How lizards survived blizzards: phylogeography of the *Liolaemus lineomaculatus* group (Liolaemidae) reveals multiple breaks and refugia in southern Patagonia and their concordance with other codistributed taxa

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Abstract

Patagonia was shaped by a complex geological history, including the Miocene uplift of the Andes, followed by volcanism, marine intrusions, and extreme climatic oscillations during Pliocene–Pleistocene glaciation–deglaciation cycles. The distributional patterns and phylogenetic relationships of southern patagonian animals and plants were affected in different ways, and those imprints are reflected in the seven phylogeographic breaks and eight refugia that have been previously proposed. In this study, we estimated time-calibrated phylogenetic/phylogeographic patterns in lizards of the *Liolaemus lineomaculatus* group and relate them to historical Miocene-to-Pleistocene events of Patagonia and the previously proposed phylogeographic patterns. Individuals from 51 localities were sequenced for the mitochondrial marker (cyt-b) and a subsample of individuals from each mitochondrial lineage was sequenced for one nuclear (LDA12D) and one slow evolving mitochondrial gene (12S). Our analyses revealed strong phylogeographic structure among lineages and, in most cases, no signal of demographic changes through time. The *lineomaculatus* group is composed of three strongly supported clades (*lineomaculatus, hatcheri* and *kolengh + silvanae*), and divergence estimates suggested their origins associated with the oldest known Patagonian glaciation (~7 Ma); subsequent diversification within the *lineomaculatus* clade coincided with the large Pliocene glaciations (~3.5 Ma). The *lineomaculatus* clade includes nine strongly genetically and geographically structured lineages, five of which are interpreted as candidate species. Our findings suggest that some *Liolaemus* lineages have persisted in situ, each of them in a different refugium, through several glaciation–deglaciation cycles without demographic fluctuations. We also summarize and update qualitative evidence of some shared phylogeographic breaks and refugia among plants, rodents and lizards.

Keywords: biogeography, Liolaemidae, nuclear and mitochondrial genes, Patagonia, refugia

Received 19 February 2012; revision received 29 August 2012; accepted 1 September 2012

Introduction

The Patagonian region of southern South America became a large arid landscape during the Miocene (23.8 –5.3 Ma), after the elevation of the Andes blocked moisture-bearing air masses coming from the Pacific (Compagnucci 2011). Miocene subtropical savannas were replaced by today’s arid steppes, and ancient forests retreated to the Andean slopes before and during the aridification (Rabassa 2008; Tambussi 2011). The oldest known Patagonian glaciation took place ~7–5 million
years ago (Ma) during the Middle Pliocene and was followed by large Late Pliocene glaciations (~3.5 Ma) (Rabassa et al. 2005). The Pleistocene (1.8–0.01 Ma) was characterized by a temperature decrease of 6–7 degrees relative to the present (Markgraf et al. 1995; Moreno 1997; Hulton et al. 2002), during which several glaciation–deglaciation cycles are identified. These include the Great Patagonian Glaciation (~1.68–1.016 Ma), the Coldest Pleistocene Glaciation (~0.7 Ma), the Last Southern Patagonian Glaciation (~0.180–0.140 Ma) and the Last Glacial Maximum (LGM, ~0.025–0.016 Ma) (Rabassa et al. 2005). During glacial advances, the Patagonian coastline shifted east about four degrees, and much of the original extra-Andean landscape cooled to form permafrost (frozen soil for most of the year, except for short periods when surface ice may be absent; Trombotto 2000). These sea level shifts and the in situ formation of periglacial refugia (first proposed by Premoli 1998; and summarized in Sérsic et al. 2011) exposed suitable habitats for organisms to shift distributions to more equitable climates (Hulton et al. 2002; Jakob et al. 2009; Fontanella et al. 2012a).

Past environmental changes in Patagonia have modified ecosystems (Rabassa et al. 2005) and species distributions, while population contractions-recovery dynamics, with concordant range contractions and expansions, and the formation of secondary contact areas, have shaped today’s phylogeographic patterns (Sérsic et al. 2011). Potential refugia can be identified by a genetic signature of allopatric fragmentation, high species and genetic diversity and distinct genetic structure, while recently colonized areas show a signature of range expansion and sometimes secondary contact and hybridization (e.g. Zemlak et al. 2008; Tremetsberger et al. 2009; Cosacov et al. 2010; Nicolas et al. 2011; Olave et al. 2011).

Sérsic et al. (2011) summarized the literature on phylogeographic patterns of terrestrial vertebrates and plants from Patagonia (Argentina and Chile) and synthesized available information on glacial refugia, phylogeographic breaks, range expansions and colonization routes. In southern Patagonia (mainly Santa Cruz province), eight refugia and seven phylogeographic breaks have been proposed based on plant studies (e.g. Premoli 1998; Muellner et al. 2005; Jakob et al. 2009; Tremetsberger et al. 2009; Cosacov et al. 2010; Mathiasen & Premoli 2010), whereas only two phylogeographic breaks were proposed for terrestrial vertebrates. The small number of published terrestrial vertebrate studies have been based exclusively on rodents (summarized in Pardinas et al. 2011), but population sampling has been sparse and restricted mainly to mountain habitats. Several lizard phylogenetic phylogeographic studies (all based on various clades of Liolaemus) have been conducted in northern Patagonia and Chile (e.g. Morando et al. 2003, 2004, 2007; Avila et al. 2006; Victoriano et al. 2008), and these studies have recovered signals of both demographic expansion and stability.

Lizards from the Liolaemus lineomaculatus group (Etheridge 1995; Squamata: Liolaemidae) provide an excellent model to test the phylogeographic hypotheses proposed for plants and rodents because of its widespread distribution throughout southern Patagonia. The lineomaculatus group was thought to include four species (L. lineomaculatus, L. silvanae, L. hatcheri and L. kolengh), but a recent molecular phylogenetic study (Breitman et al. 2011a) resolved three genetically distinct lineages within the species L. lineomaculatus, two of which were recently described as new species (L. avilae and L. morandae; Breitman et al. 2011b). While L. lineomaculatus, L. avilae and L. morandae are distributed throughout most of southern Patagonia, L. kolengh and L. silvanae are endemic to Lago Buenos Aires Plateau, and L. hatcheri is confined to high elevation areas of Asador Plateau in northwestern Santa Cruz (Cei 1986; Etheridge 1998; Christie 2002; Abdala & Lobo 2006).

