Current genetic isolation and fragmentation contrasts with historical connectivity in an alpine lizard (*Cyclodomorphus praealtus*) threatened by climate change

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ABSTRACT

Assessing levels of genetic diversity, connectivity and historical demography for threatened species provides important information for conservation management. We used a combination of the mitochondrial ND4 gene and seven microsatellite markers to examine both historical and recent population genetic structure and demography of the threatened alpine she-oak skink, *Cyclodomorphus praealtus*. This species is restricted to the "sky islands" of the Australian alpine region. Based on mtDNA, the New South Wales and Victorian regions are reciprocally monophyletic and highly divergent, with among population variation of 0.9 and net sequence divergence of 4.28%, which suggests that they should be considered separate Evolutionary Significant Units for management purposes. The mtDNA data also indicate historical connectivity between the three Victorian populations. However, a model-based clustering analysis of microsatellite genotypes identified strong population structure in Victoria, with three distinct populations that have no current inter-population gene flow. This suggests that the Victorian populations are effectively isolated from each other, and is indicative of very low dispersal capacity and a high degree of habitat specialisation. This is reinforced by the substantially lower genetic diversity within the lowest elevation population compared to the other higher elevation populations. We found no genetic signature of major changes in effective population size. These data provide a baseline for assessing future impacts of climate change on the genetic structure of this alpine endemic species.

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1. Introduction

Understanding demography and patterns of gene flow is crucial for the design of effective conservation strategies for threatened species (Moritz, 1995; Hedrick, 2001; Avise, 2004). Many threatened species are small, elusive and sparsely distributed, so it is often difficult to obtain information on population demographics using conventional field-based methods. Consequently, researchers are increasingly applying genetic methods to establish patterns of genetic variation and to infer the population structure and demographic history of threatened species (Moritz, 1995; Hedrick, 2001; Avise, 2004).

Genetic methods have been used to inform conservation policies using concepts such as the Evolutionarily Significant Unit (ESU), which provides a framework for identification of separate, intra-specific management units for conservation prioritisation (Moritz, 1994; Crandall et al., 2000; Fraser and Bernatchez, 2001;

Moritz, 2002). ESUs are most commonly identified using patterns of genetic variation; however interpretation of these patterns can be complicated by factors such as genetic drift, retention of ancestral polymorphism, selection, fluctuations in effective population size and mating systems (Bjorklund, 2003; Pabijan and Babik, 2006). These factors can influence the genetic markers used to detect variation in different ways. Mitochondrial DNA markers have a fourfold smaller effective population size than biparentally inherited nuclear DNA markers as they are haploid and maternally inherited, and so, like small populations, are more susceptible to the effects of genetic drift (Moritz et al., 1987; Sunnucks, 2000; Avise, 2004). Thus inferences on species' genetic diversity and structure, which inform conservation decisions, should ideally be based on multiple types of molecular markers due to the potential for discrepancies among estimates of genetic differentiation obtained using different markers (e.g. Brito, 2007; Canestrelli et al., 2007).

In the present study we use both microsatellite and mtDNA markers to infer the historical phylogeography and current population genetic structure of the alpine she-oak skink, *Cyclodomorphus praealtus*. Microsatellite markers are ideal markers to detect current population structure due to their rapid mutation rate, whereas mtDNA has traditionally been the marker of choice for detecting

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historical genetic structure (Sunnucks, 2000). Historical phylogeography can shed light on how palaeoclimatic events have shaped a species' current distribution and genetic structure and may be informative for conservation management (Moritz, 1995; Avise, 2004; Frankham and Briscoe, 2004). For example, high genetic diversity within a region may indicate that the species has maintained a large, stable population size within an area of historically suitable habitat (Avise, 2004). Conversely, low genetic diversity and strong phylogeographic structure can indicate severe historical population contraction and fragmentation into multiple refugial populations (Schneider and Moritz, 1999). This type of information is important for identifying historical refugia of high conservation value and for assessing a species' sensitivity to climate fluctuations.

Alpine species are of particular interest when assessing species' responses to historical climate fluctuations because they are likely to be particularly susceptible to climate change (Brereton et al., 1995; Hughes, 2003). These species are confined to "sky islands" of suitable alpine habitat, surrounded by a "sea" of warmer valleys. In contrast to the majority of species, which occupy mid to low elevations, alpine species may experience range expansion during glacial periods and range contractions during warmer interglacials (e.g. Knowles, 2000; DeChaine and Martin, 2005; Assefa et al., 2007; Browne and Ferree, 2007). Species found in the alpine region are often locally endemic and physiologically adapted to cold climates (e.g. Billings, 1974; Osborne et al., 2000) and are therefore particularly sensitive to temperature increases (Green and Osborne, 1994; Callaghan et al., 2004). Consequently, alpine species potentially show signatures of very recent population contraction in response to anthropogenic climate change (Sanz-Elorza et al., 2003; Hari et al., 2005) in addition to signatures of historical changes in genetic structure associated with either glacial or interglacial periods (e.g. Knowles, 2000; DeChaine and Martin, 2004; Smith and Farrell, 2005; Assefa et al., 2007; Browne and Ferree, 2007; Popp et al., 2008).

The alpine she-oak skink is an ideal model for assessing both current and historical demography and population genetic structure and the conservation implications of climate-driven range limitations. C. praealtus is endemic to the "sky islands" of mainland alpine Australia (Schulz and Mansergh, 1997; Wilson and Swan, 2008). Recent surveys of all known areas and habitats in which C. praealtus are found (tree-less or very lightly treed areas that contain tussock grasses, low heath or a combination of grasses and low heath higher that 1500 m above sea level), as well as areas peripheral to this habitat has resulted in the detection of the species at only four sites: Lankey Plain, Mt Hotham, the Bogong High Plains in Victoria and Mt Kosciuszko in New South Wales (Clemann, 2006, Fig. 1). Due to the secretive and cryptic nature of this species, little was known about its demography or ecology until detailed surveys were carried out by the Victorian Department of Sustainability and Environment between 2005 and 2007. Currently, the species is listed as threatened under the Victorian state *Flora & Fauna Guarantee Act* and is listed as endangered by the Victorian Department of Sustainability and Environment (DSE, 2007) due to the putative impacts of threatening processes, low numbers and restricted geographic range. It faces continued loss and degradation of habitat due to climate change and the development of infrastructure for ski resorts (Clemann, 2003).

