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Geography and past climate changes have shaped the evolution of a widespread lizard from the Chilean hotspot

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ABSTRACT

The complex orogenic history and structure of Southern South America, coupled with Pleistocene glacial cycles, have generated paleoclimatic and environmental changes that influenced the spatial distribution and genetic composition of natural populations. Despite the increased number of phylogeographic studies in this region and given the frequent idiosyncratic phylogeographic patterns, there is still the need to focus research especially on species that are currently distributed within a wide range of bioclimatic regimes, and that historically have been subject to contrasting scenarios. *Liolaemus tenuis* is a widely distributed lizard species inhabiting latitudinally in almost 1000 km through central and southern Chile. Here we describe the geographical patterns of genetic variation and lineage diversification within *L. tenuis*, and their association with geography and Pleistocene glaciations, using sequences from one mitochondrial and two nuclear genes, and five microsatellite loci, and covering most of the species distributional range. Our results revealed a high diversity both within and among populations, as well as two phylogeographic breaks, which are consistent with two of the larger rivers of central Chile, the Maipo and Biobío Rivers. *Liolaemus tenuis* is characterized by several allopatric lineages, especially in its north and central range, which suggest a history of multiple vicariance processes. Conversely, populations found in the southern range, south of the Biobío River, show signatures of recent decreases in effective population sizes, coupled with recent range expansions and secondary contact. Niche "envelope" data are consistent with patterns of genetic variation; both suggest a history of discontinuous areas of relatively stable populations throughout all of the distribution of *L. tenuis*. These data are also consistent with higher probabilities of habitat suitability north of the Maipo River (ca. 33°S), in both coastal areas and the "Intermediate Depression" between 34° and 37°S, as well as in the southern Coastal Cordillera between the Biobío and Araucanía regions. Interestingly, both molecular and niche envelope modeling data suggest that some populations may have persisted in fragmented refugia in Andean valleys, within the limits of the ice sheet. Finally, our results suggest that several populations of *L. tenuis* colonized glaciated regions from refugial areas in lowlands and coastal regions, and in the southern distribution, historic migration events would have occurred from refugial areas within the limits of the ice sheet.

1. Introduction

Geological events, coupled with past climatic changes have played a key role in shaping current patterns of genetic diversity (Hewitt, 1996). Temporal and spatial environmental heterogeneity are among the main drivers of intraspecific differentiation (e.g., Levy et al., 2012; Wang and Yan, 2014) and ultimately of species diversification (e.g., Brown et al., 2014). Consequently, a positive association between habitat

discontinuity (e.g., landscape roughness and/or the occurrence of barriers) and beta levels of intraspecific diversity (population genetic structure and/or structured lineage distribution) is expected. For example, several studies have shown the importance of large rivers acting as barriers to gene flow and delimiting the distributions of closely related subspecies or species of tropical birds (Capparella, 1991; Cheviron et al., 2005; Maldonado-Coelho et al., 2013; Ribas et al., 2011; Smith et al., 2014; Voelker et al., 2013), lizards (Pellegrino et al., 2005;

Torres-Pérez et al., 2007), and mammals (Link et al., 2015; Nicolas et al., 2011; Patton and da Silva, 1998). In the same context, historically restricted stable habitats, which allow populations to remain in place over time, are positively correlated with high levels of intrapopulation genetic variability and demographic equilibrium. The opposite situation is expected for areas that have been repeatedly exposed to climatic fluctuations, as the Pleistocene glaciations, during which populations exhibit signatures of recent colonization and demographic expansion suggested, due to, for example, a lack of mutation-drift equilibrium and high historical levels of gene flow (e.g., Ding et al., 2011; Zhang et al., 2008a, 2008b).

One example of dynamic and heterogeneous scenario is western temperate South America, where the uplift of the Andes and Pleistocene glaciation cycles would have had important evolutionary consequences for the biota. The Andes, whose uplift started approximately 23 million years ago, have acted as a barrier between the current western slope and the Argentine Patagonia, and have shaped a high topographic heterogeneity along the western slope (Gregory-Wodzicki, 2000). This orogenic event history would have facilitated evolutionary diversification, ultimately forming much of the biota to what is now referred to as the "Chilean Biodiversity Hotspot" (Antonelli et al., 2010; Myers et al., 2000).

Along the north-south axis of the western Andean range, there is a pronounced altitudinal gradient, which is characterized by high altitudes and more heterogeneity in relief from south to north. This topographical gradient is also characterized by an increasing latitudinal gradient of fragmentation of the Chilean "lowlands" that lay between the Andes and the Pacific Coastal Cordillera (Fig. 1). The opposite topography is observed in the Andean Cordillera as it extends to the south; overall elevations are lower and intervening valleys are more connected.

Over this orogenic history, and interacting with it, four Pleistocene climatic events occurred that had important demographic consequences (McCulloch et al., 2000; Rabassa and Clapperton, 1990; Ruzzante et al., 2008). Such cycles had a greater impact along the southern Andes. During the Last Glacial Maximum (LGM) the ice sheet coverage increased in extension towards the south, also extending onto low altitude areas (Hulton et al., 2002; Markgraf et al., 1995; Villagrán, 1991; Villagrán et al., 1995). In the western slope of the Andes in Central Chile, the northern limit of the ice sheets reached approximately the latitude of 33°S at high Andean regions and covering all the land up the Pacific Coast south of the 42°S. According to this, the coastal mountain range, as well as coastal areas north of 41°S, would have constituted stable areas where species would have survived during the LGM (Armesto et al., 1994; Sérsic et al., 2011; Villagrán, 2001; Villagrán et al., 1995). As such, in the western slope of the Andes the intensity of the glacial effects on the biota would have increased towards the south (Amigo and Ramírez, 1998; Heusser, 2003; Smith-Ramirez, 2004), reducing effective population sizes (Ne) mainly in Andean and Southern populations (e.g., Lessa et al., 2010; Vera-Escalona et al., 2012; Victoriano et al., 2008).

Another distinctive feature of Chile is the series of parallel steep, high gradient, east-to-west flowing rivers (e.g., Aconcagua, Maipo, Maule, Biobío). These rivers may also act as barriers to gene flow structuring intraspecific genetic diversity in Chilean vertebrates and plants (Chesser, 1999; Lamborot et al., 2003; Sallaberry-Pincheira et al., 2011; Torres-Pérez et al., 2007; Unmack et al., 2009; Vásquez et al., 2013; Viruel et al., 2014). Nevertheless, no study has formally evaluated the combined effect of rivers, topographic relief, and shifting climates (glacial advances and retreats) in structuring the intraspecific genetic variation/phylogeographic history of any Chilean species. The north-south axis of over 4000 km of the Chilean Andes, combined with its west-east elevational gradient (sea level to above 6000 m), coupled to multiple ice sheet glacial advances and retreats in addition to simultaneous increases in river water volumes due to melting ice, would have dramatically impacted terrestrial (as well as aquatic) communities

in this region. As such, widely distributed Chilean species constitute a good system to assess the joint effects of geographical complexity, glacial cycles, and fluctuating river volumes, on intraspecific population differentiation and phylogeographic structure.

Liolaemus is a highly diverse (ca. 250 species; Uetz et al., 2016) and widely distributed genus of South American lizards. *Liolaemus tenuis* (Duméril and Bibron, 1837) is a broadly distributed species that encompasses a latitudinal range of 1000 km, from the Chilean regions of Coquimbo (ca. 30°S) to Los Ríos (ca. 40°S; Fig. 1), with peripheral populations also present on some eastern Andean slopes in the Argentinean province of Neuquén. The altitude range of this species extends from sea level up to 1800 m (Donoso-Barros, 1966; Pincheira-Donoso and Nuñez, 2005; Victoriano et al., 2008), and occurs in distinct habitat types ranging from "Mediterranean" shrubland to sub-Andean forests (Di Castri, 1968). As such, the species distribution encompasses both stable and topographically heterogeneous areas in the north, as well as southern latitude landscapes that were impacted by Pleistocene glaciations. Victoriano et al. (2008) suggested that the microevolutionary history of *L. tenuis* was influenced by Pleistocene glacial cycles, but the inferred demographic changes were not dated, nor were the locations of putative stable areas (refugia) identified and subsequent colonization routes hypothesized.

The aim of this study is to evaluate the phylogeographic structure of *L. tenuis* across most of its distributional range in order to test the following predictions. (1) Populations that now occur in previously glaciated areas, mainly distributed in the Andes, will show genetic signatures of demographic expansion and lower levels of genetic structure and diversity than populations that occur in areas that were free of ice during glaciations. (2) During glacial advances, refugial areas would have been located near the Coastal Cordillera in the Southern range of *L. tenuis*, and post-glacial population expansions would have occurred in a predominantly northwest to southeast direction. (3) The east-to-west flowing rivers have acted as barriers to gene flow, shaping distribution genetic structure of clades along a north-south latitudinal arrangement.

