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Allopatric divergence drives the genetic structuring of an endangered alpine endemic lizard with a sky-island distribution

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Abstract

Anthropogenic climate change is causing a world-wide reduction of alpine habitat, leaving many high-elevation species restricted to sky-islands and vulnerable to extinction. Understanding the genetic parameters of these populations provides key insight into species diversity, dispersal capacity and vulnerability to disturbance. We examined the impact of past climatic variation on a threatened alpine endemic lizard, the Guthega skink, *Liopholis guthega*. We analysed SNP and mtDNA data to determine the population structure and phylogeny within this species, providing an understanding of the species' relatedness, dispersal and viability. We identified significant genetic structure, with the split between populations in Koscuiszko National Park, New South Wales (NSW) and the Bogong High Plains, Victoria (VIC) consistent with Plio-Pleistocene divergence. However, we also detected evidence of possible historical introgressive hybridization between some NSW populations and the VIC populations. Marked within-site population structure and significant population differentiation among sites within each state were found, indicating a limited dispersal capacity. Higher levels of genetic diversity within NSW support the correlation between elevation and diversity and implicate Kosciuszko National Park as a historic refugia. Low contemporary habitat availability, little to no capacity for elevational progression and low genetic diversity, particularly in VIC, leaves *L. guthega* highly vulnerable to threatening processes associated with climate change. Conservation management should consider genetic rescue as a potential method to enhance genetic diversity across this species' range.

Introduction

Alpine regions exhibit high biodiversity and endemism, but are one of the most vulnerable ecosystems due to anthropogenic climate warming (Dirnböck, Essl & Rabitsch, 2011; McCain & Colwell, 2011). The relatively recent emergence of modern alpine species coincided with widespread cooling since the late tertiary period (Hewitt, 2000). Frequent climatic oscillations during Pleistocene glacial periods repeatedly induced distributional shifts, increasing the frequency of population expansion, contraction and isolation of montane habitats and driving the divergence of genomes (Hewitt, 2000; Chapple, Keogh & Hutchinson, 2005). Yet, despite being shaped by climatic variation, fundamental characteristics of the alpine fauna may heighten their vulnerability to climate warming. Firstly, they are adapted to one of the coldest biomes on earth, with distributions of alpine endemics restricted to the windblown landscapes that prevail above the treeline (Green & Osborne, 2012). These regions will additionally become more limiting as climate change progresses (Pauli, Gottfried & Grabherr, 2014). Secondly, they occur in topographically variable regions with naturally fragmented habitats, where numerous 'sky-islands' of suitable alpine habitat are interspersed amongst a sea of inhospitable warmer lowland regions (McCormack *et al*., 2009).

Being confined to these often small, fragmented sky-islands means many alpine species face challenges as a result of current population allopatry (McCormack *et al*., 2009). In habitat specialist montane species with characteristically poor dispersal ability (McCain & Colwell, 2011), this current isolation inhibits migration and gene flow, promoting inbreeding and genetic drift (Sumner, 2005; Haines *et al*., 2017). Consequently, this homogenizes genetic diversity within sky-islands (Browne & Ferree, 2007), while producing genetic structure among islands (Mitrovski *et al*., 2007), increasing rates of both diversification and extinction (Hedrick & Kalinowski, 2000; Koumoundouros *et al*., 2009; Sato *et al*., 2014b). In extreme cases, this lack of contemporary connectivity can erode genetic diversity sufficiently within fragments to reduce population-level fitness, which elevates extinction risk (Maron *et al*., 2015). Small range size and the subsequent negative fitness effects of inbreeding depression and loss of adaptive potential associated with small population size, is the single best predictor of extinction risk for terrestrial organisms (Sekercioglu *et al*., 2008). Thus, alpine species are at particular risk from global climate change.

To address this risk, we need fundamental information on the potential risk of alpine species to ongoing anthropogenic climate change. In this context, genome-wide measures of intra-population genetic diversity are important as they allow us to determine population viability and susceptibility to disturbance (Demos *et al*., 2015), as well as to identify and protect important habitat corridors to ensure ongoing gene flow (Coulon *et al*., 2004). Attaining genetic insight at higher resolutions can identify processes that have shaped the structure of allopatric populations and allow for more informed conservation management of alpine endemic species (Koumoundouros *et al*., 2009; Haines *et al*., 2017). Such knowledge is increasingly important as climate warming reduces the extent of the alpine zone, further isolating populations and enhancing the likelihood of species extinctions (Dirnböck *et al*., 2011).

The Australian alpine fauna may be particularly vulnerable to climatic warming, through a combination of inherent sensitivity of alpine endemics and their increased exposure due to the topographic characteristics of the Australian alps (Tatarnic, Umbers & Song, 2013). Furthermore, the limited extent of the alpine zone (~0.15% of the total land area) and the ubiquity of eroded, flat-topped mountains (max. 2228 m) (Williams & Costin, 1994; Byrne, 2008) means that whilst many alpine species on other continents have exhibited altitudinal migration to track their preferred thermal niche (Parmesan, 1996; Chen *et al*., 2011), the potential for such distributional shifts in Australia is restricted. Threats to fauna across Australia's highly disjunct alpine region may be further exacerbated by additional human-mediated stressors that enhance habitat fragmentation, including alpine tourism (Pickering, Harrington & Worboys, 2003), altered fire regimes (Green & Sanecki, 2006) and the degradation and erosion of sensitive alpine habitats from overgrazing and trampling by invasive herbivores (Dawson, 2009).

Here, we use mitochondrial (mtDNA) and nuclear single nucleotide polymorphisms (SNPs) to investigate the phylogeography and population structure of Australia's highest elevation reptile, the Guthega skink, *Liopholis guthega*. This medium-sized, viviparous lizard is restricted to two geographically isolated sky-islands above 1600 m in Australia: the Bogong High Plains (BHP) in Victoria (VIC) and Kosciuszko National Park (KNP) in New South Wales (NSW; Fig. 1; Atkins *et al*., 2018). As a result, *L. guthega* is listed as endangered at the national level (*Environmental Protection and Biodiversity Conservation Act 1999*) and critically endangered in the state of Victoria (DSE, 2013). Threats include the recession of alpine habitat associated with climate change (Green, Mansergh & Osborne, 1992) and alpine tourism – the species is restricted to Australia's highest peaks and is distributed among ski resorts (Sato *et al*., 2014a). Within its highly limited range, *L. guthega* exhibits high site fidelity to burrow systems, living in small, discrete colonies (Atkins, Clemann & Robert, 2015). A previous phylogeographic study using a single mitochondrial gene and extremely limited sampling across the species range, suggests that *L. guthega* may be highly structured throughout its range (Chapple *et al*., 2005). The topographic variability of the Great Diving Range that separates *L. guthega* populations has also been shown to influence the population structure of other Australian sky-island taxa (Mitrovski *et al*., 2007; Koumoundouros *et al*., 2009; Tatarnic *et al*., 2013; Slatyer *et al*., 2014). Due to limited connectivity among Australian sky-island regions, coupled with the reduced extent of the Australian alpine zone, we predicted that *L. guthega* show high genetic divergence between geographically separated populations and exhibit low levels of genetic diversity within populations.

