Phylogeographic analysis and species distribution modelling of the wood frog *Batrachyla leptopus* (Batracylidae) reveal interglacial diversification in south western Patagonia

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**Background.** The evolutionary history of southern South American organisms has been strongly influenced by Pleistocene climate oscillations. Amphibians are good models to evaluate hypotheses about the influence of these climate cycles on population structure and diversification of the biota, because they are sensitive to environmental changes and have restricted dispersal capabilities. We test hypotheses regarding putative forest refugia and expansion events associated to past climatic changes in the wood frog *Batrachyla leptopus* distributed along ~ 1000 km of length including glaciated and non-glaciated areas in southwestern Patagonia.

**Methods.** Using three mitochondrial regions (*D-loop*, *cyt b*, and *col*) and two nuclear loci (*pomc* and *crybA1*), we conducted multilocus phylogeographic analyses and species distribution modelling to gain insights of the evolutionary history of this species. Intraspecific genealogy was explored with maximum likelihood, Bayesian, and phylogenetic network approaches. Diversification time was assessed using molecular clock models in a Bayesian framework, and demographic scenarios were evaluated using approximate Bayesian computation (ABC) and extended Bayesian skyline plot (EBSP). Species distribution models (SDM) were reconstructed using climatic and geographic data.

**Results.** Population structure and genealogical analyses support the existence of four lineages distributed north to south, with moderate to high phylogenetic support (Bootstrap > 70%; BPP > 0.92). The diversification time of *B. leptopus*’ populations began at ~ 0.107 mya. The divergence between A and B lineages would have occurred by the late Pleistocene, approximately 0.068 mya, and divergence between C and D lineages was approximately 0.065 mya. The ABC simulations indicate that lineages coalesced at two different time periods, suggesting the presence of at least two glacial refugia and a postglacial colonization route that gave originated two southern lineages (p = 0.93, type I error: <0.094, type II error: 0.134). EBSP, mismatch distribution and neutrality indexes suggest sudden population expansion at ~0.02 mya for all lineages. SDM infers fragmented distributions of *B. leptopus* associated with Pleistocene glaciations. Although the present populations of *B. leptopus* are found in zones affected by the last glacial maximum (~0.023 mya), our analyses recover an older history of interglacial diversification (0.107 - 0.019 mya). In addition, we hypothesize two glacial refugia and three interglacial colonization routes, one of which gave rise to two expanding lineages in the south.
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Abstract

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diversification (0.107 - 0.019 mya). In addition, we hypothesize two glacial refugia and three
interglacial colonization routes, one of which gave rise to two expanding lineages in the south.

Introduction

The southern South American landscape is characterised by dynamic transformations
resulting from tectonic processes and climatic cycles (Ortíz-Jaureguizar & Cladera, 2006; Le
Roux, 2012). In particular, geological studies (Mercer, 1972; Rabassa & Clapperton, 1990; Clark
et al., 2009) have demonstrated that in the southwestern part of Patagonia have occurred at least
four Pleistocene glaciations, including the most extensive Andean glaciation (1.1 mya), the
coldest Pleistocene glaciation (0.7 mya), the last southern Patagonian glaciation (180 kya), and
the Last Glacial Maximum (LGM; 20,500 and 14,000 years BP). It has been hypothesized that
these climatic cycles, re-organized the ecosystems structure, altered species abundance and
changed distribution patterns of many Patagonian taxa (Sérsic et al., 2011; Giarla & Jansa,
2015). It is also recognized that some areas served as climate refugia in a vast inhospitable
region, and that those refugia provided habitat to species to persist and from which they
expanded when environmental conditions were suitables (Keppel et al., 2012).

Phylogeographic studies in vertebrates and plants in this area (Sérsic et al., 2011) have
highlighted the importance of such glacial refugia, where species survived through glacial
maxima, and which today harbour high levels of genetic diversity and differentiated genetic
clusters (Ruzzante et al., 2006; Vidal-Russell, Souto & Premoli, 2011; Zemlak et al., 2011).
Postglacial colonization pathways have been also hypothesized for a range of species (Victoriano
et al., 2008; González-Ittig et al., 2010; Gallardo et al., 2013; Vidal et al., 2016) to explain how
extant populations are connected and how genetic diversity is spatially distributed.
Amphibians have attracted considerable attention on Pleistocene refugia hypotheses, largely due to their restricted dispersal capabilities that tend to promote allopatric differentiation (Fitzpatrick et al., 2009; Carnaval et al., 2014). Further, amphibians are highly sensitive to habitat disturbances owing to complex life histories, permeable skin, and exposed eggs (Beebee, 1996; Prohl, Ron & Ryan, 2010).

In Southwestern Patagonia, most of the amphibian species are endemic (70%) and strongly associated with humid Valdivian forest (Formas, 1995). These forests contracted into smaller fragments during the more arid phases of the Pleistocene, leading to the isolation and allopatric diversification of forest-associated taxa (Suárez-Villota et al., 2018). One example is the grey wood frog *Batrachyla leptopus* Bell 1843. This small amphibian (30–35 mm snout vent length) lays the eggs (diameter of ova 3–4 mm) in clusters of 93–146. Clutches are fertilized at the edges of small pools, amidst vegetation or under fallen logs and rocks on the ground, where embryonic development takes place (Busse, 1971; Úbeda & Nuñez, 2006). When autumnal rains flood the area (March-June), water stimulates hatching, and larvae metamorphose in 5-7 months (Formas, 1976). *Batrachyla leptopus* has one of the broadest distributions of any Chilean frog (Cuevas & Cifuentes, 2010), and is threatened by habitat deterioration in most of its geographic range. Furthermore, most of its current distributional area was intensively glaciated during the LGM but its genetic structure and the impact of the habitat lost are poorly known (Heusser & Flint, 1977; Paskoff, 1977).

