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Phylogeography and morphological variation of the northernmost distributed species of the *Liolaemus lineomaculatus* section (Liolaemini) from Patagonia

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Abstract. Lizards from the *Liolaemus lineomaculatus* section are endemic to Patagonia, southern South America. Three main groups are recognized within this section, one of which, the *L. kingii* group includes eleven species. The two northernmost distributed species of this group, *L. somuncurae* and *L. uptoni*, are endemic to a small area that partly overlaps with the Provincial Protected Area Somuncurá Plateau (within the Somuncurá massif). Knowledge available for these species is based on limited sample sizes, and mostly limited to their original descriptions; also a recent molecular phylogenetic study showed evidence for a closely related candidate species (*Liolaemus* sp. 4). In this paper we morphologically and genetically characterize the species *L. somuncurae*, *L. uptoni*, and *L.* sp. 4, and present past demographic hypotheses. We studied eighty lizards, and collected morphological and genetic data for almost all of them. The specific status of *L. somuncurae* and *L. uptoni* is supported by molecular, morphological, and distributional evidence, as well as the status of *L.* sp. 4; for which we recommend further morphological comparisons with other species of the *L. kingii* group. We also identified two novel lineages from restricted areas south of the Chubut River that we propose as candidate species. We extend previously published evidence (from plants and rodents) supporting the role of the Chubut River as an allopatric barrier. Also, in agreement with previous results based on plants, we found evidence for two refugia in northwestern Chubut, for which we encourage conservation efforts.

Keywords: biogeography, cryptic diversity, cytochrome-*b*, *Liolaemus somuncurae*, *Liolaemus uptoni*, morphology, Somuncurá massif.

Introduction

The *Liolaemus lineomaculatus* section includes 22 species (Abdala et al., 2014) and is one of the most conspicuous groups of endemic Patagonian vertebrates. Recent molecular (Breitman et al., 2011) and morphological (Breitman, Morando and Avila, 2013) evidence support three main species groups within the section: *L. lineomaculatus*, *L. magellanicus*, and *L. kingii*. The species of the *Liolaemus kingii* group have been classified (based on a multilocus data set) into: the *L. kingii* clade (or the *kingii* + *archeforus* group) including 11 species and mainly distributed in southern Patagonia, and the *L. somuncurae* group including two species distributed in northern Patagonia (Breitman et al., 2011; Breitman, Morando and Avila, 2013). Divergence time between these groups, estimated with mitochondrial cytochrome-*b* gene, dated to the Pliocene (~4.25 Mya, 95% HPD = 3.17-5.48; rate of molecular evolution; Breitman et al., 2011) or late Miocene (~7 Mya; fossil calibrations; Schulte, 2013). Breitman et al. (2011) based on a single specimen and using nuclear and mitochondrial genes, found evidence of a candidate species (*Liolaemus* sp. 4) geographically located in between the *L. kingii* clade and the *L. somuncurae* group. The taxonomic status of *Liolaemus* sp. 4 was ambiguous, as it was recovered within the *L. somuncurae* group with a traditional concatenation approach, but nested within the *L. kingii* clade

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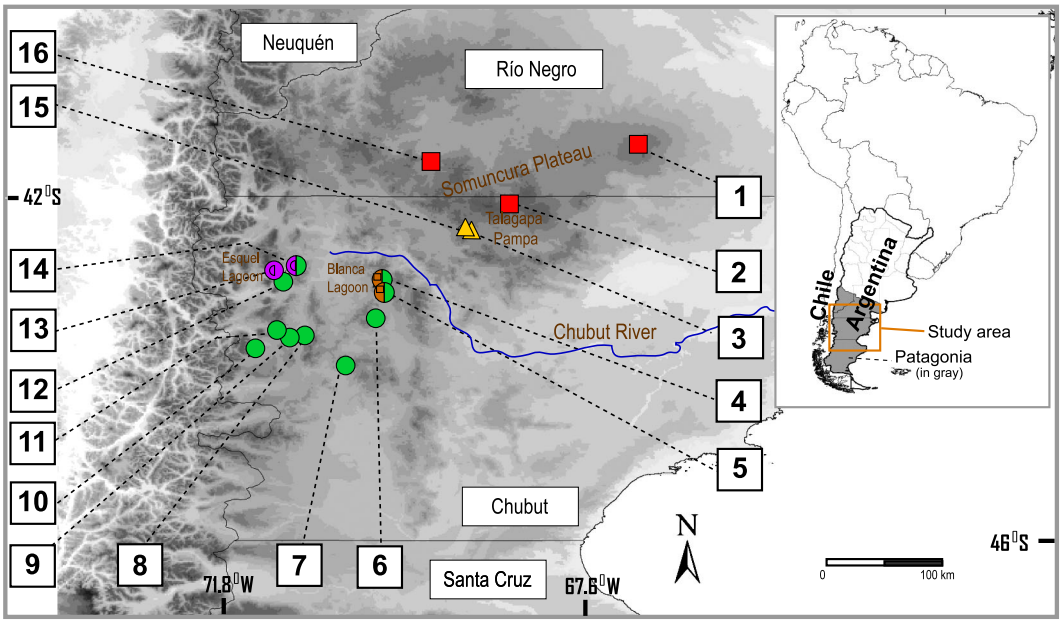

Figure 1. Distribution map of *Liolaemus somuncurae* (red squares), *L. uptoni* (yellow/orange triangles), *Liolaemus* sp. 4 (green circles and semicircles), clade 1 (purple circles/semicircles with small semicircle inside), and clade 2 (brown semicircles with a square inside). Some aspects of the graphics might only be fully comprehensible in the online version where they are reproduced in color.

based on a species tree approach (Breitman et al., 2011); similar results were recently found using an extended genetic dataset (Olave et al., 2014). The few available publications including species of the *Liolaemus somuncurae* group are based on small sample sizes, and are limited to species descriptions, distributions and some morphological comparisons (Cei and Scolaro, 1981; Scolaro and Cei, 1987, 1997, 2006; Avila et al., 2007; Breitman et al., 2010; Breitman, Morando and Avila, 2013).

