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ORIGINAL ARTICLE

Plio-Pleistocene diversification and genetic population structure of an endangered lizard (the Blue Mountains water skink, *Eulamprus leuraensis*) in south-eastern Australia

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ABSTRACT

Aim Although climatic fluctuations occurred world-wide during the Pleistocene, the severity of glacial and drought events – and hence their influence on animal and plant biogeography – differed among regions. Many Holarctic species were forced to warmer-climate refugia during glacial periods, leaving the genetic signature of recent expansion and gene flow among modern-day populations. Montane south-eastern Australia experienced less extreme glaciation, but the effects of drier and colder climatic conditions over this period on biotic distributions, and hence on the present-day genetic structure of animal and plant populations, are poorly known.

Location South-eastern Australia.

Methods The endangered Blue Mountains water skink (*Eulamprus leuraensis*) is a viviparous lizard known from fewer than 40 isolated small swamps at 560–1060 m elevation in south-eastern Australia. We conducted molecular phylogenetic, dating and population genetics analyses using the mitochondrial NADH dehydrogenase 4 (*ND4*) of 224 individuals of *E. leuraensis* sampled across the species' distribution.

Results Ancient divergences in haplotype groups between lizards from the Blue Mountains and the Newnes Plateau, and strong genetic differences, even between swamps separated by only a few kilometres, suggest that the species has persisted as a series of relatively isolated populations within its current distribution for about a million years. Presumably, habitat patches similar to current-day swamps persisted throughout glacial–interglacial cycles in this region, allowing the development of high levels of genetic structuring within and among present-day populations.

Main conclusions Our results suggest that less extreme glacial conditions occurred in the Southern Hemisphere compared with the Northern Hemisphere, allowing cold-adapted species (such as *E. leuraensis*) to persist in montane areas. However, additional studies are needed before we can assemble a comprehensive view of the impact of Pleistocene climatic variation on the phylogeography of Southern Hemisphere taxa.

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Keywords

Australia, montane species, *ND4*, phylogeography, Pleistocene climate variation, refugia, reptile, Scincidae, spatial structure.

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INTRODUCTION

World climates have undergone major variation over the periods during which current-day species have originated and diversified, and understanding the impact of such climatic fluctuations can provide important insights into current species distributions. For example, many cool-climate areas have experienced long periods of glaciation, drastically reducing the availability of habitats able to sustain plants and animals (e.g. Taberlet *et al.*, 1998). Hence the present-day occurrence of biota in such areas must reflect either post-glacial colonization from some relatively distant warmer-climate area, or expansion from fragmented refugia spread across the previously glaciated region (Taberlet *et al.*, 1998; Hewitt, 2000, 2004a,b; Bhagwat & Willis, 2008). Extensive research on Northern Hemisphere (Holarctic) taxa reveals a general pattern of southern refugia during periods of unfavourable conditions and northerly recolonization during periods of favourable conditions (Hewitt, 2000, 2004a,b). In contrast to this well-studied situation, potential impacts of glaciation on species from the Southern Hemisphere are poorly understood. Within Australia, for example, the most detailed DNA-based analyses of historical biogeography come from tropical rather than high-latitude areas (e.g. the Wet Tropics of north-eastern Australia; Hugall *et al.*, 2002; Moritz, 2002; Hewitt, 2004a,b).

In south-eastern Australia, widespread forests were replaced with more open vegetation at the end of the Pliocene (2 Ma; Bowler, 1982; Markgraf *et al.*, 1995; Gallagher *et al.*, 2001, 2003; Byrne *et al.*, 2008). During this period the climate fluctuated between cool–dry and warm–wet, with a general cooling–drying trend during the Pliocene, and intensified climatic oscillations during the Upper Pliocene to Lower Pleistocene (0.7–2 Ma), reaching a maximum during the Upper Pleistocene (0.7–0.01 Ma; Bowler, 1982; Frakes *et al.*, 1987; Markgraf *et al.*, 1995; Gallagher *et al.*, 2003; Byrne *et al.*, 2008). Although we might expect such environmental variation to induce multiple cycles of expansion, contraction and fragmentation of the temperate biota in south-eastern Australia (Bowler, 1982; Markgraf *et al.*, 1995), palynological data suggest that the impact of climatic variation on the vegetation was weaker than in the Northern Hemisphere due to factors such as the lower amplitude of variations, the absence of large ice sheets, and the failure of full-glacial environments to persist through interglacial periods (Markgraf *et al.*, 1995).

Nonetheless, those glacial–interglacial cycles may have had significant impacts on the fauna of south-eastern Australia. For example, Chapple *et al.* (2005) documented a deep phylogeographical break in lizards of the *Egernia whitii* group during the late Miocene–Pliocene, with structuring within lineages consistent with the effects of Plio-Pleistocene glacial–interglacial cycles. Similar population structuring has also been reported in frogs (e.g. the *Litoria citropa* species group – Donnellan *et al.*, 1999; *Crinia signifera* – Symula *et al.*, 2008; *Limnodynastes* – Schaüble & Moritz, 2001).

Molecular phylogeographical analyses thus have proved useful in clarifying historical patterns of expansion and contraction in the temperate fauna of south-eastern Australia. In addition, phylogeographical studies can reveal cryptic species and clarify historical processes responsible for present-day patterns of genetic variation. Results from these analyses also can be used to identify distinct genetic units that warrant separate conservation (King, 2009). Management decisions also need to account for population-level processes contributing to local adaptation (Crandall *et al.*, 2000; McKay & Latta, 2002; King, 2009).

The Blue Mountains water skink (*Eulamprus leuraensis* Wells and Wellington, 1984) is a medium-sized (to 20 cm total length) viviparous scincid lizard. It is restricted to montane areas west of Sydney in south-eastern Australia. The species is known from fewer than 40 isolated small swamps at 560–1060 m elevation, distributed in two distinct patches separated by about 20 km (in the Blue Mountains and Newnes Plateau; Fig. 1). This species is classified as 'endangered' under the Threatened Species Conservation Act (NSW, 1995) and the Environmental Protection and Biodiversity Conservation Act (Commonwealth, 1999), on the basis that it is an ecological specialist, with severely reduced populations subject to substantial ongoing threats (e.g. some areas of suitable habitat have been severely affected by urbanization: NSW National Parks & Wildlife Service, 2001a). Because the distribution of the species is strictly limited to montane areas, it may be under significant risk from global climate change. Models of climatic variation predict that the area inhabited by *E. leuraensis* will become both warmer (by up to 5 °C) and drier (by up to 40%) within the next century (http://www.climatechangeinaustralia.gov.au). Such changes might affect both the skink's habitat (e.g. reduced rainfall and thus seepage might dry out the hanging swamps) and the lizard itself, as well as rendering these sites vulnerable to further increases in the frequency and intensity of fire. Despite these threats and its 'endangered' classification, the ecology of *E. leuraensis* is poorly known (Shea & Peterson, 1985; LeBreton, 1996), and its genetic diversity and population-level differentiation have never been studied. An understanding of these topics can provide a valuable basis from which to manage the remnant populations, as well as providing significant insights into the biogeography of south-eastern Australian taxa.

