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Para: DS Lista Sitios; Marco Mendez; Gabriel Lobos; Nicolas Rebolledo; Pablo Fibla
Asunto: Información sitio SP2-119 Rinconada de Caquena

Estimados,

A través de este correo electrónico, enviamos antecedentes del sitio:

- SP2-119 Rinconada de Caquena

Esperamos que estos antecedentes sean de utilidad para la valoración y protección de este sitio. Quedamos atentos a aportar en lo que estimen necesario.

Saludos cordiales,
Paola A. Sáez
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Phylogeography of high Andean killifishes *Orestias* (Teleostei: Cyprinodontidae) in Caquena and Lauca sub-basins of the Altiplano (Chile): mitochondrial and nuclear analysis of an endangered fish

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ABSTRACT

From the early Miocene, the uplift of the Andes Mountains, intense volcanic activity and the occurrence of successive periods of dryness and humidity would have differentially influenced the modification of Altiplano watersheds, and consequently the evolutionary history of the taxa that live there. We analyzed *Orestias* populations from the Caquena and Lauca Altiplanic sub-basins of northern Chile to determine their genetic differentiation and relationship to their geographical distribution using mitochondrial (D-loop) and nuclear (microsatellite) molecular markers and to reconstruct its biogeographic history on these sub-basins. The results allowed reconstructing and reevaluating the evolutionary history of the genus in the area; genic diversity and differentiation together with different founding genetic groups suggest that *Orestias* have been spread homogeneously in the study area and would have experienced local disturbances that promoted isolation and diversification in restricted zones of their distribution.

Subjects Biogeography, Genetics, Molecular Biology, Zoology, Freshwater Biology

Keywords *Orestias*, Killifishes, Phylogeography, Altiplano, D-loop, Microsatellite, Fish, Endangered, Freshwater ecosystem

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INTRODUCTION

Mitochondrial and nuclear markers analyzed in a geographic context have been used to elucidate the relationship between geological events, species distribution and driving speciation mechanisms, as well as to infer the evolutionary history of populations, deepening the knowledge generated by phylogeography since it was pointed out by Avise in 1987 (*Avise, 1998*; *Hickerson et al., 2010*). In addition, this type of analysis can be used to know and explain the structure and genetic diversity of populations in a more efficient way (*Yi et al., 2020*). The information that they can generate is useful when making conservation decisions around those populations where the delimitation of species has not been well

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defined considering that the diversification of species can be explained by population-level evolutionary processes and making inferences from geographic distributions of genealogical lineages (*Katongo et al., 2005*; *Rissler, 2016*). Also, considering small and isolated populations that face a high degree of threat such as loss and fragmentation of habitat, contamination or introduction of invasive species and whose small population sizes make them more likely to generate inbreeding, loss of genetic diversity and therefore the decrease in their ability to adapt to future changes (*Frankham, 2005*; *Rubinoff et al., 2020*), as those freshwater fishes that inhabit environments prone to desiccation.

Phylogeographic studies in freshwater fishes have shown that they have a marked phylogeographic structure that is strongly linked to historical changes in the ecology and geology of the systems they inhabit and can be a good study model to analyze speciation processes associated with divergence adaptive and reproductive isolation (*Beheregaray, 2008*). In addition, it is known that some groups of organisms have an unusually high rate of speciation, reason why they are ideal for studying genetic differentiation processes in shorter times (*Kornfield & Smith, 2000*; *Salzburger et al., 2005*; *Mehner et al., 2010*; *Bezault, Mwaiko & Seehausen, 2011*; *Marques, Meier & Seehausen, 2019*).

"Killifishes" are Cyprinodontiforms (*Berg, 1940*), freshwater fishes that have been widely studied due to their morphological specialization, life history traits and geographic distribution that includes America, the Mediterranean, Southeast Asia and Africa (*Parker & Kornfield, 1995*; *Capobianco & Friedman, 2018*). The monophyly of the Cyprinodontiform group and its subdivision into suborders Aplocheiloidei and Cyprinodontoidei has been supported by morphological and genetic studies (*Parenti, 1981*; *Setiamarga et al., 2008*; *Pohl et al., 2015*), however, genealogical relationships within each of these groups remain controversial since their fossil record is still scarce; it has been postulated that their present continental radiation has been mediated by a large-scale vicariance or dispersion radiation (*Capobianco & Friedman, 2018*). The current presence of this group on both sides of the Atlantic Ocean can be explained by their particular physiology, life history and behavior, which have allowed them to explore habitats with ephemeral waters and adapt to them (*Parenti, 1981*; *Capobianco & Friedman, 2018*; *Vrtílek et al., 2018*). In the family Cyprindontidae (Cyprinodontoidei) the genera *Orestias* (*Parenti, 1984a*) and *Pseudorestias* (*Arratia et al., 2017*) inhabit exclusively aquatic systems in the Andes Highlands, including the extensive inter-Andean plain or Altiplano between 15–27°S whose average altitude exceeds 3,000 m (*Isacks, 1988*; *Muñoz & Charrier, 1996*). The aquatic systems of the Altiplano and their associated biota are exposed to extreme environmental conditions including altitude, wide ranges of salinity, radiation, wind and high daily temperature variation. In these systems there are places where surface waters freeze at night and thaw during the day. There is a negative hydrological regime with well-defined wet (January–March) and dry seasons (April to December) that constantly modulate the superficial water courses (*Kött, Gaupp & Wörner, 1995*; *Rundel & Palma, 2000*; *Lambrinos, Kleier & Rundell, 2006*; *Márquez-García et al., 2009*; *Demergasso et al., 2010*; *Scott, 2010*; *Vila et al., 2013*; *Scott et al., 2015*).

Geomorphologic and climatic changes occurred during the Miocene and more strongly in the Plio-Pleistocene (~5–2 million years ago or MyBP) with the rise of the Andes

Range and the major elevation of the Altiplano, produced significant disturbances in South American hydrography; only those taxa which adapted to these variations survived. It is believed that the *Orestias* cyprinodontoid ancestor colonized northern South America 80–100 MyBP (late Jurassic-Cretaceous) and reached the territory we know today as the Altiplano before its uplift. Survival and dispersion after the uplift would have been favored given its tolerance to environmental conditions such as altitude and salinity. The existence of large water bodies that were progressively subdivided into several paleo-lakes facilitated the disaggregation and reproductive isolation of the ancestral population (*Villwock, 1983*) resulting in new genetic diversity. Lake Titicaca, today one of the largest lakes in South America, has been postulated as the center of radiation of the genus, mainly due to its effluent Desaguadero River that flows south to Lake Poopó (*Villwock, 1983*; *Lüssen, Falk & Villwock, 2003*; *Scott, 2010*; *Vila et al., 2013*).

Although different approaches have been used to obtain information about genetic, ecological and evolutionary characteristics of *Orestias* populations (*Lüssen, Falk & Villwock, 2003*; *Vila, Pardo & Scott, 2007*; *Maldonado et al., 2009*; *Peña, 2010*; *Esquer-Garrigos et al., 2011*; *Morales, Vila & Poulin, 2011*; *Vila et al., 2013*; *Cruz-Jofré et al., 2014*; *Cruz-Jofré et al., 2016*; *Esquer-Garrigos et al., 2015*; *Guerrero-Jiménez et al., 2015*; *Arratia et al., 2017*; *Morales, 2018*), aspects like its evolutionary history and biogeography are still poorly understood.

Orestias has been described as a species flock (*Parenti, 1984b*; *Northcote, 2000*; *Esquer-Garrigos, 2013*) and so far, 47 species have been described in the highland systems of Peru, Bolivia and Chile (*Fricke, Eschmeyer & Fong, 2021*); seven of them inhabit the Chilean Altiplano between 17–27°S (*Arratia et al., 2017*) whose conservation status on the Red List of Threatened Species of the International Union for Conservation of Nature and Natural Resources (IUCN) includes "data deficient", "vulnerable" and "near threatened" (*Esmaeili, Asrar & Gholamifard, 2018*). Other authors have indicated them as "endangered" given the fragility of the ecosystems that inhabit and the threats to which they are exposed (*Vila, Pardo & Scott, 2007*; *Vila et al., 2007*). In Chile, the Altiplano basins are subdivided into sub-basins depending on the tributaries that carry out the drainage towards the main river course (*Dirección General De Aguas (DGA), 2014*). In this study we examined the Caquena and Lauca sub-basins, which are the nearest area to Lake Titicaca in the Chilean Altiplano, which is why their *Orestias* populations have special relevance. Studies on the gastropod Biomphalaria which co-inhabits with *Orestias* indicated differentiation between populations of the Lauca and Caquena sub-basins, each belonging to a different lineage with a divergence time of 0.68–0.34 million years. Recent isolation processes of aquatic systems would have occurred within Caquena compared to those within the Lauca sub-basin (*Collado, Vila & Méndez, 2011*; *Collado, Salinas & Méndez, 2014*). A phylogeny of *Orestias* populations of Chilean species (*Vila et al., 2013*) constructed from mitochondrial DNA showed four differentiated clades which are spatially segregated, suggesting a pattern consistent with a differentiation process by vicariance and found that individuals belonging to the locality of Umaqui (Caquena sub-basin) appear in a separate clade along with those of southern sub-basins of the Chilean Altiplano, however, there is no evidence about the processes of genetic differentiation of the genus in this sub-basin. All

species described in Lauca sub-basin are grouped in one clade. However, the study of *Peña (2010)* in Lauca National Park (LNP) highlighted the genetic coherence (mitochondrial markers) between specimens from the northern and middle regions of the sub-basin, suggesting divergence from the southeastern region. In addition, *Guerrero-Jiménez et al. (2017)*, using mitochondrial (D-loop) and nuclear DNA (8 microsatellites) found that the genetic patterns of differentiation would correspond to an incipient speciation in allopatry due to the signs of population expansion and the mixed genetic patterns. Their results were associated with the processes of fragmentation of the systems in the sub-basin during the Pleistocene-Holocene, whose main detonator would have been the collapse of Parinacota volcano's old cone 12,500 years before present (hereinafter yBP), dating that has been adjusted to 8,800 yBP (*Jicha et al., 2015*), which would have caused a significant transformation in the landscape, changed the direction of the main river course and produced three systems that remain until today: Lake Chungará, Cotacotani Lagoons and the Lauca River (*Sáez et al., 2007*; *Giralt et al., 2008*).

The Caquena and Lauca sub-basins are separated by a volcanic cord of Pleistocene origin whose summits rise above 4,000 m; both being part of larger basins shared with the countries of Bolivia and Peru and both lithological covers have been described as Tertiary superior to Quaternary origin (*Niemeyer, 1982*), but they have different characteristics despite their geographical proximity. The area of Caquena sub-basin is 1,268 Km^2; it is limited by the slopes of adjacent terraces, has an average elevation of 4,230 m.a.s.l. and is widely covered by wetlands (bofedales); the Caquena River course goes from south to north. Lauca sub-basin has a surface of 2,406 Km^2 with an average elevation of 4,295 m, has varied types of aquatic systems including lakes, lagoons, and wetlands (bofedales); the Lauca River runs from north to south (*Salazar, 1997*). This area is interesting to analyze evolutionary relations of *Orestias*, especially considering its geographical proximity to the proposed center of radiation of the genus and also, that given its rapid speciation characteristics and to the history of the geological, hydrological and climatic changes that have occurred in the Altiplano since its formation (*Morales, Vila & Poulin, 2011*). Differentiation processes linked to adaptation during short evolutionary periods has been evidenced in fishes of the *Cichlidae* family that inhabit Victoria Lake in Africa, or Apoyeque Lake in Nicaragua where a sympatry process could explain the high diversity of species within the same lake (*Elmer et al., 2010*; *Takuno et al., 2019*). Furthermore, some species of cichlids have shown similar morphological features (*Kocher, 2004*) where has been postulated a radiation of species related to colonization events by different lineages due to allopatry. This vicariant process would be related to substantial changes in the climate and rainfall regimes that would have fragmented the water systems on drought periods. According to this, we consider that the genus *Orestias* represents an excellent model to probe phylogeographic hypothesis on a historically and successively fragmented populations.

Given the high specialization of *Orestias* to the different aquatic systems of the Altiplano and that Caquena and Lauca sub-basins conform differentiated hydrological units, we hypothesized that genetic variation in *Orestias* populations would be reflected in these two units and could be a response to geological events that affected their evolutionary history. Thus, the objective of this study was to analyze the phylogeographic relationships

of *Orestias* populations in the Caquena and Lauca sub-basins using mitochondrial and nuclear molecular markers (mitochondrial D-loop and microsatellites) to provide evidence of its evolutionary history.

MATERIALS & METHODS

Sampling sites

Fish samples were captured from Caquena and Lauca sub-basin, during the years 2016–2017. The Caquena sub-basin area considers three locations: Caquena, Colpa and Umaqui. The southern zone of the Lauca sub-basin considers two locations: Ancuta and Paquisa (GenBank accession numbers BankIt2361457: MW149163–MW149238). To complement the information of the geographical distribution of the genus, data from *Guerrero-Jiménez et al. (2017)* were included (GenBank accession numbers BankIt1931003: KX498091–KX498358) from four representative localities of genetic diversity in northern zone of the Lauca sub-basin: Chuviri, Copapujo, Lauca and Misituni. These locations were selected from the pool of available data after a previous analysis that allowed us to choose those populations that turned out to be more informative for the representation of the genetic diversity of the northern zone of the Lauca sub-basin (data not shown). The geographic data of the locations considered in this work are shown on Fig. 1 and Table 1.

Sampling and preservation of samples

The fish were obtained according to research fishing permit number 1103-2015 of the Chilean Undersecretary of Fisheries (see S1) and were captured with manual fishing nets. The number of samples obtained did not significantly affect the conservation status of the populations. Euthanasia was performed with 100 mgl-1 of tricaine methanesulfonate. They were preserved in 95% ethanol and stored at the Laboratorio de Limnología of Universidad de Chile.

The identification of individuals was carried out through a review of specialized literature and consultation with experts, being classified as *Orestias* sp. for the Caquena sub-basin locations and as *Orestias cf laucaensis* for Ancuta and Paquisa. Regarding the other locations, they had been classified by *Guerrero-Jiménez et al. (2017)* as *Orestias laucaensis* for those individuals from Lauca and Misituni. For those locations of Chuviri and Copapujo, they were identified as *Orestias* sp.

DNA extraction

DNA was extracted from muscle tissue obtained by dissecting the area on the pectoral fin. The muscle is dried and then pulverized, after which it is subjected to the action of proteinase K and is treated with repeated cycles of immersion in saline dilutions and centrifugation according to the procedure indicated in *Aljanabi & Martinez (1997)*. After extraction, the purity and concentration of the DNA samples was determined by spectrophotometry with the Thermo Scientific NanoDropTM 1,000 spectrophotometer.

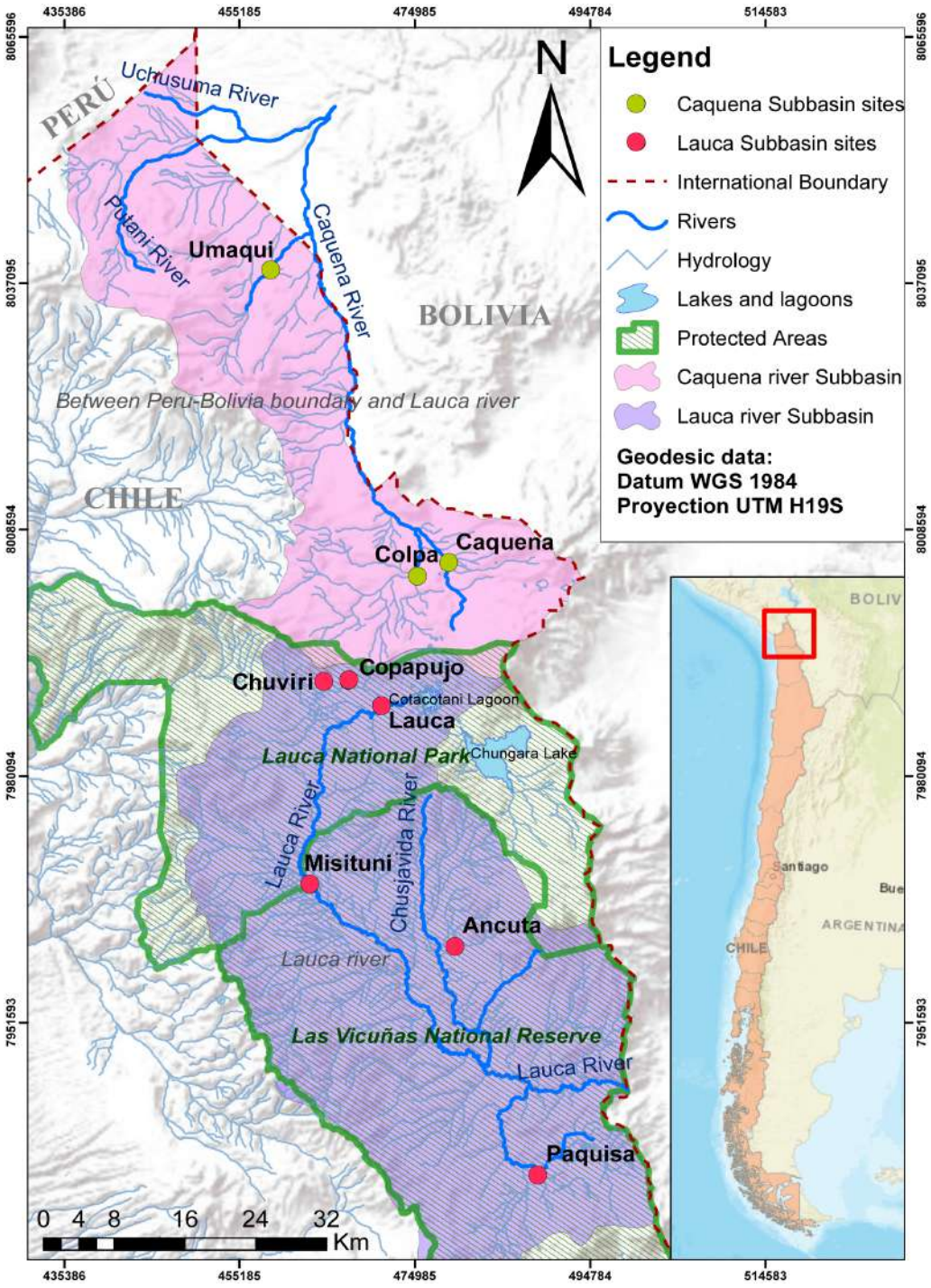

Figure 1 Map of localities of Caquena and Lauca sub-basins.

Full-size DOI: 10.7717/peerj.11917/fig-1

Table 1 Geographic coordinates of the locations studied.

Sub-basin	Location	Abbreviation	Coordinates (°)	
			South	West
Caquena	Umaqui	UMA	17.73926	69.39064
	Caquena	CAQ	18.04527	69.20166
	Colpa	COL	18.05816	69.23445
	Chuviri[a]	CHU	18.16925	69.33481
	Copapujo[a]	COP	18.16925	69.30785
Lauca	Lauca[a]	LAU	18.19379	69.27360
	Misituni[a]	MIS	18.38074	69.34920
	Ancuta	ANC	18.44611	69.19486
	Paquisa	PAQ	18.68510	69.10563

Notes.
[a]Data from *Guerrero-Jiménez et al. (2017)*.

Amplification/genotyping and data analysis

Mitochondrial DNA

The control region of mitochondrial DNA (D-loop) was amplified by PCR using specific primers for the genus: Forward 5′ ACC CCT AAC TCC CAA AGC T 3′; Reverse 5′ TGA TAG TAA AGT CAG GAC CAA 3′ (*Morales, Vila & Poulin, 2011*). The PCR reaction was standardized in a total volume of 25 µl: 2.5 µl 10 X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 2 µl MgCl2 50 mM, 2 µl of each primer 10 pm/µl, 2 µl dNTP 2.5 mM, 0.5 µl Taq (Invitrogen), 9 µl ultrapure water, 1 µl DMSO and 4 µl of 10 ng/µl DNA.

The PCR cycle consisted of an initial denaturation for 4 min at 94 °C, followed by 38 cycles of 45 s at 94 °C, 90 s at 66 °C (65.2 °C for Caquena samples, 63.5 °C for Colpa samples) and 90 s at 72 °C, with a final extension of 10 min at 72 °C. PCR products were visualized in 2% agarose gel stained with SYBR. Amplification products were sent for sequencing to Macrogen Inc. (South Korea). D-loop sequences were aligned, edited and assembled using the Proseq v.2.9.1 program (*Filatov, 2002*) and Bioedit Sequence alignment editor version 7.2.6 software (*Hall, 1999*).

Using the mitochondrial D-loop control region, genetic diversity indices were calculated for each locality, including number of haplotypes (K), number of polymorphic sites (S), haplotype diversity (H), average number of differences between pairs of sequences (Π) and nucleotide diversity (π) in DNA Sequence Polymorphism (DnaSp) software version 5.10.01 (*Librado & Rozas, 2009*).

To estimate the level of genetic differentiation between pairs of locations we used the Φ_{ST} and F_{ST} indices in Arlequin software version 3.5.1 (*Excoffier & Lischer, 2010*). The significance of F_{ST} and Φ_{ST} was tested with 10,000 permutations. To evaluate the existence of phylogeographic structure G_{ST} (genetic differentiation) and N_{ST} (genetic differentiation considered as genetic distance between haplotypes) values were calculated in PERMUT version 2.0 (*Pons & Petit, 1996*; *Burban et al., 1999*) based on the null hypothesis $G_{ST} = N_{ST}$. If N_{ST} is significantly greater than G_{ST} this hypothesis is rejected and suggests the existence of phylogeographic structure. A total of 1,000 permutations to determine significance of this comparison were tested in the same software.

Coalescent analysis was based on single locus; the genealogical relationships between haplotypes were graphed by constructing a haplotype network using the median-joining algorithm implemented in the Network version 4.501 software (*Bandelt, Forster & Röhl, 1999*). To detect possible deviations from mutation-drift equilibrium (under the Wright-Fisher model) that could indicate population expansions or bottlenecks, in each genetic group Tajima and Fu tests were performed under assumption of neutrality in Dnasp version 5.10.01 (*Librado & Rozas, 2009*). Although both account for the occurrence of demographic pressures in the past, their predictive power varies according to the type of event (*Ramírez-Soriano et al., 2008*), so the results of the tests were compared.

For demographic analysis, a mutational rate was estimated. Considering the evidence of "time dependency" of mitochondrial DNA evolutionary rates in freshwater fishes (*Ho et al., 2005*; *Ho & Larson, 2006*; *Burridge et al., 2008*; *González-Wevar et al., 2015*), the substitution rate for the demographic analysis of *Orestias* was estimated using the phylogenetic relationships and divergence times on BEAST software version 1.8.4 (*Drummond et al., 2012*). A Bayesian framework which allowed for variable divergence time estimations among lineages was used and an uncorrelated relaxed clock with a lognormal distribution model was used as prior based on the relevance of evolutionary rates. We used a total of 254 sequences where 61 was from of Chilean *Orestias* phylogeny on *Vila et al. (2013)* with GenBank accession numbers JX134506.1–JX134566.1 and 117 from *Guerrero-Jiménez et al. (2017)* with the GenBank accession numbers KX498242.1–KX498358.1. We added 76 new sequences from this work. Despite not having fossil record, the analysis was calibrated according geological/ hydrological events that modified the aquatic systems of Altiplano (Data S2). We used three different models to evaluate past demographic changes. First, mismatch distribution and demographic expansion values were calculated based on the instant growth model of *Schneider & Excoffier (1999)* available in Arlequin; Tau τ is the time the expansion began measured in mutational steps, θ_0 corresponds to $2N\mu$, Ne0 refers to the theoretical effective population size before expansion and θ_1 is the effective population size after expansion. Second, we reconstructed the demographic history of each population using (LAMARC) version 2.1.9 (*Kuhner, 2006*). This program constructs coalescence trees using maximum likelihood; several population parameters are estimated from these trees. The growth rate is g and the values of θ_0 and θ_t indicate the population sizes at present and at time t, respectively. Finally, we infer demographic history of populations performing a Bayesian Skyline analysis available on BEAST program version 1.10.4 (*Suchard et al., 2018*) with an uncorrelated relaxed clock and a random starting tree. The analysis was performed with MCMC of 10,000,000–2,000,000 iterations depending on the number of sequences analyzed with 10% as a burn-in. The value of the estimated mutational rate was assumed as the "mean" value of the uncorrelated relaxed clock model with a standard deviation of 10% on priors setting. The results were analyzed using Tracer program version 1.7.2 (*Rambaut et al., 2018*) where a Bayesian skyline plot was constructed. Bayesian skyline plots shows changes through the time of the product of effective population size (Ne) and generation time (t).

Table 2 Microsatellite loci characteristics and MIX assigned. Modified from *Esquer-Garrigos et al. (2011)*.

Locus	Primer (5′–3′)	Dye label	Temperature Alignment (°C)	MIX
A9a	F: CAGGAAGGAATCTCAGGAATG R: ACGCACCGTTTCATAGTAAGG	FAM	58	
A106	F: TGGCTGATGGTATTGGTTG R: AGCACACCTTCACAGGATG	VIC	60	
B1	F: TACAAACACATCCATCTCAGTC R: AACACTCCTATCATCCATCATC	PET	58	1
C102	F: TTCCAAACCACATTTTAGATCC R: CAGCCTTTTGATTATGGAGGT	NED	63	
A116	F: TCGCTACTTACTCCGACCTC R: AAATCACAATGGCTTTCTCTG	PET	54	
B104	F: ACCGTAGTTGCCTGGTTACA R: AGGGTGCTGTCAGAGATGAG	VIC	64	
C105	F: AGCAAGACCAGTTTGAAATCT R: GTTGCCCTGCGATGTAC	NED	58	2
D110	F: ATCACAAGACGAGGTTCTCAC R: GATTGGAGCAAGGGACTG	FAM	59	
B103	F: TATTATCCACTCCTGGTCAGTC R: GTTGAAGCGTTTCCAAGAT	FAM	51	3

Nuclear DNA

We amplified eight microsatellite loci, originally optimized in *O. agassii* of Bolivia by *Esquer-Garrigos et al. (2011)* (Table 2). Amplification of the loci was performed in three mixes according to affinity of alignment temperatures, they were MIX1: A106, B1 and C102; MIX2: A116, B104, C105 and D110; MIX3: B103. The PCR reaction volume was standardized at 10 µl: 2 µl 5X PCR Buffer (50 mM KCl, 10 mM Tris-HCL, pH 8.0), 0.9 µl 25 mM MgCl2, 1 µl 0.1 mg/l BSA, 0.5 µl of each 50 pm/µl primer, 0.8 µl 2.5 mM dNTP, 3.2 µl ultrapure water, 0.1 µl Taq (according to MIX number) and 1 µl 50 ng/µl DNA. Multiplex PCR Master Mix (QIAGEN) was used for MIX 1 and 2 and Taq platinum (ThermoFisher Scientific®) was used for MIX 3. The PCR program was different for each MIX used, with annealing temperatures that varied between 51–60 °C (see Table S3). Amplification products were sent for genotyping to Macrogen Inc. (South Korea). The genotyping results were processed using GeneMarker version 2.6.3 program (SoftGenetics). To review and correct the existence of null alleles, stuttering errors and alleles with large peaks, Micro-Checker version 2.2.3 program (*Van Oosterhout et al., 2004*) was used.