Thus, while the humid ecological requirements of *B. leptopus* might in part explain its low abundance and patchy distributional pattern, Quaternary glaciations likely have generated a phylogeographic history linked to glacial refugia. In fact, previous studies of *B. leptopus* (Formas & Brieva, 2000; Vidal et al., 2016) have revealed high levels of population divergence as a result
of past climatic oscillations. For example, based on allozyme data Formas and Brieva (2000) inferred that the lack of correlation between genetic and geographical distances among \textit{B. leptopus} populations could be the result of postglacial recolonization. On the other hand, Vidal et al. (2016) hypothesized that populations of \textit{B. leptopus} originated from geographically differentiated gene pools, and specifically that post-glacial population expansions came from at least two refugia.

One important caveat for studies on refugial hypotheses is that they are often tested by revealing patterns of intraspecific relatedness of present-day biota and placing variation and divergence into a single evolutionary context. These approaches entail an inductivist point of view, that is, the view that researchers should first observe and analyse the present-day pattern and only then might explanations emerge in terms of historical processes (Andersson, 1996; Arroyo-Santos, Olson & Vergara-Silva, 2014; Segovia & Armesto, 2015). From an epistemic perspective, these approaches cannot progress beyond being speculative first attempts to understand the evolutionary history of a group, because they tend to generate, rather than test, hypotheses (Crisp, Trewick & Cook, 2011; Papadopoulou & Knowles, 2016). Approximate Bayesian computation (ABC) methods (Beaumont, Zhang & Balding, 2002) have introduced novel model comparison and parameter estimation in population genetic and phylogeographic studies (Csilléry et al., 2010; Sunnåker et al., 2013; Inoue et al., 2014). These methods provide an approximation of the posterior distribution of model probabilities and/or parameter values by simulating data with parameters drawn from specified prior distributions, and retaining values that produce data sets similar to the observed data to test alternative hypotheses (Robinson et al., 2014; Freeland, 2020).
Progress in phylogeographic studies has been further extended by the incorporation of species distribution modelling (SDM; Phillips et al., 2017). SDM approaches has been widely applied to assessment of species ranges, and evaluate special and temporal hypothesis about current and past species occurrence (Gavin et al., 2014). Accessibility and easy data requirements of correlative SDMs, coupled with the improved availability of paleoclimate simulations, has the special advantage of permitting prediction of distributional potential across scenarios of environmental change. These models are particularly relevant for understanding the effects that ongoing human-caused global climate change will have on biodiversity (Wiens et al., 2009), including the study of glacial refugia (Gavin et al., 2014).

In this work, we use a multilocus phylogeographic approach and species distribution modelling to test two independent hypotheses regarding putative forest refugia and expansion events in *B. leptopus*. We first test the hypothesis that *B. leptopus* postglacially colonized the southern area of its current distribution assessing whether temporal and demographic patterns are consistent with such a scenario. If *B. leptopus* expanded postglacially from the LGM, then populations should exhibit the genetic signature of recent rapid expansion, and the divergence time between populations present in non-glaciated and glaciated areas should be consistent with such postglacial expansion. Second, we test the hypothesis that *B. leptopus* colonized southwestern Patagonia from a single refuge; if *B. leptopus* expanded southward from a single refuge, then genetic variation in southernmost populations is expected to be low. Alternatively, if *B. leptopus* expanded from multiple refugia, then greater genetic diversity would be expected in some populations representing suture zones where genetic admixture may have occurred, and several coalescent points should be detected.
To this aim, we first estimated the genetic structure among *B. leptopus* populations and reconstructed its phylogeographic relationships under maximum likelihood and Bayesian inference. Second, we estimated divergence time and temporal changes in population size to determine if these were consistent with late Pleistocene events. Then, we examined the demographic history of this species by simulating alternative Pleistocene glaciation scenarios in an ABC framework. Finally, we combined demographic inferences with species distribution modelling in *B. leptopus*.

**Materials & Methods**

**Sample collection**

Between 2009–2018 we collected 130 individuals and buccal swabs (most samples) from 19 localities throughout the distributional range of *B. leptopus* in south western Patagonia (Table 1; Fig. 1). Each sampling site was geo-referenced with a GPS Garmin GPSmap 76CSx. Eight individuals of *B. taeniata* were used as outgroup. This study was carried out under supervision and approval of the Bioethics and Biosecurity Committee of the Universidad Austral de Chile (UACH, Resolutions No. 236/2015 and 61/15), and the Servicio Agrícola y Ganadero (SAG, Resolution No. 9244/2015).

**DNA extraction, amplification, and sequence alignment**

Whole genomic DNA was extracted either from liver tissues or buccal swabs according to Broquet et al. (2007), using the manufacturer’s recommended protocol for the Qiagen DNeasy tissue kit (Cat. No. 69506). We amplified three mitochondrial regions: a segment of the Control region (*D-loop*; Goebel, Donnelly & Atz, 1999) Cytochrome *b* (*cyt b*; Degnan & Moritz, 1992),
and Cytochrome oxidase subunit I \((col; \text{Folmer et al., 1994})\), and two nuclear regions: Propiomelanocortin \((pomc; \text{Gamble et al., 2008})\) and \(\beta\) Crystallin A1 \((crybA1; \text{Dolman & Phillips, 2004})\), via polymerase chain reaction (PCR). Reaction cocktails for PCR were according to Suarez-Villota et al. (2018). PCR products were sequenced in Macrogen Inc. (Seoul, Korea) and at the DNA Sequencing Center at Brigham Young University (Provo, USA). To transform sequence data to haplotypes we used PHASE v2.1.1 (Stephens & Donnelly, 2003) with the default model for recombination rate variation (Li & Stephens, 2003). We aligned sequences using the automatic assembly function in Sequencher v. 4.8 (Gene Codes Corp.), and inspected the aligned sequences by eye, and made corrections manually.

