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Multilocus phylogeography of the Patagonian lizard complex *Liolaemus kriegi* (Iguania: Liolaemini)

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This study presents a detailed phylogeographical analysis of one of the most conspicuous groups of lizards in northwestern Patagonia, the *Liolaemus kriegi* complex. This region is geographically very complex as a result of Andean orogeny and subsequent volcanism coupled with a long history of glaciations and climatic changes. For 247 individuals we sequenced one mitochondrial gene (cytochrome *b*) and for a subset we sequenced another mitochondrial gene [12S ribosomal RNA (12S)] and two nuclear fragments [kinesin family member 24 (KIF24) and BA3 ribosomal RNA (BA3)]. We obtained gene trees and mitochondrial and nuclear haploytpe networks, and estimated genetic distances between the main lineages and basic molecular diversity indices. We also performed spatial analysis of molecular variance (SAMOVA) and Bayesian Skyline Plot (BSP) analyses, and concordant patterns from different lines of evidence permitted delimitation of seven lineages: two described species, *Liolaemus buergeri* and *Liolaemus tregenzai*; four candidate species, *Liolaemus* sp. A, *Liolaemus* sp. B, *Liolaemus* sp. C, and *Liolaemus* sp. D; and one lineage that includes all individuals from the geographical range of *Liolaemus ceii* and *L. kriegi*, referred to as *L. kriegi* + *L. ceii*. We discuss the evolutionary processes that may contribute to the origin of these lineages and their taxonomic and conservation implications. *Biological Journal of the Linnean Society*, 2014, **113**, 256–269.

ADDITIONAL KEYWORDS: *Liolaemus kriegi* complex – mitochondrial gene – northwestern Patagonia – nuclear gene.

INTRODUCTION

Northwestern Patagonia (southern Mendoza, Neuquén, and western Río Negro Provinces in Argentina) is a geographically complex region characterized by mountains higher than 4500 m, large volcanic fields, deep canyons, and high plateaus. These features are the products of a long Andean orogenic history coupled with sporadic volcanic eruptions and a history of glacial advances and retreats that produced pronounced climatic changes throughout the last Myr (Rabassa & Clapperton, 1990; Ramos & Kay, 2006; Ramos & Ghiglione, 2008; Martínez & Kutschker, 2011; Ramos & Folguera, 2011). As a result, the landscape is an intricate physiography that probably fostered multiple population-divergence processes across different geographical and temporal scales (Morando *et al*., 2013) and which may explain the unusually high number of lizard species in the region (Corbalán *et al*., 2011; Avila, Martinez & Morando, 2013).

During the last 40 years, lizard field surveys in southern Mendoza have revealed a high number of endemic species with restricted geographical ranges, with some taxa confined to a single mountain or plateau. Almost half of the lizard species from this area are endemic (Corbalán *et al*., 2011) and it has been proposed as the centre of origin for several lizard genera, including *Pristidactylus* Fitzinger, 1843

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(Lamborot & Díaz, 1987; Scolaro, Videla & Cei, 2003), *Leiosaurus* Duméril & Bibron, 1837, *Diplolaemus* Bell, 1843 (Cei, Scolaro & Videla, 2003), and *Phymaturus* Gravenhorst, 1838 (Scolaro *et al*., 2003; Díaz Gómez, 2009). *Liolaemus* is the most species-rich genus in this region, with more than 30 species distributed along a relatively narrow band of the Andes from southern Mendoza to northern Chubut Province, and also including the Altos Andes and Patagonian steppe eco-regions. This diversity is represented in several groups, including the *Liolaemus kriegi* complex; species in this complex are characterized by large stout bodies and they are usually saxicolous, viviparous, and omnivorous (Cei, 1986).

Here we present a detailed phylogeographical analysis of the *L. kriegi* complex, which extends from 37°S (near Vergara Pass in VII Region, Chile, and Malargüe Department, Mendoza Province, Argentina), to its southern distributional limit at the northern edge of Chubut province at 42°S (Morando, Avila & Sites, 2003; Pincheira-Donoso & Núñez, 2005) (Fig. 1). This complex traditionally included three morphologically distinct species, namely *Liolaemus buergeri*, *L. kriegi*, and *Liolaemus ceii* (Cei, 1986). Some authors have included *Liolaemus cristiani* in the *L. kriegi* complex (Nuñez, Navarro & Loyola, 1991; Lobo, Espinoza & Quinteros, 2010), based on few morphological characteristics, but others have considered *L. cristiani* as part of the *Liolaemus neuquensis* group (Cei & Videla, 2002, 2003; Pincheira-Donoso & Núñez, 2005), and in ongoing molecular studies, C. D. Medina (unpubl. data) recovered *L. cristiani* within the *Liolaemus elongatus* complex and showed that *Liolaemus tregenzai* is very closely related to this complex; thus, there is no strong evidence to include *L. cristiani* as part of the *L. kriegi* complex. In an earlier mitochondrial DNA (mtDNA)-based study, Morando *et al*. (2003) identified three lineages within this complex – *Liolaemus* sp. A, *Liolaemus* sp. B, and *Liolaemus* sp. C – and was not able to differentiate *L. kriegi* from *L. ceii*. In a recent morphological study that included individuals from the type localities of *L. buergeri*, *L.* sp. A, and *L.* sp. C, Medina, Avila & Morando (2013) showed that these samples were morphologically different lineages and added one entity (*Liolaemus* sp. D) to this complex. However, taxonomic knowledge of this complex is still limited, species limits are not well defined, and no detailed phylogeographical study exists for all species presently included in this complex. In this paper we examined the genetic structure and patterns of genetic variation of the *L. kriegi* complex across its distribution range, including eight taxa (four described species and four different lineages), based on two mitochondrial genes [cytochrome *b* (cyt *b*) and 12S ribosomal RNA (12S)] and two nuclear fragments [BA3 ribosomal RNA (BA3) and kinesin family member 24 (KIF24)].