Allele frequencies observed, heterozygosity (Ho) and expected heterozygosity (He) were calculated for each locus. F_{IS} was calculated and its significance was tested with 10,000 permutations in Genetix version 4.05.2 (*Belkhir et al., 2004*). To evaluate genetic and phylogeographic structure, F_{ST} between pairs of locations were determined in Arlequin version 3.5.1 (*Excoffier & Lischer, 2010*); their significance was evaluated by 10,000 permutations. A reassignment test was performed in the different genetic groups to assess the agreement between the assignment or exclusion of reference populations as a possible origin of the individuals based on multilocus genotypes (*Paetkaud et al., 1995*)

Table 3 Genetic diversity indexes of D-loop sequences of *Orestias* from studied locations.

Sub-basin	Location	N	S	K [rarefaction]	Hd	∏	π
Caquena	UMA	15	26	8 [6.450+/-0.827]	0.895 +/- 0.053	9.467 +/- 4.604	0.012 +/- 0.006
	CAQ	13	14	5 [4.30+/-0.665]	0.692 +/- 0.119	6.128 +/- 3.118	0.008 +/- 0.004
	COL	10	16	6	0.844 +/- 0.103	6.689 +/- 3.448	0.008 +/- 0.005
Lauca	CHU[a]	23	11	9 [5.552+/-1.087]	0.755	1.621	0.00182
	COP[a]	11	4	4	0.600	1.055	0.00118
	LAU[a]	37	3	4 [1.89+/-0.76]	0.158	0.213	0.00024
	MIS[a]	48	6	7 [2.467+/-0.924]	0.340	0.368	0.00041
	ANC	20	0	1 [1+/-0]	0.000 +/- 0.000	0.000 +/- 0.000	0.000 +/- 0.000
	PAQ	18	11	8 [8+/-0]	0.641 +/- 0.130	1.320 +/- 0.862	0.002 +/- 0.001
TOTAL		195	54	40	0.727	6.285	0.00771

Notes.

N, sample size; S, polymorphic sites; K, haplotype number; [], rarefaction of number of haplotypes; Hd, haplotype diversity; Π, mean number of differences between pairs of sequences; π, nucleotide diver.

[a]Data from *Guerrero-Jiménez et al. (2017)*.

in the Geneclass 2 software (*Piry et al., 2004*). Finally, a Bayesian analysis was performed to infer the number of genetic groups (K) of the individual genotypes in Structure version 2.2 (*Evanno, Regnaut & Goudet, 2005*), using the parameters cited in *Guerrero-Jiménez et al. (2017)*.

RESULTS

Mitochondrial DNA

Genetic diversity

195 sequences of 815 bp in 9 locations were obtained. The genetic diversity indices are shown in Table 3. The Ancuta population showed only one haplotype, with the lowest diversity of all analyzed localities. The Umaqui population presented the highest values of nucleotide and haplotype diversity.

Differentiation among populations and haplotype characterization

F_{ST} and Φ_{ST} analyses (Table 4) show highly significant differentiation ($p < 0.0001$), except for the Lauca-Chuviri and Misituni-Chuviri comparisons, whose values were not significant.

The haplotype network showed an extended pattern with some haplotypes shared between Caquena and LNP populations.

A clear differentiation is observed between the locations of LNP and Ancuta-Paquisa, however, they share a star-like net form (Fig. 2).

As found by *Guerrero-Jiménez et al. (2017)*, the mismatch distribution was unimodal for the LNP localities and also for Paquisa (Fig. 3); this is consistent with the results of the indexes of Tajima and Fu, which were significant and negative for all these locations (Paquisa Fu: -2.14, $p < 0.01$/Tajima: -2.80, $p < 0.02$; LNP Fu: -2.27, $p < 0.01$/Tajima: -4.3, $p < 0.02$), indicating evidence of demographic expansion.

The mismatch distribution for Caquena sub-basin locations was multimodal (Fig. 3), and the Tajima and Fu indexes were positive but not significant (data not shown). It was

Table 4 Genetic differentiation for locations studied based on haplotype differences (F_{ST}) and number of differences between pairs of sequences (Φ_{ST}) based on D-loop marker.

F_{ST}	Caquena sub-basin			Lauca sub-basin					
	UMA	CAQ	COL	CHU	COP	LAU	MIS	ANC	PAQ
UMA	–	***	NS	***	***	***	***	***	***
CAQ	0.20378	–	**	**	**	***	***	***	***
COL	0.06675	0.23559	–	**	***	***	***	***	***
CHU	0.19611	0.19986	0.22456	–	NS	***	***	***	***
COP	0.35877	0.34594	0.41186	0.09172	–	NS	NS	***	***
LAU	0.57288	0.56382	0.64004	0.25104	0.01357	–	NS	***	***
MIS	0.53883	0.51535	0.59371	0.21757	−0.00669	−0.00072	–	***	***
ANC	0.5959	0.71229	0.6884	0.62099	0.8751	0.89908	0.84069	–	***
PAQ	0.23615	0.33546	0.26941	0.31605	0.48565	0.66046	0.62494	0.69262	–
Φ_{ST}									
UMA	–	*	*	***	***	***	***	***	***
CAQ	0.19778	–	NS	***	**	***	***	***	***
COL	0.11547	0.07463	–	***	***	***	***	***	***
CHU	0.64063	0.66901	0.74684	–	NS	***	***	***	***
COP	0.57269	0.61894	0.70361	0.02789	–	NS	NS	***	***
LAU	0.74491	0.78465	0.85076	0.10726	0.04145	–	NS	***	***
MIS	0.7765	0.81096	0.87065	0.11986	0.04306	0.01728	–	***	***
ANC	0.59147	0.70938	0.72001	0.92075	0.97654	0.98285	0.97868	–	***
PAQ	0.50848	0.56525	0.56178	0.88715	0.90679	0.94745	0.9508	0.81725	–

Notes.

Over diagonal is the significance of each comparison

NS non-significant.

* $p < 0.05$.

** $p < 0.001$.

*** $p < 0.0001$.

Line separates the locations of each sub-basin.

not possible to perform this analysis for Ancuta, because only one haplotype was found. Considering this result, the models of demographic history of populations were tested only on those locations where evidence of population expansion was found.

The permutation analysis to evaluate the existence of phylogeographic structure showed a G_{ST} value lower than N_{ST} but it was not significant ($p > 0.05$) therefore it can be inferred that there is no phylogeographic structure between sub-basins.

Demographic analysis

Corresponding to parameters estimated by the demographic analyses performed, the calculation of the expansion time in years was based on numbers of mutational steps with a mutational rate of 3.99% per million years (Data S2).

According to the model of *Schneider & Excoffier (1999)* the time since the expansion for Paquisa is 14,800 yBP, while in the Kuhner model (2006), it corresponds to 9,250 yBP (Table 5). The times estimated since expansion for LNP locations were 5,245y BP and 7,750 yBP respectively.

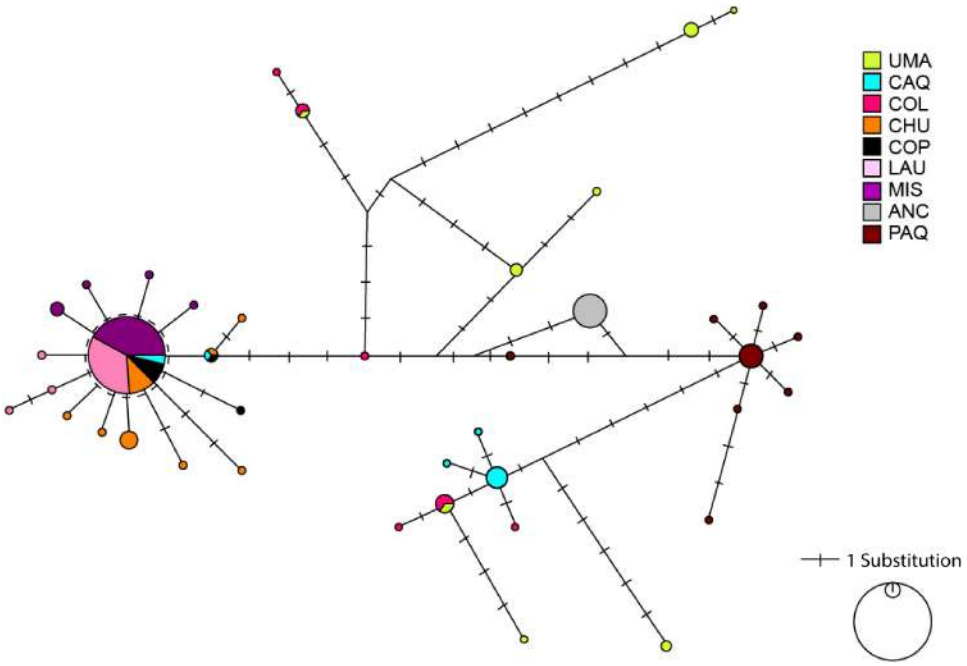

Figure 2 Haplotype network based on a median joining criterion of the studied locations of Lauca and Caquena sub-basins. Circles represent haplotypes, while the length of the main lines is proportional to the number of mutational steps between haplotypes. Short black lines that intercept the main lines correspond to mutational steps between haplotypes.

Full-size DOI: 10.7717/peerj.11917/fig-2

Regarding the Bayesian skyline analysis performed, the mean of the uncorrelated relaxed clock model was, as mentioned, the value of the estimated rate per millions of years ($3.99*10^{-6}$) and the prior distribution was computed as normal with an interval of $3.136*10^{-6}$– $4.664*10^{-6}$. The Bayesian Skyline Plots of populations showed the same patterns found on mismatch distributions. While historical N_{et} of Caquena sub-basin populations Umaqui, Caquena and Colpa were stable over time with signals of population decline towards to recent time, Paquisa and LNP populations showed an expansion signal that began approximately 2.5–3 My BP for Paquisa population and 15-20,000 yBP for LNP populations. For Umaqui and Paquisa, the TMRCA was estimated approximately at 1My BP while for Caquena, Colpa and LNP was of 2.8My BP, 4My BP and 13,000 yBP respectively (Fig. 4).

Nuclear DNA

Genetic diversity

Multilocus genotypes of 207 individuals in 9 locations were obtained. The analysis of the data with Micro-checker did not find null alleles, stuttering errors or significant deviations from the Hardy-Weinberg equilibrium. In addition, no linkage imbalances were found among loci within populations after applying the Bonferroni correction. Descriptive indices of genetic diversity are shown in Table 6.

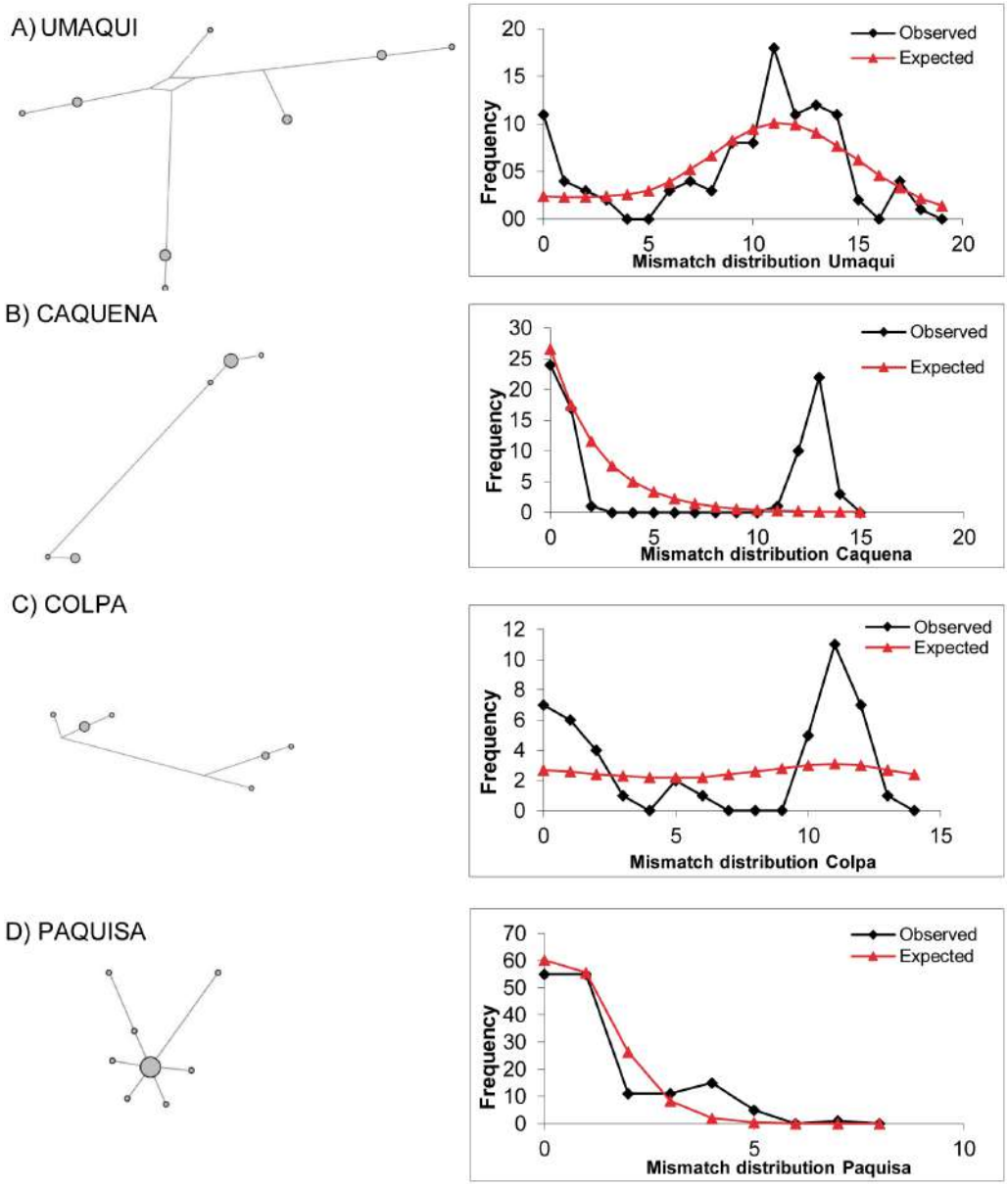

Figure 3 Haplotype network based on a median joining criterion and mismatch distribution. (A) Umaqui. (B) Caquena. (C) Colpa. (D) Paquisa. It was not possible to build this for Ancuta because it only presented one haplotype.

Full-size DOI: 10.7717/peerj.11917/fig-3

As was observed on mtDNA diversity, Ancuta showed lowest values in all the indexes analyzed. F_{IS} showed non-significant values in almost all the localities except for Misituni, where it was positive and significant.

Differentiation among populations

F_{ST} analyses (Table 7) indicated that the comparisons of locations between and within sub-basins show highly significant differentiation values ($p < 0.0001$) except for the

Table 5 Estimation of effective population size and demographic expansion time under the assumptions of the models of *Burban et al. (1999)* and *Kuhner (2006)*, evaluated in Paquisa and LNP (Lauca, Misituni, Copapujo, Chuviri).

Demographic expansion model of *Schneider & Excoffier (1999)* Arlequin v. 3.5.1				
	$\tau=2\mu t$	N_e0	N_e1	Time (years)
LNP	0.342 [0–2.02]	7546.01	16150.31	5245 [0–37653]
Paquisa	0.965 [0–2.094]	0	300546.01	14800 [0–32055]
Coalescence model of likelihood *Kuhner (2006)* LAMARC v.3.2				
	G	Ne_i	Ne_f	Time (years)
LNP	14701.9 [19292.9–11128.6]	34865.8 [76900.2–11993.3]	$3.32*10^6$ [$6.50*10^6$–$1.15*10^6$]	7750 [5750–10250]
Paquisa	12374.5 [17953.9-6510.2]	$6.59*10^6$ [$2.11*10^8$– $3.72*10^4$]	$6.42*10^8$ [$1.88*10^{10}$–$3.55*10^6$]	9250 [6250–17500]

Notes.

τ, tau; μ, nucleotide substitution rate; t, time from expansion (in mutational time units); N_e0, initial effective size; N_e1, final effective size; G, growth rate; Ne_i, initial effective size; Ne_f, final effective size; [], confidence interval.

Copapujo-Chuviri and Lauca-Misituni pairs, both from the Lauca sub-basin, where the values of the index were not significant.

Individual assignment (GeneClass) to the populations delimited by pairwise F_{ST} analysis (Fig. 5), showed a high percentage (75%) of correct assignments to their reference location. Caquena and Paquisa had 100% correct assignments, while the localities of LNP show a great percentage of individuals that were not reassigned to their reference location.

Genetic structure and cluster assignment

Bayesian analysis (Structure) to infer the number of genetic groups (K) of the individual genotypic data is shown in Fig. 6, where it was obtained, in which the appropriate number of genetic groups based on Ln (P) of the sample is five, formed by Umaqui and Colpa; Caquena; Chuviri and Copapujo; Lauca and Misituni and finally a group with Ancuta and Paquisa.

DISCUSSION

It has been proposed that the uplift of the altiplano occurred mainly during the Miocene-Pliocene period, whose greatest height would have been reached at the end of the Pliocene (∼5–2 MyBP) (*Villwock, 1983*; *Muñoz & Charrier, 1996*; *Lamb & Davis, 2003*). In this period, the Caquena and Lauca sub-basins would have risen above 4,000 m.a.s.l. During this process, together with high tectonic activity, atmospheric and oceanic circulation, it would have promoted hyper-arid climatic conditions in the area (*Lamb & Davis, 2003*), even when highly variable rainfall regimes are reported (*Feitl et al., 2019*). Our results showed an expanded haplotype network, with some shared haplotypes between Caquena and LNP populations, which suggests the existence of an ancient lineage with presence in both Caquena and Lauca sub-basins. Individuals of this ancient population of *Orestias* would have colonized the different limnic systems of the sampled area where the variation in water levels given by the alternation between wet and dry periods would have allowed the

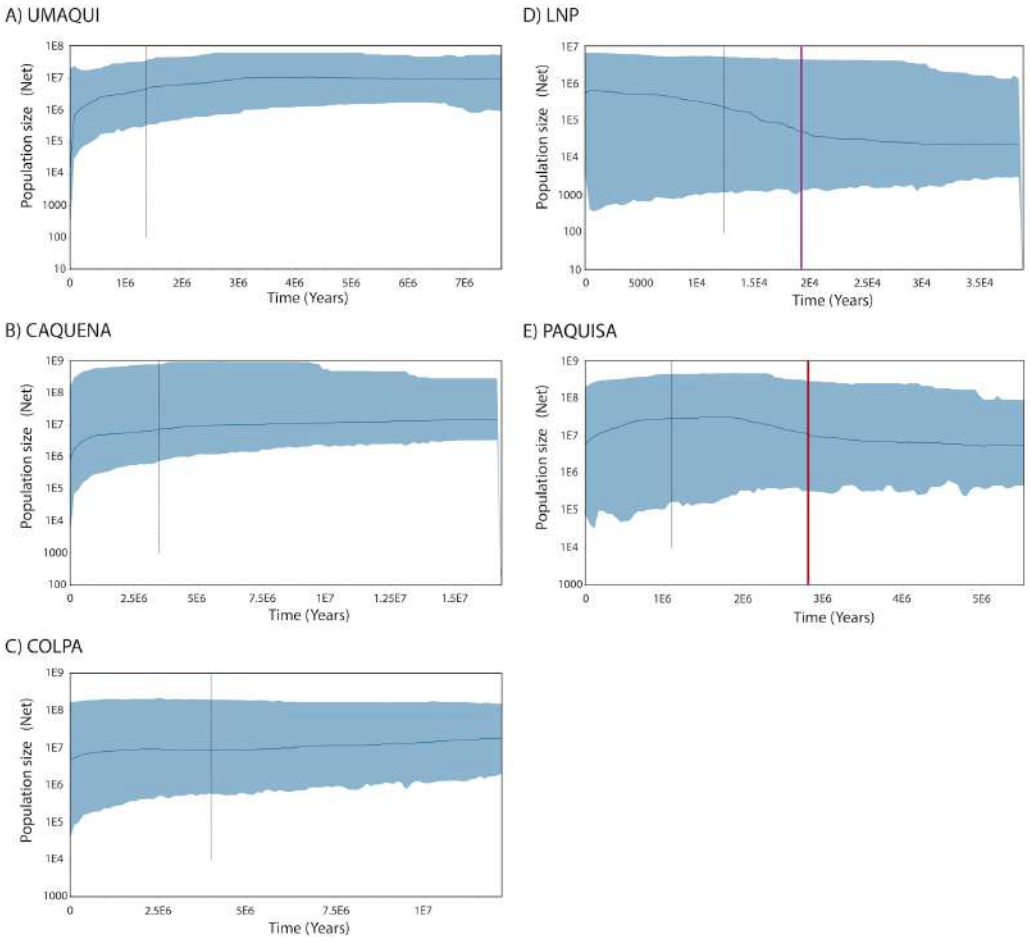

Figure 4 Demographic history from a Bayesian skyline plot analysis estimated using mitochondrial control region sequences of *Orestias* groups: (A) Umaqui, (B) Caquena, (C) Colpa, (D) LNP group and (E) Paquisa. The *x*-axis shows the Time in years and *y*-axis shows the Population size as the product of effective population size (Ne) and generation time (t). The black solid line represents the median estimate of the estimated effective population size, and 95% highest probability density (HPD) interval corresponds to the shaded blue area. The segmented line corresponds to the estimated time of the most recent common ancestor (TMRCA) and the purple and red solid lines on (D) and (E) respectively correspond to the estimated time for the expansion of that populations.

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sporadic connection of some of them. During the arid periods, isolation, local adaptation and genetic differentiation were promoted, while the wet periods facilitated gene flow (*Moreno et al., 2007*). Additionally, local pressures like volcanism and anthropic activity would have had a differentiated effect on localities over time (Fig. 7).

Caquena sub-basin

The Caquena sub-basin area is covered by wetlands (bofedales) formed by cushion plants and other azonal vegetation; several water courses flow in different directions that may or may not discharge into a main course. Pollen record studies of *Domic et al. (2018)* show that these characteristics have been present at least since 1,400 yBP and could have been

Table 6 Genetic diversity indexes of eight microsatellite of *Orestias* of the studied locations.

Sub-basin	Location	N	A	Hobs	Hexp	F_{IS}
Caquena	UMA	20	9.500	0.674	0.688	0.046^{NS}
	CAQ	20	6.125	0.543	0.548	0.035^{NS}
	COL	20	8.250	0.685	0.647	-0.034^{NS}
Lauca	CHU	15	4.375	0.453	0.451	0.038^{NS}
	COP	19	4.250	0.496	0.469	-0.012^{NS}
	LAU	34	5.250	0.520	0.513	0.004^{NS}
	MIS	42	6.625	0.493	0.538	0.097^{*}
	ANC	17	3.125	0.309	0.303	0.012^{NS}
	PAQ	20	6.125	0.519	0.523	0.033^{NS}

Notes.
N, number of individuals; A, mean number of alleles per locus; SD, standard deviation; Hobs, observed heterozygosity; Hexp, expected heterozygosity; F_{IS}, endogamy index; NS, nonsignificant; *, $p < 0.05$.

Table 7 Genetic differentiation of *Orestias* between pairs of locations (F_{ST}) based on information of 8 microsatellite loci.

F_{ST}	UMA	CAQ	COL	CHU	COP	LAU	MIS	ANC	PAQ
UMA		***	*	***	***	***	***	***	***
CAQ	0.11508		***	***	***	***	***	***	***
COL	0.03453	0.09327		***	***	***	***	***	***
CHU	0.26120	0.33904	0.29692		NS	***	***	***	***
COP	0.22754	0.31640	0.27715	0.00955		***	***	***	***
LAU	0.25316	0.30655	0.27420	0.09676	0.11751		NS	***	***
MIS	0.23674	0.29918	0.25849	0.07809	0.08711	0.00911		***	***
ANC	0.31965	0.31940	0.36195	0.50843	0.47407	0.42248	0.41178		***
PAQ	0.15498	0.17257	0.18544	0.35136	0.31075	0.32431	0.31447	0.27961	

Notes.
Above the diagonal is the significance of index values.
NS non-significant.
* $p < 0.05$.
** $p < 0.001$.
*** $p < 0.0001$.

promoted by human activity since 2,000 yBP; using the land to maintain grazing areas and water availability would have encouraged humans to maintain them and expand them across the area. The ashes found in cores indicate that volcanism did not have a significant effect on the local vegetation community, which remained stable over 7,000 yBP. When the level of water rises, this landscape characteristic allows the rapid connection for a wide area and facilitates the exchange of biota, which would have allowed it to maintain effective migrants of *Orestias* over time. Results of Fu and Tajima indexes were positive but not significant. Therefore, no evidence of demographic expansion could be probed. However, the multimodal distribution of differences between pairs of sequences of populations of Caquena sub-basin, the Bayesian skyline plots and network results are coherent with those of populations that have remained stable over time (*Slatkin & Hudson, 1991*; *Harpending et al., 1993*). This homogenization process may continue to occur today, given the seasonal wet variation within the year.