**Molecular diversity and lineage structure**

Haplotype and nucleotide diversity indexes (Nei, 1987) and their standard deviations, were estimated with DNASP v5.0 (Librado & Rozas, 2009) using all markers. The possibility of saturation in the rate of base substitutions was assessed by the method of Xia et al. (2003) using DAMBE v6.0 (Xia & Xie, 2001). Population genetic structure was examined using the package GENELAND v4 implemented in R v3.1.2 (Guillot, Mortier & Estoup, 2005), to infer the number of populations by giving a spatial model of cluster membership without prior designations. GENELAND was run with a model of uncorrelated allele frequencies for the mitochondrial locus (with all gene regions concatenated). We performed eight independent runs of \(1.5 \times 10^7\) iterations, with thinning set to 500 and a “burn in” of 20%. The number of possible clusters tested ranged from 1 to 19 (according to sampling locations). The level of population structure among the clusters obtained by GENELAND, was assessed by analysis of molecular variance (AMOVA; Holsinger & Weir, 2009) using ARLEQUIN v3.1 (Excoffier, Laval & Schneider,
for mtDNA and nDNA separately. Also, using all loci we evaluated whether the sequences evolved under strict neutrality using Tajima’s D (Tajima, 1989), Fu & Li’s D (Fu & Li, 1993), and $r^2$ (Ramos-Onsins & Rozas, 2002) tests.

### Phylogenetic trees reconstruction, split networks and divergence time estimates

Previous to phylogenetic analyses, evolutionary models and partitioning strategies were evaluated using Bayesian information criterion (BIC) scores (Schwarz, 1978) in PARTITIONFINDER v2.1.1 (Lanfear et al., 2017) (Table S1). The phylogenetic analyses were performed with mitochondrial and nuclear concatenated matrix. Partitioned maximum likelihood analyses were conducted using GARLI 2.0 (Zwickl, 2006) with 200 replicates of nonparametric bootstrap for branch support. Bayesian analyses were performed using MRBAYES v3.2 (Ronquist et al., 2012). We performed four independent MCMC runs 50 million generations, sampling every 2,000 generations. Posterior distributions for parameter estimates and likelihood scores to approximate convergence were visualized with the TRACER program v1.6.0 (Rambaut et al., 2014). The effective sample sizes (ESS) of each parameter (>200), allowed us to confirm that the analysis was adequately sampled. A maximum clade credibility tree was visualized with the program FIGTREE v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Posterior probability values $>$0.95 were taken as high statistical support for a clade being present on the true tree (Huelsenbeck & Rannala, 2004). In order to obtain additional statistical support for the best tree obtained, topologies of different trees (ML and Bayesian) were compared with the use of the Shimodaira-Hasegawa (S-H) test (Shimodaira & Hasegawa, 1999) with resampling-estimated log likelihood (RELL) and bootstrapping of 1,000 replicates, using the program PAUP*.
We are aware that phylogenetic methods may not apply at the within-species level, due to multifurcating population genealogies in which descendant alleles coexist with ancestral ones, and recombination events may produce reticulate relationships (Posada & Crandall, 2001). To consider these caveats, we constructed unrooted phylogenetic networks using the method described by Huson and Bryant (2006), implemented in SPLITSTREE v4.14.4.

To determine when major clades and lineages diverged relative to Quaternary glaciation history, we estimated time since the most recent common ancestor (TMRCA), using the reconstructed species tree from concatenated mitochondrial and nuclear sequences. For this reconstruction, we used multi-species coalescent module implemented in *BEAST of BEAST v1.8.4 (Drummond & Rambaut, 2007; Heled & Drummond, 2010), and the same models used for phylogenetic tree reconstruction found by PARTITIONFINDER. Due that it is not possible to date any of the nodes within *B. leptopus*, as there are no fossils or dated biogeographic events, we used as prior Neobatrachian mutation rates of 0.291037% per million years for COI, rate of 0.37917% per million years for each other mitochondrial markers (*dloop* and *cytb*), and a rate of 0.3741% per million years for *pomc* sequences, according to Irrisarri et al. (2012). Bayes factor analysis (Li & Drummond, 2012) indicated that species tree with strict-clock model received decisive nodal support compared to uncorrelated exponential or uncorrelated lognormal relaxed-clock models. Markov chains in BEAST were initialized using the tree obtained by MRBAYES, to calculate posterior parameter distributions, including the tree topology and divergence times. We used BEAST to estimate divergence time, based runs for 2x10^7 generations, and sampling every 1000th generation. The first 10% of samples were discarded as “burn in”, and we estimated convergence to the stationary distribution and acceptable mixing (ESS >200) using TRACER v1.6.0.
Population-size dynamics through time

Hypotheses of historical demographic expansions and dynamics through geological time of the inferred lineages were estimated by “mismatch distributions” (Rogers & Harpending, 1992), and Extended Bayesian Skyline Plots (EBSP; Heled & Drummond, 2008) respectively. We used in a complementary way both approaches due that small sample sizes apparently to fail to provide enough power to Bayesian skyline plots to detect population expansion (Grant, 2015). The smooth, unimodal distributions typical of expanding populations can be readily distinguished from the ragged, multimodal distribution “signatures” of long-term stationary populations, by means of the ‘raggedness’ of these distributions (Rogers & Harpending, 1992). Confidence intervals for these estimates were obtained by simulations using the coalescence algorithm as implemented in DNASP v5.0. To estimate population-size dynamics through geological time, we reconstructed Extended Bayesian Skyline Plots (EBSP; Heled & Drummond, 2008) implemented in BEAST for the four lineages obtained with GENELAND. This coalescent-based, nonparametric Bayesian MCMC algorithm incorporates multi-locus data to reduce error estimates associated with single genes (e.g., traditional Bayesian Skyline Plots), and increases the power to resolve alternative demographic histories (Ho & Shapiro, 2011). For each EBSP, the appropriate model of nucleotide substitution was determined using PARTITIONFINDER. Genealogies and model parameters for each lineage were sampled every 1000th iteration for 2x10^7 generations under a strict molecular clock with uniformly distributed priors and a “burn in” of 2000. Demographic patterns for each analysis were plotted in EXCEL v14.7.7.