2. Materials and methods

2.1. Sample collection

The study is based on 225 specimens of *Liolaemus tenuis* collected at 84 sites (Fig. 1). Of these, 145 specimens from 41 localities were taken from the study of Victoriano et al. (2008). The other 80 individuals were newly collected (between 2007 and 2012) at 45 localities. The new specimens were captured with collection permits granted by the Servicio Agrícola y Ganadero (authorizations SAG-1898 and SAG-4729). All captures were carried out according to the protocols approved by the Bioethics Committee of the Universidad de Concepción (Chile). Individuals newly collected were deposited in the collection of the Museo de Zoología de la Universidad de Concepción (MZUC).

Sampling sites were classified into three groups, corresponding to the main Chilean bioclimatic zones, as follows: (1) North (N), including localities north of the Maipo River, an area that corresponds to the dry Mediterranean bioclimate zone; (2) Central (C), including localities between the Maipo and Biobío rivers, which correspond to the mesic Mediterranean area; and (3) South (S), including localities south of the Biobío River, covering the wet Mediterranean area, which includes the Valdivian Forests (Di Castri, 1968; Table S1).

2.2. Laboratory procedures

Genomic DNA was extracted from muscle tissue using the commercial kit Wizard SV Genomic (Promega) following manufacturer's instructions. Detailed amplification and sequencing methods largely follow Broadley et al. (2006), Victoriano et al. (2008), and Portik et al. (2011a,b), and are available in Appendix A.
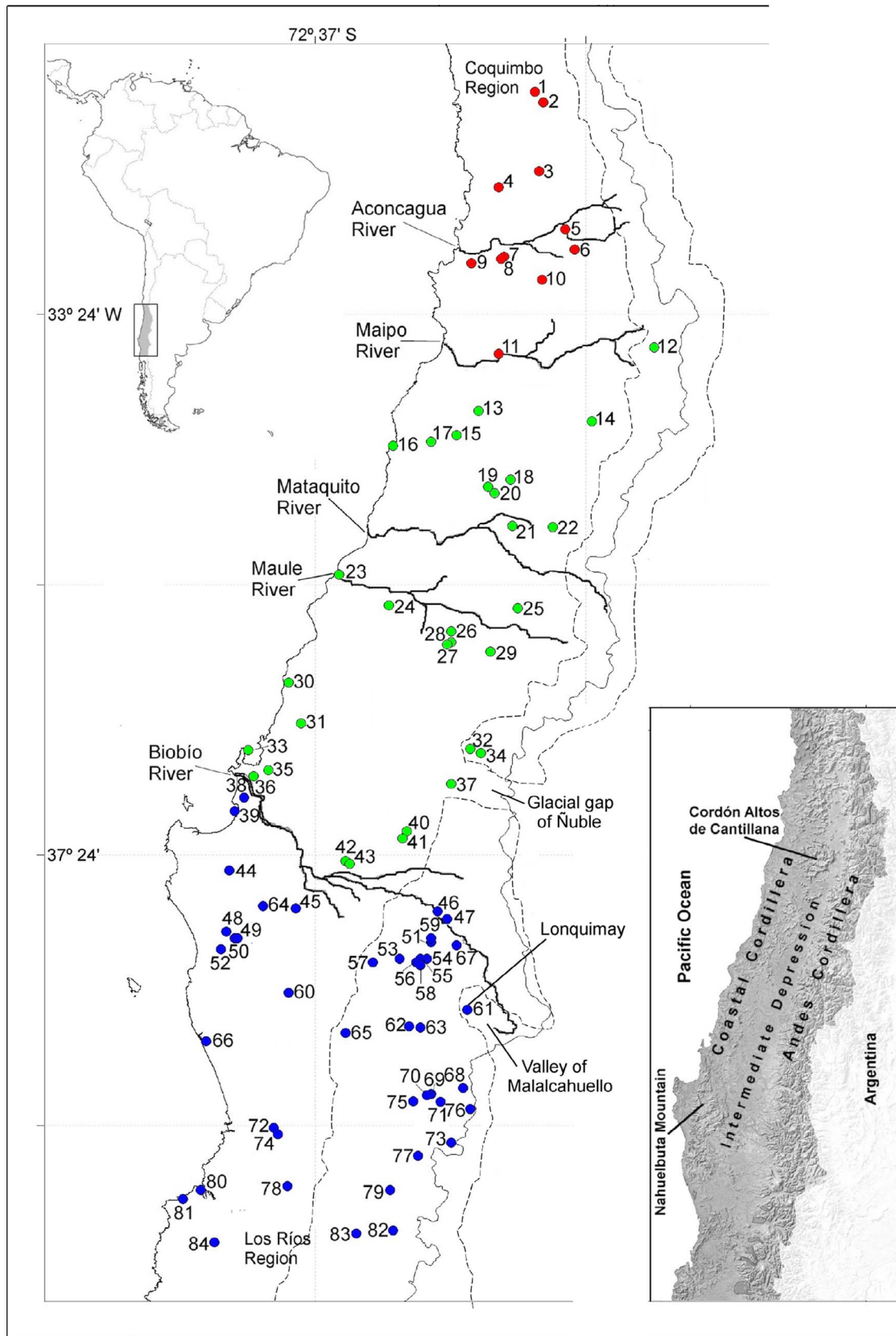
Fig. 1. Sampling sites of *Liolaemus tenuis* across its Chilean range. Numbers correspond to those of Table S1. Colors indicate their geographical assignation: red correspond to the dry Mediterranean bioclimate zone (North); green the mesic Mediterranean area (Central); and blue the wet Mediterranean area (South). The dashed line corresponds to the ice sheet's limits during the LGM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Adapted from Heusser, 2003

This study is based on DNA sequences of two protein coding genes (the mitochondrial cytochrome *b* gene: cyt *b*; the nuclear Kinesin-like protein gene: KIF24), a nuclear anonymous gene (LDB5B), and five nuclear microsatellite loci, three of which tetranucleotide repeats (TET1177, TET2216, TET1501) and two dinucleotide repeats (DI 159, DI 7938), developed for *Liolaemus fitzingerii* (Hanna et al., 2012).

Sequence editing and alignment were done using Codon Code Aligner v. 3.0.3 (Codon Code Corporation, 2009). Coding sequences (cyt *b* and KIF24) were translated into amino acids in order to corroborate the absence of stop codons. In cases where sequences from nuclear markers presented heterozygous sites, haplotypes were inferred using the coalescent-based Bayesian method implemented in Phase 2.1 (Stephens and Donnelly, 2003; Stephens and Scheet, 2005; Stephens et al., 2001). A probability threshold was first established at 0.9 but as not all haplotypes were resolved, we lowered the threshold to 0.6 following Garrik et al. (2010), who suggested that this value increases the number of resolved haplotypes with almost no increase in false positives. In addition, recombination was tested for nuclear sequences using RDP: Recombination Detection Program v3.44 (Heath et al., 2006; Martin and Rybicki, 2000; Martin et al., 2005). The resulting heterozygous sequences were split and included separately in the matrix as paternal and maternal haplotypes for subsequent analyses. The sequences obtained in this study were submitted to Dryad (http://dx.doi.org/10.5061/dryad.jk183).

Microsatellite loci were amplified by PCR for 183 specimens collected at 32 localities (Table S2). The forward primers for all loci were fluorescently labeled (Applied Biosystems, Foster City, CA, USA) and each pair of primers was used individually in amplification reactions. PCR products were checked on a SybrSafe stained 2.0% agarose gel and sent for direct fragment analysis to Macrogen Inc. (Korea). The software GeneMarker v2.6.0 (Softgenetics, State College, PA) was used for allele size identification.

2.3. Genetic diversity and genealogical analysis

For all alignments, we performed the Xia test (Xia et al., 2003) as implemented in DAMBE 5.0.11 (Xia and Xie, 2001) to evaluate the degree of sequence saturation. The test was done with 100 replicates using the proportion of invariant sites found by jModeltest 0.1.1 (Posada, 2008).

Standard indexes (haplotype diversity Hd, nucleotide diversity π) were calculated with Arlequin 3.5 (Excoffier and Lischer, 2010) both for the total dataset (for cyt *b*, KIF24 and LDB5B), and for each clade obtained from genealogical analysis (see below). Average genetic divergence values for all between-locality pairs were estimated based on p-distances using Mega 6 (Tamura et al., 2011).

Genealogical analyses were carried out separately for each gene using non-redundant matrices (i.e., including one sequence per allelic class) with MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) by means of two runs with four chains each. All analyses ran for 10 million generations and were sampled every 1000 steps; the first 25% of the data was discarded as burn-in. The mitochondrial gene tree was rooted using sequences of the congeners *L. nigroviridis, L. lemniscatus,* and *L. nitidus*. The KIF24 genealogy was rooted using alleles of the species *L. chiliensis* and *L. pictus*, and that of LDB5B with sequences of *L. lemniscatus* and *L. pictus*.