Materials and methods

Study area and sample collection

During the 2013-15 Australian summers (December-March), we collected tissue samples from 452 *L. guthega* individuals across the BHP, VIC (n = 260) and KNP, NSW (n = 192), spanning the species' known range (Fig. 1). Sampling was

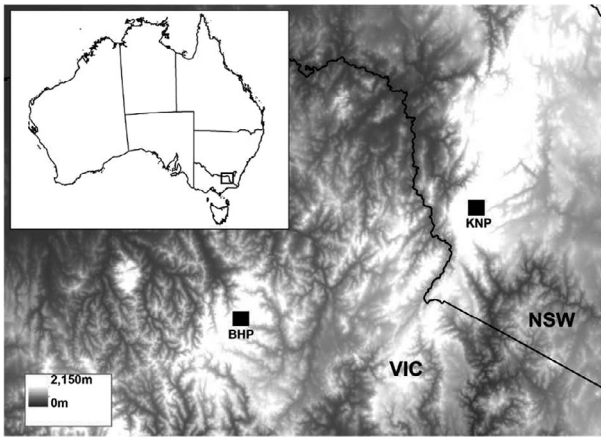

Figure 1 The allopatric distribution of *Liopholis guthega* to the two sky-islands; the Bogong High Plains (BHP) in Victoria (VIC) and Kosciuszko National Park (KNP) in New South Wales (NSW).

conducted at ~1700 m and ~1630–2170 m elevation for VIC and NSW respectively (Fig. 1). Victorian and NSW sites receive a mean annual rainfall of 1200 mm and 1750 mm respectively [http://www.bom.gov.au/australia/alpine/, accessed (14/02/2017)] and are characterized by alpine and sub-alpine grasslands, herbfields, heathlands and snow gum woodlands interspersed with granite and basalt (VIC only) boulder fields, as well as small streams and associated alpine-bog systems. Lizards were captured with the aid of a noosing pole or by hand. Tail tips ($\sim$1 cm section) were collected and stored in 70% ethanol. Captured individuals represented 32 colonies (VIC: $n = 11$, NSW: $n = 21$; see Fig. 5a and b respectively). The colonial nature of this species allowed the identification of discrete *L. guthega* aggregations, with distances between colonies ranging from 300 m (maximal short-term dispersal distance identified for the closely related *L. whitii*; Chapple & Keogh, 2005) to ~ 18 km (see Fig. 5a, b). No individual identified at one colony was recaptured at another throughout the study period.

Mitochondrial DNA

DNA extraction, amplification and sequencing

Genomic DNA was extracted from tail samples using a Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen, Hilden, Germany). A partial fragment (695 bp) of the NADH subunit 4 (ND4) mitochondrial (mtDNA) gene was amplified using the primers ND4I and tRNA-Leu (Forstner, Davis & Arévalo, 1995). PCR, PCR clean-up and sequencing was performed as per Chapple *et al*. (2013). Sequence data were edited and aligned using Geneious v6.1.2 (Biomatters, Auckland, New Zealand). Sequences were deposited in GenBank under the accession numbers MF987668 – MF987696. To investigate the diversity within the genus, additional sequences from all available representative *Liopholis* species were downloaded from GenBank and included (Table S1). *Egernia saxatilis* was included as the outgroup.

Phylogenetic analyses

We used Maximum-Likelihood (ML) and Bayesian Inference (BI) approaches. MRMODELTEST 2.3 (Nylander, 2004) was used to carry out statistical selection of best-fit models of nucleotide substitution. The appropriate model (GTR + Γ + I) was chosen based on 'goodness of fit' via the Akaike information criterion [AIC; Akaike (1974)]. The ML topology was constructed using RAxML v8.0.19 (Stamatakis, 2014). Strength of support for internal nodes of ML construction was measured using 1000 rapid bootstrap (BS) replicates. Marginal posterior probabilities (PP) for BI were calculated using MrBayes v3.2 (Ronquist & Huelsenbeck, 2003). Model parameter values were treated as unknown and were estimated. Random starting trees were used and the analysis was run for 15 000 000 generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of <0.01 was used as a guide to ensure the two independent analyses had converged. The programme Tracer v1.6 (Rambaut *et al*., 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct 'burn-in' for the analysis.

To infer the approximate timing for divergences, we used the conventional mtDNA clock calibration of 2% sequence divergence per million years (Myr) (Brown, George & Wilson, 1979), as used in other reptile studies (Chapple *et al*., 2005; Koumoundouros *et al*., 2009). Genetic divergence was calculated in MEGA6 (Tamura *et al*., 2013), using 1000 bootstrap (BS) replicates for likelihood estimation.

Demographic analyses

Estimates of genetic diversity within populations (number of haplotypes, h; haplotype diversity, Hd; nucleotide diversity, π) were calculated in DnaSP v5 (Librado & Rozas, 2009). Fluctuations in effective population size were estimated via Fu's F statistic (Fs) and Tajima's D statistic. Departures from the neutral model were used to infer population contraction/expansion. Median-joining haplotype networks (Bandelt, Forster & Röhl, 1999) were created using PopART v1.7 (Leigh & Bryant, 2015) and were based on the haplotypes determined via DnaSP.

Nuclear SNP sequencing

Data treatment

A total of 359 (KNP, $n = 186$; BHP, $n = 173$) *L. guthega* DNA extractions were sent to Diversity Arrays Technology Pty. Ltd (DArTseq™) (Canberra, Australia) for sequencing. All DNA extractions that met DArTseq recommended quantity requirements (20 μL at 50–100 ng/μL) and purity levels (>1.6 ratio of absorbance at 260 nm and 280 nm; measured via NanoDrop 2000 Thermo Fisher Scientific) were sent for analysis. DArTseq incorporates both DArT complexity reduction methods and Next Generation Sequencing (NGS) platforms (Grewe *et al*., 2015). The Pstl-SphI complexity reduction method was used as per Grewe *et al*. (2015) and followed subsequent PCR and sequence generation protocol therein. Specifications regarding sample preparation, sequencing, amplification and processing are detailed in Melville *et al*. (2017). Sequences generated from each sample of the 96-well microtitre plate were processed using proprietary DArTseq analytical pipelines (Grewe *et al*., 2015). Data analysis followed protocols detailed in (Melville *et al*., 2017).

We used BayeScan 2.1 (Foll & Gaggiotti, 2008) and OutFLANK (implemented in R) to investigate the occurrence of selection on our VIC and NSW datasets. Both methods identified <10 loci under selection and none were congruent between approaches. Therefore, outliers identified were considered to be false positives and therefore, none were removed prior to downstream analyses.

Genetic diversity and demographic analyses

Estimates of mean genetic diversity, expected (He) and observed (Ho) heterozygosity, were calculated using the

'adegenet' package (Jombart, 2008) in R (R Core Team, 2015). Fluctuations in effective population size were estimated via Tajima's D statistic. Departures from the neutral model were used to infer population contraction/expansion. Estimates of effective population size (Ne) were performed within VIC and NSW based on a linkage disequilibrium model (Waples, 2006; Jones, Ovenden & Wang, 2016) in the software package NEestimator 2.1 (Do *et al*., 2014).

Population structure

We identified genetic clusters via Discriminant Analysis of Principal Components (DAPC; Jombart, Devillard & Balloux, 2010) and a Bayesian approach implemented in fastSTRUCTURE (Pritchard, Stephens & Donnelly, 2000; Raj, Stephens & Pritchard, 2014). The number of genetic clusters supported via DAPC was determined via the Bayesian Information Criterion (BIC; (Schwarz, 1978). The most likely number of genetic clusters (K) supported by fastSTRUCTURE was determined by calculating the difference in log-likelihoods [Ln P(D)] among K values, which was then divided by the standard deviation (ΔK; (Evanno, Regnaut & Goudet, 2005). All fastSTRUCTURE runs were performed using the admixture model to allow for mixed ancestry and the detection of hybrid zones. Ten thousand initial burn-in steps were discarded, followed by a further 10 million MCMC iterations. Once the number of genetic clusters had been estimated, analysis of spatial structure was investigated using both DAPC and fastSTRUCTURE independently. As clear genetic distinction was present between VIC and NSW, hierarchical DAPC and fastSTRUCTURE were also used to identify genetic clusters within each state. DAPC was then used to determine if the geographic patterns among sampling localities were reflected in the genetic signatures of the individuals sampled. Therefore, in an independent analysis, each sampling locality within a state was assumed to be an individual population.

