Amphibian phylogeography in the Antipodes: Refugia and postglacial colonization explain mitochondrial haplotype distribution in the Patagonian frog *Eupsophus calcaratus* (Cycloramphidae)

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**Abstract**

Climatic oscillations, heterogeneity in elevation, topographical position, and isolation time in southwestern Patagonia have been important in promoting diversification of the biota. Geological studies have shown that this region had wide ice-free areas during periods of the last glacial maximum and provided forested refugia for the biota during Pleistocene glaciations. In this study, we sampled the endemic frog *Eupsophus calcaratus* from 20 localities, covering most of its distribution and including glaciated and non-glaciated regions. We collected DNA sequences for three mitochondrial regions (D-loop, cyt b, 16S), and describe patterns of variation consistent with a history of both the displacement to glacial refugia and recent recolonization to extensively glaciated regions. The inferred demographic history and divergence times of the lineages of *E. calcaratus* suggest that the Pleistocene had profound effects on the genetic patterns within this taxon in which some populations were able to survive in refugia within colder regions followed by demographic increases but without evidence of significant range expansion. The mtDNA gene tree recovers six major haploclades of *E. calcaratus*, which we consider diagnostic of species lineages. These results contribute to our understanding of how geological events, predominately glacial oscillations, have influenced current population structure of a broad-ranging, ectothermic vertebrate in the Valdivian Forest region of southern South America.

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1. Introduction

The Andean and adjacent regions in southern South America have a rich history of geological and climatic changes along broad altitudinal and latitudinal gradients, and the highly endemic biota has distributional patterns strongly linked to these histories (Hinojosa and Villagrán, 1997; Veblen, 2007). Although the Andes began to rise at the end of Cretaceous, more than 65 million years ago (Ma), it was not until the middle Miocene (11–14 Ma) that elevations exceeded 1000 m above sea level (a.s.l.; Potts and Behrensmeyer, 1992; Gregory-Wodzicki, 2000). Major orogenies occurred in the last 10 Ma, causing uplift to current elevations of greater than 4000 m a.s.l. (Gregory-Wodzicki, 2000), and forming geographic barriers with pronounced effects on the distribution and diversification of flora and fauna (Smith-Ramírez, 2004; Muellner et al., 2005; Ruzzante et al., 2006; Yoke et al., 2006). On a more recent time scale, glaciations in southernmost Patagonia began as early as the Middle Pliocene (Rabassa, 2008), in which a series of glacial advances and retreats characterized the Patagonian landscape. During the Early Pleistocene, a single, continuous montane ice sheet developed for the first time and covered broad areas of Andean South America from ~36°S to 56°S (Hulton et al., 1994; McCulloch et al., 2000). However, parts of the coastal range of western Chile remained ice free.

The dates of the recent Pleistocene glaciations in South America are well known (Rabassa and Clapperton, 1990; Ruzzante et al., 2008), and include: (1) the most extensive Andean glaciation (~1.1 Ma); (2) the coldest Pleistocene glaciation (~0.7 Ma); (3) the last southern Patagonian glaciation (180 kya); and (4) the last glacial maximum (LGM, ~25–23 Ka). These glaciers changed drainage patterns, lake distributions, and even the position of the continental divide, displacing plant and animal populations and thus providing a natural theater for examining the effects of glaciers on biodiversity patterns in this region. For example, strong genetic structure concordant with paleoclimatic shifts has been reported for endemic freshwater crabs (Xu et al., 2009), while repeated expansion and contraction events and subsequent population mixing were detected in percichthyid and galaxiid fishes (Ruzzante et al., 2006; Zemlak et al., 2008). Historical signatures are also

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evident in the phylogeographic patterns of various groups of lizards of the genus *Liolepis* (Avila et al., 2006; Morando et al., 2003, 2004, 2007, 2008; Victoriano et al., 2008). Genetic divergence between eastern and western populations of the long-tailed rodent *Oligoryzomys longicaudatus* emphasizes the role of the Andes as an effective geographic barrier to gene flow, and in range expansion from multiple interglacial refugia (González-Ittig et al., 2010). Likewise, using mtDNA sequences of 14 species of sigmodontine rodents, Lessa et al. (2010) show that postglacial colonization from lower latitudes is an important component of current Patagonian–Fuegian diversity, with *in situ* differentiation also contributing to the rodent species diversity.