Our specific goals here are to: (i) provide a well-resolved, time-calibrated phylogenetic/phylogeographic hypothesis for the lineomaculatus group and to assess clade and lineage origins within the historical Miocene-to-Pleistocene events of southern Patagonia; and (ii) qualitatively evaluate geographic concordance of refugia and phylogeographic breaks inferred from these lizards, with those proposed for plants and rodents in the same region. Because both phylogeography and species delimitation are integrative and iterative fields of study (Buckley 2009; Padial et al. 2010), this research will contribute to a foundation upon which future studies, based on additional data for these lizards and studies of codistributed species, will further clarify phylogenetic and speciation histories in this region.

Materials and methods

Sampling design

A total of 278 specimens of the lineomaculatus group were collected (Liolaemus lineomaculatus n = 109, L. kolengh n = 51, L. hatcheri n = 73, L. avilae n = 9, L. morandae n = 15 and L. silvanae n = 21) from 51 localities. Samples were collected across most of the distribution area of the group, from the Río Negro, Chubut and Santa Cruz Provinces (Fig. 1; Table 1). Liolaemus kingii, L. boulengeri, L. darwini, L. bibronii, L. gracilis, L. petrophilus and Phymaturus dorsimaculatus were used as outgroups (Table 1). Voucher specimens were catalogued in the herpetological collections of La Plata Museum, Argentina (MLP.S) and Centro Nacional Patagónico in
Puerto Madryn, Argentina (LJAMM-CNP). From this total sample, 196 individuals were used for molecular analyses.

DNA extraction, amplification and sequencing

Genomic DNA was extracted using the Qiagen® DNeasy® 96 Tissue Kit, following the protocol provided by the manufacturer. Two mitochondrial (12S and cyt-b; Kocher et al. 1989; Wiens et al. 2010; respectively) and one nuclear fragment (LDA12D, Camargo et al. 2012; the most informative selected from a screening of 36 genes; Appendix S1, Supporting information) were amplified following the PCR and sequencing protocols for mitochondrial and nuclear fragments described by Morando et al. (2003, 2004) and Noonan & Yoder (2009), respectively. The cytochrome b fragment was sequenced for 196 samples of the lineomaculatus group (3.76 per locality and 16 per lineage, on average) and several outgroups, the sequences were used to construct Bayesian and maximum-likelihood (ML) trees (Appendix S2, Supporting information; see ‘Phylogenetic relationships and clade/lineage identity’ below). To represent most of the cyt-b variation, and following the sampling design proposed by Morando et al. (2003), two to four individuals from each lineage recovered in the Bayesian cyt-b tree were selected for further amplification of 12S (n = 45) and LDA12D (n = 39) fragments.

Sequences were edited using sequencher v4.8. (™Gene Codes Corporation Inc. 2007) and checked by eye to maximize blocks of sequence identity. Alignments were performed with mafft (Katoh et al. 2002). Seven indels were identified in the 12S data set (five of one bp and two of two bp in length), and eight indels were identified in the LDA12D data set (three of one bp, three of two bp, one of three to five bp, and one of 18 bp in length). The cyt-b open reading frame was confirmed by translation into amino acids; all sequences and alignments are deposited in GenBank and in Dryad, respectively (GenBank accession nos. JX522219–JX522246; Dryad doi:10.5061/dryad.n5d5t). Nuclear gene recombination was tested using RDP v3.44 (Martin & Rybicki 2000; Heath et al. 2006). Cytochrome b was used in all the analyses described later, while LDA12D and 12S fragments were used to generate a concatenated alignment for Bayesian and Likelihood analyses, to compare tree topologies across genes and to estimate divergence time with a fossil calibration; the nuclear gene was also used to construct a haplotype network.

Phylogenetic relationships and clade/lineage identity

We used the word ‘lineage’ to identify species or candidate species (see Discussion) that are well-supported clades that include several ‘haplogroups’; haplogroups are composed of one or several individuals from one or more localities. To identify clades/lineages and relationships within the focal group, we first identified cyt-b haplotypes with collapse v1.2 (Posada 2004) and performed separate Bayesian and ML analyses for each gene (12S, LDA12D and cyt-b haplotypes), for the mitochondrial gene data set (cyt-b+12S) and for the nuclear and mitochondrial genes. The best-fit evolutionary model for each gene (cyt-b: TrN+I+G; 12S: TiM3+G; LDA12D: HKY) was selected using the corrected Akaike information criterion in jmodeltest v0.1.1 (Posada 2008). We are aware that gene trees may not recover the same topology as species tree (Maddison & Knowles 2006), but we do not evaluate species trees here due to our limited nuclear data (one locus). Bayesian analyses were
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<td>West</td>
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<td>$N_{\text{seq}}$</td>
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$N_{\text{LOC}}$, number of locality; $N_{\text{ind}}$, number of individuals collected by site; $N_{\text{seq}}$, number of individuals sequenced for Cyt-$b$; $N_{\text{hap}}$, number of haplotypes per locality. Names in parenthesis represent individuals from type localities (Lineo LT) and the new species described by Breitman et al. 2011b (L. morandae and L. avilae). “Lin.” and “pop” mean Lineage and population, respectively. Results of phylogenetic, network, SAMOVA analyses and clade identities are shown.
performed in MRBAYES v3.1.2 (Ronquist & Huelsenbeck 2003) using four heated Markov chains (default heating values) sampled at intervals of 1000 generations and run for 50 million generations. The equilibrium samples (after 25% ‘burn-in’) were used to generate a 50% majority-rule consensus tree, and posterior probabilities (PP) ≥ 0.95 were considered significant (Huelsenbeck & Ronquist 2001). Likelihood analyses were conducted using RAxML v7.0.4 (Stamatakis 2006), based on 1000 rapid bootstrap analyses for the best ML tree; strong nodal support was inferred for bootstrap values ≥ 70 (Hillis & Bull 1993; with caveats).

We used statistical parsimony to construct networks of the cyt-b and LDA12D sequences (Templeton et al. 1992) using TCS v1.21 (Clement et al. 2000) with the default connection significance (95%), and we qualitatively compared haplotype distributions between markers.