In the present study we analysed samples of *E. leuraensis* with a mitochondrial marker in order to: (1) estimate the impact of the Plio-Pleistocene climatic variations on the genetic structure within the species, and (2) identify distinctive genetic units that might warrant separate management. We also examined morphological data in order to test for concordance between genetic and morphological divergence.

MATERIALS AND METHODS

Tissue sampling and DNA extraction

We collected tissue samples of 238 individuals of *E. leuraensis* from November 2008 to April 2009 across the known geographical distribution of the species, involving 13 popula-

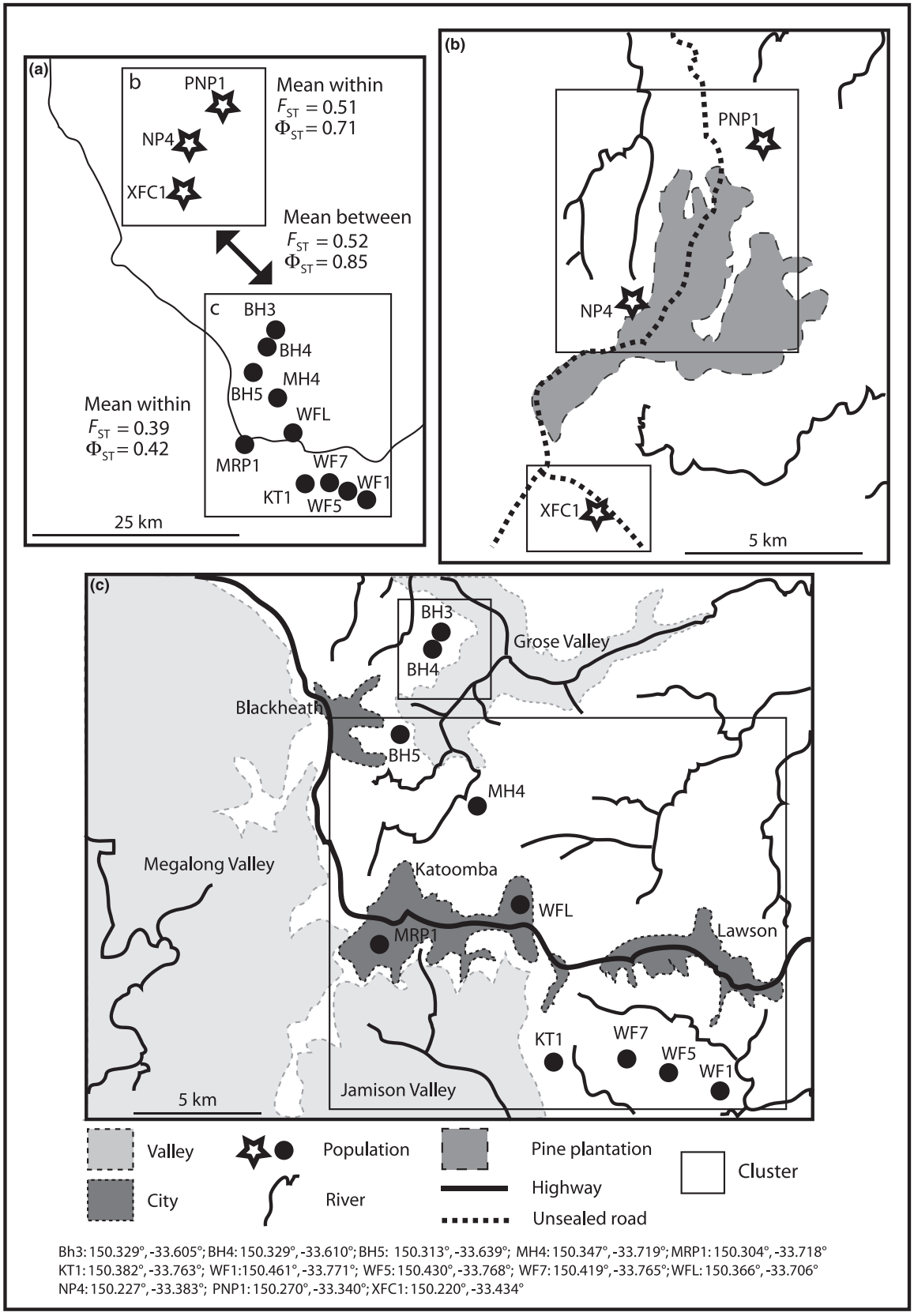

Figure 1 Sampled populations (with latitudes and longitudes in decimal degrees) of the Blue Mountains water skink (*Eulamprus leuraensis*) across its entire known geographical range (a); in the Newnes Plateau (b); and in the Blue Mountains (c), with mean F_{ST}- and Φ_{ST}-values within the Newnes Plateau and the Blue Mountains, and between the two areas, and major clusters inferred from SAMOVA.

tions. The populations sampled were selected to cover as much as possible of the species' distribution and consequently to be representative of genetic diversity. Three of the 10 known populations in the Newnes Plateau were sampled (coded as NP4, PNP1, XFC1; Fig. 1), as were 10 of the < 30 populations reported from the Blue Mountains (BH3, BH4, BH5, KT1, MH4, MRP1, WF1, WF5, WF7, WFL). Consequently, we were able to sample about 30% of the known populations within each region.

The animals were captured with funnel traps and pitfall traps, which caught individuals of all size classes and an approximately equal sex ratio (121 females:101 males), plus 16 animals of undetermined gender. Preliminary analyses revealed no significant differences between the two trapping methods in the gender or body size of animals collected (results not shown). The number of animals caught in each site varied from five (WF1) to 39 (XFC1), based on trapping sessions of about 6 days with favourable weather conditions per population. Thus, the variation in number of samples collected per population probably reflects spatial variation in population size. Each individual was sexed and measured soon after capture. Total cellular DNA was isolated from small tail clips, and tissues were placed in 200 μL of 5% Chelex containing 0.2 mg mL^{-1} proteinase K, incubated overnight at 56 °C, boiled at 100 °C for 10 min, and centrifuged at 13,300 *g* for 10 min. The supernatant, containing purified DNA, was removed and stored at −20 °C.