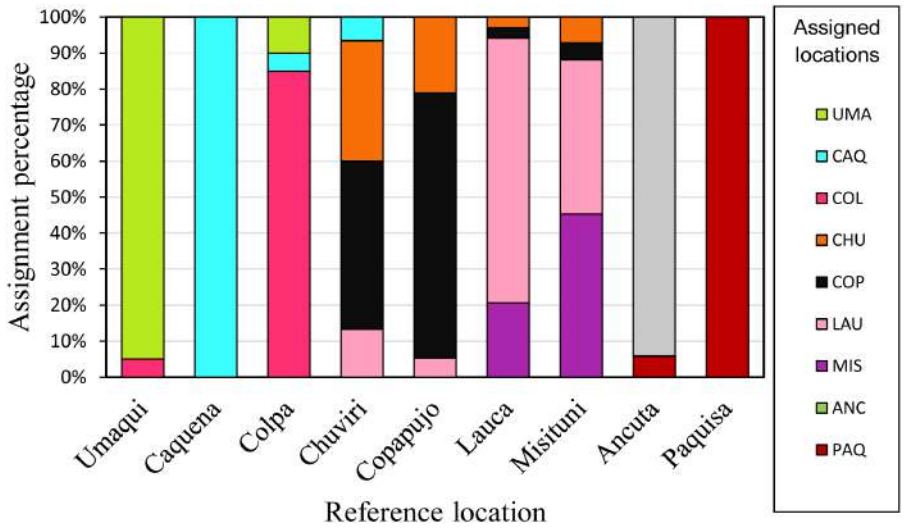

Figure 5 **Reassignment percentages of studied locations.** Reference locations are on the *X* axis and the percentage of reassignment is on the *Y* axis. Different colors of the bars represent the percentage of individuals from each assigned location. The colors of each locality are the same as in the haplotype network.
Full-size DOI: 10.7717/peerj.11917/fig-5

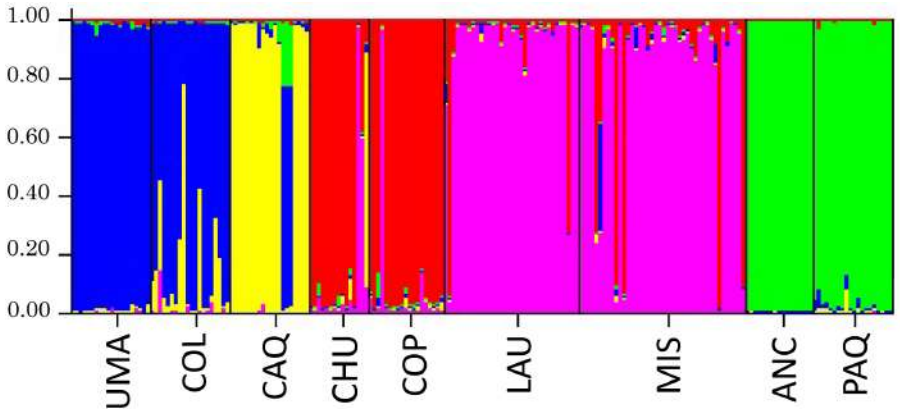

Figure 6 **Results of the analysis of genetic structuring and cluster assignment (Structure) for all locations analyzed with k = 5.** Each vertical line represents an individual, grouped according to their place of origin separated by black lines. Each color corresponds to a genetic group. The different colors in each individual represent their probability of assignment to that genetic group.
Full-size DOI: 10.7717/peerj.11917/fig-6

Lauca sub-basin

The collapse of the Parinacota volcano (8,800 yBP) is cited as one of the principal modifiers of the Lauca sub-basin area during the Pleistocene-Holocene ages; it gave origin to varied aquatic systems that remain today. This volcanic event would have had a local effect mainly with two different patches, one that reached approximately a 150 km^2 zone that was buried by an avalanche of debris (*Jicha et al., 2015*), and another given by the modification of course of the Paleo-Lauca River that changed from northerly to southerly (*Sáez et al., 2007*), affecting the connectivity of some areas of the sub-basin. It was observed the

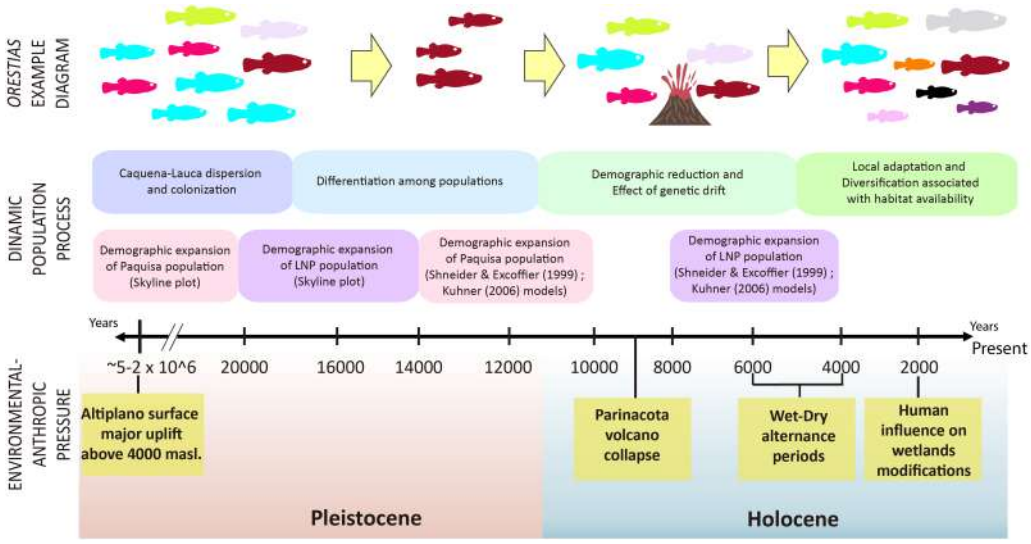

Figure 7 **Timeline and diagram of results interpretation.** Fish color icons are the same as the haplotypes used in the haplotype network of results.

Full-size DOI: 10.7717/peerj.11917/fig-7

conformation of two well-defined haplotype groups on the sub-basin, one containing the LNP localities and the other with Ancuta-Paquisa localities, however it is important to highlight that they are inserted on a wider haplotype network sharing genetic diversity with the neighbor Caquena sub-basin. Our results support that, as was demonstrated by *Guerrero-Jiménez et al. (2017)* on LNP, the fragmentation of hydrological systems, would have restructured the gene flow between the populations of *Orestias* that inhabit them. Initially by a decrease in the effective size that, due to the effect of genetic drift, decreased the number of haplotypes and later, by a demographic expansion that increased the number of individuals but with genetic diversity remaining scarce. Subject to the generation of new environmental conditions and the ability of populations to respond to them, new genetic diversity would be generated. A star-like pattern was found for the haplotype network of both LNP and Paquisa populations, both had unimodal mismatch distributions and also both showed signals of population expansion on Bayesian skyline plot. However, their starting point of demographic expansion was different. For LNP, the range of estimated dates with both *Schneider & Excoffier (1999)* and Kuhner model (2006) models were after the date of the volcanic collapse making it more likely that the demographic expansion detected could be attributable to the geological changes caused by this event. For Paquisa, on the other hand, despite the range of intervals calculated for both models was wider, the date of demographic expansion was rather before the collapse (Table 5). Yet although the timing of population expansion start time estimated by the Bayesian skyline analysis was earlier than that estimated by the other models, all three models agree on Paquisa expansion time would have been earlier than LNP populations. In addition, it is remarkable the high differentiation of F_{ST} and Φ_{ST} indexes of Paquisa-Ancuta with all other sampled locations. This high genetic distance seems to respond to an older isolation reached before the change

in the course of Lauca River and suggests their haplotypes might have not been constituted as a consequence of the volcanic collapse 8,800 yBP rather it could have been generated together with the uplift of Altiplano 5–2 M yBP (*Villwock, 1983*; *Kött, Gaupp & Wörner, 1995*; *Muñoz & Charrier, 1996*; *Lamb & Davis, 2003*).

The reconstruction of geological history of the basin, indicates a shallower northern part and a southern part which houses its oldest deposits. Over this area the existence of closed lake conditions with significant variation on water level has been probed (*Feitl et al., 2019*). This Lake (3.7 < 0.25 MyBP), was progressively replaced by rivers and ponds to several terraces fragmented the area by precursors of Lauca River and its tributaries (*Kött, Gaupp & Wörner, 1995*; *Gaupp, Kött & Wörner, 1999*). These antecedents suggest that not only the volcanic activity could have effects on the evolutionary history of the *Orestias* populations and that perhaps in the southern area (Ancuta-Paquisa) of the Lauca sub-basin, events of landscape modifications such as variations in water levels could have had greater incidence on the genetic differentiation of their populations.

A shared history?

Further analysis is required for the comprehension of *Orestias* colonization processes across the Altiplano basin, however, this study provides new evidence that contributes to the reconstruction of its evolutionary history.

The geological and climatic variations that occurred after the collapse of the Parinacota volcano, strong volcanic activity 8,000–4,000 yBP followed by arid and humid periods, gradually generated the arid and warm conditions that prevail today (*Placzek, Quade & Patchett, 2006*; *Vila et al., 2013*) and gave way to the formation of aquatic systems as we know them. This transition process would have increased geographic isolation among the sampled populations, restricting dispersion and promoting genetic diversification associated with local adaptation, which is reflected in the microsatellite results where F_{ST} comparisons (Table 7) and reassignment tests (Fig. 5) show similarities between geographically closest localities and the five genetic groups that were identified with the cluster analysis, Caquena, Umaqui-Colpa, Copapujo-Chuviri, Lauca-Misitune and Ancuta-Paquisa (Fig. 6). Nuclear marker analysis results were coherent with those obtained by mitochondrial data. These confirm the scenario of recent diversification and incipient speciation process of *Orestias* that was described by *Guerrero-Jiménez et al. (2017)* in Lauca National Park but it shows signs that it is framed on a much older evolutionary history that points to a shared history of all populations sampled before the conformation of the sub-basins, meaning that what was reported for the LNP would respond to a local disturbance. According to this, we hypothesize that first there was an original widespread population across Caquena and Lauca sub-basins due to variations in the water level and their adaptation to environmental conditions that allowed them to establish. Then this population was subjected to local disturbances that strongly affected the connectivity of some areas like isolation (Ancuta-Paquisa) and fragmentation of habitat but were not significant for others (Caquena sub-basin localities). Finally, the availability of novel habitats with different environmental characteristics resulted in genetic diversification (mainly in LNP). Additionally, we think that the high genetic differentiation found for

Ancuta-Paquisa from the rest might be the result of an older event and could be attributed to different factors. One of these explanations could be a border colonization of individuals that would have been carriers of low frequency haplotypes of an ancient population that reach this area and remained isolated even after the collapse of Parinacota volcano.

Finally, it is important to point out that the processes of connection and disconnection between populations would be associated with both past and present environmental conditions, and also that both Caquena and Lauca sub-basins are part of broader hydrological networks connected with Bolivia and Peru, which creates a framework of much greater possibilities when it comes to elucidating the possible routes that would account for the colonization of *Orestias* through the Altiplano and for the understanding of their diversification processes, thus it would be of great importance to expand these studies to the neighboring basins in Bolivia and Perú.

Implications for conservation

Altiplano aquatic systems are highly threatened and tend to desiccation and salinization because they are immersed in a desert matrix that intensifies significantly year after year (*Demergasso et al., 2010*). It is expected that current climatic conditions will be different according to the particularities of the ecosystems and specific places (*Sarricolea Espinoza & Romero Aravena, 2015*; *Sarricolea Espinoza, Meseguer Ruiz & Romero Aravena, 2017*). These highland aquatic ecosystems are faced with various threats, among which are legal factors derived from the regulatory status that Chile adopted for water resources, the overexploitation of aquifers especially associated with their high use in the mining industry, and water pollution which has created hydric stress throughout the area (*Instituto Geográfico Militar (IGM), 1983*; *Ministerio del Medio Ambiente (MMA), 2018*). Studies of genetic diversity are key to the development of conservation and management strategies to allow conservation efforts to be directed towards these most vulnerable populations and to be able to recover and maintain them in the long term, minimizing the impacts that anthropic activity could generate on them (*Ministerio del Medio Ambiente (MMA), 2018*). They are a significant source of knowledge for the understanding of the evolutionary history of species and how they have been related over time.

Despite the fact that they now inhabit separate basins, the *Orestias* populations of the Caquena and Lauca sub-basins have a shared history and represent different conservation units, whether due to the age of their haplotypes, the genetic diversification processes that continue to occur or for the uniqueness of their genetic diversity, hosting a relevant gene pool for this genus and maybe not only for it but also of the biodiversity that characterizes the entire area. However, the status of protection of these sub-basins is still in development. While the Caquena sub-basin has been considered in a national plan to protect wetlands that should be fully implemented in 2022, Lauca sub-basin is protected by a National Park and a National Reserve (Fig. 1). Even with these measures, during sampling we observed anthropic perturbations inside it such as the extraction of water to moisten the roads and the channeling of water courses for the mining industry, increasing water quality deterioration.

CONCLUSIONS

The performed analyzes allowed us to use the information obtained from mitochondrial and nuclear molecular markers to characterize genetic diversity and reconstruct part of the evolutionary history of *Orestias* in the Caquena and Lauca sub-basins. It was found that although the localities have variable genetic diversity and are differentiated from each other, they share genetic diversity. Our results revealed a scenario where *Orestias* of Caquena and Lauca sub-basins shared its evolutionary history by the presence of ancient lineages. These groups that would have dispersed throughout this area were affected by local disturbances that fragmented and isolated them. We deduced that geological and hydrological events occurred mainly during Pleistocene-Holocene ages, affected the studied sub-basins differently, and we provide some genetic evidence about how the collapse of the Parinacota volcano would have had an effect on the *Orestias* populations closest to its volcanic cone as LNP populations, but it would not have reached the most distant populations, as Ancuta-Paquisa area and Caquena sub-basin. Moreover, our results allowed us to infer that the genetic differentiation processes of the LNP populations are more recent than those occurred in the rest of the sampled localities. Further studies of taxa cohabitant with *Orestias* would allow contrasting their diversification patterns and strengthen the proposal of genetic and ecological differentiation processes in the Altiplano aquatic ecosystems.

Altiplano aquatic ecosystems harbor very unique characteristics of environmental conditions and biodiversity and they face varied types of threats to their conservation and consequently to the taxa that inhabit them. Studies of this type will provide basic information to formulate proposals for conservation and management strategies that guide decision-making regarding their protection.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Violeta Cárcamo-Tejer, Irma Vila and Claudia Guerrero-Jiménez conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Francisco Llanquín-Rosas and Alberto Sáez-Arteaga performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

The Chilean Undersecretary of Fisheries approved field studies (research fishing permit number 1103-2015).

Data Availability

The following information was supplied regarding data availability:

The D-loop sequences are available at GenBank BankIt2361457: MW149163–MW149238.

Supplemental Information

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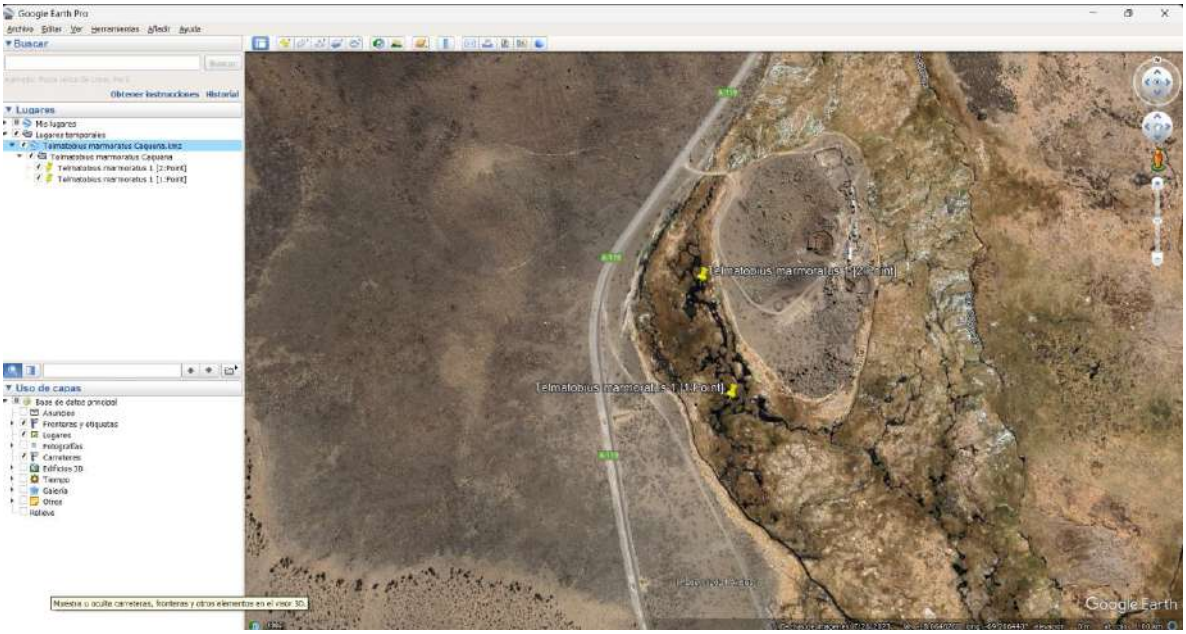
Gobierno de Chile

CONSTANCIA DE PIEZA EXCEPTUADA

Se deja constancia del ingreso, en calidad de pieza exceptuada del Expediente de la Macrozona Norte en el marco del artículo 8vo transitorio de la Ley 21.600 que mandata el proceso para el establecimiento de Sitios Prioritarios de la Estrategia Nacional y las Estrategias Regionales de Biodiversidad, a los siguientes archivos digitales recibidos a través del correo electrónico recibido el 16 de mayo del 2024, cuyo contenido es el siguiente:

- “KMZ” de nombre “KMZ T. marmoratus Caquena.kmz”



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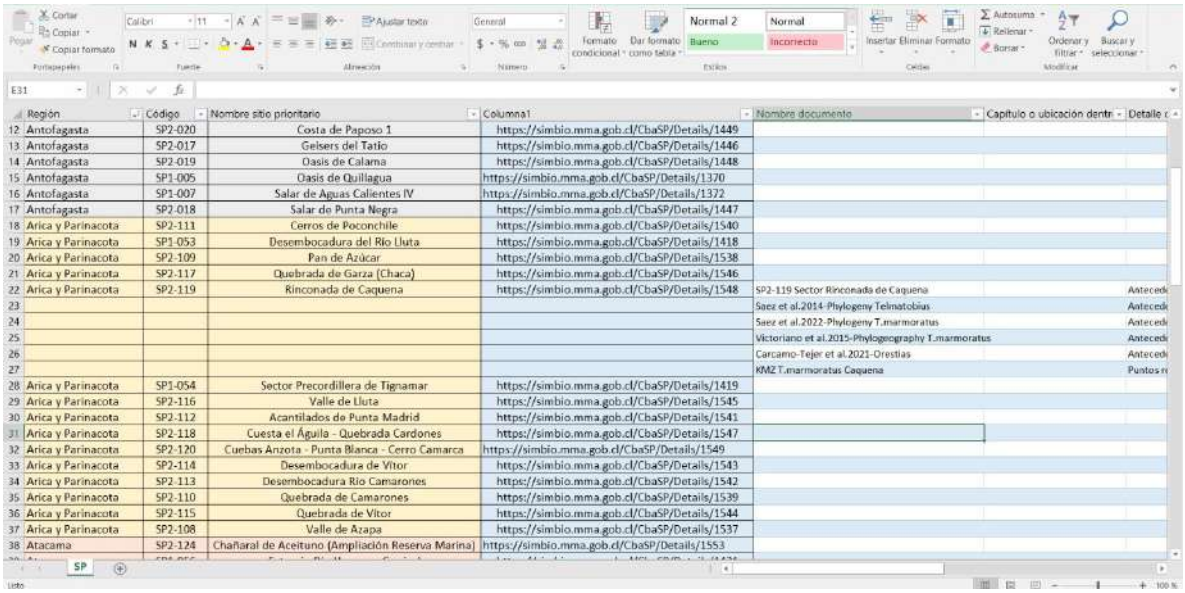
- "Recep_Ant_Sitios Prioritarios_Macrozona_Norte-RCaquena"



	Región	Código	Nombre sitio prioritario	Columna1	Nombre documento	Capítulo o ubicación dentr	Detalle
12	Antofagasta	SP2-020	Costa de Paposo 1	https://simbio.mma.gob.cl/CbaSP/Details/1449			
13	Antofagasta	SP2-017	Geisers del Tatio	https://simbio.mma.gob.cl/CbaSP/Details/1446			
14	Antofagasta	SP2-019	Oasis de Calama	https://simbio.mma.gob.cl/CbaSP/Details/1448			
15	Antofagasta	SP1-005	Oasis de Quillagua	https://simbio.mma.gob.cl/CbaSP/Details/1370			
16	Antofagasta	SP1-007	Salar de Aguas Calientes IV	https://simbio.mma.gob.cl/CbaSP/Details/1372			
17	Antofagasta	SP2-018	Salar de Punta Negra	https://simbio.mma.gob.cl/CbaSP/Details/1447			
18	Arica y Parinacota	SP2-111	Cerros de Poconchile	https://simbio.mma.gob.cl/CbaSP/Details/1540			
19	Arica y Parinacota	SP1-053	Desembocadura del Río Lluta	https://simbio.mma.gob.cl/CbaSP/Details/1418			
20	Arica y Parinacota	SP2-109	Pan de Azúcar	https://simbio.mma.gob.cl/CbaSP/Details/1538			
21	Arica y Parinacota	SP2-117	Quebrada de Garza (Chaca)	https://simbio.mma.gob.cl/CbaSP/Details/1546			
22	Arica y Parinacota	SP2-119	Rinconada de Caquena	https://simbio.mma.gob.cl/CbaSP/Details/1548	SP2-119 Sector Rinconada de Caquena		Antecede
23					Saez et al.2014-Phylogeny Telmatobius		Antecede
24					Saez et al.2022-Phylogeny T.marmoratus		Antecede
25					Victoriano et al.2015-Phylogeography T.marmoratus		Antecede
26					Carcamo-Tejer et al.2021-Orestias		Antecede
27					KMZ T.marmoratus Caquena		Puntos r
28	Arica y Parinacota	SP1-054	Sector Precordillera de Tignamar	https://simbio.mma.gob.cl/CbaSP/Details/1419			
29	Arica y Parinacota	SP2-116	Valle de Lluta	https://simbio.mma.gob.cl/CbaSP/Details/1545			
30	Arica y Parinacota	SP2-112	Acantilados de Punta Madrid	https://simbio.mma.gob.cl/CbaSP/Details/1541			
31	Arica y Parinacota	SP2-118	Cuesta el Águila - Quebrada Cardones	https://simbio.mma.gob.cl/CbaSP/Details/1547			
32	Arica y Parinacota	SP2-120	Cuebas Anzota - Punta Blanca - Cerro Camarca	https://simbio.mma.gob.cl/CbaSP/Details/1549			
33	Arica y Parinacota	SP2-114	Desembocadura de Vitor	https://simbio.mma.gob.cl/CbaSP/Details/1543			
34	Arica y Parinacota	SP2-113	Desembocadura Río Camarones	https://simbio.mma.gob.cl/CbaSP/Details/1542			
35	Arica y Parinacota	SP2-110	Quebrada de Camarones	https://simbio.mma.gob.cl/CbaSP/Details/1539			
36	Arica y Parinacota	SP2-115	Quebrada de Vitor	https://simbio.mma.gob.cl/CbaSP/Details/1544			
37	Arica y Parinacota	SP2-108	Valle de Azapa	https://simbio.mma.gob.cl/CbaSP/Details/1537			
38	Atacama	SP2-124	Chañaral de Aceituno (Ampliación Reserva Marina)	https://simbio.mma.gob.cl/CbaSP/Details/1553			

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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

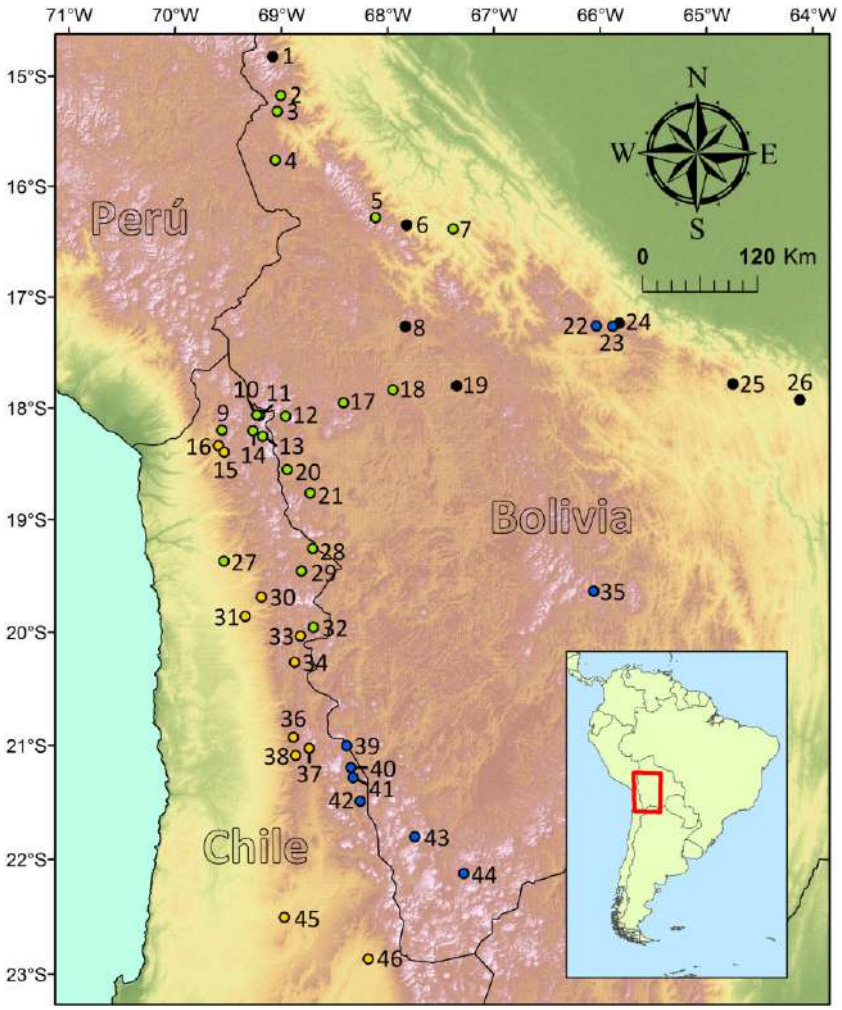


Figure 1. Geographical location of the Chilean and Bolivian *Telmatobius* populations included in this study. Numbers correspond to those in Table 1. Black circles, *Telmatobius verrucosus* and *Telmatobius bolivianus* groups; green circles, *Telmatobius marmoratus* group; blue circles, *Telmatobius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