Test of phylogeographical hypotheses with ABC
A coalescent method was used to test phylogeographic hypotheses by constraining the
genealogies to fit alternative evolutionary models, and assessing each model’s fit by comparing
the observed genetic pattern with the range of simulated patterns. Competing phylogeographic
hypotheses were compared using approximate Bayesian computation method (ABC approach),
as implemented in DIYABC v2.1 (Cornuet et al., 2014). We evaluated five demographic
scenarios to test alternative divergence times and tree topologies of the four main lineages
recovered in GENELAND and in the phylogenetic analyses. Furthermore, the divergence of the
main four lineages took place before the last glacial maximum so the divergence scenarios were
proved in such range. The refugia hypotheses correspond to the points of coalescence, with four
lineages strongly supported, the possibilities of coalescence are from 1 to 3, for which we tested
all the possibilities except scenarios with postglacial admixture because there are not paraphly
events between lineages. The prior coalescence points at time times t1 and t2 applied in the ABC
correspond those estimated by BEAST for the origin of *Batrachyla leptopus* (t2) and the
divergence of the four lineages (t1, lower and higher range of the four lineages). Thus, all
historically relevant scenarios differed only in the order of population divergence, and in the
number and timing of demographic expansion events. These alternatives were: Scenario 1 – the
null model – all four lineages coalesced at t1 with equal divergence rates. Scenario 2 – also a null
model, but all four lineages coalesced at t2 with equal divergence rates. Scenario 3 – the first
coalescence of lineages A and B at t1, whose ancestor coalesced at t2 with lineage C and D.
Scenario 4 – the first coalescence of lineages C and D at t1, whose ancestor coalesced at t2 with
lineage A and B. Scenario 5 – one split event at t1 isolated the north (lineages A and B) from the
south (lineages C and D) clades, and then a coalescence of both clades at t2 (see Results). We
tested other scenarios whose divergence time were lower and the probability was very low so
they were not considered.

Prior values of $N_e$ were set as 1,000–500,000 individuals with a uniform distribution, based on
$N_e$ calculated from MIGRATE-N v3.6 (Beerli, 2006). For this analysis, we performed maximum
likelihood using 10 short chains of 1000 steps, and two long chains of 10,000 steps, sampling
each 100 steps, and a burn-in of 10 per cent. $N_e$ was calculated using mitochondrial and nuclear
rates reported by Irisarri et al. (2012).

Prior values for divergence of the ancestral populations were based on divergence times
calculated here (see Results), and a generation time of 2-3 yr (Martin & Palumbi, 1993), using a
uniform distribution. Divergence times were set at between 20,000 - 500,000 generations ago for
t2, and 10,000 - 200,000 generations for t1.

Paleo-distribution and species distribution modelling

A total of 120 occurrence records were used for the species distribution models (SDMs) and
paleo-distribution modelling. Records were obtained from peer-reviewed literature, our sampled
sites, and online databases (GBIF: gbif.org, VetNet: vertnet.org, and iDigBio: idigbio.org). We
modeled the SDMs using the standard 19 bioclimatic variables downloaded from Worldclim
(Hijmans et al., 2005) for the current conditions (1960-1990), the Mid Holocene (Mid-Hol,
~6,000 yrs BP), the LGM (~22,000 yrs BP), and the Last Inter-glacial (LIG, ~120,000 - 140,000
yrs BP). The variables were at 30 arc-sec for the current conditions and the LIG, and at 2.5 arc-
min for the Mid-Hol and LGM. The Mid-Hol and the LGM variables were based on the
Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on
Climate (MIROC), while LIG conditions were based on Otto-Bliesner et al. (2016). We
restricted the projection of the models by creating a buffer of $2^\circ$ around the outermost occurrence records and the known distribution of *B. leptopus*. All SDMs were performed using MAXENT v3.4.0 (Phillips et al., 2017). To avoid model overfitting and account for the correlation between the variables and the presence of outliers encountered during data exploration, we reduced the number of variables to five. This was performed by retaining the variables with $|\rho| < 0.8$ that contributed the most to ten cross-validated models, as shown by a Jackknife test. These variables were Bio2 = Mean Diurnal Range, Bio4 = Temperature Seasonality, Bio5 = Max Temperature of Warmest Month, Bio13 = Precipitation of Wettest Month, and Bio17 = Precipitation of Driest Quarter.

For all models, the equal training and sensitivity threshold rule was applied and the cloglog output was selected. Extrapolation was not used and clamping was applied when hind-casting the model of the current conditions to the past. The models for the Mid-Hol and for the LGM were overlaid within each time period to identify areas of agreement and disagreement between the models for each the time period. All models were transformed to binary using the selected threshold rule (Phillips et al., 2017).

Results

Genetic structure using Bayesian clustering model

A total of 113 haplotypes were found when mitochondrial and nuclear markers were combined (Table 2). Saturation tests as a function of the genetic distance estimated under substitution model GTR showed non-significative saturation of DNA sequence alignments. The mtDNA Bayesian analysis with GENELAND yielded a modal number of four clusters (K=4), recovered from all independents runs (Fig. 2A); this is based on the highest average posterior probability.
The distribution of these four clusters, from north to south, was named as follows (Fig. 2B):

lineage A (Los Queules), lineage B (Nahuelbuta), lineage C (Bahía Mansa, Cordillera Pelada, Máfil, and Pichirropulli), and lineage D (all remaining localities shown in Table 1). The highest estimate of haplotype diversity was found in lineage A, whereas the lowest values were found in lineage B (Table 2). Highest nucleotide diversity was detected in lineage D and the lowest one in lineage B (Table 2). Negative but non-significant values for Tajima’s D and Fu’s FS neutrality test indexes were found in all lineages except for B lineage. Here the non-significant positive values likely reflected the smallest sample size for this locality. Rozas’s $r^2$ were positive for all lineages but only significant for A and B lineages, suggesting recent expansion (Table 2).

The AMOVA results using the four lineages indicate a significant genetic structure (groups defined as Lineages A, B, C, and D): (1) variation among lineages = 35.64% and variation within lineages = 45.93% for mtDNA; (2) variation among lineages = 0 % and variation within lineages = 51.12% for $pomc$; (3) variation among lineages = 4.49 % and variation within lineages = 2.74% for $crybA1$ (Table 3).