In this context of a geological history of episodic orogenies, glaciations, marine intrusions and vulcanism, the Patagonian region is now being intensively studied from biogeographic and phylogeographic perspectives as a southern-hemisphere analog of temperate continental regions of Europe and North America (Avila et al., 2006; Cosacov et al., 2010; Zemlak et al., 2010; Patterson, 2010). Across the entire “cone” of temperate South America, however, few phylogeographic studies of broadly distributed terrestrial vertebrates have been conducted in the mesic southwestern region of the Valdivian Forest (37°-45°S–43°20’S). This region was extensively glaciated (see below), and the degree to which populations were reduced in size, the extent to which they survived within refugia, and the routes through which recolonizations occurred remain unknown.

Frogs are an excellent taxon for studies of phylogeographic diversification and speciation because restricted dispersal capabilities tend to promote differentiation (Duellman, 1999; Pröhl et al., 2010), and they often show significantly greater population-genetic structure relative to species without such limitations (Johns and Avise, 1998; Semsrits et al., 2009). In fact, one of the most significant contributions to our understanding of the genetic structuring of amphibian populations was Ward et al.’s (1992) comparative study, which demonstrated that, of all the major classes of vertebrates, amphibians exhibit the highest degree of population subdivision, as measured by allozyme markers. Later studies based on microsatellite markers have confirmed that amphibians generally display a high degree of genetic structuring at all scales (Vos et al., 2001; Palo et al., 2004). Further, they are highly sensitive to climatic changes owing to complex life histories, permeable skin and exposed eggs (Fitzpatrick et al., 2009), and past climatic records from southwestern South America have the potential to shed light on population divergence and speciation processes. For example, Nuñez and Formas (2000), hypothesized that the Miocene Andean orogeny was crucial in driving divergence and adaptation of the mountain frog genus *Telmatobufo*. Uncertainties remain about the origin and diversification of endemic genera such as *Batrachyla*, *Eupsophus*, and *Rhinoderma*, but it is possible that the glacial and interglacial events from Miocene to early Holocene, with the reduction in precipitation during glacial episodes (McCulloch et al., 2000), may have been the primary drivers of population differentiation, speciation, and the evolution of some specialized breeding strategies (degrees of territoriality such as those that occur in *Eupsophus* species; Úbeda and Nuñez, 2006).

In this study, we reconstruct the phylogeographic history of *Eupsophus calcaratus* (Günther, 1881), an endemic frog of the temperate Nothofagus forests of southwestern Patagonia. This species has one of the broadest distributions of any Chilean frog (Rabanal and Nuñez, 2009), and ranges from sea level to at least 600 m, and from 39° to 55° latitude. *Eupsophus calcaratus* inhabits stream edges where it hides in holes leading to water-filled chambers. Its larvae are distinctive in being endotrophic and developing in these water-filled chambers, which connect to small streams or permanent pools (Vera Candidi et al., 2005). *Eupsophus calcaratus* is a good candidate for phylogeographic research because its distribution includes both glaciated and non-glaciated regions, is strongly ecologically linked to forest environments, has a high tolerance to cold, and a relatively old origin as inferred from Oligocene fossils (fossils assigned to this genus are known from ~ 40 mya; Schaeffer, 1949).

The goals of this study are to: (1) describe the lineage diversity and phylogeographic patterns of *E. calcaratus*; and (2) evaluate the effects of Pleistocene glaciation on the population demography in glaciated and un-glaciated regions. We are aware of the limitations of using only mtDNA, but for many reasons (Zink and Barrowclough, 2008), we consider this a good “first pass” marker with which to investigate phylogeographic history of poorly known taxa. We consider this a valid “hypothesis generating” approach (Avila et al., 2006; Morando et al., 2007; Galbreath et al., 2010), and details of the historical demography of the female *E. calcaratus* revealed in this study provide insight into how southwestern Patagonian species may have responded to climatic fluctuations of the last 20,000–25,000 years.

2. Material and methods

2.1. Sampling

Populations of *Eupsophus calcaratus* were sampled across the temperate Nothofagus forests of Southern Chile (Fig. 1; Table 1). Animals were euthanized in the field with benzocaine, following the protocol of the Animal Experimentation Ethics Committee of the Universidad Austral de Chile. Samples of liver and muscle of each specimen were stored in 95% ethanol at ~ 80 °C and deposited in the Amphibian Collection, Institute of Zoology (Universidad Austral de Chile), and M.L. Bean Life Science Museum (BYU, Provo, UT). Specimens of *E. roseus* (Lago Pellaifa, 39°36’07”S; 72°01’58”W) and *E. septentrionalis* (Los Queules, 39°36’07”S; 72°01’58”W) were used as outgroups.

