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RESEARCH ARTICLE

A complex history of introgression and vicariance in a threatened montane skink (*Pseudemoia cryodroma*) across an Australian sky island system

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Abstract Species endemic to sky island systems are isolated to mountain peaks and high elevation plateaux both geographically and ecologically, making them particularly vulnerable to the effects of climate change. Pressures associated with climate change have already been linked to local extinctions of montane species, emphasizing the importance of understanding the genetic diversity and population connectivity within sky islands systems for the conservation management of remaining populations. Our study focuses on the endangered alpine skink *Pseudemoia cryodroma*, which is endemic to the Victorian Alps in south-eastern Australia, and has a disjunct distribution in montane habitats above 1100 m a.s.l. Using mitochondrial DNA (mtDNA) and microsatellite loci, we investigated species delimitation, genetic connectivity and population genetic structure across the geographic range of this species. We found discordance between genetic markers, indicating historical mtDNA introgression at one of the study sites between *P. cryodroma* and the closely related, syntopic *P. entrecasteauxii*. Molecular diversity was positively associated with site elevation and extent of suitable habitat, with inbreeding detected in three of the five populations. These results demonstrate the complex interaction between geography and habitat in shaping the population structure and genetic diversity of *P. cryodroma*, and highlight the importance of minimising future habitat loss and fragmentation for the long-term persistence of this species.

Keywords Australian Alps · Biogeography · Lizard · Population genetics · Scincidae · Threatened species

Introduction

Originally popularized by Heald (1951), the term "sky islands" refers to mountain ranges that comprise a series of peaks and plateaux isolated both geographically and ecologically by areas of lower elevation. Sky islands have been described worldwide and include regions such as the Rocky Mountains, Ethiopian Highlands, and Western Ghats (McCormack et al. 2009). The size and connectivity of many sky island populations are thought to have fluctuated in response to Plio-Pleistocene glacial cycles (Markgraf et al. 1995; Williams et al. 1998). Specifically, sky island habitats extended downwards during periods of glaciation, facilitating gene flow between populations previously restricted to higher elevations, and then contracted again during interglacial periods. This is thought to have allowed species to extend their distributions to adjacent mountains during glacial periods (Browne and Ferree 2007; Rull and Nogue 2007) and/or diversify during interglacial periods

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(Masta 2000; Trewick et al. 2000; Petit et al. 2003; Shepard and Burbrink 2008; Schoville and Roderick 2009; Zimkus and Gvozdik 2013), both of which produce characteristic genetic signatures (Knowles and Carstens 2007). Current population genetic structure therefore provides insight into past population dynamics.

Although contemporary sky island species have survived historic fluctuations in temperature, many species are not predicted to be able to adapt fast enough in response to the current, unprecedented rates at which global temperatures are rising (Brereton et al. 1995; Sinervo et al. 2010). Climate change has already been linked to local extinctions of montane populations of lizards (Sinervo et al. 2010), frogs (Pounds et al. 1999), and mammals (Beever et al. 2011). Factors such as rising temperatures, are expected to directly impact the probability of population persistence by placing additional physiological stresses on cold-adapted species. Thermoregulation may become increasingly important, especially in viviparous ectotherms, where high female internal body temperature can harm embryonic development (Beuchat 1986). Under warmer conditions, sky island species may also experience a reduction in daily activity periods, limiting the feeding opportunities needed to counter rising energy costs (Sinervo et al. 2010; Cahill et al. 2013). In addition, climate change is likely to have indirect impacts on the survival of sky island taxa. As habitats contract, species may face increased resource competition, yet experience decreased habitat connectivity, hindering migration to more suitable habitats (Sato et al. 2014a). However, since montane species are often already restricted to the highest available habitats, more suitable areas may no longer exist. Within Australia, for example, the entire alpine zone is expected to be replaced by sub-alpine habitat with a 3 °C rise in mean annual temperature (Green et al. 1992), which could occur as early as 2050 (Hennessy et al. 2003). Thus, there is a pressing need to understand how climate change affects sky islands systems in time to incorporate it into conservation management.

Located in south-eastern Australia, the Australian Alps (1100–2228 m) formed approximately 90 mya (Vandenberg 2010) and comprise a series of sky islands that constitute the southern portion of the Great Dividing Range. Like many older sky island systems, the Australian Alps harbour high species endemism (McCormack et al. 2009), including seven vertebrates which are listed as federally threatened under the *Environmental Protection and Biodiversity Conservation Act 1999* (Atlas of Living Australia 2014). The prospects for long-term survival of these species are bleak. For example, a 1 °C rise in average temperature is predicted to lead to the extinction of the mountain pygmy possum, *Burramys parvus* (Brereton et al. 1995). Under the worst-case scenario presented by Hennessy et al. (2003), rising temperatures are predicted to result in a 377 m upwards shift in the snowline and extend the tree line past Australia's highest point Mt Kosciuszko (2228 m a.s.l.; Pickering 2007). In addition to climate change, the construction and maintenance of ski resort infrastructure also poses a significant threat to species survival (Sato et al. 2014a, b). Population genetics research has provided information critical for conservation management and, in the case of the alpine she-oak skink *Cyclodomorphus praealtus*, has contributed to a successful submission for federal endangered species status. Despite this progress, relatively few studies have focused on sky island species from the Australian Alps (but see Osborne et al. 2000; Mitrovski et al. 2007; Morgan et al. 2008; Koumoundouros et al. 2009; Tatarnic et al. 2013; Slatyer et al. 2014; Endo et al. 2015).

