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Phylogeography of the Chilean red cricket *Cratomelus armatus* (Orthoptera: Anostostomatidae) reveals high cryptic diversity in central Chile

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We analysed the phylogeographical history of the red cricket *Cratomelus armatus* (Orthoptera: Anostostomatidae) from central and southern Chile using 248 mitochondrial DNA *COI* sequences. Phylogenetic analyses revealed multiple lineages that were highly structured geographically. The two main lineages (north and south) were parapatric, with a contact zone at the latitude of Concepción (~36.6°S), and have an estimated divergence time of 2 Mya. Deep divergence and a species delineation analysis suggest that these lineages should be considered as different species. The north lineage exhibited four well-supported subclades whose divergence times occurred during the Largest Patagonian Glaciation between 0.84 and 1.1 Mya. Signals of demographic expansion in southern areas indicate a more recent history for the south lineage (southern Chile). A positive correlation between latitude and genetic distances between populations suggests postglacial colonization of southern areas. Bayesian estimations of population size over time placed a bottleneck at ~150 kya. Our results support a role for glaciations in shaping contrasting patterns of genetic diversification in *C. armatus*. More intensive past glaciations may have promoted diversification in central Chile, whereas subsequent glaciations, with stronger impacts in southern areas, could have constrained diversification in southern Chile. We discuss the taxonomic implications of our findings and hypothesize a contrasting role for glaciation on patterns of genetic diversification in central and southern Chile.

ADDITIONAL KEYWORDS: cryptic diversity – glaciations – high genetic diversity – mitochondrial DNA – multiple refugia.

INTRODUCTION

Pleistocene climatic oscillations had important consequences for species distributions, genetic diversity and the evolutionary history of taxa, particularly in temperate regions (Avise, 2000; Hewitt, 2000, 2004; Sérsic *et al.*, 2011). Phylogeographical research has revealed that isolation and postglacial expansion from refugia are among the most important processes explaining genetic patterns in the Northern Hemisphere (Avise, 2000; Hewitt, 2004). In contrast to the vast phylogeographical research conducted in temperate regions of North America and Europe, phylogeographical studies on taxa from temperate regions of South America have accumulated at a much lower pace (Beheregaray, 2008;

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Turchetto-Zolet *et al.*, 2013), limiting our understanding of the processes driving biodiversity patterns in this region. Predictably, Pleistocene glaciations and the Andean orogenesis have played important roles in shaping biodiversity in southern South America (Ruzzante *et al.*, 2006; Sérsic *et al.*, 2011). However, inferred evolutionary histories of taxa in this region appear more complex than previously assumed, with variation in phylogeographical patterns indicating mixed responses to environmental change (Sérsic *et al.*, 2011; Victoriano *et al.*, 2012).

A general pattern found in several phylogeographical studies conducted in central and southern Chile is a decrease of genetic diversity towards higher latitudes (e.g. Rodríguez-Serrano, Cancino & Palma, 2006; Himes, Gallardo & Kenagy, 2008; Victoriano *et al.*, 2008). This pattern has supported the idea of glacial refugia in northern areas with more stable climatic conditions that allowed the persistence of taxa during the Last Glacial Maximum (LGM) and recolonization and expansion into southern areas after glaciations (Palma *et al.*, 2005; Lessa, D'Elía & Pardiñas, 2010). Although this pattern has been observed in several taxa (see Sérsic *et al.*, 2011), some studies have found patterns that are in contrast to this general trend and seem to reveal a more complex scenario. For instance, studies analysing patterns of genetic diversity in fish (Unmack *et al.*, 2009), lizard (Vera-Escalona *et al.*, 2012) and freshwater crustacean (Xu *et al.*, 2009) species have shown weak or no relationship between genetic diversity and glacial impact. Indeed, some of these studies (Xu *et al.*, 2009) have also found high genetic diversity in areas that were putatively covered by the ice sheet, suggesting a more complex scenario, with potential cryptic refugia within the hypothesized boundaries of the ice sheet.