2.2. DNA extraction, amplification, and sequence alignment

Whole genomic DNA was extracted using the QIAGEN DNeasy kit (Qiagen, Valencia, CA) according to the standard protocol. Air-dried DNA pellets were eluted in distilled water, followed by electrophoresis of 5.0 μL of extraction product on 1% agarose gel to estimate the quality and amount of genomic DNA. Sample dilutions were performed, when necessary, prior to polymerase chain reaction (PCR) amplifications. Three mitochondrial gene regions were amplified via PCR in a cocktail containing 2.0 μL of template DNA, 8 μL dNTPs (1.25 mM), 4 μL 10 × Taq buffer, 4 μL each primer (10 μM), 4 μL MgCl2 (25 mM), 22 μL distilled water, and 0.25 μL Taq DNA polymerase (5 U/μL; Promega Corp., Madison, WI). Cycling profile for PCR amplifications was 3 min at 94 °C, 30 s, 49 °C, 1 min 47–53 °C and 60 s, 72 °C (35 cycles), followed by a final extension of 7 min at 72 °C. Sequence and PCR annealing temperature for each primer pair are given in Table 2. PCR products were checked by electrophoresis on a 1% agarose gel stained with SYBR-Safe (Invitrogen), purified using a MultiScreen PCR 96 (Millipore Corp., Billerica, MA) and directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA). Excess of Dye Terminator was removed with CentriSep spin columns (Princeton Separations, Inc., Adelphia, NJ), and sequences were generated on an ABI Prism 3730 capillary autosequencer at the DNA Sequencing Center at Brigham Young University.

Complementary chromatograms were assembled and annotated in Sequencher™ v4.7. Sequences were aligned using MAFFT v.6.0 (Katoh et al., 2005) under the iterative method of global pairwise alignment (G-INS-i), and default settings were chosen for all the parameters involved. The data set was collapsed to unique
haplotypes by use of COLLAPSE v.1.2 (Posada, 2009) to facilitate all subsequent phylogenetic analyses. Sequences were submitted to National Center for Biotechnology Information (NCBI) database, under Accession Numbers HQ710833–HQ711306.

2.3. Phylogenetic analyses

We performed a partition-homogeneity test using 1000 replicates as implemented in PAUP 4.10b (Swofford, 2002) in order to
examine whether the three analyzed regions (D-loop, Cyt b, and 16S) could be combined into a larger data matrix (Farris et al., 1995). Because the result of the partition-homogeneity test was not significant, further analyses were performed on the combined data.

For phylogenetic analyses, the best-fitting model of nucleotide substitution was selected using the Akaike information criterion (AIC) following the procedure outlined by Posada and Buckley (2004), and implemented in jModelTest v0.1.1 (Posada, 2008). These results give a best fit for the general time-reversible (GTR) nucleotide substitution model with gamma-distributed rate heterogeneity among sites (gamma shape = 0.723), and a proportion of invariable sites (p-inv = 0.554). Likelihood analyses (ML) were performed using GARLI 0.96 (Zwickl, 2006), and were terminated after 10,000 generations without an improvement in the overall tree topology. Two ML analyses were performed to ensure convergence. Support was evaluated using 300 bootstrap repetitions (Felsenstein, 1985), with each repetition terminated after 5000 generations without a topology improvement. We also conducted partitioned Bayesian analyses using MrBAYES v3.1.2b (Ronquist and Huelsenbeck, 2003) in which the GTR + model was applied to each gene fragment. Each Markov chain was started from a random tree and run for 1.0 × 10^7 generations with every 1000th tree sampled from the chain. Stationarity was checked as suggested in Nylander et al. (2004). All sample points prior to reaching the plateau phase were discarded as “burn in” and the remaining trees combined to find the a posteriori probability estimate of phylogeny. Branch lengths were estimated as means of the posterior probability density. Trees were visualized using the FigTree v1.1.2 program, available at http://tree.bio.ed.ac.uk/software/figtree/. Eupsophus roseus and E. septentrionalis were used as outgroups in all analyses, based on their sister-group relationship to E. calcaratus (Correa et al., 2006).