The age of the most recent common ancestor (MRCA) of the main clades of *L. tenuis* was estimated with Beast 1.8.2 (Drummond et al., 2012) using the cyt *b* matrix. Because the cyt *b* rate of evolution for *L. tenuis* was unknown, it was estimated with Beast 1.8.2 using a relaxed molecular clock in a phylogenetic framework. To place the mean priors in the tree we used two fossils from the subgenus *Eulaemus*, both from Argentina. One fossil corresponds to 18.5–20 Mya (Albino 2008) while the second is a recent fossil associated with the origin of *Liolaemus multimaculatus* 70,000 ya (Albino 2005; Etheridge 2000). The model used for this analysis was the TrN + I + G with a relaxed uncorrelated

log normal clock selected using Bayes factor in Tracer 1.6 (Rambaut et al., 2013). To ensure convergence, analyses were run four times using a randomly generated starting tree and a Yule process to speciation. The length of the chain was 40 x10^6 steps, sampling parameters every 1000 steps and discarding the first 25%. We obtained a substitution rate of 4.72% per site per million years.

As another way to visualize relationships among sequences, a haplotype network was inferred separately for mtDNA (cyt *b*) and each nuclear locus (KIF24 and LDB5B) using statistical parsimony algorithms (SP; Crandall and Templeton, 1993) as implemented in TCS 1.21 (Clement et al., 2000). Ambiguities (loops) within the network were resolved following the criteria of Crandall and Templeton (1993).

2.4. Genetic structure

The three genes matrices (cyt*b*, KIF24 and LDB5B), each was used separately, were used to infer the most likely number of population clusters with the Bayesian clustering method implemented in Geneland version 4.0.3 (Guillot et al., 2005) in R version 3.0.2 (R Development Core Team, 2009). We performed a preliminary run where K (the number of populations or clusters) was allowed to vary from 1 to maximum number of sampled localities, in order to determine the modal number of clusters, using 10 replicates with 5×10^5 Markov chain Monte Carlo iterations. All runs were conducted using the spatial Dirichlet model for the priors in allele frequencies. Five runs with a fixed K number were performed for the spatially explicit model, and for each run, the posterior probability of subpopulation membership was computed for each pixel of the spatial domain (100×100 pixels). The Markov Monte Carlo (MCMC) chain was run for 500,000 generations, thinning was set at 100, and the burn-in period was set at 200 iterations. Once the clusters were determined, we used Arlequin 3.5 (Excoffier and Lischer, 2010) to calculate Fst (Weir and Cockerham, 1984) values among all pairwise combinations of the inferred population clusters.

Distinct hierarchical analyses of the distribution of genetic diversity of *L. tenuis*, for three genes (cyt *b*, KIF24 and LDB5B), were conducted in the form of analysis of molecular variance (Amova) using Arlequin 3.5 (Excoffier and Lischer, 2010). Hierarchical levels were defined on the basis of sampling localities, results of the Geneland analyses, and river locations. Amova groups were constructed as follows: comparison 1, north of Maipo River vs. south of Maipo River; comparison 2, north of Maipo River vs. between Maipo and Biobío rivers vs. south of Biobío River; comparison 3, north of Biobío River vs. South Biobío River; and comparison 4, among clusters inferred by Geneland.

Considering that the cyt *b* clusters resolved by Geneland suggest genetic structure associated both to bioclimate and to the Maipo and Biobío rivers (see below), gene flow between these clusters was estimated under a Bayesian coalescent framework implemented in Migrate-n version 3.6.4 (Beerli, 2006; Beerli and Felsenstein, 2001). In order to obtain the posterior distribution of the number of immigrants per generation (Nm), four possible models of gene flow were tested (Table S3). The marginal likelihood of each model was estimated by ranking the Bayes factor (Beerli and Palczewski, 2010). A starting UPGMA tree was used and the initial theta and M values were derived from the Fst calculation. Static heating was applied to all four independent chains using the temperature settings of 1.0, 1.5, 3.0 and 1,000,000.0. A total of 500,000 steps were run and recorded every 100 generations, from which 12,500 were discarded as the burn-in. Stationarity was assessed by examining the effective sample size (ESS) and distribution of each parameter in Tracer 1.6 (Rambaut et al., 2013).

2.5. Historical demographic changes

The demographic history of each main clade was inferred by pairwise mismatch distribution analyses (Rogers and Harpending, 1992) and Ramos-Onsis and Rozas's R2 test (Ramos-Onsins and Rozas, 2002)

Table 1

Measures of genetic diversity and demographic expansion for mtDNA and nuclear genes (KIF24 and LDB5B) clades. Numbers in bold indicate statistical significant values. Numbers in parentheses indicate standard deviations. N: sequence numbers. $R2$: Ramos-Onsis Roza's test.

Gene	Phylogroup				Genetic variability		Demographic expansion		
Cytb	Clade	N	S	H	Hd	π	Fs' Fu	D	R2
	I	140	245	82	0.989	0.0615	**−16.27**	−0.084	0.062
	A	45	117	23	0.954	0.0298	−0.41	−0.831	0.094
	B1	25	67	15	0.943	0.0186	−0.36	−1.114	0.088
	B2.2.1	23	68	14	0.97	0.0304	−1.56	−1.216	0.119
	B2.2.2	22	75	14	0.922	0.0313	1.77	0.411	0.152
	B2.2.3	23	47	14	0.933	0.0105	**−5.14**	−1.073	0.092
	II	85	20	14	0.472	0.0015	**−9.31**	**−2.188**	**0.024**
		225	261	96	0.931	0.0725	−5.92	0.714	0.049
KIF24	Clade	N	S	H	Hd	π	Fs' Fu	D	
	1	26	16	19	0.942	0.0072	**−14.61**	0.505	
	1.3	5	3	5	1	0.0031	**−3.304**	1.573	
	1.4	19	12	12	0.895	0.005	**−8.332**	−0.112	
	2	132	60	85	0.985	0.0132	**−24.7**	−0.884	
	2.1	72	35	40	0.962	0.0078	**−25.67**	−1.21	
	2.2	26	24	20	0.951	0.0075	**−13.37**	−1.048	
	2.3	5	1	2	0.4	0.0007	0.09	−0.817	
	2.4	4	1	2	0.667	0.0011	0.54	1.633	
	2.6	15	17	13	0.971	0.0069	**−7.97**	−0.95	
	2.12	2	4	2	1	0.0069	1.39	0	
		158	71	104	0.988	0.0151	**−24.44**	−0.818	
LDB5B	Clade	N	S	H	Hd	π	Fs' Fu	D	
	1	9	20	7	0.917	0.0142 (0.0082)	0.13	0.442	
	3	147	50	39	0.922	0.0164 (0.0084)	−6.012	0.21	
	3.1	51	28	16	0.89	0.0054 (0.0032)	**−5.63**	**−1.622**	
	3.3	74	9	7	0.745	0.004 (0.002)	1.258	0.522	
	3.4	14	10	9	0.923	0.0064 (0.0038)	−2.39	0.454	
	3.6	4	3	3	0.833	0.0026 (0.0023)	−0.2876	−0.754	
	4	2	1	2	1	0.0017 (0.0024)	0	0	
		200	71	59	0.94	0.0172 (0.0088)	**−20.95**	−0.544	

was computed under the sudden expansion model. Both implemented in DNASP. Two neutrality tests implemented in Arlequin, Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989), were used to detect departures from the mutation–drift equilibrium, which would be indicative of changes in historical demography or selective sweeps.

Population size fluctuations through time were estimated for each main clade, identified by Mrbayes, using the Bayesian Skyline Plot (BSP) method implemented in Beast (Drummond and Rambaut, 2007). Given Ho (2007) suggests the use of a different substitution rate for multispecific tree and intraspecific processes, we follow Fontanella et al., 2012 for BSP. We used a substitution rate of 1.6% for cyt *b*, which has been previously used for intraspecific analysis in *Liolaemus* (Morando et al., 2007). We ran 50×10^6 generations and discarded the first 10% as "burn in". Estimated distributions were used to generate confidence intervals representing phylogenetic uncertainty and coalescence (Drummond et al., 2005), and we used Tracer 1.6 (Rambaut et al., 2013) to monitor parameters estimates and ESS values.

2.6. Microsatellite data analysis

Microsatellite summary statistics, including average number of alleles per locus and number of private alleles for each locus, observed (Ho) and expected (He) heterozygosity, and Shannon's index, were calculated in GenAlEx 6.5 (Peakall and Smouse, 2006, 2012).

