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A new endemic lineage of the Andean frog genus *Telmatobius* (Anura, Telmatobiidae) from the western slopes of the central Andes

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The amphibian genus *Telmatobius* is a diverse group of species that inhabits the Andes. This study analysed the phylogenetic relationships of 19 species described from the central Andes of Chile and Bolivia, and 12 undescribed populations of Chile. A molecular phylogeny based on mitochondrial DNA *16S* and *cytochrome b* shows that the Chilean species belong to three groups: (1) the *Telmatobius marmoratus* group, widespread in the Chilean and Bolivian Altiplano; (2) the *Telmatobius hintoni* group, including the species *Telmatobius philippii*, *Telmatobius fronteriensis*, and *Telmatobius huayra*, occurring in the south-western Altiplano of Chile and Bolivia, and (3) the *Telmatobius zapahuirensis* group, a new clade which also includes *Telmatobius chusmisensis*, *Telmatobius dankoi*, and *Telmatobius vilamensis*, restricted to western slopes of the Andes, and which was recovered as more closely related to the *T. hintoni* group than the *T. marmoratus* group. The divergence times between clades were traced to the late Pleistocene. The molecular phylogeny also confirmed that the groups of the Altiplano and western Andes slopes form a clade separated from the species that inhabit the eastern Andes (*Telmatobius verrucosus* and *Telmatobius bolivianus* groups), supporting the forest origin of the Altiplano groups proposed by several previous authors.

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ADDITIONAL KEYWORDS: Altiplano – Amphibia – mitochondrial DNA – molecular clock – systematics.

INTRODUCTION

The amphibians of the genus *Telmatobius* Wiegmann, 1834, are a typical component of the Andean batrachofauna. The genus is currently composed of 61 aquatic and semi-aquatic species (Frost, 2013) distributed in the Andean ecosystems of Ecuador, Peru, Bolivia, Argentina, and Chile. In the Altiplano (or Puna) and western range of the central Andes around 20 species of *Telmatobius* have been described, of which eight are endemic to Chile.

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The Chilean species of *Telmatobius* are mostly aquatic (Veloso *et al*., 1982) and inhabit rivers, streams, and springs in an altitudinal range from 2260 to approximately 4500 m (Formas, Veloso & Ortiz, 2005). Geographically, these species are associated with two regions of the central Andes: the western slopes (or forearc) (e.g. *Telmatobius vilamensis*) and the Altiplano (e.g. *Telmatobius fronteriensis*). Few populations of *Telmatobius* are known in Chile (e.g. Benavides, Ortiz & Formas, 2002; Formas, Benavides & Cuevas, 2003). This is probably because of a lack of exploration as in recent years new populations have been found in this region. This suggests that the distribution and/or diversity of these endemic amphibians may be greater than what has been described so far in this region of the Andes.

Most systematic studies of the genus *Telmatobius* have included a small number of species and/or a small geographical area. These studies have mainly used morphological characters (e.g. Aguilar & Pacheco, 2005; Aguilar & Valencia, 2009). Recently, the value of morphological characters has been questioned, including osteological traits, for the study of the systematics and taxonomy of the genus *Telmatobius* because of the high degree of intraspecific variation (Trueb, 1979; Wiens, 1993; Sinsch, Hein & Glump, 2005; De la Riva, García-París & Parra-Olea, 2010; but see De la Riva, Trueb & Duellman, 2012 and Barrionuevo, 2013). To date, the most complete study using DNA sequences is that of De la Riva *et al*. (2010), who reviewed the taxonomic status and phylogenetic relationships of 12 species of *Telmatobius* from the Bolivian central Andes. Their results revealed the existence of three clades in this region; the *Telmatobius verrucosus* group and the *Telmatobius bolivianus* group, both of the forests and inter-Andean valleys of the eastern range, and the Altiplano group. The last species group has two subgroups, *Telmatobius hintoni* and *Telmatobius marmoratus*, whereas the latter is a paraphyletic group that also includes *Telmatobius gigas*. This species was initially described by Vellard (1969, 1970) as one of the many subspecies of *T. marmoratus*, but later was elevated to the species level by De la Riva (2002), considering morphological evidence.

It was suggested that the Chilean species of *Telmatobius* are divided into two geographical groups: northern and southern (Formas *et al*., 2003). However, these categories do not correspond to natural groups, and the discovery and description of *Telmatobius chusmisensis* in an intermediate geographical area suggests that this pattern was produced by a low sampling effort (Formas, Cuevas & Nunez, 2006). The only molecular systematic study that has incorporated Chilean *Telmatobius* species is Correa *et al*. (2006). In this study the three species included (*T. marmoratus*, *Telmatobius zapahuirensis*, and *T. vilamensis*) formed a robust clade. Nevertheless, the low number of taxa included was inadequate to evaluate the phylogenetic relationships of the species of *Telmatobius* present in Chile. As the species present in this region have not been included in any of the systematic studies of the genus, the phylogenetic relationships and the origin of Chilean *Telmatobius* are unknown.

The Altiplano was formed by complex geological and climatic processes, which included orogenesis, volcanism, and cycles of formation and reduction of large ancient lakes (Gregory-Wodzicki, 2000; Babeyko *et al*., 2002; Schmitt *et al*., 2002; Rigsby *et al*., 2005; Placzek *et al*., 2006). The origin and diversification of the anurans of the central Andes has been the subject of various biogeographical and evolutionary studies, and several authors have proposed that the processes that influenced the formation of the Altiplano have played a key role in promoting speciation and diversification of the species in this region (e.g. Duellman, 1979; Cei, 1986; Lynch, 1986). Recently, De la Riva *et al*. (2010) found patterns concordant with the hypothesis proposed by Duellman (1979), Cei (1986), and Lynch (1986) of the forest origin of the Altiplano species of *Telmatobius*. De la Riva *et al*. (2010) suggested that the diversification of the species of the Altiplano of Bolivia is closely associated with the elevation of the Andes in the late Pliocene and Pleistocene, and with the climatic events that occurred during the Pleistocene. Recent studies in other taxa that inhabit the Altiplano and western Cordillera of the Andes suggest that in this region there has been genetic differentiation mediated by peripatric mechanisms, such as for rodents of the genus *Phyllotis* and *Abrothrix* (Palma, Marquet & Boric-Bargetto, 2005) and the anuran *Rhinella spinulosa* (Correa *et al*., 2010); and by allopatric mechanisms, as in the case of snails of the genus *Biomphalaria* (Collado, Vila & Méndez, 2011) and fishes of the genus *Orestias* (Vila *et al*., 2013). In the case of *Telmatobius* only broad diversification patterns have been proposed (e.g. Cei, 1986; De la Riva *et al*., 2010).