Lastly, using the cyt-b data set, we searched for genetically homogenous groups of populations (K) that were maximally differentiated from other groups using SAMOVA v1.0 (Dupanloup et al. 2002). Two independent runs were performed for the lineocamalactus and hatcheri clades, analyses were performed with ‘K’ values ranging from 2 to 20 and 2 to 8, respectively; the kolengh + silvanae clade was not analysed because samples were collected from only two localities. Analyses were conducted using 5000 independent annealing processes, and the best grouping option was identified based on the highest FCT score (Dupanloup et al. 2002).

**Genetic differentiation and divergence time analysis**

Cytochrome b genetic distances between the main lineages were estimated using the Arlequin program (Excoffier et al. 2005). Divergence times among main clades and lineages were calculated using BEAST v1.6.1 (Drummond & Rambaut 2007) and likelihood ratio tests (LRT) were performed using JModeltest to assess deviation from a strict molecular clock. The importance of calibrations in molecular dating is considered essential (Inoue et al. 2010; Parham et al. 2012), and calibration points are very informative when fossil data are placed close to the root of the focal taxa (Drummond et al. 2006). For Liolaemus, there is one recently available fossil [lizard dentary bones; MPEF 1442 and MLP 90-II-13-47, Museo Paleontológico Egidio Feruglio (MPEF), Museo de La Plata (MLP), both in Argentina], the taxonomic status of the fossil was identified as a Liolaemus on the basis of the following characters: open Meckel channel, extended splenial, the absence of posterior extension of dentary and tricuspid teeth (Albino 2008). The presence of remains of the genus Liolaemus from the Early Miocene of Gaiman (Sarmiento Formation, Chubut, Argentina) suggests a minimum age of ~20 to 18.5 Ma (Albino 2008) for the genus, according to the calibration of the Colhuehuapian Age proposed by Madden (2004). We therefore performed divergence dating analyses using this fossil calibration point and all gene sequences (Appendix S3, Supporting information).

A data set, including the three gene sequences for one to four individuals from each mitochondrial lineage and the above-mentioned outgroups, was utilized to perform the divergence time analyses (Appendix S3, Supporting information). This analysis was run using a relaxed uncorrelated lognormal clock model (cyt-b data do not conform to the strict molecular clock, LRT = 66.780625, P < 0.015), implemented in BEAST v1.6.2 (Drummond & Rambaut 2007; Ho & Phillips 2009). The fossil information was placed on the node representing the most recent common ancestor of the two Liolaemus subgenera (‘Tmra Liolaemus’) with the prior set to a lognormal distribution (mean: 1, standard deviation: 1.5, offset 18.5; Ho 2007). Two independent analyses were performed for 200 million generations and sampled every 1000 generations, with a GTR+G evolutionary model for the mitochondrial genes and a HKY model for the nuclear gene (selected by jModeltest), and assuming a Yule tree prior. Trees were summarized (discarding 10% as burn-in) using TREEANNOTATOR v1.6.1 (Drummond & Rambaut 2007). Convergence of estimated parameters was verified when effective sample sizes (ESS) were >150, using TRACER v1.5.0 (Rambaut & Drummond 2009).

Dating the divergence times for the clades and lineages allowed us to hypothesize past phylogeographic histories in a temporal context (McCormack et al. 2010), and while these types of inferences are important, we recognize the limitations of our approach (e.g. Graur & Martin 2004) and interpret our results cautiously as specific hypotheses for further testing.

**Genetic signature of past demographic histories**

To characterize past demographic events for the clades and lineages recovered in our analyses, summary statistics were calculated as follows using the cyt-b data set. We calculated standard molecular diversity indices (number of haplotypes: h; number of segregating sites: S; average number of differences between two random sequences: k; haplotype diversity: Hd; and nucleotide diversity: π) using DNASP v5.0 (Librado & Rozas 2009). Tajima’s D and Fu’s Fs (Tajima 1989; Fu 1997) are classical neutrality tests used to assess population demographic history, and both assume that populations have been in mutation–drift balance for a long period of time (Nei & Kumar 2000); when this is not the case due to sudden expansion, these indices usually have negative
values (although they do not provide information about the shape of the change). The $R_2$ test is considered a sensitive indicator for detecting demographic growth using small sample sizes (Ramos-Onsins & Rozas 2002). Tajima’s $D$, Fu’s $F_s$, and $R_2$ were calculated and the significance of these values was examined using 5000 samples simulated under a coalescent algorithm in DnaSP.

Date and shape of past lineage dynamics were estimated using Bayesian Skyline Plots (BSLP) (Drummond et al. 2005). Separate analyses were run using the HKY model (selected with JModeltest), with an uncorrelated relaxed clock (Drummond et al. 2006), and a mutation rate of 0.0223 substitutions per lineage per site per million years (Fontanella et al. 2012b), using BEAST. The number of groups ($k$) that the program required to run was chosen based on the number of samples ($n$) and taking into account that $k'$ could not be higher than $n$ (thus when $n = 4-5$, $k = 2-3$; $n = 8-29$, $k = 5$; $n = 59$, $k = 10$; Heled & Drummond 2008); Metropolis Coupling of Markov chains simulations were run with 30 million iterations twice for each lineage. Genealogies and model parameters were sampled every 1000 iterations, and after 10% burn-in, results were combined in LogCombiner and summarized results as BSLP after verifying convergence in Tracer, where all parameters had ESS values $>$200. Even though BSLP are widely used and seem to perform accurately (Minin et al. 2008; Ho & Shapiro 2011), choosing an excessive number of groups ($k$) can increase the error estimation (Heled & Drummond 2008). Bayesian skyline methods (Minin et al. 2008) were developed to provide an alternative model of demographic history with fewer parameters assuming that demographic sizes changed gradually over time (Ho & Shapiro 2011). Few empirical studies have used both methods together, and we generated Bayesian skyline plots (BSRP) using the same data and settings described previously. Our use of both methods permits assessment of the influence of different assumptions on the results of these analyses.