We used a Bayesian clustering approach, Structure version 2.3.4 (Pritchard et al., 2000), a non-spatial model that ignores geographic proximity. The number of assumed genetic clusters (K) was set from 1 to 32 (corresponding to the number of sampled localities), and 10 runs with 100,000 MCMC iterations were performed for each K following a burn-in of 50,000 iterations. The admixture model was run with correlated allele frequencies. The optimal value of K was determined depending on ΔK value (Evanno et al., 2005) using the Structure Harvester version 0.6.93 application (Earl and vonHoldt 2012). Consensus

analyses were performed using Clumpp 1.1.2 version (Jakobsson and Rosenberg, 2007) on the average scores for the inferred K value. Visualization of clustering outcomes was performed using the program Distruct 1.1 (Rosenberg, 2004). A membership coefficient (q) above 0.6 has been considered a feasible cut-off membership value to assign individuals to a population with confidence, since more than 50% of the genome is assigned to a group and therefore suggests inferred ancestry (Coulon et al., 2008; Pelletier et al., 2012).

We implemented an analysis of molecular variance (Amova) to determine if rivers play a significant role in the genetic spatial structuring of *L. tenuis*. Three Amova hierarchies were constructed: comparison 1, north of Maipo River vs. south of Maipo River; comparison 2, north of Maipo River vs. between Maipo and Biobío rivers vs. south of Biobío River; comparison 3, north of Biobío River vs. South Biobío River.

2.7. Present and past distribution modeling

We modeled *L. tenuis* ecological niche "envelopes" to identify potential refugial areas during the LGM. Distribution models were generated for the present and the LGM (21 kya) from a total of 146 occurrence records, based on 19 bioclimatic variables at a 2.5′ spatial resolution (WorldClim v1.4; Hijmans et al., 2005) in MAXENT v 3.1.0. (Phillips et al., 2006). We used the default convergence threshold (10^{-5}) and maximum number of iterations (500), using 25% of the localities as "burn-in" for model testing. As suggested by Waltari et al. (2007), we chose a presence threshold to render each projection into a binary form, and considered grid cells with a cumulative probability of more than 10 (from a range of 0–100) as suitable. Model performance was evaluated using the area under the curve (AUC) (receiver operating characteristic) calculated by Maxent. Values between 0.7 and 0.9 indicate good discrimination (Swets, 1988).
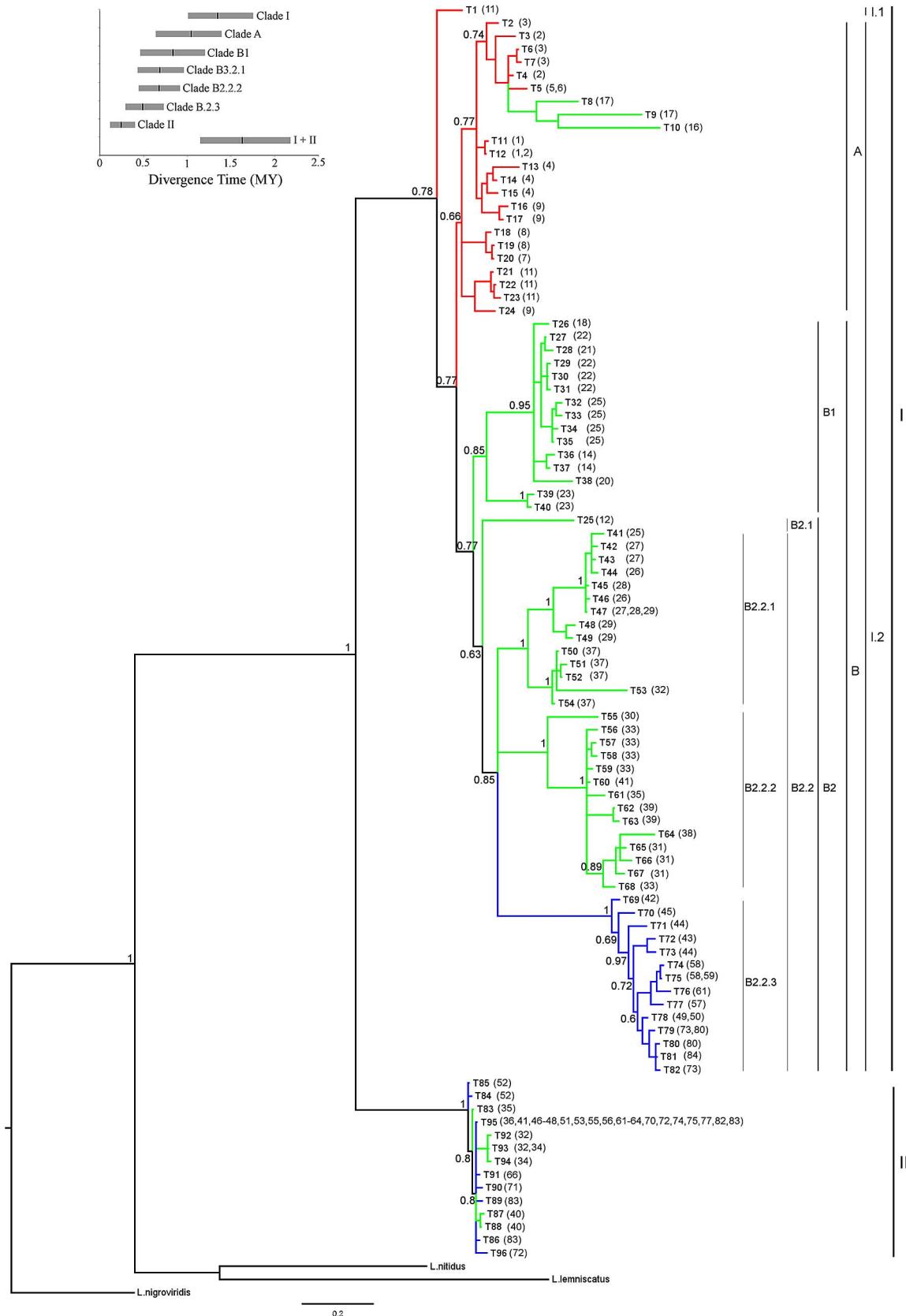
Fig. 2. Genealogical relationships of cyt *b Liolaemus tenuis* haplotypes as inferred by Bayesian analysis. Branch colors correspond to the colors depicted in the map of Fig. 1. Numbers on branches represent Bayesian posterior probabilities. Haplotype numbers on tips are the same as in Fig. 3. Numbers within parentheses refer to the locality numbers detailed in Table S1. Vertical black bars correspond to the main clades. Divergence times (in million years) for each clade are shown in the upper left side. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Genetic diversity and genealogical analysis

We obtained 726 base pairs (bp) of mtDNA sequences for 225 individuals, the matrix of the nuclear gene KIF24 is composed by 158 sequences of 580 bp and we obtained 196 sequences for the nuclear gene LDB5B with 611 bp. Variable sites, mean haplotype and mean nucleotide diversity, for each gene are shown in Table 1. Most of the shared cyt *b* haplotypes among localities occur in the southern range (36–40°S). In general, p-distance values were correlated with the geographical distance between localities (Table S4a).

The topology of the cyt *b* gene tree generally corroborates the previous study of Victoriano et al. (2008); two well supported deep clades (Fig. 2) were obtained. Clade I includes haplotypes distributed throughout the whole distribution range of *L. tenuis* (between 31° and 40°S), while clade II includes most haplotypes south of the Biobío River. Within clade I, main subclades are generally distributed allopatrically, and several of these are delimited by rivers. Clade I included six subclades: two of them extends from the northern limit of the distribution of *L. tenuis* to the Maipo River (subclades I.1 and A). Subclade B1 is restricted to the area between the Maipo and Maule rivers. Subclades B2.2.1, which includes localities distributed in the Andes, and B2.2.2, mainly distributed in the Coastal range, are restricted to the area between the Maule and Biobío Rivers. Finally, subclade B2.2.3 occurs mainly south of the Biobío River, where it is sympatric with the main clade II. The divergence of the main clades of *L. tenuis* occurred during the Pleistocene (Fig. 2). The age of the crown group *L. tenuis* is ca. 1.63 Ma, while crown ages of the main clades are: Clade I ~1.34 Ma, and Clade II ~0.24 Ma. Haplotype networks were highly congruent with the Bayesian genealogy. We recovered two main haplogroups corresponding to mtDNA clades I and II, separated by 50 mutational steps (mp values in Fig. 3). The two southernmost haplogroups (subclades B2.2.3 and II in Fig. 2) are sympatric in the wet zone in the south, one of them with a star-like shape (subclade II), and with a high frequency haplotype (Fig. 3).

In the Bayesian genealogies, the nuclear data failed to recover all the phylogroups detected by the mtDNA dataset. However both nuclear genes tend to recover the northern clade A of the mitochondrial gene (Figs. S1 and S2). The haplotype networks for both nuclear genes were in general concordance with the cyt *b* network (Figs. S3 and S4).