**Phylogenetic tree reconstruction, split networks, and lineage divergence time**

The models selected for the ML and Bayesian analyses are described in Table S1. Because the Bayesian analyses recovered a maximum clade credibility tree similar to the best ML tree, and the Shimodaira-Hasegawa test showed that topological disagreements were restricted to “low-support” nodes, we show only the Bayesian tree (Fig. 3A). The same four lineages recovered by GENELAND were recovered in the phylogenetic reconstruction with moderate to high support values. Lineages A and B were recovered as sister groups (bootstrap = 96%; BPP = 0.99), as were the C and D lineages, but with moderate support (bootstrap 74%; BPP = 0.95).
Mitochondrial phylogenetic analyses recovered similar results to concatenated datasets, but the nuclear phylogeny was highly polytomized (Fig. S1). The split networks (Fig. 3B) recovered the same four lineages obtained by the ML and Bayesian analyses. The fit index was 94.96, meaning that only 5.04% of the distances in the distance matrix are not represented by the network. Most of the internal splits have bootstrap support between 68 and 100%.

Divergence dating indicates that the A-B and C-D clades separated during the late Pleistocene, approximately 0.107 mya [95% confidence interval (CI) = 0.020–0.278 mya]. The divergence between A and B lineages would have occurred by the late Pleistocene (approximately 0.068 mya; 95% CI = 0.036–0.147 mya) and divergence between C and D lineages was approximately 0.065 mya (95% CI = 0.056–0.092 mya) (Fig. 3A).

Demographic patterns of the inferred clusters

Results of mismatch distribution analyses (Fig. 4A-D) revealed a single primary peak for lineage A, but with a non-significant raggedness index (r=0.0230, P>0.1). Similarly, unimodal patterns were observed in lineages C (r=0.003, P<0.001) and D (r=0.0008, P<0.001). The small sample size (n = 7) and haplotype numbers (H = 2) precluded this analysis on lineage B. Reconstruction of the demographic histories by means of Extended Bayesian Skyline Plot (Fig. 4E-H) suggested population expansions for all lineages except B (Fig. 4F). EBSP further resolved sequential demographic expansions from the oldest, lineage D (c. 18,000 years bp), then lineage A (c. 11,000 years bp), and most recently, lineage C (c. 5,000 years bp).

Hypothesis testing with ABC
Logistic regression analysis with DIYABC identified Scenario 4 as most strongly supported among the five tested (Fig. 5D), with a high posterior probability (0.93; Table 4); all other scenarios had much lower support (0.0–0.04). Moreover, the Type I and Type II error rates estimated for Scenario 4 were the lowest in both cases (0.14; 0.014–0.196; Table 4).

Scenario 4 placed the first divergence as the split between lineages A, B, and the ancestor of the southern clade (lineages C and D) at t2, and the second split between lineages C and D at t1 (Fig. 5D). The effective population size \((N_e)\) and divergence time parameters, in terms of the number of generations \((t)\), estimated for this divergence scenario (Table S2), corroborate the population expansions inferred by EBSP and mismatch distribution for lineages C and D.

SDMs and paleo-distribution models

The predicted distribution models of \(B. leptopus\) under four periods (last inter-glacial to current) are shown in Fig. 6. The model for the current conditions showed that the distribution of this species is mostly encompassed by the county-based current distribution mapped by the IUCN, with a high AUC value (0.961, SD = 0.013) (Fig. 6A). The two circulation models for the Mid-Hol showed different distributions: the MIROC-based model showed a continuous distribution similar to the current conditions model, while the CCSM-based model resolved disjunct distributions for the northern region of the current distribution model (Fig. 6B). Both LGM models showed clear disjunct distributions with concordance around Nahuelbuta mountain range (Fig. 6C). The MIROC based model indicated a distribution to the north of the current distribution model, and the CCSM resolved a highly fragmented distribution. The model for the LIG showed a disjunct distribution for the species at the northern portion of the current distribution of \(B. leptopus\) (Fig. 6D).
Discussion

Our hindcasting-based approach supports the existence of four lineages in *B. leptopus* (A, B, C and D; Figs. 2, 3) distributed discontinuously along the narrow, ~1000 km long Patagonian region of southern Chile. AMOVA confirmed the strongly different patterns of variation among the *Batrachyla leptopus* populations. In our study, most of the mtDNA genetic differences are present among localities and within lineages (Table 3), with less differentiation among lineages. This indicates the presence of high local genetic structure and high interpopulation differentiation. These results might suggest that although *B. leptopus* has a wide range of distribution, long-range dispersal is highly unlikely, which is in agreement with historical data. On the other hand, most of the variation in nuclear markers were observed within localities, while the values of both genes were not significant (Table 3).

The basic topology of the concatenated ML and Bayesian trees were similar, consequently we used the Bayesian tree as our primary hypothesis of relationships among *B. leptopus* populations (Fig. 3A). We recovered two main clades (named south and north) and four lineages (A, B, C, and D) strongly supported by bootrap, and posterior probabilities. The nuclear phylogeny was highly polytomized (Fig. S1A, B) and it did not inform on lineages relationships. In contrast, the mtDNA phylogeny (Fig. S1C) was highly informative, separating the same clades of the concatenated data set. Consequently, mtDNA variation was a leading indicator of population differentiation and phylogenetic relationships relative to nuclear loci in *B. leptopus*, indicating that mitochondrial markers can be more sensitive in revealate lineage divergence than any single nuclear gene, probably because a lower effective population sizes than nuclear genes (Hung, Drovetski & Zink, 2016).
Biogeographic structure of the lineages

The two northernmost lineages (A and B) are currently separated by two major river systems (Itata and Bío Bío; Fig. 1); these boundaries also coincide with a region with sparsely distributed forest patches. These two lineages are genetically well-differentiated from each other (Fig. 2b); lineage A is restricted to the Los Queules Reserve, and lineage B are limited to a small area in Nahuelbuta range (Locality 2) near to the Butamalal River. The evolution of distinct lineages or genetic clusters is often attributed to population isolation during glacial advances, which geographical isolation in combination with different selection pressures and/or genetic drift would drive population divergence (Hewitt, 2004). Similarly, low levels of current genetic diversity as observed in lineage B (Nahuelbuta; Table 2), suggest a recolonization history of founder effects, small population size, and genetic bottlenecks (Hewitt, 2004).