2.4. Divergence dating and historical demography

Haplotype (HD) and nucleotide (π) diversity as well as the number of segregating sites (S) were calculated to estimate DNA polymorphism using DnaSP v5.0 (Librado and Rozas, 2009). To infer the date of origin for each lineage without relying on a strict molecular clock and considering uncertainty in tree topology and branch length (i.e. the relaxed phylogenetic method) we used BEAST 1.5.4 (Drummond and Rambaut, 2007). Partitioned phylogenetic estimates were constructed using the best-fitting model of nucleotide substitution, which was selected using the AIC with an uncorrelated lognormal tree prior and the Yule speciation process. Analyses were run for 20 million generations and sampled every 1000th iteration, following a pre-burnin of 2000 generations. No independent fossil or geological evidence is available to calibrate a local molecular clock for populations of Eupsophus, so external calibrations were used. Estimates of amphibian protein-coding mtDNA mutation rates have been shown to vary slightly depending on gene regions and taxonomic group (Tan and Wake, 1995; Macey et al., 1998; Crawford, 2003). For anurans, Crawford (2003) estimated ND2 to evolve at a rate of 0.957% per lineage per million years, based on a recalibration of Macey et al.’s
(1998) Eurasian Bufo dataset. We used this divergence rate (with a standard deviation of 0.05) as it is in agreement with estimates for several other vertebrate species (Moritz et al., 1987). An implicit assumption is that our study species has comparable DNA divergence rates to those estimated by Crawford (2003) for Bufo. We fully recognize that this approach is far from ideal (Graur and Martin, 2004), but a beginning exploration of phylogeographic histories of the endemic Patagonian species will in our view benefit from provisional estimates.

To examine whether E. calcaratus populations are at equilibrium, we calculated Fu’s Fs (Fu, 1997) implemented in DnaSP v.5.0. We chose this test because of the increased statistical power in detecting significant changes in population size when using small sample sizes (Ramos-Onsins and Rozas, 2002). Under the assumption of neutrality, negative values are expected in populations that have undergone recent expansion because rare alleles are more numerous than expected. Positive values occur if rare alleles are eliminated from populations following genetic bottlenecks (Tajima, 1989).

The validity of the assumed stepwise expansion model and the significance of Fs values were calculated by constructing 1000 coalescent simulations in DnaSP. Mismatch distributions were also calculated for each diagnosed population lineage to infer changes in population size. Under an infinite sites model, population expansion would be depicted as a unimodal distribution whereas the distribution expected for population stability is ragged and multi-modal (Harpending et al., 1998). The fit of the observed data was compared using the sum of squared deviations between observed and expected values estimated from 10,000 coalescent simulations in DnaSP. Although the raggedness index (r) is often used to assess significance of mismatch plots, multi-modal distributions that fit sudden-expansion models indicate structuring within populations or populations that are stable or shrinking (Excoffier and Schneider, 1999; Rogers and Harpending, 1992; Rogers et al., 1996). Therefore we do not consider the good fit of sudden-expansion models to unimodal mismatch distributions strong evidence of population expansion. However, multi-modal distributions can be inferred for populations that have undergone recent expansion but were recently subdivided, subjected to substantial migration, and/or have undergone historical contractions (Bertorelle and Slatkin, 1995; Marjoram and Donnelly, 1994; Ray et al., 2003; Castoe et al., 2007).

Although Fu’s Fs and mismatch distributions can provide insights into whether or not populations have undergone expansion, they do not provide information about the shape of population growth over time. Non-significant negative values of Fs and multi-modal mismatch distributions are indications that populations have not undergone expansive growth relative to a null hypothesis of population stability. However, such values are agnostic as to whether populations are expanding slowly, contracting or remaining relatively constant in size. Therefore, to estimate the shape of population growth through time we constructed Bayesian skyline plots (BSP) implemented in BEAST 1.5.4 (Drummond and Rambaut, 2007). For each BSP, the appropriate model of nucleotide substitution was determined using jModeltest. Genealogies and model parameters for each lineage were sampled every 1000th iteration for 20 million generations under a strict molecular clock with uniformly distributed priors and a pre-burnin of 2000. Demographic plots for each analysis were visualized in Tracer v 1.5 (Rambaut and Drummond, 2007).