In this paper, we investigate population genetic structure within one of Australia's sky island endemics, the alpine bog skink, *Pseudemoia cryodroma*. This species is a small (SVL ~55 mm), viviparous lizard restricted to mountain plateaux above 1000 m in Victoria. *Pseudemoia cryodroma* is listed as endangered at the state level (Department of Sustainability and Environment 2013); though, it may be eligible for federal threatened species status, given that it has a patchy distribution exclusively within areas of higher elevation in the state of Victoria. Nevertheless, critical information regarding population structure and demography is not available. Habitat loss and fragmentation, which are driven primarily by a combination of climate change (Green et al. 1992; Brereton et al. 1995; Hennessy et al. 2003) and anthropogenic habitat disturbance (Sato et al. 2014a, b), constitute major threats to the persistence of *P. cryodroma*. Shrinking habitats not only decrease available resources but may also increase competition between ecologically similar taxa (Parmesan and Yohe 2003; Sinervo et al. 2010).

One factor complicating assessment of the conservation status of *P. cryodroma* is its relationship with the morphologically and ecologically similar congeners, *P. entrecasteauxii* and *P. pagenstecheri*, with which its entire distribution is sympatric (Hutchinson and Donnellan 1992; Wilson and Swan 2013). Although *P. cryodroma* is considered a distinct species (Hutchinson and Donnellan 1992), variable morphology and ongoing introgression with ecologically and morphologically similar species *P. entrecasteauxii* and *P. pagenstecheri* pose challenges to conservation management (Haines et al. 2016). Previous genetic work using nuclear markers and allozymes indicated that *P. cryodroma* and *P. entrecasteauxii* form a sister group to *P. pagenstecheri*. However, this was incongruent with mtDNA analyses, which show *P. cryodroma* as two distinct lineages (Hutchinson and Donnellan 1992; Haines et al. 2014). Within the most current mtDNA phylogeny, the majority of *P. cryodroma* populations formed a clade within *P. pagenstecheri*, which is most likely explained by historic mitochondrial introgression from *P. pagenstecheri* into *P.*

cryodroma. In contrast, the second lineage of *P. cryodroma* comprised a single population from Lake Mountain, Victoria, in the westernmost part of its range and was undifferentiated from *P. entrecasteauxii*. It is unclear if this is due to more recent mitochondrial introgression from *P. entrecasteauxii* into *P. cryodroma* or contemporary hybridization between these two species (Haines et al. 2014). Thus, our initial aim in this study is to determine whether there is current gene flow between *P. cryodroma* from Lake Mountain and *P. entrecasteauxii* and/or *P. cryodroma* from the rest of its range and examine evidence of potential hybrids within additional *P. cryodroma* populations using microsatellite loci. Secondly, we assess genetic structure within *P. cryodroma* using both mtDNA and microsatellites. In particular, we investigate genetic diversity within isolated populations and examine genetic signatures of fluctuation in population size. Since populations are restricted to sky islands that have expanded and contracted in response to glacial cycles, we predict that each population will be genetically distinct and will have undergone past population bottlenecks. We then compare historic and contemporary population connectivity across *P. cryodroma* populations to other Australian alpine endemics. Lastly, we discuss the application of our findings for future conservation management.

Materials and methods

Sampling

Tissues samples from 144 *P. cryodroma* individuals were included in this study from six sites across the range of *P. cryodroma* in Victoria, Australia (Fig. 1): Lake Mountain (LM; n = 29), Mt Baw Baw (BB; n = 25), Mt Buller (BL; n = 15), Mt Stirling (ST; n = 15), Mt Higginbotham (HT; n = 15), and Bogong High Plains (n = 45; see Supplementary Table S1 for sampling details). Most samples (n = 138) were collected during the Austral summer from 2011 to 2013. Additional individuals from the type locality BB (n = 4) were subsampled from the Australian Biological Tissue Collection housed at the South Australian Museum. All samples collected for this study were obtained over multiple days per year for multiple years.

DNA extraction, mtDNA sequencing, and genotyping

Genomic DNA was extracted from liver and tail tip samples using a Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen, Hilden, Germany), a GenCatch Blood and Tissue Genomic DNA Miniprep Kit (Epoch Life Sciences, Sugar Land, Texas, USA) or a modified high-salt method (Aljanabi and Martinez 1997). A 794-bp section of the mitochondrial DNA (mtDNA) gene ND4, including partial tRNAs, was amplified and sequenced for a subset of individuals (n = 103) using the protocol described in Haines et al. (2014). Samples were sent to Macrogen, Inc (Seoul, South Korea) for sequencing. Resulting sequences were visually inspected and trimmed to a consistent length in GENEIOUS v. 6.1.2 (Biomatters, Auckland, New Zealand, available at: http://www.geneious.com/). Sequences were aligned using the default clustering algorithm and translated to amino acids, revealing no premature stop codons. All sequences have been deposited in GenBank (see Table S1, Supporting Information).