DNA amplification

Double-stranded DNA amplifications of NADH dehydrogenase 4 (*ND4*) were performed with the primer pairs ND4Leu (5′-GTCCTAGCAGCTATTTTACT-3′)/LEULeu (5′-CCTAAGGCTAACGGATAGAC-3′; developed for this study). Amplification conditions included a hot start denaturation of 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 50 °C annealing temperature for 45 s, 72 °C for 90 s, and a final extension of 72 °C for 7 min. Amplified products were genotyped with a 3130 xl Genetic Analyzer (Applied Biosystems) using BioEdit (Hall, 1999).

Phylogenetic analyses

We aligned sequences by eye. Tests were conducted on the total fragments (790 bp); all codon positions were used. Additional sequences of closely related species within the *Eulamprus quoyii* group (water skinks; Skinner, 2007) were included within our analyses (*Eulamprus kosciuskoi*, DQ915340; *E. quoyii*, AY169660; and *Eulamprus heatwolei*, AY520462), as well as different species of the infraorder Scincomorpha. The latter group included two species of Lacertidae (*Lacerta viridis*, AM176577; *Takydromus takydromoides*, AB080237), one species of Xantusiidae (*Lepidophyma flavimaculatum*, AB162908), one species of Cordylidae (*Cordylus warreni*, AB079613), and one species of Scincidae (*Eumeces egrerius*) as in Albert *et al.* (2009; see Molecular dating section for more details). All the non-*Eulamprus* species were used as outgroups. Maximum parsimony (MP) analyses were performed using PAUP* 4.0b10 (Swofford, 2001) with 100 random additions of sequences followed by tree bisection and reconnection (TBR) branch-swapping, and retaining at most 100 trees at each replicate. Branch support was estimated using 1000 bootstrap resamples using the same heuristic settings. For maximum likelihood (ML), models of DNA substitution were selected using jMODELTEST 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). The TIM1 + G model (Posada, 2003) best fitted the dataset with Akaike's information criterion (AIC; Lset base = 0.3583 0.3014 0.0955 0.2494; nst = 6; rmat = 1.0000 7.8098 0.5603 0.5603 5.3869 1.0000; rates = gamma; shape = 0.3480).

We performed ML heuristic searches and bootstrap analyses (1000 replicates) using PHYML (Guindon & Gascuel, 2003). Bayesian analyses (BA) were conducted with the GTR model (N_{ST} = 6), using MRBAYES ver. 3.1.2.1 (Huelsenbeck *et al.*, 2001). Two independent runs were performed, each consisting of four parallel Markov chain Monte Carlo (MCMC) chains of 3 million generations, allowing a good convergence of the independent runs (the average standard deviation of split frequencies being lower than 0.01). Trees were sampled every 100 generations. Burn-in was assessed by comparing the mean and variance of log likelihoods, both by eye and using the program TRACER ver. 1.4 (Rambaut & Drummond, 2007). Tree parameters reached stationarity after a burn-in period of 300,000 generations. Optimal trees were then sampled every 100 generations to obtain the final consensus BA tree and associated posterior probabilities.

Molecular dating

Because our study taxa evolved at equal rates (likelihood ratio test: $\Delta \ln L$ = 39.8, d.f. = 38, $P > 0.05$ n.s.), we applied a strict molecular clock for the estimation of divergence times in BEAST 1.4 (Drummond & Rambaut, 2006) and a coalescent tree prior (adequate to study intraspecific diversification: Drummond *et al.*, 2007). In addition, the standard deviation of the uncorrelated lognormal relaxed clock (ucld.stdev parameter) was < 1 with a frequency histogram abutting 0, hence failing to reject a strict molecular clock (Drummond *et al.*, 2007).

Because no reliable calibration points are available for Australian skinks, we used secondary calibration points from the recent study by Albert *et al.* (2009) on the phylogeny of squamate reptiles. That study was based on complete mitochondrial genome data and 12 reliable internal time constraints based on fossil data, and two different molecular dating methods (BA, Bayesian and PL, penalized likelihood). Consequently, to calibrate our molecular dating, we included in our dataset members of the Scincomorpha infraorder (including scincid lizards) as used by Albert *et al.* (2009): two Lacertidae (see phylogenetic analyses above), one Xantusiidae, one Cordylidae, and one Scincidae. As secondary calibration points, we used the divergence dates estimated from the BA

and PL analyses: that is, between (1) the two Lacertidae [BA: 61 Ma (95% highest posterior density, HPD: 81–41); PL: 70 Ma (95% HPD: 77–63)]; (2) Xantusidae and Lacertidae [BA: 140 Ma (95% HPD: 165–115); PL: 162 Ma (95% HPD: 174–150)]; (3) Cordylidae and Scincidae [BA: 168 Ma (95% HPD: 190–146); PL: 177 Ma (95% HPD: 187–167)]; and (4) the four families [BA: 190 Ma (95% HPD: 215–165); PL: 203 Ma (95% HPD: 213–193)].

The analysis was performed with two independent chains and 20 million generations for calibration based on BA and PL from Albert *et al.* (2009); chains were sampled every 1000 generations with a burn-in of 2 million generations. Additional simulations were run with the same dataset and the same models, but strictly based on two different plausible rates of divergence (1.3% and 2.3% Myr^{-1}) derived from other studies on reptiles. The lower limit (1.3%) was derived from Zamudio & Greene's (1997) work on viper mtDNA and from Macey's *et al.* (1999; also used in Torres-Carvajal & de Queiroz, 2009) work on anguid lizards. The upper limit (2.3% Myr^{-1}) was based on the work of Brown & Pestano (1998) and Brown *et al.* (2008) from Canary Island skinks and Balearic Islands lacertids (mean rate of 2.05% Myr^{-1}).

Population genetic analyses

We conducted four types of analysis to test the hypothesis of recent population growth from low-diversity founder populations within each of the two allopatric areas where this taxon is found (the Newnes Plateau and Blue Mountains), as well as within all the populations. Three methods were implemented in Arlequin (Excoffier *et al.*, 2005) and one in DnaSP (Rozas *et al.*, 2003). The first method, Ramos-Onsins & Rozas's (2002) R_2-statistic, is based on the difference between the number of singleton mutations and the average number of nucleotide differences. Lower values of R_2 are expected under a scenario of recent population growth. The second method, Fu's (1997) F_S-statistic, tests the probability of having no fewer than the number of observed alleles in the sample, given that q (heterozygosity per sites) = p. This statistic tends to be negative when there is an excess of recent mutations (or rare alleles). The third method, Tajima's (1989) D-statistic, tests the null hypothesis that two estimates of the neutral mutation parameter, one derived from the average number of pairwise nucleotide differences and the other based on the number of segregating sites in the sample, are equal. In the fourth test, pairwise mismatch distributions among individuals were plotted and tested for goodness-of-fit against a model of sudden expansion using parametric bootstrapping with 1000 replicates (Schneider & Excoffier, 1999).