In contrast to these well-differentiated/low variability lineages, those in the southern distribution (lineages C and D) are geographically more heterogeneous. Lineage C is widely distributed from Máfil in the Los Ríos region (Locality 3 in Fig. 1) to Bahía Mansa, Los Lagos region (Locality 6 in Fig. 1), suggesting that certain landscape features, such as extensive forests, would have allowed dispersal among breeding groups over long timescales. Moreover, the encompassed areas of lineage C also include large mountains (e.g. Bahía Mansa and Cordillera Pelada; Fig. 1), suggesting that such orography could represent barriers to gene flow in *B. leptopus*, as reported in some co-distributed vertebrates and plants (Sérsic et al., 2011). The combined dataset suggests that lineage D is widespread throughout the rest of the species’ range, with few phylogeographical subdivisions.

Lineage divergence time
Divergence time estimates suggest that diversification of *B. leptopus* lineages may have occurred earlier than reported in other frogs such as the ground frog *Eupsophus calcaratus* (Nuñez et al., 2011), although co-distributed populations (e.g. Bahía Mansa) appear to have diverged later in time (0.025 mya for *B. leptopus* (Fig. 3A), and 0.065 mya for *E. calcaratus* (Nuñez et al., 2011; Fig. 2). Vidal et al. (2016) point out that some *B. leptopus* populations from Chiloé Island and the mainland (included in Lineage D in our study) diverged approximately 1.1 mya. Our results indicated that the initial split of *B. leptopus* into the North and South clades was during the Late Pleistocene (~0.107 mya; Fig. 3). Moreover, the overall pattern suggests that *B. leptopus* has undergone several rounds of fragmentation, followed by successive radiations within each clade. Further, at least two more recent series of fragmentation events are inferred within each of these clades. Our calibrations place the split between lineages A and B at ~0.068 mya, and between lineages C and D at ~0.065 mya.

The discrepancy in divergence times between our results and those of the Vidal et al. (2016) may be due to the use of different mutations rates (0.8%) and a single marker (mitochondrial *cyt b*). It is well known from population genetics theory that the stochastic nature of the genealogical process implies a significant amount of variance associated with parameter estimation. In fact, Nabholz et al. (2009) suggest that for inferring divergence dates should imperatively use statistical phylogenetic methods accounting for substitution rate variation across lineages. Indeed, analysis of mtDNA sequence data can be enhanced if they are collected in conjunction with nuclear sequences, because they provide an independent estimate of phylogenetic relationships, mitigating the inherent random effects of the randomness inherent to genetic drift, and the significant amount of variance associated with parameter estimation (Carstens & Dewey, 2010). Interpretations derived of the divergence time analyses also need to take into account the
largely overlapping confidence intervals of the results for each lineage divergence. For example, if only the median values are considered, the results suggest coalescence of the lineages C and D at t1 (around 0.066 mya), and coalescence of the lineages A and B at t2 (around 0.107 mya). But if we consider the confidence intervals, the divergence of both clades, North and South could be closer in time than what is hypothesized in such reconstruction.

**Hypothesized refugia and post-glacial expansion**

Studies on past contraction-expansion climate cycles in Patagonian landscapes suggest that a rapid population expansion should occur in the biota affected by these processes, as habitats became more available (Fraser et al., 2012). Despite the geomorphological differences in Patagonian landscapes, population genetics theory points out that a population undergoing rapid expansion can be characterized by low genetic diversity, since each new founder population represents only a fraction of the ancestral population (Nichols & Hewitt, 1994; Hewitt, 2000; Hewitt, 2004; Waters, Fraser & Hewitt, 2013).

The last two Pleistocene glaciations in southwestern South America (180 kya and 20 kya) covered the Andes with large ice fields reaching the Pacific Ocean south to 39ºS, where the ice sheet decreased in elevation to sea level, and extending further to the southern tip of South America (Rabassa, 2011). Consequently, Late-Pleistocene divergence time estimates (0.107 mya) for first diversification of *B. leptopus* populations separating the North and South clades (Fig. 3), are consistent with a hypothesis of Pleistocene isolation followed by interglacial dispersal. In fact, the divergence of lineages A and B suggests that this interglacial dispersal from the ancestral population occurred rapidly across the current range of the species.
The existence of two suitable areas for the species is supported by the SDM for the LIG (0.120 – 0.140 mya) in that Los Queules population (Locality 1, Table 1) showed the highest genetic diversity (Table 2). This is typical for refugial populations that have been stable over time (Fraser et al., 2012). This evidence and the agreement of the two circulation models for Los Queules area as a suitable habitat for the species during the LGM (Fig. 6C), suggest that it is highly probable that now Los Queules location is a remnant of the northern refuge, derived from the last southern Patagonian glaciation (180 kya).

Demographic reconstruction in *B. leptopus* using an ABC framework also supports the hypothesis of two putative refugia at different time during the Pleistocene (Scenario 4, Fig. 5D, Table 4). This scenario suggests that the South clade populations (lineages C and D) are likely descended from a divergence event approximately 65 Kya. This scenario is concordant with the predicted patchy distribution of the species during the Mid-Holocene (~6,000 years BP; Fig. 6B).

On the other hand, the various demographic analyses show that the genetic structure of lineages C and D contains signatures of demographic expansion consistent with Pleistocene glacial retreat. In fact, strong support for recent population expansion is represented by significantly negative Fu’s Fs values, unimodal mismatch distributions with low raggedness indexes, and EBSP’s depicting rapid expansion following the retreat of the Patagonian ice sheet after 15 Kya (Table 2; Fig. 4). This hypothesis is also reinforced with SDMs produced by both circulation models, although when viewed separately, the MIROC circulation model is the only one predicting this scenario.