MATERIAL AND METHODS

SAMPLING

Collecting localities were selected with the aim of covering the full distributional range of the *L. kriegi* complex. We obtained samples from all eight identified lineages (Fig. 1), including a total of 247 individuals from 55 localities ranging from southern Mendoza

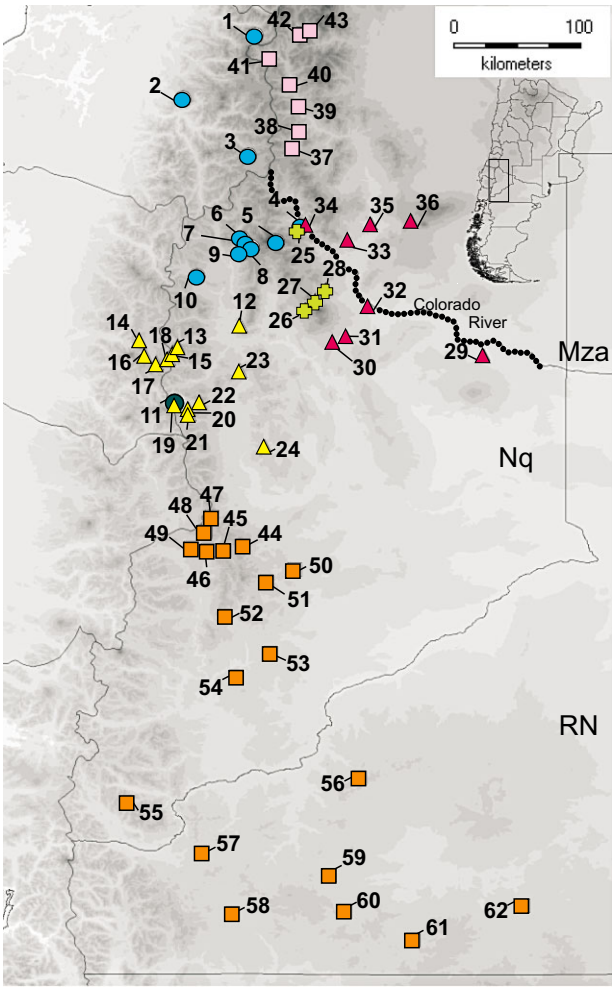

Figure 1. Localities sampled for *L. kriegi* complex are indicated as follows: *L.* sp. A, light-grey triangles (12–24); *L. tregenzai*, black circle (11; same locality as 19 of *L.* sp. A); *L.* sp. C, light-grey crosses (25–28); *L.* sp. D, light-grey squares (37–43); *L.* sp. B, dark-grey triangles (29–36); *L. kriegi* + *L. ceii*, dark-grey squares (44–62); *L. buergeri*, dark-grey circles (1–10). Mza, Mendoza Province; Nq, Neuquén Province; RN, Río Negro Province. The numbers correspond to those given in Appendix 1, and the colours match those given in Figures 3 and 4.

(35°05′S) to Río Negro (41°45′S) Provinces in Argentina, and seven localities from the complex's small distributional range in Chile. The specimen's voucher numbers with locality details are listed in Appendix 1. Specimens were collected by hand and were killed by pericardial injection of sodium pentothal. Liver samples were extracted for molecular analyses; specimens were fixed in 20% formalin and later transferred to 70% ethanol. Voucher specimens and tissues were catalogued in the herpetological collection Centro Nacional Patagónico in Puerto Madryn (LJAMM-CNP), Argentina (http://www.cenpat.edu.ar/nuevo/colecciones03.html). We included two tissue samples from the Miguel I. Christie (MIC) personal collection, two samples each from type localities of *L. elongatus* and *Liolaemus petrophilus* (which represent phylogenetically closely related groups), and one sample of *Liolaemus bibronii* to root the tree.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted using the Qiagen DNeasy 96 Tissue Kit for animal tissues, following the protocol provided by the manufacturer. Although mitochondrial sequences have, until recently, been the most common data used for these types of analyses (Zink & Barrowclough, 2008; Camargo, Sinervo & Sites, 2010), the discovery of nuclear markers with sufficient variability for phylogeographical analysis has expanded the power of these studies (Brito & Edwards, 2009; Camargo *et al*., 2010; Leaché *et al*., 2013). For this study, we sequenced two mitochondrial [12S (~853 bp, 29 individuals) and cyt *b* (~800 bp, 247 individuals)] and two nuclear [KIF24 (~490 bp, 18 individuals) and BA3 (~265 bp, 14 individuals)] DNA fragments. The protocols used for polymerase chain reaction (PCR) amplification and sequencing were those of Morando *et al*. (2003, 2004) and Noonan & Yoder (2009) for the mtDNA and nuclear fragments, respectively. All sequences were edited and aligned using Sequencher v4.10 (Gene Codes Corporation Inc. 2007) and were checked by eye to maximize blocks of sequence identity (12S). Missing data were coded as '?' and haplotype sequences were deposited in GenBank (Appendix 1; accession nos: KJ493945–KJ494247). The cyt *b* fragment was sequenced for all individuals, and the 12S and the two nuclear markers were subsampled to include genetically distinct representatives of each haploclade. The complete cyt *b* matrix was used for all analyses described below, the complete 12S matrix was used to obtain a gene network, and the KIF24 and BA3 markers were used to obtain single-gene haplotype networks and a combined multilocus nuclear gene network (see Appendix 1 for details).

Gene tree analyses and networks

The cyt *b* gene tree was constructed from non-redundant haplotypes identified using DnaSP 5.10 (Librado & Rozas, 2009), and a genealogy was constructed from this matrix with Bayesian inference (BI), after selection of the best-fitting evolutionary model using the corrected Akaike information criterion in JModelTest v0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). Analyses were conducted using MrBayes v3.2 (Ronquist & Huelsenbeck, 2003), run for 5×10^7 generations, and used equilibrium samples (after 25% burn-in) to generate a 50% majority-rule consensus tree; posterior probabilities (PP) were considered significant when ≥ 0.95 (Huelsenbeck & Ronquist, 2001). To identify lineages within the *L. kriegi* complex, we looked for clades that contained individuals from each of the type localities of the described species and clades that included individuals from localities assigned to candidate species by Morando *et al*. (2003) and Medina *et al*. (2013). For the complete cyt *b*, 12S and for nuclear BA3 and KIF24 phased-haplotype matrices, we used the program DnaSP (Librado & Rozas, 2009) to generate statistical parsimony haplotype networks under the 95% probability criterion using the software TCS 1.21 (Clement, Posada & Crandall, 2000). We also generated a multilocus network by converting the distance matrices for haplotypes from each separate nuclear gene (BA3 and KIF24) into an organismal matrix using the program POFAD v1.03 (Joly & Bruneau, 2006). The reconstructed organism network was then visualized using the NeighborNet algorithm implemented in SplitsTree v4.6 (Huson & Bryant, 2005), according to Leaché (2009). We also performed two further analyses. In the first analysis, we estimated a concatenated nuclear gene tree based on BI using MrBayes v3.2, we ran 1×10^7 generations and used equilibrium samples (after 25% burn-in) to generate a 50% majority-rule consensus tree; PP were considered significant when ≥ 0.95 (Huelsenbeck & Ronquist, 2001). In the second analysis, we estimated a concatenated mitochondrial network under the 95% probability criterion using the software TCS 1.21. Recombination was tested for the nuclear gene regions using RDP v3.44 (Martin & Rybicki, 2000; Heath *et al*., 2006).