Lizards from the *Liolaemus lineomaculatus* section span a large area of Patagonia, which had a complex geomorphologic history. Northern and Southern Patagonia were differentially affected by Pliocene and Pleistocene glaciations (although permafrost was continuous throughout Patagonia); while glaciers covered vast regions in Southern Patagonia, they covered smaller portions of the Andes in Northern Patagonia (Tomobotto, 2002; Rabassa, 2008). Further, these two regions differ in topographic structure and by the presence of the Somuncurá massif in Northern Patagonia (fig. 1), which is inhabited by lizards from the *L. somuncurae* group. The massif (which partially overlaps the Somuncurá Plateau) was formed by layers of basaltic Cenozoic lavas, was not covered by the Miocene marine transgressions (Ardolino et al., 2008; Malumian and Náñez, 2011), and was volcanically active until the early Pliocene (central and western locations; Rabassa, 2008). The Somuncurá Plateau includes at least 29 endemic taxa and the northwestern part of the system was declared a Protected Area by the government of Río Negro Province (Official bulletin number 356/1986). Although the biodiversity of this plateau and the surrounding areas is unique, knowledge regarding genetic and morphological variation of this biota is still very limited. Among the endemic taxa (see online supplementary text S1 for species names and references) there are five plant species, at least fourteen invertebrate species, and ten vertebrate species, including the lizard species *Liolaemus somuncurae* (Cei and Scolaro, 1981) and *Lio-*

laemus uptoni (Scolaro and Cei, 2006) from the *L. lineomaculatus* section.

Several phylogeographic studies of Patagonian plants and animals have been published (reviewed in Sérsic et al., 2011), and based on these, areas of stability, fragmentation, secondary contact, and routes of colonization have been hypothesized. Several refugial areas have been identified along the Andean mountains of northern Patagonia, and nine phylogeographic breaks for animals and five for plants have been hypothesized (three of them shared among both; Sérsic et al., 2011).

In northern Patagonia, lizards are the animal group that has received most of the attention in terms of phylogeographic studies. Those studies were focused on five of the ten species' complexes of the genus *Liolaemus* (*L. bibronii*, *L. elongatus-L. kriegii*, *L. darwinii*, and *L. melanops*; Morando, Avila and Sites, 2003; Morando et al., 2004, 2007; Avila, Morando and Sites, 2006; Olave et al., 2011; Fontanella et al., 2012; Camargo et al., 2013), and one species group of the *L. lineomaculatus* section (Breitman et al., 2012; although this group's distribution is restricted to a small western area). Evidence of population expansions have been reported in several of these studies, in some cases from central Neuquén towards the South, in others from southern Mendoza to eastern Río Negro, and in others from northeastern to central-southern Chubut. Some of those contributions also hypothesized that the Somuncurá plateau acted as a geographic barrier promoting divergence in other lizard groups (Morando, Avila and Sites, 2003; Avila, Morando and Sites, 2006; Morando et al., 2007; Fontanella et al., 2012).

Besides the species phylogeny of the *Liolaemus lineomaculatus* section, little is known about the biology and morphological patterns of the northernmost species of the *L. kingii* clade, namely the two included in the *L. somuncurae* group. There is also no information about species limits, phylogeographic patterns, or past demographic histories. Thus, the main goal of this paper is to present extensive morphological analyses and phylogeographic patterns, and to propose evolutionary and demographic hypotheses for these two species and the candidate species *Liolaemus* sp. 4. Given the high endemicity of the study area, our work provides results that will be useful for conservation planning in the Somuncurá area.

Materials and methods

Sampling design

Sixteen localities (including type localities of *Liolaemus somuncurae* and *L. uptoni*), spanning the distributional area of the northernmost species of the *L. lineomaculatus* section (fig. 1), were used for this study. Field trips were completed between 2004 and 2010, and voucher specimens (and tissues) are deposited in the LJAMM-CNP herpetological collection of the Centro Nacional Patagónico in Argentina. A total of 80 specimens were studied, including 29 *L. somuncurae*, 12 *L. uptoni*, and 39 without taxonomic identification, but from within the distributional range of *Liolaemus* sp. 4. Specimens of *L. kingii*, *L. magellanicus*, *L. lineomaculatus*, *L. bibronii*, *L. petrophilus*, *L. darwinii*, and *Phymaturus dorsimaculatus* were used as outgroups (Breitman et al., 2011, 2012).

Of the 80 ingroup specimens, 60 individuals were sequenced and 77 were used in morphological analyses (60 adults and 17 juveniles, 41 females and 36 males; online supplementary table S2). Some specimens were used in our previous publications [Breitman et al., 2011 (GenBank accession numbers for ingroup sequences JF272790, JF272785, JF272795, for outgroups JF272778-81, JF272767, JF272771, JF272789); Breitman, Avila and Morando, 2013]; in this paper we present 56 novel sequences and new morphological data from 50 individuals.

Genetic data and analyses

Genomic DNA was extracted from liver using the Qiagen® DNeasy® 96 Tissue Kit, following the manufacturer's protocol. The mitochondrial cytochrome-*b* fragment (Kocher et al., 1989) was amplified using the PCR and sequencing protocols of Morando, Avila and Sites (2003). Sequences were edited in SEQUENCHER v4.8 (TMGene Codes Corporation Inc. 2007) and translated to amino acids to confirm open reading frame. Alignments were done using MAFFT (Katoh et al., 2002). Sequences are deposited in GenBank (KR072564-KR072620).

Gene trees were estimated using Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. The best-fit evolutionary model (TIM2 + I + G) was selected under the corrected Akaike information criterion in jModeltest v0.1.1 (Posada, 2008). Bayesian analyses were performed using four heated Markov chains (default heating values) sampled at intervals of 1000 generations, and run for 50 million

generations in MrBAYES v3.1.2 (Ronquist and Huelsenbeck, 2003). Convergence was verified when effective sample sizes > 200 using Tracer v1.5.0 (Rambaut and Drummond, 2009). A 50% majority-rule consensus tree was generated (after 25% 'burn-in'); posterior probabilities were significant when >0.95 (Huelsenbeck and Ronquist, 2001). Likelihood analyses were conducted in RaxML v7.0.4 (Stamatakis, 2006), based on 1000 rapid bootstrap analyses for the best ML tree, using a GTRGAMMA model of nucleotide substitution; nodal support was significant when bootstrap > 95 (Felsenstein and Kishino, 2003), and moderate when > 70 (Hillis and Bull, 1993). Cytochrome-*b* pairwise genetic distances (corrected pairwise differences = intergroup distance − intragroup distance) were calculated in Arlequin v3.11 (Excoffier, Laval and Schneider, 2005). Different genetic lineages were hypothesized by identification of haplotype clades with >3% uncorrected cytochrome-*b* genetic distances among them (Breitman et al., 2012). Haploclades that included individuals collected at type localities (or identified as candidate species in previous contributions) were named as the nominal species (Breitman et al., 2011, 2012).