Considering that the Chilean and Bolivian Altiplano species of *Telmatobius* have contiguous geographical distributions with many hydrological connections amongst them, we hypothesized that the Chilean species would be more closely related to the lineages that include the species of the Bolivian Altiplano (*T. marmoratus* and *T. hintoni* groups) than to the lineages that include the species that inhabit the eastern Cordillera of the central Andes (*T. verrucosus* and *T. bolivianus* groups) proposed by De la Riva *et al*. (2010). The goal of this study was to establish the phylogenetic relationships of the species of *Telmatobius* present in the central Andes of Chile and Bolivia, with emphasis on the Chilean species. We used partial sequences of the mitochondrial *16S* and *cytochrome b*

(*Cytb*) genes published by De la Riva *et al*. (2010) together with new sequences of eight species present in Chile and 12 previously unknown Chilean populations. We also estimated the divergence times for the species of *Telmatobius* of this region in order to establish a time framework of the diversification.

MATERIAL AND METHODS

Taxon sampling and laboratory procedures

Samples of one to six individuals of *Telmatobius* were obtained from the type localities of eight of the ten species present in Chile. Identification of specimens was based on original descriptions and the taxonomic key of Formas *et al*. (2003) for adult *Telmatobius*. *Telmatobius halli* and *Telmatobius pefauri* were not found in spite of an extensive sampling effort. Additionally, samples were obtained from 12 previously unknown Chilean populations. The localities included in this study are indicated in Table 1 and Figure 1. Animals were captured with a fishing sieve from under vegetation and amongst rocks in the watercourses.

DNA was obtained using buccal swabs (following Gallardo *et al*., 2012) or a small piece (approximately 3 mm^3) of interdigital membrane. To obtain the samples, animals were anaesthetized using 0.2% tricaine methanesulphonate (modified from Mitchell, 2009). Buccal swabs samples were conserved in buffer solution (100 mM Tris-HCl pH 7.5; 100 mM ethylenediaminetetraacetic acid, 100 mM NaCl, 0.5% sodium dodecyl sulphate) until analysis in the laboratory. In a few cases we used muscle tissue. Membrane and tissue samples were conserved in absolute ethanol until analysis. Total DNA was isolated using the salt extraction method (modified from Jowett, 1986).

Molecular markers

Partial sequences of the mitochondrial genes *16S* (± 560 bp) and *Cytb* (± 900 bp) were amplified with the same pairs of primers used by De la Riva *et al*. (2010), except for CytbAR-H (5′-TAWAAGGGTCTTCTACT GGTTG-3′; Goebel, Donnelly & Atz, 1999). This was used instead of the primer MVZ18 (Moritz, Schneider & Wake, 1992). PCR conditions were the following (values separated by an en-dash indicate the differences between the protocols for a primer pair); 2.5–3.0 mM $MgCl_2$, 100 μM deoxyribonucleotide triphosphates, 0.67–0.83 μM primers, 1.0 U Taq and 50 to 200 ng total DNA in 30 μL total. The thermal profile was 3–1 min initial denaturation at 94 °C, 35–41 cycles of 30–60 s, denaturation at 94 °C, 45–50 s, annealing at 58–56 °C, and 45–50 s extension at 72 °C, with a final extension of 10–8 min at 72 °C.

Alignment of sequences, phylogenetic analyses, and genetic divergence

Sequences were aligned and edited in the BioEdit v. 7.2.0 program (Hall, 1999) using ClustalW option (implemented in BioEdit), and then reviewed by visual inspection. Phylogenetic reconstruction used the maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods. The MP and ML analyses were performed in the PAUP v. 4.0 program (Swofford, 2002) using heuristic search with the tree bisection-reconnection and branch-swapping options. Statistical support for the nodes was estimated by a bootstrap with 1000 and 100 pseudoreplicates (Felsenstein, 1985), respectively. The BI analysis was performed in the MrBayes v. 3.1.2 program (Ronquist & Huelsenbeck, 2003). Ten million generations were run with four Markov chains, sampled every 1000 generations. The first 2500 trees were discarded. For the ML and BI analyses we used the general time-reversible (GTR) + gamma model, which was selected by the JModelTest program (Posada, 2008) under the Akaike and Bayesian information criteria, respectively.

Trees were rooted using the outgroup method. Initially we used sequences of four species of sister groups of the Telmatobiidae (revalidated by Pyron & Wiens, 2011): *Rhinoderma darwinii* (GenBank accession numbers DQ864561 and KJ562948), *Insuetophrynus acarpicus* (GenBank accession numbers DQ864558 and KJ562949) (Rhinodermatidae), *Batrachyla taeniata* (GenBank accession numbers KJ562950 and KJ563018), and *Atelognathus salai* (GenBank accession numbers DQ864547 and KJ562951) (Batrachylidae). In later analyses we used the *T. verrucosus* group (De la Riva *et al*., 2010) as outgroup. In the ingroup were included the sequences of the *16S* and *Cytb* genes of 12 species of *Telmatobius* present in Bolivia published by De la Riva *et al*. (2010) (GenBank accession numbers GU060549–GU060618), eight species present in Chile, and 12 Chilean previously unknown populations (GenBank accession numbers KJ562873–KJ563017 and JX442356–JX442365).

First, the data of the *16S* and *Cytb* markers were analysed independently. After using the test of homogeneity of partitions implemented in PAUP v. 4.0 (incongruence length difference test; Swofford, 2002), we combined the matrices in a total evidence analysis.

