia), *P. rothschildi* (JQ042134-S204, Rosemary Island, Dampier Archipelago) and *P. lateralis* West Kimberley race (JQ042130) – were used for comparison. Control region of *P. lateralis lateralis* (AF348675-S207, Ningaloo) was also used, along with *P. rothschildi* (not published) and *P. lateralis* West Kimberley race (AF348688) for comparison. To ensure validity of DNA sequences and to overcome ancient DNA damage, multiple-sequence datasets were created and an overall consensus was drawn for use in the final phylogenies. Topology analysis was conducted using the Bayesian phylogenetic program MrBayes (Huelsenbeck and Ronquist 2001), a plug-in application provided through GENEIOUS 5.4.3. The MrBayes analysis extended over 1 million iterations, and genealogies and model parameters were sampled every 1000 iterations with 10% burn-in. An HKY85 substitution model was imposed and an invariance gamma model for among-site variation was chosen according to the simulations run in MrjModel Test (Nylander 2004). Results were visualised and examined in FigTree ver. 1.2.2.

Results

MtDNA analysis of Cytochrome b and control region

The cytochrome *b* sequence alignment of 975 bp of the DI rock-wallaby specimen to the two reference species, *P. l. lateralis* and *P. rothschildi*, consisted of 99% and 93% of identical sites, respectively. Among-site variation between the DI rock-wallaby and *P. l. lateralis* revealed 10 single-nucleotide polymorphisms (SNPs), nine transitions (7 C → T and 2 G → A) and one transversion (A → C). Between the DI rock-wallaby and *P. rothschildi* 69 SNPs were identified, 58 being transitions (48 C → T and 10 G → A) and 11 transversions (7 A → C and 4 A → T).

The control region (Domain 1) alignment of 200 bp of the DI rock-wallaby specimen and *P. l. lateralis* and *P. rothschildi* consisted of 92.3% and 63.2% identical sites, respectively. Among-site variation revealed 15 SNPs between the DI rock-wallaby and *P. l. lateralis*, eight transitions (2 G → A and 6 C → T) and seven transversions (2 A → C, 4 A → T and 1 G → C). Among-site variation between the DI rock-wallaby and *P. rothschildi* consisted of 41 SNPs, 16 transitions (13 C → T and 3 G → A) and 25 transversions (3 A → C, 13 A → T, 3 G → C and 6 T → G).

Phylogenetic analysis

Mitochondrial DNA sequences were positively identified on the basis of congruent phylogenies for both the cytochrome *b* gene and control region. This is a strong indication that sequences were not artefacts of nuclear copies. Modelling the concatenated consensus sequence data for both cytochrome *b* and control region was carried out using Bayesian phylogenetic methods. The *Thylogale* (pademelon) was chosen as the outgroup for this study. Phylogenetic analysis shows clear support that the unidentified rock-wallaby specimen collected from DI is most similar to *P. l. lateralis* at the mtDNA level (Fig. 2). *P. l. lateralis* consistently forms a sister clade with the DI specimen (100% posterior probabilities) in both phylogenies to the exclusion of *P. rothschildi* and other closely neighbouring rock wallabies such as *P. lateralis* West Kimberley (Fig. 2).