Phylogeographical analyses

We defined groups of geographically homogeneous populations that are maximally differentiated from each other (*K*) and simulated an annealing procedure that maximizes the proportion of total genetic variance as a result of differences between groups, using SAMOVA v1.0 (Dupanloup, Schneider & Excoffier, 2002). We performed analyses with *K* values ranging from 2 to 13 (Appendix 2) for the complete cyt *b*

matrix of the *L. kriegi* complex, using 500 independent annealing processes. The best grouping option for each *K* value was selected based on the among-group component (F_{CT}) of the overall genetic variance. Genetic distances (pairwise corrected and uncorrected) between lineages, previously estimated in the gene-tree analyses, were estimated using Arlequin v3.1 (Excoffier, Laval & Schneider, 2005). Following the criterion described for amphibians by Fouquet *et al*. (2007), which controls for genetic distances combined with lineage allopatric distributions, we used the specific uncorrected cyt *b* genetic distance estimated for *Liolaemus* by Martínez (2012) and Breitman *et al*. (2012), which is an average of 3% for pairwise comparisons. Thus, genetic distances higher than 3% from geographically isolated areas were considered as evidence for candidate species. We used the cyt *b* matrix to estimate divergence times between the main lineages of the *L. kriegi* complex based on the best-fitting evolutionary model and performed a likelihood ratio test (LRT) using JModeltest v0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) to test for deviation from a strict molecular clock. We used the 2.23^{-2} cyt *b* rate of evolution estimated by Fontanella *et al*. (2012) for the *Eulaemus* clade calibrated with one fossil, and BEASTv1.6.1 to estimate gene trees under a strict molecular clock model (Drummond & Rambaut, 2007). Two independent analyses were performed for 100 million generations and sampled every 1000 generations with an HKY + I + G model of nucleotide substitution and assuming a Yule tree prior. The effective sample sizes (ESS) for parameter estimates and convergence were checked using Tracer v1.5 (Rambaut & Drummond, 2009).

Demographic analyses

For each main lineage recovered in the gene tree, we calculated basic molecular diversity indices: number of polymorphic sites (*S*), number of haplotypes (*H*), haplotype diversity (*Hd*), and nucleotide diversity (*Pi*). We performed Tajima's neutrality test (D) and Ramos-Onsins and Rozas test (R^2) to evaluate possible temporal changes in population sizes; all estimates were calculated using the program DnaSP. We also generated Bayesian Skyline Plots (BSP) to estimate changes in effective population size, through time, for *L. buergeri*, *L. kriegi* + *L. ceii* (based on gene tree results, see below), *L.* sp. A, *L.* sp. B, and *L.* sp. D. We used a strict molecular clock and a specific model of molecular evolution for each lineage, and ran 2×10^7 iterations, with sampling every 1000 iterations, and analyzed parameter convergence using Tracer v1.5 (Rambaut & Drummond, 2009). This analysis was not performed for *L. tregenzai* because of its small sample size.

RESULTS

Gene-tree analyses and networks

We found 122 non-redundant cyt *b* haplotypes from the 247 original sequences collected for the *L. kriegi* complex. The best-fit model of nucleotide substitution selected by ModelTest was TPM3uf + I + G (nst = 6 rates = gamma). Based on the haplotype matrix, we recovered a gene tree with high support [PP support = 1 for the *L. kriegi* complex (Fig. 2) including *L. tregenzai*]. Seven lineages with high statistical support were recovered within this complex: six corresponded to previously recognized taxa (*L. buergeri, L. tregenzai*, *L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D; Fig. 2); and the seventh included all samples corresponding to the *L. ceii* and *L. kriegi* terminals that are interdigitated with no differentiation and thus we refer to this lineage as *L. kriegi* + *L. ceii*. *Liolaemus* sp. C and *L.* sp. D are recovered as sister taxa with strong support, but there is no support for any deeper relationships among the main lineages. Although the lineages *L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D were previously proposed as candidate species (Morando *et al*., 2003; Medina *et al*., 2013), our treatment of these taxa as separate *L.* sp. units is not an endorsement of their recognition as distinct species but simply a stronger proposal for candidate species status (Vieites *et al*., 2009) that deserves further integrative studies to assess their taxonomic status.

With the complete cyt *b* data set, we recovered five separate haplotype networks, with a connection limit of 11 steps, corresponding to *L. buergeri*, *L. tregenzai*, *L.* sp. B, *L. kriegi* + *L. ceii*, and *L.* sp. A + *L.* sp. C + *L.* sp. D (Fig. 3). Network D included *L.* sp. A, *L.* sp. C, and *L.* sp. D, separated by five/six steps (Fig. 3D); although these lineages are geographically close, they are allopatric over a complex altitudinal variable landscape (indicated by the range of grey shades on the map), and even *L.* sp. D is separated from the other two lineages by the Colorado River. Also, *L.* sp. D is geographically closer to *L. buergeri* (Fig. 3A) than to *L.* sp. A and *L.* sp. C, and this last lineage is geographically closer to *L.* sp. B (Fig. 3B) than the other two lineages recovered in the same network.

Although individuals from *L. ceii* and *L. kriegi* type localities do not share haplotypes (Fig. 3E, stars), there is no apparent differentiation between these two species with this marker. For the 12S data set (Fig. 4A) we obtained one network, with clusters of haplotypes that correspond approximately to the cyt *b* haploclades. The two separate mitochondrial gene networks are fully concordant with the combined mitochondrial gene network (Appendix 4). The BA3 nuclear haplotype network with a connection limit of six steps (Fig. 4A), showed that although most of the haplotypes are species specific, three are shared