The percentage of genetic divergence was used as an indicator of different species of the genus *Telmatobius*. To evaluate the degree of genetic divergence amongst *Telmatobius* species we used the *Cytb* gene because it proved to be more informative than the *16S* gene. We calculated the genetic distance corrected by the Kimura two-parameter nucleotide evolution model using the MEGA v. 4.0 program (Tamura *et al*., 2007).

Table 1. Localities of the sequences of nominal taxa included in this study. Localities in bold are Chilean *Telmatobius* type localities. Locality numbers match those in Figure 1

Nominal taxon	Locality	Locality number	Author
Telmatobius espadai	Choquetanga	19	De la Riva *et al*., 2010
Telmatobius sanborni	Pelechuco	1	De la Riva *et al*., 2010
Telmatobius verrucosus	Rio Chairo	8	De la Riva *et al*., 2010
Telmatobius bolivianus	Río Unduavi	6	De la Riva *et al*., 2010
Telmatobius yuracare	Incachaca	24	De la Riva *et al*., 2010
Telmatobius sibiricus	Siberia	25	De la Riva *et al*., 2010
Telmatobius simonsi	La Hoyada	26	De la Riva *et al*., 2010
Telmatobius culeus	Lago Titicaca	4	De la Riva *et al*., 2010
Telmatobius marmoratus	Río Charazani	2	De la Riva *et al*., 2010
T. marmoratus	Charazani-Escoma	3	De la Riva *et al*., 2010
T. marmoratus	Zongo	5	De la Riva *et al*., 2010
T. marmoratus	La Cumbre	7	De la Riva *et al*., 2010
T. marmoratus	Kkota Pata	Not on map	De la Riva *et al*., 2010
T. marmoratus	Colpa	10	This study
T. marmoratus	Caquena	11	This study
T. marmoratus	Sajama	12	De la Riva *et al*., 2010
T. marmoratus	Chungará	13	This study
T. marmoratus	Lauca	14	This study
T. marmoratus	Comanche	17	De la Riva *et al*., 2010
T. marmoratus	Laguna Macaya	20	De la Riva *et al*., 2010
T. marmoratus	Río Pacokhaua	21	De la Riva *et al*., 2010
T. marmoratus	Cancosa	32	This study
Telmatobius gigas	Huayllamarca	18	De la Riva *et al*., 2010
Telmatobius peruvianus	Putre	9	This study
Telmatobius hintoni	Corani	23	De la Riva *et al*., 2010
T. hintoni	Tunari	22	De la Riva *et al*., 2010
T. hintoni	Río San Juan	35	De la Riva *et al*., 2010
Telmatobius huayra	Pastos Grandes	43	De la Riva *et al*., 2010
T. huayra	Sol de Manana	44	De la Riva *et al*., 2010
Telmatobius fronteriensis	**Puquios**	39	This study
Telmatobius philippii	**Quebrada Amincha**	40	This study
Telmatobius chusmisensis	**Chusmiza**	30	This study
Telmatobius dankoi	**Las Cascadas**	45	This study
Telmatobius vilamensis	**Vilama**	46	This study
Telmatobius zapahuirensis	**Zapahuira**	16	This study
Telmatobius sp.	Ascotán	42	This study
Telmatobius sp.	Quebrada Choja	38	This study
Telmatobius sp.	Loanzana	31	This study
Telmatobius sp.	Belén	15	This study
Telmatobius sp.	Carcote	41	This study
Telmatobius sp.	Quebrada Chijlla	37	This study
Telmatobius sp.	Copaquire	36	This study
Telmatobius sp.	Huasco	34	This study
Telmatobius sp.	Isluga	28	This study
Telmatobius sp.	Piga	33	This study
Telmatobius sp.	Quebe	29	This study
Telmatobius sp.	Quebrada Tana	27	This study

Estimation of divergence times

The divergence times of the species of *Telmatobius* were estimated in the BEAST v. 1.7.2 program (Drummond *et al*., 2012). This program performs a Bayesian inference based on the molecular clock hypothesis (Zuckerkandl & Pauling, 1965). As there are no fossils calibrated for *Telmatobius*, we used the calibration