Population structure across the study area and between all sampling sites was assessed by calculating Φ_{ST}, which takes into account haplotype frequencies and the genetic distance between haplotypes in Arlequin (Excoffier *et al.*, 1992). For the genetic model, we used the Kimura two-parameter genetic distance (K2P; Kimura, 1980). We also calculated population structure by means of F-statistics (F_{ST}; Wright, 1951) using only haplotype frequencies. Significance values for the two methods of computation of population structure were obtained after 1000 permutations.

To test for isolation by distance (IBD), we calculated the correlation between genetic and geographical distances for all populations using a Mantel test in Arlequin (Excoffier *et al.*, 2005).

Spatial analysis of molecular variance using the program samova 1.0 (Dupanloup *et al.*, 2002) was used to characterize population structure and to define groups of populations using genetic criteria. Given an *a priori* number of groups (K), samova uses a simulated annealing procedure to define the group composition in which populations within a group are as genetically homogeneous as possible (F_{SC} minimized) and groups are maximally differentiated from each other (F_{CT} maximized). The analysis was run for $K = 2$ to $K = 12$ and the significance of fixation indices was tested by 1000 permutations.

The method of statistical parsimony (Templeton *et al.*, 1992), implemented in tcs ver. 1.21 (Clement *et al.*, 2000), first defines the uncorrected distance (p) above which there is a > 5% probability that the parsimony criterion is violated (parsimony limit). All connections are then established among haplotypes, beginning with the smallest distances and ending either when all haplotypes are connected or when the distance that corresponds to the parsimony limit has been reached.

Morphology

We captured a total of 238 individuals, determined their genders (by hemipenal eversion), and measured the length of the head, front limbs and rear limbs, interlimb distance, and snout-to-vent length (SVL). Neonates ($n = 16$) were not included in the morphological analyses. We used jmp ver. 7.0 (SAS Institute, 2007) to perform multivariate analysis of covariance (MANCOVA) with gender and location (Blue Mountains or Newnes Plateau) as factors, SVL as the covariate, and morphological traits (length of head, front limbs and rear limbs, and interlimb distance) as dependent variables, as well as ANCOVA on each morphological trait, with the same design.

RESULTS

Phylogenetic analyses

Of the 238 samples of *E. leuraensis* analysed, we obtained reliable sequences for 224 samples. The 224 sequences showed 31 different haplotypes (H1–H31; GenBank accession numbers GU046454–GU046485) of 790 bp. The entire dataset (including outgroups) contained 473 variable sites, of which 336 were parsimony-informative. No insertions or deletions were observed within *E. leuraensis*, and no haplotypes were shared between lizards from the Newnes Plateau versus the Blue Mountains. As the three phylogenetic methods yielded similar arrangements of the main branches, Fig. 2 shows the

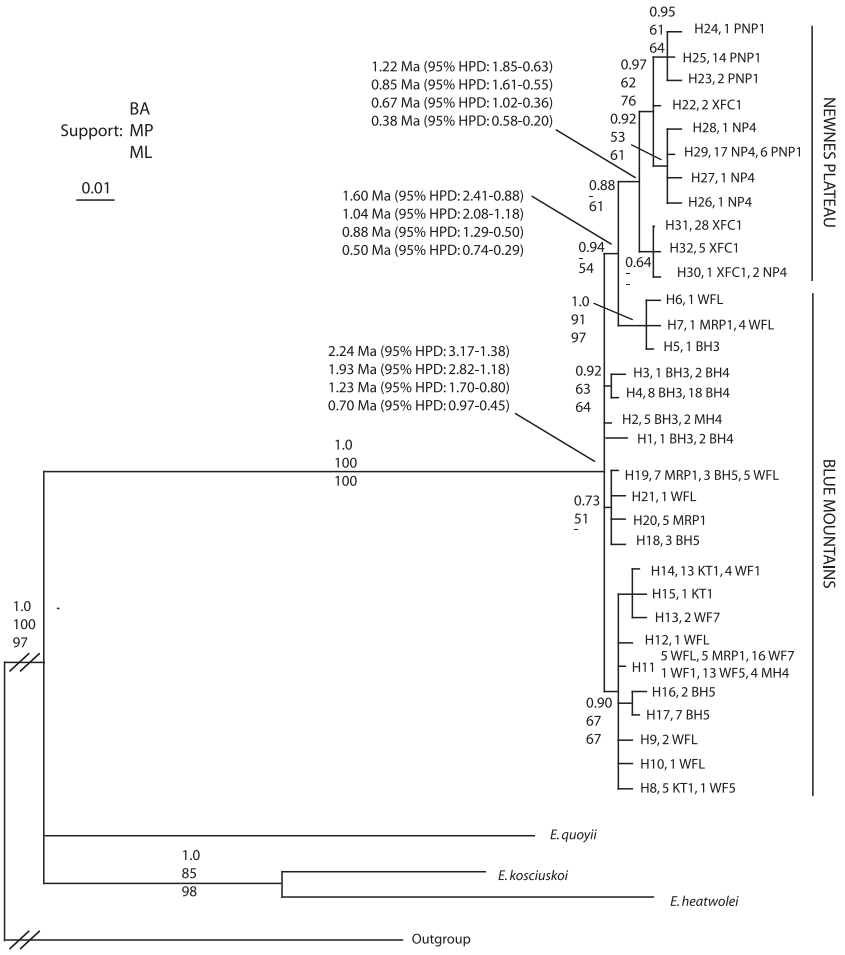

Figure 2 Phylogeny of the *ND4* fragment of *Eulamprus leuraensis* in southern Australia analysed using a Bayesian analysis (BA) procedure. Values in branches are indices of support for the major branches for BA, maximum likelihood (ML) and maximum parsimony (MP) analyses (percentage of 1000 replications for ML and MP; posterior probabilities based on 3,000,000 generations for BA), and dating of the major splits in Ma from the BEAST analyses, with (i) secondary calibration points from Albert *et al.* (2009) respectively PL-based and BA-based, and (ii) the divergence rates of 1.3 and 2.3% Myr^{-1}, respectively. For clarity, only the relationships between *Eulamprus* taxa are shown.

relationship between haplotypes only for the BA analysis. The genus *Eulamprus* as well as the samples of *E. leuraensis* formed a monophyletic unit (support values for ML, MP, BA: 97, 100, 1.0 and 100, 100, 1.0, respectively).