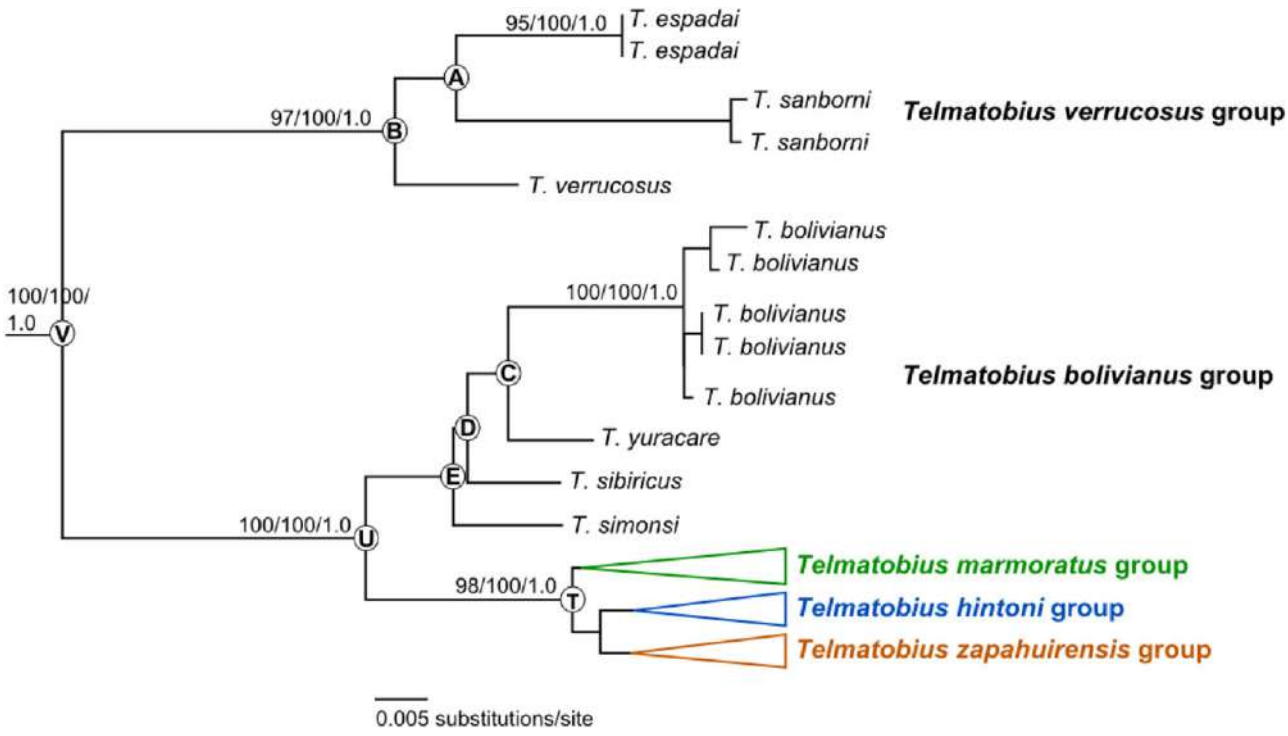
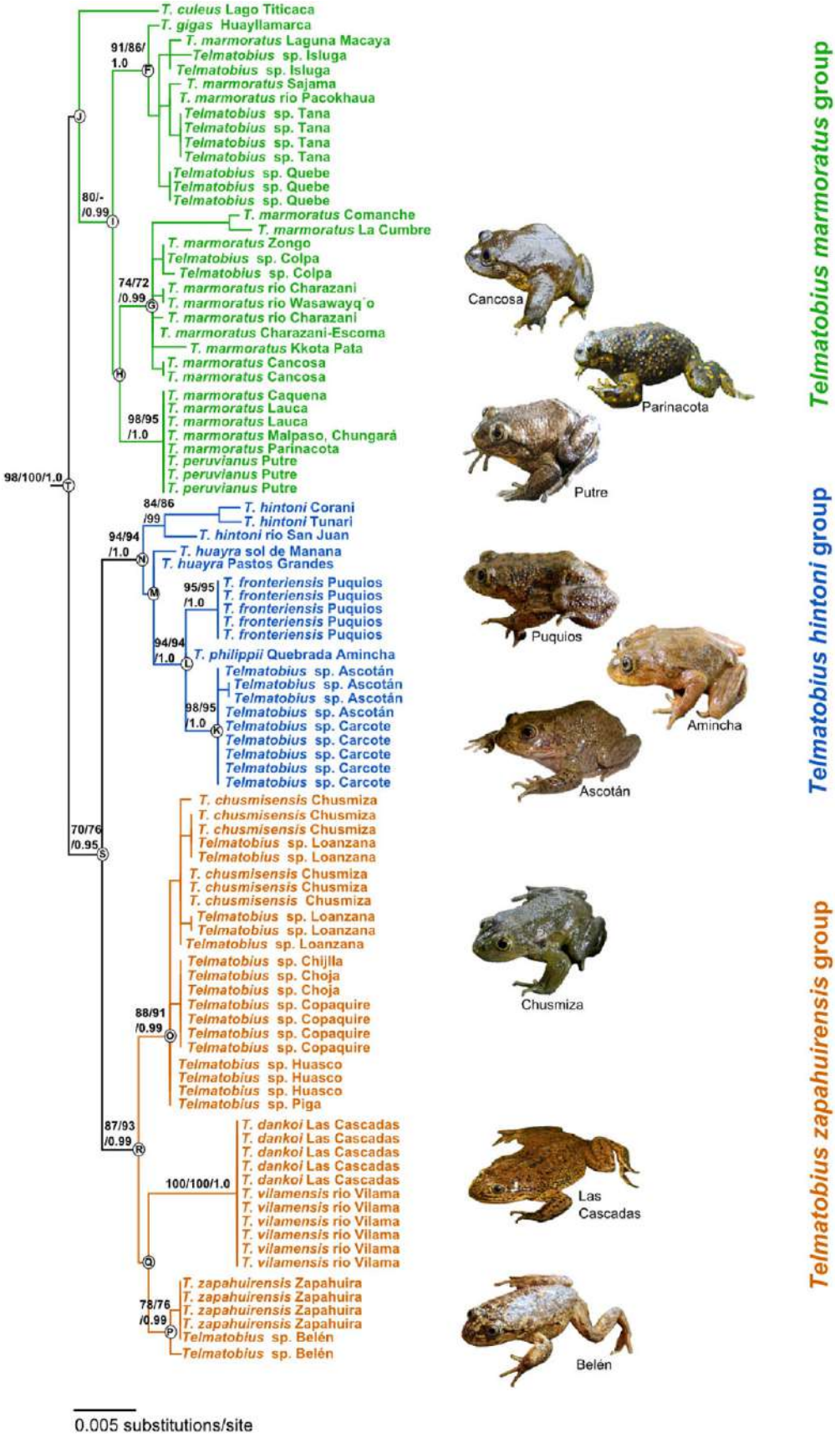


Figure 2. Maximum likelihood tree (-ln likelihood 3667.32) obtained using the concatenated *16S* and *cytochrome b* mitochondrial markers of 19 nominal taxa of *Telmatobius* in Chile and Bolivia and 12 undescribed Chilean localities. Values above the nodes are, from left to right, the bootstrap values 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.

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Phylogeny of *Telmatobius marmoratus* complex (Anura, Telmatobiidae) reveals high cryptic diversity in the Andean Altiplano

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ABSTRACT

Telmatobius is the most diverse group of anurans in the Andean Altiplano (highlands) Morphologically, these amphibians have a generally conserved morphology but in turn present large intraspecific variation, which has led to a complex taxonomy and systematics. *T. marmoratus* has the widest distribution of the genus and forms a complex composed of at least two *Telmatobius* species. Partial systematic studies based on molecular evidence reveal the existence of three lineages with a complex spatial distribution. However, these studies did not include the entire distribution of *T. marmoratus*. Our study aims to reassess the current systematic scenario including the complete distribution of the complex. For this, we used a multilocus approach based on mitochondrial (16S, Cytb) and nuclear (RAG1-1, BFIB) DNA sequences to build a phylogenetic hypothesis based on Bayesian inference, maximum likelihood and maximum parsimony. Subsequently, we performed single-locus (ABGD and PTP) and multilocus (STACEY) species delimitation analyses to verify the diversity of nominal species within the complex. The analyses suggest seven non-sibling lineages and 6–10 candidate species within the *marmoratus* complex. Only one of the two lineages restricted to the central northern plateau correspond to *T. marmoratus sensu stricto*. South-central marbled water frogs belong to completely new lineages closer to *T. gigas* and *T. culeus*, evidencing the polyphyletic condition of the *marmoratus* complex. The findings of several sympatric lineages in some localities reveal a complex history of ancient water connections in south-central Altiplano.

1. Introduction

Telmatobius Wiegmann, 1834 is a genus of amphibians of Gondwanic origin (Cei, 1986), which has 61 described species (Frost, 2021) and represents the most diverse group of anurans endemic to the Central Andes. In the Andean (or South American) Altiplano, *Telmatobius* diversified more successfully than other genera (e.g., *Pleurodema*, *Gastrotheca* and *Rhinella*), being the most speciose group of anurans inhabiting the intermontane basins of this region. From the perspective of the environmental niche occupied by the *Telmatobius* species that inhabit the highland region, these taxa could be considered extremophilic organisms because they have an almost exclusively aquatic life cycle, adapted to conditions of low atmospheric pressure and oxygen, high aridity and evaporation and with enormous daily temperature fluctuations. These environmental characteristics are typical of this high-altitude ecosystem (Vellard, 1951). These extreme environmental pressures have probably led to its general morphology being highly conserved (Benavides et al., 2002). Despite this, *Telmatobius* species also

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present a large intraspecific variation that has been sparsely studied (Trueb, 1979; Wiens, 1993; Barrionuevo, 2013). This variability could be related to microhabitat differences, which would also be influenced by the isolation between them as a result of the region's intricate geography (Vellard, 1951), but it could also be related to the differential development of osteological traits (Barrionuevo 2013). This pattern has led to an overlapping of morphological characters between the different species, which has historically hindered establishing the boundaries between taxa of the genus *Telmatobius*. Evidence of this is that the first taxonomic studies in the basins of the Altiplano failed to establish specific conclusive species boundaries that inhabit this region (see Benavides et al., 2002). In the Lake Titicaca basin, studies based on morphology recognized four species: *Telmatobius albiventris* Parker, 1940, *T. culeus* Garman, 1876, *T. marmoratus* Duméril and Bibron, 1841 and *T. crawfordi* Parker, 1940; and a high number of subspecies (Parker, 1940; Vellard, 1953, 1960, 1991; Cei, 1986). However, when morphological traits were analyzed in conjunction with alloenzymatic molecular traits, only two entities were recognized in this system: *T. culeus*, confined to Lake Titicaca and nearby interconnected lagoons, and *T. marmoratus*, present in streams and pools around Lake Titicaca (Benavides et al., 2002). When Benavides (2005) analyzed the populations of *T. culeus* and *T. marmoratus* associated with Lake Titicaca, using the molecular marker cytochrome b, he observed an absence of reciprocal monophyly. But, subsequently, Victoriano et al., (2015), using the same molecular marker (but a different fragment), observed a difference of 10 mutational steps between *T. culeus* and *T. marmoratus*, when including *T. marmoratus* populations from other localities.

More recently, molecular phylogenetic studies that covered a greater number of high Andean basins grouped *Telmatobius* species into three lineages in this area: *T. pefauri*, *T. hintoni* and *T. marmoratus*, which diverged ~4.8 Ma from the species that live at lower elevations (±2000-3000 masl) and in less cold and more humid environments in central Bolivia (*T. bolivianus* group; Departments of La Paz, Cochabamba and Chuquisaca) (De la Riva, 2005; De la Riva et al., 2010; Sáez et al., 2014). The *T. pefauri* group (which also includes *T. chusmisensis*, *T. dankoi*, *T. vilamensis* and *T. halli* [although recently, *T. dankoi* and *T. vilamensis* have been considered junior synonyms of *T. halli* by von Tschirnhaus and Correa 2021]) has an exclusive distribution on the western slopes of the Andes in Chile (Sáez et al., 2014; Fibla et al. al., 2017; von Tschirnhaus and Correa 2021), and the *T. hintoni* group (which also includes *T. huayra*, *T. philippii* and *T. fronteriensis*) is distributed in the high valleys between the eastern and western Andes in the southern region of the Altiplano (De la Riva et al., 2010; Sáez et al., 2014). The *T. marmoratus* group (which includes *T. gigas* and *T. culeus*) is distributed in the center and northern Altiplano, in the Lake Titicaca basin and adjacent areas (De la Riva et al., 2010), including the western slope the Andes in Chile (Sáez et al., 2014; Victoriano et al., 2015).

Telmatobius marmoratus Duméril and Bibron, 1841 (marbled water frog) is a medium-sized frog (55–63 mm) that inhabits streams and small lagoons in high Andean wetlands, which has the widest distribution of the genus, between 13° and 19° S of latitude, covering a linear extent ~800 km (De la Riva, 2005). This large range is striking due to the low vagility of these species due to their high dependence on aquatic systems. The original description of the species was based on individuals collected north of Lake Titicaca (Duméril and Bibron, 1841). However, today, their distribution is known to encompass the upper areas of the hydrographic basins of the Lluta and Camarones rivers and the highland basins in Chile; the intermontane valleys of the Departments of Oruro and La Paz, extending north to the basins of the Desaguadero, Poópo and Lake Titicaca rivers, in Bolivia and Peru, to the region of Cusco; and has even been described in the *Puna* (arid high plateau) of the Amazonian flank adjacent to the Cordillera Real (Vellard, 1951; Capurro, 1953; De la Riva, 2005; Lavilla and Barrionuevo 2005; Sáez et al., 2014; Victoriano et al., 2015). The taxonomic history of this species is not alien to the typical problems of the genus (see above) and has a long list of synonyms in its early history (see Lavilla, 2005). Based on morphological traits, seven subspecies have been described (*T.m. marmoratus* [Duméril and Bibron, 1841], *T.m. angustipes* [Cope, 1877], *T.m. microcephalus* [Vellard, 1953], *T.m. pseudojelskii* [Vellard, 1960], *T.m. pustulosus* [Cope, 1877], *T.m. riparius* [Vellard, 1953] and *T.m. rugosus* [Vellard, 1953]), which were later invalidated by De la Riva (2005), justifying that the recognition of these subspecies is unsustainable due to a high degree of morphological overlap. This is due to high levels of homoplasy in this type of trait (Sinsch et al., 1995). For these reasons, *T. marmoratus* has been recognized as a complex of closely related species, and morphologically, two entities are recognized: *T. marmoratus* and *T. gigas*, the latter distributed only in the Cordillera de Huayllamarca, Bolivia (De la Riva, 2002; De la Riva et al., 2010). However, due to the enormous distribution of *T. marmoratus*, compared to other species of the genus which are mostly known only in their type localities, its low dispersion capacity, and especially the difficulty in identification based on its morphological traits, it is possible that this complex contains a greater number of entities.

Regarding the systematics of this complex, molecular approximations recognized three lineages with recent divergence (~0.92 Ma) in the southern distribution: 1) a lineage distributed between Sajama National Park, Bolivia and the Tarapacá Region, Chile (Isluga, Quebrada Tana and Quebe), which is the brother of *T. gigas*; 2) a second lineage would be found exclusively in Lauca National Park, Chile (Parinacota, Lauca, Chungará and Caquena, Arica and Parinacota Region); and 3) a third lineage distributed between the Department of La Paz (Bolivia) and the Tarapacá Region in the localities of Cancosa and Colpa, Chile (Sáez et al., 2014). Subsequently, Victoriano et al. (2015) analyzed the phylogeographic processes in the species of the *T. marmoratus* complex, revealing the absence of reciprocal monophyly between the Chilean and Bolivian populations. This suggests a high recent connectivity, which would be explained by a historical flow between the western slope of the Andes and the center of the Altiplano (Victoriano et al., 2015). This genetic pattern shows a complex spatial distribution among the lineages that could present a sympatric distribution. Therefore, the problems in this group are not only due to morphology but also due to their genetic and spatial patterns. Additionally, to date, all studies related to *T. marmoratus* have considered only the southern portion of the distribution of this complex, so including the northern portion of the distribution is essential to understand the complex evolutionary history of this particular group of highland *Telmatobius*.

Based on all of the above, our main objective is to analyze the systematic scenario including the complete distribution of the complex and to evaluate the current taxonomic arrangement, which will allow us to delimit the *T. marmoratus* complex. For this, we used a multilocus approach based on mitochondrial and nuclear DNA sequences to construct a phylogenetic hypothesis, and then we performed a species delimitation analyses to contrast with the nominal species within the complex.

2. Materials and methods

2.1. Frogs collection

During 2017 and 2018, a comprehensive sampling on the *T. marmoratus* complex was carried out in the whole distribution of the species from 13° to 19° south latitude, between approximately 3300 to 4500 m.a.s.l. (Table 1, Fig. 1). Additionally, we added samples from *T. pefauri* and *T. hintoni* groups, which together with *T. marmoratus* complex conform a well-supported clade (Sáez et al., 2014: here assigned as clade MPH). All the type localities of the subspecies were explored but we did not find presence of the species in all of them (Table 1). The animals were captured with a fishing net from under the vegetation and between the rocks from small caves under the ground at the edges of the hydrological systems. Because *T. marmoratus* is a threatened species (Vulnerable A3cde, IUCN SSC Amphibian Specialist Group, 2020), a non-invasive method was used to obtain DNA samples.

Table 1
Species and sampling sites considered in this study. † = localities associated with subspecies. * = published by De la Riva et al., (2010). NP = National Park. NR = National Reserve. NM = Natural Monument.

N	Species	Sampling site	Region/Country	Latitude (S)	Longitude (W)
1	*T. marmoratus*	Tambo Real	Cusco, Perú	13 28 39,8	72 14 15,9
2	*T. marmoratus*	Cachimallo, Hatunmallo river	Cusco, Perú	13 29 04	72 03 30,7
3	*T. marmoratus*	Huancaro, river	Cusco, Perú	13 32 54,5	71 59 17,1
4	*T. marmoratus*	Sicuani, Vilcanota river†	Cusco, Perú	14 18 03,7	71 12 26,8
5	*T. marmoratus*	Espinar	Cusco, Perú	14 44 41,64	71 26 07,51
6	*T. marmoratus*	Azángaro	Puno, Perú	14 55 35,5	70 14 25,2
7	*T. marmoratus*	Wasawayqo, river*	La Paz, Bolivia	15 10	68 59
8	*T. marmoratus*	Charazani, river*	La Paz, Bolivia	15 10	69 0
9	*T. marmoratus*	Moho†	Puno, Perú	15 18 50,6	69 33 36,9
10	*T. marmoratus*	Sillustani	Puno, Perú	15 42 40,1	70 09 01,3
11	*T. marmoratus*	Lagunillas	Puno, Perú	15 44 15,3	70 37 01,6
12	*T. culeus*	Chinta, river	Oruro, Bolivia	15 57 25,33	68 42 28,51
13	*T. marmoratus*	Platería	Puno, Perú	15 57 27,7	69 49 57,0
14	*T. marmoratus*	Juli†	Puno, Perú	16 14 46,0	69 29 04,2
15	*T. culeus*	Lago Titicaca*	La Paz, Bolivia	16 15	68 44
16	*T. marmoratus*	Kkota Pata*	La Paz, Bolivia	16 16	68 4
17	*T. marmoratus*	Zongo*	La Paz, Bolivia	16 16	68 6
18	*T. marmoratus*	Pomata†	Puno, Perú	16 19 29,3	69 18 45,1
19	*T. marmoratus*	La Cumbre	La Paz, Bolivia	16 20	68 2
20	*T. marmoratus*	Copani	Puno, Perú	16 22 55,8	69 03 46,1
21	*T. marmoratus*	Aurincota	Puno, Perú	16 32 09,6	69 23 16,2
22	*T. marmoratus*	Achocalla	La Paz, Bolivia	16 34 22,9	68 10 42,1
23	*T. marmoratus*	Marquiviri	La Paz, Bolivia	16 35 09,6	68 09 55,1
24	*T. marmoratus*	Comanche	La Paz, Bolivia	16 57 35,79	68 25 14,84
25	*T. marmoratus*	Corocoro	La Paz, Bolivia	17 07 25,84	68 27 35,74
26	*T. marmoratus*	Chojñokho	Oruro, Bolivia	17 13 18,8	68 08 56,3
27	*T. marmoratus*	Puerto Liqueta	Oruro, Bolivia	17 48 04	68 01 40,7
28	*T. marmoratus*	Totora, San Pedro de	Oruro, Bolivia	17 48 47	68 03 49,9
29	*T. marmoratus*	Khancoyo	Oruro, Bolivia	17 49 11,82	68 28 37,90
30	*T. gigas*	Huayllamarca*	Oruro, Bolivia	17 50	67 57
31	*T. marmoratus*	Culta	Oruro, Bolivia	18 00 52,68	68 41 14,65
32	*T. marmoratus*	Tomarapi, river; NP Sajama	Oruro, Bolivia	18 01 0,47	68 51 27,78
33	*T. marmoratus*	Colpa	Arica-Parinacota, Chile	18 03 29,2	69 13 52,4
34	*T. marmoratus*	Caquena	Arica-Parinacota, Chile	18 03 55,1	69 12 25,9
35	*T. marmoratus*	Parinacota; NP Lauca	Arica-Parinacota, Chile	18 12 11,8	69 16 03,6
36	*T. marmoratus*	Bofedal de Laguna; NP Sajama	Oruro, Bolivia	18 13 12,39	68 55 56,19
37	*T. marmoratus*	Chungará; NP Lauca	Arica-Parinacota, Chile	18 18 40,54	69 08 02,39
38	*T. marmoratus*	Chirigualla; NR Las Vicuñas	Arica-Parinacota, Chile	18 20 37,0	69 10 48,6
39	*T. marmoratus*	Lauca sur, river; NP Lauca	Arica-Parinacota, Chile	18 22 51,03	69 20 59,85
40	*T. marmoratus*	Mogachi; NP Sajama	Oruro, Bolivia	18 25 57,9	68 53 23,4
41	*T. marmoratus*	Ancuta; NR Las Vicuñas	Arica-Parinacota, Chile	18 26 44,9	69 11 40,7
42	*T. marmoratus*	Lauca Vichuta; NR Las Vicuñas	Arica-Parinacota, Chile	18 30 37,5	69 13 54,9
43	*T. marmoratus*	Laguna Macaya*	Oruro, Bolivia	18 33	68 56
44	*T. marmoratus*	Pacokhaua*	Oruro, Bolivia	18 45	68 41
45	*T. marmoratus*	Avaroa	Oruro, Bolivia	18 52 02,62	67 18 56,10
46	*T. marmoratus*	Llachu; NM Salar de Surire	Tarapacá, Chile	18 52 36,8	69 02 50,8
47	*T. marmoratus*	Surire; NR Las Vicuñas	Tarapacá, Chile	18 54 22,1	69 05 22,5
48	*T. marmoratus*	Sajama*	Oruro, Bolivia	18 6	68 59
49	*T. marmoratus*	Paserijo; NP Volcan Isluga	Tarapacá, Chile	19 09 24,1	68 55 57,1
50	*T. marmoratus*	Enquelga; NP Volcan Isluga	Tarapacá, Chile	19 13 44,3	68 49 18,7
51	*T. marmoratus*	Isluga, river	Tarapacá, Chile	19 16 47,2	68 40 33,4
52	*T. marmoratus*	Quebe	Tarapacá, Chile	19 27 29,3	68 48 46,8
53	*T. marmoratus*	Toroni	Tarapacá, Chile	19 30 08,9	68 42 52,8
54	*T. marmoratus*	Tambo Cancosa	Tarapacá, Chile	19 51 29,4	68 35 23,2
55	*T. pefauri*	Murmuntani	Arica-Parinacota, Chile	18 21 08	69 33 48
56	*T. chusmisensis*	Chusmiza	Tarapacá, Chile	19 41 03,38	69 11 2,29
57	*T. fronteriensis*	Puquios	Antofagasta, Chile	21 00 4,6	68 23 11,2
58	*T. philippii*	Quebrada Amincha	Antofagasta, Chile	21 11 38.5	68 20 46.5
59	*T. dankoi*	Las Cascadas	Antofagasta, Chile	22 30 17,4	68 58 02,1
60	*T. vilamensis*	Vilama, river	Antofagasta, Chile	22 51 59,5	68 10 55,6
61	*T. huayra*	Campamento Khastor	Bolivia	22 02	66 08
62	*T. hintoni*	Tolota	Bolivia	17 31	65 58

After capture, the animals were anesthetized using benzocaine ([ethyl 4-aminobenzoate]; 300 mg/L) diluted in water from the same source where they were captured and then they were released at the same collection site immediately after full recovery from anesthesia. Buccal cell samples were obtained with swabs (FLOQSwabs™, Copan) or a piece of interdigital membrane were used as the DNA source (juvenile and adults). For tadpoles, a piece of tail was used (3 mm^3 approx.). Tissue samples were stored in absolute ethanol or in nucleic acid protection and stabilization solution (RNAlater™, Invitrogen), until laboratory analysis.