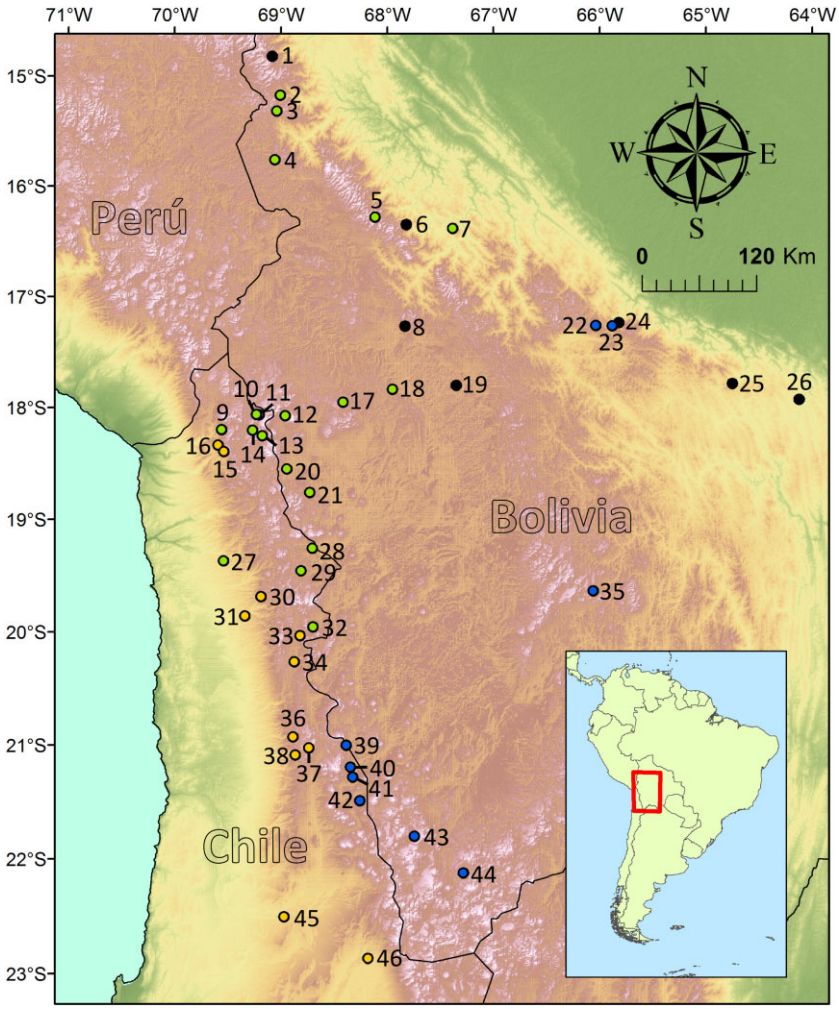


Figure 1. Geographical location of the Chilean and Bolivian *Telmatobius* populations included in this study. Numbers correspond to those in Table 1. Black circles, *Telmatobius verrucosus* and *Telmatobius bolivianus* groups; green circles, *Telmatobius marmoratus* group; blue circles, *Telmatobius hintoni* group; orange circles, *Telmatobius zapahuirensis* group.

performed by De la Riva *et al*. (2010). These authors used the *Cytb* gene and a mutation rate of 2%. This procedure allowed us to compare our estimate with De la Riva *et al*. (2010). The nucleotide substitution model utilized was the GTR + gamma model obtained in JModelTest (Posada, 2008).

We used two speciation models, Yule (Edwards, 1970) and Birth and Death (Yang & Rannala, 1997). Under these two speciation models we evaluated three models of the distribution of evolution between branches: strict molecular clock, lognormal relaxed molecular clock (Kishino, Thorne & Bruno, 2001), and exponential relaxed molecular clock (Drummond *et al*., 2006). For each model the Markov chain was run for 100 000 000 generations, discarding the first 10% of the trees obtained. The Bayes factor was then calculated for each speciation model under the three molecular clock models evaluated, in order to select the model that best fits the data (Kass & Raftery, 1995; Suchard, Weiss & Sinsheimer, 2001).

RESULTS

Phylogenetic relationships

The analyses of the *16S* gene partition showed high resolution at the base of the tree and little resolution in the terminal nodes, whereas the analyses with the *Cytb* gene showed high resolution at both levels (data not shown). The phylogenetic relationships recovered by the two concatenated mitochondrial markers (1279 nucleotide sites) showed the same level of resolution as the *Cytb* partition; thus, we use the results of the concatenated analyses hereafter. The phylogenetic relationships recovered using MP, ML, and BI were highly

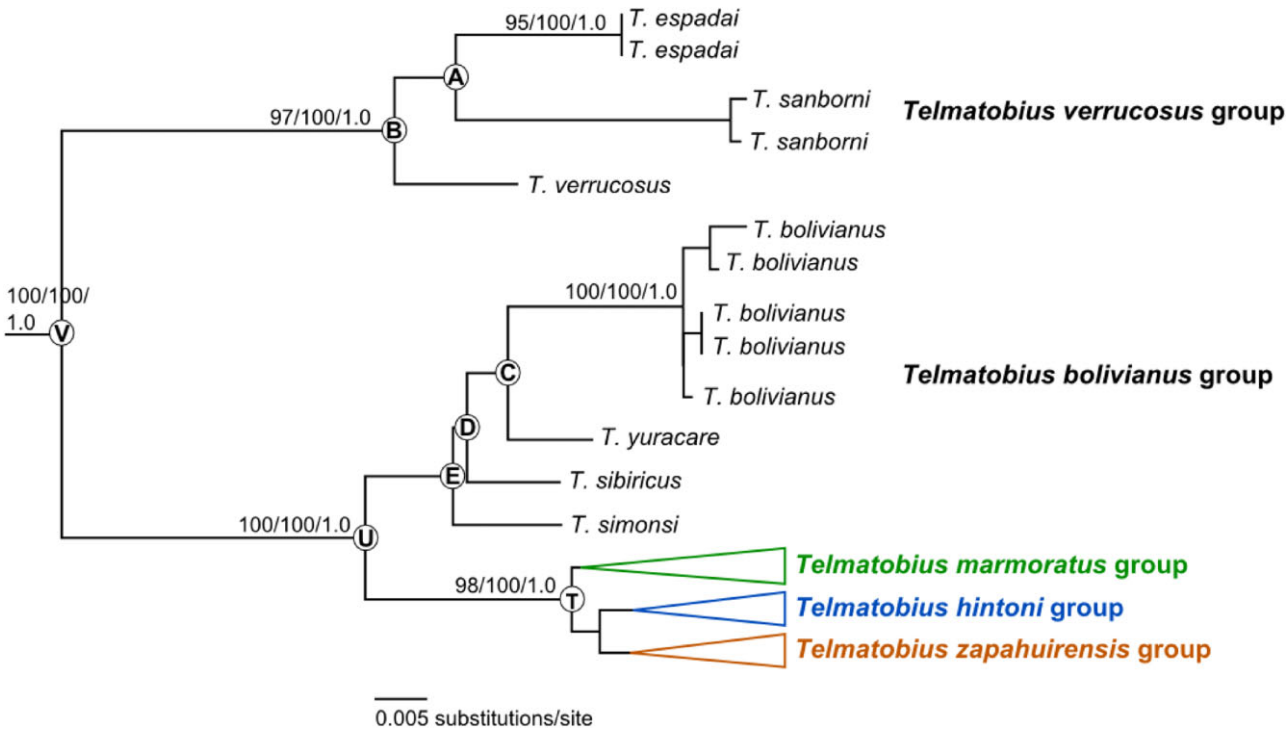
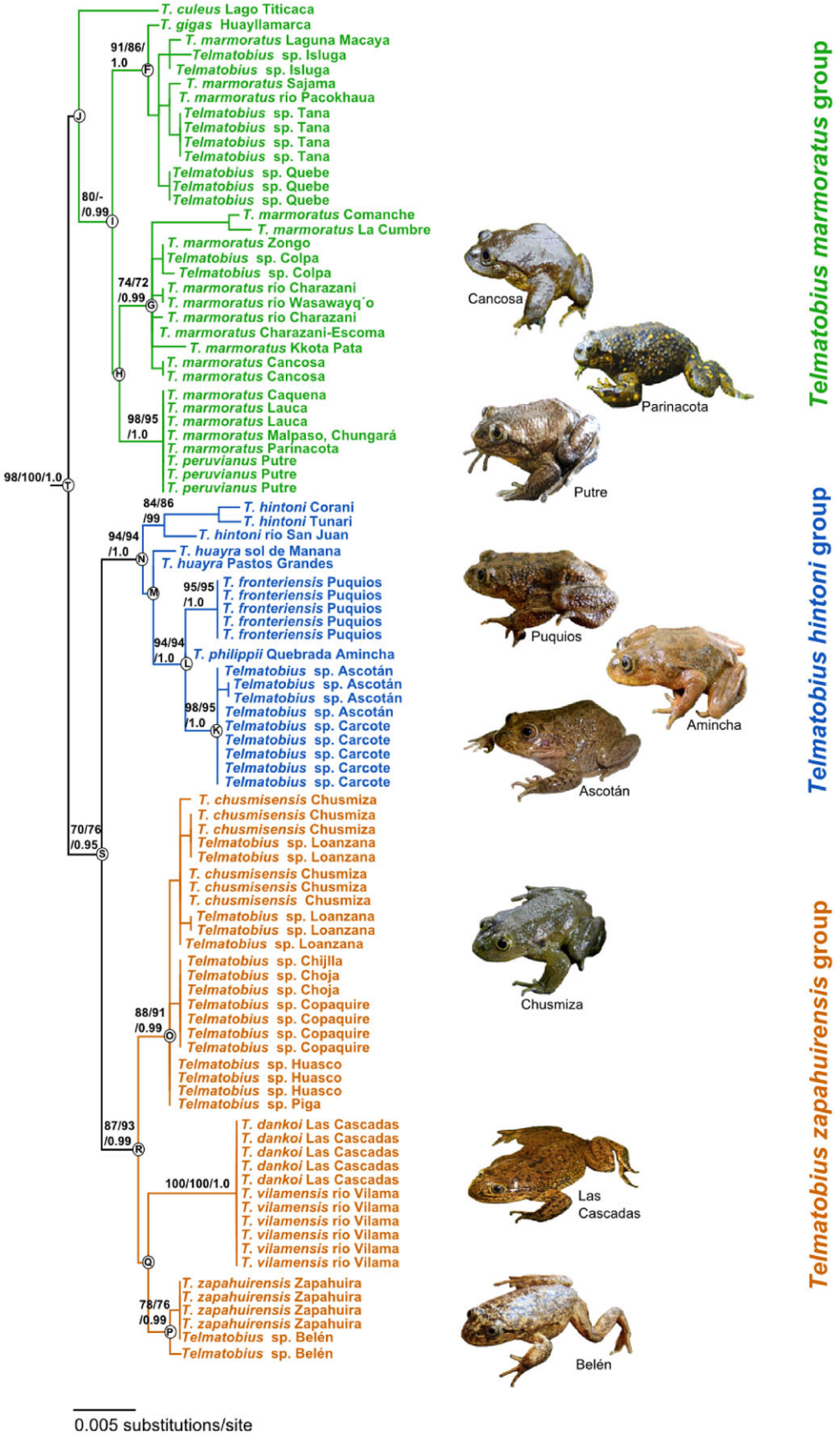