These contrasting patterns only emphasize that it is still too soon to make generalizations, and more research needs to be conducted to gain a better understanding of the phylogeographical complexity of the region. In addition, the strong taxonomic bias in phylogeographical studies conducted in the region, which have focused on terrestrial vertebrates and plants (Beheregaray, 2008; Sérsic *et al.*, 2011; Turchetto-Zolet *et al.*, 2013), has limited a more general understanding of the historical processes impacting diversity in southern Chile. In particular, the most diverse group of animals on Earth, the insects, has so far received little attention in temperate areas of South America (but see Zúñiga-Reinoso *et al.*, 2016).

In this study, we examined the phylogeography of a large cricket that is widely distributed in central and southern Chile, the Chilean red cricket or 'grillo rojo' (
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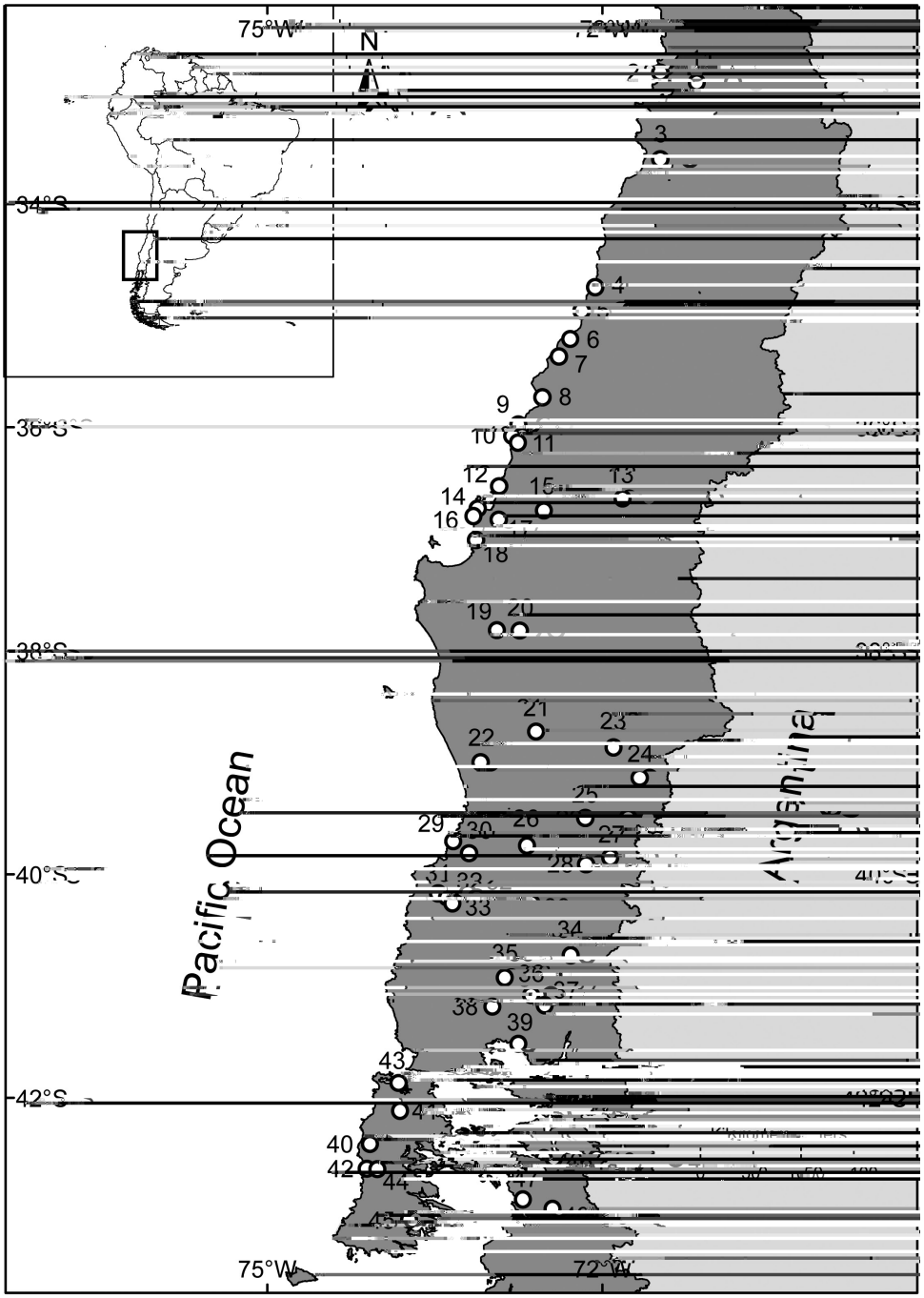