3. Results

3.1. Phylogeny of Eupsophus calcaratus

One hundred sixty-two sequences of 2224 aligned base pairs were obtained for Eupsophus calcaratus and two outgroup taxa. The absence of any internal stop codons in the Cyt b gene region suggested that the sequences were mitochondrial and not nuclear-integrated copies or pseudogenes (Zhang and Hewitt, 1996). The combined partitioned analyses produced a 50% majority-rule consensus tree with a harmonic mean of Log likelihood (LogL) of –13329.46 following a burnin of 20,000 generations. Because ML and BI analyses produced highly congruent estimates of the phylogenetic relationships, only the Bayesian consensus phylogram is presented with the posterior probabilities (P) and non-parametric ML bootstrap values for the shared branches (Fig. 2).

Six strongly-supported haplotype lineages were recovered within the E. calcaratus complex (Fig. 2), all with P > 1.0. Samples from the northern Villarrica region (lineage A; locality 1, Fig. 1) formed the sister group to all others, with the next interior lineage B, including all haplotypes from Reserva Valdivia (loc. 2). Lineage C comprised all haplotypes from Bahia Mansa (loc. 3), while samples collected from Guapo Island (loc. 11) were recovered as two lineages (D and E), which together formed the sister group of clade F. This clade includes all samples from the glaciated areas (localities 4–5, 6–10, and 12–20), and the most southern haplotypes, particularly from localities 7 to 20 (see Table 1), were randomly distributed with no geographic structure.

3.2. Divergence times and historical demography

Genetic diversity indices (Hd, S, and π) for the well-supported lineages are summarized in Table 3. Among all lineages, Hd = 0.999 and π = 0.048, indicating a high haplotype but relatively low nucleotide diversity. No haplotypes were shared among lineages A, B, C, E, and F, and most haplotypes are confined to a single locality within A, B, C, or E.

Results of the dating analysis suggest that each of the divergences occurred during the Pleistocene with the initial split occurring 188 kya with a 95% credible interval of 96–290 kya (Fig. 2). Subsequently, each of the extant lineages shared a most recent common ancestor during the early Pleistocene ranging from 16.7 to 70 kya (Table 3). The recent dates for these lineages suggest that the LGM may have played a major role in generating the extensive genetic diversity within E. calcaratus. Additionally, each of the lineages would have responded to the retreat of the most recent Patagonian glacial cycle. Despite the high level of nodal support, the small samples size (n = 2) prevented us from conducting demographic analysis on lineage D.

Fu’s Fs statistic was significant and negative (p = 0.05) for lineages A, E, and F, indicating an excess of low-frequency haplotypes while lineages B and C were not significant. Likewise, the mismatch distributions showed relatively unimodal patterns with low raggedness values for lineages A and E and multi-modal distributions and high raggedness values for lineages B and C (Fig. 3). The mismatch distribution for lineage F appears multi-modal, but the raggedness index is low (r = 0.009). Multi-modal distributions that fit sudden-expansion models can be an indication of structuring within populations (Castoe et al., 2007), a pattern consistent with the substructuring present in the tree (Fig. 2). The effective sample size (ESS) for each of the Bayesian skyline analyses was greater than 200, suggesting that the 50 million generations were sufficient to estimate the demographic history for each lineage. None of the plots showed any evidence of genetic bottlenecks, recent subdivisions or significant historical population contractions (Fig. 3). Significantly negative Fs values, low raggedness indices for the mismatch distributions, and Bayesian skyline plots depicting growth provide evidence that lineages A, E and F have undergone recent expansions.
4. Discussion

4.1. Phylogeography of E. calcaratus

This study currently represents the most comprehensive analysis of the phylogeographic history of an endemic Patagonian frog throughout most of its distributional range. The mtDNA gene tree (Fig. 2) recovers six major haploclades of E. calcaratus, which we consider diagnostic of species lineages. Four of these lineages occur at lower elevations along the coastal areas and outside the extent of the major ice sheet during the LGM (lineages B, C, D, and E), and two (A and F) are confined to higher previously glaciated montane environments (Fig. 1). A general pattern of greater genetic diversity along the coastal regions of Chile coupled with larger, more homogeneous population lineages throughout the Andes (Table 3) is consistent with previous phylogeographic studies of