Figure 2. Maximum likelihood tree (-ln likelihood 3667.32) obtained using the concatenated *16S* and *cytochrome b* mitochondrial markers of 19 nominal taxa of *Telmatobius* in Chile and Bolivia and 12 undescribed Chilean localities. Values above the nodes are, from left to right, the bootstrap values of maximum likelihood, maximum parsimony (above 70%), and Bayesian inference (above 0.95). Capital letters in the nodes correspond to the letters in Table 3.

Figure 3. Maximum likelihood tree (-ln likelihood 3667.32) obtained from the concatenated mitochondrial *16S* and *cytochrome b* markers of the *Telmatobius marmoratus*, *Telmatobius hintoni*, and *Telmatobius zapahuirensis* groups. Values above the nodes are, from left to right, the bootstrap values of maximum likelihood, maximum parsimony (above 70%), and Bayesian inference (above 0.95).

congruent; these are shown in Figures 2 and 3. These results indicate that the 19 described species of the genus *Telmatobius* included in this study belong to two main clades: a smaller clade formed by the Bolivian *T. verrucosus* group (which also includes *Telmatobius espadai* and *Telmatobius sanborni*; Node B, Fig. 2) recognized by De la Riva *et al*. (2010) and a larger clade that groups the remaining 16 species (Node U, Fig. 2). This latter clade contains two subclades; the first includes four species endemic to Bolivia that compose the *T. bolivianus* group (*sensu* De la Riva *et al*., 2010; Node E, Fig. 2), whereas the second is composed of the species from the Altiplano and the western slopes of the Andes (Node T, Fig. 2).

Within the clade that groups the Altiplano species there are three main groups: the *T. marmoratus* group (Node J, Fig. 3), the *T. hintoni* group (Node N, Fig. 3), and a new group that includes the species associated with the western slopes of the Andes that we tentatively designated as the *T. zapahuirensis* group (Node R, Fig. 3). The *T. marmoratus* group is formed by the *T. marmoratus* complex (Node I, Fig. 3) and *Telmatobius culeus*, although this relationship was poorly supported by the bootstrap values (Node J, Fig. 3). Within the *T. marmoratus* complex three subclades well supported by bootstrap values and Bayesian posterior probabilities were formed, in which all the populations traditionally recognized in Chile as *T. marmoratus* (Caquena, Chungará, Lauca, Parinacota, but not Cancosa) constitute one of the subclades together with *Telmatobius peruvianus* from the locality of Putre (Fig. 3; *T. marmoratus* group I). The second subclade of the *T. marmoratus* complex was formed by the Bolivian populations of the Departamento de La Paz (Comanche, Cumbre, Zongo, Charazani, Wasawayq'o, Kkota Pata) and by the Chilean populations of Cancosa and Colpa (Node G, Fig. 3) (*T. marmoratus* group II). The Bolivian populations from Parque Nacional Sajama



(Sajama, Laguna Macaya, and Río Pacokhaua) formed a monophyletic group together with the Chilean populations from Isluga, Quebrada Tana, and Quebe and with *T. gigas* from Huayllamarca, Bolivia (Node F, Fig. 3) (*T. marmoratus* group III).