Alpine vegetation in Australia currently comprises 0.15% of the Australian land surface and is one of the communities most threatened by climate change as high altitude refuges are extremely limited (Hughes, 2003). Snow cover has decreased by 40% since 1960 (Hennessy et al., 2007) and trees have begun to encroach into sub-alpine grasslands in the Victorian alps (Wearne and Morgan, 2001) with consequent changes in the distribution of alpine fauna (Green and Pickering, 2002). Based on future climate scenarios,

these trends are likely to accelerate (Hennessy et al., 2007). In addition to informing conservation management, current measures of genetic structure and diversity provide an important baseline for assessing future impacts of climate change. In this paper we describe the population genetic structure of C. praealtus in order to better understand the processes that shaped its distribution, both currently and historically, and to identify populations of importance for conservation. Specifically, we assess patterns of current and past gene flow using microsatellite and mtDNA markers, respectively. We also test for genetic signatures of major changes in effective population size. We interpret our results in light of historical changes in the distribution of alpine vegetation in Australia.

2. Materials and methods

2.1. Study area and field sampling

C. praealtus tissue samples (n = 112) were collected during late spring, summer and early autumn between 2005 and 2008 from three sites in Victoria and one in New South Wales (NSW). The Victorian samples were collected on the Bogong High Plains, Mt Hotham and Lankey Plain, and the NSW samples were collected from the Kosciuszko region (Fig. 1). This sampling covers all sites known to have extant populations of C. praealtus.

Transect lines consisting of ten terra cotta tiles, with approximately 5 m between each tile, were used as artificial shelters to lure the lizards, facilitate capture then obtain tissue samples from toe clips (Clemann, 2006). Transects were located in potential C. praealtus habitat (areas of alpine heathland, tussock grassland and herb-fields). Occasionally during capture and handling the lizards would autotomise portions of their tails. When this occurred the shed tail was collected and the lizards were not toe-clipped. Unfortunately the frequency and number of recaptures of marked individuals along transects was insufficient to permit calculation of population sizes (N. Clemann pers. comm.).

2.2. DNA extraction and mtDNA sequencing

We extracted genomic DNA using the chloroform–isoamyl alcohol method described by Sambrook et al. (1989). The mtDNA NADH dehydrogenase subunit 4 (ND4) gene was chosen for sequencing because it has shown useful levels of variability in related skink groups (Chapple and Keogh, 2004). An 850 base pair region of ND4 was amplified using 20 μL reactions containing 2 μl DNA, 10 μL Gotaq (Promega), 0.5 μL 10 nM forward primer ND4I (sequence: 5′TGACTACCAAAAGCTCATGTAGAAGC′3) 0.5 μL 10 nM reverse primer tRNA-leu (sequence: 5′TACTTTTACTTGGATTTGCAC-CA′3; (Chapple and Keogh, 2004) and 7 μL H$_2$O. The polymerase chain reaction (PCR) amplification was performed using a touchdown thermal cycle on a Corbett Palm Pilot PCR machine. The DNA was initially denatured at 95 °C for 5 min followed by two cycles of 95 °C for 30 s, an annealing step of 65 °C for 30 s and 72 °C for 45 s. This cycle was repeated with annealing temperatures of 60 °C, then 55 °C (two cycles each), followed by a final annealing temperature of 50 °C for 30 cycles. The final extension step occurred at 72 °C for 7 min. The remaining product was purified using the Sure Clean Plus protocol (Bioline, UK), and the purified PCR product was sent to Macrogen (Seoul, Korea) for sequencing.

Forward and reverse sequences were assembled using SeqMan II version 5.07 (Lasergene; DNA Star, Inc., Madison, Wisconsin) and manually aligned in paup* beta version 4.0b10 (Swofford, 2000). Alignment was checked using mega version 4 (Tamura et al., 2007). Only the first 803 base pairs of the sequences were used for the analysis as the last 47 base pairs could not be aligned due to the length-variable loops and stems of the tRNA genes.
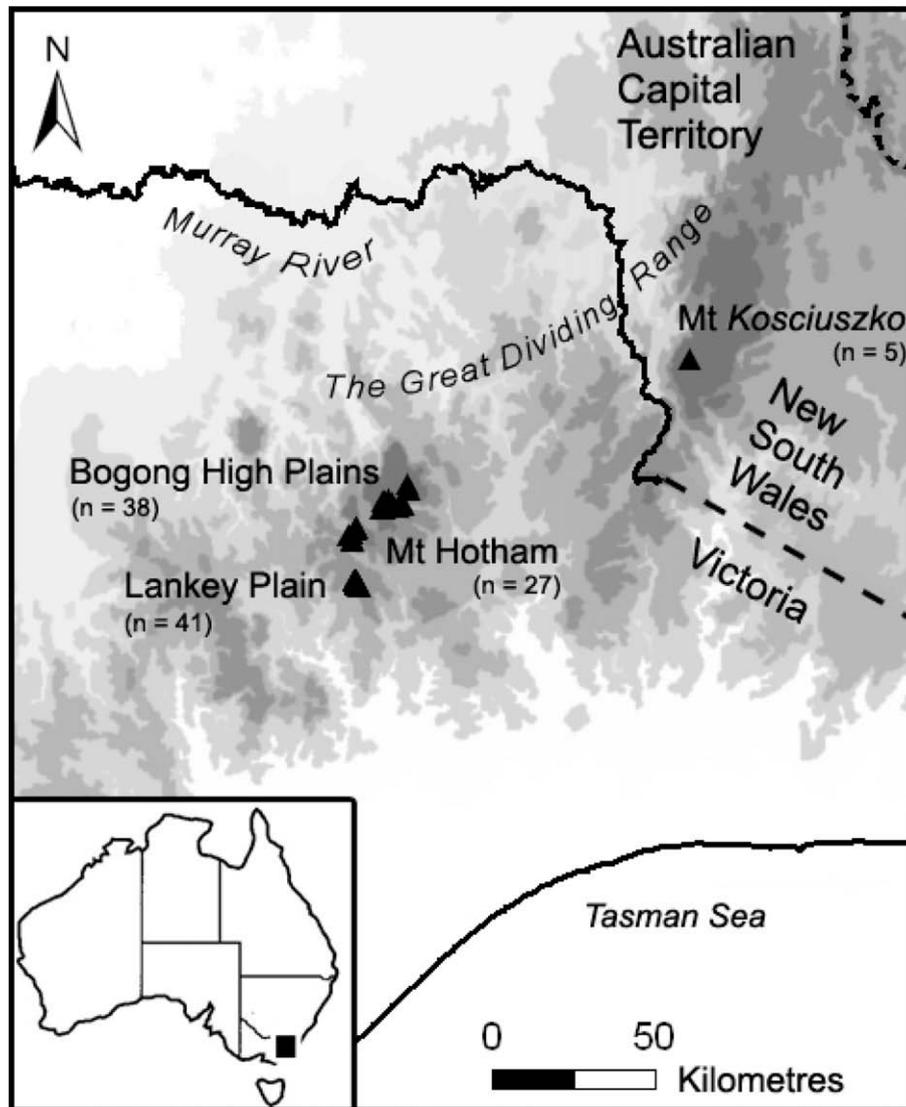
Fig. 1. Elevation map showing collection sites (triangles) for *Cyclodomorphus praealtus* in the alpine region of mainland south-eastern Australia. Number of tissue samples collected at each site is shown in brackets.