Spatial patterns in the genetic variability of individuals were investigated via Spatial spatial principal component analysis (sPCA) (Jombart *et al*., 2010) using the 'adegenet' package (Jombart, 2008) in R. A matrix of relative allele frequencies was calculated and analysed against a spatial weighting matrix based on the latitude and longitude of each individuals sampling site.

Genetic differentiation and degree of inbreeding

Genetic differentiation among populations from VIC and NSW inferred from DAPC and fastSTRUCTURE analyses were estimated independently using pairwise calculations of both Jost's D (Jost, 2008) and Hedrick's G'st (Hedrick, 2005), a standardized analogue of F_{ST}, via the 'mmod' package (Winter, 2012) in R (R Core Team, 2015). Thousand permutations were run to calculate 95% confidence intervals and associated p-values. The inbreeding coefficient was calculated for VIC, NSW and swNSW (populations defined based on population structure results), using the 'adegent' package in R. We conducted hierarchical Analysis of Molecular Variance (AMOVA; (Excoffier, Smouse & Quattro, 1992) to investigate the partitioning of genetic variation among inferred populations and sampling localities. Significance levels were calculated by 999 permutations.

Isolation by distance

We tested for a correlation between genetic and geographic distances using a mantel test conducted in the ade4 package in R. Genetic distances were calculated using each individuals latitude and longitude data and were based on the relative allele frequency matrix used in sPCA.

Population divergence

To estimate the level of divergence among the three main populations identified in our study (BHP, KNP and swKNP), we calculated per cent divergence estimates using SNAPP (Bryant *et al*., 2012) in BEAST 2.4 (Bouckaert *et al*., 2014). Three a priori groups were set (BHP, KNP and swKNP) and ten independent runs were performed, each running for 1 000 000 generations, sampling every 1000 steps. Log files were viewed in Tracer 1.6 to assess convergence (Rambaut *et al*., 2014).

Results

Phylogenetic analyses

All *L. guthega* individuals grouped into a highly supported monophyletic clade based on 695 base pairs of the mitochondrial ND4 gene (BS = 100, PP = 1; Fig. 2). This clade formed an unresolved polytomy with *L. whitii*, *L. montana* and *L. personata* clades (BS = 53, PP = 0.99). Phylogenetic structure was evident in *L. guthega*, with three groups corresponding to geographic area, but with low support values (BS = ≤ 57, PP = ≤ 0.83). The clade composed of individuals from NSW [excluding the three haplotypes from southwest KNP (swKNP) (H8, H9 & H11)] was weakly supported by BI (PP = 0.83); however, the ML topology (Fig. S1) showed no support for this clade (BS = 22).

Based on mtDNA, genetic divergence between the geographically isolated BHP VIC and KNP NSW sky-island systems was 1.9% (± 0.4), while divergence between KNP and the nearby swKNP region was 1.8% (± 0.4). Despite the comparable geographic distance to KNP, the divergence between the BHP and swKNP region and was only 0.8% (± 0.2).

Demographic analyses

Low Hd and π were recorded across both regions, with diversity highest in the KNP *L. guthega* population (BHP: Hd = 0.419 ± 0.032, π = 0.00069 ± 0.00006; KNP: Hd = 0.582 ± 0.041, π = 0.00464 ± 0.00059). Analyses of historical population expansion/contraction using the mtDNA dataset revealed significant departures from neutral expectations in regards to the KNP population, supporting population

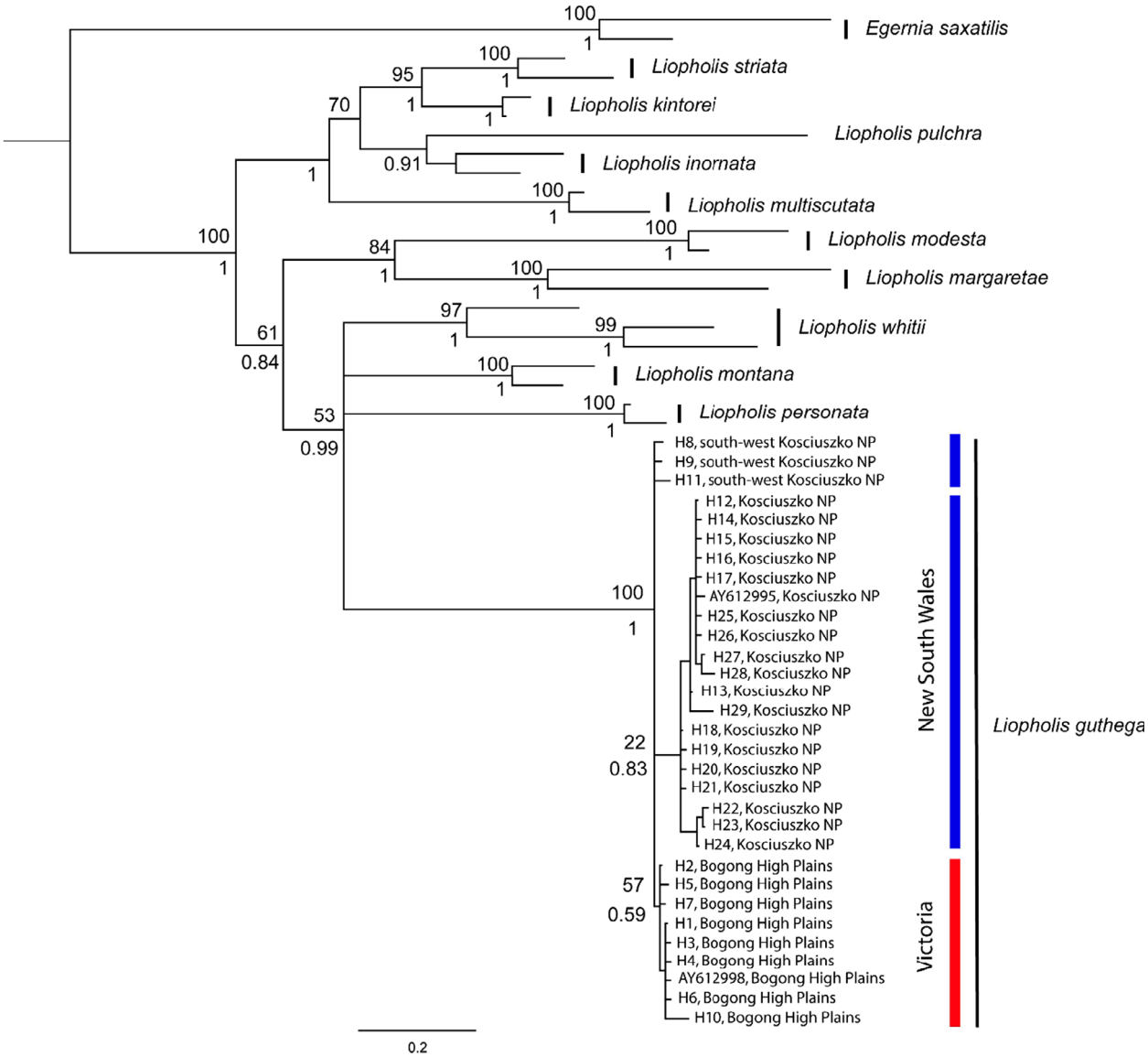

Figure 2 Bayesian topology depicting the inferred relationships among members of the genus *Liopholis*. ML and BI phylogenetic analyses based on 695 base pairs of the mitochondrial ND4 gene. MLbootstraps (above) and Bayesian posterior probabilities (below) are listed next to internal nodes. [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com]

expansion (Fu's Fs: −4.529, $P < 0.02$; Tajima's D: −1.80752, $P < 0.05$). The BHP population showed a negative, although non-significant, deviation from neutrality, suggesting relatively stable genetic diversity in VIC (Fu's Fs statistic: −3.039, $P > 0.05$; Tajima's D: −1.19361, $P > 0.10$). These patterns were supported by analysis of our SNP dataset which showed evidence of population expansion in KNP (Tajima's D: −2.727, $P = <0.01$) but no evidence for population expansion in the BHP, indicating a relatively stable population (Tajima's D: −0.693 ($P = >0.4$).