A statistical parsimony algorithm was used to construct networks (Templeton, Crandall and Sing, 1992) using TCS v1.21 (Clement, Posada and Crandall, 2000) with default connection significance (95%) and excluding outgroups. Standard molecular diversity indices were calculated for each lineage (number of haplotypes, number of segregating sites, average number of differences between two random sequences, haplotype diversity, and nucleotide diversity) using DnaSP v5.0 (Librado and Rozas, 2009).

Divergence times among main lineages were estimated using a smaller data set of cytochrome-*b* and a fossil calibration (Albino, 2008; Inoue, Donoghue and Yang, 2010). The data set included one to four individuals of each main lineage and outgroups (see details in online supplementary text/figure S3). Analyses were run in BEAST v1.6.1 (Drummond and Rambaut, 2007) using a relaxed uncorrelated lognormal clock model. The fossil information was placed in the node representing the most recent common ancestor of the two *Liolaemus* subgenera with a prior' lognormal distribution (mean: 1, standard deviation: 1.5, offset 18.5; Ho, 2007). Two independent analyses were performed for 20 million generations and sampled every 1000 generations, with a HKI + I + G evolutionary model (from jModeltest), and assuming a Yule tree prior. Trees were summarized (after 10% burn-in) using TreeAnnotator v1.6.1 (Drummond and Rambaut, 2007). Convergence was verified using Tracer v1.5.0. Dating the divergence events among lineages frames past phylogeographic histories in a temporal context; and while these types of inferences are important, we recognize the limitations of our approach (e.g. Graur and Martin, 2004; Rutschmann, 2006) and interpret our estimated dates as hypotheses for future testing.

Morphological data and analyses

Variation in morphology was estimated from formalin-fixed lizards. We evaluated variation in fourteen morphometric (only in adults), ten meristic (scale counts), and seven qualitative characters (only in adults) representing squamation and patterns of body coloration (e.g., Abdala, 2007; Breitman, Morando and Avila, 2013; sample sizes in online supplementary table S4). Measurements were made with a Schwyz® electronic digital caliper to the nearest 0.1 mm, and scale counts were performed under a stereoscopic microscope, usually on the individuals' right side. Measurements, scale terminology, and chromatic states followed Smith (1946) and Breitman, Morando and Avila (2013). Sex was determined by the presence of precloacal pores (males only) and cloacae shape, while adults were identified by size and coloration patterns (Cei, 1986; Breitman, Morando and Avila, 2013).

Morphometric characters used in this study included: snout-vent length (SVL), tail length (TL), distance between fore and hind limbs (DFH), foot length (FOL), tibia-fibula length (TFL), radius-ulna length (RUL), hand length (HAL), head height (HH), head width (HW), head length (HL), rostral-nasal distance (RND), rostral height (RH), rostral-eye distance (DRE), and auditory meatus height (AH). Meristic characters used in this study were: scales in contact with the interparietal (SCI), lorilabial scales (LS), supralabial scales (SS), infralabial scales (IS), midbody scales (MS), dorsal scales (DS), ventral scales (VS), infradigital lamellae of the third finger (IL3), infradigital lamellae of the fourth toe (IL4), and number of precloacal pores (PP). Qualitative characters included: dorsal stripe pattern, referring to the shape and size of dorsal bands (perpendicular to the body axis), including four categories of bands: (1) complete or slightly broken, (2) dotted, (3) irregular, (4) indistinct or almost indistinct (these variables were illustrated and respectively described as 0-20, 40, 60 and 80-100 in Scolaro and Cei, 1987); presence/absence of a single vertebral line; presence/absence of dorsolateral lines; presence/absence of ventral variegation (black and white spots); ventral melanism, including six categories: (m0) absent, (m1) only present in the gular zone, (m2) only present in the belly, (m3) present in all body regions except cloacal region and limbs, (m4) present in all body except the limbs, (m5) present in all the body; presence/absence of red/orange scales; presence/absence of differentiation in head coloration relative to body. All characters (meristic, morphometric and qualitative) are described in Breitman, Morando and Avila (2013).

Meristic and morphometric data sets were analyzed separately. Variation was evaluated at intraspecific (between sex within each species; sexual dimorphism) and interspecific levels (among species, separated by sex when intraspecific variation was observed). Morphological statistical tests were performed in *Liolaemus uptoni*, *L. somuncurae* and *L.* sp. 4 (small sample sizes for other lineages, see results). Qualitative data were transformed to percentages to reflect prevalence, and were qualitatively compared within and among lineages.

Intraspecific analyses: morphological variation was summarized through standard statistics (mean, range, and standard deviation). Sexual dimorphism was evaluated (1) for each variable with either Student's *t* or Kruskal-Wallis tests (non-parametric test; Kruskal and Wallis, 1952), and (2) for

all variables using MANOVA analyses (in data sets where the number of variables exceeded the number of individuals, two independent runs were performed after dividing the data set in half, due to the fact that MANOVA analyses require number of individuals to exceed number of variables; Scheiner, 2001). Assumptions of equal variance and normality were evaluated in morphometric (raw and standardized, divided by sex) and meristic data sets by using Levene and Shapiro-Wilks tests, respectively (Montgomery, 1991).

Interspecific analyses: one-way Analysis of Variance (ANOVA) using DGC comparisons (the Di Rienzo, Guzmán and Casanoves test; Di Rienzo, Guzmán and Casanoves, 2002) was used to statistically evaluate differences in variables among species. When assumptions (same as for MANOVA analyses) were not met Kruskal-Wallis tests with comparisons were used (Kruskal and Wallis, 1952). All morphological analyses were performed in INFOSTAT® 2011 (Di Rienzo et al., 2011). The morphometric data set was divided by sex and analyzed independently due to observed sexual dimorphism (see results, Vukov et al., 2006; Breitman, Morando and Avila, 2013). Significant differences in SVL among species were observed (see results), thus a standardized morphometric data set (by dividing by SVL; Breitman, Morando and Avila, 2013) was assembled and analyzed independently.