2.3. Microsatellite amplification and genotyping

Twenty-two microsatellite loci, isolated from related species (Cooper et al., 1997; Gardner et al., 2007), were tested on eight *C. praealtus* samples. Some of the markers had previously been tested on other *Cyclodomorphus* species with promising results (Chapple et al., 2006). Seven loci were found to amplify reliably, had useful amounts of variation, and were easy to score (Table 1). DNA samples of all Victorian individuals ($n = 95$) were amplified at those seven microsatellite loci, using fluorescently labelled primers. As there were too few NSW individuals ($n = 5$) for adequate statistical power, these were not genotyped. The PCRs were performed using 10 μL reactions containing 1 μL DNA, 5 μL Promega master mix, 0.5 nM forward primer, 0.5 nM reverse primer and 4 μL H$_2$O. A touchdown thermal cycle was used, similar to that used for the ND4 amplification, but with annealing temperatures of 65°, 60°, 55°, 50° and 45 °C. The PCR products were sent to the Australian Genome Research Facility, where they were sized on an AB 3730 platform and analysed using GeneMapper 3.7 (Applied Biosystems). Chromatograms were checked by eye for accuracy. The resulting genotype data were checked for errors, large jumps in al-

lele size, and samples with identical genotypes using microsatellite toolkit version 3.1 for Microsoft Excel (Park, 2001).

2.4. Molecular diversity

For the mtDNA, we calculated haplotype (H) and nucleotide diversity (π) within populations based on uncorrected pairwise differences using arlequin version 3.1 (Excoffier et al., 2005). For the microsatellite data, we estimated the genetic diversity of the microsatellites, the number of alleles per locus, the number of private alleles, the observed heterozygosity (H_O), and unbiased gene diversity (H_E) using microsatellite toolkit and generated the probability of identification statistic (the probability of obtaining two individuals with the same alleles at each loci) using genalex. We also tested for the presence of null alleles, stuttering and small allele dominance using microchecker (Oosterhout et al., 2004). We analysed the data both as a single population and separated into the three sampling localities. The data were then examined for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between loci using genepop version 4.0.7 (Rousset, 2007). These analyses all use the Fisher exact test to eval-

Table 1
Microsatellite molecular diversity of Cyclodomorphus praealtus. The total number of alleles and range of allele sizes for all samples genotypes, and the number of alleles (A), private alleles (P), expected heterozygosity (H_E) and observed heterozygosity (H_O) for each of the three sampled population in the Victorian alps. Primer sequences can be found in Cooper et al. (1997) and Gardener et al. (2007).

Locus	All samples		Bogong High Plains (n = 34)				Mt Hotham (n = 23)				Lankey Plain (n = 26)			
	No. of alleles	Allele size (bp)	A	P	H_E	H_O	A	P	H_E	H_O	A	P	H_E	H_O
Trl1	28	245–289	19	6	0.88	0.78	15	3	0.91	1	11	5	0.82	0.76
Trl9	8	80–94	7	1	0.7	0.53	5	0	0.72	0.43	7	1	0.8	0.58
Trl14	19	128–166	14	3	0.86	0.76	12	3	0.75	0.82	16	2	0.79	0.8
Trl19	31	164–239	26	12	0.95	0.94	12	0	0.89	0.87	16	2	0.89	1[*]
Trl27	3	118–124	3	0	0.54	0.44	2	0	0.16	0.09	3	0	0.54	0.39
Trl29	29	132–194	16	8	0.88	0.74[*]	7	2	0.83	1	16	10	0.89	0.83
Tr3.2	19	144–214	15	1	0.91	1	13	2	0.89	1	15	1	0.92	0.78[*]
Mean ± SD			14.29 ± 7.57	4.43 ± 4.43	0.82 ± 0.06	0.74 ± 0.03	8.85 ± 4.67	1.43 ± 1.39	0.74 ± 0.10	0.74 ± 0.03	11.14 ± 4.95	3 ± 3.46	0.81 ± 0.05	0.74 ± 0.03

[*] Significant departures from Hardy–Weinberg equilibrium ($P < 0.01$) using "exact HW test"

uate significance, and estimate P values by applying the Bayesian Markov Chain Monte Carlo method (MCMC). The parameters used for these analyses were a burn-in period (dememorisation in genepop) of 10,000, 100 batches and 1000 iterations. We used sequential Bonferroni corrections (Rice, 1989) to adjust significance values for multiple comparisons to correct for type I error.

2.5. Population genetic structure and gene flow

To infer the relationship between mitochondrial ND4 haplotypes, we created a statistical parsimony network in tcs (Clement et al., 2000), using a 95% statistical confidence limit for the maximum number of nucleotide substitutions between two haplotypes. We used modeltest version 3.6 (Felsenstein, 1985) to determine which of the 56 potential models of DNA sequence evolution was most suitable for our data. The TVM+I+G model of evolution was selected as the most appropriate model using the Akaike information criterion (AIC; (Akaike, 1974). mrbayes version 3.1.1 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) was used to conduct a Bayesian MCMC phylogenetic analysis using five million generations sampled every 100 generations, with two chains sampling independently and default prior distributions. The parameter values and the sampled trees were summarised, disregarding all topologies that were generated before the MCMC reached a stable state.

To further explore how genetic diversity was partitioned within and between populations we calculated the percentage net sequence divergence (D_A) based on mtDNA and conducted an Analysis of Molecular Variance (AMOVA) to test the hierarchical population structure using the program arlequin version 3.1 (Excoffier et al., 2005). This analysis divides levels of variation into: among regions (NSW and Victoria), among populations and within populations. Among population variation was estimated as Φ_{ST} (an analogue of Wright's fixation index F_{ST}) for each pair of populations based on mtDNA. The number of permutations for each test was 1000.