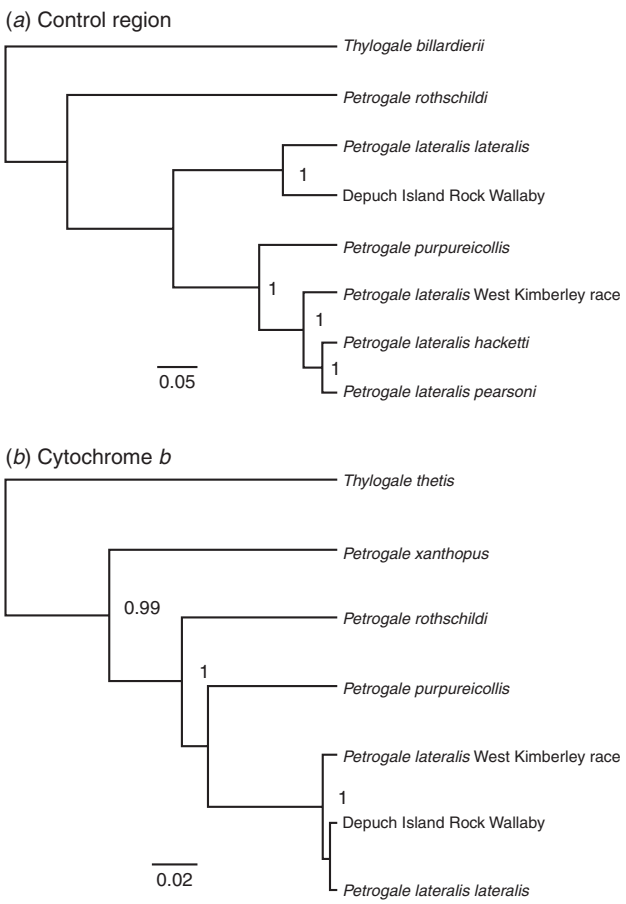

Fig. 2. Bayesian phylogenetic tree showing the relationship of the Depuch Island rock-wallaby sequence with other available rock-wallaby sequence data: (*a*) based on 200 bp of control region and (*b*) based on 975 bp of cytochrome *b* sequence. Posterior probabilities greater than 90 are shown on nodes. The tree was built using a HKY85 model and invariant gamma was assumed and imposed with a relaxed molecular clock.

Discussion

Mainland Australia has recorded the world's highest rate of recent mammal extinctions (Short and Smith 1994) and mainland *Petrogale* populations have not been immune to these changes. Having undergone severe population retractions, the remaining animals are highly vulnerable to introduced predators (Kinnear *et al*. 1988, 1998), inbreeding depression and a multitude of interacting anthropogenic factors that further threaten population viability. Offshore islands have provided a refuge for many species, and currently harbor 14 species of Australian mammals (Eldridge *et al*. 1999) that are now extinct on the mainland. Such isolated islands can offer valuable insights into speciation and adaptive radiation and provide a refuge and conservation site for terrestrial mammal species (Maxwell *et al*. 1996; Abbott 2000). To date, species such as *Bettongia lesueur*, *Lagorchestus fasciatus*, *Perameles bougainville*, *Pseudomys fieldi*, amongst others, would now be extinct had they not been conserved on Western Australian islands (Burbidge *et al*. 1997).

DI is currently isolated, with the land bridge connecting it to the mainland being permanently under water, creating a barrier to mammal dispersal to the island. Prior to this separation it was connected by ~3 km of mud flats, exposed at low tide, which enabled access to the island and therefore gene flow and migration of several species, including introduced predators such as the red fox. Rock-wallaby (*Petrogale* spp.) populations were noted to exist on the island at one time and on other offshore islands of Western Australia, such as Salisbury and Barrow Island (Hall and Kinnear 1991), which have remained fox free, mainly due to their isolation (Eldridge *et al*. 2001). The species of rock-wallaby on DI, however, was never identified before it became locally extinct.

Our ancient DNA study has established that *P. l. lateralis* is most closely related to the DI rock-wallaby population and therefore the most probable rock-wallaby taxon that once inhabited DI. Having determined this makes future reintroduction attempts possible, with the exclusion of other potential rock-wallaby taxa like the *P. lateralis* 'West Kimberley' and *P. rothschildi* as potential candidates.

Previous attempts to identify unknown taxa using traditional morphological criteria have sometimes been challenging when dealing with morphologically cryptic species such as *Petrogale* (Loupis and Eldridge 2001). Other researchers have shown that although karyotypes can be useful in differentiating rock-wallaby taxa (Eldridge *et al*. 1991*a*), a major limitation is the necessity of living tissue that can then be cultured (Eldridge and Spencer 1997). The advancement of molecular biology techniques and ancient DNA techniques means that even samples that have been subjected to severe degradation can be used as a valuable source of information to investigate past populations.

Petrogale l. lateralis is classified as Vulnerable by the *Environment Protection and Biodiversity Conservation Act 1999* and the *Western Australian Wildlife Conservation Act*. To ensure survival of this species in the face of the continuing decline of mainland populations (Hall and Kinnear 1991; Mason *et al*. 2011), we would advocate the relocation of a viable population of *P. l. lateralis* onto the now fox-free DI, which would provide an insurance population for the species, at least for the short term. Recent studies by Mason *et al*. (2011) and others (Frankham 1997; Frankham *et al*. 2002) highlight the importance of preserving fragmented mainland populations of rock-wallabies rather than translocating populations to islands, because of the increasingly low levels of genetic diversity. However, given that *P. l. lateralis* was the original inhabitant of DI, repopulating it will be a significant step in restoring the island's past biodiversity and one that is likely to have positive effects on the island's ecosystem. Additionally, a sound understanding of the genetic diversity of mainland populations is a necessary prerequisite to enhance the reintroduced population's survival in the new island environment (Mason *et al*. 2011). The ultimate aim must be that, following successful reintroductions, such island sanctuaries can in the future be used as sources for returning species back onto the mainland, and, in doing so, restore at least some of the lost biodiversity of the recent past.

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