2.2. DNA extraction and genotyping

Total DNA was isolated using the salt extraction method (modified from Jowett, 1986) and the commercial kit ReliaPrep™ gDNA Tissue Mini System (Promega) (for interdigital membrane and buccal swabs, respectively). Verification of DNA extraction and quality was performed by agarose gel electrophoresis (2 %) with GelRed™ staining (Biotium)

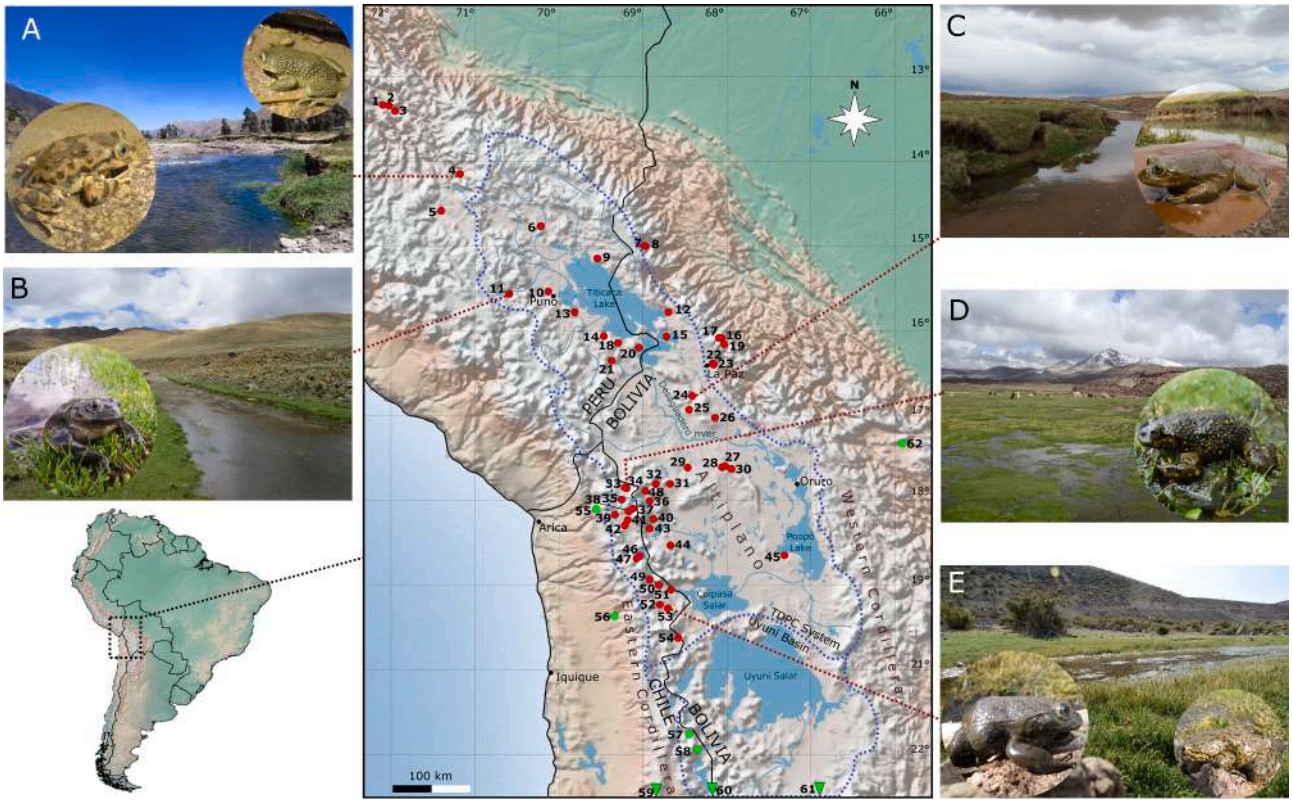

Fig. 1. Map of the sampling sites of the *Telmatobius marmoratus* complex and altiplanic species of the sister group. The numbers correspond to the names of Table 1. Photographs of some habitats are shown: (A) Sicuani, Vilcanota river (Cusco, Perú); (B) Lagunillas (Puno, Perú); (C) Comanche (La Paz, Bolivia); (D) Parinacota (Arica-Parinacota, Chile); (E) Toroni (Tarapacá, Chile).

visualized with UV light. DNA quantification was performed on a NanoDrop Lite™ spectrophotometry kit (Thermo Scientific).

For the construction of the DNA matrix, partial sequences of the mitochondrial genes 16S (±550 bp) and Cytb (±930 bp) were amplified with the same pairs of primers and PCR conditions used by Sáez et al., (2014). Partial sequences of two nuclear markers were also used: the recombination activating gene 1 (RAG-1 ± 850 bp; San Mauro et al., 2004), and intron 7 of the β-fibrinogen gene (BFIB ± 440 bp; Prychitko and Moore 1997), following the PCR conditions suggested by the authors of each primer.

Additionally, we looked for available sequences from previous studies in GenBank and included in the analyses (see supplementary material S1). The DNA sequence of each sample was reviewed, and all sequences were aligned using the Clustal W algorithm implemented in BioEdit version 7.0.5.3 (Hall, 1999) and then manually checked for inconsistencies. In addition, Xia's test (Xia et al., 2003) implemented in DAMBE6 (Xia, 2017) was used to evaluate the saturation of each gene matrix.

2.3. Phylogenetic reconstruction

Phylogenetic reconstructions were performed with the concatenated 16S, Cytb, RAG1 and BFIB sequences. Trees were rooted with *T. bolivianus* as the outgroup and altiplanic species of *T. pefauri* and *T. hintoni* groups were included as a sister of *T. marmoratus*. The GenBank accession numbers of the sequences used for rooting are shown in supplementary material (S1).

We performed phylogenetic reconstructions using the Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) algorithms. The MP reconstructions were performed in PAUP* 4.0 (Swofford, 2003), using a heuristic search with 100 replicates and adding each taxon randomly and with tree bisection reconnection (TBR). Node support was obtained using bootstrap with 1,000 pseudoreplicates, and we constructed a 50 % majority rule consensus tree. The Maximum Likelihood (ML) phylogenetic reconstruction was performed using the software IQ-TREE 1.6 (Nguyen et al., 2015) implemented on CIBIV web server (Trifinopoulos et al., 2016). For this, the prior substitution model was estimated by Model Finder (Kalyaanamoorthy et al., 2017) implemented in the same software for molecular characters. The best scheme for data partitioning considering nucleotide substitution models under AIC criterion and codon position was the follow: 16S, BFIB = K2P + I (partition 1: 1–552 2337–2776\3), Cytb = TN + F + G4 (partition 2: 553–1483\3) and RAG1 = TPM2u + F + I (partition 3: 1484–2336\3). Node support was obtained using SH-aLRT and ultrafast bootstrap (Hoang et al., 2018) with 1,000 replicates each one, where values ≥ 80 % and ≥ 95 % respectively are considered of high clade support (Guindon et al., 2010; Nguyen et al., 2015). Finally, we ran the BI tree with the program Mr.Bayes3 (Ronquist and Huelsenbeck, 2003) implemented on the web server CIPRES Science Gateway 3.3 (Miller et al., 2010). We first selected for each partition the best model with the Bayesian Information Criterion (BIC) in the program jModelTest 0.1.1 (Posada, 2008). The best models for the sequences were K80 for 16S, TrN + I + G for Cytb, TPM2 + I for RAG1 and K80 + I for BFIB; these models were used for each partition in BI analyses. We conducted four independent runs, with a setting of four chains, starting with a random tree, running for 100,000,000 million generations, and sampling every 10,000 trees. The initial 25 % of the resulting trees was discarded as burn-in. Once convergence of the four independent runs was confirmed by the average standard deviation of split frequencies and the potential scale reduction factor, results from the runs were combined to obtain a total of 30,004 trees. Finally, a consensus tree was constructed by a 50 % majority rule; the nodes were evaluated using posterior probabilities.

2.4. Species delimitation

We performed two different approaches for species delimitation: i) based on a single-locus, the mitochondrial cytochrome b gene; and ii) a multilocus analysis, based on two mitochondrial molecular markers (16S, Cytb) and two nuclear (RAG1, BFIB). For single-locus analysis, we constructed a matrix based on Cytb with representative haplotypes of each clade (31 haplotypes), based on Bayesian Inference. We used two different analyses to delimitate the lineages: first, we ran Automatic Barcode Gap Discovery software (ABGD), which statistically partition the samples into candidate species based on a barcode gap (i.e., a gap in the pairwise genetic distance distribution, presumably between intra-specific and interspecific distances, Puillandre et al., 2012). ABGD was performed through the web server of ABGD (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using Kimura 2-parameter model. A range between 0.001 and 0.25 of prior intraspecific divergence values was assayed (in 100 steps), applying a relative gap width of 0.5. Secondarily, we ran three different approaches of Poisson Tree Process: normal PTP, maximum likelihood PTP (mlPTP) and Bayesian PTP (bPTP). The original PTP can determine the transition point among the processes occurring between species and within a species using a two-parameter model, a parameter for speciation, and another for coalescent processes, so the adjustment of both parameters delimits the species in a given topology (Zhang et al., 2013). Finally, for the PTPs analyses, a ML tree was reconstructed in IQ-TREE (Nguyen et al., 2015) using the TN + F + G4 model of nucleotide substitution which was calculated by the same software. To perform the normal PTP we used the mPTP web server (https://mptp.h-its.org/), while for mlPTP and bPTP we use the bPTP web server (https://species.h-its.org/).

For the multilocus analysis we use the STACEY (Jones 2015, 2017) package from BEAST2 (Bouckaert et al., 2019). STACEY is a Bayesian method for inferring both species delimitations and species trees under the multispecies coalescent model (Rannala and Yang, 2003) under the birth–death-collapse tree prior and without the requirement of a guide tree and requires no a priori assignment of individuals to species. The analysis generates a 'species or minimal cluster tree' (SMC tree), where the tips represent minimal clusters of individuals (Jones et al., 2015) that can merge but not split to form potential species (candidate species). For this analysis we used a matrix of four loci: two mitochondrial (16S and Cytb) and two nuclear (RAG1 and BFIB) (sequences used are indicated in bold in Fig. 2). We incorporated species from the *Telmatobius marmoratus* complex, species from *T. pefauri* and *T. hintoni* groups (sister group), and *T. bolivianus* as an outgroup. This scheme allowed us to set a species delimitation threshold considering not only the ingroup, but also several other species from the genus. For each locus, the nucleotide substitution model obtained in jModelTest 0.1.1 (Posada, 2008) was selected (16S = HKY, RAG1 = JC, Cytb and FIB = GTR). A strict clock was defined for all loci using the default rate (1.0). MCMC run was performed with 500 million generations sampling trees and model parameters every 50,000 generations. Run was carried out on the web server CIPRES Science Gateway 3.3 (Miller et al., 2010), run convergence was checked in Tracer v1.7.2 (Rambaut et al. 2018). Trees were summarized onto a single target tree discarding first 25 % of each posterior sample using TreeAnnotator v2.6.7 (Bouckaert et al. 2014). Candidate species (or minimal clusters) were delimited from.trees file using the package SpeciesDelimitationAnalyser (speciesDA.jar) (burnin 25 %, -collapseheight = 0.0001, -simcutoff = 1.0), and plotted using the plot.simmatrix.R script (Jones et al., 2015) modified by Simon Crameri (https://github.com/scrameri/smtools/tree/master/SpeciesDelimitation) in R environment (R Core Team 2022) considering a PP. threshold = 0.1.

3. Results

The concatenated matrix contained a total of 132 sequences with 2776 bp (see details in supplementary material S2) (GenBank Accession

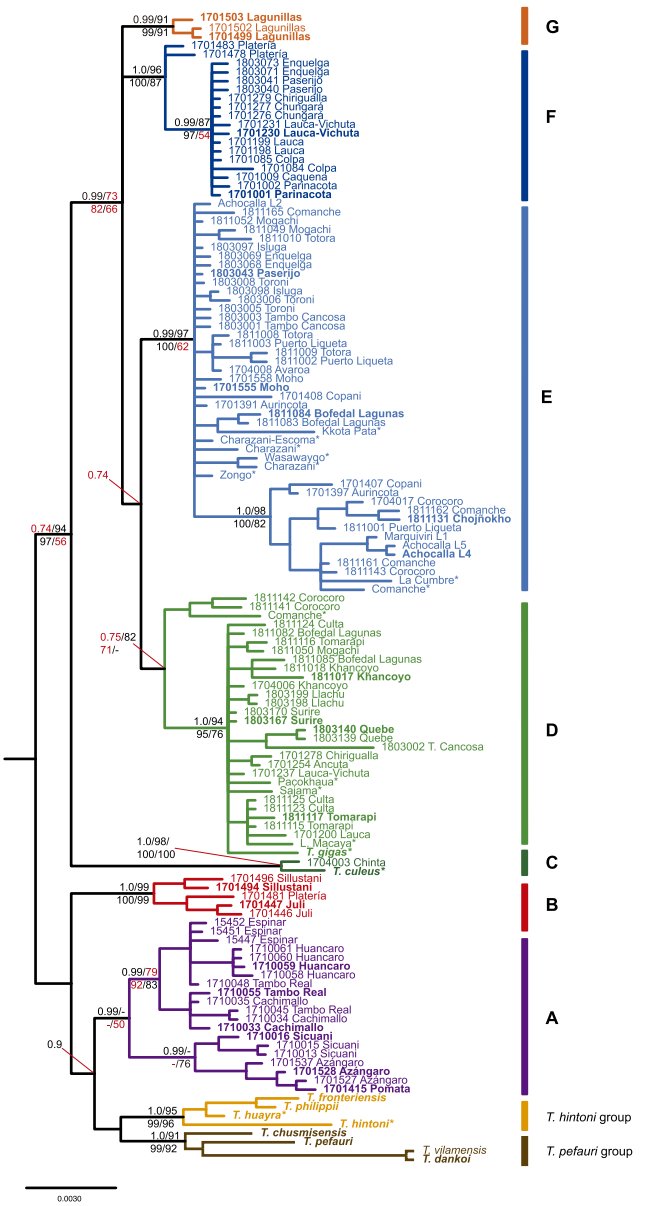

Fig. 2. Phylogenetic hypotheses for the *Telmatobius marmoratus* complex obtained using Bayesian Inference (BI) with mitochondrial and nuclear genes combined. The numbers above and below the nodes correspond to the support values of posterior probabilities of BI, SH-aLRT and ultrafast bootstrap of Maximum Likelihood and Maximum Parsimony, respectively. Letters A to G correspond to the lineages recovered in this study. Low support values are shown in red. The outgroup was removed from the figure. * = samples only with mtDNA obtained from GenBank (see supplementary material S1). Tips in bold = samples used in species delimitation analyses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Numbers 16S: OP099972-OP100079; Cytb: OP125646-OP125753; RAG1: OP163327-OP163413; BFIB:XXXX). In cases where a sequence was not available for any of molecular markers, it was treated as missing data, in particularly for the sequences of the nuclear genes of Bolivian species *T. bolivianus*, *T. hintoni*, *T. huayra* and some of *T. marmoratus* obtained from GenBank for which only the mitochondrial sequences were available (supplementary material S1). Additionally, the sequences of the four molecular markers showed low substitution saturation, therefore they are suitable for phylogenetic analyzes (supplementary material S1).

3.1. Phylogenetic reconstruction

The MP analysis considered 118 informative characters, and the search tree yielded more than 1,000 equally parsimonious reconstructions. The quality indices of the MP analysis were as follows: tree length = 264; consistency index (CI) = 0.4811; homoplasy index (HI) = 0.5189; retention index (RI) = 0.8933; rescaled consistency index (RC) = 0.4297. These indices reveal high levels of homoplasy. The ML (-ln likelihood = −5908,651) and BI analyses each produced a tree with similar topology, recovering the same lineages as the MP analysis.

The phylogenetic analyses of the samples of interest recovered seven lineages that were named with the letters A-G (Fig. 2). Lineage A is formed by two well-supported subgroups by BI and MP analysis: one formed by the samples of Sicuani, Azángaro and Pomata and another formed by the samples of Espinar, Huancaro, Tambo Real and Cachimallo. All these samples correspond to localities located in the Cusco region, except for Azángaro and Pomata, which are found in the Puno region, Peru (Fig. 1). Lineage A is closely related to the highland species groups *T. hintoni* and *T. pefauri* (*sensu* De la Riva et al., 2010; Fibla et al., 2017), although this relationship is weakly supported. Lineage B includes samples from three localities located near the western shore of Lake Titicaca: Sillustani, Platería and Juli (Puno, Peru) (Fig. 1) and would be related to the grouping formed by lineage A and the *hintoni* and *pefauri* groups. Both lineages, A and B, would be exclusive to the northern region of the Altiplano in Peru. Lineage C is formed exclusively by the species *Telmatobius culeus* and includes a sample obtained on the eastern shore of Lake Titicaca, Chinta River (Fig. 1).

The D-G lineages form a grouping well supported by the phylogenetic reconstruction methods used in this study, which is independent of the other clades assigned to *T. marmoratus* (Lineages A and B, Fig. 2). Lineage D is formed by two subgroups: a well-supported subgroup that includes *T. gigas* (De la Riva et al., 2010) and samples from 15 localities of the western and central regions of the Altiplano: Lauca, Ancuta, Chirigualla (Arica-Parinacota, Chile), Cancosa, Quebe, Surire, Llachu (Tarapacá, Chile), Tomarapi, Culta, Khancoyo, Bofedal de Laguna, Mogachi, Laguna Macaya, Sajama, Pacokhaua (Oruro, Bolivia) (Fig. 1). The other subgroup of lineage D is formed by samples from the locality of Corocoro and Comanche (Oruro, Bolivia); however, these grouping lacks support, as does its relationship with the rest of this lineage.

Lineage E (Fig. 2) was the most widely distributed since consists of samples from more than 20 locations: Copani, Aurincota (Puno, Peru), located near the southwestern shore of Lake Titicaca; Achocalla, Marquiviri (La Paz, Bolivia), located south of Lake Titicaca; and Comanche, La Cumbre, Corocoro, Puerto Liqueta, in the center of the plateau in the Andean Altiplano,central-southern highlands: Aurincota, Copani, Moho (Puno, Peru), Bofedal de Laguna, Mogachi, Puerto Liqueta, Totora, Avaroa, Comanche (one sample) (Oruro, Bolivia), Zongo, Charazani, Wasawayqo, Kkota Pata (La Paz, Bolivia), one sample from Achocalla (La Paz, Bolivia), Tambo Cancosa, Toroni, Isluga, Paserijo, Enquelga (Tarapacá, Chile) (Fig. 1).

Lineage F (Fig. 2) recovered samples from 10 localities present almost exclusively in southwestern Altiplano: Parinacota, Caquena, Colpa, Lauca sur, Lauca Vichuta, Chungará, Chirigualla (Arica y Parinacota, Chile), Paserijo, Enquelga (Tarapacá, Chile), except for Platería (Puno, Peru), which is located on the western shore of Lake Titicaca (Fig. 1).

Finally, the G lineage (Fig. 2) is composed only of samples from the town of Lagunillas, located in the vicinity of a series of three lagoons (Lagunillas, Sara Cocha and Ululunasa), approximately 70 km west of Lake Titicaca (Fig. 1).

The topological differences between the phylogenetic reconstructions are mainly found in the internal relationships of the lineages: i.e in the ML analysis the *T. hintoni* and *T. pefauri* groups are the sister of C to G lineages (supplementary material S3), whereas the MP analysis that relations are not resolve at all (supplementary material. material S4). In the ML analysis, lineage A was not monophyletic, but appears in three different groups (supplementary material S3). However, each of these three groups is in agreement with observed groupings within lineage A in the Bayesian inference analysis (Fig. 2).

In summary, phylogenetic reconstruction groups all the samples of interest into three main groups (A, B and D to G lineages), where all the samples from the region of Cusco, Peru are in the A and B lineages (Fig. 2), and only Group A is associated to *T. hintoni* and *T. pefauri* groups; but the vast majority of the samples of interest of the study area are found forming a well-supported clade that includes the D - G lineages, closely related to *T. culeus* (Lineage C, Fig. 2) and *T. gigas* (Lineage D, Fig. 2).

3.2. Species delimitation

Figure 3 (See Fig. 3) summarizes the results derived from the four analyzed of single-locus approaches (PTP, mlPTP, bPTP and ABGD) and multi-locus analysis (STACEY) in comparison with the ML tree obtained from the Cytb marker. In our analyses, the lineage of the *T. hintoni* group (orange) and *T. pefauri* (brown) was not considered candidate species because they do not belong to the focus taxon. The bPTP analysis reached the highest number of candidate species (CS), suggesting 14 hypothetical entities, while the PTP, mlPTP and ABGD results were consistent with each other, generating 11 CS each. The differences between these analyses lie in lineage A, where the bPTP analysis recognizes five entities, *versus* three, of the other analyses (Fig. 3), and in lineage G, where the bPTP analysis recognizes two CS, unlike the other analyses that recognize only one (Fig. 3). These results reveal ten CS (excluding *T. culeus*) within the *T. marmoratus* complex of which eight would be new entities.

The SMC tree obtained in STACEY recovered nine clusters excluding the outgroup *T. bolivianus* (Fig. 4a), three of which correspond to *T. culeus* and to the *Telmatobius hintoni* and *T. pefauri* groups and all others belong to the *Telmatobius marmoratus* complex. The similarity matrix obtained in SpeciesDelimitationAnalyser shows six candidate species for the *T. marmoratus* complex, one of those correspond to *T. gigas* (Fig. 4b).

4. Discussion

Our results suggest an alternative scenario for what has been proposed thus far for the *Telmatobius marmoratus* complex, and supports the discovery of new lineages that correspond completely or partially with the currently recognized taxa.

Telmatobius marmoratus was described from the locality of Guasacona, near Azángaro, Puno, Peru, based on specimens collected in expeditions by the geographer and naturalist J.B Pentland between 1830 and 1833. The original description is succinct and highlights the following morphological characters: hind limbs “extending beyond the end of the snout”, “males with larger front limbs than females”, “skin perfectly smooth”, “males with a gland on the first digit”, and “black marble on a gray background that tends toward light brown” (Duméril and Bibron 1841). This last characteristic gave the name to the species. Subsequent studies expanded its distribution and showed great morphological variability through a long list of synonymy and subspecies, which has made its identification difficult (see Section 1). More recently, De la Riva (2005) proposed a key in which *T. marmoratus* (adult) can be distinguished (in the field) by presenting the following set of characters: “rounded snout, ventral surfaces of limbs never yellow or orange, plantar surfaces never with keratinized spicules; and body size up to 66.5 mm”. These characteristics were observed in the adult specimens sampled in this study and that constitute lineage A (Fig. 2) (however these are not unique to this lineage). Therefore, based on these descriptions and their type locality, the naming of *T. marmoratus sensu stricto* (*s.s*) would correspond to a lineage that inhabits only the north-central region of the Andean Altiplano (Lineage A, Fig. 2), which is related to a new lineage that lives exclusively on the western shore of

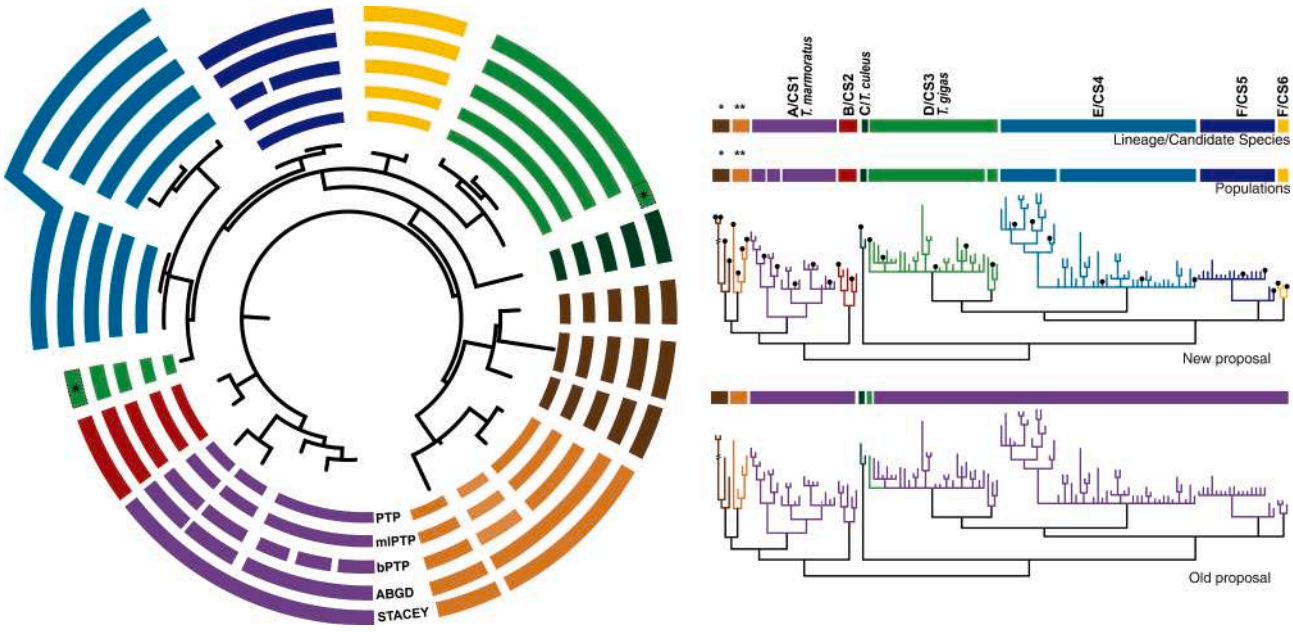

Fig. 3. Candidate species of the *Telmatobius marmoratus* complex according to the species delimitation analyzes of PTP, mlPTP, bPTP,ABGD and STACEY. Old proposal = current taxonomy. New proposal = candidate species (1–11). * = *Telmatobius pefauri* group. ** = *Telmatobius hintoni* group.

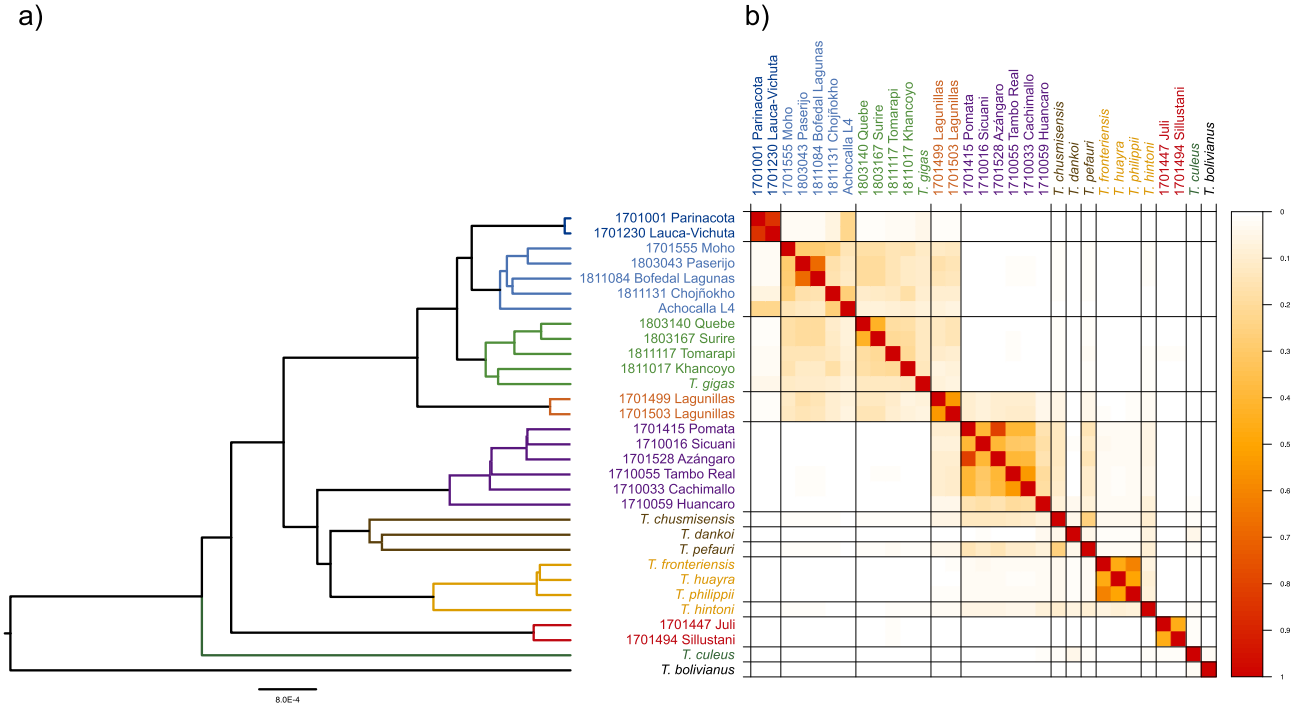

Fig. 4. a) Minimal Cluster Tree based on two mitochondrial (16S and Cytb) and two nuclear (RAG1 and BFIB) loci with b) similarity matrix obtained in Species Delimitation Analyser. The color scale shows the posterior probability of two pairs of individuals to belonging to the same cluster (species). Red is for 1.0 posterior probability and white for 0.0. The colors of the tips correspond to the phylogeny groups in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Lake Titicaca (Lineage B, Fig. 2), but how are related is not fully clear.