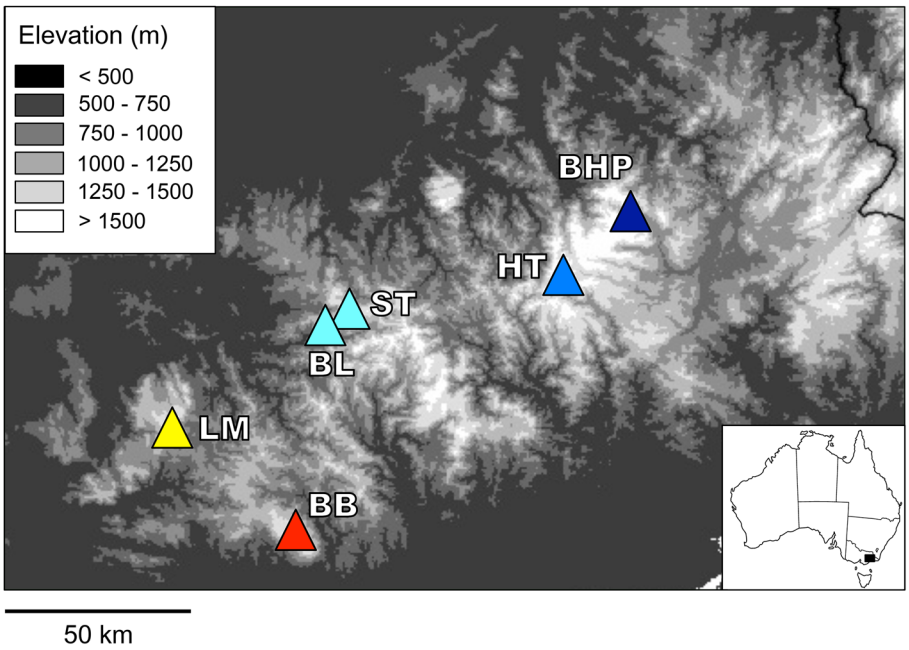

Fig. 1 Elevation map of the Victorian alpine region of southeastern Australia showing study sites for *Pseudemoia cryodroma*. *LM* Lake Mountain, *BB* Mt Baw Baw, *BL* Mt Buller, *ST* Mt Stirling, *HT* Mt Higginbotham, *BHP* Bogong High Plains. (Color figure online)

The full dataset of 144 individuals was screened at 13 microsatellite loci developed by Stapley et al. (2003) and Haines et al. (2016) using the protocols detailed in Haines et al. (2016). The PCR products were sent to Macrogen, Inc (Seoul, South Korea) and analysed on an AB 3730 platform using a LIZ-500 size standard. Chromatograms were scored in Geneious 6.1.6 (Biomatters) and manually checked for accuracy. Samples were checked for identical genotypes using Microsoft Excel and evidence of stutter products, large allele dropout, and null alleles in MICROCHECKER version 2.3.3 (Van Oosterhout et al. 2004).

Statistical analyses

Population structure and gene flow

Mitochondrial DNA sequences were analysed using a Bayesian framework in MrBayes 3.2 (Ronquist and Huelsenbeck 2003) to construct phylogenetic trees. Duplicate haplotypes from the same geographic region were removed from the analysis. Using Partition-Finder (Lanfear et al. 2012), we assessed partitioning schemes and models of best-fit based on Akaike Information Criterion (AIC). Bayesian analyses consisted of two independent runs, each with four chains of Markov Chain Monte Carlo (MCMC), and were run for five million generations with a burn-in of one million generations. Chains were sampled every 500 generations and chain convergence was confirmed using an assessment of average standard deviation of split frequencies (<0.01) and effective sampling size (>200). Uncorrected mean pairwise genetic distances were calculated between the main geographic localities in MEGA 5.2.2 (Tamura et al. 2011) and Φ_{ST} (an analogue of Wright's fixation index F_{ST}) in Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

Since past and present introgression has been found between the closely relates species *P. cryodroma, P. entrecasteauxii*, and *P. pagenstecheri* (Haines et al. 2014, 2016), where *P. cryodroma* was sympatric with *P. entrecasteauxii* and *P. pagenstecheri*, microsatellite loci were first analysed in STRUCTURE version 2.3.4 (Pritchard et al. 2000) to establish a genetically pure ($Q \geq 0.90$) *P. cryodroma* dataset based on nuclear DNA. Specifically, we addressed the following questions: (1) Do morphologically identified *P. cryodroma* from Lake Mountain grouped with *P. cryodroma* from the rest of its range or with *P. entrecasteauxii*, as it does based on mitochondrial DNA; and (2) Is evidence of hybridization between *P. cryodroma* and *P. entrecasteauxii* or *P. pagenstecheri* at sites that had not been previously examined: BB, BL, ST, and LM. For the analysis, we included morphologically identified *P. cryodroma* from the sites listed above and compared genetic results against known pure *P. cryodroma* (n = 60), *P. entrecasteauxii* (n = 66), and *P. pagenstecheri* (n = 61) from HT and BHP, and *P. entrecasteauxii* and *P. pagenstecheri* only from the Dargo High Plains as determined previously by Haines et al. (2016). Since potential hybrids from areas of sympatry (Bogong High Plains and Mt Higginbotham) had already been removed and the additional *P. cryodroma* sites were geographically isolated, an ancestry model with no admixture was applied. Species assignments were used for the LOCPRIOR and assumed allele frequencies were correlated. To estimate the true number of clusters in the dataset (K), K was set to K = 1–8 and five independent runs per K were conducted. Each run comprised a burn-in of 10^4 followed by 10^6 MCMC iterations and showed a convergence of key summary statistics, indicating this was a sufficient number of runs to accurately determine K (Pritchard et al. 2009). In Structure Harvester (Earl and von Holdt 2012), the most likely number of clusters was estimated by examining where values for the mean posterior probability (Ln *Pr* (X|K)) reached a plateau (Pritchard et al. 2009) and using the second-order derivative of the natural log-likelihood (ΔK) as described by Evanno et al. (2005). The highest support was observed for K = 3, with each cluster corresponding to a different species (see Results). Hence the K = 3 results were used to identify pure *P. cryodroma* individuals ($Q \geq 0.90$ for the cluster corresponding to *P. cryodroma*). Two individuals initially classified as *P. cryodroma* were found to be hybrids and removed from subsequent analyses. All remaining *P. cryodroma* individuals had $Q \geq 0.99$.

To examine recent genetic structure within *P. cryodroma*, the STRUCTURE analyses were repeated with only pure *P. cryodroma*. The same parameters were implemented as in the three species analysis, except that sites were used for the LOCPRIOR. The most likely number of clusters was determined in Structure Harvester using the methods described above. This process was repeated for each cluster until no sub-structure was observed. Individual runs were combined in CLUMPP (Jacobsson and Rosenberg 2007) and visualized in MS EXCEL. We used GENODIVE 2.0b25 (Meirmans and Van Tienderen 2004) to estimate genetic differentiation ($F_{ST)}$ between clusters identified in the hierarchical STRUCTURE analyses and the inbreeding coefficient (F_{IS}) within clusters. F_{IS} was only calculated for loci that were found to be in Hardy–Weinberg equilibrium. While inbreeding itself can cause loci to be out of Hardy–Weinberg equilibrium, so can the presence of null alleles, which can which can artificially inflate F_{IS} (Pemberton et al. 1995). Thus, we chose to exclude the few loci that deviated from Hardy–Weinberg equilibrium for a given population in our calculations in order to obtain less biased F_{IS} values.