Past population dynamics and current conservation significance
During the Cenozoic climatic oscillations included multiple glaciations in southern South America (Rabassa & Clapperton, 1990; Coronato, Martínez & Rabassa, 2004). These geological events have been hypothesized as causes of the retreat and advance of temperate *Nothofagus* forests and conifers (Villagrán & Hinojosa, 1997; Premoli, Kitzberger & Veblen, 2000; Tremetsberger et al., 2009). Accordingly, several phylogeographic hypotheses suggest that Pleistocene glaciations had profound effects on the population genetic structure and variability of Patagonian fauna. For example, glaciated populations of some fish species display molecular diversity (high haplotype diversity and low nucleotide diversity) significantly correlated with latitude (Ruzzante et al., 2008; Cosacov et al., 2010). The same genetic patterns have been observed in reptiles (Breitman et al., 2011; Fontanella et al., 2012), amphibians (Nuñez et al., 2011), and mammals (Himes, Gallardo & Kenagy, 2008; Lessa, D'Elía & Pardiñas, 2010).

In agreement with these previous studies, the location of glacial refugia and postglacial expansion identified here, indicate that the climatic niche of *B. leptopus* is likely to be related to an increase in the availability of suitable habitat in the southern part of its current distribution. Reconstruction of the potential distribution area of *B. leptopus* (Fig. 6) suggests that suitable habitats underwent expansions and contractions during the glacial periods. In addition to the inferences of past population dynamics, predictions about the ability of species to respond to future climate change play an important role in alerting potential risks to biodiversity. In fact, many studies have investigated the response of biodiversity to climate change, and most of them indicate that current and future rates of these changes may be too fast for the ecological niche to evolve (Fraser et al., 2012; Rolland et al., 2018). This is particularly critical in species with low dispersal capacity such as amphibians, which makes them potentially less able to respond to changes induced by climate and, consequently, more vulnerable to
557 extinction (Duan et al., 2016). In this context, the genetic impoverishment at northern area of the
558 distributional range of *B. leptopus* is of great concern, given a climate change scenario based on
559 increases in temperatures and aridity in central-southern Chile.

560
561 Conclusions

562 Our study on genetic diversity throughout the geographic range of *B. leptopus*, supporting the
563 existence of four lineages distributed along ~ 1000 km of length in southwestern Patagonia,
564 including glaciated and no glaciated areas by the LGM. The two northernmost lineages are
565 present in a region with poorly preserved forest patches, whilst the southern lineages are
566 geographically more heterogeneous, suggesting that extensive forests would have allowed
567 dispersion among breeding groups over time scales. Late Pleistocene divergence estimates for
568 the first diversification of the *B. leptopus*, that separate the North and South clades, also
569 supported by the SDM for the LIG, are consistent with a Pleistocene isolation followed by
570 interglacial dispersion. The ABC analyses also supported the hypothesis of two putative refugia
571 at different times during the Pleistocene, concordant with a patchy distribution of the species
572 during the Middle Holocene. In addition, northern populations of *B. leptopus* showed the highest
573 degree of isolation, deserving special attention since considering an increase of the temperatures
574 and aridity in the center-south of Chile, and that they do not have connectivity with nearby
575 populations, they could end up disappearing.

576

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Figure 1

Location map of 19 localities of *B. leptopus* sampled throughout the species’ range in Southwestern Patagonia.

The dashed line corresponds to the limits of the LGM. The full names of the populations are provided in Table 1.
Figure 2

Genetic structure analyses of *Batrachyla leptopus* populations.

(A) number of populations simulated from the posterior distribution obtained with GENELAND v4.0; (B) spatial distribution of clusters. Black points correspond to sample sites. Lineage A to D are represented by light grey, orange, light green and dark green, respectively.
Figure 3

Phylogenetic reconstruction of *Batrachyla leptopus*.

**A** Bayesian tree of pooled data. Strongly supported lineages are represented with different colours and as lineages A–D. Branch support is based on ML bootstrap resampling and Bayesian posterior probabilities above the branch, and point estimates of selected divergence estimates are below. **B** Genealogical relationship based on unrooted phylogenetic networks showing the north and south clades.
Figure 4

Historical demographic analysis for each lineage of *Batrachyla leptopus*.

*(left)* Mismatch distribution of observed frequencies of pairwise differences among *B. leptopus* lineages for concatenated data. *(right)* Extended Bayesian skyline plots analysis. (A, E) Lineage A. (B, F) Lineage B. (C, G) Lineage C. (D, H) Lineage D. X-axe corresponds to time (Ma) and y-axe corresponds to $Ne$, the product of effective population size and generation length in years.
Figure 5

Highest probable ancestral connectivity observed using Approximate Bayesian Computation analysis among the four lineages of *B. leptopus*.

**(A)** Scenario 1: as a null model all lineages coalesced at t1 in a single refuge. **(B)** Scenario 2: all lineage coalesced at t2 in a single refuge. **(C)** Scenario 3: starting at 0, lineage A coalesced at t1 in a single refuge with the lineage B. AB lineage ancestor coalesced at t2 with lineage C and D. **(D)** Scenario 4: lineage C and D coalesced at t1. CD lineage ancestor coalesced at t2 with lineage A and B in a single refuge. **(E)** Scenario 5: starting at 0, lineage A coalesced at t1 in a single refuge with the lineage B, same situation with lineages C and D. In the same way, AB and CD lineage ancestors coalesced at t2 in a single refuge.
Figure 6

Geographical distribution for climatically predicted areas for the occurrence of *B. leptopus* based on current and past bioclimatic variables.

Potential distribution under: (A) current conditions (1960-1990). (B) Mid Holocene (Mid-Hol, ~6,000 yrs BP). (C) Last Glacial Maximum (LGM ~ 22,000 yrs BP). (D) Last Interglacial Period (LIG, ~130,000 years before present). Red, orange, and purple represent areas with high probability of *B. leptopus* for each period.
Sampling locations of *B. leptopus*, its coordinates, sample size for each location (N), and lineage structure according to GENELAND.
Table 1: Sampling locations of *Batrachyla leptopus* with its coordinates, sample size for each location (N), and lineage structure according to GENELAND.