For the microsatellite data, we used structure version 2.2 (Pritchard et al., 2000) to identify the number of genetically distinct clusters (or populations; K) in the microsatellite data set and to test whether the geography of the sampling sites was reflected in the genetic structure of the individuals. This program uses the MCMC method to probabilistically assign individuals to the number of K populations, based on their genotypes, in a way that minimizes linkage disequilibrium and departures from Hardy–Weinberg equilibrium. We chose a model that allowed for correlated allele frequencies between populations and admixture (an approach allowing for mixed ancestry of individuals); letting the program infer the degree of admixture from the data. The program was run for 10^6 steps, after a burn in of 10,000. These parameters were found sufficient for accurate determination of K by establishing that values of key summary statistics converged (Pritchard et al., 2000). We conducted five independent runs for each value of K ($K = 1$–8). To determine the true number of populations (K) we used both the maximal value of $L(K)$ returned by structure and we calculated a second-order derivative of the natural log-likelihood (ΔK; as explained in Evanno et al., 2005). We used both methods to determine the real K as Evanno et al. (2005) observed in their simulations that in most cases, once the real K is reached, $L(K)$ at larger Ks plateaus or continues increasing slightly (as mentioned in the manual; Pritchard et al., 2007) and the variance between runs increases. We then repeated the simulations using the same parameters, but with information on the sampling site for each individual. This allowed the program to calculate the probability that each individual arose from its sample population vs. neighbouring populations (Clark et al., 2007). We also ran the simulation independently for data within each of the three sampling localities in order to detect any further population substructure.

We tested the hierarchical population structure using AMOVA implemented in arlequin version 3.1 (Excoffier et al., 2005). We estimated among population variation (F_{ST}) and the inbreeding coefficient (F_{IS}) to determine whether there was evidence of inbreeding in any of the populations. We calculated F_{IS} without TrL9, a locus that was found to contain null alleles, as a high F_{IS} value might result from the presence of null alleles rather than from inbreeding.

2.6. Signatures of fluctuation in population size

We tested for signatures of major changes in effective population size based on mtDNA using Fu's F statistic (F_S) and mismatch distributions calculated in arlequin. Fu's F_S tests if a population has deviated from evolutionary equilibrium based on the probability of the observed number of haplotypes occurring under conditions of neutrality (Fu, 1997). The more negative the F_S value, the greater the population's departure from the neutral model, hence a sign of population expansion. The mismatch distribution looks at pair-wise nucleotide substitution differences among haplotypes and tests how well their distribution fits with that expected under the unimodal demographic expansion model (Rogers and Harpending, 1992). The raggedness index (RI) provides an indication of how different the observed distribution is to that under the expansion model.

For the microsatellite data, we used bottleneck (Piry et al., 1999) to test for heterozygosity excess, indicative of population size reduction. A rapid decrease in the effective population size causes rare alleles to be lost in a population more rapidly than common alleles, which leads to an excess of expected heterozygosity (Maruyama and Fuerst, 1985). This test was performed using a one-tailed Wilcoxon test, for three different mutation models: the stepwise mutation model (SMM); the infinite alleles model (IAM); and the two-phase model (TPS). The TPS model was tested with several different parameter settings for the proportion of multi-step mutations and the mean size of multi-step mutations. These parameters were set to 0.1 and 0.05; 0.1 and 0.15; 0.2 and 0.05; 0.2 and 0.15; as well as 0.3 and 0.3, respectively.

3. Results

3.1. Molecular diversity

The ND4 region of mtDNA was sequenced for 101 individuals (GenBank accession numbers: FJ195806 to FJ195907). There were 25 mtDNA haplotypes, four from NSW and 21 from Victoria. Nucleotide diversity (π) was relatively low but haplotype diversity (H) was moderate to high in all populations (NSW $\pi = 0.0065 \pm 0.0044$, $H = 0.9 \pm 0.16$; Bogong High Plains $\pi = 0.0059 \pm 0.0033$, $H = 0.907 \pm 0.029$; Mt Hotham $\pi = 0.007 \pm 0.0039$, $H = 0.68 \pm 0.054$; Lankey Plain $\pi = 0.0009 \pm 0.0008$, $H = 0.577 \pm 0.065$). Nucleotide diversity was markedly lower in Lankey Plains than in the other three populations.

Based on the microsatellite data, two pairs of samples, one pair from Lankey Plains and one pair from Bogong High Plains, were recaptures as they had identical alleles at all loci and the same capture locality. We assume that these samples came from the same individual as the probability of two individuals having identical alleles at all seven loci for the Lankey Plains population is 5.5×10^{-8} and 6.9×10^{-9} for the Bogong High Plains population. One of each pair was excluded from all analyses (including mtDNA analyses). Thus 93 samples were scored at seven microsatellite loci retrieving 98.6% of alleles. The seven loci displayed variable levels of genetic diversity within the areas sampled, different subsets of alleles between the areas sampled, and different levels of observed and ex-

pected heterozygosity (Table 1). All loci were independent as there was no significant linkage disequilibrium ($P > 0.02$, after Bonferoni adjustment). Departures from HWE were found in three cases: (i) Bogong High Plains for locus TrL29; (ii) Lankey Plain for TrL19 and (iii) Lankey Plain Tr3.2 (Table 1). Locus TrL9 consistently displayed a significant amount of homozygote excess across all three sampling localities (Bogong High Plains: 10.5 expected homozygotes, 16 observed homozygotes $p = 0.025$; Mt Hotham: 6.78 expected, 13 observed, $p = 0.01$; and Lankey Plain: expected 7.72, observed 15, $p = 0.025$), indicating that null alleles may be present at this locus. Because null alleles can lead to overestimates of genetic distances when gene flow between populations is low (Chapuis and Estoup, 2007), as is the case with these populations, we repeated all analyses of microsatellite data with and without the TrL9 locus. The results with and without TrL9 were qualitatively the same, so we present the results with all seven loci included. There were also general excesses of homozygotes for other loci, which may indicate inbreeding.

3.2. Population structure and gene flow

The Bayesian consensus tree (Fig. 2) revealed that the NSW individuals form a well-supported monophyletic group (bootstrap 99%). The statistical parsimony network indicates that there are two well-supported clades in Victoria (Fig. 3A). One of these clades is composed of individuals from Mt Hotham and Bogong High Plains, whilst the other clade has individuals from Mt Hotham and Lankey Plains. The presence of individuals with mtDNA haplotypes from both major clades within the Mt Hotham population is indicative of historical contractions of populations into refugia and subsequent expansion and admixture between the Victorian populations.