Table 3
Sample size (N), haplotype diversity (Hd), number of segregating sites (S), nucleotide diversity (π), Fu’s Fs, raggedness index (r), and time to most recent common ancestor (TMRCA, years) for five of the six lineages of Eupsophus calcaratus. Lineage labels correspond to clades in Fig. 2.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>N</th>
<th>Hd</th>
<th>S</th>
<th>π</th>
<th>Fs</th>
<th>Raggedness index</th>
<th>TMRCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>4</td>
<td>1.00</td>
<td>91</td>
<td>0.021</td>
<td>2.001</td>
<td>0.1</td>
<td>25,900 (7.62-5.10)</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.900</td>
<td>66</td>
<td>0.015</td>
<td>3.703</td>
<td>0.19</td>
<td>19,700 (7.86-3.48)</td>
</tr>
<tr>
<td>A</td>
<td>12</td>
<td>1.00</td>
<td>39</td>
<td>0.006</td>
<td>-3.87</td>
<td>0.02</td>
<td>70,700 (3.64-1.16)</td>
</tr>
<tr>
<td>E</td>
<td>21</td>
<td>0.995</td>
<td>34</td>
<td>0.003</td>
<td>-14.156</td>
<td>0.03</td>
<td>16,700 (6.63-2.98)</td>
</tr>
<tr>
<td>F</td>
<td>114</td>
<td>0.998</td>
<td>316</td>
<td>0.024</td>
<td>-34.073</td>
<td>0.009</td>
<td>38,750 (2.49-5.35)</td>
</tr>
</tbody>
</table>

* P = 0.05.
other taxa across this region, such as the magnoliopsid Hypochaeris palustris (Muellner et al., 2005) and the Southern beech genus Nothofagus (Marchelli and Gallo, 2006; Azpilicueta et al., 2009; Mathiasen and Premoli, 2010). The initial divergence within E. calcaratus occurred in its northern distributional range during the Pleistocene, approximately 188 kya, a period associated with

Fig. 3. Bayesian skyline plot for each mtDNA lineage of E. calcaratus. The solid line represents the median value for the log of the population size (log Ne) and the dashed lines represent the upper and lower 95% credible intervals. The x-axis measures time in substitutions per site per million years.
a time of global cooling (Bonan, 2002). The position of this deep lineage (clade A, Fig. 2) within the inferred boundaries of the LGM, suggests the possible existence of a small refugium within the ice field, a phenomenon found in similarly heterogeneous environments in other temperate regions (Rowe et al., 2004; Anderson et al., 2006). The subsequent divergence events, concurrent with a time within the Llanquihue Glaciation (Llanquihue I, ~70,000 years ago; Rabassa and Clapperton, 1990), occurred southward along the Chilean coast, followed by an eastward expansion into the Andes presumably following glacial retreat. Lineages B and C are confined to the Valdivian Coastal range, an area considered to be one of the most important refugia during Pleistocene glaciations (Premoli, 2005). For example, fossils indicate that some tree species (Notothamnus, Fitzroya) survived locally in this area throughout the Pleistocene, and subsequent increases in the pollen record at 11,000 YBP indicate a rapid expansion of these species after ice retreat (Villagrán and Armesto, 2005). Thus, Valdivian forests that currently occur in these areas should all be early Holocene in age (10,000 YBP). Alternatively, if ice sheets in Patagonia were not so extensive and continuous as has been proposed (Fig. 1), we can infer that the Coastal range populations of *E. calcaratus* may have survived locally in ice-free environments. This scenario of multiple refugia is consistent with the deep genetic divergence among the northern lineages of *E. calcaratus*.

An unexpected discovery was the documentation of two well-supported lineages (D and E) co-occurring on the previously un-glaciated and relatively small Guao Island (Fig. 1). Although lineage D contains only two samples, the deep genetic split between it and lineage E suggests a complex history of divergence in isolation, perhaps over a broad geographic area during sea-level declines during or prior to the LGM, followed by secondary contact. Lineage F consists entirely of identical or minimally divergent haplotypes from localities in previously glaciated regions, extending from southern Chile north through the southern Andes to Antillanca (latitude of 40°45′31″). This scenario is also consistent with the levels of divergence and genetic variation detected for the lizard *Liolaemus tenuis* (widely distributed in central-southern Chile), which showed lower values both for the haplotype and nucleotide diversity for the clade distributed from 38°S to the southern edge of its distribution (Victoriano et al., 2008).