A total of 29 mtDNA haplotypes were identified, with 21 unique to KNP and eight unique to BHP (Fig. 3). Haplotypes were only shared by individuals from the same region. Within each region, haplotypes were shared among individuals from neighbouring colonies at a local scale (Fig. S2). However, three haplotypes (H8, H9, H11 – marked with an asterisk), only identified in two south-west *L. guthega* colonies in KNP (Southern Ramshead Range and Thredbo), formed a third grouping, each separated from the nearest (VIC) haplotype by a minimum of four mutations (one inferred/unsampled) (Fig. 3). These two colonies are over 5 km from the closest *L. guthega* individuals sampled in KNP main range, separated by the headwaters of the Snowy River (Fig. 5b).

Population structure

A total of 35 519 SNPs were obtained, with an average genotype call rate of 0.92, a scoring reproducibility of

99.6% and an average PIC of 0.095. Six thousand and seventy SNPs had a 100% genotype call rate, while 25 501 SNPs had a call rate of 95%. Three datasets were made using only SNPs > 95% call rate: VIC and NSW samples combined, VIC samples only and NSW samples only. After minor allele frequency (MAF = 0.005) filtering and associated genotype pruning, the total SNPs used in analyses were: VIC and NSW combined = 14 965, VIC only = 5837, NSW only = 14 444. Of these SNPs, <1.6% and <0.4% were reported as outliers from VIC and NSW respectively using two independent analyses. No SNPs were identified by both outlier detection analyses; therefore, none were discarded from our dataset.

Analyses of the full SNP dataset supported two distinct genetic clusters using both fastSTRUCTURE (K = 2; Fig. 4) and DAPC (Table S2). These corresponded to both allopatric sampling regions: VIC and NSW. All individuals were strongly assigned to a single genetic cluster (Q > 0.99) in fastSTRUCTURE, with the exception of the three southern-most populations in NSW [Colonies 30–32 (Southern Ramshead Range, Thredbo and Mt. Kosciuszko)], where admixture was evident and individuals averaged 84% assignment to the NSW cluster and 16% to the VIC cluster. Both G_{ST} and Jost's D between VIC and NSW were significantly different (G_{ST} = 0.595821, Jost's D = 0.185069), which also supports clear genetic differentiation between the two allopatric regions (P=<0.001).

Subsequent analysis of each genetic cluster revealed additional genetic subdivisions. Within VIC, four distinct clusters were supported by both fastSTRUCTURE (Fig. 4b) and DAPC (Fig. S3), with all individuals strongly assigned (Q > 0.90) to their respective population. For NSW, the greatest support using both fastSTRUCTURE (Fig. 4c) and DAPC (Fig. S4) was found for K = 3 (Table S2), with the three southern-most colonies (30, 31 and 32) assigned to a single cluster (Cluster 3; Q > 0.98) and the remaining NSW colonies split between two additional clusters (Fig. 4c). Cluster 1 comprised sites in the central part of the sampling area (colonies 12–20) and showed strong individual assignment (Q > 0.94), while cluster 2 included all the eastern colonies (colonies 21–29) and showed moderate levels of admixture (Q > 0.85; Fig. 4c).

Within VIC, the greatest admixture was evident in the cluster corresponding to the central region (colonies 3–9) and colony 10 (Fig. 4b; Fig. 5a). Clusters 3 (colony 10) and 4 (colony 11) each comprised a single colony separated by an alpine stream, while cluster 1 (colonies 1 and 2) comprised two colonies in the north-east region of the BHP (Fig. 5a). Greater admixture was evident between colonies in NSW (Fig. 4c; Fig. 5b). Cluster 1 comprised colonies 12–20 in the central main range region of KNP, which showed low-moderate admixture with cluster 2, encompassing colonies 21–29 in the north-east region. Cluster 2 showed evidence of gene flow with both cluster 1 and cluster 3. In contrast, cluster 3, located in swKNP (identified as having admixture with VIC), showed little admixture with other *L. guthega* colonies in KNP (Fig. 4c; Fig. 5b). Specifically, colonies 31 and 32 showed no admixture, suggesting low gene flow out of these sites and no gene flow into these colonies from other KNP regions.

Spatial patterns of genetic divergence within states

Among VIC individuals, a strong gradient in genetic signal was evident along latitude and longitude, with colonies that were further apart being more genetically differentiated (Fig. 6a). Analyses of Gst and Jost's also supported genetic

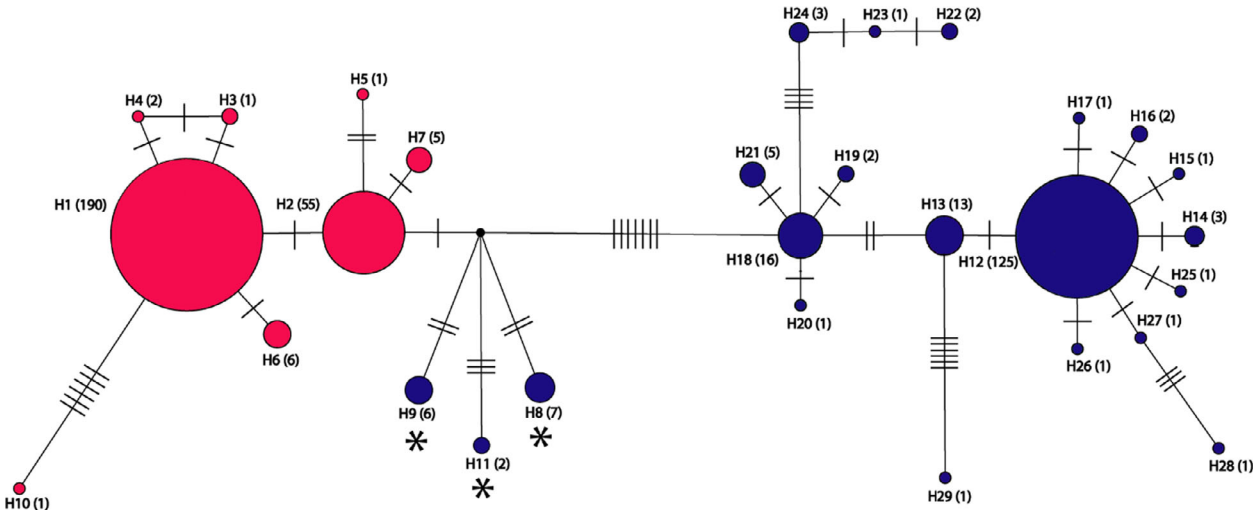

Figure 3 Statistical parsimony network of 29 *Liopholis guthega* mtND4 haplotypes of individuals from the Bogong High Plains (BHP; Red) and Kosciuszko National Park (KNP; Blue). Circles represent unique haplotypes (numbered H1-29), with size proportional to haplotype frequencies (*n* within parentheses). Line breaks on branches represent number of base pair differences between haplotypes and black circles denote inferred/unsampled mutation. The unique New South Wales haplotypes from the southern-most colonies in Kosciuszko National Park are marked with an asterisk (*). [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com]