Figure 1. Sampling localities of *Cratomelus armatus* used in the study. The numbers represent the identification of localities (see Table 1).

distribution of the species, which extends over 1200 km, from the Cardenal Caro Province in the north (O'Higgins, 34°S) to the southern Palena Province (Los Lagos, 42°S; Fig. 1). For species identification, we followed Gorochov (1999). The collected individuals were fixed and stored in 99% ethanol and deposited in the repository of the Laboratorio de Genética y Evolución, Universidad de Chile, Chile (GEVOL), the collection of Laboratorio de Entomología Ecológica, Universidad de La Serena, Chile (LEULS) and the Phoenix group collection, Massey University, New Zealand.

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

DNA was extracted from muscle tissue using a modified salt extraction method (Jowett, 1986;

Sunnucks & Hales, 1996). We amplified partial sequences of the mtDNA gene cytochrome oxidase I (*COI*) using primers C1-J-2183 (5′-CAACATTTATTTTGATTTTTTGG-3′) and TL2-N-3014 (5′-AATTCCGCACATTGCCTAATCATTA-3′) (Simon *et al.*, 1994). Previous studies with related crickets have shown that this marker provides good resolution for phylogeographical analysis (e.g. Trewick & Morgan-Richards, 2004; Pratt, Morgan-Richards & Trewick, 2008; Brettschneider *et al.*, 2009; Chappell, Trewick & Morgan-Richards, 2012). The reaction mixture included 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 1 U Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 50–100 ng total DNA. The thermal protocol for the PCR was 94 °C for 5 min, followed by 36 cycles of 94 °C for 45 s, 45–50 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 2 min. The PCR products were visualized in agarose gels and sequenced using the same primers. DNA sequences were edited and aligned in BIOEDIT v7.0.5.2 (Hall, 1999) and translated into amino acids in Mega 6 to check for codon stops and frame shifts that could indicate alignment errors and the potential presence of nuclear copies of the mitochondrial gene (Numts; Song, Moulton & Whiting, 2014). Levels of substitution saturation were analysed with Xia's test (Xia *et al.*, 2003) in DAMBE, version 5.1.5 (Xia & Xie, 2001). All sequences are available in GenBank (MG202165–MG202417).

Time-calibrated genealogy and species delimitation analysis

A dated genealogical reconstruction was estimated by Bayesian inference with the program BEAST version 2.4.4 (Bouckaert *et al.*, 2014). We included all the sequences of *C. armatus* and the sequences of the congeneric species *C. integer* as an outgroup (MG202413–MG202417). The HKY + G evolutionary model was selected as the best-fit model using the program jModelTest version 0.1.1 (Posada, 2008) using the Bayesian information criterion. The strict-clock model was selected as the molecular clock prior, using a substitution rate of 0.017 substitutions per site per million years, following recently published rates for insects (Papadopoulou, Anastasiou & Vogler, 2010) and orthopterans (Allegrucci, Trucci & Sbordoni, 2011; Kaya & Çiplak, 2016).

We conducted two independent analyses to check for consistency in the results. Each analysis was run for 50 million generations, sampling trees every 10 000 generations. The program Tracer v. 1.6 (Rambaut & Drummond, 2009) was used to visualize the traces of the Markov chain Monte Carlo runs and to check that the effective sample size of model parameters were > 200 (indication of convergence). Sampled trees were summarized using the maximum clade credibility criteria in TreeAnnotator v2.4.4 (distributed as part of BEAST), discarding the first 20% of the trees as burn-in. The summarized tree was visualized and edited for illustration purposes using Figtree v1.4.2 (Rambaut, 2008). Complementarily, to examine haplotype relationships and the frequency distribution across space we constructed a haplotype network using the median joining algorithm (Bandelt, Forster & Röhl, 1999) implemented in PopArt v1.7.1 (Leigh & Bryant, 2015).