Results

Phylogenetic relationships and clade/lineage identity

Three genes were amplified for this study: cyt-b (length: 659 bp; 180 informative sites), 12S (length: 771 bp; 91 informative sites) and LDA12D (length: 627 bp; 40 informative sites). A total of 112 haplotypes were recovered from our original cyt-b matrix ($n = 196$). Gene trees recovered from both mitochondrial gene regions separately and combined were topologically concordant across phylogenetic analyses with no well-supported conflict, except for the position of two samples in the nuclear tree when compared with the mitochondrial tree (see following paragraph). Relationships recovered using the concatenated matrix of the nuclear plus mitochondrial markers were similar to those recovered in the mitochondrial gene tree (see support values in Fig. 2). Three clades were recovered within the *lineomaculatus* group (Fig. 2): *lineomaculatus*, *kolengkap + silvane* and *hatcheri*, all with high support ($PP > 0.95$; ML bootstrap $\geq 70$), but with poor resolution for the phylogenetic position of *L. hatcheri* (only Bayesian posterior probability for the nuclear plus mitochondrial genes was significant). The *lineomaculatus* clade includes nine lineages: one lineage represents the type locality of the species [lineage 2], two are referred to *L. avilae* [lineage 5] and *L. morandae* [lineage 7] in the study described by Breitman et al. (2011b), and three lineages are each comprised of singletons (Fig. 2, Table 1). The lineages divergence order within the *lineomaculatus* clade is recovered in a pectinate topology with the northernmost (lineage 9) sister to all others, which form successively more southern-derived lineages. Lineages 3 and 4 were the most southerly distributed ones and were recovered in a sister relationship to clade (lineage 1 + lineage 2), which are the most easterly distributed in Santa Cruz province.

Recombination was excluded for the nuclear gene (LDA12D), whose tree (not shown) recovered some concordant patterns relative to the mitochondrial tree (similar results between Bayesian and ML analysis): three well-supported clades were recovered in a tricotomy representing the *kolengkap + silvane* clade, the *lineomaculatus* clade and the *hatcheri* clade; although two individuals of *L. hatcheri* (9562 from locality 13 and 7264 from locality 34) were recovered interdigitated in the *lineomaculatus* clade, this could be caused by incomplete lineage sorting or introgression. Nested in the *lineomaculatus* clade we also recovered a monophyletic group formed by the individuals with the northernmost distribution (lineage 9 + lineage 8 + singleton 9093).

Network analysis performed on the cyt-b data set recovered ten different networks and two singletons (Fig. 3; Table 1) within the *lineomaculatus* group. Networks were concordant with the lineages recovered in the mitochondrial tree, with the exception of network 5 in which lineages 3 and 5 were connected by singleton 9545. Networks showed evidence of strong genetic structure, including no star-like connections, several haplotypes connected by more than 1–2 steps, high number of different haplotypes per locality; six localities (6, 23, 26, 34, 42 and 43) were characterized by more than five haplotypes. TCS analysis performed on the nuclear gene recovered two networks (Fig. 2): a small one composed of almost all the individuals from...
the *hatcheri* clade, and a bigger network composed of all the individuals from the *lineomaculatus* group, the *kolengh* + *silvanae* clade and two individuals from the *hatcheri* clade. In the big network, individuals from the *kolengh* + *silvanae* clade were recovered in a group highly differentiated from the rest of the individuals; a close relationship among individuals of the northernmost clade of the *lineomaculatus* clade (lineages 8, 9 and singleton 9093) was recovered, as was a close relationship among individuals from lineages 2, 3 and 4. Finally, a highly differentiated haplotype was identified from the southernmost lineage of the *lineomaculatus* clade (individual 7223, from locality 29). Two haplotypes (individual 7264 'locality 34' and individual 9562 'locality 13') belonging to the *hatcheri* clade were recovered mixed among haplotypes of the *lineomaculatus* clade.

Results for the *samova* analyses recovered nine lineages within the *lineomaculatus* clade and four haplogroups within the *hatcheri* clade (Appendix S4, Supporting information, Table I). Genetic structure was high among these clades (*amova*, permutation test $P < 0.00001$), most of the variation was partitioned among groups (78.27% and 69.51% in the *lineomaculatus* and *hatcheri* groups, respectively), and a small portion of variance was contained among-populations within groups (10.55%, 7.61%) or within populations (11.18%, 22.89%; Appendix S4, Supporting information). Using *samova*, four haplogroups were identified in the *hatcheri* clade (I, II, III and IV) and all the samples belonging to locality 37 were recovered in *hatcheri* 'I' haplogroup. In contrast, *cyt-b* phylogenetic tree reconstruction did not recover members of this locality in a clade; in other
words, except for individuals of locality 37, similar grouping patterns were found between SAMOVA and the cyt-b phylogenetic reconstruction. This was not surprising because SAMOVA groups populations (defined as group of individuals from one locality) and not individuals as phylogeny and network reconstructions do (Dupanloup et al. 2002). Thus, following the phylogenetic results, we assigned individuals of locality 37 to each hatcheri population according to the mitochondrial tree (individual 9493 belonging to hatcheri population ‘b’, 9494 to hatcheri population ‘c’ and 9495-97 to hatcheri population ‘a’; Appendix S2, Supporting information, Table 1, Fig. 3).

For the lineomaculatus clade, SAMOVA grouping was concordant with network analysis for all lineages except for lineages 5 and 6, which were recovered in one network (Figs. 2 and 3, Table 1); two singletons were recovered within Group 5, and singleton 9093 was recovered within Group 9 (Appendix S4, Supporting information, Table 1).

Genetic differentiation and divergence time analyses

Most of the pairwise genetic distances within each lineage were small. Distances among main lineages of the lineomaculatus clade were much higher than 3%, except for distances between lineages 1 vs. 2 (2.28%) and 5 vs. 6 (2.88%). Distances within hatcheri and kolengh + silvanae clades were ~<2% (mean = 1.47, min = 1.05, max = 2.01) (Appendix S5, Supporting information).

Divergence times estimated from the fossil calibration between main clades were inferred to be in the Miocene, the divergence between hatcheri and (kolengh + silvanae) clade was ~9.98 Ma [95% HPD = 5.78–14.49], while divergence between the kolengh + silvanae clade and lineomaculatus clade occurred ~8.13 Ma [95% HPD = 4.64–12.25]. Splits among most of the lineages of the lineomaculatus clade and within hatcheri and kolengh + silvanae clades occurred during the Late Pliocene; lineages 1 and 2 (within the lineomaculatus clade) diverged during the Pleistocene ~1.33 Ma [95% HPD = 0.43–2.47] (Fig. 2; Appendix S3, Supporting information).