3.2. Genetic structure

The mtDNA based Geneland analysis identifies seven clusters (K = 7; Figs. 4A; S5), with smaller distributions towards the north and central parts of the species range, and with cluster boundaries defined by the main rivers (e.g., N1 and N2 separated by the Aconcagua River, and S6 and S7 mainly towards the south of the Biobío River). Cluster S7 extends to the southern limit of the species distribution, covering the largest geographic area and including the largest number of localities relative to all other clusters. This cluster also includes two deeply divergent mitochondrial clades within the same range (subclade B2.2.3 haplotypes and Clade II haplotypes; Fig. 2).

The nuclear KIF24 based Geneland identifies four clusters (Fig. S6). The clusters boundaries are defined by the main rivers (C1 and C2 separated by Maipo River, C2 and C3 separated by Maule River, C3 and C4 separated by Biobío River). Geneland based on gene LDB5B identified six clusters (Fig. S7). As for KIF24, the clusters boundaries are defined by the main rivers (C1 and C2 separated by Maipo River, C2 and C3–C4 separated by Maule River, C3–C4 separated to C5–C6 by the Biobío River).

For cyt *b*, geographically contiguous clusters showed lower Fst values than comparisons involving distant clusters (Table S4b). High Fst values were observed for comparisons between clusters north and south of the Maipo River as well as north and south of the Biobío River.

For the cyt *b*-based Amova, all values were significant (p < 0.001). The largest fraction of the observed genetic variation was reached when localities are grouped according to the seven cyt *b* Geneland clusters (45.15%); for the other grouping schemes, and independently if Maipo is considered as a barrier or not, arrangements where the Biobío River was considered a barrier showed the highest inter group values (above 43%) (Table 2a).

For the KIF24-based Amova, the largest percentage of variation for all comparisons, was attributed to among individuals within localities and only a small portion of the genetic variance was attributed to differences between groups (Table 2b). The Amova for the Maipo River as a barrier, indicated that 29.86% of variance could be attributed to differences between groups north and south of the Maipo River, compared to 46.20% for differences among populations within groups. For the Biobío River as a barrier, a lower and significant portion of variance was explained by differences between groups (21.25%), with a corresponding increase in the among-population/within group variance (51.10%), compared to the first hierarchical Amova. The variance between groups is not maximized when populations are grouped in the four Geneland groups (27.02%), as the variance among individuals within localities remains higher (52.21%).

For the LDB5B-based Amova, all values were significant (p < 0.001). The largest fraction of the observed genetic variation was reached when localities are grouped according to the six LDB5B Geneland clusters (62.02%). The other group arrangements, which involved the Maipo and Biobío rivers as barriers, always showed higher percentage of variance for hierarchy between groups (Table 2c).

When comparing migration models, the highest support was for the Biobío River being a gene flow barrier, with a log Bayes Factor difference > 10^3 units relative to the other migration models (where a difference of > 10 units provide very strong support for one model over another; Kass and Raferty, 1995). The historic migration scenario for our samples shows a prevalence of asymmetric migration rates. Low gene flow between the seven mitochondrial population clusters was estimated (Table S5, Fig. 4A). In general, there is no gene flow after we contrast all clusters north of the Maipo River (N1 + N2) versus clusters south of it. In the northern and central areas, gene flow is lower relative to rates estimated for samples in the southern range. South of the Biobío River, within cluster S7 but between physiographical zones (Andes–Coast), bidirectional gene flow was inferred, but it is highly asymmetrical in favor of more gene flow from the Andes towards the coast (Table S5, Fig. 4A).

3.3. Historical demographic changes

The Fu's, and R2 test suggest population expansion in southern clades B2.2.3 and Clade II (Table 1). The population stability of most clades, with exception of Clade II, could not be statistically rejected (Table 1, Fig. S8). The results of BSP differed between clades of different geographic distributions. At both the northern and southern distribution, larger changes in effective size were estimated in comparison to the central clades. In the north, reductions of Ne would have started about 100,000 years ago, where the northernmost clade (clade A) showed no evidence of recovery of genetic variability after such reduction. Contrarily, clade B1 showed indications of recovery of the population size after a minimum reached about 20,000 years ago. In the south, *L. tenuis* also showed signs of population reduction for the two co-distributed clades, but with an earlier onset of such reduction, about 400,000 years ago. The dates of the estimated population minimums were different for these two clades, where clade II reached a minimum about 100,000 years ago and clade B2.2.3 about 20,000 years ago. The clades of the central distribution tree of *L. tenuis* (clades B2.2.1 and B2.2.2), showed signs of greater population stability (see Fig. 5).
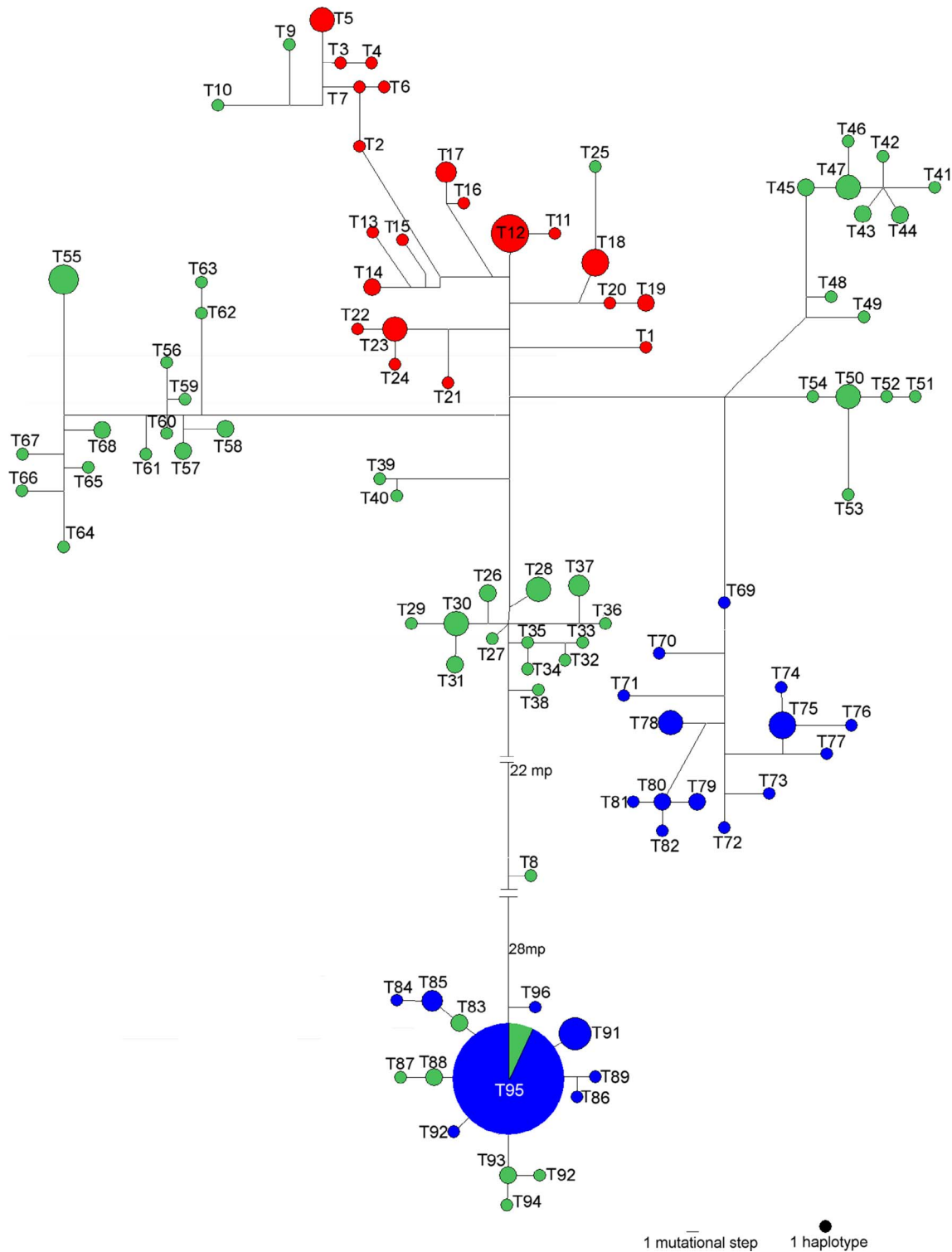
Fig. 3. Parsimony-based haplotype network of mitochondrial sequences (cyt *b*) of *Liolaemus tenuis*. Colors indicate geographic origin as depicted in Fig. 1. Table S1 gives details of collection site and group assignation for the haplotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Microsatellite data