| Location                  | Latitude      | Longitude     | N  | Lineage structure |
|---------------------------|---------------|---------------|----|-------------------|
| Los Queules (LQ)          | -35.99277778 | -72.52583333  | 12 | A                 |
| Nahuelbuta (NA)           | -37.78861111 | -72.99222222  | 7  | B                 |
| Mafil (MA)                | -39.66964722 | -72.92111111  | 2  | C                 |
| Pichirropulli (PI)        | -40.13305556 | -72.90888889  | 7  |                   |
| Cordillera Pelada (CP)    | -40.14027778 | -73.41777778  | 10 |                   |
| Bahía Mansa (BM)          | -40.56305556 | -73.73166667  | 12 |                   |
| Antillanca (AN)           | -40.66888889 | -72.16027778  | 3  | D                 |
| Alerce Andino (AA)        | -41.58027778 | -72.54083333  | 5  |                   |
|   | Location          | Latitude       | Longitude      |   |
|---|-------------------|----------------|----------------|---|
| 9 | Sarao (SA)        | -41.16416667   | -73.72722222   | 3 |
| 10| Puntra (PT)       | -42.10388889   | -73.87000000   | 11|
| 11| Pumalín (PU)      | -42.61638889   | -72.47805556   | 3 |
| 12| Tepuhueico (TP)   | -42.71833333   | -73.94138889   | 11|
| 13| Isla Lemuy (LY)   | -42.62416667   | -73.63722222   | 6 |
| 14| El Amarillo (EA)  | -42.89444444   | -72.46833333   | 14|
| 15| Yaldad (YA)       | -43.10527778   | -73.69555556   | 14|
| 16| Marín Balmaceda (MB) | -43.78388889   | -72.96388889   | 1 |
| 17| La Junta (LJ)     | -43.93944444   | -72.36361111   | 7 |
| 18| Lago Verde (LV)   | -44.23472222   | -71.84083333   | 1 |
| 19| Queulat (QU)      | -44.37694444   | -72.54138889   | 1 |
Table 2 (on next page)

Genetic diversity by lineage on *B. leptopus*.

N: Sample Size, H: Haplotype number, S: Segregating sites, Hd: Haplotype diversity, Pi: Nucleotide diversity, Neutrality test indexes (Rozas’ $r^2$, Tajima’s D and Fu’s FS).
Table 2: Genetic diversity by lineage. N: Sample Size, H: Haplotype number, S: Segregating sites, Hd: Haplotype diversity, Pi: Nucleotide diversity, Neutrality test indexes (Rozas’ $r^2$, Tajima’s D and Fu’s FS).

| Lineage | N  | H  | S  | Hd  | Pi       | $r^2$  | D       | Fu’s FS  |
|---------|----|----|----|-----|----------|-------|---------|----------|
| A       | 12 | 12 | 58 | 1   | 0.00485  | 0.150**| -1.503* | -1.813*  |
| B       | 7  | 2  | 2  | 0.476| 0.00035 | 0.238**| 0.687*  | 1.145*  |
| C       | 31 | 29 | 127| 0.996| 0.00891 | 0.082* | -1.051* | -1.779* |
| D       | 80 | 72 | 150| 0.998| 0.00999 | 0.065* | -1.267* | -1.340* |
| Total   | 130| 113| 264| 0.996| 0.01448 | 0.054* | -1.482* | -2.261* |

**$P < 0.02$, *non significant, $P < 0.10$
Table 3 (on next page)

Results of hierarchical analysis of molecular variance for *Batrachyla leptopus* lineages, over nuclear and mitochondrial markers.

Results of hierarchical analysis of molecular variance for *Batrachyla leptopus* lineages, over mitochondrial, *pomc*, and *crybA1* markers, respectively. df = degrees of freedom, SS = sum of squares, *p*-value is based on 1000 permutations.
Table 3: Results of hierarchical analysis of molecular variance for *Batrachyla leptopus* lineages, over mitochondrial, *pomc*, and *crybA1* markers, respectively. df = degrees of freedom, SS = sum of squares, *p*-value is based on 1000 permutations.

| Source of variation | df  | SS       | Variance components | Percentage of variation | *p*   |
|---------------------|-----|----------|---------------------|-------------------------|-------|
| **Mitochondrial**   |     |          |                     |                         |       |
| Among lineages      | 3   | 1781.206 | 16.16587            | 35.64715                | 0.034 |
| Among localities    | 15  | 2036.172 | 20.83160            | 45.93547                | < 0.001 |
| Within localities   | 111 | 926.981  | 8.35222             | 18.41738                | < 0.001 |
| **pomc**            |     |          |                     |                         |       |
| Among lineages      | 3   | 32.023   | 0                   | 0.00000                 | < 0.001 |
| Among localities    | 15  | 148.948  | 1.38080             | 51.12677                | < 0.001 |
| Within localities   | 111 | 164.776  | 1.48447             | 54.96532                | 0.293 |
| **crybA1**          |     |          |                     |                         |       |
| Among lineages      | 3   | 0.738    | 0.00488             | 4.49187                 | 0.212 |
| Among localities    | 15  | 1.785    | 0.00298             | 2.74255                 | 0.153 |
| Within localities   | 111 | 11.185   | 0.10076             | 92.76558                | 0.193 |
Table 4 (on next page)

Type I and Type II error rates and posterior probabilities for each scenario calculated from DIYABC.
Table 4: Type I and Type II error rates and posterior probabilities for each scenario calculated from DIYABC.

| True scenario used for simulation | Type 2 error rate | Type 1 error rate | Posterior probability (95% credible interval) |
|----------------------------------|-------------------|-------------------|-----------------------------------------------|
|                                  | Scenario 1 | Scenario 2 | Scenario 3 | Scenario 4 | Scenario 5 |                                  |
| Scenario 1                       | -        | 0.030      | 0.090      | 0.066      | 0.041      | 0.227 | 0.0438 (0.0000 - 0.4411)         |
| Scenario 2                       | 0.030    | -          | 0.053      | 0.057      | 0.057      | 0.197 | 0.0044 (0.0000 - 0.4273)         |
| Scenario 3                       | 0.022    | 0.074      | -          | 0.014      | 0.145      | 0.255 | 0.0000 (0.0000 - 0.4076)         |
| Scenario 4                       | 0.020    | 0.094      | 0.003      | -          | 0.023      | 0.140 | 0.9339 (0.9057 - 0.9622)         |
| Scenario 5                       | 0.015    | 0.009      | 0.029      | 0.196      | -          | 0.250 | 0.0178 (0.0000 - 0.4195)         |