### 4.2. Historical population demography

During the Pleistocene, glacial advances affected the physical and biological environments of the Southern Hemisphere (Villagrán and Armesto, 2005). These glaciers and the associated climatic changes drove high-latitude populations into more southern habitats in the Northern Hemisphere (Hewitt, 1999, 2004). In Patagonia the contraction–expansion model predicts that populations affected by these habitat shifts would have undergone rapid population expansion as previously unsuitable habitat became colonized, but this would occur along predominantly east–west axes due to the N–S orientation of the historical ice sheets (Fig. 1). Despite differences in geography, rapid or step-wise colonizations would be characterized by low levels of genetic diversity as each new founding population represented only a fraction of the ancestral population’s genetic diversity (Nichols and Hewitt, 1994; Hewitt, 2000).

The various demographic analyses show that the intra-clade genetic structure of *E. calcaratus* contains signatures of demographic expansion consistent with Pleistocene glacial retreat. Specifically, the demographic analysis and genetic variation for the A, E and F lineages show strong support for recent population expansions represented by significantly negative Fs values, unimodal mismatch distributions with low raggedness indexes, and BSPs (Fig. 3) consistent with estimated times of these expansions range from ~71 to ~18 kya, but with sufficient overlap in error terms to permit precise assessments (Table 3). Additionally, the high haplotype diversity and low nucleotide diversity for each of these lineages is consistent with population expansion, and for lineage F, this expansion is consistent with a history of colonization of previously glaciated or periglacial regions. The topology of this clade, with shallow branches, little internal resolution (Fig. 2), and low sequence divergence is also suggestive of a recent population expansion.

In contrast, the inferred demographic histories, current distributions, and divergence times of lineages A and E suggest that these populations were able to survive in refugia within colder regions, followed by demographic increases but without evidence of significant range expansion. It is probable that some ecological barriers, perhaps the Valdivia and Osorno paleobasins, sediment-depository since late Oligocene–early Miocene times (Muñoz et al., 2000; Jordan et al., 2001) have maintained the isolation of these populations (Premoli, 2005; Hinojosa et al., 2006). A non-exclusively alternative that is the abrupt beginning of a remarkable series of glacially-deepened lake basins at this latitude (Clapperton, 1994) probably inhibited the population expansion to other latitudes. The climate of this region is currently dominated by the seasonal winter northward migration of the austral Polar Front, which largely accounts for mean annual precipitation totals of ca. 3000–4000 mm. Despite the abundant precipitation, the low terrain and generally mild temperature inhibit the development of ice caps, and modern glaciers are confined to mountains above 2500 m (Clapperton, 1994).

The leading-edge model of population expansion predicts that lineages located at or near the edge of glacial margins should undergo rapid population expansion associated with glacial retreat. The demographic analyses for lineages B and C revealed multimodal mismatch distributions, non-significant Fs statistics and flat BSPs, suggesting that these lineages have remained relatively stable through time, but small sample sizes for both lineages require cautious interpretation. It is possible, given the low vagility and ecological specificity of *E. calcaratus*, that these two lineages did not undergo demographic expansion following the retreat of the Patagonian glacier. However, given (1) the strong evidence of population expansion for the other lineages, (2) the periglacial geographic position of lineages B and C, and (3) patterns of population expansion for other periglacial taxa in other parts of Patagonia (Xu et al., 2009; Mathiasen and Premoli, 2010), we suggest that further sampling is needed in these localities before definitive statements about the demographic histories of these lineages can be made.

### 4.3. Taxonomic implications

It is clear from the phyleogeographic patterns within *E. calcaratus* that the climatic changes throughout the Pleistocene have had profound effects on the genetic patterns within this taxon, but the role these processes may have played in the formation of new species is uncertain. For example, the high levels of mtDNA sequence divergence between the Villarrica population (lineage A) and the rest of the *E. calcaratus* populations raises questions regarding the inclusion of this population within *E. calcaratus*. Elsewhere in the genus, the sister species *E. roseus* and *E. migueli* are separated by a smaller mtDNA uncorrected genetic distance (*p* = 0.1021) than that between lineages A and the other lineages of *E. calcaratus* (*p* = 0.1228). Certainly, genetic divergence alone is a poor metric of taxonomic rank, so formal integrative taxonomic studies (sensu Dayrat, 2005) should be conducted before species boundaries can be reassessed.

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