The Newnes Plateau populations formed a monophyletic unit (support values for ML, MP, BA: 61, < 50, 0.88), sharing no haplotypes with the Blue Mountains populations. Nevertheless, all the haplotypes of Newnes Plateau populations were nested within those of the Blue Mountains animals, with the haplotypes H5–H7 of the Blue Mountains skinks (present at very low frequencies in populations BH3, MRP1 and WFL) being basal to those of lizards from the Newnes Plateau (support values for ML, MP, BA: 54, < 50, 0.94).

The mean K2P between *E. leuraensis* and its congeneric species (*E. kosciuskoi*, *E. heatwolei* and *E. quoyii*) was 16.9%. The mean distance between all the haplotypes of *E. leuraensis* was 0.8% (mean number of differences: 6.16), and between the Newnes Plateau and Blue Mountains haplotypes, 1.1% (mean number of differences: 8.36). The mean distance within the Newnes Plateau and Blue Mountains was, respectively, 0.4 (mean number of differences: 3.23) and 0.6% (mean number of differences: 4.5). Nucleotide and gene diversity, and the mean number of pairwise differences within populations, respectively, varied from 0.0005 (WF1) to 0.034 (BH5); from 0.143 (WF5) to 0.842 (WFL); and from 0.14 (WF5) to 3.93 (WFL).

Molecular dating

The dating analyses suggested an initial divergence within *E. leuraensis* about 2.24 Ma (95% HPD: 3.17–1.38; with PL-based calibration points from Albert *et al.*, 2009) to 1.93 Ma (95% HPD: 2.82–1.18; with BA-based calibration points from Albert *et al.*, 2009; Fig. 2), and about 1.23 Ma (95% HPD: 1.70–0.80; with a divergence rate of 1.3% Myr^{-1}) to 0.70 Myr (95% HPD: 0.97–0.45; with a divergence rate of

2.3% Myr^{-1}; the results of dating will be in the same order below). A subsequent split between H5 to H7 and haplotypes of the Newnes Plateau (H22–H32) occurred from 1.60 Ma (95% HPD: 2.41–0.88) to 1.04 Ma (95% HPD: 2.08-0.78) and from 0.88 Ma (95% HPD: 1.29–0.50) to 0.50 Ma (95% HPD: 0.74–0.29). The split between haplotypes within the Newnes Plateau occurred from 1.22 Ma (95% HPD: 1.85–0.63) to 0.85 Ma (95% HPD: 1.61–0.55) and from 0.67 Ma (95% HPD: 1.02–0.36) to 0.38 Ma (95% HPD: 0.58–0.20).

Population genetic analyses

The F_{ST} and Φ_{ST} between populations varied respectively from 0 (WF5–WF7) to 0.747 (WF5–BH4; overall mean = 0.441) and from 0 (KT1–WF1) to 0.914 (WF5–PNP1; overall mean = 0.596; see Table 1). F_{ST} and Φ_{ST} were, respectively, 0.507 and 0.711 within the Newnes Plateau, 0.385 and 0.417 within the Blue Mountains, and 0.517 and 0.853 between lizards from the Newnes Plateau versus the Blue Mountains.

The SAMOVA analysis revealed high F_{CT}-values (among population groups) for all the groups and small F_{SC}-values (within population group) in cases of nine to 12 groups, indicating very high population structure. For example, at $K = 9$ the majority of variation (74.87%) is among groups, although 0.43% of variation at the level of among populations within groups still represents highly significant population structuring in the remaining population groups ($P < 0.001$). At $K = 2$ (highest F_{ST} and F_{SC}), the two clusters identified were the populations of the Blue Mountains versus the Newnes Plateau.

The Mantel tests were significant when considering all the populations (F-statistic-based: $R^2 = 0.43$, $P = 0.0011$; Φ_{ST}-based: $R^2 = 0.78$, $P < 0.0001$), as well as within the Blue Mountains (F-statistic-based: $R^2 = 0.43$, $P = 0.0088$; Φ_{ST}-based: $R^2 = 0.60$, $P < 0.0002$).

The statistical parsimony network again revealed two distinct groups: the Newnes Plateau and the Blue Mountains populations (Fig. 3). Significant reticulation was apparent within the Blue Mountains, with two distinct haplogroups. The smaller of these groups included a total of three haplotypes (H5, H6, H7) and seven samples (1 MRP1, 5 WFL, 1 BH3).

We obtained non-significant P-values for the mismatch distribution test of goodness-of-fit for the Newnes Plateau (raggedness index = 0.13, $P = 0.08$) and Blue Mountains (raggedness index = 0.03, $P = 0.61$), with the frequency distribution of the mean pairwise difference between haplotypes showing multimodal distributions (Fig. 4). Fu's F_S-statistics were negative, and the P-value was non-significant for the Newnes Plateau (NP) and marginally significant for the Blue Mountains (BM) (NP: $F_S = -0.78$; $P = 0.391$; BM: $F_S = -5.89$; $P = 0.05$). Tajima's D-values were positive for the Newnes Plateau and negative for the Blue Mountains, but non-significant (NP: $D = -0.98$; $P = 0.15$; BM: $D = 0.38$; $P = 0.69$). In addition, the R_2-statistics were non-significant (NP: $P = 0.11$; BM: $P = 0.06$). Similar results were found at population level (results not shown). These results imply a scenario of non-expansion.

Morphology

To test whether the strong genetic divergence between the populations of the Newnes Plateau versus the Blue Mountains was correlated with morphological traits, we conducted MANCOVA with gender and location (Blue Mountains or Newnes Plateau) as factors, SVL as the covariate, and morphological traits (length of head, front limbs and rear limbs, and interlimb distance) as dependent variables. The MANCOVA revealed a significant effect of location on phenotype ($F_{4,208} = 9.70$, $P < 0.0001$). A lizard's morphology also was affected by its gender ($F_{4,208} = 109.87$, $P < 0.0001$) and its body length ($F_{4,208} = 745.27$, $P < 0.0001$). Only one interaction term (SVL × gender) was significant ($F_{4,208} = 13.71$, $P < 0.0001$), reflecting the fact that head length increased more rapidly with SVL in males than in females.

Given the significant overall MANCOVA, we conducted analyses on individual traits using the same design. ANOVA with gender and location as factors and SVL as the dependent variable was significant ($F_{2,219} = 5.91$, $P = 0.0032$): lizards from the Newnes Plateau were smaller than those from the Blue Mountains ($F_{1,219} = 10.45$, $P = 0.0014$). Using ANCOVA with SVL as the covariate, location effects were significant for head length ($F_{1,211} = 16.38$, $P < 0.0001$; see Fig. 5), front limb length ($F_{1,211} = 12.41$, $P = 0.0005$), and interlimb distance ($F_{1,211} = 12.7803$, $P = 0.0004$; see Fig. 5), but not for rear limb length ($F_{1,34} = 1.37$, $P = 0.24$). On average, lizards from the Newnes Plateau were smaller and more gracile (had smaller heads and front limbs, but a larger interlimb distance relative to SVL) than conspecifics from the Blue Mountains.