Genetic signature of past demographic histories

We found high haplotype diversity (except for the kolengh clade, Table 2) and intermediate to low nucleotide diversity in all lineages. The lowest values of nucleotide diversity were found in lineage 5 and 6 (0.005, 0.007, respectively), L. kolengh (0.0001), and hatcheri populations a, b, c and d (0.002, 0.001, 0.002 and 0.005, respectively). Neutrality tests were significant only for lineage 1 (n = 26; R₂ = 0.07, P = 0.02), lineage 6 (n = 11; R₂ = 0.10, P = 0.03; Fu’s Fₛ = −7.40, P = 0.0004), lineage 7 (n = 14; R₂ = 0.09, P = 0.03) and lineage 8 (n = 4; R₂ = 0.07, P = 0.01; although sample size was low).
<table>
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<th>N</th>
<th>S</th>
<th>h</th>
<th>k</th>
<th>Ho</th>
<th>± SD of Ho</th>
<th>D (95% C.I.)</th>
<th>R^2 (95% C.I.)</th>
<th>P R^2 coal</th>
<th>Fu’s F_s (95% C.I.)</th>
<th>P Fu’s F_s coal</th>
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<td>26</td>
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<td>0.008 ± 0.0017</td>
<td>−1.43 (−1.72 to 1.81)</td>
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<td>0.07 (0.07−0.19)</td>
<td>0.02</td>
<td>−1.21 (−4.80 to 5.67)</td>
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<td>10</td>
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<td>0.4</td>
<td>0.14 (−1.70 to 1.64)</td>
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<td>0.011 ± 0.0025</td>
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<td>−0.93 (−3.02 to 4.54)</td>
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<td>−0.05 (−1.84 to 1.74)</td>
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<td>0.007 ± 0.0011</td>
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<td>0.10 (0.09−0.233)</td>
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<td>0.008 ± 0.0023</td>
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<td>0.012 ± 0.0024</td>
<td>−0.84 (−0.84 to 2.07)</td>
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<td>0.06 (0.06−5.09)</td>
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<td>0.9</td>
<td>0.003 ± 0.0007</td>
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<td>0.012 ± 0.0010</td>
<td>0.73 (−1.75 to 1.79)</td>
<td>0.8</td>
<td>0.15 (0.06−1.18)</td>
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<td>13</td>
<td>3</td>
<td>2</td>
<td>0.7</td>
<td>0.26</td>
<td>0.001 ± 0.0006</td>
<td>−0.49 (−1.67 to 1.96)</td>
<td>0.32</td>
<td>0.13 (0.12−0.25)</td>
<td>0.13</td>
<td>2.03 (−2.28 to 3.64)</td>
</tr>
<tr>
<td>L. silvanae</td>
<td>16</td>
<td>23</td>
<td>7</td>
<td>8.9</td>
<td>0.86</td>
<td>0.013 ± 0.0008</td>
<td>1.19 (−1.77 to 1.73)</td>
<td>0.91</td>
<td>0.19 (0.08−0.20)</td>
<td>0.92</td>
<td>2.96 (−4.59 to 4.91)</td>
</tr>
<tr>
<td>Hatcheri clade</td>
<td>59</td>
<td>40</td>
<td>27</td>
<td>8.2</td>
<td>0.95</td>
<td>0.012 ± 0.0004</td>
<td>−0.14 (−1.64 to 1.85)</td>
<td>0.5</td>
<td>0.09 (0.05−0.16)</td>
<td>0.49</td>
<td>−5.59 (−8.19 to 7.27)</td>
</tr>
<tr>
<td>Hatcheri pop. a</td>
<td>19</td>
<td>9</td>
<td>6</td>
<td>1.6</td>
<td>0.69</td>
<td>0.002 ± 0.0008</td>
<td>−1.20 (−1.74 to 1.91)</td>
<td>0.1</td>
<td>1.13 (0.08−0.23)</td>
<td>0.48</td>
<td>−0.83 (−2.89 to 4.31)</td>
</tr>
<tr>
<td>Hatcheri pop. b</td>
<td>14</td>
<td>10</td>
<td>9</td>
<td>2.5</td>
<td>0.94</td>
<td>0.001 ± 0.0006</td>
<td>−0.75 (−1.79 to 1.85)</td>
<td>0.25</td>
<td>0.12 (0.09−0.22)</td>
<td>0.22</td>
<td>−3.80 (−3.96 to 4.05)</td>
</tr>
<tr>
<td>Hatcheri pop. c</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1.6</td>
<td>0.7</td>
<td>0.002 ± 0.0010</td>
<td>−1.09 (−1.09 to 1.64)</td>
<td>0.3</td>
<td>0.29 (0.14−0.4)</td>
<td>0.73</td>
<td>0.27 (−3.30 to 3.02)</td>
</tr>
<tr>
<td>Hatcheri pop. d</td>
<td>21</td>
<td>12</td>
<td>9</td>
<td>3.3</td>
<td>0.9</td>
<td>0.005 ± 0.0004</td>
<td>0.04 (−1.76 to 1.81)</td>
<td>0.57</td>
<td>0.13 (0.07−0.20)</td>
<td>0.54</td>
<td>−1.19 (−4.61 to 4.67)</td>
</tr>
<tr>
<td>lineomaculatus clade</td>
<td>193</td>
<td>206</td>
<td>112</td>
<td>53</td>
<td>0.98</td>
<td>0.081 ± 0.0007</td>
<td>1.66 (−1.5 to 1.88)</td>
<td>0.96</td>
<td>0.12 (0.04−0.13)</td>
<td>0.96</td>
<td>−12.1 (−19.30 to 18.45)</td>
</tr>
</tbody>
</table>

N, number of samples; S, number of segregating sites; h, number of haplotypes; k, average number of differences between two random sequences; Ho, haplotype diversity; π, nucleotide diversity and standard deviation. Tajima’s D, R^2 and Fu’s F_s values, confidence intervals and P values are shown. Enlarged and bolded fonts identify the statistically significant results.
Similar results were recovered using BSLP or BSRP, with relatively constant or only slightly different demographic size changes through time (Appendix S6, Supporting information). No strong signals of demographic expansions or declines were recovered; weak signals of demographic expansion were recovered for lineage 6 (0.07 Ma), and expansion (0.15 Ma) followed by a slight demographic decline (0.0125 Ma) was inferred for the hatcheri clade (Appendix S6, Supporting information). We are aware that sample size could be an issue here and that more sampling is needed for some lineages, but, our results seem to be robust to sample size differences across lineages. Moreover, despite variable sample sizes among lineages (mean = 16, min = 4, max = 59), no differences were found between BSLP vs BSRP results (when 95% HPD was taken into account), suggesting that both tests perform similarly although, as expected, the BSRP showed smoother curves (Minin et al. 2008; Ho & Shapiro 2011).