Although the mtDNA indicated two distinct mitochondrial haplogroups in Victoria, Bayesian clustering analysis showed that microsatellite diversity clearly falls into three populations ($K = 3$; Fig. 4A) that correspond with the geographic regions sampled in Victoria (Fig. 3B). The number of populations is estimated as occurring at the maximum L (K) scores, which occurred at $K = 3$. The Evanno et al. (2005) method also gave $K = 3$ as the estimated number of populations (Fig. 4B). The 93 individuals were consistently assigned to one of the three groups with very high probability (Fig. 3 C) for each of the five independent runs of $K = 3$. No migrant individuals were identified when simulations were run with a priori information on sampling localities. Most of the admixture disappeared in this run, except for one individual (indicated by the black arrow in Fig. 3C). There was no evidence of further population structure within populations.

The highly geographically structured pattern of genetic diversity in *C. praealtus* was confirmed by AMOVAs and estimates of net sequence divergence (Table 2). Most of the mtDNA genetic variation (78.55%) occurs at the regional level between Victoria and NSW, followed by 12.28% between populations, and 9.17% within populations. Pairwise population divergence tests indicated significant divergence between all pairs of populations ($P < 0.001$) with both the mtDNA and microsatellite DNA data (Table 2). The greatest level of divergence occurs between NSW and Victoria with the mtDNA, further evidence that these two groups have been diverging for long enough to become reciprocally monophyletic. Among the three Victorian populations, the Bogong High Plains and Mt Hotham are most genetically similar, whilst the Bogong High Plains and Lankey Plain are the most dissimilar. Significant F_{IS} values for both Lankey Plain ($F_{IS} = 0.08$, $p < 0.01$) and Bogong High Plains ($F_{IS} = 0.05$, $p < 0.05$) indicate that there are low levels of inbreeding in both populations. There is no evidence of inbreeding in the Mt Hotham population ($F_{IS} = -0.08$, $p = 0.98$).
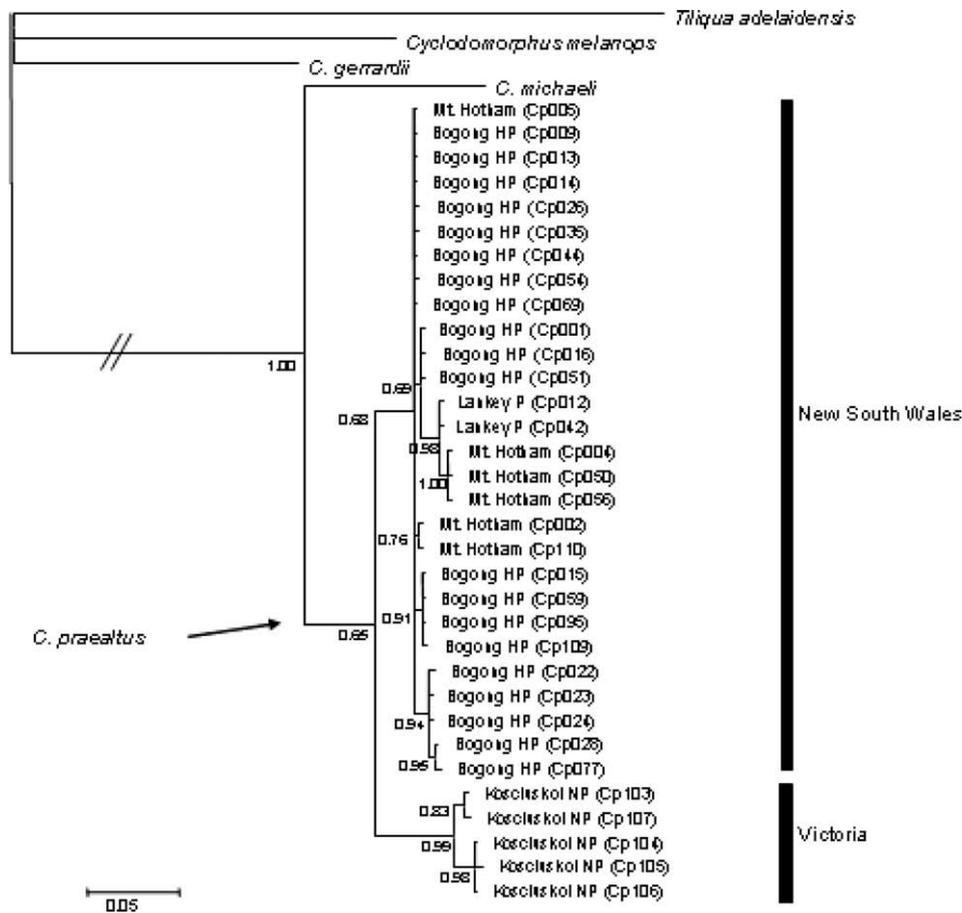
Fig. 2. A Bayesian consensus tree of the ND4 mtDNA sequences, depicting the relationship between all unique haplotypes genotyped at each locality (Bogong High Plains, Lankey Plain, Mt Hotham and Kosciuszkoi National Park) for *Cyclodomorphus praealtus*. Bootstrap values, shown at the branches, represent the level of support for the branch arrangement; bootstrap values >90% indicate that the branch topology is well supported.

3.3. Signatures of population contraction and expansion

There was little evidence for major fluctuations in effective population size based on mtDNA data. For the mtDNA, none of the Victorian populations deviated significantly from expectations of neutrality (Fu's *F* statistics, Table 3); however, based on mismatch distributions, a model of population expansion could only be conclusively rejected for the Mt Hotham population (Table 3, Fig. 5).

Analysis of the microsatellite data found no consistent indication of a recent bottleneck within any of the populations using the heterozygosity excess tests. However the IAM test detected significant deviation from mutation-drift equilibrium in all three Victorian populations although this became non-significant for Bogong High Plains following a Bonferroni correction for multiple tests (Table 3).