DISCUSSION

Biogeography of *Eulamprus leuraensis*

Given the very limited geographical distribution of this species, and its restriction to < 40 isolated swamps, the long and stable evolutionary history of its occupation of these swamps is surprising. For example, the major haplotype groups within the species diverged about 1 or 2 Ma (we estimate between 2.24 and 0.70 Ma), during the Plio-Pleistocene period (Ogg, 2004). The populations seem to have remained relatively stable over that long period, with no hint of a recent expansion detected (either within the distinct Newnes and Blue Mountains regions, or within the populations overall), consistent with the reticulated rather than star-like haplotypic network. For example, no haplotypes were shared between populations of the Newnes and Blue Mountains, two areas separated by < 30 km. This long period of genetic separation between the two major parts of the species' range is also consistent with: (1) the spatial analysis of molecular variance revealing the Newnes and Blue Mountains as the two major clusters, (2) the very high F_{ST}- and Φ_{ST}-values between these areas (0.517 and 0.853), and (3) significant differences in body shape between lizards from these two areas. The presence of different, long-diverged genetic lineages within populations (e.g. BH3:

Table 1 Populations of the Blue Mountains water skink (*Eulamprus leuraensis*) analysed in south-eastern Australia, with number of samples, geographical distances between them (metres; upper matrix), F_{ST} and Φ_{ST} (K2P) between populations (lower matrix), and nucleotide and gene diversity, and mean number of pairwise differences within population (with K2P; diagonal).

Pop	*n*	BH3	BH4	BH5	MH4	MRP1	WFL	KT1	WF1	WF5	WF7	XFC1	NP4	PNP1
BH3	16	**0.0021** **0.683** **1.66**	455	4015	12,628	12,696	11,557	18,099	22,018	20,235	19,451	21,582	26,534	30,047
BH4	22	*0.138* *0.062n.s.*	**0.0011** **0.329** **0.87**	3568	12,198	12,241	11,160	17,697	21,701	19,884	19,090	21,933	26,917	30,467
BH5	15	*0.291* *0.402*	*0.491* *0.567*	**0.0034** **0.733** **2.66**	9352	8802	8814	15,140	20,047	17,899	17,008	24,329	29,572	33,482
MH4	6	*0.301* *0.395*	*0.612* *0.646*	*0.343* *0.127n.s.*	**0.0014** **0.533** **1.07**	3950	2264	5901	12,070	9417	8405	33,679	38,909	42,659
MRP1	18	*0.292* *0.305*	*0.481* *0.496*	*0.206* *0.196*	*0.199* *0.217*	**0.0030** **0.732** **2.36**	5853	8767	15,665	12,862	11,822	32,458	37,912	42,124
WFL	19	*0.235* *0.270*	*0.424* *0.449*	*0.167* *0.155*	*0.133* *0.044n.s.*	*0.031n.s.* *0.112*	**0.005** **0.842** **3.93**	6547	11,453	9119	8203	32,989	38,068	41,558
KT1	19	*0.420* *0.688*	*0.597* *0.794*	*0.398* *0.457*	*0.499* *0.434*	*0.393* *0.558*	*0.336* *0.363*	**0.0012** **0.485** **0.93**	7346	4423	3428	39,446	44,583	48,105
WF1	5	*0.412* *0.659*	*0.653* *0.811*	*0.383* *0.368*	*0.456* *0.484*	*0.346* *0.489*	*0.277* *0.254*	*0.007n.s.* −0.0360n.s.	**0.0005** **0.400** **0.40**	2923	3937	43,553	48,274	51,059
WF5	14	*0.574* *0.695*	*0.747* *0.828*	*0.555* *0.357*	*0.199n.s.* *0.310n.s.*	*0.386* *0.505*	*0.318* *0.242*	*0.660* *0.477*	*0.734* *0.752*	**0.0002** **0.143** **0.14**	1042	41,816	46,706	49,778
WF7	19	*0.522* *0.685*	*0.688* *0.806*	*0.503* *0.372*	*0.104n.s.* *0.240*	*0.335* *0.514*	*0.271* *0.269*	*0.611* *0.395*	*0.613* *0.491*	−0.005n.s. *0.0563n.s.*	**0.0006** **0.292** **0.48**	41,021	45,958	49,117
XFC1	36	*0.500* *0.811*	*0.640* *0.870*	*0.482* *0.812*	*0.579* *0.876*	*0.474* *0.788*	*0.422* *0.692*	*0.576* *0.901*	*0.614* *0.913*	*0.697* *0.924*	*0.653* *0.913*	**0.0008** **0.382** **0.62**	5750	11,477
NP4	22	*0.467* *0.833*	*0.632* *0.887*	*0.446* *0.819*	*0.555* *0.885*	*0.439* *0.805*	*0.383* *0.716*	*0.556* *0.907*	*0.595* *0.923*	*0.702* *0.934*	*0.647* *0.923*	*0.606* *0.821*	**0.001** **0.407** **0.79**	6250
PNP1	23	*0.374* *0.814*	*0.545* *0.871*	*0.352* *0.804*	*0.437* *0.859*	*0.350* *0.788*	*0.295* *0.705*	*0.466* *0.892*	*0.475* *0.906*	*0.604* *0.914*	*0.557* *0.906*	*0.533* *0.805*	*0.383* *0.508*	**0.0013** **0.557** **1.06**

n.s., non-significant differences.