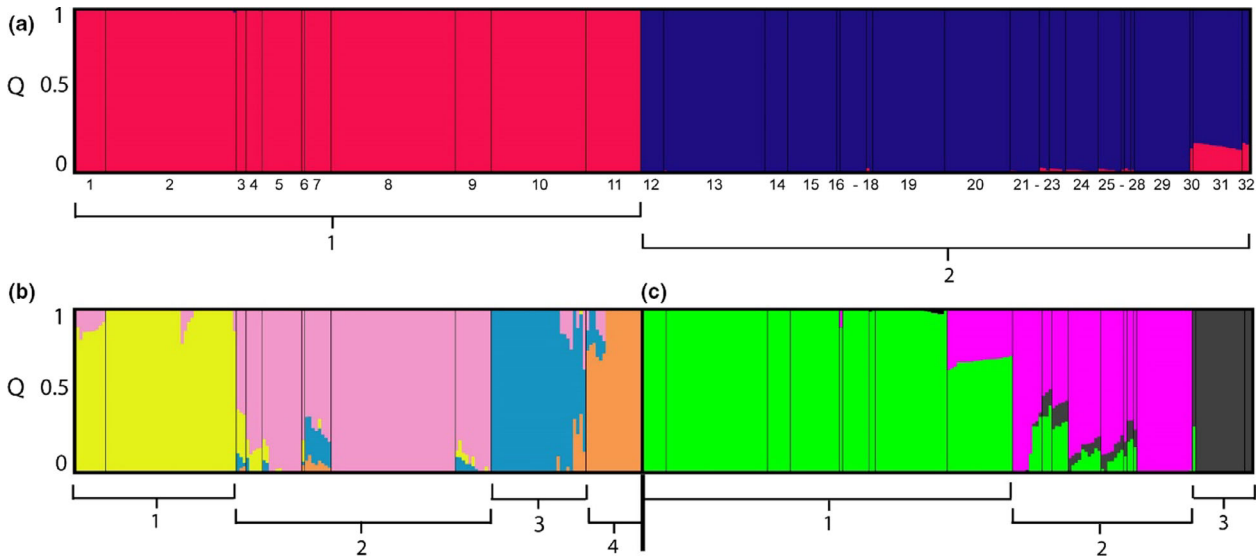

Figure 4 Individual proportion of membership (Q) for 32 *Liopholis guthega* colonies to each population cluster identified using fastSTRUCTURE. Results shown for (a) all colonies (VIC = Red; NSW = Blue), (b) VIC colonies only, (c) NSW colonies only. Different clusters denoted by different colours and numbered below. [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com]

structure among all VIC populations identified (1). The majority of individuals sampled from NSW showed evidence of isolation-by-distance, while the individuals collected from swKNP showed greater genetic differentiation from the remaining NSW population (Fig. 6b). G_{ST} and Jost's D also showed structure among NSW populations (1).

Individuals from NSW were found to have higher levels of heterozygosity than those from VIC (2), suggesting greater genetic diversity within NSW. Our analyses of heterozygosity among VIC populations identified by population structure showed that levels of heterozygosity were even among sites, although individuals from cluster one recorded the lowest average Ho and He values (3). Within NSW, we identified that individuals from the swKNP site (Cluster 3) had the highest levels of mean Ho and He, suggesting there is slightly higher genetic diversity at this site (3). This was also reflected by our estimates of effective population size (3). NSW populations were estimated to have greater effective breeding populations compared to VIC. Within VIC, cluster 3 had the lowest effective population size, ranging between as few as 9.3–15.9 breeding individuals.

The observed patterns of genetic structure at both VIC and NSW were confirmed by two independent AMOVAs. In VIC, 9.6% of the overall variation was observed among the four genetic clusters identified by STRUCTURE (AMOVA: $P = 0.001$), while variation among sites explained 8.9% (AMOVA: $P = 0.001$). The greatest variation (80.7%) was within sites (AMOVA: $P = 0.001$). No evidence of isolation-by-distance was recorded among VIC individuals ($P = 0.275$). Similarly, significant variation (10.8%) was identified among the three NSW genetic clusters identified by STRUCTURE (AMOVA: $P = 0.001$). The majority of variation (78.6%) was within sites (AMOVA: $P = 0.001$), while 7.2% of variation was explained among sites (AMOVA: $P = 0.008$). We found evidence of isolation-by-distance among NSW individuals, with genetic structure having a significant correlation with geographic distance ($P = <0.001$).

The estimated per cent sequence divergence between VIC and NSW *L. guthega* based on SNPs was 0.0277% [0.0227–0.0323 95% highest probability density (HPD)], while divergence between the KNP NSW and the swKNP region was estimated as 0.0073% (0.0005–0.0098 95% HPD) (Fig. S5).

Discussion

Here, we have shown significant genetic structure between two allopatric populations of an endangered alpine-endemic reptile. These results are consistent with previous estimates of Plio-Pleistocene divergence between populations from VIC and NSW carried out over a much smaller set of markers (Chapple *et al.*, 2005). These results suggest that the dispersal capability of *L. guthega* is restricted by habitat specificity and presence of natural and artificial barriers. At a finer scale, analyses of our SNP data support this finding, as we detected significant genetic structure among sampling localities within both states. Finally, we found evidence of significant genetic admixture between a previously unsampled new locality (swKNP, NSW; colonies 30–32) and their Victorian counterparts. Here, we found that individuals from swKNP were more similar to the VIC population based on mitochondrial DNA, but clustered strongly with the NSW in nuclear SNP-based analyses, with low levels of admixture with the VIC population evident. Our results therefore suggest that the swKNP population has experienced possible historical introgressive hybridization with the VIC population. Below we discuss the processes that may have led to these patterns as well as their implications for the conversation of this unique species.