Molecular diversity

Genetic diversity was analysed within the clusters identified in the hierarchical STRUCTURE analysis. In ARLEQUIN 3.5 (Excoffier and Lischer 2010), nucleotide (π) and haplotype (H) diversity were calculated based on the mtDNA dataset. For each microsatellite locus, the number of alleles (A) and the number of private alleles (A_P) were calculated in GENALEX 6.5 (Peakall and Smouse 2012), while allelic richness (A_R) and private allelic richness (A_{PR}) were determined based on thirteen randomly selected individuals per population in HP-RARE (Kalinowski 2005). Expected heterozygosity (H_E), observed heterozygosity (H_O), deviation from Hardy–Weinberg equilibrium, and linkage disequilibrium between loci were calculated in ARLEQUIN.

Signatures of population expansion and contraction

For each population cluster, we investigated genetic evidence of past population expansions using the mtDNA dataset and bottlenecks using the microsatellite dataset. In ARLEQUIN, we tested for historical population expansion by calculating Fu's F statistic (F_S). Negative values for F_S indicated the presence of more unique mtDNA haplotypes than expected at equilibrium (Fu 1997), with more negative values corresponding to greater population expansion. The raggedness index (RI; Rogers and Harpending 1992) was calculated to determine whether evidence of population expansion significantly deviated from a unimodal demographic expansion model. We also used Bayesian Skyline Plots to examine past changes in population size. Using BEAST 2.4 (Bouckaert et al. 2014), we implemented a HKY model with a relaxed log normal clock for each population cluster. Analyses were run for 5 million generations with 10% burn-in and sampled every 1000 iterations. Plots were visualized using Tracer 1.5 (Rambaut and Drummond 2007). The microsatellite data was used to detect signatures of genetic bottlenecks in the software programs Bottleneck 1.2.02 (Piry et al. 1999) and M-Ratio (Garza and Williamson 2001). In Bottleneck, a one-tailed Wilcoxon test was performed for a two-phase mutation model with 95% single step and 5% multiple-step mutations and 12% variance, as recommended by Piry et al. (1999) for microsatellite data. Populations undergoing recent bottlenecks are expected to have higher heterozygosity relative to allelic diversity. To test for bottlenecks that occurred further in the past (>100 generations), the ratio of the number of alleles to the range in allele size (M) was calculated and compared to the ratio expected from a population at equilibrium (M_C) using M-Ratio. Since monomorphic loci can artificially inflate the M_C (Garza and Williamson 2001), locus Pe242 was omitted from the analyses for LM. M_C was determined using a model with 90% single step and 10% multi-step mutations (average size = 3.5) with the recommended mutation rate of 5.0×10^{-4}. For each set of 10,000 simulations of M_C, we calculated 95% confidence intervals. True effective population sizes (N_e) were unknown; therefore, analyses were run using five biological plausible values: 100; 500; 1000; 5000; and 10,000. Values for M lower than M_C were interpreted as evidence of past bottlenecks.

Results

Population structure and gene flow

The mitochondrial gene region ND4 and partial tRNAs were sequenced for 103 *P. cryodroma* individuals. Of the 794 bp, there were 155 variable sites, 142 of which were parsimony informative. We observed 59 unique haplotypes, 20 of which were shared between two or more individuals. Haplotypes were only shared between individuals from the same site, with the exception of one haplotype that was shared between neighbouring BL and ST. Based on AIC calculated in PartitionFinder, the coding region was partitioned and the following partition schemes were applied: codon 1 = GTR + I, codon 2 = HKY + I, codon 3 = HKY + I, tRNAs = SYM + I. The Bayesian tree (Fig. 2), comprising only unique haplotypes, showed *P. cryodroma* to be paraphyletic, with individuals from LM forming a well-supported clade with *P. entrecasteauxii* and the remaining individuals forming a well-supported clade sister to alpine *P. pagenstecheri*. Within the main *P. cryodroma* clade, individuals formed two main geographic clades: (1) BB, BL, and ST in the west and (2) HT and BHP in the east. Mean uncorrected sequence divergence between these clades was 1.9%. Within the western clade, BB comprised two well-supported lineages, though individuals from these lineages were syntopic. In contrast, populations from adjacent BL and ST form single a clade, with a shared haplotype between individuals at both mountains. The BL/ST clade was separated from the two BB lineages by 1.2% mean uncorrected sequence divergence. The eastern clade comprises two lineages, separated by 1.4% sequence divergence. The first lineage includes individuals exclusively from BHP and the second lineage comprises individuals from both BHP and HT, indicating historical migration from BHP to neighboring HT.