The species of the *T. zapahuirensis* group formed a reciprocally monophyletic group with the *T. hintoni* group with high node supports (Node S, Fig. 3). The *T. hintoni* group (Node N, Fig. 3) also included the species *Telmatobius huayra* and the well-supported Chilean group formed by *Telmatobius philippii*, *T. fronteriensis*, and *Telmatobius* sp. from the salt pans of Carcote and Ascotán (Node L, Fig. 3). We also found that *T. fronteriensis* and *Telmatobius* sp. from Carcote and Ascotán formed a robust monophyletic group whose relationships were not resolved (Fig. 3). The *T. zapahuirensis* group (Node R, Fig. 3) described here is composed of the Chilean species *T. zapahuirensis*, *Telmatobius dankoi*, *T. vilamensis*, and *T. chusmisensis* and has three subgroups with high node support values. The first subgroup includes *T. chusmisensis* and *Telmatobius* sp. from the localities Loanzana, Quebrada Choja, Quebrada Chijlla, Copaquire, Huasco, and Piga (Node O, Fig. 3). The second subgroup includes *T. zapahuirensis* plus *Telmatobius* sp. from the locality of Belén (Node P, Fig. 3). This clade may be the sister group of *T. dankoi–T. vilamensis*; however, this relationship was not poorly supported (Node Q, Fig. 3). The species that form the third subgroup, *T. dankoi* and *T. vilamensis*, formed a monophyletic group without resolution between them (Fig. 3).

Genetic divergence

The genetic divergences between *Telmatobius* species are detailed in Table 2. The *T. marmoratus* groups I, II, and III in Table 2 correspond to subgroups recovered in the phylogeny (discussed above). We considered 1% divergence as an indicator of different species of *Telmatobius* because *T. zapahuirensis* and *T. chusmisensis* correspond to closely related species with allopatric geographical distribution and well-defined diagnostic characters. According to this criterion, *T. huayra*, *T. fronteriensis*, *T. philippii*, and *Telmatobius* sp. from Carcote and Ascotán should be conspecifics, as well as *T. marmoratus* III and *T. gigas*.

Divergence times

The model that fitted the data best was the Birth and Death speciation process under a relaxed molecular clock model with noncorrelated exponential distribution (Table S1). This model is more realistic than the model that assumes only birth (i.e. Yule), given that it considers that the sample of taxa is incomplete because taxa have been lost by extinction during the evolutionary process (Yang & Rannala, 1997; Nee, 2006).

The estimations of divergence times of the species of *Telmatobius* show that the separation between the *T. verrucosus* group and the rest of the species included in the study occurred in the late Miocene, about 9.8 Mya (Node V, Table 3). The separation of the *T. bolivianus* group and the Altiplano and western slopes of the Andes species would have occurred in the Pliocene, about 4.9 Mya (Node U, Table 3), whereas the separation of the two lineages of the Altiplano and western slopes (*T. marmoratus*, *T. hintoni*, and *T. zapahuirensis* groups) occurred about 1.9 Mya (Node T, Table 3). The separation between the *T. hintoni* group and the species of the Chilean western slopes (*T. zapahuirensis* group) would have occurred about 1.4 Mya (Node S, Table 3). However, these results should be taken with caution because the mutation rate used for the *Cytb* gene was calculated for species of the order Caudata (Amphibia) (Mueller, 2006).

DISCUSSION

Phylogenetic considerations

The phylogenetic relationships of the Bolivian *T. verrucosus* and *T. bolivianus* groups recovered here are consistent with the phylogeny proposed by De la Riva *et al*. (2010). Our results show that the Chilean species of *Telmatobius* belong to the Altiplano groups *T. marmoratus* and *T. hintoni* and to the *T. zapahuirensis* group, which is distributed exclusively in the western slopes of the Andes. This pattern is concordant with the proposal of De la Riva *et al*. (2010) for the Altiplano groups. Barrionuevo (2013) recently suggested that the Altiplano species form a group that differs in osteology from the forest and inter-Andean species of the genus. Thus, morphological evidence in this group also supports the genetic divergence of the Altiplano species.

The *T. marmoratus* complex in the Chilean–Bolivian Altiplano appears to be composed of three subclades, in which the Bolivian populations from Parque Nacional Sajama and the Chilean populations of Isluga, Quebrada Tana, and Quebe would be closely related to *T. gigas*. In spite of this, there are no records of large females for the Chilean species of this group, which is one of the principal features of *T. gigas* (109 mm; De la Riva, 2002). As neither De la Riva *et al*. (2010) nor our study recovered a reciprocal monophyletic relationship between *T. gigas* and *T. marmoratus*, the taxonomic validity and the presence of *T. gigas* in Chile are doubtful. The Chilean populations from Parque Nacional Lauca (Parinacota, Lauca, and Chungará) constitute a monophyletic group with *T. peruvianus* of Putre (Fig. 3).

Table 2. Per cent divergence between the sequences of the mtDNA *cytochrome b* gene of *Telmatobius* in Chile and Bolivia using the Kimura two-parameter model