Because results varied among neutrality tests and Bayesian demographic plots, we conservatively inferred that only lineages 6 and 7 have experienced past demographic changes, because both analyses detected signals of the same directional changes. The hatcheri populations presented low nucleotide diversity, little within population pairwise divergence, and BSLP/BSRP showed signals of demographic changes through time (for the hatcheri clade). All of these analyses indicated that these populations had experienced past demographic expansions, but as neutrality tests did not show significant signals of demographic changes, we hypothesize past demographic expansions in these populations but with caveats.

Discussion

General patterns within the Liolaemus lineomaculatus group

Three main clades, whose divergence was inferred to occur during the Miocene, are recovered within the lineomaculatus group: hatcheri, kolengh + silvanae and lineomaculatus (Fig. 2). The hatcheri clade is restricted to the Asador Plateau (Fig. 1, dark purple squares) and is genetically structured into four haplogroups (East, Central, South and West) with low to moderate genetic distances between them (Fig. 3, Appendix S5, Supporting information), these populations may have experienced recent demographic expansions. The kolengh + silvanae clade includes two nominal species (Fig. 2), both known only from type localities (Fig. 1; localities 40 and 43, respectively) northwest and northeast of the Lago Buenos Aires Plateau. Several morphological differences are diagnostic of both species (Abdala & Lobo 2006), and our time-calibrations suggest a late Pliocene (~2.26 Ma [95% HPD = 0.61–4.67]) divergence. Moreover, Liolaemus kolengh has very low haplotype and nucleotide diversity values, suggesting a very recent speciation event in a lineage that has remained relatively small, possibly under a selective regime that fostered morphological divergence in both species. Lizards of the lineomaculatus clade are present throughout the study area except in localities where L. hatcheri and L. kolengh occur. The lineomaculatus clade includes nine lineages with high genetic distances among them (except for lineages 1 and 2), and three of them have been described as different species (L. lineomaculatus, L. avilae and L. morandae). All of these lineages are allopatric except at one locality where individuals from lineages 1 and 3 were collected together (Fig. 1, locs. 24 and 33, respectively; different locality numbers were given to the same area for different lineages).

Most of our analyses were performed using the cyt-b gene, and we are aware that the nature of this marker (rapid saturation, matrilineal history) could mislead interpretations. However, a well-resolved mtDNA gene tree is an excellent starting point for phylogeographic investigations, and mtDNA data can be taken as reliable indicators of female geographic population structure and ‘first pass’ identification of ‘candidate’ species (Morando et al. 2003). The incorporation of multiple unlinked nuclear loci will increase the strength of phylogeographical inferences, but nuclear loci may or may not be concordant with mtDNA patterns, suggesting different evolutionary processes (Zink & Barrowclough 2008). Here, we incorporated one nuclear gene and found that the geographic patterns of variation in LDA12D were largely concordant with the mtDNA results.

‘Candidate species’ within the lineomaculatus clade

Liolaemus species have been recognized on the basis of morphological differences, but several recent publications have first identified ‘candidate species’ on the basis of mtDNA divergence (well-sampled and well-supported haploclades that show geographic concordance; Morando et al. 2003), which have then been described based on morphological discontinuities (for example see Breitman et al. 2011a,b,c). Those studies have used an ‘arbitrary’ percentage of >3% uncorrected mtDNA divergence to identify ‘candidate species’ that merit detailed taxonomic study. The use of an ‘arbitrary’ 3% mtDNA is justified by two features: morphological diagnosability and geographic isolation. From a morphological perspective, the mean sequence divergence between morphologically described sister species within Liolaemus is ~3.1% [based on recalculations of...
data from Martínez (2012)]. Martinez estimated a mean of 4% cyt-b divergence using several sister species pairs of *Liolaemus*, and here, we recalculated this mean excluding the pair *L. somuncurae*–*L. uptoni* because they are not sister species (Breitman et al. 2011a), and specifically, within the *L. lineomaculatus* section, the threshold of mtDNA differentiation is 2.23% (recalculated from Martínez 2012). Our adjustments suggest that sister species of *Liolaemus* are in general morphologically diagnosed with an average of ~3% mtDNA divergence. Based on our divergence estimates, 3% mtDNA differentiation requires ~1.5 million years to accumulate.

In this study, we find cyt-b distances >3% between geographically isolated lineages of the *lineomaculatus* group and hypothesize that these are ‘candidate species’ based on the arguments described in the above paragraph. Specifically, we find the highest values of mtDNA differentiation in lineage 9 (estimated to be the oldest ~6 Ma); this is the northernmost lineage and the most morphologically differentiated (M. F. Breitman, personal observation) from the others, suggesting that it is a well-corroborated candidate species. Lineages 5 and 7 were recently described as new species (*L. avilae* and *L. morandae*, respectively; Breitman et al. 2011b), and our evidence suggests that lineages 3, 4, 6 and 8 should be also considered as candidate species for which more integrative studies are needed.

**The role of Miocene-to-Pleistocene glaciations in diversification**

The oldest known Patagonian glaciation took place ~7–5 Ma in the late Miocene (Rabassa et al. 2005); this event may have promoted the origin of the three main clades of the *lineomaculatus* group. This period was followed by a warming phase, in the Pliocene, during which geodynamic events and climatic fluctuations probably favoured the diversification of many groups (Cosacov et al. 2010; Cione et al. 2011; Compagnucci 2011), especially *Liolaemus* (Albino 2008, 2011). The Pliocene was a warm period characterized by multiple large glaciations (~3.5 Ma; Rabassa et al. 2005), and this timing is correlated with our estimate of the divergence times of lineages 3–9 and within the *kolengh + silvane* and *hatcheri* clades. Our divergence estimates also suggest a Pleistocene role for the separation of sister lineages (1 ± 2). At this time, the Patagonian climate shifted to cooler conditions (Lisiecki & Raymo 2007; Compagnucci 2011), with latitudinal and altitudinal vegetation shifts in response to recurrent glacial oscillations (Quatrochcio et al. 2011).