The results of the STRUCTURE analyses on the microsatellite data indicate the presence of three distinct clusters, which correspond to *P. cryodroma, P. entrecasteauxii* and *P. pagenstecheri* (Fig. 3a; Supplementary Fig. S1). Although the mtDNA dataset for *P. cryodroma* from LM was more closely related to *P. entrecasteauxii* than other *P. cryodroma* populations, the microsatellite data clearly group the LM *P. cryodroma* population in the same

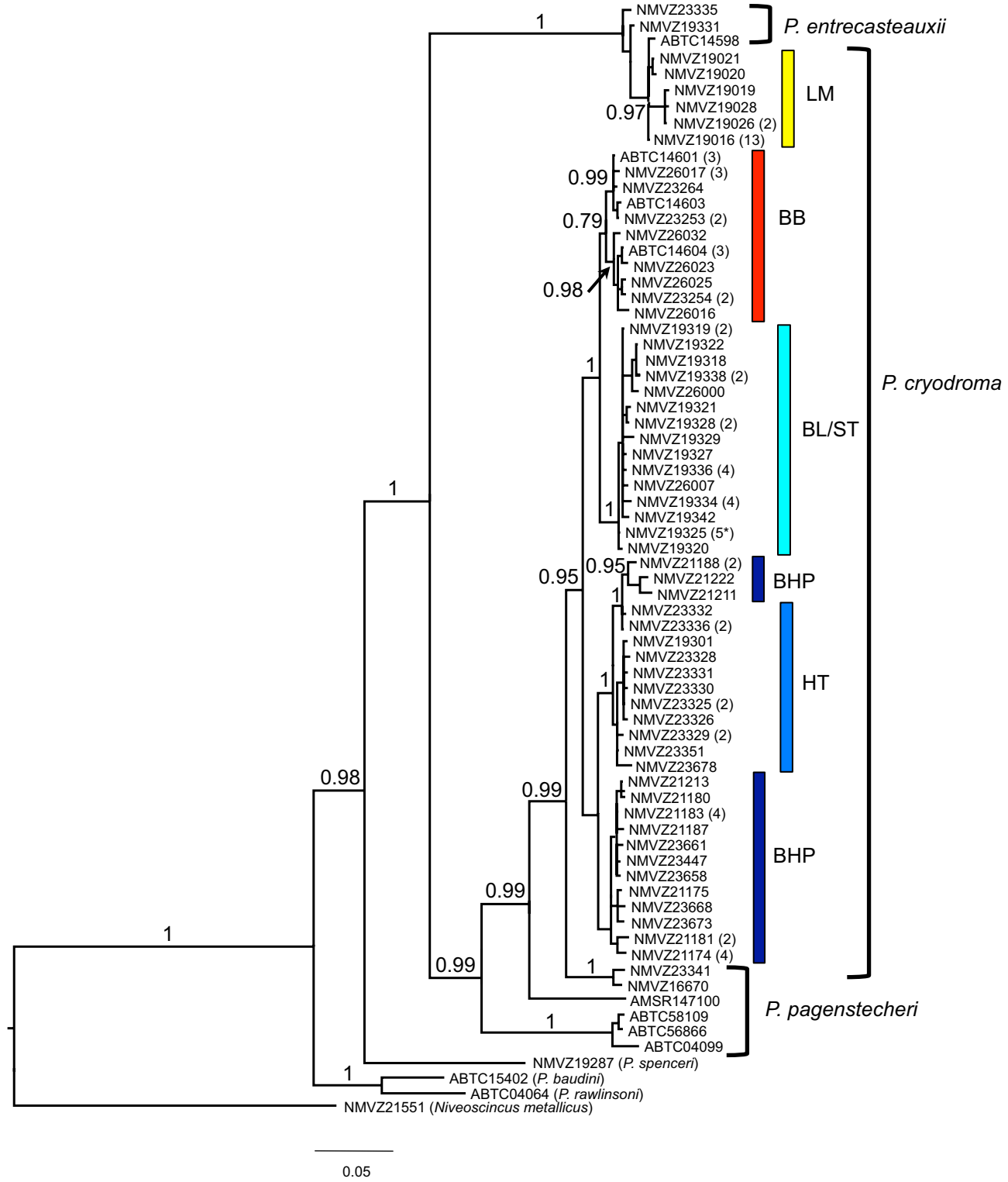

Fig. 2 Bayesian tree based on ND4 haplotypes with posterior probabilities presented above branches. Population abbreviations are: *LM* Lake Mountain, *BB* Mt Baw Baw, *BL* Mt Buller, *ST* Mt Stirling, *HT* Mt Higginbotham, *BHP* Bogong High Plains. *Colored bars* denoting haplotype clades correspond to *colors* in Fig. 1. (Color figure online)

cluster as its conspecifics (Q ≥ 0.99). Within this cluster, we observed one putative hybrid (Q = 0.42) from BB and another (Q = 0.63) from BL and excluded them from further analyses. The remaining 142 *P. cryodroma* were highly likely to belong to their respective cluster (Q ≥ 0.99), indicating pure *P. cryodroma* ancestry.

Individuals were genotyped at 13 microsatellite loci and 98.4% of all genotypes were successfully scored. Loci were polymorphic for all populations with the exception of Pe242, which was monomorphic at LM (Supplementary Table S2). The number of populations within the *P. cryodroma* dataset was estimated based on the STRUCTURE analyses (Supplementary Fig. S1). Initial analyses indicated two geographic clusters: (1) LM and BB in the southwest; and (2) BL, ST, HT, and BHP in the northeast (Fig. 3b). When cluster 1 was analysed independently, two clusters were observed with all individuals correctly assigned (Q > 0.90) for their respective localities (Fig. 3c). For cluster 2, the highest support was found for K = 3, with neighbouring mountains BL and ST forming a single group and HT and BHP remaining distinct. However, two individuals from BHP showed admixture with HT (Q = 0.81, Q = 0.83). No further structure was observed within groups. Pairwise estimates of F_{ST} revealed significant differentiation ($p < 0.001$) between all five groups (Table 1). The highest F_{ST} values were between the westernmost sampling site (LM; cluster 1) and the three eastern sampling sites (BL/ST, HT, and BHP; cluster 2). Despite HT being geographically located between BL/ST and BHP, pairwise F_{ST} estimates between each pair of sites was not significantly different. At $\alpha = 0.05$ significance level, all populations showed low but significant heterozygote deficiencies (LM: $F_{IS} = 0.046$; BB: $F_{IS} = 0.050$; BL/ST: $F_{IS} = 0.067$; HT: $F_{IS} = 0.122$; BHP: $F_{IS} = 0.069$). However, when a standard Bonferroni correction was applied to account for multiple comparisons, only F_{IS} values for BL/ST, HT, and BHP were significant.