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Telmatobius verrucosus group																				
1 *Telmatobius espadai*	–																			
2 *Telmatobius sanborni*	5.7	–																		
3 *T. verrucosus*	5.2	5.3	–																	
Telmatobius bolivianus group																				
4 *T. bolivianus*	9.5	10.5	9.9	–																
5 *Telmatobius sibiricus*	10.3	10.7	10.0	4.1	–															
6 *Telmatobius simonsi*	9.7	10.7	9.8	4.5	3.0	–														
7 *Telmatobius yuracare*	9.0	9.3	9.0	3.4	2.8	3.2	–													
Telmatobius marmoratus group																				
8 *Telmatobius culeus*	11.3	12.2	11.2	6.8	5.5	5.7	6.0	–												
9 *Telmatobius gigas*	10.8	12.0	11.3	7.0	5.7	5.5	6.2	1.8	–											
10 *T. marmoratus* I	10.8	11.7	10.7	6.7	5.7	5.5	6.2	1.8	1.0	–										
11 *T. marmoratus* II	11.1	12.2	11.4	7.0	5.7	5.9	6.5	1.9	1.4	1.1	–									
12 *T. marmoratus* III	10.9	12.1	11.4	7.1	6.0	5.5	6.2	2.1	0.4	1.4	1.6	–								
Telmatobius hintoni group																				
13 *T. hintoni*	11.7	12.5	11.8	7.1	6.2	6.5	7.1	3.3	3.4	3.1	3.5	3.6	–							
14 *Telmatobius huayra*	11.0	11.8	11.2	6.8	5.8	6.0	6.3	2.3	2.5	2.2	2.6	2.8	1.3	–						
15 *Telmatobius fronteriensis*	10.8	11.5	11.7	7.0	6.3	6.5	6.5	2.8	3.0	2.7	3.0	3.3	1.8	0.8	–					
16 *Telmatobius philippii*	11.0	11.7	11.5	6.8	6.2	6.3	6.3	2.7	2.8	2.5	2.8	3.1	1.6	0.7	0.2	–				
17 *Telmatobius* sp. Asc./Car.	11.2	11.9	11.7	7.0	6.4	6.5	6.5	2.8	3.0	2.7	3.0	3.2	1.8	0.9	0.4	0.2	–			
Telmatobius zapahuirensis group																				
18 *Telmatobius chusmisensis**	11.1	11.6	10.9	6.9	5.4	5.9	6.6	2.6	2.8	2.8	3.1	3.0	2.7	1.9	2.4	2.3	2.4	–		
19 *Telmatobius dankoi* / *Telmatobius vilamensis*	11.3	12.0	11.2	8.0	6.5	7.0	7.0	3.3	3.5	3.5	3.8	3.7	3.6	2.7	3.2	3.0	3.1	2.0	–	
20 *T. zapahuirensis*†	11.5	12.0	11.3	7.0	5.5	5.9	6.5	2.6	2.8	2.8	3.1	3.1	2.7	2.0	2.5	2.3	2.4	1.0	1.6	–

*Including populations from Loanzana, Chijlla, Choja, Copaquire, Huasco, and Piga.
†Including population from Belén.
Asc., Ascotán; Car., Carcote.

Table 3. Estimated divergence times between the species of *Telmatobius*. We used a mutation rate of 2% for the *cytochrome b* gene. Letters indicate the names of the nodes in Figures 2 and 3

Node	Mean (Mya)	Standard deviation	95% HPD superior	95% HPD inferior
A	2.673	3.366E-02	5.410	0.656
B	2.923	3.342E-02	5.729	0.794
C	3.260	5.668E-02	6.760	0.686
D	3.406	5.544E-02	6.862	0.909
E	3.424	5.473E-02	6.854	0.909
F	0.394	1.823E-03	0.726	0.133
G	0.425	2.531E-03	0.754	0.163
H	0.766	4.038E-03	1.325	0.307
I	0.920	4.526E-03	1.527	0.423
J	1.476	8.857E-03	2.544	0.618
K	0.127	6.999E-04	0.252	0.030
L	0.316	1.646E-03	0.582	0.107
M	0.679	3.036E-03	1.185	0.253
N	0.771	3.751E-03	1.281	0.330
O	0.340	1.576E-03	0.626	0.112
P	0.161	9.091E-04	0.366	0.020
Q	0.647	3.401E-03	1.168	0.229
R	0.809	3.954E-03	1.379	0.356
S	1.408	7.982E-03	2.361	0.673
T	1.997	1.334E-02	3.248	0.997
U	4.935	4.351E-02	8.503	2.213
V	9.880	0.1079	17.665	4.165

HPD, high posterior density.

This result differs from that reported by De la Riva *et al*. (2010), who suggested that these populations would be more closely related to *T. gigas* than to *T. marmoratus*. However, the individuals of Putre, which were assigned to *T. peruvianus* by Schmidt (1928), were not compared with the type series; this author suggested that these individuals 'might be a different species than *T. peruvianus*'. Thus, it is probable that the individuals captured in this locality were classified incorrectly, generating a taxonomic mistake that was repeated in later studies (e.g. Veloso *et al*., 1982). Nevertheless, the nominal taxa represent distinct morphological forms (Lynch, 1971), whose conspecificity has not been doubted previously. This reveals the need to re-evaluate the taxonomic status of the Chilean populations of *T. peruvianus*, comparing with the type material or at least with individuals from the same watershed as the holotype (valley of Río Caplina, Tacna, Peru; Schmidt, 1928). The third subclade of the *T. marmoratus* group would be formed by the Bolivian populations of the Departamento de La Paz and by the Chilean populations of Cancosa and Colpa (Fig. 3). These results would increase the distribution of the *T. marmoratus* complex to nine localities of the Chilean Altiplano (including Putre) and one locality of the western slopes (Quebrada Tana), which would be the lowest locality known for this region (1866 m a.s.l.). It is possible that the individuals found in Quebrada Tana come from populations present at greater altitudes in the Parque Nacional Isluga that are part of the same watershed. This may be facilitated by the rapid increase in precipitation during the Altiplano rainy season and/or occasional inundations that reach the eastern edge of the Atacama Desert (Nester *et al*., 2007). It is also interesting that the genetic divergences observed between these three clades were similar to those found between different species with allopatric distributions (e.g. *T. dankoi–T. vilamensis* vs. *T. zapahuirensis*). Thus, both *T. gigas* and the *T. marmoratus* complex should be evaluated using other lines of evidence (e.g. nuclear markers and karyotypes) to validate their taxonomic status, as according to the evidence presented here and that of De La Riva *et al*. (2010) it is possible that the *T. marmoratus* complex is formed by more than one species.