This has as an immediate consequence that the lineages thus far known and attributed to *T. marmoratus* and that inhabit the central-southern high Andean region (De la Riva et al., 2010; Sáez et al., 2014; Victoriano et al., 2015) correspond to a set of independent lineages not related to *T. marmoratus s.s* and whose monophyly is well supported (lineages D-G, Fig. 2). These lineages are more related to the lacustrine species *T. culeus*, a species that has been validated with different types of evidence (e.g., Benavides et al., 2002; Victoriano et al., 2015). This group consists of four lineages (lineages D-G, Fig. 2), three of which are partially congruent with previous molecular studies under the name of the *T. marmoratus* complex (Sáez et al., 2014; Victoriano et al., 2015). The other lineage is completely new and inhabits exclusively in Lagunillas on the western shore of Lake Titicaca (Lineage G, Fig. 2).

Additionally, our results suggest that part of the sampled populations of Sajama National Park (Bofedal de Laguna, Tomarapi River, Mogachi; Fig. 1), as well as other populations located on the western slopes of the Andes (Lauca River [the southern part], Chirigualla, Surire, Llachu, Quebe, Tambo Cancosa; Fig. 1), some of which were previously assigned to *T. marmoratus* (De la Riva et al., 2010; Sáez et al., 2014), would correspond to *T. gigas* (Lineage D, Fig. 2). Although De la Riva et al. (2010) showed that the populations of Sajama National Park are phylogenetically closer to *T. gigas* than to *T. marmoratus*, they do not recognize these populations as *T. gigas*, alluding to the fact that there are differences in external morphology. However, the authors do not show comparative data between these populations that support these differences. Although our study also does not show morphological data, due to the problems discussed above (see Section 1), the species delimitation analyses support this hypothesis (Fig. 3). The differences between *T. gigas* and other individuals of lineage D could be interpreted as intraspecific polymorphisms, ontogenetic differences or phenotypic plasticity. Considering that the populations grouped in lineage D correspond to *T. gigas*, the lineages that inhabit the central-southern high Andean region, E - G, are closer to *T. gigas* than to *T. marmoratus s.s* (Fig. 2). Field observations support the closeness of these lineages with *T. gigas* due to the finding of a large female specimen (Fig. 4-H: 105 mm SVL [Snout-vent length]) in the locality of Toroni (Tarapacá, Chile; Fig. 1). This large form has been described for *T. gigas*, but as already mentioned, there is great variability in the external morphology that hinders its differentiation from *T. marmoratus*, which is why it was initially described as a subspecies of *T. marmoratus* (Vellard, 1969). Examples of these variations are observed in Fig. 5-G and Fig. 5-H, both specimens collected in the locality of Toroni.

It is interesting to observe that in some areas of the western slopes of the Andes, it is possible to recover several sympatric lineages. An example of this is the Salar de Coipasa basin and its tributaries (Isluga and Tambo Cancosa Rivers), where individuals belonging to the D, F and G lineages are observed. The lower part of this area has been flooded

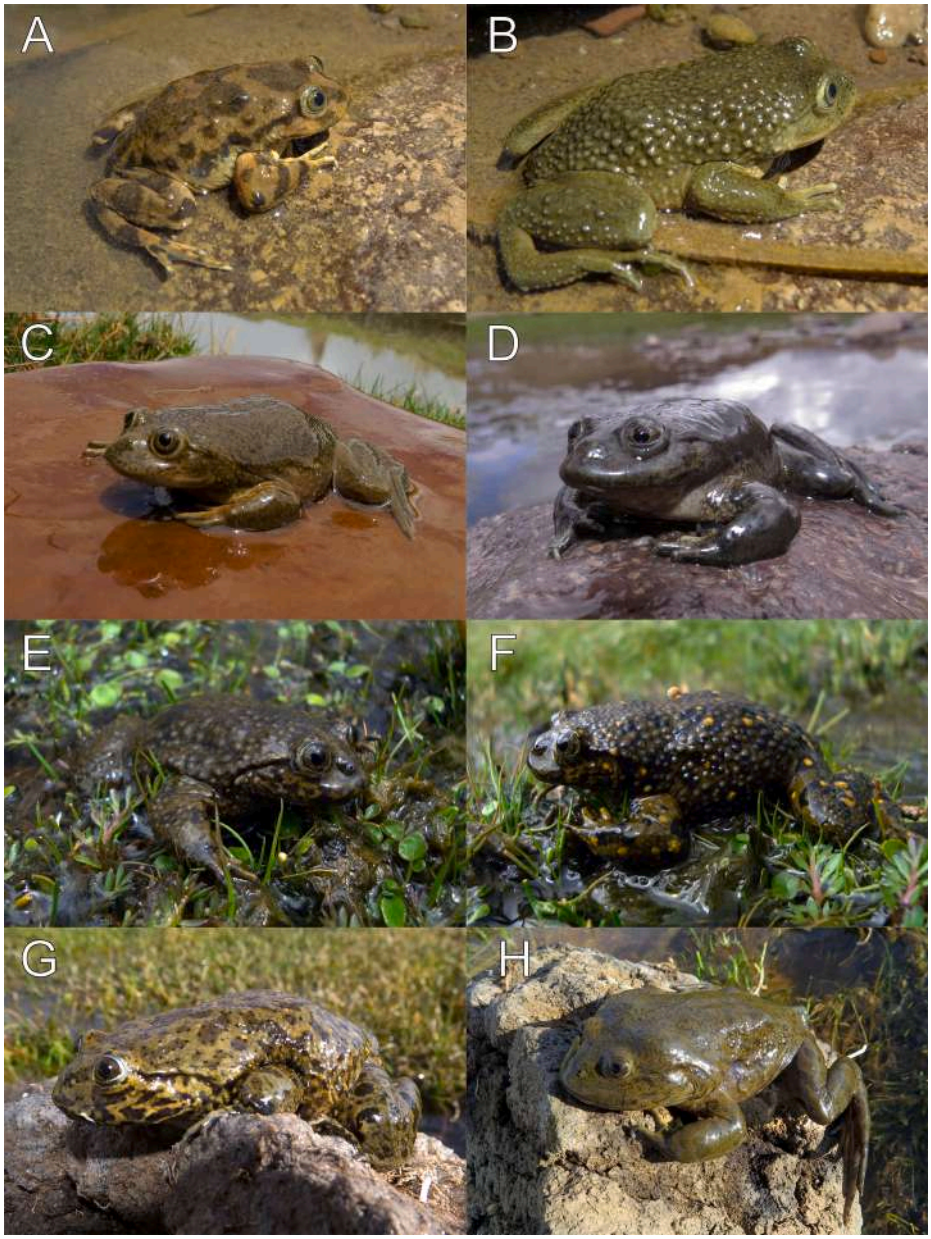

Fig. 5. Overview of variability in body color of *Telmatobius marmoratus* observed in different habitats from: (A, B) Sicuani, Vilcanota river (Cusco, Perú); (C) Comanche (La Paz, Bolivia); (D) Lagunillas (Puno, Perú); (E, F) Parinacota (Arica-Parinacota, Chile); (G, H) Toroni (Tarapacá, Chile). Photos autorship: A-C, Paola Sáez; D-H, Pablo Fibla.

successively by cycles of paleolakes in the last 130 ky BP (e.g., Ouki, Tauca and Coipasa) (Placzek et al. 2013) and constitutes a volcanic zone with evidence of activity cycles during the last 1.7 My (Cascante, 2015; Wörner et al., 2000.), events that have fragmented and/or connected aquatic systems on a recurring basis. A similar pattern is observed in the Lauca River basin, where individuals of lineages G and D are observed. In addition, lineages D, E and F account for connections between the western slopes of the Andes and the center of the Altiplano (Corocoro, Comanche).

It has been suggested that one of the main factors shaping the distribution patterns of the freshwater biota of the Andean Altiplano has been the hydrological history of the region. The existence of climatic processes that resulted in wet periods alternating with dry periods during the Pleistocene formed expansive lacustrine systems, which connected in the center and south of the region (Lavenu et al., 1984; Fornari et al., 2001; Fritz et al., 2004; Rigsby et al., 2005; Placzek et al., 2006, 2013). Thus, these variations in the levels of the lacustrine systems added to the repeating connecting and isolating would have induced vicariance processes (Northcote 2000; Lüssen et al., 2003; Vila et al., 2013; Collado et al., 2011), but would also have facilitated secondary contact events (Vila et al., 2013). Our evidence supports these isolation-connection patterns, suggesting multiple colonization events to or from the Lake Titicaca basin and/or connections between the center of the Altiplano and the western slopes, corroborating the pattern suggested by Victoriano et al. (2015), who suggests a historical migratory flow from the western Andes to the east in Bolivia. Thus, the localities that have individuals belonging to different lineages (see above) are interesting from the biogeographical point of view, since they would represent remnants of the ancient connections of the aquatic systems, but they are also interesting from a conservation point of view, because they are places of interest for maintaining genetic diversity, since they are capable of sustaining different sympatric lineages. Under our evidence, we cannot discard event of secondary contact between clades, particularly the closest lineages with most recent divergences, which some of the nucleotide sequences are geographically shared.

Although our phylogeny is the most detailed with molecular data for the highland *Telmatobius* species, it still presents gaps when establishing the deepest relationships in the tree, which generates inconsistencies between the topologies obtained with the ML, MP and BI analyses (see supplementary material S3 and S4 for ML and MP, respectively). For example, the differences observed for the lineage A suggest: a) the need to carry out new specific field work to obtain new material from these localities, and b) the need to add more characters, including new molecular markers and other types of evidence. As well as the relationships within this clade formed by the groups *T. marmoratus*, *T. pefauri* and *T. hintoni* are still ambiguous. This could be explained because it is a partial phylogeny that lacks the *Telmatobius* species in its southern distribution in Argentina and in its northern distribution in Peru and Ecuador, which could help to resolve the oldest relationships between the groups of highland species. However, our molecular evidence supports the current taxonomic conception that the *T. hintoni* and *T. pefauri* groups are part of the *T. marmoratus* group, as suggested by De la Riva (2005, 2010) and Barrionuevo (2017).

To date, the most complete phylogenetic hypothesis for *Telmatobius* is based on morphological characters and includes 43 species (Barrionuevo, 2017). This phylogeny recovers three of the four groups of *Telmatobius* recognized in previous molecular studies, *T. verrucosus*, *T. bolivianus* and *T. marmoratus* (*sensu* De la Riva et al., 2010), but obtained a low phylogenetic resolution in some clades and/or species. In the case of highland species, this study fails to recognize the *T. hintoni* and *T. pefauri* groups and places them within the *T. marmoratus* group, where most of the internal relationships are not resolved (Barrionuevo, 2017). Apparently, the morphological characters thus far addressed in different studies lack a phylogenetic signal. Even the karyotype seems to be highly conserved among *Telmatobius* species (e.g., Veloso et al., 1982; Northland et al., 1990; Formas et al., 1999; Cuevas and Formas, 2002; Formas et al., 2003), demonstrating how difficult it is to find traits that can help elucidate the evolutionary history of this group of amphibians.

Molecular evidence has been one of the most efficient tools for the delimitation of *Telmatobius* lineages; therefore, there is a clear decoupling of genetics and morphology due to the polyphyletic condition of *T. marmoratus*. This evidence fits well with the phenomenon of cryptic species (Bickford et al., 2007). One of the explanations for the morphological overlap of this group of highland *Telmatobius* species could be explained by a recent divergence (Bickford et al., 2007; Struck et al., 2017). In this sense, the morphological decoupling between related species would be strongly related to genetic divergence (Struck et al., 2017). On the other hand, as a consequence of extreme environmental conditions and the action of stabilizing selection, convergence/evolutionary parallelism is a factor producing morphological crypsis (Bickford et al., 2007; Zuñiga-Reinoso and Méndez, 2018). This is because the ancestor sets the key characters for survival in extreme environments and is subsequently conserved by the descendants, allowing diversification in that extreme environment (Zuñiga-Reinoso and Méndez, 2018; Zúñiga-Reinoso and Predel, 2019). Maintaining ancestral traits would also be related to the tendency of the lineages to maintain their ancestral ecological niche (phylogenetic niche conservatism; Wiens, 2004), and this could contribute to explaining the existence of geographically isolated lineages (e.g., lineages A-B and lineages C-G).

Traditionally, the Andean Altiplano region has been considered an extreme environment due to the climatic conditions associated with the altitude, and if we add to this that it has been a historically unstable zone due to the recent climatic oscillations of the Plio-Pleistocene, then it is the perfect setting for the cryptic diversification of this recent clade of *Telmatobius*. Apparently, diversification in cryptic species is a relatively common pattern in high Andean systems (e.g., Collado et al., 2013, 2016; Zuñiga-Reinoso and Méndez, 2018), and this is one more piece of evidence that supports this pattern. Additionally, mainly based on the presence of the diagnostic characters in all populations historically considered to be *T. marmoratus,* the morphological convergence toward the morph of *T. marmoratus* has occurred repeatedly within the phylogeny. Probably the most recent common ancestor of the clade that brings together these highland species has had this morph, while those different from *T. marmoratus* could be the evolutionary novelties, as in the case of *T. culeus*, a form adapted to aquatic life at depth. Therefore, future morphological studies should evaluate the similarities of the diagnostic traits in these genetically distinguishable groups to corroborate this pattern.

In comparison with the current taxonomy, the results of the species delimitation analyzes are dissimilar since, to date, only two entities have been described within the complex: *T. marmoratus* and *T. gigas*; and one closely related species, which is part of the lineage of the group *T. marmoratus sensu* De la Riva et al., (2010): *T. culeus* (Fig. 3). Our results suggest between six to eleven candidate species (CS) within the *T. marmoratus* complex (excluding *T. culeus*) depending of the approach used for the species delimitation. We suggest to keeping a more conservative perspective of our results (the multilocus approach) which fit with the main lineage found in the phylogenetic tree. Therefore, we used these results for a new systematic proposal in the *T. marmoratus* complex. However, the single-locus species delimitation analysis highlights a remark genetic diversity structure at the intraspecific level: intraspecific structure or populations (i.e POP). Under this scenario, the lineages describe for the phylogenetic tree match with the CS (Fig. 4). Thus, the lineage A belongs to the CS-1, with three well-structured populations: POP-1 which would correspond to *T. marmoratus* s.s from Azángaro, POP-2 from Sicuani and Vilcanota River and POP-3 from Espinar, Huancaro, Tambo Real and Cachimallo. Both single-locus and multilocus approaches recognized lineage B as a single CS-2, which is present in Sillustani and Juli localities. In the central and southern regions of Altiplano, four CS are recognized, where CS-3 corresponds to *T. gigas* which contain to well-structured populations (D lineage). Therefore,

three new species are proposed in this region: CS-4 has a large distribution on the southernmost part of the distribution of the *T. marmoratus* complex, this CS also present two populations (E lineage). The CS-5 is present in the Andes of Parinacota and Lauca river (F lineage). Finally, CS-4 being exclusive to the locality of Lunulas (G lineage) (Figs. 3 and 4).

Some considerations are necessary to discuss concerning the species delimitation proposed. We like to emphasize that we nominate a lineage as CS, we propose a species hypothesis that must contrast with complementary (new) evidence. For instance, if we consider geographic criteria and monophyly, only 3 of the 6 (CS 1, 3 and 5) CS are clearly distinguishable. Therefore, its validation depends on the use of other lines of evidence. Interestingly, several of these CS proposed corresponds to subspecies described for *T. marmoratus*. For example, POP-2 of the CS-1 would correspond to the subspecies *T. m. pseudojelskii* (Vilcanota River); CS-2 would partially correspond to the subspecies *T. m. riparius* (Juli); and the POP-1 of the CS-6 partially corresponds to *T. m. rugosus* (Moho). All these subspecies were described based on morphological traits; therefore, this finding reveals correspondence with the genetic evidence that should be analyzed in new studies incorporating a more detailed analysis of these populations.

The finding of crypsis also has important consequences for the conservation of *Telmatobius* because the vast majority of species of the genus are under some category of threat. According to our results, some of the current conservation statuses should be reevaluated. For example, *T. marmoratus s.s* would have a considerably more limited distribution, making it more susceptible to events that threaten its subsistence. In contrast, the distribution range of *T. gigas* (Endangered, IUCN, 2020) would expand toward the western slopes of the Andes in Chile, which implies such distant populations could be genetically differentiated and constitute different management units and should therefore be treated independently. In addition, the different entities found (CS) could require specific conservation strategies, especially considering that the high Andes presents different threats, including the introduction of exotic species (in particular trout, *Oncorhynchus mykiss* and *Salmo trutta*; Lobos et al., 2020), the presence of agriculture and livestock, the absence of wastewater treatment plants, the strong demand for water for human consumption, and especially for mining activity, as is the case in Chile (IUCN, 2021, Méndez et al., 2020). In addition, there is the stressor of climate change, since forecasts for the end of the century include increases in temperature, frequency of extreme events, change in the rainy periods and reduced soil moisture (Valdivia et al., 2013). Therefore, the new challenge is to investigate other characteristics that allow defining the entities proposed here and thus formally describe the diversity of *Telmatobius* species present in the Andean Altiplano.

5. Conclusions

The comprehensive genetic diversity sampling of the *T. marmoratus* complex allowed us present a systematic scenario that requests the development of further studies for these marbled water frogs. The traditional concept of *T. marmoratus* was an artificial proposal that is not recovered as a monophyletic group in our phylogenetic analyses. In the whole distribution of this species complex (from 13° to 19°S), the species *marmoratus*-like appear several times in the phylogeny and are related to other species of the genus. Apparently, there is a tendency to the morphological convergence in the Altiplano lineages and this may explain the known taxonomic problems. Additionally, the recent speciation and the ancient water connections have important roles in the cryptic pattern found. The next challenges will be to perform accurate morphological studies to find the diagnostic characters of the candidate species suggested, and a biogeographic approach for a better understanding of the Altiplano history. Finally, conservation policies must be re-evaluated since the different units found may be subject to different threats and therefore require differential management.

CRediT authorship contribution statement

Paola A. Sáez: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Álvaro Zúñiga-Reinoso:** Writing – original draft, Validation, Formal analysis. **Pablo Fibla:** Formal analysis, Writing – review & editing. **Franco Cruz-Jofré:** Writing – review & editing. **César Aguilar:** Writing – review & editing. **James Aparicio:** Writing – review & editing. **Juan Carlos Cusi:** Writing – review & editing. **Katherin Otálora:** Writing – review & editing. **Marco A. Méndez:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data will be available in GenBank Database

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Appendix A. Supplementary material

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SP2-024 Sector Rinconada de Caquena, Región de Arica y Parinacota

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ANTECEDENTES

En el humedal altoandino ubicado en el sector de Rinconada de Caquena se ha registrado una especie de rana acuática, *Telmatobius marmoratus* (Sáez et al. 2014, Victoriano et al. 2015, Sáez et al. 2022, ***se entregan los manuscritos***). Esta especie tiene la distribución más amplia de los anuros del género *Telmatobius* en el Altiplano Andino (13º- 19º S de latitud, aproximadamente), sin embargo, ha sido escasamente estudiada y existen dudas sobre la afiliación taxonómica de las poblaciones que habitan en Chile (Sáez et al. 2022). De acuerdo con el reglamento de Clasificación de Especies del Ministerio del Medio Ambiente de Chile *T. marmoratus* se considera como Vulnerable (DS N°42/2011), mientras que la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza (UICN, 2020) la considera En Peligro.

Fotografía 1. Humedal de Caquena.

Fotografía 2. Humedal de Caquena.

Fotografía 3. *Telmatobius marmoratus* fotogradiados en Humedal de Caquena.

En el humedal de Caquena también se ha registrado a la especie de anfibio *Pleurodema marmoratum* (Cei 1957), clasificada como En Peligro-Rara por el Reglamento de Clasificación de Especies (DS N°50/2008), y Vulnerable por la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza (UICN, 2020).
Otra especie que se ha asociado a este humedal altoandino es el pez de agua dulce *Orestias* sp. (Cárcamo-Tejer et al. 2021).

PROPUESTA DE CONSERVACIÓN

El humedal altioandino de Rinconada de Caquena es de alta relevancia para la protección de especies amenazados de Chile. La falta de acceso a este sector, ha permitido mantener las buenas condiciones ambientales, por lo que estudios más profundos de la flora y fauna deberían entregar más antecedentes para la presencia de otras especies (felinos, carnívoros y roedores). Se adjunta archive.KMZ referencial.

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Symposium Article

Evolution and Conservation on Top of the World: Phylogeography of the Marbled Water Frog (*Telmatobius marmoratus* Species Complex; Anura, Telmatobiidae) in Protected Areas of Chile

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Abstract

The Andean Altiplano has served as a complex setting throughout its history, driving dynamic processes of diversification in several taxa. We investigated phylogeographic processes in the *Telmatobius marmoratus* species complex occurring in this region by studying the geographic patterns of genetic variability, genealogies, and historical migration, using the cytochrome b (cyt-b) gene as a marker. DNA sequences from *Telmatobius gigas* and *Telmatobius culeus*, Bolivian species with an uncertain taxonomic status, were also included. Additionally, we evaluated the phylogenetic diversity (PD) represented within Chilean protected areas and the complementary contribution from unprotected populations. Phylogenetic reconstructions from 148 cyt-b sequences revealed 4 main clades, one of which corresponded to *T. culeus*. *T. gigas* was part of *T. marmoratus* clade indicating paraphyletic relationships. Haplotypes from Chilean and Bolivian sites were not reciprocally monophyletic. Geographic distribution of lineages, spatial Bayesian analysis, and migration patterns indicated that *T. marmoratus* displays a weaker geographic structure than expected based on habitat distribution and physiological requirements. Demographic and statistical phylogeography analyses pointed out to a scenario of recent population expansion and high connectivity events of a more recent age than the post Last Glacial Maximum, probably associated to more humid events in Altiplano. PD of *T. marmoratus* populations within protected areas represents 55.6% of the total estimated PD. The unprotected populations that would contribute the most to PD are Caquena and Quebe (21%). Recent evolutionary processes and paleoclimatic changes, potentially driving shifts in habitat connectivity levels and population sizes, could explain the phylogeographic patterns recovered herein.

Resumen

El altiplano andino ha funcionado como un complejo escenario a través de su historia, el cual ha involucrado procesos dinámicos de diversificación en varios taxa. Nosotros investigamos los procesos filogeográficos en el complejo de especies *Telmatobius marmoratus* que existen en esta zona, mediante el estudio de patrones geográficos de variabilidad genética, genealogías y migración histórica, mediante el uso del gen mitocondrial citocromo-b (cit-b). También incluimos secuencias de ADN de *Telmatobius gigas* y *T. culeus*, especies bolivianas con un estatus taxonómico incierto. Adicionalmente, evaluamos la diversidad filogenética (DF) representada dentro de las áreas protegidas de Chile y la contribución por complementariedad de parte de áreas no protegidas. Las reconstrucciones filogenéticas de 148 secuencias de cit-b recuperaron cuatro clados principales, uno de los cuales correspondió a *Telmatobius culeus*. La especie *T. gigas* fue parte del clado *T. marmoratus*, sugiriendo una relación parafilética. Los haplotipos de localidades chilenas y bolivianas no conformaron grupos recíprocamente monofiléticos. La distribución geográfica de linajes, análisis bayesianos geográfiamente explícitos y los patrones de migración, sugirieron para *T. marmoratus* una menor estructura genética que la esperada en función de su distribución de habitat y requerimientos ecológicos. Análisis demográficos y de filogeografía estadística reconstruyeron un escenario de expansión poblacional reciente y eventos de alta conectividad de edad menor que el término del Último Máximo Glacial, probablemente asociados a períodos más lluviosos en el altiplano. La DF de las poblaciones de *T. marmoratus* dentro de las áreas protegidas representan un 55,6% del total estimado para Chile. Las poblaciones no protegidas que contribuirían con mayor DF son Caquena y Quebe (21%). Cambios paleoclimáticos recientes habrían modulado variaciones en la conectividad del hábitat y en los tamaños poblacionales, lo que podría explicar el patrón filogeográfico recuperado.

Subject areas: Conservation genetics and biodiversity; Population structure and phylogeography
Key words: Andean Altiplano, Chile, Phylogenetic Diversity, *Telmatobius*

Geological history and paleoclimatic fluctuations can be determinants of the current patterns of genetic diversity and the geographic distribution of lineages within a species (Sérsic et al. 2011). Tectonic uplift events that result in the formation of mountain ranges generally lead to the occurrence of complex reliefs that could subsequently act as barriers. Nevertheless, climatic fluctuations during the Quaternary have been recognized as the main historic processes influencing spatial patterns of genetic diversity (Hewitt 1996, 2004). According to Gregory-Wodzicki (2000), the Altiplano Plateau extends from 15°S to 24°S at over 3700 m.a.s.l. and about 250 km wide (Figure 1). The Andean Altiplano is the second highest plateau on Earth and, while it originated during the Cretaceous period, it did not reach its current height until the Plio-Pleistocene (Gregory-Wodzicki 2000; Moon 2008). Therefore, it has long existed as an element that, combined with contrasting and cyclic climates (Ochsenius 1986), would have acted as a dynamic generator of species diversity. Evidence for the latter is the high degree of species richness within the fish genera *Orestias* and *Trichomycterus* (Vila et al. 2011, 2013) and in aquatic gastropods (Collado et al. 2013). Although high species diversity has been noted, the evolutionary mechanisms related to the origin of this diversity remain poorly understood. Previous studies suggest allopatric speciation associated to vicariant events and historical fragmentation of previously continuous aquatic environments as the main drivers of this diversification (e.g., Vila et al. 2011; Collado et al. 2013). Unfortunately, few studies have explored the biodiversity and diversification processes that occur in the Andean Altiplano system, despite its interesting geological history and its peculiarities. Recent studies have uncovered the occurrence of cryptic diversity in mollusks within this region (Collado et al. 2013), suggesting that hidden diversity could also be present in other biological groups in this area.