Results

Genetic results

Forty-seven haplotypes were recovered from the ingroup's cytochrome-*b* matrix ($n = 60$; 742 bp in length; 118 informative sites). Gene trees were topologically concordant across phylogenetic reconstructions (BI and ML) without well-supported conflicts (fig. 2). Five main lineages were recovered corresponding to: *Liolaemus somuncurae* (three localities on the Somuncurá Plateau, fig. 1), *L. uptoni* (two localities on the southern edge of the Plateau, fig. 1), the previously recognized candidate species *L.* sp. 4 (several localities in Chubut, fig. 1), and two novel lineages (each one including three individuals), here referred as clades 1 and 2 (allopatric with each other, but mostly sympatric with the northernmost localities of *L.* sp. 4; fig. 1). Lineages were recovered in the following well-supported pectinate topology (*L. somuncurae* (*L. uptoni* (*L.* sp. 4 (clade 1, clade 2)))); all main lineages had strong support ($Pp > 0.95$; ML bootstrap > 95), except for the monophyly of *L.* sp. 4 and the clade (*L.* sp. 4 (clade 1, clade 2)) where support was strong with BI but moderate with ML (>70).

Haplotypes were recovered forming five separated networks plus one singleton (fig. 3), concordant with haploclades recovered in the gene tree (fig. 2). Several haplotypes were recovered within *Liolaemus somuncurae*, including one singleton from locality #2, and very distinct haplotypes in locality #16 (fig. 3). Three haplotypes were recovered in each one of the networks corresponding to *L. uptoni* and clades 1 and 2 (fig. 3). Individuals of *Liolaemus* sp. 4 were recovered in a network that had very distinct haplotypes in the north and signals of demographic expansion (star-like connections) in the south (fig. 3). Cytochrome-*b* pairwise genetic distances within lineages were small to moderate (0.27-1.09%); uncorrected cytochrome-*b* genetic distances among lineages were higher than 3% (3.10-8.62%) (table 1). High haplotype diversity and relatively low nucleotide diversity values were found in all lineages (table 2).

Divergence time (text/figure S3) between *Liolaemus somuncurae* and *L. kingii* was dated to the Pliocene ~ 5.38 Mya [95% HPD = 2.38-6.09]; falling within the range of previously estimated divergence rates for these taxa (~7 Mya from Schulte [2013] and ~4.25 Mya from Breitman et al. [2011]). The occurrence of the most recent common ancestor between *L. somuncurae* and other species of the ingroup was dated to the Pliocene ~ 3.91 Mya [95% HPD = 2.38-6.09]. The split between *L. uptoni* and (*L.* sp. 4 plus clade 1 + 2) was dated to the Late Pliocene (~2.65 Mya [95% HPD = 1.58-4.24]), while the divergence between *L.* sp. 4 and clade 1 + 2 was dated to the Early Pleistocene (~1.43 Mya [95% HPD = 0.81-2.35]), and between clades 1 and 2 to the Pleistocene (~0.89 Mya [95% HPD = 0.42-1.54]) (text/figure S3).

Morphological results

Morphological differentiation was evaluated within and among *Liolaemus somuncurae*, *L.*

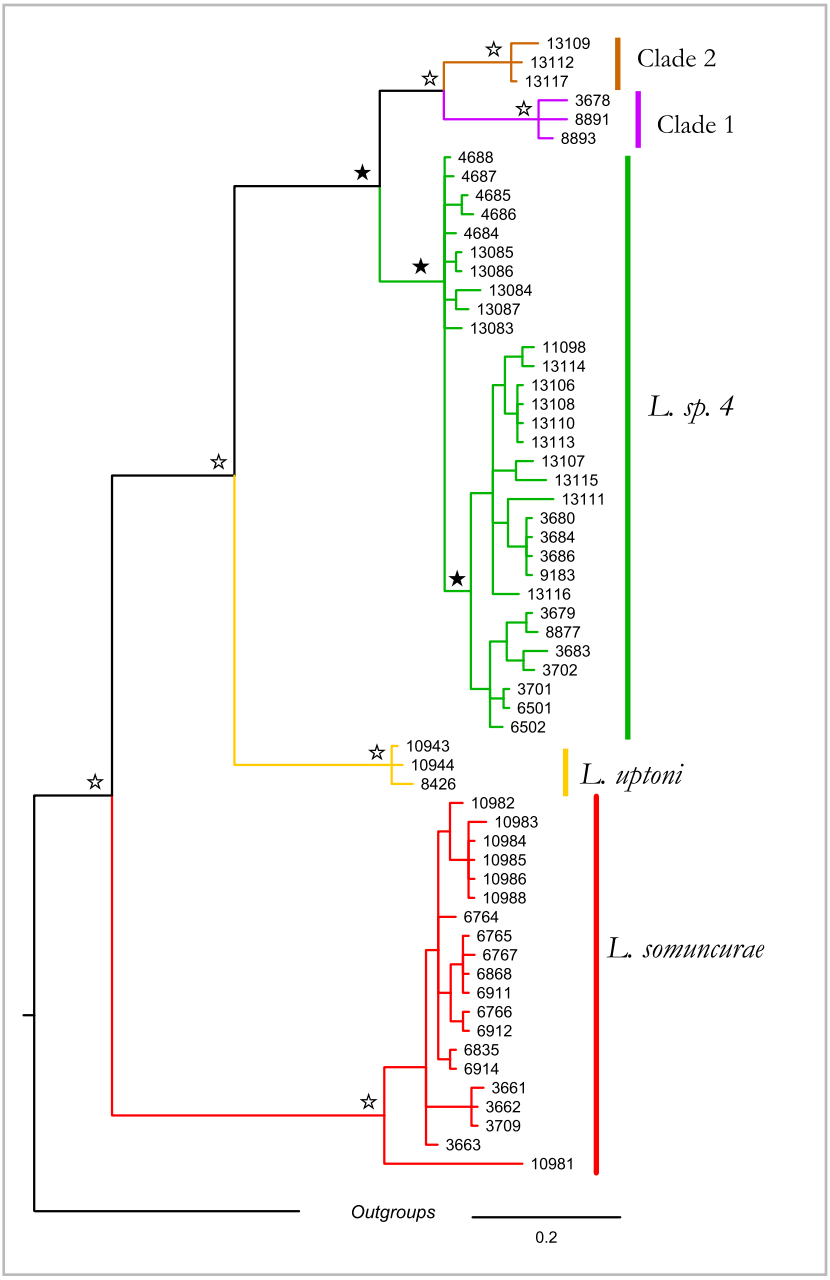

Figure 2. Cytochrome-*b* gene tree. Sample numbers shown on the tree; white stars represent significant Bayesian and ML support values ($Pp > 0.95$ and bootstrap > 95%), black stars represent significant Bayesian support values but moderate ML support values ($Pp > 0.95$ and bootstrap > 70%). *Liolaemus kingii*, *L. magellanicus*, and *L. lineomaculatus* were used as outgroups. Some aspects of the graphics might only be fully comprehensible in the online version where they are reproduced in color.