During the full glacial expansions of the Pliocene and Pleistocene, water flows were significantly higher than at the present, and they endured for several thousands of years. The capacity for erosion and the magnitude of water transported were also increased by lowered sea level; followed by shorter periods called ‘terminations’ representing the abrupt ending of the main glacial events. During these ‘terminations’, large volumes of water were released as a result of the intensive melting of the Cordilleran ice sheets (Martínez & Kutschker 2011). These past events are inferred from depositions of rocks called ‘Rodados Patagónicos’, for which there is extensive evidence along the Río Chico and Río Coig (Río Coyle on some maps) basins in central-south Santa Cruz (Martínez & Coronato 2008; Martínez & Kutschker 2011).

**Fig. 4** Phylogeographic breaks and refugia from southern Patagonia, modified and updated from Sérsic et al. (2011; Fig. 2), with the addition of lizard data from this study. Phylogeographic breaks (lines), hypothesized refugia (grey areas) and Lago Buenos Aires and Asador plateaus (LBAP and AP dotted lines, respectively) are shown. Breaks and refugia areas inferred for species of the *Liolaemus lineomaculatus* group are shown with ‘L’. Breaks ‘R’ and ‘P’ correspond to those proposed for rodents and for plants, respectively. The unnumbered break was not proposed in Sérsic et al. (2011), and the number of taxa for each break is given in parentheses (names are preserved from Sérsic et al. 2011). All refugia shown here were proposed based on plants, one of which is also supported by the lizards studied here (marked with the asterisk). Breaks that are concordant with water courses are: Break 10 following the Magellan strait, Break 9p following the Río Coig Basin and Break 8 following the Río Chico basin; these water courses were strong glaciofluvial rivers during the Pliocene and Pleistocene and have almost certainly been major causes of fragmented habitats and influenced diversification in some animals and plants from Patagonia. Lago Buenos Aires and Asador plateaus are both proposed as refugia based on this study.
2011). The volume and velocity of water in these two basins (breaks 8 and 9, in Fig. 4) during the sea level draw down followed by termination events, would provide long periods of time during which both rivers were much larger than today. We hypothesize that fragmented habitats influenced relatively recent patterns of diversification in several groups of southern Patagonia; these include lizards (this study), plants (Mathiasen & Premoli 2010; Sede et al. 2012), rodents (Pardiñas et al. 2011) and freshwater fishes (Ruzzante et al. 2011); see also Sérsic et al. (2011) for a summary of patterns.

Shared phylogeographic patterns among codistributed taxa in southern Patagonia

Relative to northern Patagonia, few phylogeographic studies have focused on southern Patagonian clades. Sérsic et al. (2011) summarized all phylogeographic studies of Patagonian plants and terrestrial vertebrates and mapped all proposed phylogeographic breaks and refugia (their Fig. 2). Figure 4 presents an update of a section of the Sérsic et al. (2011) map with our results for the lineomaculatus group. In Fig. 4, we number these breaks following Sérsic et al. (2011) and identify each by taxon: lizards (‘L’), rodents (‘R’) and plants (‘P’). In southern Patagonia, two breaks for terrestrial vertebrates (rodents, breaks 9, 10) and five breaks for plants (5, 6, 8, 9, 10) were previously proposed; here, we show that five of these also represent phylogeographic breaks for lizards (5, 6, 8, 9P, 9R). The number of taxa for which each break is resolved is shown in subscripted parentheses: (1) 9/LR1;2, at latitude 45°S; (2) 5/LP1;3, 43°S; (3) 6/LP1;1, 50°S; (4) 8/LP1;1, following the Río Chico basin; and (5) 9/LP1;1, following the Río Coig basin. In this study, we present evidence of a new phylogeographic break at ~47°S (break with no number in Fig. 4) recently proposed for the tree Nothofagus pumilio (Mathiasen & Premoli 2010). There is one other proposed phylogeographic break for the Strait of Magellan, 10/RP1;1, which is not included in the distribution of our focal group (Fig. 4).

Within the lineomaculatus clade a north-to-south diversification pattern was recovered, which is concordant with other Liolaemus clades, rodents and plants (Morando et al. 2003, 2004; Cosacov et al. 2010; Lessa et al. 2010; reviewed in Sérsic et al. 2011). Moreover, only two studies (to our knowledge) have presented divergence estimates for the phylogeographic breaks of southern Patagonian taxa included here, those estimations were made for breaks 8 and 5, by Cosacov et al. (2010), for the annual herb Calceolaria polyrhiza, and Premoli et al. (2012), for trees of the genus Nothofagus, respectively. For break 8, Cosacov et al. (2010) inferred a divergence time of ~1.16–1.45 Ma; this is slightly younger that our estimate (~2.48 Ma [95% HPD = 1.1–4.2]) between the lineages (1 + 2) and (3 + 4), which are separated by this same break. Across break 5, our estimates of divergence between lineages 9 and 8 (5.97 Ma [95% HPD = 3.2–9.36]) were again slightly older that the 2.96–4.6 Ma estimates for Calceolaria from northern and southern Patagonia (Cosacov et al. 2010). However, both of these estimates for break 5 are much younger than the 32 Ma estimated for Nothofagus by Premoli et al. (2012). This older estimate is in the line with the ‘pre-Quaternary’ fragmentation and isolation estimated for trees of this region by other authors (Mathiasen & Premoli 2010; Soliani et al. 2011). These results suggest that differentiation in Liolaemus may have predated differentiation in Calceolaria, while differentiation in Nothofagus is much older than both.

Despite these differences in timing of divergence, the shared patterns of divergence between the annual plants and lizards suggest the possible presence of ‘suture zones’ (Remington 1968) in some regions (breaks 5, 6, 8, 9P, 9R). A suture zone is defined as a cluster of hybrid zones, contact zones or phylogeographic breaks (as extended by Swenson & Howard 2005), which may be associated with present-day physiographic features. Such regions are natural laboratories for the studies of adaptation, divergence and speciation (Moritz et al. 2009), and future phylogeographic studies can now focus on dense sampling of multiple taxa in these regions. Future studies, when coupled with GIS tools (either for spatial analyses of genotypes to resolve the ‘fine structure’ of contact zones, or the inclusion of niche models), and molecular analyses can provide deeper resolution of the spatial components of evolutionary processes (Swenson 2008; Moritz et al. 2009).