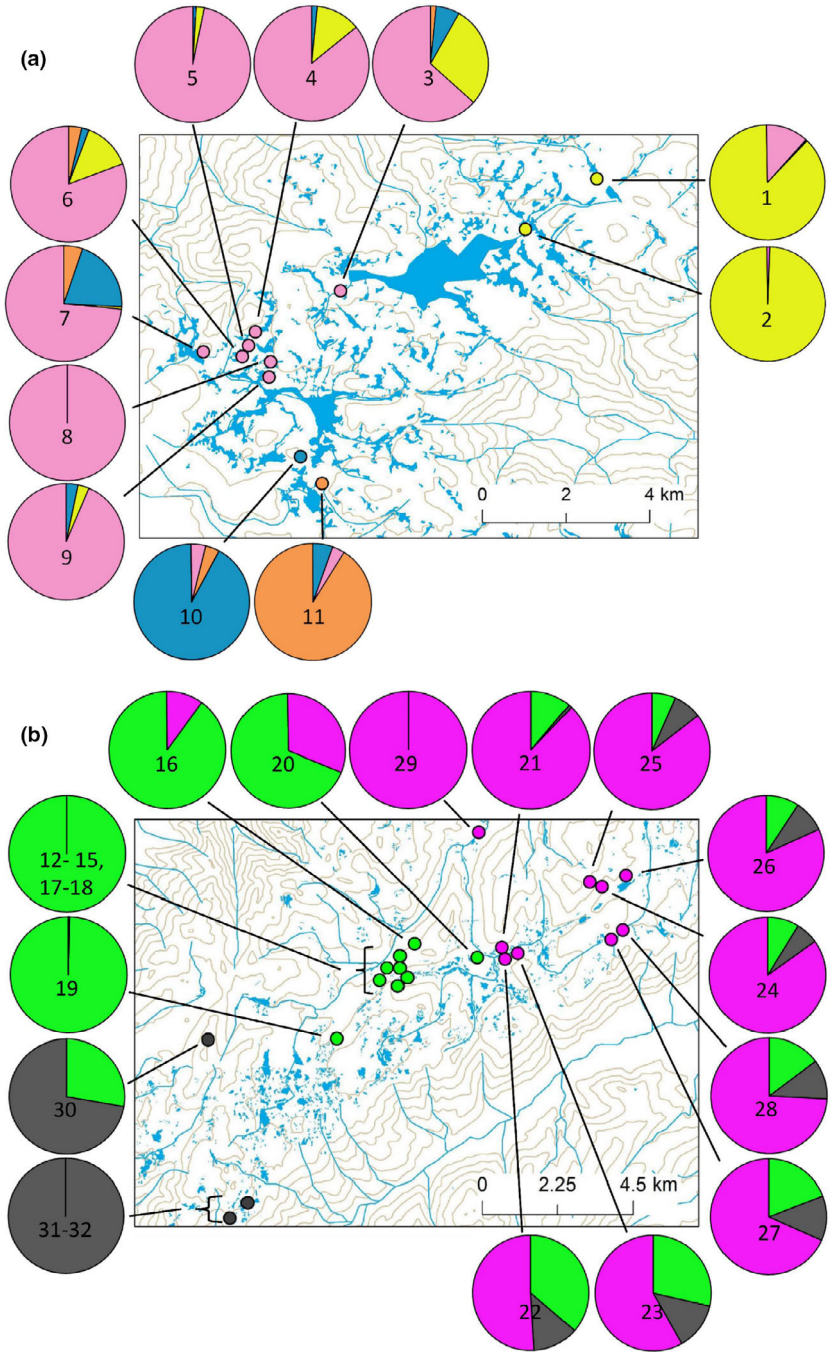

Figure 5 The location and admixture proportions of *L. guthega* colonies in A) Victoria and B) New South Wales, with colony colours based on population clusters identified by fastSTRUCTURE. Pie charts represent degree of genetic admixture between population clusters. Alpine bog systems/tributaries (blue), the headwaters of the Snowy River (dashed circle) and contour lines (grey) also presented. Note colonies 30–32 (grey) represent the swKNP population. [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com]

Phylogenetics

Our analyses demonstrate clear genetic structure between *L. guthega* from VIC and NSW. These findings mirror those on the genetic structure in other Australian alpine taxa (e.g. alpine she-oak skink, Koumoundouros *et al*., 2009; mountain pygmy possum, Mitrovski *et al*., 2007; several invertebrate species; Tatarnic *et al*., 2013; Slatyer *et al*., 2014). The modern-day allopatric distribution and genetic differentiation between these sky-island systems has likely been driven by

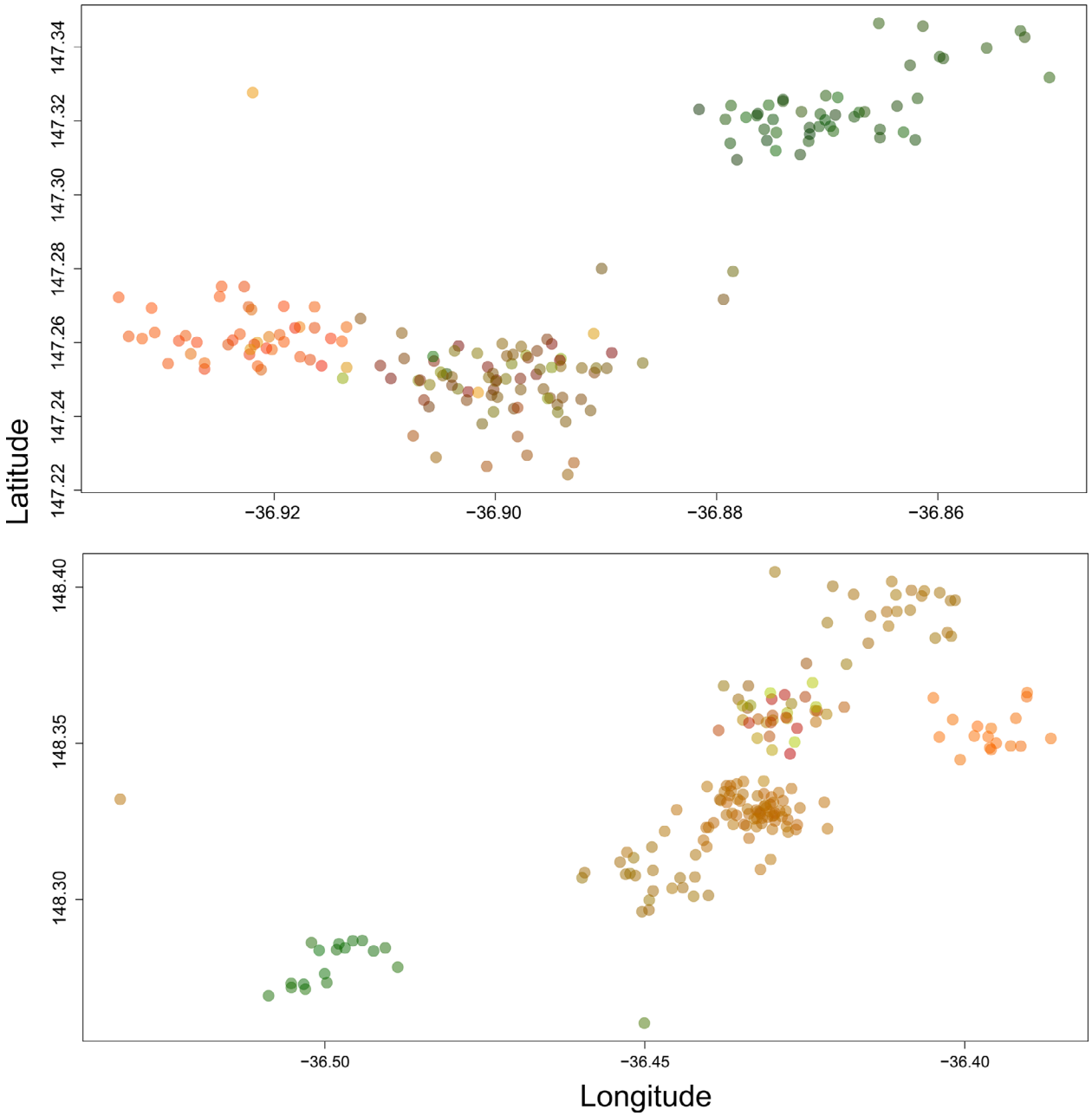

Figure 6 Spatial Principal Component Analysis of *Liopholis guthega* individuals sampled in (a) Victoria and (b) New South Wales. Individuals are plotted according to their spatial location. Individuals are coloured according to a relative scale for genetic differentiation (with extremes represented by green and orange). [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com]

past climatic cycles, as suggested for other Australian alpine endemic taxa (Hewitt, 2000; Chapple *et al*., 2005; Haines *et al*., 2017). A cooler climate during past Plio-Pleistocene glacial periods meant that alpine habitats existed at lower elevations. During this period, alpine regions were relatively expansive, allowing population continuity across a greater area than modern alpine habitats (Chapple *et al*., 2005; Byrne, 2008; Slatyer *et al*., 2014). A warming climate during more recent interglacial periods resulted in alpine habitats retreating to areas of high elevation, transforming the habitat of many taxa to sky-island systems, with unsuitable habitat and dispersal barriers inhibiting migration and gene flow (Chapple *et al*., 2005; Slatyer *et al*., 2014). As a result, highly structured populations, despite close geographical proximity, are a feature of Australian sky-island systems (Slatyer *et al*., 2014). The genetic structure between VIC