Molecular diversity

We examined genetic diversity within each of the five clusters identified in the STRUCTURE analyses: LM, BB, BL/ST, HT and BHP. For the mtDNA, nucleotide diversity was relatively low (0.0033–0.0219), especially at LM, BB, and BL/ST; whilst haplotype diversity was generally high (>0.9) across all sites, except for LM, which had roughly 50% less diversity (Table 2). The low haplotype diversity at LM suggests that this population may have experienced historic population bottlenecks and/or inbreeding. BHP, which had the greatest sample size, had the most alleles (A), yet BL/ST had the highest number of private alleles (A_P). Allelic richness (A_R), based on a sample size of thirteen randomly selected genotypes, was substantially lower at LM compared to the other sites, mirroring

Table 1 Pairwise differentiation for each *P. cryodroma* population based on mtDNA (Φ_{ST}; top) and microsatellites (F_{ST}; bottom)

Population	LM	BB	BL/ST	HT	BHP
LM	–	0.263	0.252	0.260	0.252
BB	0.083	–	0.065	0.049	0.060
BL/ST	0.171	0.102	–	0.050	0.061
HT	0.155	0.081	0.061	–	0.045
BHP	0.136	0.083	0.039	0.051	–

All values were significant at $p < 0.01$

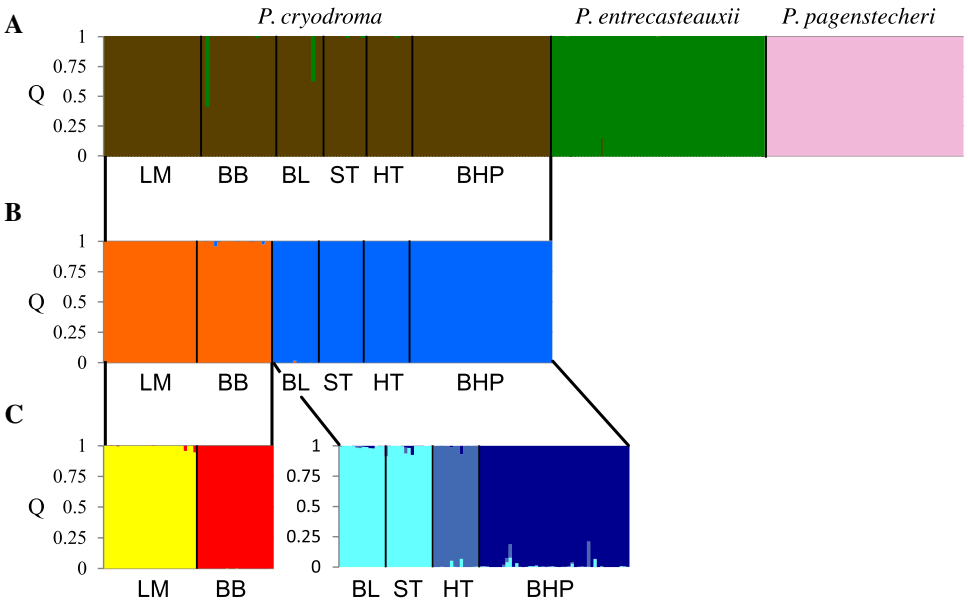

Fig. 3 Individual proportion of membership (Q) of *Pseudemoia cryodroma, P. entrecasteauxii*, and *P. pagenstecheri* to each population cluster (K) using STRUCTURE. Results are given for the best-supported model using **a** the entire dataset (K = 3), **b** *P. cryodroma* only (K = 2), and **c** individuals within each *P. cryodroma* cluster (K = 2, K = 3). *Separate colors* are used to denote clusters and sampling *site colors* correspond to those in Fig. 1. Sampling site abbreviations are: *LM* Lake Mountain, *BB* Mt Baw Baw, *BL* Mt Buller, *ST* Mt Stirling, *HT* Mt Higginbotham, *BHP* Bogong High Plains. (Color figure online)

Table 2 Molecular diversity of *P. cryodroma* by population

	LM	BB	BL/ST	HT	BHP
mtDNA					
π	0.003 ± 0.002	0.007 ± 0.004	0.005 ± 0.003	0.022 ± 0.012	0.012 ± 0.006
H	0.538 ± 0.133	0.936 ± 0.032	0.934 ± 0.026	0.967 ± 0.037	0.945 ± 0.030
Microsatellites					
A	8.54 ± 1.45	10.54 ± 1.65	13.00 ± 1.85	8.92 ± 1.22	13.62 ± 2.17
A_R	6.67 ± 1.04	8.59 ± 1.29	9.65 ± 1.27	8.53 ± 1.15	9.26 ± 1.29
A_P	0.46 ± 0.18	0.92 ± 0.18	1.85 ± 0.44	0.54 ± 0.18	1.54 ± 0.57
A_{PR}	0.44 ± 0.16	1.11 ± 0.20	1.48 ± 0.28	1.00 ± 0.23	1.06 ± 0.27
H_E	0.64 ± 0.08	0.73 ± 0.06	0.73 ± 0.08	0.71 ± 0.08	0.76 ± 0.07
H_O	0.59 ± 0.09	0.65 ± 0.08	0.68 ± 0.08	0.63 ± 0.08	0.67 ± 0.07

Mean nucleotide diversity (π), haplotype diversity (*H*), number of alleles (A), allelic richness (A_R), number of private alleles (A_P), private allelic richness (A_{PR}), expected heterozygosity (H_E), observed homozygosity (H_O) for pure individuals ($Q \geq 0.90$ in Structure) of each species per site. All values are ±SE

the low nucleotide and haplotype diversity observed in the mtDNA. While locus Pe305 deviated significantly from Hardy–Weinberg Equilibrium ($p < 0.001$) for LM, BB, and BHP, eliminating this locus from the analyses did not greatly impact the results. Furthermore, departure from Hardy–Weinberg Equilibrium is not necessarily a result of null alleles and could also be due to inbreeding or non-random mating (Pemberton et al. 1995). Following a standard Bonferroni correction, linkage disequilibrium was only detected for a combined total of eight pairs of loci across the five populations. Since each population did not have the same loci in linkage disequilibrium, we chose not to exclude any loci from further analyses.