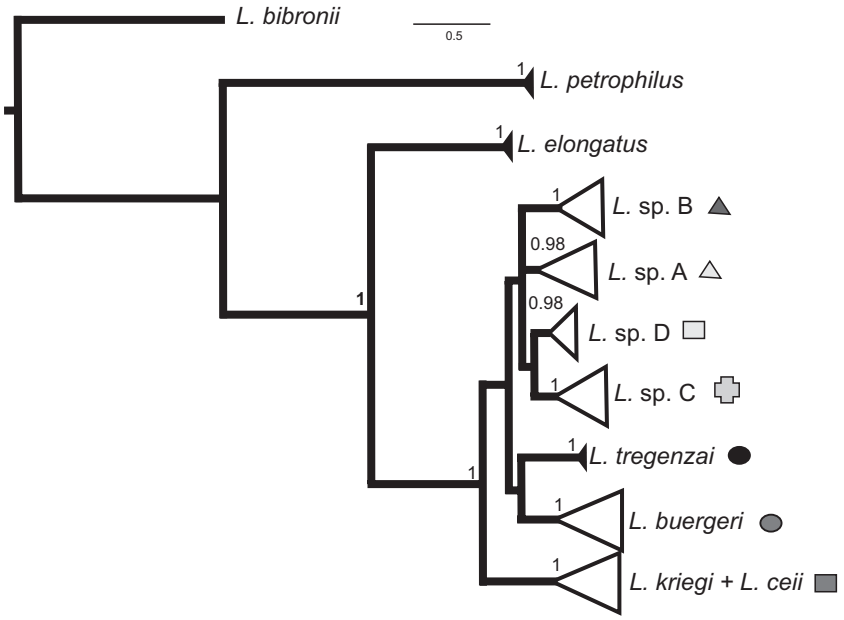

Figure 2. Bayesian 50% majority rule consensus cytochrome *b* gene tree. The numbers above branches are posterior probability values, and the symbols on each terminal correspond to those in Figure 1.

between some taxa: (1) *L.* sp. D–*L. kriegi* + *L. ceii*; (2) *L.* sp. D–*L.* sp. A; and (3) *L.* sp. A–*L. kriegi* + *L. ceii*–*L. buergeri*. The KIF24 nuclear haplotypes network, with a connection limit of nine steps (Fig. 4D), showed that all haplotypes are species specific and some were recovered outside the main network, including all from *L.* sp. B and *L. tregenzai* and two from *L.* sp. A. The two-locus genetic network summarizes average genetic distances among specimens (Fig. 4B): similar genetic distances are shown among three lineages (*L. buergeri, L. kriegi* + *L. ceii*, and *L.* sp. A), whereas different genetic distances are shown among the other four (*L. tregenzai*, *L.* sp. B, *L.* sp. C, and *L.* sp. D). The concatenated nuclear gene tree only recovered *L. tregenzai* and *L.* sp. A as well-supported clades (Appendix 5).

Phylogeographical analyses

The optimal partitioning of genetic diversity by spatial analysis of molecular variance (SAMOVA) was obtained when samples were grouped into the seven groups (*L. buergeri*, *L. kriegi* + *L. ceii*, *L. tregenzai*, *L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D) recovered in the cyt *b* gene-tree analysis; $K = 7$ ($F_{CT} = 0.29882$; Appendix 2). Table 1 shows all pairwise uncorrected genetic distances for the seven recognized lineages. *Liolaemus kriegi* + *L. ceii* had uncorrected distances higher than 3% (minimum = 3.79; maximum = 4.47) compared with all other lineages. *Liolaemus tregenzai* had uncorrected distances higher than 3% compared with all other lineages, except for *L.* sp. C (2.94%). Smaller uncorrected distances were found among *L.* sp. A , *L.* sp. B and *L.* sp. C (minimum = 1.64; 1.77; maximum = 2.12; 2.27).

Figure 5 shows the time-calibrated cyt *b* gene tree; the origin of the *L. kriegi* complex is near 1 Myr, and all other estimated times between recognized lineages are late Pleistocene.

Demographic analysis

The lineage with the highest number of sequences, polymorphic sites, and haplotypes was *L. kriegi* + *L. ceii* (Table 2). *Liolaemus tregenzai* had the smallest sample size, the fewest polymorphic sites and haplotypes, and the lowest haplotype and nucleotide diversity. *Liolaemus* sp. A showed the highest haplotype diversity and *L.* sp. C showed the highest nucleotide diversity. The only lineages that showed evidence of no neutrality with both Tajima's and Onsins & Rozas tests were *L.* sp. A and *L. kriegi* + *L. ceii*. With BSP we also detected a change in population size for *L. kriegi* + *L. ceii* (Appendix 3) and for *L. buergeri,* for which the neutrality tests did not detect departure from neutrality. For *L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D, no change in population size was detected with BSP (Appendix 3), in agreement with the neutrality test results.

DISCUSSION

The aim of this work was to study the genetic structure and phylogeography of all recognized lineages