Our phylogenetic hypothesis suggests a common origin of the Altiplano species of the *T. hintoni* group and the western slopes species of the *T. zapahuirensis* group (Fig. 3). *Telmatobius hintoni* would be an entity genetically differentiated from the rest of the species of this group, whereas *T. huayra* showed little divergence from the Chilean species of the group (Table 2). Our analyses also show greater genetic variation amongst the individuals of *T. hintoni* than amongst the

analysed samples of *T. fronteriensis*, *T. philippii*, and *Telmatobius* sp. from Carcote and Ascotán (Fig. 3). The Chilean species of the *T. hintoni* group and *T. huayra* inhabit desert environments of the south-west part of the Altiplano, whereas *T. hintoni* inhabits the Altiplano and the dry intermountain valleys of central Bolivia and whose distribution is relatively distant from the rest of the species of this group. In particular, these species of *Telmatobius* of the Chile–Bolivia border are geographically close, have relatively similar morphology (rounded head, flared lips, copper-orange colour on the belly, and ventral surfaces of limbs), and the genetic divergence observed amongst the individuals of these localities was small; thus, studies with other types of characters are required to re-evaluate their taxonomic status.

The phylogenetic evidence suggests that the populations of Loanzana, Quebrada Chijlla, Quebrada Choja, Copaquire, Huasco, and Piga all belong to *T. chusmisensis*, which would increase the distribution range of this species, known up to now only from the type locality (Chusmiza; 19°41′S, 69°13′W). Furthermore, the individuals from Zapahuira (type locality of *T. zapahuirensis*) and Belén correspond to the same species. Additionally, *T. dankoi* and *T. vilamensis* showed identical sequences, which is coincident with the morphological similarity of these two species. The similarity between *T. dankoi* and *T. vilamensis* occurs even in some diagnostic characters: both are medium-sized, have well-developed postfemoral folds, lack of vomers, maxillary and premaxillary teeth. and tadpoles with dark coloration on distal extreme of tail (see Formas *et al.,* 1999, 2003). Despite differences in the degree of ossification of the cranium, other differences between these species correspond to traits that show variation within a species (e.g. skin texture; see Barrionuevo & Baldo, 2009; Sinsch & Lehr, 2010; Barrionuevo, 2013). According to these antecedents, we suggest that *T. dankoi* and *T. vilamensis* correspond to the same species.

In this study we observed incongruences between the nominal species and the phylogenetic evidence. Although this is a plausible result, given that different characters may diverge in different moments during the process of evolutionary change (De Queiroz, 2007), it must be considered that the taxonomy of the genus *Telmatobius* is difficult. This is due mainly to the fact that the level of intraspecific variation in morphological species has not been established (Barrionuevo, 2013), and thus the delimitation of the species of *Telmatobius* based on these characters is problematic (De la Riva *et al*., 2010). Therefore it is necessary to re-evaluate the taxonomy of the Chilean species integrating new lines of evidence, for instance using an approximation known as integrative taxonomy (e.g. Dayrat, 2005; Padial *et al*., 2010; Puillandre *et al*., 2012).

Biogeographical scenario of divergence amongst *Telmatobius*

The divergence time estimated for the separation of the *T. verrucosus* group and the other species of *Telmatobius* included in this study is congruent with that of De la Riva *et al*. (2010), who proposed an ancient divergence between these groups and supported the forest origin of the Altiplano species proposed by Duellman (1979), Cei (1986), and Lynch (1986). Our results also show that the species of the *T. marmoratus* and *T. hintoni* Altiplano groups would have originated during the Pleistocene, corroborating the suggestion of De la Riva *et al*. (2010). The species of the *T. zapahuirensis* group from the western slopes showed a temporal origin similar to the Altiplano groups, which would indicate that the differentiation processes of *Telmatobius* in the region occurred more or less simultaneously. Although the calibration method used in this study should be considered with caution for the estimation of divergence times amongst species, studies performed in the same area as our study on other taxa have also shown a Pleistocene origin of the species that inhabit the Altiplano. This is the case for snails of the genera *Heleobia* (0.8–0.28 Mya; Kroll *et al*., 2012) and *Biomphalaria* (0.84–0.28 Mya; Collado *et al*., 2011), and fishes of the genus *Orestias* (0.88–0.37 Mya; Lüssen, Falk & Villwok, 2003; Vila *et al.,* 2013). Thus, although these taxa show different biogeographical histories to *Telmatobius*, they suggest that species of the Altiplano would have diversified at about the same time.

Vicariance has been proposed as the main process that generated the diversification of the fauna in the Altiplano region (e.g. Cei, 1986; Northcote, 2000; Lüssen *et al*., 2003; Vila *et al*., 2010, 2013; Collado *et al*., 2011), probably stimulated by processes such as the elevation of the central Andes (Gregory-Wodzicki, 2000), climatic cycles in the last 0.9 Myr (Potts & Behrensmeyer, 1992), intense volcanic activity (Babeyko *et al*., 2002; Schmitt *et al*., 2002), and multiple cycles of palaeolakes that are thought to have occurred between 1.6 Mya (Mataro formation; Lavenú, 1995) and 13–11 Kya (formation of Coipasa; Placzek, Quade & Patchett, 2011). Comparing our results in *Telmatobius* with codistributed populations of fishes of the genus *Orestias* we observed a similar topological pattern (Vila *et al*., 2013), suggesting that a similar vicariant speciation pattern also occurred in *Telmatobius*.

At a global level, freshwater species are considered to be amongst the most threatened (Ricciardi & Rasmussen, 1999; Saunders, Meeuwig & Vincent, 2002). One of the main risks for species that inhabit these ecosystems in Chile is the decrease in the levels of water and the loss of aquatic systems, which is intensified by the growing pressure exerted on these systems by mining activities (Keller & Soto, 1998; Vila, 2006; Vila

et al., 2007; Morales, Vila & Poulin, 2011). In spite of this imminent threat, the conservation status of the majority of the Chilean species of *Telmatobius* has not been evaluated, placing them in the category of 'Data Deficient' because of the lack of information on their distribution and abundance of their populations. This study has revealed that the distribution of the Chilean species is greater than previously known. However, in the Altiplano and western Chilean slopes there are still unexplored areas where undescribed *Telmatobius* populations may exist. Therefore, further efforts are required for a better understanding of the diversity and evolution of these amphibians in the Andes.

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SUPPORTING INFORMATION

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

Table S1. Bayes Factor ($\log_{10}$) of the speciation and molecular clock models evaluated. Positive values indicate better fit of the model in the row compared with that in the column, and conversely. S.D., standard deviation.