Historic population expansion and contraction

Analyses showed some evidence of historic population expansion at BL/ST and more recent population bottlenecks at BB and HT. Based on the mtDNA, Fu's F_S significantly deviated from equilibrium only at BL/ST, indicating past population expansion (Table 3). In contrast, based on the raggedness index (RI), demographic expansion could not be rejected for any locality because the observed mtDNA data for each locality did not significantly differ from a unimodal demographic expansion model. The Bayesian skyline plots indicate that all populations have remained stable through time (Supplementary Fig. S2). The microsatellite dataset was used to examine signatures of past population bottlenecks. Tests for excess heterozygosity in the program Bottleneck were not significant, indicating no recent bottlenecks within any population. By contrast, evidence of bottlenecks was detected in the more distant past using M-Ratio, though this was highly dependent on the value of N_e (Table 3). When N_e was set to 100, all populations fit the criteria for bottlenecks yet N_e of 10,000 consistently indicated

Table 3 Tests for signatures of populations expansions in mtDNA and population bottlenecks in microsatellite loci in each of the five populations of *P. cryodroma*

	LM	BB	BL/ST	HT	BHP
mtDNA					
Fu's F_S	0.396	−1.404	−5.369*	0.063	−2.010
RI	0.178	0.040	0.017	0.021	0.027
Microsatellites					
M	0.720	0.658	0.777	0.645	0.798
M_C for N_e =					
100	**0.842**	**0.845**	**0.850**	**0.845**	**0.849**
500	**0.787**	**0.788**	**0.789**	**0.784**	0.794
1000	**0.753**	**0.751**	0.754	**0.742**	0.760
5000	0.685	**0.676**	0.689	0.637	0.710
10,000	0.657	0.641	0.660	0.583	0.695

Bold values denote M-ratios at equilibrium (M_C) for a given effective population size (N_e) that are higher than the observed M-ratio (M)

*$p < 0.05$

that no bottlenecks had occurred. The strongest evidence for a bottleneck was at BB, which had $M < M_C$ when $N_e \leq 5000$. As an alternative to manipulating N_e, Garza and Williamson (2001) suggest using $M_C = 0.68$ for datasets with more than seven loci. Using that criterion, there is support, though weak, that BB and HT have both undergone bottlenecks.

Discussion

Relationship between *P. cryodroma* and congeners

Although *P. cryodroma* are polyphyletic in their mtDNA, the microsatellite data clearly show that all *P. cryodroma* populations form a single cluster, to the exclusion of both *P. entrecasteauxii* and *P. pagenstecheri*. A similar pattern of incongruence between mtDNA and microsatellite analyses was uncovered in studies on alpine populations of chamois, *Rupicapra* spp. (Rodríguez et al. 2010), and chipmunks, *Tamias alpinus* (Rubidge et al. 2014), in which the mtDNA separated populations based on geography whilst the microsatellite clusters closely corresponded to current taxonomy. Based on nuclear protein-coding and microsatellite loci, *P. cryodroma* and *P. entrecasteauxii* are more closely related and this group is sister to *P. pagenstecheri* (Haines et al. 2014; this study). However, in mtDNA *P. cryodroma* from all sites (except LM) form a clade within *P. pagenstecheri* and *P. cryodroma* from LM is undifferentiated from *P. entrecasteauxii*. This discordance can partly be explained by historic introgression of *P. pagenstecheri* mitochondrial haplotypes into *P. cryodroma*, followed by independent evolution in both species. While the lack of resolution between *P. cryodroma* from LM and *P. entrecasteauxii* in mtDNA and nuclear protein-coding loci (Haines et al. 2014; this study) can be explained by incomplete lineage sorting, the clustering of all *P. cryodroma* populations to the exclusion of *P. entrecasteauxii* in the more quickly evolving microsatellite data provides strong evidence of historical mitochondrial introgression. The larger effective population size in microsatellites, as consequence of their faster substitution rate, makes the patterns observed in the genetic data less likely to be caused by drift and more likely to show evidence of stronger, true divergence between species. Thus, our data, combined with previous genetic analyses by Haines et al. (2014), indicate that *P. cryodroma* is currently a distinct, independently evolving species, despite past hybridization with sympatric congeners.

Phylogeographic structure

Geographic structuring was observed among localities within the mtDNA and microsatellite datasets. We expected that genetic relatedness and gene flow would be dependent on habitat connectivity and geographic distance, as *P. cryodroma* is an alpine endemic and likely disperses only tens of metres throughout its lifetime (Sato et al. 2014a). Consistent with our expectations, the closest locations geographically (BL and ST), which were approximately 6 km apart, were admixed in both the mtDNA and microsatellite analyses, suggesting ongoing interbreeding between individuals at the two sites. The next closest pair of sampling sites (BHP and HT), which were separated by 23 km, shows evidence of female migration from HT to BHP and subsequent introgression of a HT haplotype into BHP in the mtDNA. However, the strong separation in the microsatellite data indicates that populations at each of these locations are now genetically isolated. In contrast, BB comprised two well-supported clades in the mtDNA, yet formed a single cluster based on the microsatellite data. Changes in habitat connectivity may also explain why the mtDNA analysis grouped individuals from centrally located BL/ST grouped with those from BB in the west, whereas the microsatellite analysis grouped BL/ST with the eastern localities of BHP and HT.