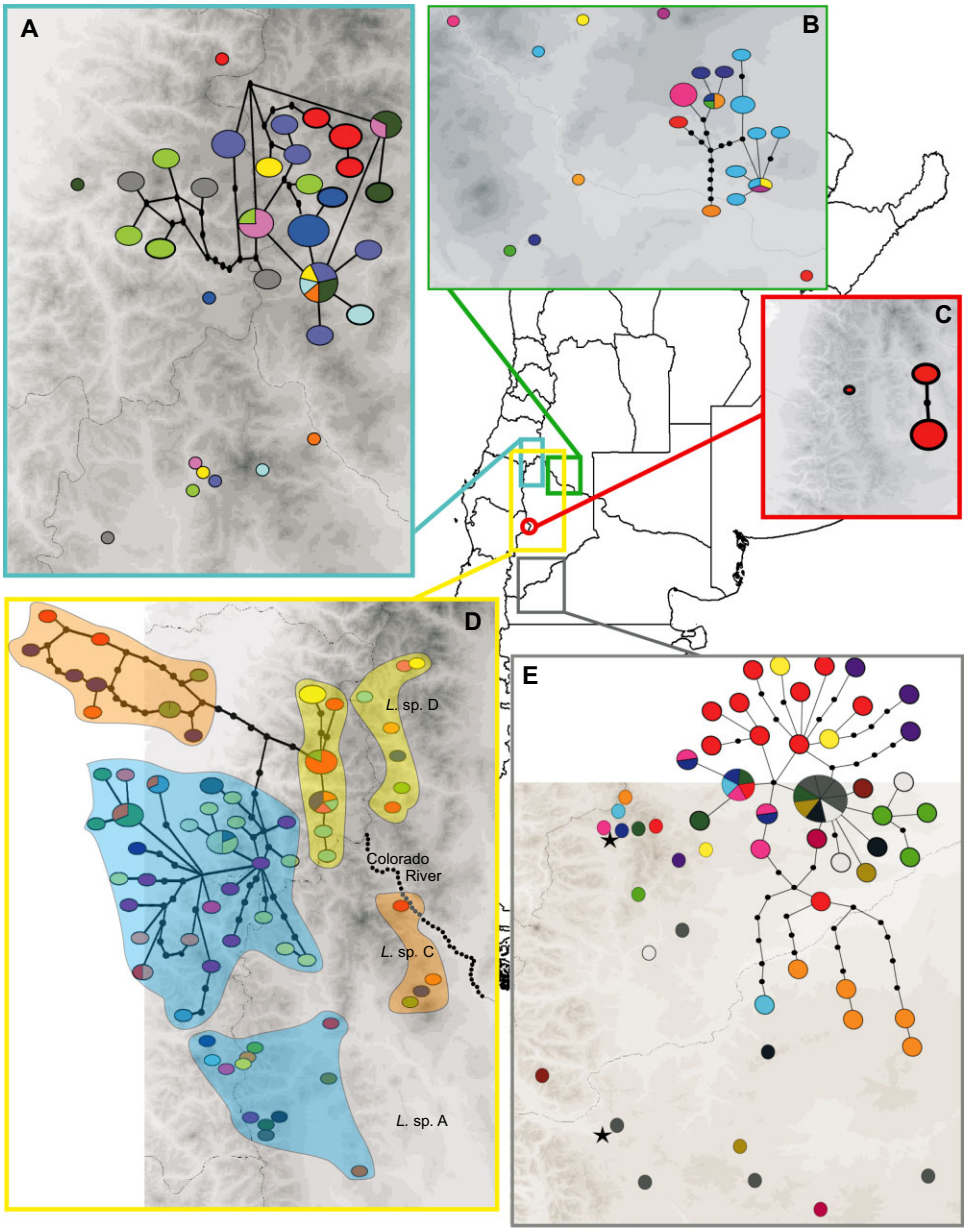

Figure 3. Cytochrome *b* statistical parsimony haplotype networks. Each lineage appears with its respective geographical distribution, and each haplotype is colour-coded according to its geographical distribution. (A) *L. buergeri*; (B) *L.* sp. B; (C) *L. tregenzai*; (D) *L.* sp. A (blue area), *L.* sp. C (orange area), and *L.* sp. D (yellow area); (E) *L. kriegi* + *L. ceii*.

within the *L. kriegi* complex using two mitochondrial and two nuclear gene sequences. All of our analyses were concordant in identifying seven lineages: two described species (*L. buergeri* and *L. tregenzai*), four candidate species (*L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D), and one lineage that included all individuals from the geographical range of *L. ceii* and *L. kriegi* (referred to as *L. kriegi* + *L. ceii*). We discuss the phylogeographical history of these lineages and then consider the taxonomic and conservation implications in the light of previous phylogeographical and biogeographical studies that included the distribution of this complex.

PHYLOGEOGRAPHICAL HISTORY

The northwestern most distributed lineage of the *L. kriegi* complex is *L. buergeri*, and its geographical range spans the southern Andean Cordillera and a topologically complex landscape that includes high

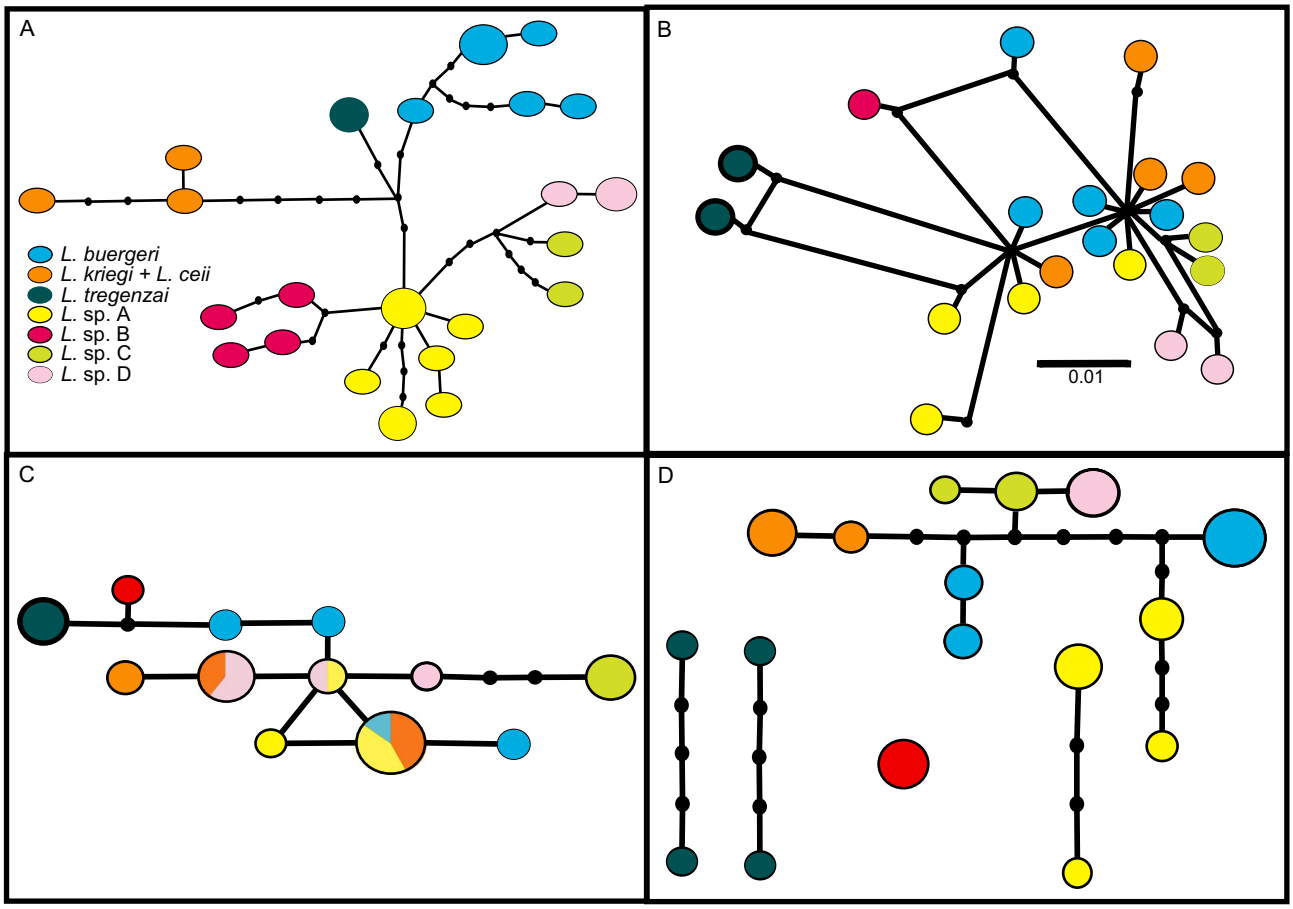

Figure 4. Statistical parsimony haplotype networks based on: (A) the mitochondrial 12S region; (B) the nuclear multilocus network (BA3 and KIF24); (C) the BA3 network; and (D) the KIF24 network. Haplotypes are colour-coded for each recognized lineage within the *L. kriegi* complex.