uptoni, *L.* sp. 4, clades 1 and 2. For the first three lineages sampling was adequate (table S4), but it was limited for clades 1 and 2 (tables S2, S4), thus meristic and morphometric (divided by sex) data sets were statistically analyzed only for the first three lineages. The qualitative data set was analyzed for all the species, but results for clades 1 and 2 are interpreted as hypotheses

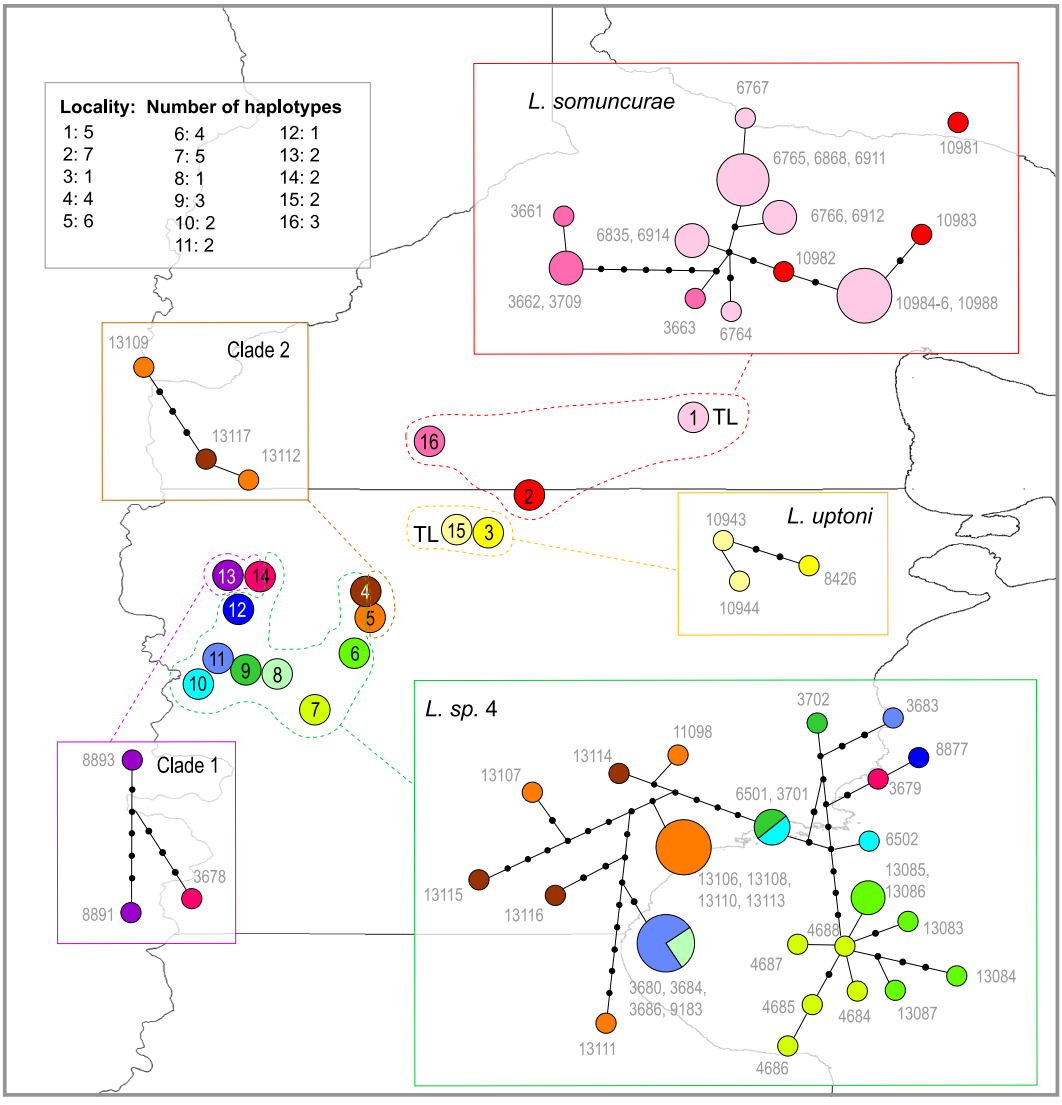

Figure 3. Networks recovered for the ingroup, colored by locality and discriminated by lineage. Type localities indicated by 'TL', specimen numbers shown in the networks, and quantity of haplotypes per locality shown in the inset (black dots on lines connected colored circles record the number of nucleotide differences between haplotypes). Singleton #10981 was recovered in locality #2. Some aspects of the graphics might only be fully comprehensible in the online version where they are reproduced in color.

deserving further testing (due to small simple sizes). Means, standard deviations and ranges of meristic and morphometric data (separated by sex for each species), tests and assumptions of sexual dimorphism, and values of qualitative variables, are summarized in online supplementary tables S5, S6 and S7, respectively.

In the qualitative data set of *Liolaemus somuncurae*, *L. uptoni*, and *L.* sp. 4 (excluding red scales and vertebral line, where no inter- or intraspecific differences were found) several intra- and interspecific differences were found (table S7). Ventral variegation was sexually dimorphic in all the species (present in most of the males and only in half of the females), but not among species. Melanism was not sexually dimorphic in any species; some interspecific differences were observed (mainly in more than

Table 1. Cytochrome-*b* pairwise genetic distances (expressed in percentage) among species/lineages. Intragroup distances (on the diagonal), uncorrected intergroup distances (below diagonal), and corrected intergroup distances (above diagonal).