Genetic diversity, based on the mtDNA and microsatellite data, may be partially explained by population elevation. Since LM has the lowest elevation (1381–1441 m a.s.l.), suitable habitat at LM would have been relatively smaller and more isolated during Plio-Pleistocene interglacials compared to the other localities. Potentially, these habitat features would have had a negative effect on genetic diversity by increasing the likelihood of inbreeding and decreasing opportunities for gene exchange with nearby populations. In comparison, HT had the highest elevation (1739–1814 m a.s.l) and correspondingly had the greatest nucleotide and haplotype diversity in the mtDNA dataset. For the microsatellite data, the locality with the highest allelic richness (A_R) was also found at one of the higher sites (BL/ST; 1617–1789 a.s.l.). The BL/ST population also had the most private alleles (A_P) and private allelic richness (A_{PR}). However, the high genetic diversity at BL/ST may also be due to this region comprising two areas of high elevation that likely formed a larger, continuous habitat during glacial periods compared to the other study sites. The correlation between higher elevation and greater genetic diversity has been observed in alpine she-oak skinks (Koumoundouros et al. 2009), but was not supported for mountain pygmy possums (Mitrovski et al. 2007).

Overall, the genetic structuring in the mtDNA and microsatellite datasets is consistent with those observed

in sympatric, alpine restricted taxa. Mitochondrial introgression was detected between *P. cryodroma* populations at neighboring BHP and HT in concordance with genetic studies on the mountain pygmy possum (Osborne et al. 2000; Mitrovski et al. 2007), alpine she-oak skink (Koumoundouros et al. 2009), and several invertebrate species (Tatarnic et al. 2013; Slatyer et al. 2014; Endo et al. 2015). These observations withstanding, based on microsatellite data, contemporary introgression was not detected between populations of *P. cryodroma*, mountain pygmy possums, alpine she-oak skinks, or alpine grasshoppers, *Kosciuscola tristis*, from BHP and HT. While mtDNA analysis of the alpine grasshopper also revealed a grouping of BL/ST and BB, unlike in *P. cryodroma*, this pattern was also observed in the microsatellite data (Slatyer et al.). Lastly, using the microsatellite data, pairwise estimates of F_{ST} between HT and BHP populations were comparable across lizards, mountain pygmy possums, and grasshoppers (Osborne et al. 2000; Mitrovski et al. 2007; Koumoundouros et al. 2009; Slatyer et al. 2014).

Conservation implications

Though *P. cryodroma* likely comprises refugial populations that experienced fluctuations in population size in response to glacial cycles, evidence of inbreeding as well as population expansion and contraction was only observed for a subset of sites. High F_{IS} values, which were detected at BL/ST, HT, and BHP, often result from geographic isolation coupled with limited dispersal and has been recorded in other alpine species, such as the mountain pygmy possum (Osborne et al. 2000; Mitrovski et al. 2007); northern corroboree frog (Morgan et al. 2008), and alpine she-oak skink (Koumoundouros et al. 2009). Though this is commonly associated with inbreeding, we were unable to distinguish inbreeding from other causes of high homozygosity, such as genetic drift. Based on Fu's F statistic (F_S), only BL/ST had a significant excess of unique mtDNA haplotypes compared to the expected amount at equilibrium, indicating population expansion. There was no evidence of recent bottlenecks in any population based on analysis using the program Bottleneck and only weak support for historic bottlenecks in BB and HT populations using M-Ratio. Nevertheless, it is important to monitor inbreeding, genetic drift, and potential genetic bottlenecks in *P. cryodroma* because these factors can cause a reduction in genetic diversity (Hedrick and Kalinowski 2000), which may hinder a population's ability to adapt to rapidly changing environmental conditions (Reed and Frankham 2003; Willi et al. 2006). In order to provide *P. cryodroma* with the greatest chance of adapting to climate change, other existing or potential threats, such as development of habitat, damage to habitat by feral species such as horses and deer, and elevated predation rates from feral species or native predators moving into the alps, need to be urgently mitigated. Further research should investigate the potential benefits of conservation translocations ('genetic rescue' *sensu* Frankham 2015), which may help alpine endemics to cope with pressures such as a warming climate (Mansergh et al. 2013).

Conclusion

This study confirms that lizards from Lake Mountain identified morphologically as *P. cryodroma* are in fact *P. cryodroma*, not a morphological variant of *P. entrecasteauxii*. Our findings elucidate both historic and recent population structure and demography within this understudied sky island species. Although we observed discordance in species groupings between the mtDNA and microsatellite datasets, this is likely due to mitochondrial introgression of *P. entrecasteauxii* into the LM *P. cryodroma* population. Additionally, the clustering of populations from HT and BHP is consistent with studies on sympatric species (Osborne et al. 2000; Mitrovski et al. 2007; Koumoundouros et al. 2009; Tatarnic et al. 2013; Endo et al. 2015). The microsatellite analysis shows no evidence of recent gene flow between the mtDNA lineages, indicating that populations are not only geographically isolated but also genetically isolated. As global temperatures rise, alpine habitats are expected to become more fragmented (Green et al. 1992; Hennessy et al. 2003; Pickering 2007), splitting contemporary *P. cryodroma* populations and creating stronger barriers to gene flow. Already, potential inbreeding has been detected in multiple *P. cryodroma* populations and is likely to increase with decreased habitat size. Habitat conversion for ski tourism is also expected to negatively impact population demography by reducing existing habitat size and population connectivity (Sato et al. 2014b). These factors contribute to decreased genetic diversity within *P. cryodroma* populations, making *P. cryodroma* increasingly susceptible to local extinction. Creating new isolated patches of suitable habitat is unlikely to offset local extinctions, unless translocation is considered, as small skinks have low dispersal ability (Sato et al. 2014a). Maintaining existing habitat connectivity through responsible management of the Australian Alps and mitigation of existing and potential threats will therefore be critical to long-term persistence of *P. cryodroma*.

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