Table 1. Pairwise differences between the seven recognized lineages of *Liolaemus*

	L. kriegi + *L. ceii*	*L. tregenzai*	*L. buergeri*	*L.* sp. B	*L.* sp. D	*L.* sp. A	*L.* sp. C
L. kriegi + *L. ceii*	–	4.4691	4.056	4.2401	3.8828	3.7883	4.3418
L. tregenzai	3.8727	–	3.4189	3.7029	*2.937*	3.0918	3.1519
L. buergeri	3.0475	2.7202	–	3.5726	2.9413	2.9441	3.27
L. sp. B	3.3021	3.0747	2.5324	–	2.3768	2.2795	2.7693
L. sp. D	3.2052	2.5692	2.1615	1.6674	–	1.7739	1.6442
L. sp. A	2.9201	2.5334	1.9736	1.3794	1.1343	–	2.1285
L. sp. C	3.4043	2.5242	2.2302	1.800	0.9353	1.229	–

Above diagonal: average number of pairwise differences corresponding to the cytochrome b gene fragment between lineages of *Liolaemus*; below diagonal: corrected average pairwise differences (interlineage distance – intralineage distance).

peaks (2452 m; Fig. 1, locality 4) and deep valleys. The eastern edge of the *L. buergeri* distribution is geographically close to that of *L.* sp. D, but separated by the Andean Cordillera, whilst its southern margin is close to that of *L.* sp. A, and its easternmost locality (Fig. 1, locality 4) is parapatric with *L.* sp. B and *L.* sp. C (Fig. 1, localities 34 and 25, respectively). Individuals from *L. buergeri* were recovered as a cohesive group in the mtDNA gene tree (Fig. 2) and mitochondrial haplotype networks (Figs 3, 4), and

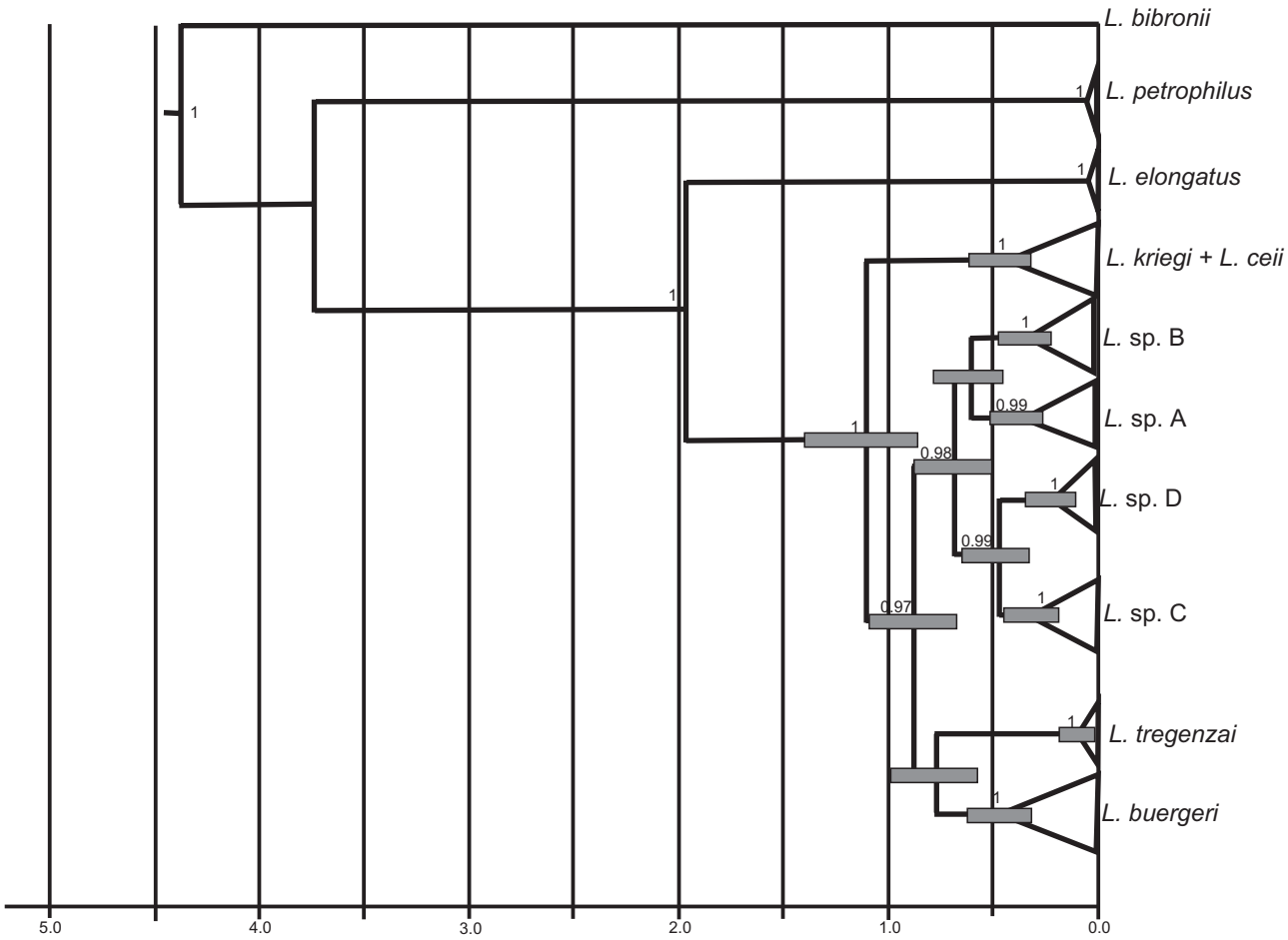

Figure 5. Estimated divergence times on the cytochrome *b* gene tree, marked with light grey, based on BEAST analyses. The *x*-axis is in millions of years (Myr) and numbers on nodes are PP > 0.95 from the Bayesian analysis.