All five microsatellite markers tested are polymorphic, with a total of 50 alleles identified, but loci vary both in terms of number of alleles (from 5 in DI7938 to 15 in TET1177) and number of private alleles (from 0 in DI7938 to 8 in TET1177). The TET1177 was both the most variable and the most informative locus for population discrimination (I = 1.008), whereas DI7938 was the least variable and least informative marker. Mean values of observed heterozygosity over all samples were lower than expected under Hardy-Weinberg (HW) equilibrium at each locus (Table S6). Mean allelic richness, calculated only for populations with at least 5 individuals (rarefaction sample size = 5), ranged from 4.0 in the northern locality of San Felipe (population 3 in Fig. 1) to 1.2 at Corral in the south (population 80), with an average value of 2.65. Mean allelic richness by locus, standardized by sample size, was higher in northern and central zones (0.17 and 0.15
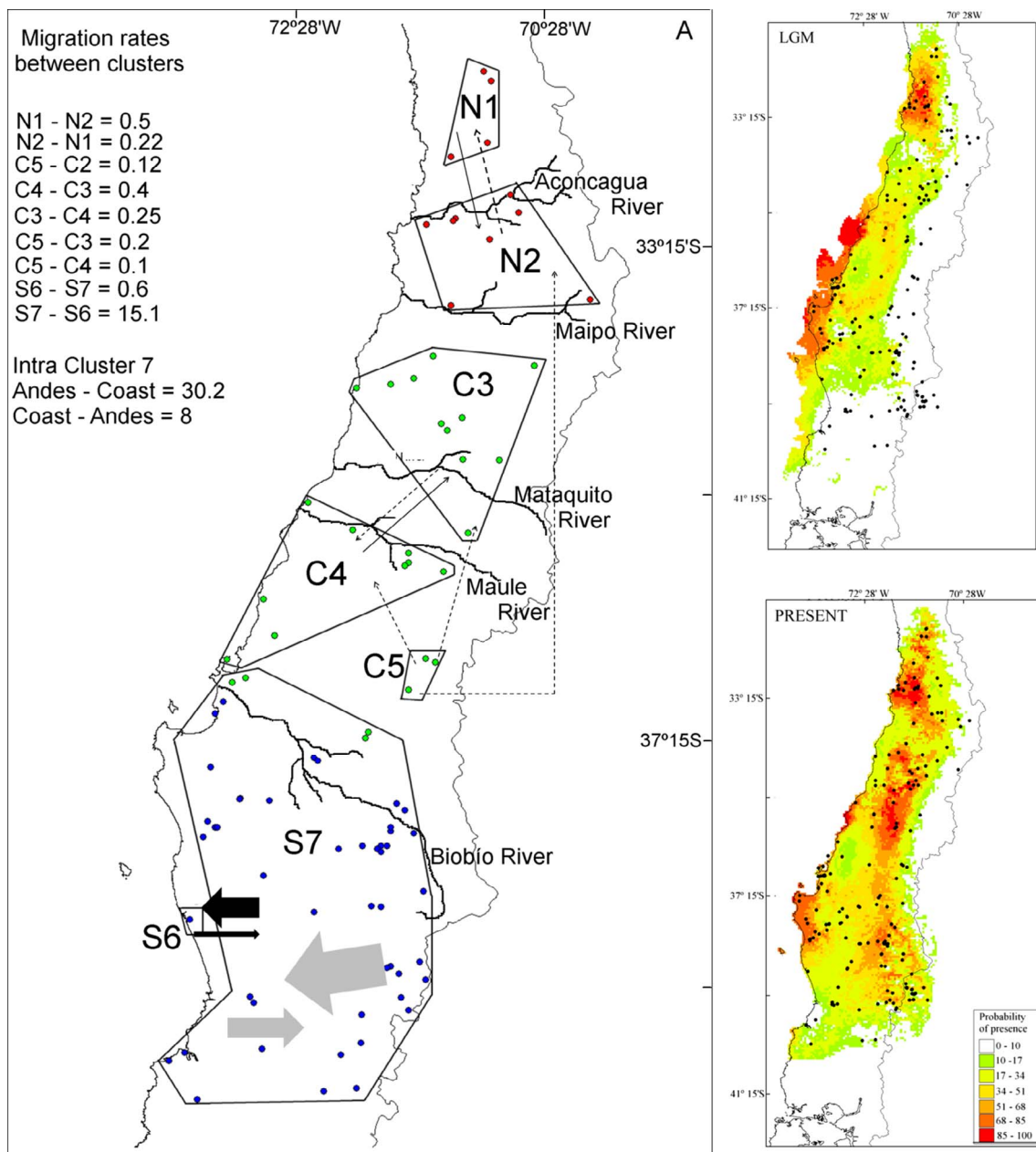
Fig. 4. (A) Population clusters (polygons) of *Liolaemus tenuis* identified by Geneland, based on cyt *b* sequences. Colors of site circles correspond to those depicted in Fig. 1. Black arrows represent direction and migration rates between clusters, and grey arrows represent direction and migration rates between Andes–Coast intra Cluster S7. Migration rate values for selected cluster pairs are shown in the upper left corner. (B) Habitat suitability map for *L. tenuis* during the Last Glacial Maximum (upper right) and the present (lower right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively) than in the south (0.09). Private alleles were detected in 11 localities, whose means after standardization by number of analyzed localities ranged from the highest value in the northern cluster (1.0 private alleles/locality) to the lowest in the south (0.4 private alleles/locality; values not shown).

Implementing methods developed by Evanno et al. (2005) to determine the greatest change in K, Structure (Pritchard et al., 2000) analysis identified two populations clusters (K = 2; Fig. S9). The consensus membership coefficients, implemented in Clumpp version 1.1.2 (Jakobsson and Rosenberg, 2007) are shown in Fig. S9. Although many individuals show signs of admixture (mean Q values of cluster 1 = 0.75 and cluster 2 = 0.85), mostly individuals north of Biobío River (i.e. from Salamanca to Concepcion) associated more strongly with cluster 1 while individuals South of Biobío River (i.e. Curanilahue to Valdivia) were associated more strongly with cluster 2.

In the multi-locus Amova for all comparisons, the largest percentage of variation was attributed to among individuals within localities and only a small portion of the genetic variance was attributed to differences among groups (Table 2d). The Amova for the Maipo River as a barrier, indicated that 6.36% of variance could be attributed to differences between groups north and south of the Maipo River, compared to 23.16% for differences among populations within groups. For the Biobío River as a barrier, a higher and significant portion of variance was explained by differences between groups (10.39%), with a corresponding decrease in the among-population/within group variance (19.39%), compared with the first hierarchical AMOVA.

3.5. Niche modeling

The predictive ability of ecological niche modeling was significantly

Table 2

Amova results for (a) cyt *b*; (b) KIF24; (c) LDB5B and (d) microsatellites. Toponymic for each group corresponds to river names.

a	MtDNA		
Groups	Among groups	Among population within groups	Within populations
(North of Maipo) vs (South of Maipo)	30.44[**]	58.79[**]	10.77[**]
(North of Maipo) vs (Maipo to Biobío) vs (South of Biobío)	43.83[**]	44.68[**]	11.49[**]
(North of Biobío) vs (South of Biobío)	43.87[**]	45.46[**]	10.68[**]
Among clusters of Geneland	45.15[**]	42.82[**]	12.03[**]
b	KIF24		
Groups	Among groups	Among population within groups	Within populations
(North of Maipo) vs (South of Maipo)	29.86[**]	23.93[**]	46.20[**]
(North of Maipo) vs (Maipo to Biobío) vs (South of Biobío)	27.33[**]	22.26[**]	50.41[**]
(North of Biobío) vs (South of Biobío)	21.25[**]	27.65[**]	51.10[**]
Among clusters of Geneland	27.02[**]	20.77[**]	52.21[**]
c	LDB5B		
Groups	Among groups	Among population within groups	Within populations
(North of Maipo) vs (South of Maipo)	50.62[**]	36.35[**]	13.03[**]
(North of Maipo) vs (Maipo to Biobío) vs (South of Biobío)	46.26[**]	36.49[**]	17.25[**]
(North of Biobío) vs (South of Biobío)	41.69[**]	41.21[**]	17.11[**]
Among clusters of Geneland	62.06[**]	19.04[**]	18.90[**]
d	Microsatellite		
Groups	Among groups	Among population within groups	Within populations
(North Maipo) vs (South Maipo)	6.36[*]	23.16[**]	70.48[**]
(North of Maipo) vs (Maipo to Biobío) vs (South of Biobío)	9.96[**]	18.75[**]	71.28[**]
(North of Biobío) vs (South of Biobío)	10.39[**]	19.39[**]	70.22[**]

* $p < 0.05$.
** $p < 0.001$.

higher than expected under a random model (AUC 0.983). Some populations north of 34°S would have remained disconnected from the rest, at areas of the Maipo River basin and Cordón Altos de Cantillana mountain range (Fig. 1). During the LGM the distribution of *L. tenuis* was considerably reduced south of 38°S and along the Andes (Fig. 4B). South of 37°S the distribution of the species would have been restricted to the Nahuelbuta region and the coastal area. At the same latitude in the Andes, we identified small stable habitats around volcanic areas (38°S). Previous evidence of glacial refuges around volcanoes has been suggested for other parts of the world (Fraser et al., 2014).