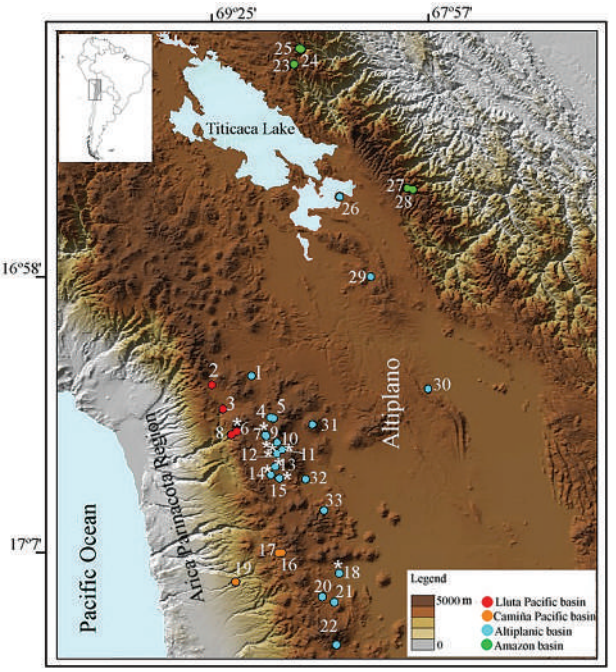

Figure 1. Sampled locations for the *Telmatobius marmoratus* species complex considered in this study. The numbers correspond to the site numbers detailed in Table 1. Color codes correspond to basin systems, which are detailed in Table 1. *Locations within protected areas as detailed in Table 1.

Table 1. Sites of sampled mtDNA cyt-b haplotypes for the *Telmatobius marmoratus* species complex, grouped by country, region/district, site name and geographical coordinates, and basin category

Site	Country	Región/district	Locality	Latitude	Longitude	Basin	*N*	Cluster	Haplotype
1	Chile	Arica y Parinacota	Umaqui	17°44'	69°23'	Altiplanic basin	3	2	**H29**, H31
2	Chile	Arica y Parinacota	Surapalca	18°43'	69°25'	Pacific basin	4	2	H7
3	Chile	Arica y Parinacota	Allane	17°59'	69°37'	Pacific basin	10	2	H5
4	Chile	Arica y Parinacota	Colpa	18°03'	69°13'	Altiplanic basin	2	2	**H1**
5	Chile	Arica y Parinacota	Caquena	18°03'	69°12'	Altiplanic basin	6	2	H14
6	Chile	Arica y Parinacota	Pacollo[a]	18°10'	69°30'	Pacific basin	5	2	**H18**
7	Chile	Arica y Parinacota	Lauca[a]	18°32'	69°09'	Altiplanic basin	4	2	**H3, H18**
8	Chile	Arica y Parinacota	Putre	18°11'	69°33'	Pacific basin	4	2	**H3**
9	Chile	Arica y Parinacota	Parinacota[a]	18°12'	69°16'	Altiplanic basin	12	2	**H3**
10	Chile	Arica y Parinacota	Malpaso[a]	18°15'	69°10'	Altiplanic basin	1	2	**H3**
11	Chile	Arica y Parinacota	Chungará[a]	18°18'	69°8'	Altiplanic basin	9	2	**H3**
12	Chile	Arica y Parinacota	Chiriguaya[a]	18°20'	69°10'	Altiplanic basin	10	2	**H12**, **H18**, H21, H30
13	Chile	Arica y Parinacota	Ancuta[a]	18°26'	69°11'	Altiplanic basin	5	2	**H3**, **H12**, H18, H29
14	Chile	Arica y Parinacota	Lauca Vichuta[a]	18°30'	69°13'	Altiplanic basin	1	2	H11
15	Chile	Arica y Parinacota	Lauca Sur[a]	18°32'	69°09'	Altiplanic basin	2	2	H25
16	Chile	Tarapacá	Pumiri	19°6'	69°8'	Pacific basin	5	4	**H2,H8**
17	Chile	Tarapacá	Toculla	19°7'	69°10'	Pacific basin	3	4	**H2,H8**
18	Chile	Tarapacá	Isluga[a]	19°15'	68°42'	Altiplanic basin	20	3	**H1**,H10,**H12**,H13,H22
19	Chile	Tarapacá	Quebrada Tana	19°22'	69°32'	Pacific basin	4	4	**H2,H8**
20	Chile	Tarapacá	Quebe	19°27'	68°48'	Altiplanic basin	16	4	H4,H24
21	Chile	Tarapacá	Toroni	19°30'	69°42'	Altiplanic basin	2	4	**H1**,H9
22	Chile	Tarapacá	Cancosa	19°57'	68°41'	Altiplanic basin	8	4	H6,**H17**
23	Bolivia	La Paz	Rio Wasawayqo	15°10'	68°59'	Amazon basin	1	1	**H16**
24	Bolivia	La Paz	Charazani	15°10'	69°0'	Amazon basin	1	1	**H17**, **H16**
25	Bolivia	La Paz	Charazani Escoma	15°13'	69°2'	Amazon basin	2	1	H20
26	Bolivia	La Paz	Lago Titicaca	16°15'	68°44'	Altiplanic basin	1	1	H15
27	Bolivia	La Paz	Zongo	16°16'	68°6'	Amazon basin	1	1	**H1**
28	Bolivia	La Paz	KkotaPata	16°16'	68°4'	Amazon basin	1	1	H28
29	Bolivia	La Paz	Comanche	16°58'	68°25'	Altiplanic basin	1	1	H19
30	Bolivia	Oruro	Huayllamarca	17°50'	67°57'	Altiplanic basin	1	1	H27
31	Bolivia	Oruro	Sajama	18°6'	68°59'	Altiplanic basin	1	1	H23
32	Bolivia	Oruro	Lago Macaya	18°33'	68°56'	Altiplanic basin	1	2	H26
33	Bolivia	Oruro	Rio Packohaua	18°45'	68°41'	Altiplanic basin	1	3	**H18**
							148		

Bold haplotypes are present in more than one site.
Cluster, genetic group assigned by Geneland; *N*, sample size.
[a]Locations within protected areas as indicated in Figure 1.

Genetic variation is widely recognized as one of several currencies for the evaluation of diversity (Ehrlich & Wilson 1991; Humphries et al. 1995), and the protection of diversity is incorporated into many national and international conventions (Moritz & Faith, 1998). For single species conservation, it is important to identify the evolutionary relationship between populations in order to retain the maximum genetic diversity and to incorporate information on historical population processes (Avise 1989, Moritz 1994, 1995). The use of molecular data in phylogeographic studies has led to the discovery of many cryptic phylogeographic lineages (Daniels et al. 2003), usually not reflected in morphological variation. By combining the resulting gene trees with the geographic location at which each individual was sampled, one can elucidate the geographical distributions of major gene lineages that comprise a gene tree (Arbogast and Kenagy 2008). This is a key issue for the delimitation of evolutionary significant units and for defining intraspecific biodiversity units for conservation purposes. Moritz (1999) suggested that in order to define conservation units one should identify and protect groups of historically isolated evolutionarily significant units (ESUs) in the first place, and that groups that would maximize the potential for evolutionary adaptation are protected for each ESU (Fraser and Bernatchez 2001). The phylogenetic diversity (PD) (Faith 1992a) is a quantitative measure of phylogenetic diversity. Larger PD values are expected to correspond to greater feature diversity (Faith 1992b; 1994). This index allows a priority system to be established that reflects the value of the taxonomic diversity, which is very important when resources are limited or when the goal of conservation is to maintain the most hierarchical variation (Faith 1992a); PD can also be used to measure the complementary diversity of a taxon (or taxa) that is not covered by a reference set of taxa (Faith 1992b). For instance, for a set of populations distributed outside protected areas, it is possible to determine which of them would provide greater phylogenetic diversity complementary to those already protected and prioritize their conservation.

The genus *Telmatobius* Wiegmann 1834 constitutes a very diverse genus that is associated with Andean landscapes, and distributed from Ecuador, throughout the Bolivian and Peruvian highlands, reaching southwards into Argentina and Chile (Aguilar and Valencia 2009). These amphibians have aquatic and semi-aquatic habits, occurring in

lakes, river systems, and shallow wetlands between 1000 and 5200 m.a.s.l. (De la Riva 2005). About 10 *Telmatobius* species are known to occur in northern Chile, from which 8 are endemic to Chile. According to recent studies (Sáez et al. 2014), the species group that occurs in Chile is composed of 3 clades that does not form a reciprocal monophyletic unit relative to the *Telmatobius* species from neighboring Andean regions. The latter makes the *Telmatobius* species an ideal model for the inference of diversification processes and for the reconstruction of evolutionary relationships. One of the northern Chilean clades within this genus includes *T. marmoratus* (Duméril and Bibron 1841), a species with a wide distribution range in the highlands that extends to the Bolivian provinces of Oruro and La Paz. Its taxonomic history, characterized by a long list of synonyms, indicates that this is a taxon whose delimitation has been challenging and it is currently under development. Systematic uncertainties have led researchers to include within this clade several putative species that show a low degree of divergence from *T. marmoratus*, such as *T. culeus* (Garman 1875) and *T. gigas* Vellard 1969 from Bolivia (Benavides et al. 2002; De la Riva et al. 2010). As a result, it becomes compulsory to consider *T. marmoratus* as a species complex. According to recent results (Sáez et al. 2014), some Chilean populations whose evolutionary relationships were uncertain, belong to the *T. marmoratus* clade, *T. gigas* is apparently part of *T. marmoratus*, and *T. culeus* could be the sister species of *T. marmoratus*. As such, investigating the degree of genetic differentiation between *T. marmoratus sensu stricto* and weakly differentiated species like *T. culeus* and *T. gigas*, as well as the genealogical relationships within *T. marmoratus sensu lato*, becomes a very interesting undertaking.

From a phylogeographic viewpoint, it would be interesting to characterize patterns of diversity, both inter- and intrapopulations belonging to this species complex and associate these with geological and climatic historical processes that have occurred in the Andean Altiplano. As *Telmatobius* species are aquatic, their distribution are strongly associated with water bodies. The habitat range occupied by the *T. marmoratus* complex in the Altiplano is highly fragmented and shows low levels of connectivity. In general, amphibians display low levels of vagility relative to other vertebrates, and many species are philopatric (Correa et al. 2010), frequently generating high levels of structure even in areas separated by narrow or moderate distances. Additionally, this habitat can be assumed to be historically unstable due to the dynamic paleoclimatic history of these highlands that were subjected to recurrent glacial events and to cyclical dry and wet periods. Unstable environments are often associated with low levels of local genetic variability due to recurrent decreases in effective population size (Carnaval et al. 2009). Therefore, based on the factors described above, we predict low levels of local genetic variability and high levels of structure coupled with low levels of historical gene flow among populations of the *T. marmoratus* complex.

Regarding its conservation status, *T. marmoratus* is listed as vulnerable as its population is expected to decline by more than 30% between 2010 and 2020 (Icochea et al. 2010). One of the major threats for this species is chytridiomycosis, an infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* that is believed to be responsible for the decline of some Peruvian populations (Seimon et al. 2007). Another relevant threat for the Chilean populations of *T. marmoratus* is habitat alteration due to increased mining, groundwater withdrawals, and intensive grazing by domestic livestock. The IUCN Red List provides scientific decision-making guidelines to assign species into categories of threat based on threshold values of population parameters, such as distribution range and population decline (Mace and Lande 1991). However, these criteria do not ensure the preservation of intraspecific biodiversity. For example, many species with a broad distribution and high number of records are not listed as threatened. But in these cases, this system of classification does not consider the geographic distribution of their genetic variability, or the evolutionary uniqueness of their populations. Many species differ substantially in the amount of unique genetic information they embody (Crozier 1997), which is not commonly considered as a criterion in conservation measures.

The major goal of this study is to investigate from a phylogeographic standpoint, the spatial patterns of genetic diversity and lineage distribution within the *T. marmoratus* species complex, and the historical levels of connectivity among the populations assignable to this species based on variants of a mitochondrial gene. Additionally, we evaluate to which extent the patterns of PD are represented inside the National System of Protected Areas of Chile (Sistema Nacional de Áreas Protegidas de Chile, SNASPE), and which unprotected populations would contribute the most in increasing the amount of protected PD if their range were to be included in the SNASPE.

Materials and Methods

Sample Collection

We analyzed a total of 148 sequences from specimens of the *Telmatobius marmoratus* complex distributed in Chile and Bolivia. From these, 136 corresponded to individuals collected in 22 sites between the Arica and Parinacota Region and the Tarapacá Region. DNA samples were obtained from buccal swabs, interdigital membranes, or both. In some cases, muscular tissue was extracted and preserved in absolute ethanol. All samples were catalogued with the geographic coordinates of the sampling site recorded by GPS (Table 1; Figure 1). For analyses, sites were grouped in 3 basin categories: Pacific, Altiplano, and Amazona basins (Table 1; Figure 1). The Bolivian samples of *T. marmoratus* studied here are the same that De la Riva et al. (2010) included in their work with Bolivian *Telmatobius*. (GenBank accession codes GU060589 – GU060612). In this work, we complemented the sampling coverage performed in Sáez et al. 2014 by taking samples from sites outside of the Lauca and Isluga National Parks and including samples from the Las Vicuñas National Reserve, which results in a relatively continuous sampling of *T. marmoratus* specimens along the high Andean zone of the Arica y Parinacota and Tarapacá regions. It is important to point out that this work extends the actual known distribution of *T. marmoratus* in Chile (from 11 to 22 localities; see Sáez et al. 2014).

Data Archiving

All new sequences were deposited in GenBank (accession codes KT156848--KT156960). We have deposited the primary data underlying these analyses in Dryad following data archiving guidelines (Baker 2013).

Laboratory Protocols

Genomic DNA was extracted using the commercial Kit Wizard SV Genomic (Promega). Subsequently, extraction was verified by electrophoresis in a 2% agarose gel using 3 microlitres of extraction products, followed by staining of the gel with SYBR Safe®. A fragment of the mitochondrial gene (mtDNA) cytochrome b (cyt-b) was amplified using the primers MVZ15 (GAA CTA ATG GCC CAC ACW WTA CGN AA) (Moritz et al. 1992) and CYTBAR-h (TAW AAG GGT CTT CTA CTG GTT G) (Goebel et al. 1999). DNA

amplification was performed by polymerase chain reaction (PCR) in a final volume of 30 µL containing: 1.5 U of Taq polymerase (Invitrogen), 3 mM of $MgCl_2$, 0.12 mM of each dNTP, 0.1 µM of each primer, and 10–50 ng of genomic DNA. The PCR reactions were performed using the following conditions: initial denaturation at 94 °C for 2 min, followed by 40 cycles (45 s at 94 °C, 1 min at 53 °C, and 1.3 min a 72 °C), and a final elongation at 72 °C for 10 min.

PCR products were visualized using a 2% agarose gel stained with SYBR Safe® and purified using a MultiScreen $PCR_{\mu 96}$ Filter plate (Millipore) according to the protocol provided by the manufacturer. PCR products were sequenced on both directions through automatic sequencing using the equipment ABI3730XL of Macrogen (Korea). Sequences were aligned and edited using Codon Code Aligner v. 3.0.3 (Codon Code Corporation 2007), and later translated into amino acids in order to corroborate the absence of stop codons.

Saturation levels of the matrix were evaluated following the protocols described in DAMBE v. 5.0.11 (Xia and Xie 2001). Tests were performed with 100 iterations using the proportion of invariant sites informed by the software jModeltest v. 0.1.1 (Posada 2008).

Phylogenetic Analyses

Phylogenetic relationships were inferred using the Bayesian approach implemented in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Four Markov chains were run for 5 million iterations and sampled at intervals of 1000 generations. The first 1250 trees (25%) were discarded as burn-in. Posterior probabilities were obtained from 50% majority-rule consensus tree. The best-fit model of sequence evolution for each gene was selected with JModeltest 0.1.1 (Posada 2008). The species *Telmatobius bolivianus* was selected as the outgroup (accession number of GeneBank GU060588).

The haplotype network was inferred using statistical parsimony in TCS (Clement et al. 2000). Ambiguities within the network were solved according to the criteria of Crandall and Templeton (1993): 1) Frequency criterion: haplotypes are more likely to be connected to high-frequency haplotypes than to other sequences with lower frequencies; 2) Topological criterion: haplotypes are more likely to be connected to interior haplotypes than to tip haplotypes; and 3) Geographic criterion: haplotypes are more likely to be connected to other sequences belonging to the same population or region, than to haplotypes occurring in distant populations.

Neutrality Test, Population Genetic, and Historical Demographic Analyses

To asses if cyt-b gene sequences behave neutrally (Ballard and Whitlock 2004), the McDonald and Kreitman (1991) neutrality test was performed using the program DnaSP v. 5 (Librado and Rozas 2009). In order to detect deviations from a constant population size under a neutral model, we applied Tajima's D test (Tajima 1989) implemented in the program DnaSP, and Fu's Fs (Fu 1997) using the Arlequin program (Excoffier et al. 2005). Negative and statistically significant values for these statistics suggest an excess of low-frequency mutations, relative to what is expected under a neutral model (e.g., strict selective neutrality of variants and constant population size). Additionally, analyses of pairwise haplotype comparisons ("mismatch distribution") were performed using Arlequin. To test if the data deviate from what is expected under an expansion model, the Raggedness index was calculated. In order to determine the most likely number of populations (cluster), we used a Bayesian approach implemented in the program Geneland 4.0.3 (Guillot et al. 2005a, Guillot et al. 2005b, Guillot 2008, Guedj and Guillot 2011, Guillot et al. 2012). This program provides a frequency distribution graphic that indicates the most probable number of populations and their approximate geographic limits. We performed a preliminary run where *K* was allowed to vary from 1 to 33 (number of localities collected) to determine the modal number of clusters. All runs were conducted using the spatial Dirichlet model for the priors in allele frequency and 5 runs with fixed *K* were performed for the spatially explicit model, and for each run, the posterior probability of subpopulation membership was computed for each pixel of the spatial domain (100 × 100 pixels). The Markov chain Monte Carlo (MCMC) repetitions were set at 500,000, thinning was set at 100, and the burn-in period was set at 200 iterations. Because use of Geneland with nonrecombining DNA sequence data could incur a considerable loss of information, it should not be viewed as a substitute for methods that model the genealogy of genes (Guillot et al. 2012). For this reason we also use coalescent methods to estimate the historical population connectivity. Four possible models of gene flow were tested using Migrate-n v. 3.6 (Beerli 2006) (see Supplementary Table A online). The marginal likelihood of the model was estimated followed by a ranking of the Bayes factor of each one (Beerli and Palczewski 2010). The starting genealogy was taken from a UPGMA tree and initial theta and *M* values were derived from the FST calculation. Static heating was applied to 4 independent chains using temperature settings of 1.0, 1.5, 3.0, and 1 000 000.0. A total of 500 000 steps were run, recorded every 100 generations, of which 12 500 were discarded as the burn-in. Stationarity was assessed by examining the effective sample size and distribution of each parameter in Tracer v 1.5 (Drummond and Rambaut 2007).

Genetic distance between and within clusters was calculated using MEGA 5 (Tamura et al. 2011), while haplotype diversity (Hd; Nei 1987), nucleotide diversity (π; Nei 1987) and the average number of nucleotide differences (*k*; Tajima 1989) were estimated using the program DnaSP.

Based on the results from the phylogenetic and gene flow analyses, a hypothesis of postglacial gene flow was proposed and tested against the alternative hypothesis of ancestral polymorphism retained from an earlier divergence using a model-based phylogeographic approach. These hypotheses were tested by performing coalescent simulations in the program Mesquite ver. 3.01 (Maddison and Maddison 2008) to produce thousands of expected genealogies under 2 models representing the 2 hypotheses being tested. For each simulated genealogy, the s statistic (Slatkin and Maddison 1989) was calculated and its frequency distribution from each of the 2 models was then compared with the s-value obtained from the empirical dataset. The s statistic is used here as a measure of departure from reciprocal monophyly. The higher the value, the higher the lack of monophyly. Support for a given model is assessed based on where the observed *s*-value falls relative to the distribution of the simulated *s* values, as the different models may produce different expectations regarding the degree of reciprocal monophyly. Simulations were initially performed for 2 models differing in the time since divergence (14 000 ybp vs. 7000 ybp) (Figure 2). However, based on the preliminary results, additional divergence times were also simulated (1000 and 400 ybp). For simplicity, we test our models with populations from cluster C2, which we divided in 2 subpopulations based on the basin in which they were collected (west: Lluta River basin or east: Altiplano basin). Simulations were performed assuming a population size of 10 000 individuals for each model. Models were

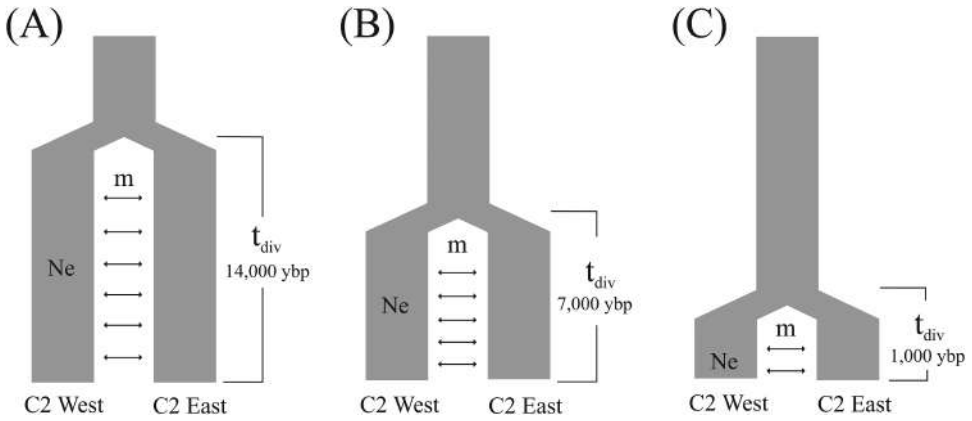

Figure 2. Models of coalescent simulations performed in the program Mesquite ver. 3.01. Ne, effective sample size; tdiv, time since divergence; m, migration rate between basins; C2 West, localities from cluster C2 collected in Lluta Pacific basin (west basin); C2 East, localities from cluster C2 collected in Altiplano basin.

also run assuming 2 scenarios of migration, a without-migration scenario ($m = 0$) and a with-migration scenario ($m = 0.001$; percent of migrants per generation). These values of m are close to those obtained by Migrate, and we selected a slightly larger m value for the with-migration scenario to account for the larger uncertainty in the estimation of this parameter when estimated from mtDNA only.

Phylogenetic Diversity and Its Representation in Protected Areas in Chile

In order to evaluate how much of the *T. marmoratus* diversity is sheltered by protected areas in Chile, we calculated the PD index (Faith 1992b) for all populations within the SNASPE, and compared it to the PD of populations outside protected areas. Protected and unprotected sites are showed in Figure 1. Additionally, we determined which of the currently unprotected populations, if included in the protected areas system, would contribute the most to raise the total phylogenetic diversity contained in the SNASPE. This approach enables the prioritization of those populations that could contribute the most toward the PD while minimizing the number of populations to protect. These analyses were conducted using the Picante package for R (Phylocom Integration, Community Analyses, Null-models, Traits and Evolution in R) (Kembel et al. 2010) and the R function "Phylorare" (Nipperes and Matsen 2013). The "Phylorare" function calculates mean rooted phylogenetic diversity and can be used to standardize a set of samples to a particular level of sampling effort. This allows comparing PD between different sets (protected and unprotected) with unequal sample size.

Results

The final alignment consisted of 148 sequences representing an 815 bp fragment of the gene coding for cyt-b, from 33 sampling sites. All sequences (including the outgroup) showed low levels of saturation according to Xia's test. None of the cyt-b sequences presented gaps or stop codons, which indicates they represent functional copies of mtDNA. Neutrality test indicated the sequences evolve neutrally instead of evolving as a response to selective processes. A total of 60 segregating sites were found among 31 haplotypes.

Phylogenetic Analyses

The phylogenetic tree obtained from Bayesian analysis is shown in Figure 3. Four major clades can be delimited (A, B, C, and D), but the relationship among them are not resolved, represented by polytomies and low geographical structure. Clade A shows a strong support (0.99) and it includes 10 haplotypes, from both one of the 2 Pacific basins and from part of the Altiplano basin. The specimen from Huayllamarca, Oruro, which was putatively assigned to *T. gigas* in De la Riva et al. (2010), is nested within this group. Clade B is represented by only one haplotype and corresponds to specimens from Allane in the Lluta Pacific basin, whereas clade C is constituted only by the specimen from Titicaca Lake, which was designated as *T. culeus* prior to these analyses. Clade D was weakly supported (PP = 0.57) and includes sequences from specimens collected in most of the sampling sites in both Chile and Bolivia, from Lluta Pacific basin, Altiplano, and Amazon basin. Within this group, clade E includes haplotypes from Arica-Parinacota (Pacific basin), and samples from part of the Altiplano basin. Clade F is also strongly supported (PP = 0.98) and comprises haplotypes from a wide geographic area, in which the haplotypes from the Amazon basin are also included. In summary, the Bayesian genealogy shows a lack of genetic structure and no reciprocal monophily according to each river system.

The haplotype network showed 3 main haplogroups (Figure 4), with low geographical structure. The haplogroups were designated using the same codes as the Bayesian phylogenetic tree (Figure 3). The haplotype from clade B (from the Lluta Pacific basin) is closely associated with the haplogroup F that is distributed both in the Altiplano and Amazon basins. Haplogroup E shows a clear star-like shape with an internal high-frequency haplotype broadly distributed in the Lluta Pacific basin and Altiplano. Both clades A and F present a wider geographic distribution with no star-like shape, and without a clear dominant haplotype. Within clade F, one haplotype (H1) presented the widest geographic distribution and was also the most frequent. Overall, the network shows connections with few mutational steps with the exception of the unique haplotype forming clade C (preliminarily assigned to *T. culeus*), which is separated from the nearest haplogroup (F) by 10 mutational steps.