	L. somuncurae	*L. uptoni*	*L.* sp. 4	Clade 1	Clade 2	Clade 1 + 2
L. somuncurae	0.94	7.63	7.19	7.15	7.92	6.81
L. uptoni	8.23	0.27	5.37	5.97	6.02	5.27
L. sp. 4	8.21	6.05	1.09	3.26	3.06	2.43
Clade 1	8.07	6.56	4.26	0.90	2.43	–
Clade 2	8.62	6.38	3.83	3.10	0.45	–
Clade 1 + 2	8.34	6.47	4.04	–	–	2.13

Table 2. Standard molecular diversity indices. "k" average number of differences between two random sequences.

	Sample size	# segregating sites	# haplotypes	Haplotype diversity	Nucleotide diversity ± SD	k
L. somuncurae	20	39	12	0.94	0.01015 ± 0.00256	7.00
L. uptoni	3	4	3	1.00	0.00359 ± 0.00134	2.67
L. sp. 4	31	46	23	0.97	0.01143 ± 0.00068	8.48
Clade 1	3	10	3	1.00	0.00898 ± 0.00254	6.67
Clade 2	3	5	3	1.00	0.00449 ± 0.00175	3.33
Clade 1 + 2	6	33	6	1.00	0.02200 ± 0.00382	16.40
Ingroup	60	135	44	0.99	0.05316 ± 0.00275	36.60

half of the individuals of *L. somuncurae* and *L.* sp. 4, but absent in *L. uptoni*). Dorsal stripe pattern was sexually dimorphic in *L. somuncurae* and *L. uptoni* (most females with complete or slightly broken bands, while > 50% of males presented almost indistinct bands); *L.* sp. 4 show differences in this character (most individuals had complete or dotted bands) relative to the other species. Dorsolateral bands were sexually dimorphic in *L.* sp. 4 (70% of females and 30% of males presented the character); interspecific variation was observed in females of *L.* sp. 4 when compared to *L. somuncurae* and *L. uptoni* (band was absent in most of these individuals). Head color differentiation was not sexually dimorphic; interspecific differences were observed in *L.* sp. 4 (lower frequency relative to the other species). For clades 1 and 2 sexual dimorphism was present in ventral variegation, melanism, and dorsal stripe pattern, while interspecific differences were found in dorsolateral bands and head color differentiation (table S7).

Sexual dimorphism was absent in the meristic data set (MANOVA; *Liolaemus somuncurae* $P = 0.52$, *L. uptoni* $P = 0.28$ and 0.39, *L.* sp. 4 $P = 0.19$, online supplementary tables S5, S6, and S8). Morphometric sexual dimorphism was significant for a different number of variables depending on the species (table S6): *L. somuncurae* showed sexual dimorphism in almost all characters (except SLV, DFH, AH, and TL), *L.* sp. 4 in several characters (all except SVL, DFH, HAL, RND, DRE, and TL), and *L. uptoni* in two characters (HL and AH). Multivariate sexual dimorphism in the morphometric data set (MANOVA; table S8) was also significant for *L. somuncurae* (raw data $P = 0.002$, standardized $P = 0.002$) and *L.* sp. 4 (raw $P = 0.004$ and 0.001, standardized $P = 0.03$ and 0.001), but absent for *L. uptoni* (raw $P = 0.76$ and 0.45, standardized $P = 0.23$ and 0.77).

Univariate tests showed significant differences in meristic (online supplementary table S9) and morphometric characters (supplementary table S10) among *Liolaemus somuncurae*, *L. uptoni* and *L.* sp. 4 (summarized in table 3). For the meristic data set, *L. somuncurae* differed from *L. uptoni* in six characters (SCI, MS, DS, LS, IL3, IL4), while *L.* sp. 4 differed in three characters from *L. so-*

Table 3. Meristic (above diagonal) and morphometric (raw and standardized by SVL; below diagonal) differentiation among species (ANOVA or Kruskal-Wallis tests). For the morphometric data (sexual dimorphism was present) results are discriminated by: "♀ and ♂" when differences were present in both sexes, "♀" when present only in females, and "♂" when present only in males.

	L. somuncurae	*L. uptoni*	*L.* sp. 4
L. somuncurae		SCI, MS, DS, LS, IL3, IL4	MS, DS, IL3
L. uptoni	♀ and ♂: FOL, HAL ♀: SVL, DFH, TFL, RUL, RUL/SVL, HH, HW, HW/SVL, HL, HL/SVL, RND, RND/SVL, DRE, AH, AH/SVL		LS, DS, IL4
L. sp. 4	♀ and ♂: SVL, DFH, FOL, TFL, HAL, HW, HL ♀: RUL/SVL, HH, RND/SVL, AH ♂: FOL/SVL, RUL, DRE, RND, RH/SVL, AH/SVL	♀ and ♂: RUL ♀: RND, AH ♂: HL, DRE	

muncurae (MS, DS, IL3) and from *L. uptoni* (LS, DS, IL4) (tables S5, S9; table 3). For the morphometric data set, (1) *L. uptoni* differed from *L.* sp. 4 in females (RUL, RND, AH) and males (RUL, HL, DRE); (2) *L. somuncurae* and *L. uptoni* showed significant differences in females (SVL, DFH, FOL, TFL, RUL, RUL/SVL, HAL, HH, HW, HW/SVL, HL, HL/SVL, RND, RND/SVL, DRE, AH, AH/SVL) and males (FOL and HAL); and (3) *L.* sp. 4 and *L. somuncurae* showed significant differences between females (SVL, DFH, FOL, TFL, RUL/SVL, HAL, HH, HW, HL, RND/SVL, AH) and males (SVL, DFH, FOL, TFL, HAL, HW, HL, FOL/SVL, RUL, DRE, RND, RH/SVL, AH/SVL) (table 3).

Discussion

Species boundaries and genetic characterization

The northernmost distributed Patagonian lizards of the *Liolaemus kingii* group were recovered as five well-supported independent lineages based on the cytochrome-*b* gene tree (*L.* sp. 4 was well supported in BI and moderately supported in ML reconstructions), network analysis, and cytochrome-*b* genetic distances. Morphological differences also supported the specific status of *L. somuncurae*, *L. uptoni*, and *L.* sp. 4. Two mtDNA lineages represent the species *L. somuncurae* and *L. uptoni*, and three represent candidate species. One candidate species was previously identified as *L.* sp. 4 (Breitman et al., 2011), and two presenting restricted distributions are identified in this study.