Table 2. Demographic analyses of the seven recognized lineages of *Liolaemus*

Lineage	*NS*	*S*	*H*	*Hd*	*Pi*	Tajima's D	*P* (D ≤ Dt)	R^2	*P* (R^2 ≤ Ri)
L. buergeri	44	30	23	0.950	0.0079	–0.7938	0.2246	0.0800	0.1768
L. sp. A	44	46	29	0.960	0.0076	–1.8042	0.0126	0.0474	0.0028
L. sp. B	30	26	13	0.900	0.0082	–0.5879	0.3132	0.0977	0.2702
L. sp. C	12	15	09	0.940	0.0085	0.6178	0.7732	0.1649	0.6446
L. sp. D	18	08	06	0.800	0.0032	–0.2721	0.4244	0.1293	0.4078
L. kriegi + *L. ceii*	93	58	36	0.840	0.0052	–2.1733	0.0000	0.0292	0.0000
L. tregenzai	06	02	02	0.333	0.0009	–	–	–	–

All statistics (Tajima's D and associated *P* values, Ramos-Onsins and Rozas' R^2 and associated *P* values) were calculated from a fragment of the cytochrome *b* mitochondrial gene in lineages of *Liolaemus*. The sample size of *L. tregenzai* was too small for calculation of Tajima's D and Ramos-Onsins and Rozas' R^2. H, haplotypes; Hd, haplotype diversity; NS, number of sequences; Pi, nucleotide diversity; S, polymorphic sites.

uncorrected cyt *b* distances averaged 3% divergence from all other haploclades (Table 1). This species is also characterized by relatively high haplotype and nucleotide diversity, neutrality tests were not significant (Table 2), and only small changes in effective population size were evident in the BSP plots (Appendix 3). Our divergence estimates suggest that *L. buergeri* originated during the late Pleistocene,

which was impacted by multiple glacial cycles (Rabassa, Coronato & Salemme, 2005). Geological evidence suggests, however, that the geographical range of this species was not covered by ice, and this region has been hypothesized to be a stable refugium for plants and vertebrates during this time (Sérsic *et al*., 2011). The genetic 'signature' we detected for this taxon (population stability, high haplotype, and nucleotide diversity) is congruent with expectations of this refugium hypothesis. We suggest that the ancestral (late Pleistocene) populations of *L. buergeri* persisted *in situ* during these glacial cycles, with population size changes mainly driven by temperature and humidity fluctuations associated with glacial cycles (Rowe, Heske & Paige, 2006; Stone *et al*., 2012; Marske, Rahbek & Nogués-Bravo, 2013), which may have promoted peripheral isolation and differentiation of the other closely related taxa recognized within this complex.

The lineage *L.* sp. D is distributed along a north–south gradient in southwestern Mendoza Province (Fig. 1, light-grey squares), *L.* sp. C is distributed along the same longitudinal axis but south of the Colorado River (Fig. 1, light-grey crosses), and to the southwest of this lineage is *L.* sp. A (Fig. 1, light-grey triangles); this is a low-elevation 'foothills' area of the Andes. These three lineages are recovered as separate lineages in the cyt *b* gene tree (Fig. 2), and although they are included in one network with mitochondrial (Fig. 3D and Fig. 4A) and nuclear (Fig. 4B, C, D) markers, they are structured in different parts of the networks and their nuclear haplotypes are mostly exclusive. The uncorrected genetic distances among these three lineages are the lowest of the complex (< 2.13, Table 1); *L.* sp. D had the lowest number of polymorphic sites and nucleotide diversity, whereas *L.* sp. A had the highest haplotype diversity and significant signals of range expansion (Table 1). Our results are congruent with those for two other lizard species (Morando *et al*., 2003, 2007) co-distributed in this area, for which there is evidence of range expansion; most probably after retreat of the Last Glacial Maximum (LGM) ice sheet these populations colonized new areas.

Liolaemus sp. A is found in sympatry with *L. tregenzai* at localities 11 and 19 (Fig. 1). Although the sample size of *L. tregenzai* was small ($N = 6$), which precluded detailed analyses, it was recovered as an independent lineage across all methods (Figs 1–4). This species is known only from this locality on a mountain top (2020 m); thus, the low diversity indexes (Table 2) may represent the signature of a small isolated population.

Liolaemus sp. B is distributed along both sides of the Colorado River (Fig. 1, black triangles) and is recovered as an independent lineage within the *L. kriegi* complex in all of our analyses (Figs 1–4). This result is concordant with a previous study based only on mtDNA and one sampled locality (Morando *et al*., 2003). The phenotypic appearance of this lineage is almost identical to that of *Liolaemus austromendocinus*, a species belonging to the *L. petrophilus* group, and a morphometric analysis showed no statistically supported differences between the two (Feltrin, 2013). Phylogenetic analyses based on 16 nuclear loci recovered *L.* sp. B within the *L. petrophilus* group (Feltrin, 2013), and one of our nuclear haplotype networks (Fig. 4D) recovered this taxon as exclusive, whereas the other nuclear network (Fig. 4C) recovered *L.* sp. B nested within the main network. These conflicting results between several independent markers (mitochondrial, nuclear, and morphological) suggest either interspecific hybridization or hybrid origin of *L.* sp. B as the most plausible explanations for these patterns. The complex topographic characteristics of this area, coupled with climatic cycles, could have facilitated secondary contact between previously isolated populations. A detailed analysis, including denser geographical sampling, more high-resolution molecular markers, and paleo-niche model approaches, are needed to evaluate these alternative hypotheses fully.

Liolaemus kriegi + *L. ceii* is the southernmost distributed lineage, and all haplotypes were recovered as a well-supported lineage in the gene tree (Fig. 2) and as a separate network with cyt *b* data (Fig. 3E). The two nuclear gene networks (Fig. 4C, D) showed that haplotypes for these individuals are very similar or identical and are closely related to *L. buergeri*, *L.* sp. A, and *L.* sp. D, as also shown by the multilocus distance network (Fig. 4B). All of our results showed a signature of range expansion for this lineage (Table 2, Appendix 3), which is congruent with previous results of other lizards (*L. elongatus*, Morando *et al*., 2003; *L. bibronii* haploclade 4, Martínez, 2012) and a rodent species inhabiting this area (*Loxodontomys micropus*, haploclade N2, Cañon *et al*., 2010). The northern distributional limit of this lineage coincides approximately with the vertebrate and plant phylogeographical breaks proposed by Sérsic *et al*. (2011; Fig. 2A break 5; Fig. 2B, break 4, respectively); thus, the isolation of this lineage as the southernmost of this complex probably resulted from processes that affected the regional biota in similar ways.

Taxonomic and conservation implications

In closely related species complexes, different lineages can go undetected using traditional taxonomic methods (Bickford *et al*., 2007; Dasmahapatra *et al*., 2010; Sistrom, Donnellan & Hutchinson, 2013);

therefore, genetic patterns are a useful tool for the identification and delimitation of different evolutionary units and cryptic species (DNA taxonomy), as well as for the assignment of unknown specimens to known taxa (e.g. Sites & Marshall, 2004; Hebert & Gregory, 2005; Pons *et al*., 2006; Shaffer & Thompson, 2007; Vogler & Monaghan, 2007; Marshall *et al*., 2011).