Table 1. Pairwise G_{ST} (below left) and Jost's D (above right), based on inferred STRUCTURE groups, among *Liopholis guthega* populations from the Bogong High Plains, Victoria (VIC) and Kosciuszko National Park, New South Wales (NSW)

VIC					
		1	2	3	4
	1	X	0.05328473*	0.04405980*	0.0899197*
	2	0.2299378*	X	0.03806154*	0.03178967*
	3	0.2160551*	0.1787850*	X	0.07721598*
	4	0.3580048*	0.1583999*	0.3088008*	X
NSW					
		1	2	3	
	1	X	0.05030121	0.03741632	
	2	0.2019325*	X	0.06038359	
	3	0.1766799*	0.2366985*	X	

*Population numbers refer to those obtained via fastSTRUCTURE analyses (see 4b). Asterisks correspond to a significance level of < 0.001 as measured via confidence intervals after 1000 permutations.

Table 2. SNP-based estimates of expected heterozygosity (He), observed heterozygosity (Ho) and inbreeding coefficient (Fis) among *Liopholis guthega* populations from the Bogong High Plains, Victoria (VIC), Kosciuszko National Park, New South Wales (NSW) and south-west Kosciuszko National Park, New South Wales (swKNP). Standard error is reported in parentheses

Population	He (±SE)	Ho (±SE)	Fis
Victoria	0.237 (±0.002)	0.223 (±0.002)	0.501
NSW	0.280 (±0.003)	0.253 (±0.002)	0.499
swKNP	0.276 (±0.003)	0.255 (±0.002)	0.507

and NSW populations in our study is consistent with a Plio-Pleistocene divergence (although we also find evidence for a subsequent hybridization event in swKNP – discussed later) and suggests the once connected lowland valleys separating these two regions are a contemporary barrier to dispersal for *L. guthega*.

We also found weak phylogeographic structure among *L. guthega* populations. Individuals from VIC and NSW formed two distinct clades, however, the support values on these clades were lower than a previous study investigating *L guthega* phylogenetics. (Chapple *et al*., 2005). Unlike Chapple *et al*. (2005) our study included individuals from an intermediate geographic locality (swKNP) which may have resulted in the *L. guthega* clade being unresolved. Although individuals from VIC and NSW formed distinct clades, those from swKNP formed an unresolved polytomy. We, therefore, can only be confident that all *L. guthega* individuals sampled belong to a single species/clade and place little weight on internal phylogenetic structure. Constructing phylogenies based on data from a single marker, especially mtDNA, may not be appropriate as the result can often reflect a gene tree rather than the true species tree (Edwards & Bensch, 2009; Toews & Brelsford, 2012). Our mtDNA-based divergence estimates suggest that the VIC and NSW *L. guthega* populations are less divergent [1.9% (0.7 –1.1 Mya)] than the 2.2% (1.1–1.7 Mya) reported by Chapple *et al*. (2005). The collection of more individuals (n = 452 vs. n = 5), across a wider geographical area has improved our understanding of the phylogeography of this species. The addition of sequences from the recently identified swKNP population shows that *L. guthega* populations separate into three clear sub-groups that correspond to three regions: BHP, KNP and swKNP NSW. These groups were further supported by our SNP-based divergence estimates, with greatest nucleotide divergence identified between the VIC and NSW populations and less sequence divergence between the two NSW regions. However, due to the absence of adequate calibration data, our divergence times should only be considered rough estimates.

Historic population connectivity

Our SNP-based analysis identified substantial admixture between *L. guthega* from the BHP and three sampling localities from swKNP, suggesting possible recent gene flow between these two regions. This is particularly interesting as the remaining colonies from KNP are much more distinct from BHP than the swKNP location. Paleoclimatic data suggest that approximately 25 000 years ago, during the height of the last Pleistocene glacial period, the winter snow line was ~400 m lower than what it is today (Barrows, Stone & Fifield, 2004). *Liopholis guthega* may have been able to survive at lower elevations and maintained a wider distribution at this time. With a more expansive alpine zone during this period, populations from the BHP and swKNP were likely sympatric and connected by a suitable montane habitat corridor, permitting migration and gene flow (Slatyer *et al*., 2014). This exact scenario was proposed by Mitrovski *et al*. (2007) to explain the genetic admixture between populations of the sympatric mountain pygmy possum and is well supported in the fossil record (Ride, 1956). Interestingly, mtDNA analyses suggests that individuals from swKNP are genetically more similar to those from VIC. Alternatively, analyses of our SNP dataset show that individuals from swKNP are most similar to other NSW individuals, however, a small amount of admixture is present with VIC. Differing signals between mtNDA and nuDNA are relatively common and in cases where nuclear data show greater levels of biogeographic structure, as we see here, hybridisation after isolation and secondary contact is often responsible (Toews &

Table 3. SNP-based estimates of expected heterozygosity (He), observed heterozygosity (Ho), inbreeding coefficient (Fis) and effective population size (Ne) of *Liopholis guthega*

	Cluster	He (±SE)	Ho (±SE)	Ne (95% CI) Lowest allele frequency 0.05	Ne (95% CI) Lowest allele frequency 0.01
VIC	1	0.174 (±0.002)	0.205 (±0.003)	18.3 (18.2-18.4)	41.6 (41.5-41.8)
	2	0.231 (±0.002)	0.234 (±0.002)	36.3 (36.2-36.5)	51.2 (51.0-51.3)
	3	0.198 (±0.002)	0.224 (±0.003)	9.4 (9.3-9.5)	15.9 (15.8-15.9)
	4	0.192 (±0.002)	0.211 (±0.002)	13.6 (13.5-13.7)	18.3 (18.2-18.5)
NSW	1	0.230 (±0.002)	0.239 (±0.002)	85.3 (85.2-85.4)	93.9 (93.8-94.1)
	2	0.266 (±0.002)	0.254 (±0.003)	39.0 (38.9-39.0)	48.7 (48.6-48.8)
	3	0.276 (±0.002)	0.255 (±0.002)	26.2 (26.1-26.3)	29.6 (29.4-29.7)

He and Ho standard errors (SE) and Ne confidence intervals (CI) are presented in parentheses. Values are based on inferred STRUCTURE groups, among populations from the Bogong High Plains, Victoria (VIC) and Kosciuszko National Park, New South Wales (NSW).

Brelsford, 2012; Amor *et al*., 2019). Furthermore, mtDNA is likely to reflect more recent patterns that nuDNA, especially considering our SNP data were found to be neutrally evolving. We therefore consider the genetic signal from our analyses of genome wide SNP data to reflect the historical population structure present within *L. guthega* and show that mtDNA is a useful marker for providing supporting evidence for more recent introgression events, in our case the secondary contact between VIC and NSW individuals at swKNP. The lack of evidence of hybridization between BHP and the remainder of the KNP suggest that a landscape feature may have been impeded dispersal and subsequent admixture (discussed below) or alternatively, that these differences between swKNP and KNP reflect historical separation into different refugia with more recent gene flow. Ultimately, more detailed sampling and a more complex quantitative approach to the data (i.e. Approximate Bayesian Computation framework analyses, see Yang *et al*., 2018) is needed to tease apart these explanations.