For *Liolaemus*, uncorrected cytochrome-*b* genetic pairwise distances of ∼3% between clades is considered a valid threshold towards identification of putative species (Breitman et al., 2012), calculated based on morphologically described sister species (Breitman, 2013). We found values $> 3\%$ among all lineages pairs, suggesting that the three undescribed lineages represent candidate species. Divergence times among lineages ranged from Pliocene to Early Pleistocene (text/figure S3). The specific status of *L. somuncurae* and *L. uptoni* was reinforced by morphological differences (meristic, morphometric and coloration), relatively older divergence times, and geographic isolation, greatly extending previous evidence (Scolaro and Cei, 2006; Breitman et al., 2012, 2013).

Liolaemus sp. 4 is geographically isolated (figs 1, 3), and genetically ($>3\%$ cytochrome-*b* genetic distance; table 1) and morphologically (table 3; table S7) different from *L. somuncurae* and *L. uptoni* (tables 1, 3). Although mitochondrial DNA placed *L.* sp. 4 within the *L. somuncurae* group, nuclear DNA and species tree approaches recovered this candidate species nested within the *L. kingii* clade (BEST, *BEAST; Breitman et al., 2011; Olave et al., 2014). This pattern, in addition to the intermediate geographic position of *L.* sp. 4 (northwestern Chubut, which is north and south of the *L. kingii* clade and *L. somuncurae* group distributions, respectively) suggests a scenario of

past hybridization with asymmetrical mitochondrial DNA gene flow (Breitman et al., 2011), or a possible mitochondrial capture event. Further studies are needed in order to test these hypotheses. Although we were tempted to formally describe *L.* sp. 4 in the present contribution, in the light of the uncertain taxonomic position of *L.* sp. 4 (Breitman et al., 2011; Olave et al., 2014), we consider that morphological variation should be evaluated against other species of the *L. kingii* clade before attempting a formal description.

Individuals with haplotypes recovered in clades 1 and 2 have restricted and allopatric distributions in northwestern Chubut, in the surroundings of Blanca and Esquel lagoons (fig. 1). Clades 1 and 2 diverged in the early Pleistocene (table S2), are characterized by high genetic differentiation (table 1), geographic separation (although overlapped with the northernmost *Liolaemus* sp. 4 populations; figs 1-3), and preliminary morphological differences (see next section); suggesting that these lineages should be considered candidate species in need of further study.

Morphological variation

This is the first study to evaluate morphological variation throughout the known distributions of the northernmost lizards of the *Liolaemus lineomaculatus* section. Two previous contributions, based on a small number of individuals from type localities, described morphological variation in *L. somuncurae* and *L. uptoni* (Scolaro and Cei, 2006; Breitman, Morando and Avila, 2013). The original species description of *L. uptoni* (Scolaro and Cei, 2006) was based on five individuals and although several morphological differences with *L. somuncurae* ($n = 16$) were listed (*L. uptoni* had longer hind limbs, fewer midbody scales, less ventral melanism, and a more prevalent vertebral/paravertebral line, relative to *L. somuncurae*; sexual dimorphism was absent in *L. uptoni*, but present in *L. somuncurae*), further analyses were encouraged by the authors.

Recently, a morphological review including all species of the *Liolaemus lineomaculatus* section was published (Breitman, Morando and Avila, 2013). This review presented an extended data set (14 morphometric, 10 meristic, and 10 qualitative characters) and increased the sample size for *L. uptoni* ($n = 11$). This study supported previous observations of *L. uptoni* (relative to *L. somuncurae*) as being characterized by a more defined vertebral/paravertebral line, fewer midbody scales, no sexual dimorphism, and less melanism; this last difference probably related to *L. uptoni* inhabiting sandy environments and *L. somuncurae* basaltic substrates (Scolaro and Cei, 2006). Scolaro and Cei (2006) found that *L. uptoni* had longer hind limbs than *L. somuncurae* (although these differences were not statistically significant), but opposite results were obtained later by Breitman, Morando and Avila (2013), in which *L. somuncurae* had longer limbs than *L. uptoni*. We think that this difference might be due to different techniques since Scolaro and Cei (2006) included the femur in their measurements, but not Breitman, Morando and Avila (2013). More significant morphometric (females: RUL/SVL, HAL/SVL, HW/SVL; males: DFH/SVL, HH/SVL) and meristic (only between males: SS, IS, VS) differences between *L. somuncurae* and *L. uptoni* were reported by Breitman, Morando and Avila (2013), most probably due to the extended data set used by these authors.

The results from our paper are based on larger sample sizes ($n = 12$ for *Liolaemus uptoni* and $n = 29$ for *L. somuncurae*) collected throughout the species' ranges, which allowed considerable extension of the available morphological information. While sexual dimorphism is statistically rejected in *Liolaemus uptoni*, it is present in *L. somuncurae* (table S8), particularly in morphometric variables related to limb and head lengths but not to body size (SVL or DFH). In several *Liolaemus* species differences in these characters were detected as these relate to sexual or natural selection; in males "bite force" is the character selected, while for females "prey se-

lection" is the focal character (Vanhooydonck et al., 2010; also discussed in Breitman, Morando and Avila, 2013). Sexual dimorphism in *Liolaemus* limb length has been associated with substrate use (Tulli, Abdala and Cruz, 2011); but further ecological studies are needed to test the hypothesis of whether male and female *L. somuncurae* are partitioning the habitat. In addition to support most of the previously published differences (see previous paragraph) between *L. uptoni* and *L. somuncurae*, we found interspecific variation in other meristic (SCI, DS, LS, IL3, IL4) and morphometric variables (only for females: SVL, DFH, TFL, RUL, HH, HW, HL, HL/SVL, RND, RND/SVL, DRE, AH, AH/SVL; table 3) related to body, head, and limb size. However, we did not find evidence of interspecific differences in some previously reported variables (SS, IS, VS; and only for males DFH, HH). We also report for the first time, inter- and intraspecific differences in hand and foot length, probably reflecting adaptations to different environments (Scolaro and Cei, 2006; Tulli, Abdala and Cruz, 2011).