4. Discussion

4.1. Phylogeographic pattern and barriers

Phylogeographic studies focused on the biota of South America and the Southern Hemisphere in general are still scarce (Beheregaray, 2008; Turchetto-Zolet et al., 2013; see also Sérsic et al., 2011). However, this scenario is changing, as prominent phylogeographic contributions have been made for terrestrial species from the Brazilian Atlantic coast (e.g., Carnaval et al., 2009; Valdez and D'Elía, 2013), Patagonia (e.g., Breitman et al., 2015; Fontanella et al., 2012; Lessa et al., 2010; Morando et al., 2004), and areas west of the Andes in Chile (e.g., Núñez et al., 2011; Sallaberry-Pincheira et al., 2011; Vera-Escalona et al., 2012; Victoriano et al., 2015). Even when several phylogeographic studies have focused on vertebrates from Central Chile, none has centered on an ectothermic species broadly distributed in more than one bioclimatic zone. This has prevented the evaluation of paleoclimatic history of contrasting and topographically complex scenarios within the same species. Moreover, most phylogeographic analyses base their conclusions on only one genetic marker. Our study constitutes a novel contribution given its wide geographic coverage, its wide character sampling (mitochondrial and nuclear loci) and an integrative approach.

Our results suggest a complex phylogeographic history for *L. tenuis*, with contrasting population behaviors according to latitude, with signals of greater stability, structure and levels of genetic variability in populations from the northern and central range, compared to southern populations. Evidence for co-distributed deeply divergent clades in the South suggests two recent invasions to the area resulting in a large region of secondary contact, where populations lack structure and show evidence of range expansion. In addition, our results point to some phylogeographic breaks consistent with large Chilean rivers that bisect the country from east to west. Results indicate a reduced gene flow among populations south and north of the Maipo River, which has been previously identified as a significant barrier to dispersal in other reptiles, such as *L. monticola* (Torres-Pérez et al., 2007) and *Philodryas chamissonis* (Sallaberry-Pincheira et al., 2011). The second break for *L. tenuis* is located approximately between 37°S and 38°S, and is associated with the Biobío River basin, one of the largest Chilean rivers. This river has been suggested to act as a geographic barrier to the distribution of other vertebrates, including low vagility birds of the genus *Pteroptochos* (Chesser, 1999). Although other rivers could have an important role limiting the gene flow in *L. tenuis*, the Maipo and Biobío rivers seem to be those that generated the greatest impact on the observed pattern of genetic variability of this species.

4.2. Historical demography and refugia

Demographic analysis supports an historical decrease of Ne both for the southern and northern populations, as well as a relatively higher stability for the populations in the central range of the species. However, changes in population sizes were not synchronous for populations of *L. tenuis*. In addition, the beginning of the size reductions in the populations of the extreme north, and the minimum reached by those of the southern clade II, around 100,000 years, do not coincide with the LGM, but with the onset of the Llanquihue glaciations (Rabassa
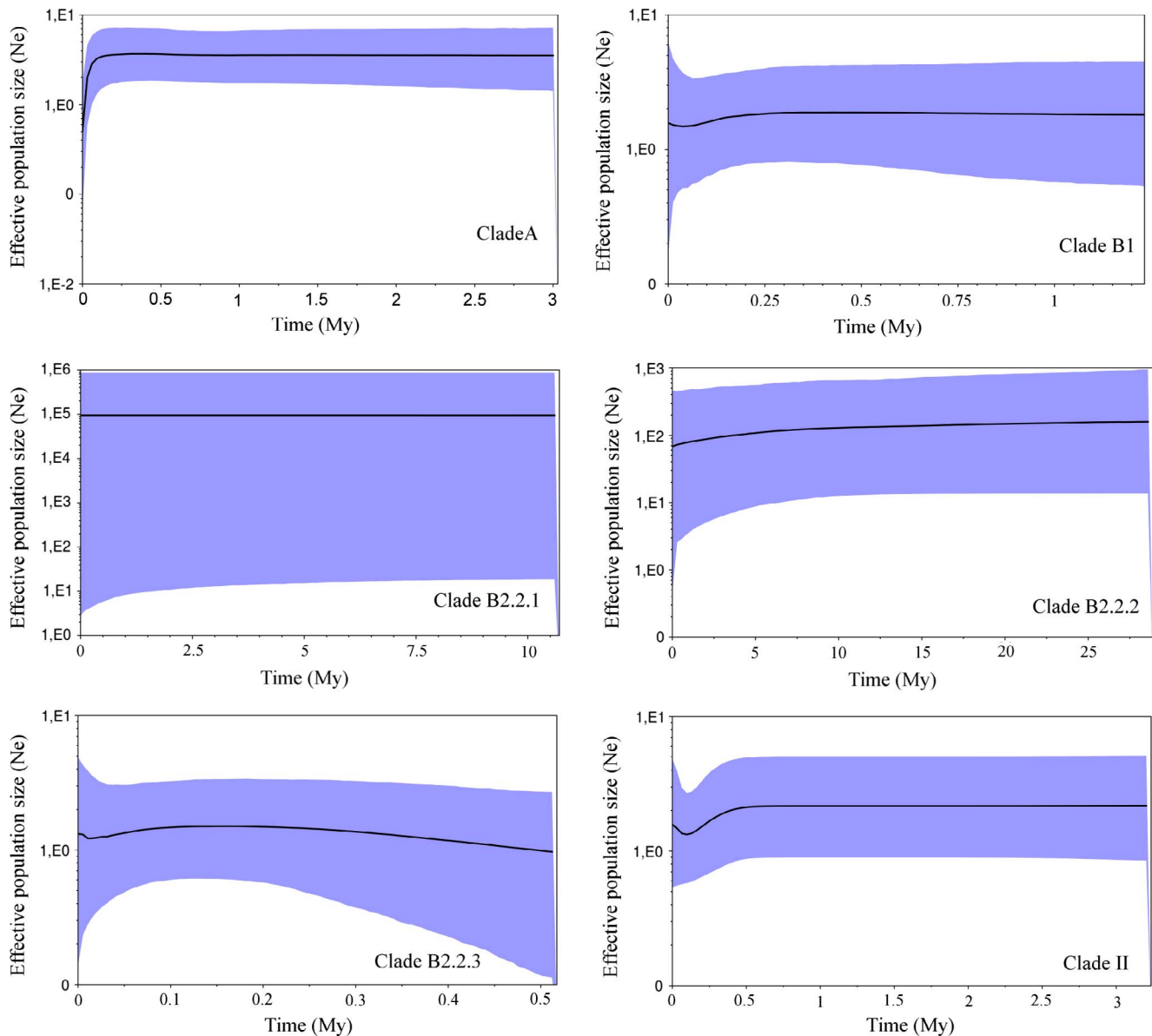
Fig. 5. Bayesian Skyline Plots (BSP) representing recent demographic trends of each of the six main phylogroups of *Liolaemus tenuis* based on mitochondrial sequences (cyt *b*). Light-blue area represents 95% confidence intervals for Ne.

and Clapperton, 1990). In contrast, the lowest effective size values, both in the north-central (B1) and southern (2.2.3) clades, occurred about 20,000 ago, coinciding with the LGM. This may have been due to the different populations of *L. tenuis* having different biological attributes, or because the environmental conditions during glaciations were different in different areas of the distribution of the species. Historical demographic reduction in clades distributed in the northern and southern range areas, suggests stronger past changes in those environments; in particular, the decrease in Ne was of greater magnitude in populations at higher latitudes where glaciations had a larger impact in habitats, as suggested also for other congeneric lizards such as *L. pictus* (Vera-Escalona et al., 2012). For *L. tenuis*, the occurrence of broadly distributed haplogroups in the south, less genetic diversity, mismatch and R2, star-like haplogroups and broadly distributed redundant haplotypes found with high frequency in populations distributed in glaciated areas (especially for clade II), is evidence of a history of recent colonization of the southern species range. In contrast, a greater demographic constancy, current distribution and times of divergence of haplotypes outside from the LGM ice borders, suggest more historical stability in areas without glacial effects. However, in the north end of its

current distribution, although far from glacial ice sheets, where the direct glacial effects were supposedly smaller, this area would have been exposed to dynamic environmental conditions throughout its history, probably conditioned by periglacial changes, such as variation in vegetation and/or precipitation (Villagrán 1991; Villagrán et al., 1995). In summary, populations distributed in both northern and southern ends of the range would have highly reduced their Ne, in contrast with central populations that would have remained more stable.