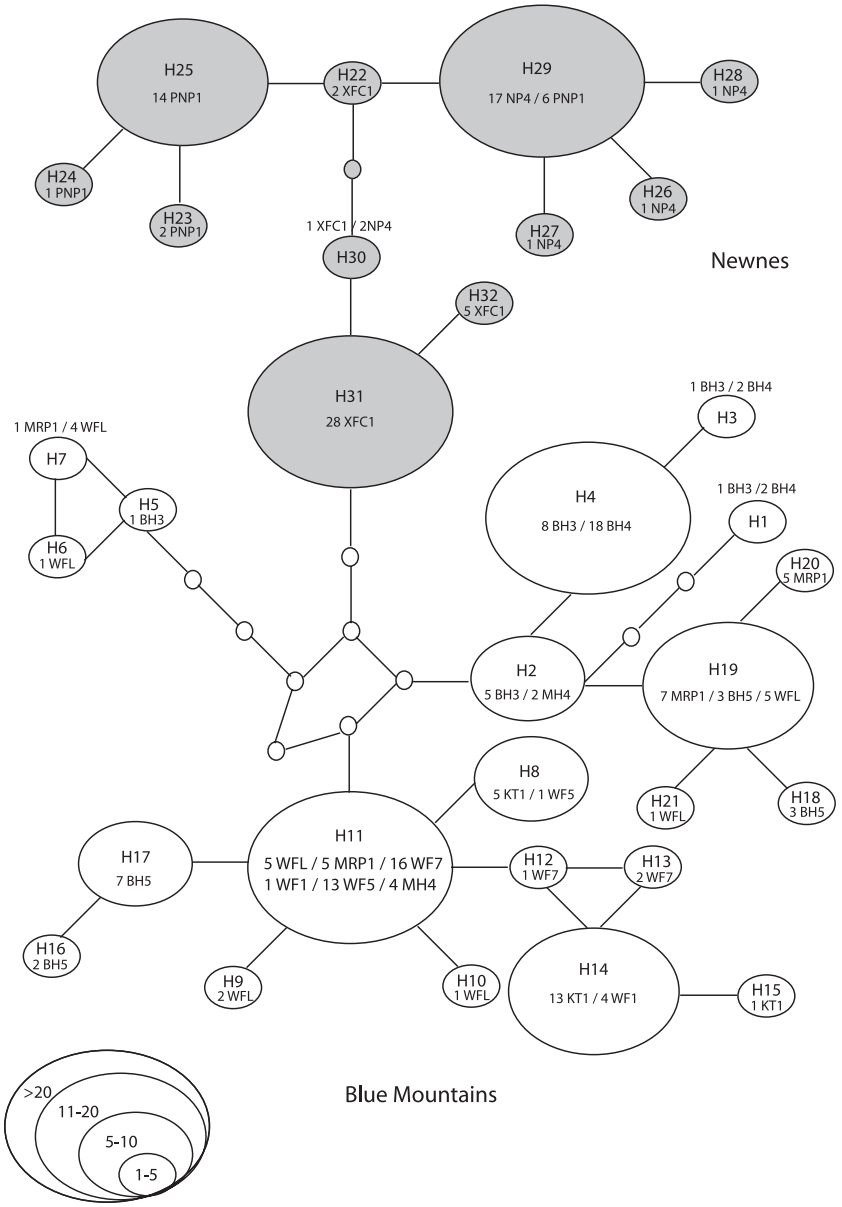

Figure 3 Statistical parsimony network of *Eulamprus leuraensis* haplotypes (grey circles, Newnes Plateau; open circles, Blue Mountains).

2.24–0.70 Ma) is also consistent with long-term stability in distributions, but alternatively might be due to more recent gene flow between populations harbouring different genetic lineages.

The overall pattern of stable ancient divergences among populations is not consistent with a model of population contraction to warm-climate areas during colder and drier periods, followed by reinvasion of the main range after unsuitable cold and dry habitats recede. Instead, patches of favourable habitat (presumably similar to present-day swamps, because these are the only known current habitat for *E. leuraensis*) must have persisted and provided refugia from unsuitable conditions, even during the Last Glacial Maximum (LGM: 21000 ± 2000 years), when aridity was widespread and locally extreme in Australia (Byrne *et al.*, 2008), with temperatures colder than the present by up to 10 °C (Williams, 2000; Byrne *et al.*, 2008). According to the same authors, extensive areas were treeless, and streams draining the Eastern Highland ferried seasonally substantial volumes of sand and gravel.

Such refugia have been described for many species of animals and plants in the Holarctic, but rarely for reptiles (perhaps reflecting the reliance of this latter group on suitable thermal regimes). Of the European species studied to date in this respect, the two most cold-tolerant reptile taxa (the adder *Vipera berus* and the sand lizard *Lacerta agilis*) probably survived glacial periods in non-Mediterranean refuges, such as central France and the Carpathian Basin (Ursenbacher *et al.*, 2006a; Joger *et al.*, 2007). A glacial refuge in France also has been inferred for the aspic viper *Vipera aspis* (Ursenbacher *et al.*, 2006b) and the common lizard *Lacerta* (*Zootoca*)

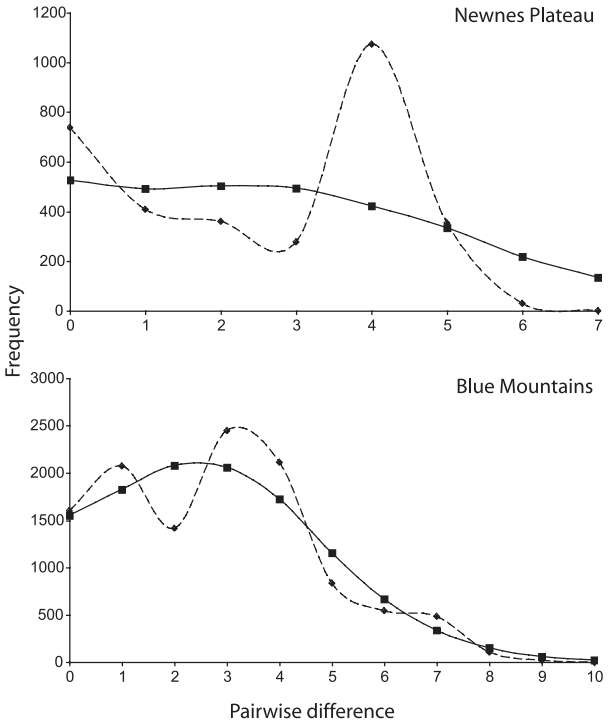

Figure 4 Observed (dotted line) and expected (solid line) mismatch distributions (frequency distribution of the mean pairwise difference between haplotypes) for a sudden expansion of Newnes Plateau and Blue Mountains samples of *Eulamprus leuraensis*, determined with Arlequin (Excoffier *et al.*, 2005).