Following the results from the phylogenetic analyses, we conducted a barcode gap analysis to look for evidence of species-level differences between major clades using the automatic procedure ABGD described by Puillandre *et al.* (2012). This analysis uses the barcode gap, which is the gap observed when divergence among individuals of the same species is smaller than divergence among individuals from different species, to automatically find groups that might correspond to different potential species. A range of prior intraspecific divergence values from 0.001 to 0.1 was assayed (in ten steps), applying a relative gap width (X) of 1.5 and using three options for genetic distance (JC69, K80 and simple distance). This analysis was performed through the Web server of ABGD (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html).

Analyses of genetic diversity and demography

To estimate genetic diversity across the distribution of *C. armatus*, we estimated several diversity statistics, namely haplotype diversity (*Hd*), nucleotide diversity (π) and genetic distance among geographically close populations for all sampling localities, using the program DnaSP v5.10.01 (Librado & Rozas, 2009). Given that phylogenetic analyses revealed multiple lineages, demographic analyses were conducted separately for each of the two main lineages that were supported

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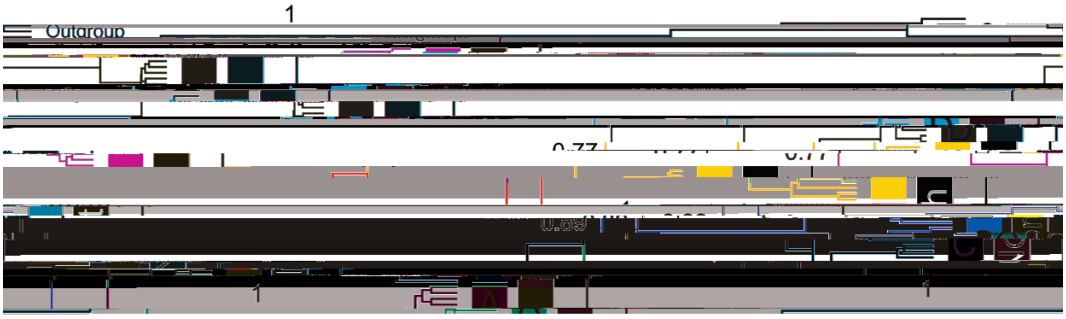

Figure 2. Bayesian phylogenetic tree for *Cratomelus armatus* based on mitochondrial DNA. Numbers on nodes are Bayesian posterior probabilities, and the colour of branches represents the different lineages (see Fig. 3).

parameters to reconstruct the effective population size (N_e) over time using a Bayesian statistical approach. Priors and settings were kept as in the dated genealogical analyses, but using the coalescent Bayesian skyline option as the tree prior. We ran 100 million iterations, sampling every 10 000 iterations to generate 100 000 parameter estimates for two independent runs. Likelihood values of demographic plots for each genetic group were visualized with the program Tracer v1.5.0 (Rambaut & Drummond, 2009).

Complementarily, a Mantel test (Mantel, 1967) was also performed to compare patterns of isolation by distance between the north and south lineages. Impacted areas that are not under equilibrium will show weak or no correlation between geographical distance and genetic structure, whereas areas that have been more stable will show a stronger correlation. The Mantel test was conducted in Alleles In Space (AIS; Miller, 2005).

Testing the impact of glaciations on genetic diversity and structure

We conducted various tests to assess the impact of glaciation on genetic diversity. We performed correlation tests between measures of genetic diversity and latitude. The general model suggests that the extent of glaciation increased with latitude, predicting a decrease in genetic diversity from north to south. We first looked for correlation between genetic diversity and latitude with all localities, as the distribution of populations strongly follows a latitudinal gradient. However, the presence of distinct lineages in our phylogenetic analyses suggested that these should be treated separately, so we then focused the analysis on populations of the southernmost clade (south of 37°S), which presented different degrees of exposure to glacial coverage.