4. Discussion

4.1. Historical and recent population genetic structure and gene flow

The ND4 mtDNA sequence analysis revealed deep genetic divergence between the Victorian and NSW populations of *C. praealtus*. This divergence is not surprising considering the biogeographic barriers that are currently found between the Victorian and NSW populations: the deep valleys of eucalypt forests and the Murray River. The sequence divergence of 4.28% between the NSW and Victorian populations is comparable to subspecies-level divergence in other taxa (Chapple and Keogh, 2004; Gamauf et al., 2005). Using the conventional mtDNA clock calibration of 2% sequence diver-

gence per million years (Avise, 2004), an estimate of the divergence time between the two regions is around two million years. This coincides with the end of the rapid cooling and drying that occurred during the Miocene and Pliocene, 5–2 million years ago, which is thought to be when the alpine biota developed (Frakes et al., 1987; Green and Osborne, 1994). A series of warming and cooling cycles then followed over the last two million years, resulting in changing habitat boundaries in the Australian alps (Frakes et al., 1987). Studies on other alpine vertebrate fauna have detected comparable levels of divergence between Victorian and NSW populations: the Guthega skink, *Egernia guthega*, has a 2.2% sequence divergence, suggesting a divergence time of 1.1–1.7 million years ago (Chapple et al., 2005), and the mountain pygmy possum, *Burramys parvus*, has a sequence divergence of 1.1% between NSW and Victorian populations (Mitrovski et al., 2007).

Our divergence time calculation for *C. praealtus* is tentative as molecular clocks should be calculated independently from multiple markers (Pamilo and Nei, 1988) and ideally, calibrated with fossil records (Bromham and Penny, 2003). The divergence time of two million years may be an overestimate as it is based on one mtDNA marker, which is vulnerable to the effects of genetic drift. Genetic drift has a disproportionate influence on small fragmented populations, causing them to become reciprocally monophyletic faster than larger populations (Templeton, 1980; Neigel and Avise, 1986; Canestrelli et al., 2007). Nevertheless, the deep divergence in the mtDNA sequences indicates that the NSW and Victorian populations of *C. praealtus* should be considered separate Evolutionarily Significant Units. The data reported here meets part of Moritz's (1994) criteria for ESU's, which requires strong geographic parti-
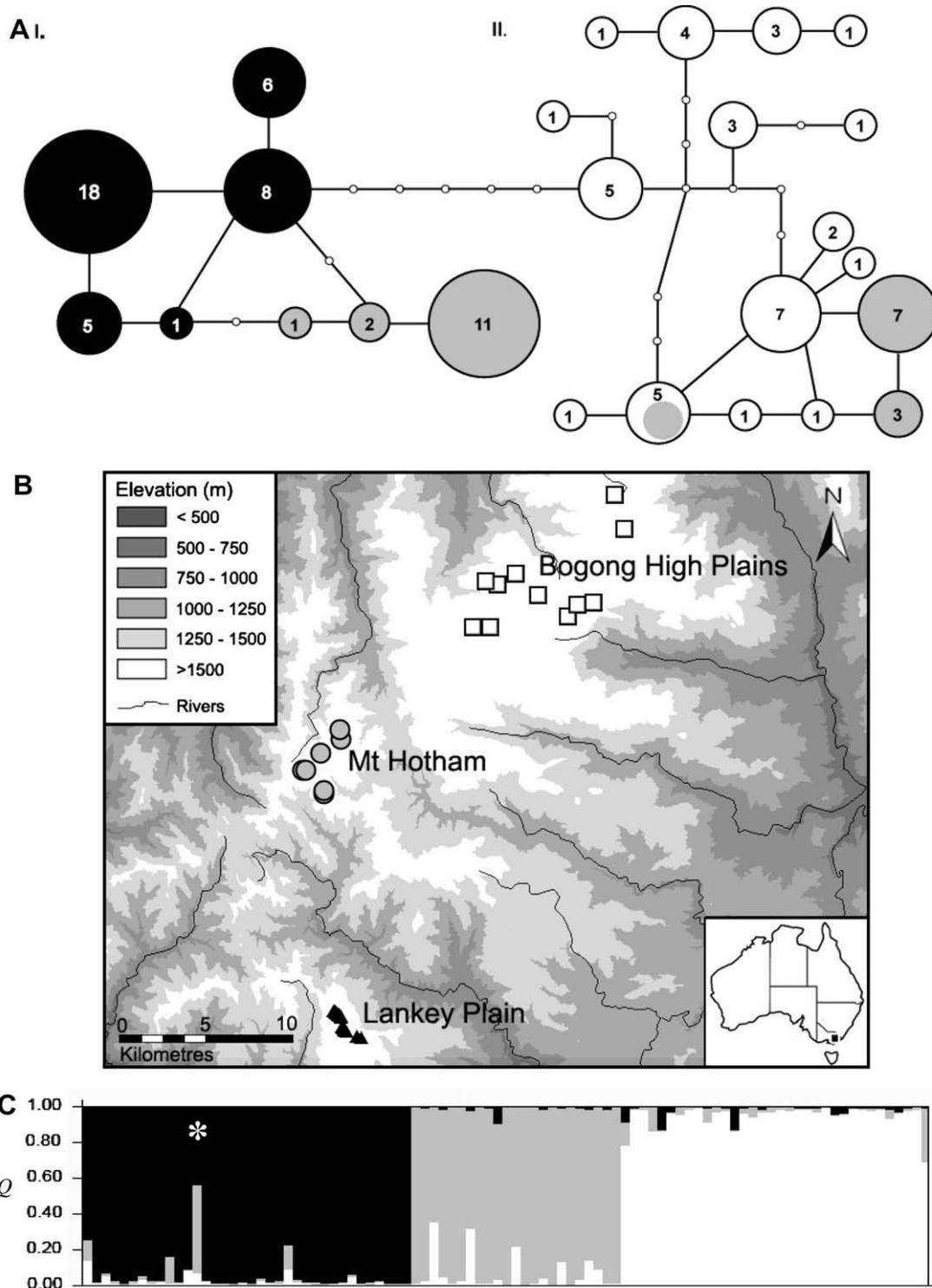
Fig. 3. Population structure of Victorian *Cyclodomorphus praealtus*. Black indicates Lankey Plain, grey indicates Mt Hotham and white indicates Bogong High Plains. (A) Haplotype network of the ND4 mtDNA displays two Victorian haplogroups (I and II). Each circle represents a unique haplotype, with the circle size indicative of frequency and sample sizes within each circle. Empty circles represent missing haplotypes and differ by one base pair from the closest haplotype. (B) Elevation map of the Victorian alpine region of south east Australia indicating location of transects where *C. praealtus* were sampled. (C) Bayesian population assignment analysis using structure. Each column represents *q* values, the proportions in which a given genotype belongs to a cluster of the given colour. The asterisk indicates the only individual that still shows substantial admixture after the sampling site of origin is taken into account.

tioning, reciprocal monophyly of mtDNA haplotypes and significant divergence of allele frequencies at nuclear loci. Levels of divergence at nuclear loci in *C. praealtus* are required to confirm that this substantial divergence is not only present in the mtDNA.