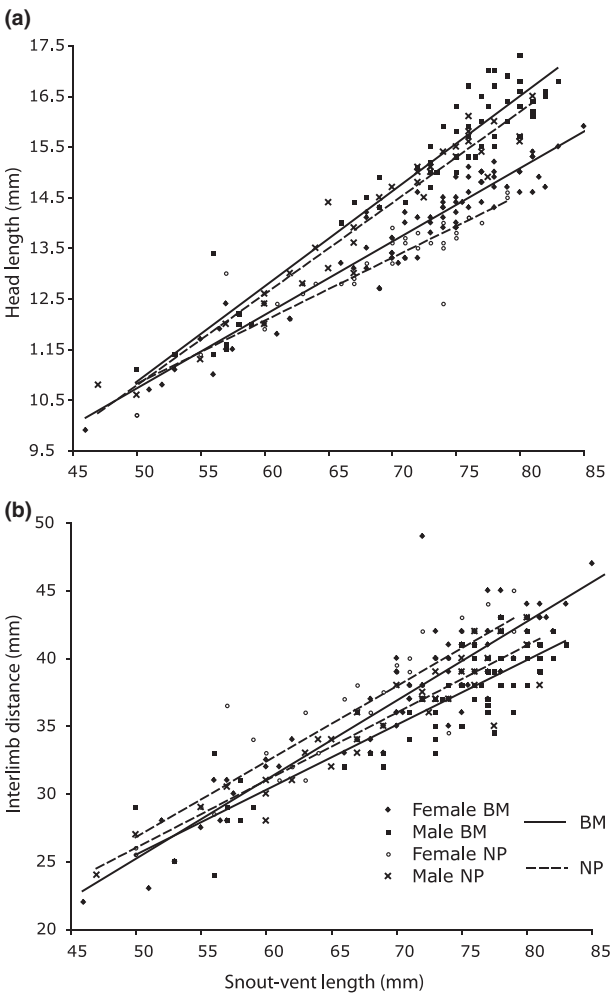

Figure 5 Relationship between snout-to-vent length (SVL) and (a) head length and (b) interlimb distance in female and male water skinks (*Eulamprus leuraensis*) from the Blue Mountains (BM) and Newnes Plateau (NP).

vivipara (Guillaume *et al.*, 2000). Likewise, a glacial refuge in the Carpathian Basin has been suggested for cold-tolerant species of non-squamate lineages (*Rana arvalis* – Babik *et al.*, 2004; *Capreolus capreolus*, *Cervus elaphus*, *Sus scrofa*, *Vulpes vulpes* – Sommer & Nadachowski, 2006; *Microtus agrestis* – Jaarola & Searle, 2002).

The persistence of stable populations of *E. leuraensis* over a 1-Myr period encompassing major climatic variation suggests that this area of montane south-eastern Australia provided opportunities to escape glacial extremes, perhaps reflecting its proximity to the Pacific Ocean. Consistent with that inference, the same area that supports *E. leuraensis* also houses endemic plant species (e.g. Wilson, 1996; NSW National Parks and Wildlife Service, 2001b; Turton & Melick, 2001). Palynological data from a swamp of the Newnes Plateau reveal the presence of wet-adapted species such as ferns (*Gleichenia*) and mosses for at least 14,200 yr BP (the entire study period of Black & Mooney, 2006), confirming a high stability of the swamps in this area. More generally, the Greater Blue Mountains is one of the three most diverse areas on Earth for scleromorphic plant species, and is a centre of plant endemism. It contains almost 1500 species: 10% of Australia's vascular plants, the highest diversity of any of its temperate zones (UNEP–WCMC, 2007). This diversity largely reflects the region's exceptional geological stability and intricate topography, which has allowed some environments and their biota to persist for millennia as refugia from climatic change (UNEP–WCMC, 2007). The most famous example is the discovery in 1994 of a 'living fossil', the Wollemi pine (*Wollemia nobilis*), represented by fewer than 100 adult trees, known only from an inaccessible canyon in the Greater Blue Mountains (Jones *et al.*, 1995; Hill, 1996).

Our data on haplotypes suggest a scenario of long-term stability, with *E. leuraensis* populations persisting in small pockets of suitable habitat over long periods. One plausible alternative would be of genetic connectivity among the swamps, such that frequent local extinctions have been balanced by dispersal and hence re-establishment of the species in most suitable patches of habitat. This metapopulation model is not well-supported by our data, however, because local populations are highly distinct genetically, even in cases where they are separated by only a few kilometres (e.g. WF5–WF1: distance = 2.9 km, $F_{ST} = 0.73$; or BH4–BH5: distance = 3.6 km, $F_{ST} = 0.49$). The strong pattern of IBD and the frequent presence of unique haplotypes in populations or small groups of populations also suggest very limited gene flow among populations.

In summary, this low-vagility species seems to have persisted in small, semi-isolated habitat fragments within its current range over very long periods of evolutionary time: periods that have seen major climatic fluctuations, and that have included significant cold and dry events such as the LGM (Bowler, 1982; Frakes *et al.*, 1987; Markgraf *et al.*, 1995; Gallagher *et al.*, 2003; Byrne *et al.*, 2008). Climatic fluctuations during the Pleistocene thus have left relatively little signature on the genetic structure of populations of this endangered lizard species. This situation poses a strong contrast to the situation with Holarctic species (including reptiles) that faced more extreme glacial events over the same period (e.g. Hewitt, 2000, 2004a,b; Joger *et al.*, 2007; Bhagwat & Willis, 2008). Taxa from the Northern Hemisphere clearly exhibit a strong impact of Plio-Pleistocene climatic variation, involving a general pattern for populations to contract to southern refugia during glacial periods followed by subsequent northern expansion during the interglacials, with only few cold-tolerant species subsisting in northern refugia.

Previous studies have detected the impact of the climatic variations on south-eastern Australian biota, but that impact is mostly reflected in deep phylogeographical breaks and structuring within lineages, rather than by clear signs of population contraction and expansion as seen in the skinks of the *Egernia whitii* group (Chapple *et al.*, 2005) or the froglets *Crinia signifera* (Symula *et al.*, 2008). These results suggest that less extreme glacial conditions in the Southern Hemisphere, compared with the Northern Hemisphere, allowed cold-adapted species (such as *E. leuraensis* would appear to be, based on its present distribution) to persist in montane areas. However, additional studies are needed before we can assemble a comprehensive view of the impact of Pleistocene climatic variation on the phylogeography of Southern Hemisphere taxa.

Implications for the conservation of *Eulamprus leuraensis*

The genetic distinctiveness of local populations of *E. leuraensis* has clear implications for conservation of this endangered taxon. Clearly, the Newnes Plateau and Blue Mountains populations comprise phenotypically and genetically distinctive units that warrant separate status in any management plan. Even within the Blue Mountains, many local populations are highly distinctive genetically. Thus the loss of a population (through habitat degradation or predation by feral mammals, for example) would result in a loss of genetic diversity within the species overall, and gene flow between populations is so rare that recolonization is likely to be slow or non-existent. Even if colonists were introduced from another population, their genetic characteristics inevitably would differ from those of the previous population. Land managers thus should accord high priority to preservation of the unique montane swamps that house these lizards, by preventing urban development in the catchments of swamps housing *E. leuraensis*, and controlling non-native weeds that might otherwise invade these swamps and modify their structure (NSW National Parks & Wildlife Service, 2001a).

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BIOSKETCHES

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