How lizards survived blizzards? Refugia hypotheses

New studies have clarified some of the geological and climatic events that have shaped Patagonia and since then several phylogeographic refugia (i.e. in situ persistence during Pleistocene glaciations) have been proposed based mainly on plants (Sérsic et al. 2011). Studies of several species complexes of Liolaemus inhabiting northern Patagonia have revealed signals of demographic expansion and recent colonization (Morando et al. 2003, 2004, 2007; Avila et al. 2006), particularly in central Chubut and northern Santa Cruz. In contrast, our study recovers signals of genetically structured lineages that have persisted in situ in the absence of expansions. Interestingly, Fontanella et al. (2012a) working on the northern species L. petrophilus, found that the species was structured in two haploclades, one of which had a signature of LGM stability and its sister haplode showed a signal of demographic expansion.
The Asador Plateau [Fig. 1 (dark purple squares), Fig. 4] has been proposed as a refuge for in situ survival of plants and rodents (Cosacov et al. 2010; Lessa et al. 2010; Mathiasen & Premoli 2010; Sede et al. 2012; Villa-Martínez et al. 2012); our evidence of the hatcheri clade age (3.44 Ma [95% HPD = 1.2–6.5]), combined with a demographically stable genetic signature (high genetic structure, high haplotype diversity) suggests that the survival of a common ancestor may have been possible there. In contrast, a demographic expansion signature within each one of the hatcheri populations suggests that population increases may have occurred after diversification (~1.8–2 Ma [95% HPD = 0.52–3.67]). Sampling of additional populations and genes and further studies are needed to understand the evolutionary history of these lizards that inhabit a hypothesized glacial refugium.

The Lago Buenos Aires Plateau (Fig. 4) is inhabited by the kolengh + silvanae clade, available geological data on this Plateau suggest that it was partially affected by several glacial advances, with the first being dated ~1.3 Ma and followed by at least six glacial advances between 0.0227 and 0.0144 Ma (Rabassa et al. 2011). Glaciations were present in this area surrounding the Plateau, the Buenos Aires and the Pueyrredón lakes and their valleys were covered by glaciers and permafrost was present on the plateau; moreover, because the mean annual air temperature of the plateau is below 0 °C, permafrost is nowadays present on several places of the Lago Buenos Aires Plateau (Hubbard et al. 2005; Trombotto 2008). The age of this clade and its geographical restriction to this plateau suggest that it may have survived in situ through multiple glaciation–deglaciation cycles.

The north–south zone along the eastern flank of the Andes (between 47°S and 51°S) has been identified as a refugial area for plants of the genera Calceolaria, Fitzroya, Hypochaeris and Nothofagus (Sérsic et al. 2011), but not for terrestrial vertebrates. Our findings (marked with asterisk in Fig. 4) suggest that this area (between Argentino and Viedma lakes) was also a refugium for in situ persistence of some populations of the lineomaculatus clade. Several terminal moraines were mapped in the valley of the Argentino Lake indicating the occurrence of at least nine glacial advances from the Pliocene to the LGM (Rabassa 2008). Glaciations occurred in the period 2.1–1.0 Ma, in the valleys surrounding the lakes Viedma and Argentino (Rabassa et al. 2011).

Finally, we found weak signals of demographic expansion only for lineages 6 and 7 dating to about 0.07 and 0.05 Ma, respectively, between the last southern Patagonian glaciation (0.140–0.180 Ma) and the LGM (~0.025–0.016 Ma; Rabassa et al. 2005).

More detailed comparative studies are needed to test these hypotheses, to assess how geological and climatic events have influenced patterns of diversification observed in other co-distributed taxa, to find the locations of proposed refugia and to estimate the genetic structure and divergence time of each lineage that may have persisted through multiple glacial cycles. In southern Patagonia, several organisms appear to have survived in situ through glacial advances; thus, it will not be surprising if future studies find further evidence for shared refugia and phylogeographic breaks.

Conclusions

This is the first phylogeographic study of lizards from southern Patagonia using nuclear and mitochondrial data in a multispecies framework. We found that geological and climatic conditions affecting Patagonia since the Miocene seems to have strongly influenced the diversification of the lineomaculatus group. In contrast with other species of lizards from northern Patagonia, most of the lineages included in this study are genetically and geographically structured and do not show evidence of demographic expansions, suggesting in situ survival for several lineages throughout glaciation–deglaciation cycles. If true, these patterns suggest considerable thermoregulatory plasticity in these lizards, because regional environments were dominated mainly by permafrost and arid conditions (Trombotto 2000). Within the lineomaculatus clade, some candidate species were identified. We present the first evidence that many of the phylogeographic breaks and refugia previously proposed for plants and rodents in southern Patagonia are also present in lizards, and we predict that wider taxonomic sampling of other terrestrial groups will reinforce these patterns.

Acknowledgements

We thank J. Bagley for help with data analyses and graphics, and C.H.F. Perez, N. Feltrin, N. Frutos, M. Kozykariski, I. Minoli, M. Nicola, R. Martínez, C. Zanotti, D.J. Alvarez and S. Quiroga for assistance in field collections, A. Camargo for help in the laboratory, T. Dozo for help in the fossil description, and J. Rabassa, J. Strelin and D. Trombotto for help with permafrost information. We also thank other members of the Grupo de Herpetología Patagónica for assistance in animal curation procedures. This research benefited from valuable discussions and comments from J. Bagley, A. Cosacov, I. Minoli, C. Medina, M. Nicola and two anonymous reviewers. Financial support was provided by the following grants: ANPCYT-CONICET (LJA) PICT 2006-506, ANPCYT-CONICET 33789 (MM), and a doctoral fellowship (MFB) from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The NSF-PIRE award (OISE 0530267) supported collaborative research on Patagonian Biodiversity to the following institutions (listed alphabetically): Brigham Young University, Centro Nacional Patagónico (AR), Dalhousie University, Instituto Botánico
Darwinion (AR), Universidad Austral de Chile, Universidad de Concepción (CH), Universidad Nacional del Comahue (AR), Universidad Nacional de Córdoba (AR) and University of Nebraska. We also thank the Chubut Provincial authorities (MFB) for financial support; and the BYU Kennedy Center for International Studies, Department of Biology, and the Bean Life Science Museum, for supporting study-abroad student exchanges and field work. We thank the fauna authorities from Chubut, Santa Cruz and Rio Negro Provinces for collection permits.

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Screened genes.
Appendix S2 Bayesian cyt-b tree for all samples.
Appendix S3 Divergence time analysis.

Appendix S4 SAMOVA.
Appendix S5 Genetic distances.
Appendix S6 Bayesian Skyline and skyride plots.

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