Liolaemus sp. 4 was recovered with molecular data, and here we present the first morphological characterization of this lineage, and a comparison with *L. somuncurae* and *L. uptoni*. Several morphological variables (table 3) were statistically different between *L.* sp. 4 and *L. somuncurae* (morphometric: SVL, DFH, FOL, TFL, HAL, HW, HL; only females: RUL/SVL, HH, RND/SVL, AH; only males: FOL/SVL, RUL, DRE, RND, RH/SVL, AH/SVL; meristic: MD, DS, IL4), and between *L.* sp. 4 and *L. uptoni* (morphometric: RUL; only females: RND, AH; only males: HL, DRE; meristic: LS, DS, IL4). A greater number of differences was found between *L.* sp. 4 and *L. somuncurae*, relative to *L.* sp. 4 and *L. uptoni*; suggesting that either *L.* sp. 4 and *L. uptoni* are closely related (also supported by the mitochondrial tree, but not the nuclear tree), or that the similarity in the species phenotypes is the result of similar ecological pressures (both species inhabit similar habitats). Individuals of *L.* sp. 4 have a thick and well-defined vertebral line blended with perpendicular well-defined lines that are wider in the vertebral portion of the body, but become narrow in the lateral zone; this pattern is very distinct from other species of the *L. lineomaculatus* section (described in Breitman, Morando and Avila, 2013). For *Liolaemus* sp. 4 we also found evidence of sexual dimorphism in meristic and morphometric characters related to limb length and head size (tables S5, S6), which most probably are associated with different selection pressures on males and females (as previously discussed for *L. somuncurae* and *L. uptoni*), and have been previously reported for species of the *Liolaemus kingii* group (Breitman, Morando and Avila, 2013).

Although small sample sizes in clades 1 and 2 precluded statistical tests to quantify morphological variation, differences in coloration patterns (adult individuals have a black and brown "tabby" pattern formed by transversal bands) suggest that they may be morphologically different from other lineages of the *Liolaemus lineomaculatus* section. Thus, we recommend field surveys in these and adjacent areas for detailed morphological and genetic analyses, in order to clarify the status of these candidate species.

Biogeographic scenarios

The northernmost lineages of the *Liolaemus kingii* group were recovered in a pectinate topology in the gene tree (fig. 2), which (based on current species sampling) suggests a north-to-south colonization pattern (as previously hypothesized by Breitman et al., 2011). The oldest speciation event that most probably isolated *L. somuncurae* from the other lineages was dated to be in the Pliocene ($\sim$ 3.91 Ma). This may be associated with changes in climate (due to the end of the Late Pliocene glaciations $\sim$ 3.5 Ma) that promoted southern population expansion, and also with volcanic activity (present in this area until the Pliocene-Pleistocene limit, and predominant on the plateau and its western areas; Rabassa, 2008), that may have pro-

moted isolation by raising topographic barriers to lizard dispersion. Evidence supporting this expansion scenario was found from other unique lizards lineages (including *L. bibronii*, *L. melanops*, and *L. petrophilus*; Morando et al., 2007; Fontanella et al., 2012) found in this area (Talagapa Pampa), whereas the Somuncurá plateau is proposed to be an allopatric barrier for some dispersal routes. Statistical analysis evaluating temporal and spatial congruence of phylogenetic breaks will clarify the role of the environment in shaping the distribution of these species (e.g. Bagley and Johnson, 2014).

The Chubut River (present from the Miocene) runs from the Andes to eastern Chubut Province. Some authors have hypothesized this drainage to be a barrier to permafrost during the last glacial periods (Kim et al., 1998), but others considered the maximum advance of the permafrost to be further north, and during the Last Quaternary Glaciation (1.2 Mya) extending at least to the parallel 38° (Trombotto, 2002). Evidence from moraines suggests that during at least the last three major glaciations (350.000-275.000 ya; 250.000-130.000 ya; 90.000-15.000 ya; J. Rabassa, personal communication), the Chubut River acted as a strong glaciofluvial drainage (much bigger in magnitude that the actual river) that discharged water towards the Atlantic Ocean (Rabassa, Coronato and Martínez, 2011). Further, evidence from rodados patagónicos suggests that during the interglacial periods the Chubut River had a permanent high water discharge regimen, probably similar to the current volume (J. Rabassa, personal communication), thereby eroding and forming the present-day Chubut Valley (Martínez and Kutschker, 2011). Although evidence pre-dating these last three glaciations is not available, the caudal of the river had probably been similar (J. Rabassa, personal communication). The allopatric distributions of *Liolaemus uptoni* and the clade (*L.* sp. 4 + clade 1 + 2), north and south of the Chubut River (fig. 1), in addition to their divergence times concordant with the Pliocene-Pleistocene limit (∼2.65 Ma), suggest that better climatic conditions might have promoted the dispersal of the populations towards the south and that the Chubut River could have been a geographic barrier between these species. Evidence from rodents and plants support the hypothesis that the Chubut River was a topographic barrier to gene flow among populations (Kim et al., 1998; Sérsic et al., 2011). Further sampling coupled with paleoclimatic niche modelling analysis should be performed in order to test the role of the Chubut River in shaping the observed multitaxon phylogeographic patterns (e.g. Wiens and Graham, 2005).

Diversification of *Liolaemus* sp. 4 and clades 1 and 2 (∼1.43 Ma) might have been associated with the Great Patagonic Glaciation (∼1.68-1.016 Ma; Rabassa, Coronato and Salemme, 2005), followed by later southern expansion of *L.* sp. 4 (fig. 3). We recognize two areas characterized by high genetic variation that might have served as refugia during the last period of glaciations. One of these is the Talagapa Pampa area (fig. 1) south of the Somuncurá massif (localities #2, 3, 15; figs 1, 3), where differentiated haplotypes and one ancestral haplotype of *Liolaemus somuncurae* are found (singleton #10982; fig. 2), along with very distinct lineages of mammals and plants (see discussion above). The second is the region of the Blanca Lagoon (localities 4 and 5; figs 1, 3), characterized by high genetic variation in *L.* sp. 4 and clade 2. The Somuncurá Plateau has also been proposed as a refugium (reviewed in Sérsic et al., 2011), and is characterized by the presence of several endemic species. Although the Somuncurá Plateau is protected, the Talagapa Pampa and the areas surrounding the Blanca Lagoon are not (fig. 1). Recent molecular studies focused on plants (*Anarthrophyllum desideratum*, *Mulinum spinosum*, *Nassauvia* sp. and *Calceolaria polyrhiza*; Cosacov et al., 2012) have identified the area around the Blanca Lagoon as a conservation priority, and our results also support this recommendation.

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