Our phylogeographical results identified seven well-distinguished lineages within the *L. kriegi* complex: *L. buergeri*, *L. tregenzai*, *L. kriegi* + *L. ceii*, *L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D. All were recovered as well-supported mitochondrial haploclades that are geographically cohesive, mostly allopatric, and most are exclusive with at least one nuclear haplotype (KF24, panel 3). A recent morphological analysis including most of the species of this complex found several statistically significant differences between *L. buergeri* and lineages A, C, and D (Medina *et al*., 2013). The morphologically most similar species were *L.* sp. C and *L.* sp. D, but their distributional ranges are separated by the Colorado River, which has been identified as a barrier for gene flow for other lizard species (Morando *et al*., 2007; Feltrin, 2013). Similarly, small, but significant, morphological differences were detected between these two taxa and *L.* sp. A (Medina *et al*., 2013). Thus, the distributional, molecular, and morphological evidence suggests that these three lineages may represent evolutionary significant units aiming for conservational implications. Alternatively, they may represent one species but with moderate morphological differentiation and strong structured populations. Geographical sampling between the distribution areas of these lineages is needed to test these hypotheses.

Liolaemus sp. B and *L. tregenzai* are statistically different from each other in some morphological characters, as well as from all other lineages of this complex (C. D. Medina, L. J. Avila, M. Morando, unpubl. data). The taxonomic status of *L.* sp. B is more uncertain as a result of conflicting results in nuclear, mitochondrial, and morphological data sets. These taxa could represent a peripheral geographical subset of *L. austromendocinus* (to which they are morphologically very similar) that in the past experienced massive mitochondrial introgression from a *L. buergeri* lineage, or alternatively it could represent a species with a hybrid origin.

Morando *et al*. (2003) suggested that *L. ceii* and *L. kriegi* represented one lineage based on cyt *b* data, and our analyses, from a greater sampling of localities, individuals, and markers, strongly support this earlier conspecific hypothesis for these taxa. Individuals from the two type localities are morphologically different, and individuals from the intermediate area between these two localities are more similar to those from the *L. ceii* type locality than to those from the *L. kriegi* type locality (C. D. Medina, L. J. Avila, M. Morando, unpubl. data). This phenotypic variation could be the result of different environments; the *L. ceii* type locality and the other localities with phenotypically similar individuals are located in the Patagonian Steppe phytogeographical region, whilst the *L. kriegi* type locality is located in or near the Andean Patagonian forest phytogeographical region (Cabrera, 1994). Based on these observations and the available evidence, we propose two hypotheses: (1) they constitute two species, for which different environments prompted relatively rapid and recent morphological divergence with insufficient time for molecular differentiation; or (2) they are conspecific and show clinal morphological variation owing to local adaptations. Although available data seem to favour the second hypothesis, we suggest that a high-resolution molecular study associated with a detailed morphological study along a densely sampled transect is needed to settle these alternatives fully.

Most of the divergence within this complex has occurred during the last half Myr, most probably favoured by the complex topological landscape and climatic cycles associated with late Pleistocene glacial events. The genetic and geographical distinctness we detected for *L.* sp. A, *L.* sp. B, *L.* sp. C, and *L.* sp. D, associated with small, but significant, differences in morphology (Medina *et al*., 2013), indicate that they represent microendemics, regardless of whether we consider them full species under the unified species concept (de Queiroz, 2005), or as significantly differentiated intraspecific lineages. The picture these lineages suggest, together with the introgression/hybridization hypotheses proposed for *L.* sp. B, reflects how dynamic evolutionary processes can be and how they can impact biodiversity studies.

The geographical distribution of the *L. krieigi* complex corresponds to northwestern Patagonia, for which a recent phylogeographical review (Sérsic *et al*., 2011) included Patagonian lizards, small mammals, and plants, and identified a region high in genetic diversity that probably persisted as a refugium during Pleistocene glacial cycles. More recent plant phylogeographical studies on other taxa (*Nassauvia,* Nicola, 2013; *Mulinum spinosum,* Sede *et al*., 2012) have also documented exclusive, highly divergent haplotypes in this area and recognized the need for detailed studies with dense sampling across many species to document fully the level of diversity within it. In agreement with the phylogeographical approaches, a biogeographical review based on Argentinean continental fishes (López & Miquelarena, 2005) found that although the Patagonian region has 15 native freshwater species (a relatively low number), six are endemic and most of these are endemic to the same geographical area.

At broader geographical and taxonomic scales, endemism analyses of 426 South American beetle species (Carabidae, Casagranda *et al*. 2009) identified four areas of endemism within the Patagonia biogeographical region, all of which overlap geographically with this lizard complex. Similarly, Domínguez *et al*. (2006) quantified endemicity in an orthopteran family (Tristiridae) and in five coleopteran families (Carabidae, Curculionidae, Tenebrionidae, Geotrupidae, and Scarabaeidae) distributed over the Patagonian Steppe, and resolved endemic areas that are also congruent with the distribution of the *L. kriegi* complex. These findings reinforce the hypothesis that this region, proposed as a biodiversity 'hotspot' with the highest percentage of identified priority and irreplaceable conservation areas from Patagonia (Chehébar *et al*., 2013), harbors taxa highly differentiated from other Patagonian areas. Our results complement the above studies by taking a narrowed taxonomic focus based on a dense sampling scheme that resolved different evolutionary lineages of recent origin. To our knowledge there are no other genetic studies with dense sampling from this area; therefore, we encourage researchers to follow this approach and we predict that microendemisms will be found for many other taxa. Furthermore, if the candidate species are supported by additional lines of evidence in 'integrative taxonomic' studies (e.g. in *Liolaemus*, Aguilar *et al*., 2013), then biodiversity conservation planning will need to focus on the small geographical ranges of these relatively young species. These results have important conservation implications, as efforts should be directed at establishing reserve networks that capture the adaptive diversity within species or closely related lineages, as well as ecological and evolutionary processes that generate and sustain this diversity (Crandall *et al*., 2000).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix 1. Specimens sampled.
Appendix 2. Results of SAMOVA analyses.
Appendix 3. Results of Bayesian Skyline Plot BSP analyses.
Appendix 4. Concatenated mitochondrial gene network result.
Appendix 5. Concatenated nuclear gene phylogeny result.