Within Victoria, the ND4 haplotype network shows that the mtDNA is divided into two haplogroups. The Mt Hotham popula-

tion comprises haplotypes with affinities to those found at both Bogong High Plains and Lanky Plains, indicating historical connectivity between the three populations. This is reflected in levels of mtDNA diversity, which is greatest at Mt Hotham. During the last glacial period (approximately 25 – 18 000 y.b.p.), alpine habitat would have extended into the valleys connecting the mountain
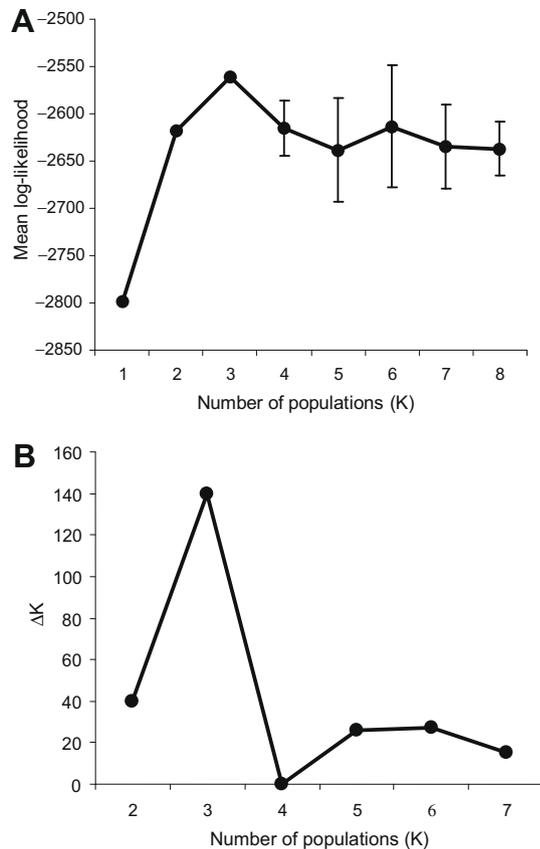
Fig. 4. Two methods for estimating *K*, the number of populations, in *Cyclodomorphus praealtus*. (A) The mean log-likelihood for *K* between 1 and 8 for five runs using structure 2.2. *K* is estimated as three populations, the maximal mean log-likelihood value before the values plateau. (B) The number of populations (*K*) vs. the second-order derivative of structure's natural log-likelihood output (Δ*K*; as described in Evanno et al., 2005). The highest Δ*K* indicates the most likely number of populations.

Table 2
Measures of pairwise population divergences between *Cyclodomorphus praealtus* populations. Percentage net sequence divergence (D_A), estimates of among population variation for mtDNA (Φ_{ST}) and microsatellite (F_{ST}) data. BH = Bogong and Hotham, HL = Hotham and Lankey, BL = Bogong and Lankey, NV = NSW and Victoria. $P < 0.001$ for all values.

		BH	HL	BL	NV
mtDNA	D_A	0.35	0.43	0.866	4.28
	Φ_{ST}	0.35	0.56	0.73	0.9
Microsatellites	F_{ST}	0.09	0.08	0.07	–

tween the three Victorian populations, the microsatellite data show little evidence of recent gene flow, with only one individual from Lankey Plain identified as having mixed ancestry with the Mt Hotham population. Thus, despite Victoria having three populations within 100 km, *C. praealtus* may be prone to local extinctions, as any reduction in population size will not be compensated by dispersal. Further fragmentation of the populations, for instance due to additional ski resort development, may also increase risk of local extinction and inbreeding depression. Unfortunately, the population at Mt Hotham is probably already fragmented by development within the species' habitat for alpine resort and recreational infrastructure. Further development in this area is slated. Thus, it is important to maintain connectivity within each of the remaining extant populations. Now that a baseline for genetic diversity has been established for *C. praealtus*, future monitoring should be better able to detect changes in genetic diversity.

4.2. Historical and recent population demography

We found no consistent evidence for deviations from neutral expectations based on mtDNA. However, based on mismatch distributions, a model of population expansion could only be rejected for the Mt Hotham population. Indeed, our results suggest that the mismatch distribution was multimodal at Mt Hotham, indicating an ancient splitting of gene genealogies at that locus, and thus a population that has remained relatively stable in size (Reich et al., 1999). Alternatively, the multimodal pattern could indicate that the Mt Hotham skinks are descended from individuals from multiple refugial populations (Schneider and Moritz, 2007).

We also found no strong evidence for a more recent change in effective population size for any of the populations based on microsatellites. Although the IAM detected significant heterozygosity excess in all populations, contradicting results of the two alternative models, the IAM is known to be prone to Type I error when used with microsatellite markers (Luikart and Cornuet, 1998; Busch et al., 2007). The power of detecting relatively recent bottlenecks increases with the number of loci genotyped and simulations indicate that the number of loci used should, ideally, be higher than we have used here (Cornuet and Luikart, 1996). The ability to detect a population bottleneck also decreases over time as the allele frequencies relax into a new equilibrial state (Cornuet and Luikart, 1996). In addition, populations suffering a reduction in census size may not suffer a severe reduction in effective population size (a genetic bottleneck) if the historical effective population size has always been low due to fluctuations in population size, mating system dynamics (e.g., polygyny or inbreeding), or metapopulation structure involving local extinctions and recolonisations (Pimm et al., 1989). It may well be that the effective population size of the alpine she-oak skink is historically low due to repeated cycles of warmer weather forcing populations into small refugial areas. We also found some evidence of inbreeding within populations on the Bogong High Plains and Lankey Plain, which may be due to small effective population size and limited dispersal ability in this species.