Three measures of genetic diversity were also used to assess for geographical correlation, namely nucleotide diversity (π), haplotype diversity and the average number of pairwise differences between populations. Analysis using pairwise genetic distances and latitude used only pairwise distances of one decimal degree or less (~111 km) to represent local diversity. We used the midpoint between the compared localities to obtain a single latitude value for pairwise comparisons. Finally, to visualize the variation of genetic distances across the distribution of the species better, we performed a genetic landscape shape interpolation analysis with the program AIS (Miller, 2005).

RESULTS

We obtained a 765 bp alignment of mtDNA *COI* gene sequences comprising 167 variable sites and a total of 117 haplotypes from 248 individuals. The sequences presente Tc te0 Tc -0.04 Tw T*c te0aR.5 (e)0.5 (a(Tc tew1 (.)l

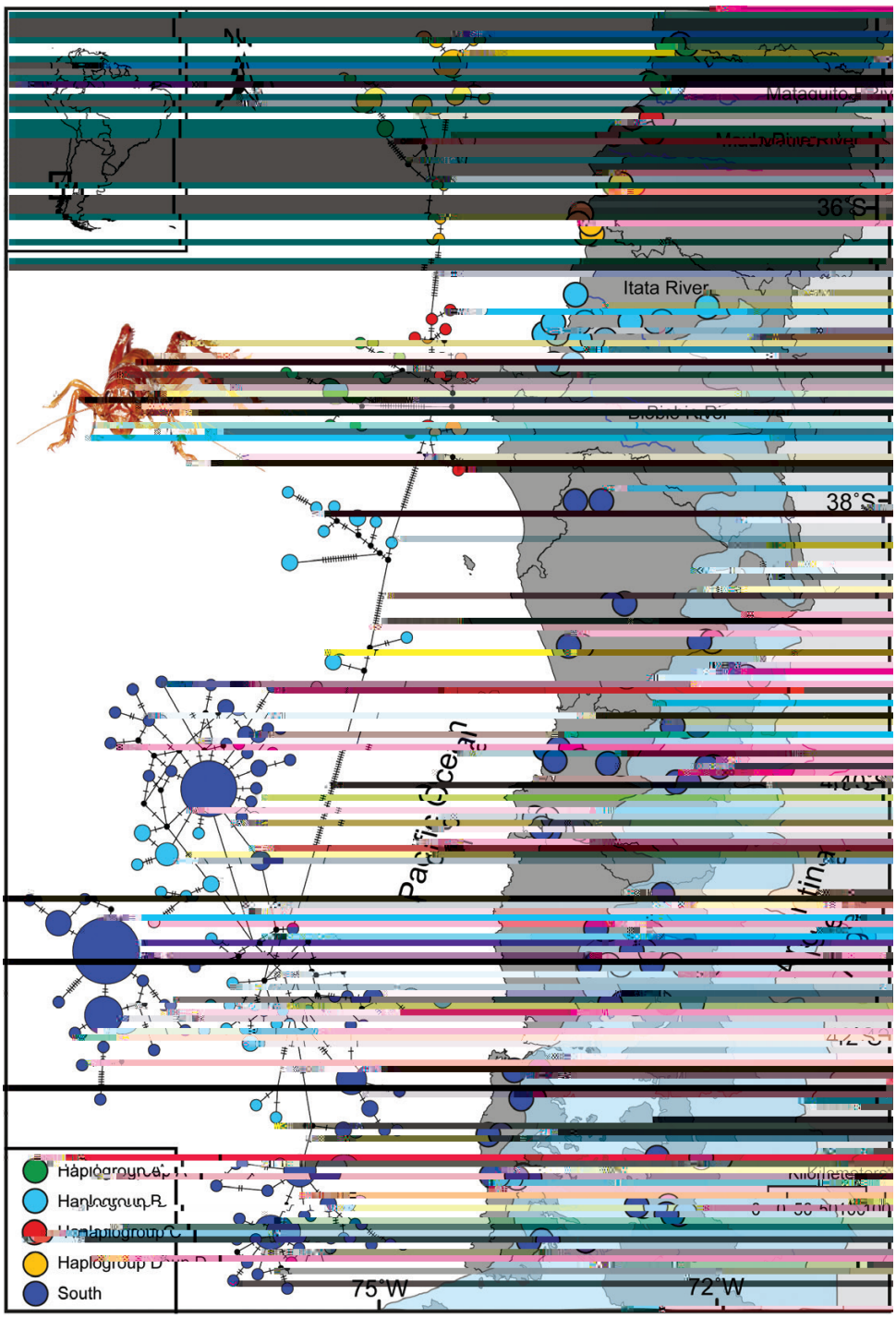

Figure 3. Median joining network of mitochondrial DNA of *Cratomelus armatus* from central and southern Chile, showing geographical distribution of haplogroups. The haplotypes are represented by circles whose sizes are proportional to their frequencies. Colours represent the different geographical groups where haplotypes are present. The light blue shape shows the extent of the ice cover during the Last Glacial Maximum (Clapperton, 1993).

distributed across a larger geographical area. The estimated age of the southern lineage was ~50% younger than the age of the northern lineage, with a time to the MRCA of 0.53 Mya (95% HPD: 0.38–0.67 Mya).

The haplotype network was consistent with the genealogical reconstruction and showed a complex structure with five main haplogroups separated by a large number of nucleotide substitutions (Fig. 3).

Cratomelus armatus with haplogroup B haplotypes came from localities north of the Biobio River, whereas those with group D haplotypes were associated with localities south of the Maule River (between the Maule and Itata rivers). Haplogroup C was associated with localities south of the Mataquito River, whereas haplogroup A was associated with localities north to the mouth of the Mataquito River. Individuals collected in localities south of the Biobio River (37–43°S) belonged to the south lineage. The four haplogroups north of the Biobio River were represented by haplotypes at similar frequencies that did not form star-like topologies. In contrast, the south lineage included a few haplotypes at high frequency, each with numerous rare derived haplotypes differing by one to a few nucleotide substitutions. This star-like topology is often associated with recent demographic expansions.