Population structure

Genetic structure was observed within the BHP and KNP, which suggests *L. guthega* has a limited dispersal ability. Geographic isolation coupled with limited dispersal is a feature of many Australian species that occur at high elevation (e.g. Mitrovski *et al*., 2007). Reduced dispersal capacity in reptiles is commonly associated with habitat specificity (Dubey & Shine, 2010) and has previously been documented in sympatric reptile species occurring at high elevation (Sato *et al*., 2014a; Haines *et al*., 2017). Indeed, *L. guthega* is a habitat specialist, found predominantly in granite outcrops, which permit the formation of extensive warren systems (Atkins *et al*., 2015).

The genetic structure observed within *L. guthega* correlates with the presence and distribution of bog systems and water bodies. Bog systems are a common feature of Australia's alpine environments, occurring in valley bottoms, along streams and drainage lines in waterlogged soils; they constitute~ 10% and 2.5% of the high elevation region of the BHP and KNP respectively (Williams & Costin, 1994). The presence of water bodies has been shown to be a barrier to dispersal for many reptile species (Smissen *et al*., 2013; Harradine *et al*., 2015) and the genetic distinction of colonies/clusters despite very close geographic proximity (<500 m) suggests that the presence of water bodies may affect movement of *L. guthega*. This is particularly evident in KNP, where the headwaters of the Snowy River and the associated bog systems separate the south-west (colonies 31–32) and central *L. guthega* population clusters (colonies 12–20) and is potentially a landscape feature that has restricted gene flow between these regions. The low levels of admixture between the south-west and more distant north-east colonies (21–29) suggest that movement has likely been facilitated by tracking the bog-free high elevation perimeter of the Ramshead Range. The lower population structure observed in KNP *L. guthega* is consistent with broader genetic studies on sympatric alpine taxa, attributed to the larger, more contiguous alpine habitat within this region (Koumoundouros *et al*., 2009; Slatyer *et al*., 2014). Additionally, the reduced density of bog systems within KNP in comparison to the BHP likely facilitates greater movement, and in turn admixture, of *L. guthega* within this region (OEH, 2012).

Genetic diversity and population demographics

Although genetic diversity varied between the two *L. guthega* regions, in both regions, it was low in comparison to studies on other Australian alpine reptiles [Alpine She-oak skink: $\pi = \sim 0.006$, Hd = $\sim$ 0.9 - Koumoundouros *et al*. (2009); Alpine Bog skink: $\pi = 0.0033–0.0219$, Hd = > 0.9 - Haines *et al*. (2017)]. One explanation for this is that within their sympatric range, the area occupied by *L. guthega* is smaller than that of these other two alpine lizards (Clemann *et al*. in prep.) due to their restriction to specific (and more limited) habitats (Atkins *et al*., 2015). Alternatively, the lower genetic diversity and effective population size identified in *L. guthega* in our study suggests that it may have experienced a greater bottleneck during earlier climate-mediated distributional shifts and may leave the species more susceptible to disturbance (Haines *et al*., 2017).

The higher levels of genetic diversity and greater size of the effective breeding population in KNP may be explained by historical introgressive hybridization (as SNP-based analyses indicated that swKNP had low levels of admixture with the Victorian population), elevation and associated habitat

availability. The Kosciuszko alpine region contains the continent's highest elevations (Mt Kosciuszko; 2228 m) and was the only region on Australia's mainland to experience glaciation during the Pleistocene glacial period (Ehlers *et al*., 2011). As a result, this region is believed to have formed a larger, more continuous habitat during these cool periods, as well as providing refuge for montane species during warmer interglacial cycles (Slatyer *et al*., 2014; Haines *et al*., 2017). Larger habitat size and connectivity favours higher genetic diversity, more likely avoiding genetic drift, reductions in population size, inbreeding depression and local extinctions more commonly associated with smaller, fragmented populations (e.g. Dubey & Shine, 2010). In contrast, the low genetic diversity at BHP is likely a product of its lower elevation and much smaller habitat size (total available areas of approximately 50 km^2; Williams & Ashton, 1987; vs 160 km^2 for the KNP, NSW Scientific Committee, 2017) although within these sky-islands, the specific habitat actually occupied by this species is a small fraction of this area (Clemann *et al*. in prep.). Small range size is the single best predictor of extinction risk for terrestrial species (Sekercioglu *et al*., 2008). Not only does *L. guthega* have a small, isolated range, it also has almost no scope for further uphill migration. The inability to disperse in the face of these pressures will leave *L. guthega* populations reliant on their ability to adapt, which is constrained by already low genetic diversity.

Conservation strategies and conclusions

The strong isolation and limited connectivity between sky-island systems identified in this study highlight how *L. guthega* and other sympatric alpine-endemics, have effectively had their populations cut in half following the recession of alpine habitats to modern-day high elevations. As many high elevation specialist species are at a high risk of extinction (e.g. Pereira *et al*., 2010), it is imperative that existing areas are protected to prevent further fragmentation. Effective management will be particularly important because warming temperatures will further constrain and fragment alpine species distributions (Laurance *et al*., 2011). Vulnerability will be exacerbated by novel trophic pressures arriving from lower elevations (Pickering, 2007), which ultimately threaten to disrupt species interactions and ecosystem processes (e.g. Fussmann *et al*., 2014). In the case of *L. guthega*, the two populations each warrant consideration as discrete Evolutionary Significant Units for conservation (Moritz, 1994); however, this may not be the best management strategy in the face of threating processes (Harrisson *et al*., 2016). Managed human-assisted gene flow could be implemented in order to boost genetic diversity and adaptive potential. Such 'genetic-rescue' is currently being undertaken on the sympatric mountain pygmy-possum, with considerable success (Weeks *et al*., 2017). Improving adaptive potential, in conjunction with conservation initiatives like habitat supplementation (e.g. Goldingay & Newell, 2017), may increase habitat availability and connectivity, while simultaneously enhancing population viability. The incorporation of multiple conservation strategies is vital to mitigate climate change impacts on alpine species. However, further assessing how evolutionarily distinct these regions are and evaluating the potential risks of outbreeding depression (as per criteria outlined in Frankham *et al*., 2011), is an important step in formulating such management strategies. Thus, we suggest genetic trials be thoroughly investigated in captivity before genetic rescue is employed *in situ*.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. GenBank Accession numbers and locality data for taxa used in this study.

Figure S1. Maximum likelihood depicting the inferred relationships among members of the genus Liopholis based on 696 base pairs of the mitochondrial ND4 gene. Haplotype locality information and GenBank Accession numbers available in Table S1.

Figure S2. Statistical parsimony network of 29 Liopholis guthega mtDNA haplotypes with sites coloured based on population clusters identified by fastSTRUCTURE (Fig. 4).

Table S2. Marginal likelihood (lnL) and BIC values for structure runs (K1-10).

Figure S3. Discriminant Analysis of Principal Components based on 5837 SNPs. Four genetic clusters composed of L. guthega individuals sampled within Victoria, Australia were identified.

Figure S4. Discriminant Analysis of Principal Components based on 14 444 SNPs.

Figure S5. Percentage divergence estimate of the three identified Liopholis guthega populations within the SNP dataset using SNAPP BEAST 2.0.