Variation in estimated effective population sizes and genetic diversity of these populations does not reflect the area of suitable habitat. Mt Hotham has the greatest mtDNA genetic diversity of the three Victorian populations and is the only population in Hardy–Weinberg equilibrium, but it currently has the smallest area of suitable habitat. Bogong High Plains offers the largest area of suitable habitat, and has a similar level of microsatellite diversity to Mt Hotham, suggesting that these populations have relatively larger effective population sizes than Lankey Plain (Avise, 2004). During warmer climatic periods *C. praealtus* is most likely to have persisted in the highest altitude areas, and become extinct at lower altitudes. The Bogong High Plains and Hotham Heights at-

peaks, allowing gene flow between the three extant Victorian sites. There is evidence that the snowline extended 400 m lower during this time (Frakes et al., 1987) and the subalpine-alpine boundary (where large woody plants cannot survive) was 850 m lower at 1 100 m in the southern highlands (Hope, 1987). This is supported by fossil evidence for *B. parvus*, which currently has a very similar alpine restricted distribution to *C. praealtus*, but which is present in the fossil records throughout most of south-eastern Australia in the Pleistocene glacial period (Ride, 1956; Wakefield, 1967, 1972; Hope, 1987). As the climate warmed, conditions became suitable for eucalyptus forests to move into higher altitude areas, isolating the once connected alpine meadows (Frakes et al., 1987).

Currently, the three Victorian "sky island" sites are isolated by deep "seas" of forested valleys, habitat in which these skinks have never been found. Despite evidence for historical connectivity be-

Table 3
Tests for a signature of population expansion in mtDNA and for a population bottleneck in microsatellite DNA genotypes, for each of the Victorian populations of *Cyclodomorphus praealtus*. *P < 0.001 (see Fig. 5). The values shown for the bottleneck tests (for the infinite alleles model, IAM; stepwise mutation model, SMM; and two-phase model, TPM) are one-tailed p values, from the Wilcoxon tests for excess of heterozygotes. The parameters for TPM (in brackets) refer to the proportion of multi-step mutations and variance in the multi-step mutations for the heterozygosity excess test.

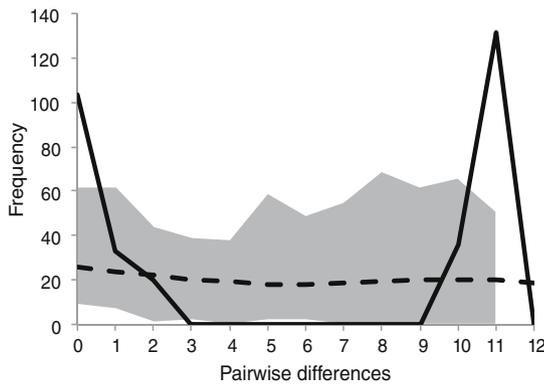|		Bogong High Plains	Mt Hotham	Lankey Plain
mtDNA	RI	0.03	0.32[*]	0.07
	Fu's F_S	−1.37	7.7	0.71
Microsatellites	IAM	0.039	0.012	0.004
	SMM	0.988	0.961	0.961
	TPM (0.1, 0.05)	0.988	0.813	0.961
	TPM (0.1, 0.15)	0.988	0.656	0.813
	TPM (0.2, 0.05)	0.988	0.766	0.852
	TPM (0.2, 0.15)	0.945	0.594	0.344
	TPM (0.3, 0.3)	0.711	0.235	0.289

Fig. 5. The pairwise mismatch distribution for the Mt Hotham *Cyclodomorphus praealtus* population. The solid line represents observed values and the dashed line represents expected values under a demographic expansion model. The 95% confidence interval of the expected distribution is indicated by the shaded area.

tain elevations of around 1800 m, whilst Lankey Plain reaches 1600 m (Holdgate et al., 2008). Both geographic and genetic diversity estimates support the possibility that Bogong High Plains and Mt Hotham were refugial populations (they have almost an order of magnitude greater genetic diversity than Lankey Plain). Although there were only five samples, the nucleotide diversity of individuals from the Kosciuszko area was similar to that of Mt Hotham and the Bogong High Plains. Again, this is consistent with higher elevation areas maintaining refugial populations as this area reaches higher elevations than the Victorian sites. Co-distribution of other alpine restricted species can be indicative of shared history (Avise, 2004). Other alpine endemic species occur on the Bogong High Plains, Mt Hotham and Mt Kosciuszko. For example, the mountain pygmy possum, *parvus*, occurs on the Bogong High Plains, Mt Higginbotham/Loch (Hotham area), Mt Buller and Mt Kosciuszko (Mitrovski et al., 2007), and the alpine water skink, *Eulamprus kosciuskoi*, occurs on Dinner Plain, Davies Plain, Bogong High Plains and the Mt Kosciuszko area (Meredith et al., 2001). Similar patterns are also evident in the skinks *E. guthega* and *E. montana* (Wilson and Gerry, 2003; Chapple et al., 2005). Studies indicate phylogeographic concordance among species, with all showing deep divergences between NSW and Victorian populations (Osborne et al., 2000; Chapple et al., 2005; Mitrovski et al., 2007; Sumner unpublished data).

5. Conclusions

C. praealtus comprises two distinct ESUs, one containing the Victorian populations, and the other the NSW populations. Despite historical connectivity the three known Victorian populations are effectively isolated, reflecting very low dispersal ability and a high degree of habitat specialisation. This is reinforced by the high genetic diversity of the three highest elevation populations (the Bogong High Plains, Mt Hotham and Mt Kosciuszko) and the comparatively low genetic diversity of the lower elevation Lankey Plain population.

Alpine specialist species such as *C. praealtus* are precariously positioned as early indicators of how ecosystems will respond to future climate change. The species is known only from specific tree-less alpine vegetation communities which rely on a certain climatic envelope that includes a consistent, sustained cover of snow in the winter. There is growing evidence that alpine vegetation is changing rapidly, and contracting to higher altitude areas as temperatures increase (Sanz-Elorza et al., 2003; Edmonds et al., 2006). As a result, climate change is expected to further reduce *C. praealtus* habitat. Future snow forecasts indicate that in mainland Australia, the area with more than one day a year of snow cover is likely to be 10–40% smaller by 2020 and 22–85% smaller by 2050 (Hennessy et al., 2007). In the 'worst case scenario' the only places within the known range of *C. praealtus* where substantial snow will still fall is the Bogong High Plains and Kosciuszko regions, as well as a few small isolated peaks (Whetton et al., 1996). *C. praealtus* has the greatest chance of persisting into the future in the higher altitude refugial areas of alpine habitat on Mt Hotham, the Bogong High Plains, and Kosciuszko National Park. We caution that responsible management of the species' habitat in these areas will be crucial for this persistence, particularly on Mt Hotham where the area of available habitat is extremely limited. Further loss and/or degradation of habitat in this area will compromise the most genetically diverse population of this species.

Acknowledgements

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