On the other hand, it is interesting the likely low connectivity promoted by landscape roughness and high altitude, both in the Coastal and Andean areas at northern and central latitudes. According to this, overall Fst values indicated a high genetic differentiation between clusters that suggest a higher structure in the northern-central distribution of *L. tenuis* (Balloux and Lugon-Moulin, 2002). One example of an altitudinal barrier is Cordón Altos de Cantillana (> 2000 m; cca. 33°S; Fig. 1), which could add to the barrier effects of the Maipo River. Geological data from this region shows that the glaciations in the Pleistocene period were important over 600 m.a.s.l. at this latitude. During the LGM, glaciers in the Maipo Valley reached the Intermediate

Depression (Brüggen, 1950; Vuilleumier, 1971). Although there is evidence that the western slope of the Cordillera de la Costa in its lower areas remained free of ice during the Pleistocene (Formas, 1979; Heusser, 1966), highlands in these mountains were affected by glaciations, probably excluding lizard populations. Evidence suggests that glaciers have acted as barriers with a greater magnitude towards the Andes, rather than the Cordillera de la Costa, but in Central Chile, in transversal and coastal mountains with high altitudes, glaciations could affect populations nonetheless. Although mtDNA data suggest a strong genetic structure for *L. tenuis*, both nuclear sequences and the microsatellite data do not corroborate that result. Several other studies have also found greater population differentiation using mitochondrial rather than nuclear markers (e.g. Castella et al., 2001; Scribner et al., 2001), but others have found the reverse (e.g. Bracken et al., 2015; Johnson et al., 2003; Piertney et al., 2000). Many explanations can account for differences between nuclear and mtDNA markers, including different intensities of selection on each marker, different movement patterns between females and males, as well as the difference in Ne of maternally inherited markers (such as mtDNA) from the those of biparentally inherited markers (e.g. microsatellites), among others (Frankham et al., 2002). Amova of the cyt *b* dataset indicated significant high variance among the main groups. In contrast, analyses of nuclear microsatellite data recovered a low among-groups variance. Variance between populations is usually higher in mtDNA than microsatellite data because of the high intrapopulation variance in microsatellites, and because the higher effective size of the nuclear genome (Naidoo et al., 2016). In addition, mitochondrial genetic structure in the presence of low levels of nuclear genetic structure may indicate a higher female philopatry. Interestingly, differences in genders' social behavior in *L. tenuis* have been suggested by Vidal et al. (2008).

Despite the fact that we have found differences in the number of clusters retrieved by Geneland when using mitochondrial DNA (7 clusters) and microsatellites (2 clusters), there is not total conflict between them, as they can all be classified in northern (north of the Biobío River) and southern (south of the Biobío River). Finally, our results based on microsatellite loci should be considered with caution because our number of loci (five) is relatively low to be able to conclude about population processes.

Refugia outside the ice sheet limits and close to the Pacific coast in Central-Southern Chile have been proposed for plants (Sérsic et al., 2011), which is consistent with the geographic distribution of genetic variation and demographic estimations for *L. tenuis*. Interestingly, in addition to the classical coastal refugia in the south, we detected probable refugial areas within the boundary of the LGM ice sheet. There is evidence pointing to the occurrence of small refugia at Andean areas for other species. For example, the glacial gap of Ñuble (36°30′S) and the valley of Malalcahuello and Lonquimay would have been free of ice during the LGM (Heusser, 2003). As such, distinct Andean refugia have been suggested for the lizard *L. pictus* (Vera-Escalona et al., 2012), crabs of the genus *Aegla* (Xu et al., 2009), and for the frog *Eupsophus calcaratus* (Núñez et al., 2011). In *L. tenuis* we have evidence for refugial areas south of the Biobío River, near the Valley of Lonquimay in the Andes (~38°S). High haplotype diversity, the occurrence of private haplotypes, and suitable habitat during the LGM in such area suggest that populations persisted there over time. Moreover, the results for the historical migration of *L. tenuis* suggest that in the southern range, and consistent with probable Andean refugia, gene flow was lower W-E than E-W. Evidence for a similar migration pattern was inferred for the lizard *L. pictus* (Vera-Escalona et al., 2012). It is likely then that the Andean populations have acted as a source of recolonization, adding variability to coastal populations in the same latitude. Although genetic variation suggests intra-Andes refugia for *L. tenuis*, we cannot exclude the possibility that in addition to refugium conditions, the Valley of Lonquimay constitutes a secondary contact zone of lineages previously differentiated in distinct refugia (Hewitt, 1996; Mraz et al., 2007; Petit et al.,

2002). The results obtained here partially agree with previous proposals (e.g., Sérsic et al., 2011); in addition to the classical coastal refugia in Central-Southern Chile, we provide evidence for the persistence of intraglacial refugia during the LGM. Although the concept of refugium refers broadly to areas where biota could grow and survive locally under unfavorable regional conditions, these may have several definitions depending on spatial scale and location. We have followed some authors who have clarified the definitions of the different types of refugia. For the case of *L. tenuis*, the concept of coarse-scale or macro-refugia with a temporal dynamics according to temperate-adapted species (Aschcroft, 2010; Birks, 2015; Stewart et al., 2010) is applicable for most of the stable areas. However, given their limited geographic extent and location, those areas where *L. tenuis* persisted within the LGM limits may well correspond to the concept of microrefugia and/or cryptorefugia (Stewart et al., 2010; Rull, 2010). In summary, *L. tenuis* has behaved as a species sensitive to climate changes associated with glacial cycles, to orographic and fluvial barriers. Demographic changes seem to have been more pronounced south of the Biobío River and mainly at Andean areas. In the northern fraction of the distribution, populations also were negatively affected, likely due to changes in vegetation cover. Meanwhile, populations of the central range have remained more structured and stable.

4.3. Taxonomic implications

Müller and Hellmich (1933) proposed two subspecies for *L. tenuis* based on color patterns and distribution (Donoso-Barros, 1966): *L. t. tenuis*, with type locality in Santiago de Chile, distributed from the northern limit in Coquimbo (ca. 30°S) south to Los Ríos (ca. 40°S) (Donoso-Barros, 1966; Vidal et al., 2004); The second subspecies, *L. t. punctatissimus* whose type locality is Lota (ca. 37°S), distributed in the coastal Biobío Region. According to the above, both subspecies overlap their distributions, which is incompatible with the concept of subspecies, which, to be recognized need to be allopatric. Vidal et al. (2002, 2004, 2005) integrated morphology and population genetic structure to assess the validity of these subspecies, concluding that the color pattern varies clinally within a single species with no subspecies. Nevertheless, the high levels of molecular divergence between both main lineages of *L. tenuis* recovered in this study (I and II), cast doubts on the taxonomic scenario proposed by Vidal et al. (2002, 2004, 2005). For example, other species of the genus, such as *L. fitzingerii* and *L. chehuachekenk* diverge from each other by a lower value of genetic distance (approx. 4%; Avila et al., 2008) than that observed between the major clades of *L. tenuis* (Clades I and II show a p-distance higher than 9%). These values may indicate that *L. tenuis* as currently understood is a species complex. According to our results, there is some concordance with the sub-species distribution, however the *L. t. punctatissimus* distribution should be further evaluated (molecular and mophological approach) to see if there is a possible agreement with our results. The hypothesis about the occurrence of more than one species within *L. tenuis* should be tested with an integrative taxonomic approach.

5. Conclusions

Based on thorough sampling of the species *Liolaemus tenuis*, and using both mitochondrial and nuclear markers, we conclude that both landscape heterogeneity and Pleistocene glaciations have shaped this species phylogeographic history. We found evidence of recent colonization of the southern range of the species, as well as signs of higher stability, genetic structure and higher haplotype diversity in the central (as in the Intermediate Depression), and coastal ranges, relative to the southern populations. Our results point to phylogeographic breaks concordant with some large Chilean rivers, mainly, the Maipo (~34°S) and the Biobío (~37–38°S) rivers, which have been previously identified as significant barriers to dispersal for other vertebrates. Finally,

based on p-distance and Fst values, as well as on the occurrence of co-distributed divergent clades in the southern range of the species, we predict that *L. tenuis* probably constitutes a species complex with cryptic diversity. Therefore, we posit that this taxon merits a thorough taxonomic revision in order to determine if the evolutionary lineages reported in this study merit full species status.

Acknowledgements

Appendix A

PCR protocols. Three genes were amplified through the polymerase chain reaction (PCR): a fragment of the mitochondrial Cytochrome *b* (cyt *b*), an exon of the nuclear Kinesin-like protein gene (KIF24), and the anonymous nuclear gene LDB5B. Amplification and sequencing of cyt *b* was performed using primers GluDG-L (Palumbi, 1996) and WWR (Broadley et al., 2006) following the protocol of Victoriano et al. (2008). Nuclear genes were amplified following the protocol published by Portik et al. (2011a) using primers B5BF, B5BR, KIF24F, and KIF24R (Portik et al., 2011b). Amplifications occurred in 25uL volume reactions initiated at 95 °C for 2 min followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s and 72 °C for 1 min 35 s (with extension increasing 4 s per cycle). All PCR products were checked using a SybrSafe stained 1.5% agarose gel. Both forward and reverse strands of each amplicon were sequenced at Macrogen Inc. (Korea).

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.08.016.

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