Genetic differentiation and species delimitation analysis

Genetic distances between the north and south clades and between subclades within the north clade were fairly high (Table 3), revealing high cryptic diversity within the nominal species *C. armatus*, especially in its northern range (from the Biobio River to the north). The average (uncorrected) number of pairwise differences between the north and the south lineages was 52.34 (p-distance = 0.068). The average number of pairwise differences between c

Demographic analyses indicated different demographic histories for the north and south lineages. For the north lineage, both neutrality tests, *Fs* and Tajima's *D*, showed no significant departures from zero, indicating stable population size and long-term population history (Table 4). The Mantel test also showed a high and significant correlation between geographical and genetic distances ($R = 0.72$, $P < 0.001$) for the north lineage. In contrast, for the south lineage the *Fs* and Tajima's *D* tests showed negative and significant departures from zero, suggesting past bottlenecks and a more recent population history (Table 4). Consistently, the Mantel test for this group showed a weak (although significant) correlation between genetic distance and geographical distance ($R = 0.17$, $P < 0.001$).

The Bayesian skyline plot analyses showed consistent patterns with the above tests. For the north clade, the analysis inferred constant population size over time (Supporting Information, Fig. S5A), whereas for the south clade it inferred a demographic expansion over time (Supporting Information, Fig. S5B) that started ~100–150 kya and coincided with the OIS 6 glacial event, the most extensive in the past half million years (Mortyn *et al.*, 2003). Altogether, these results strongly suggest a more recent demographic

rather than displacing its habitat, facilitating population isolation and divergence. In contrast, more continuous and widespread ice coverage in southern areas may have prevented isolation in multiple isolated refugia; hence, it prevented the diversification of the southern lineage. Interestingly, our dated genealogy indicates that divergence times for all major clades of the northern lineage (clades A–D; Fig. 2) date back to 1.1–0.84 Mya (Table 2), matching the period of the most extensive Andean glaciation (Largest Patagonian Glaciation; Singer, Ackert & Guillou, 2004). These

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