Neutrality Test, Population Genetics, and Historical Demographic Analyses

Analyses of population genetic structure (Geneland) indicated that the most probable number of population units is $K = 4$, which were largely, but not completely concordant with the basins limits (Figure 5). Cluster 1 (C1 in Figure 5) represented all sites from La Paz. This unit shows a wide north to south extension, suggesting that in La Paz there is a low genetic differentiation among

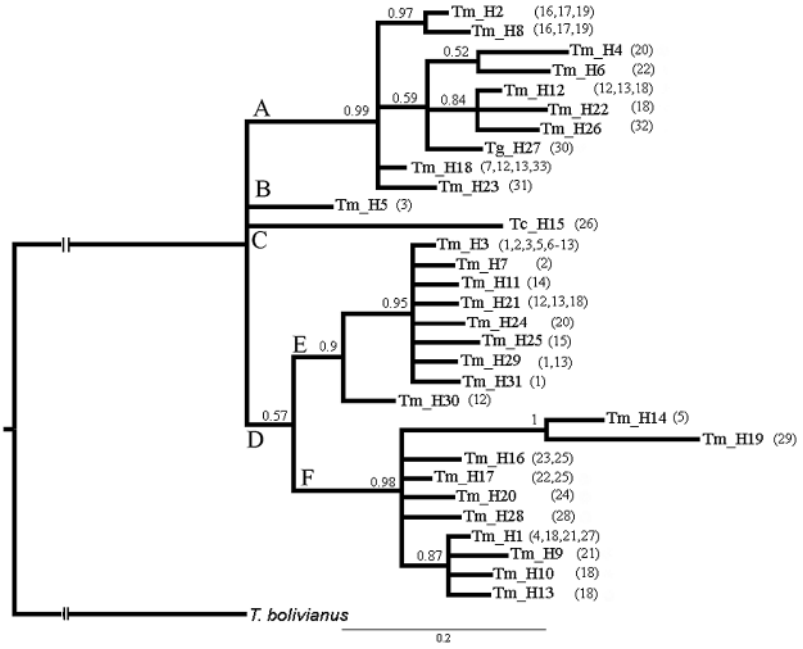

Figure 3. Bayesian majority-rule consensus tree depicting relationships of the *Telmatobius marmoratus* species complex based on sequences of the cyt-b gene. Numbers above nodes represent Bayesian posterior probability values, and numbers between parentheses indicate the corresponding site number according to Table 1. Tm = *Telmatobius marmoratus*. Tg = *Telmatobius gigas*, according to De la Riva 2010. Tc = *Telmatobius culeus*, according De la Riva 2010. H = haplotype codes according to Table 1.

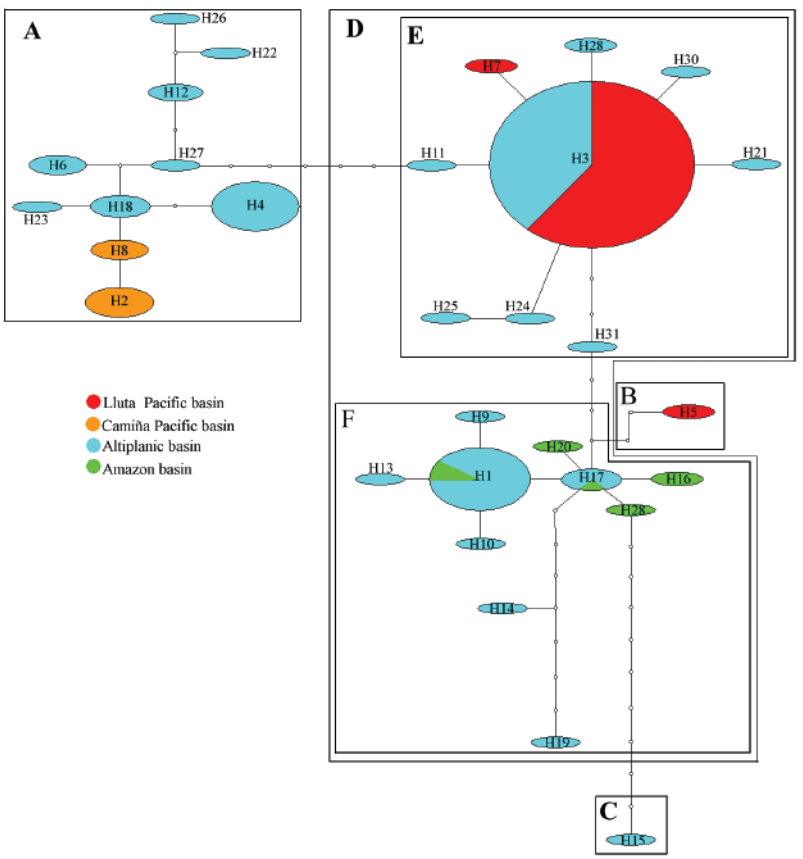

Figure 4. Haplotype network inferred using cyt-b gene sequences by statistical parsimony (TCS), for the *Telmatobius marmoratus* species complex. Color codes correspond to basin systems as shown in Figure 1. White circles indicate mutational steps, for details of haplotype codes see Table 1.

local populations. Cluster C2 included localities mainly from Arica-Parinacota, with the exception of a bordering site adjacent to Chile, in the Oruro province (Site 32, Macaya Lake). The C3 group included only 2 sites, one from Tarapacá, near the Bolivian border (site 18, Isluga), and the Bolivian site of Río Packohaua (site 33). Cluster C4 included only sites in Tarapacá, Chile.

Haplotype (Hd) and nucleotide diversity (π) for all cyt-b sequences was Hd = 0.8148 and π = 0.0076, respectively (Table 2). Diversity estimators were also calculated separately for each population inferred by Geneland. Higher haplotype and nucleotide diversity was observed for the group C1 which is distributed in La Paz with a wide geographic range, and the lowest estimated value corresponded to cluster C3, composed by only 2 border localities between Chile and Bolivia. Mostly, the above results were congruent with distance values within and between clusters (Table 3); generally, such genetic distance values were low (between 0.005 and 0.013). Pairwise

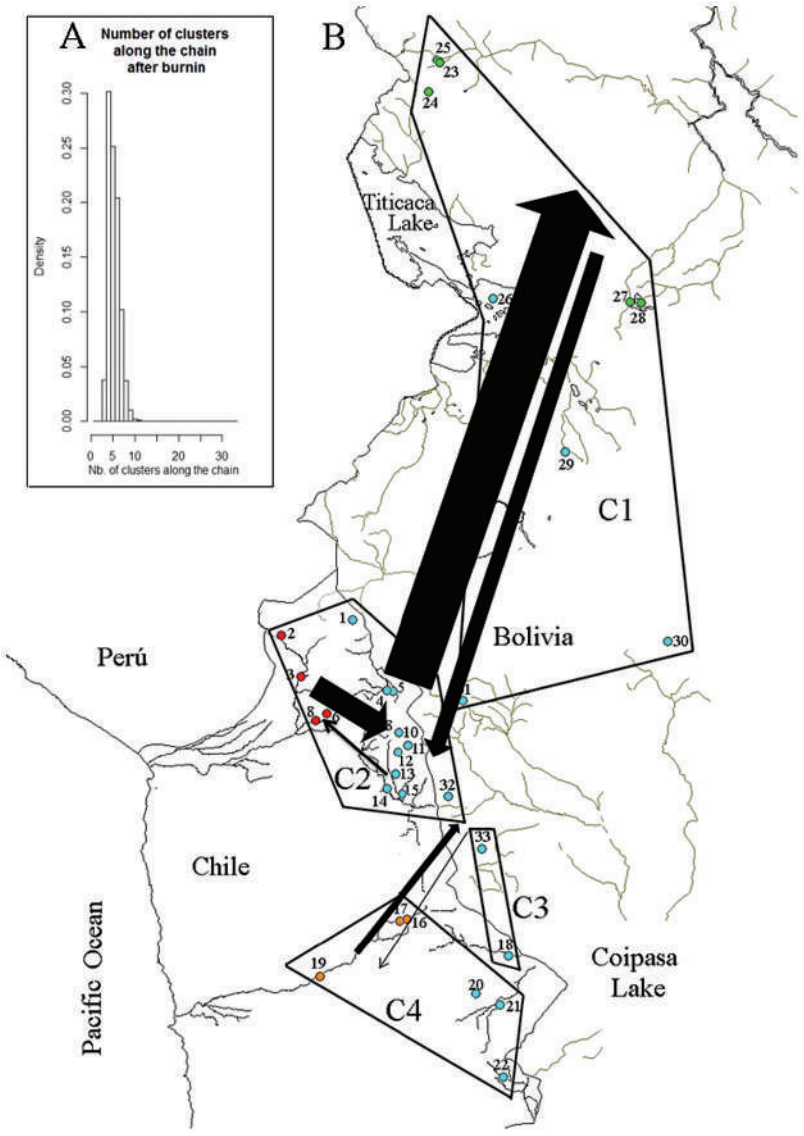

Figure 5. (**a**) Probability density for the number of populations simulated and obtained with Geneland. (**b**) Spatial distribution of each cluster (C1 to C4), defined by Geneland at $K = 4$. Cluster codes and basins included in each cluster are detailed in Table 1. Arrows show the Migrate gene flows between basins (see details in Table 4).

Table 2. Nucleotide diversity statistics, tests for neutrality, and demographic expansion of mtDNA cyt-b sequences for the clusters retrieved by Geneland in the *Telmatobius marmoratus* species complex

	N	*S*	*H*	*k*	Ragg	Hd	π	Fs Fu	D Tajima
Cluster 1	9	26	8	6.611111	0.15509259	0.9722	0.009391	−1.98453	−1.53992
Cluster 2	80	34	15	2.910759	0.20216452	0.4734	0.003058	−2.77868	**−1.82096**
Cluster 3	21	15	6	2.1	0.28272109	0.4286	0.002211	−0.04573	**−1.80962**
Cluster 4	38	23	9	6.042674	**0.16540371**	0.7895	0.006347	2.77071	0.3518
Total	148	60	32	7.291046	**0.04313333**	0.8148	0.007659	−3.55073	−0.99927

Basins included in each cluster are detailed in Table 1.

H, number of haplotypes; Hd, haplotype diversity; *k*, average number of nucleotide differences; *N*, number of sequences; π, nucleotide diversity; Ragg, Raggedness index; *S*, variable sites.

Bold values represent significance at $P < 0.05$.

distance average within each group was lower than between groups, except when comparing group C1 to group C3.

According to Fu's Fs values and Tajima's D, only groups C2 ($D = -1.82096$ from Arica-Parinacota) and C3 ($D = -1.80962$) show signals of recent bottlenecks and are currently experiencing a demographic expansion, as suggested by the significant negative values of one or both indicators (Table 2). As suggested by mismatch graphics, although some populations showed a tendency toward a multimodal distribution, a significant deviation from the expected curve under a demographic expansion model was only observed for one of the 4 haplogroups (see Raggedness values in Table 2). The mismatch graphics showing pairwise distances mostly in low values (Figure 6) correspond to clusters C2 and C3. The former is in agreement with the significant Fu's Fs value.

Migration values between clusters are shown in Table 4 and represented in Figure 5. Comparison of migration models revealed that the Bisected stepping stone model had the highest support from the data, with a log Bayes Factor difference > 10^5 units relative to the other migration model (where a difference of > 10 units provide very strong support for one model over another; Kass and Raferty 1995). According to these results, the historic migration scenario for the analyzed populations shows a prevalence of asymmetric migration rates. Higher migration magnitudes correspond to gene flow from C3 to C1, from C4 to C1, and from C2 to C1. Therefore, the cluster that historically acts as a sink is cluster C1, distributed in the La Paz highlands. Cluster C3 (formed by 2 sites, one in Oruro and the other in Tarapacá) could have acted primarily as a source. Cluster C4 distributed in Tarapacá, Chile, appears to have functioned more as a source than as a sink, although at a lower scale when compared to cluster C3.

Because patterns of historical migration can be commonly confounded with the process of incomplete lineage sorting, we performed coalescent simulations to test how likely the later scenario could be for a number of models with different population splitting times. These analyses indicated that the low genetic structure found in *Telmatobius marmoratus* was likely not due to incomplete lineage sorting, but rather to migration. Simulations at 3 different times of population splitting and no migration between the basins (Figure 7, upper row) showed that all simulated gene genealogies produced *s* values lower than the observed *s* value. Additional simulations showed that only after 400 years it was possible to obtain *s* values equal or higher than the observed *s* value in at least 5% of the cases (data not shown). On the other hand, simulations that incorporated the effect of migration produced a range of *s* values that were not significantly different from the observed *s* value ($P > 0.5$), regardless of the time of population splitting (1000, 7000, and 14000 ybp; Figure 7, lower row). All together, these results indicate that only the models incorporating migration account for the lack of reciprocal monophyly observed across populations.

Table 3. Pairwise sequence divergence for cyt-b haplotypes between clusters of the *Telmatobius marmoratus* complex, retrieved by Geneland

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1	**0.01 (0.002)**	0.011	0.007	0.013
Cluster 2	(0.003)	**0.003 (0.001)**	0.009	0.012
Cluster 3	(0.002)	(0.003)	**0.004 (0.001)**	0.013
Cluster 4	(0.003)	(0.003)	(0.003)	**0.008 (0.002)**

Basins included in each cluster are detailed in Table 1.

The values on the diagonal (in bold) represent mean differences within clusters, while those above the diagonal represent mean differences between clusters. Standard deviation values are shown in parentheses.

Phylogenetic Diversity and Its Representation in Protected Areas in Chile

Faith's Phylogenetic Diversity Index (1992b) was estimated with the "phylorare" function in R and the PICANTE package for R, including all *T. marmoratus* populations in Chile. The Phylorare analysis with $m = 110$ showed that the PD for all Chilean populations was 1.87. When considering only populations sampled from protected areas, the index value decreases considerably to 1.04, representing 55.6% of Chile's phylogenetic diversity for *T. marmoratus*. We obtained the

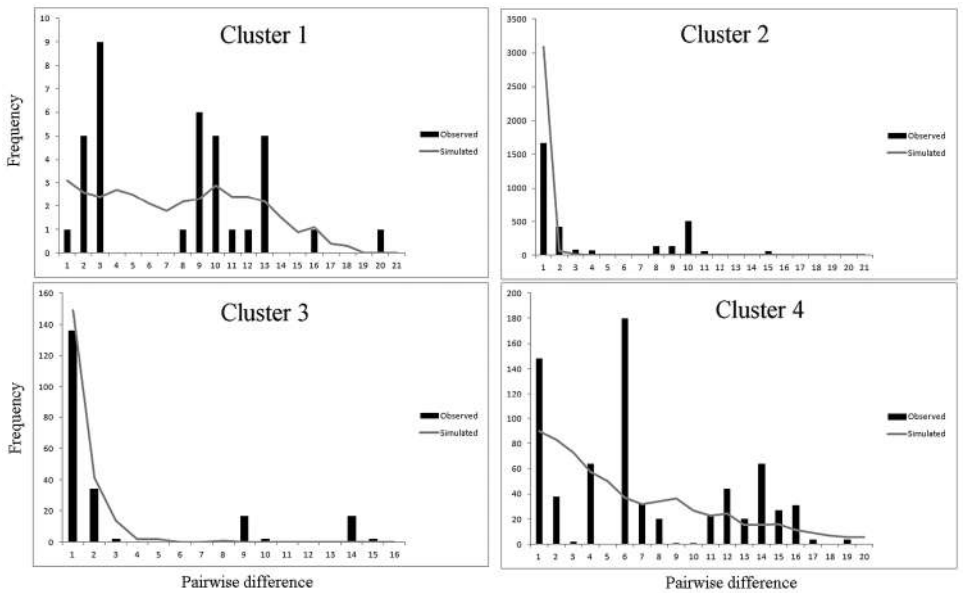

Figure 6. Mismatch distribution of haplotype lineages for each cluster (C1 to C4) in the *Telmatobius marmoratus* species complex. Raggedness index values are detailed in Table 2.

Table 4. Long-term female effective migration rates (ΘM) among basins, estimated by MIGRATE-N

	Donor basin			
Receiver basin	Amazon	Altiplanic	Lluta Pacific	Camiña Pacific
Amazon	*0.20 (0.04–0.20)*	3.21 (0–35.1)	—	—
Altiplanic	1.88 (0–337)	*0.12 (0.06–0.19)*	2.27 (0–28.1)	0.89 (0–5.38)
Lluta Pacific	—	0.38 (0–13.8)	*00.02 (0–0.09)*	—
Camiña Pacific	—	0.05 (0–1.83)	—	*0.01 (0–0.07)*

The 95% confidence interval is constructed from minimum and maximum estimates of 0.025 and 0.975 percentiles.
Theta values (Θ) for each basin is given on the identity diagonal in italics.

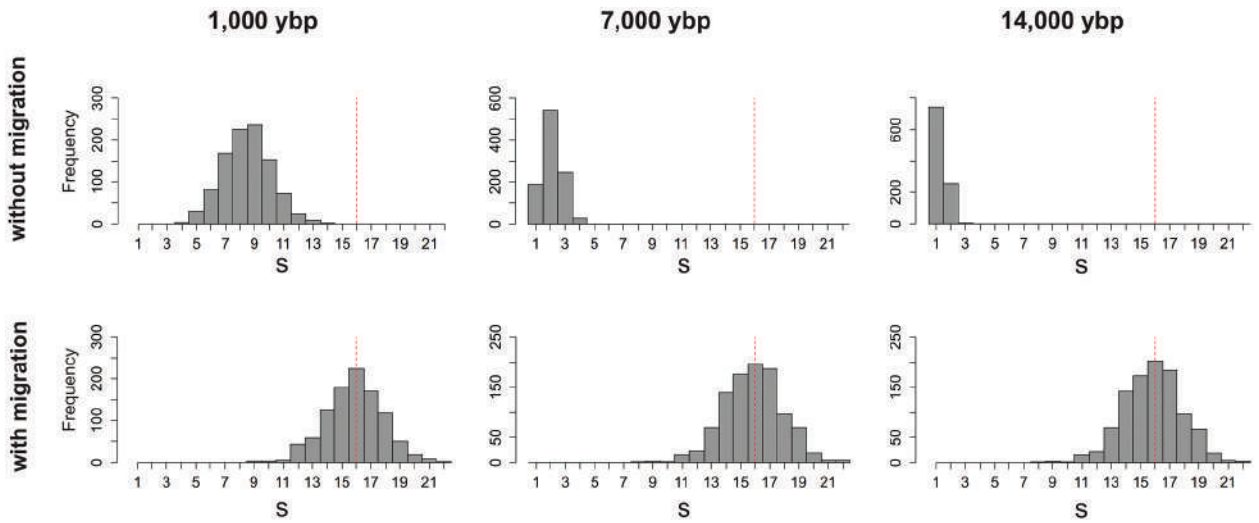

Figure 7. Comparison of the *s* value from the reconstructed gene tree (observed for groups explained in Figure 2) to the expected distributions of s from gene trees simulated by neutral coalescence with (lower row) and without (upper row) migration ($m = 0.001$ and $m = 0$, respectively) and Ne = 10000, over a range of different times of population splitting (measured in generations). Vertical dashed line indicates the observed *s* value.

same results with the PICANTE package for R. The population from Caquena, currently unprotected, was retrieved as the most likely to complement at the highest degree the phylogenetic diversity included in protected areas (see Supplementary Table B online). This population is represented by one haplotype (H14) that, together with H19, would form a sister-clade relationship with haplotypes from the locality of Isluga, 150 km away (H1, H10, and H13). If this population was to be included, the phylogenetic diversity of protected areas would increase from 55.6% to 66.3%. Another population that would also contribute to phylogenetic diversity is that located in Quebe. This locality alone has a high phylogenetic diversity (PD = 0.53), potentially due to the presence of 2 haplotypes belonging to well-differentiated clades (H4 and H24). Providing protected status to the populations in Caquena and Quebe would raise the phylogenetic diversity in protected areas to 76%.

Discussion

Although not all clades from our inferred phylogenetic tree display high support values, it is interesting to analyze the position of *T. culeus* and *T. gigas* in the tree topology and in the haplotype network. From both species, only *T. culeus*, from the Titicaca Lake, appears as a well-differentiated clade relative to the rest. On the other hand, the specimen assigned to *T. gigas* prior to analyses shows a relationship to the *T. marmoratus* clade that includes specimens from the localities of Oruro, Arica-Parinacota, and Tarapacá. The strong evolutionary relationship between *T. marmoratus* and *T. gigas* has been noted in previous studies (e.g., Benavides 2005). De la Riva et al. (2010), when analyzing Bolivian *Telmatobius* species, could not find a relationship of reciprocal monophyly between samples of *T. marmoratus* and *T. culeus* from Titicaca. These authors also found evidence of relatively recent divergence between *T. marmoratus* and *T. gigas* (no more than 300000 to 600000 ybp), which could explain the absence of complete lineage sorting in both taxa. In conclusion, relative to taxonomy, the cyt-b gene suggests that *T. gigas* could be cospecific with *T. marmoratus*, while *T. culeus* is clearly a different species. However, the genetic variability analyzed in this study relied on only one gene of uniparental inheritance. In order to produce more robust results, a broader sampling in Bolivia, and an integrative approach is needed in order to establish the definite characterization of *T. gigas* as a separate species, including the use of nonlinked genomic markers (nuclear genes) and other characteristics such as morphological markers (Sites and Marshall 2004).

Contrary to our expectations of high structured populations, we found patterns of low genetic structure that suggest the occurrence of a complex scenario in terms of lineage distribution, genetic variability, and historic migrations within the *T. marmoratus* species complex. Based on our results, we were not able to statistically corroborate our prediction of highly structured populations within *T. marmoratus*. Overall, *T. marmoratus* presented moderate levels of population structure and low levels of genetic variation. We have recovered considerable levels of historic gene flow among some of

its populations. Nonetheless, Bayesian analyses using Geneland suggest the existence of 4 populations units or clusters, which although presenting low support values, serves as an indicator of the existence of moderately differentiated geographic groups. According to the star-like shape of the cluster C2 from Arica-Parinacota, and the significant negative values for the Fu's Fs and Tajima's D statistic, this population unit is expected to have undergone a recent population size reduction, and it would currently be at a phase of demographic expansion. Although not all groups showed significant values for expansion or for bottleneck indicators, 2 clusters exhibited significant negative Tajima's D values, both distributed in the Arica-Parinacota region and border areas between Chile and Bolivia. In this regard, only the localities included in the C4 cluster, which are mainly distributed in Tarapacá, Chile, showed a significant Raggedness value relative to the expected distribution under an expansion model. Below we discuss the plausible mechanisms that could explain the lack of population structure found.

The Andean highlands are believed to have first appeared around the Late Cretaceous, while the Western Cordillera and the Eastern Cordillera arose later, during the Miocene. Subsequently, during the Plio-Pleistocene, the Andean mountain range rose to nearly its current height, at approximately 4000 m (Moon 2008). Therefore, the Andean highlands have existed as an ecosystem for a long enough time to have generated dynamic evolutionary processes that promoted diversification in several biological groups, probably associated to vicariant events and fragmentation of aquatic environments (e.g., Collado et al. (2013)). At an intraspecific level, Vila et al. (2013) conducted a phylogeographic analysis for *Orestias ascotanensis*, a fish species that now inhabits only the Ascotan saltmarsh, but with isolated populations in nonconnected watersheds. The authors found highly differentiated populations when comparing those from distant watersheds, but less differentiation and more diversity between populations from watersheds closer together. Although this suggests a history of low connectivity for some isolated aquatic habitats, this also suggest that, in the past, water levels may have connected some other geographically closer populations that are currently isolated. Evidence for low genetic structure in amphibians from the same area considered in this study was also found for *Rhinella spinulosa*, suggesting high connectivity in the recent past. Correa et al. (2010) found a unique widely distributed haplotype for this frog. De la Riva and collaborators (2010) suggest that the current distribution of the *T. marmoratus* species complex could have arisen from refugial populations from lowlands, during cold and dry periods. Individuals could have dispersed from these refugia and could have quickly colonized territories at higher elevations during wetter periods, resulting in present-day populations. According to our results, most of the historical migrations (see Migrate results), would have been directional and strongly asymmetric originating from the high western Altiplano in Chile, towards the east in Bolivia. The haplogroup from Arica-Parinacota (C2) occurs in basins clearly topographically separated from adjacent basins in Chile, where the Tarapacá populations (C4) exist. This could be explained because some of these basins flow into Bolivian territory, allowing a greater connectivity among Chilean and Bolivian populations than between both Chilean groups. The occurrence of the some broadly distributed haplotypes, as in the case of the haplotypes from the Arica-Parinacota region, could be taken as evidence for recent dispersal events. One explanation for the genetic variation and haplotype distribution recovered for the *T. marmoratus* species complex is the probable influence of climate shifts and recent glacial events. The wide distribution exhibited by some haplotypes and the low structure found could be explained by events that resulted in increased habitat connectivity during postglacial melting. Glacial processes would have had an important impact on the biodiversity of the highlands, through a cycling of dry and wet periods (Ochsenius 1986). According to Maldonado and Rozas (2008), during the Last Glacial Maximum (LGM), temperatures dropped while rainfall increased in the north of Chile. A wet cycle would have occurred 14 000 to 11 000 years ago, whereas around 7000 years ago the climatic conditions would have been extremely arid. At the end of each glacial cycle, following the melting of the ice-sheet, lakes would have suffered an increase in both depth and extension. The former suggests that during these periods, aquatic environments could have been saturated with water, becoming the most common habitat, covering a larger area, and hence being closer to each other. According to Villagrán (1993), during the early Holocene (between 8000 and 4000 years), climatic conditions would have been more unstable with cycles of dry and wet periods taking place in different regions of South America. As a consequence, paleoenvironmental changes in the Andean highlands would have repeatedly caused reductions in the suitable habitat for *T. marmoratus* (Duellman 1982), especially during dry periods. This could have resulted in a reduction in population size, therefore leading to periods of fragmented populations and low connectivity which would have alternated with periods of glaciation, with lower temperatures and more rainfall (Maldonado and Rozas 2008). In turn, connectivity would have been reestablished during postglacial periods, when melting ice would have raised the water level of lakes, extending their coverage (Ochsenius 1986) and probably flooding or saturating wide areas that currently do not form water bodies or wetlands. This phenomenon could have increased connectivity levels for the *Telmatobius* populations among wide areas, leading to secondary contact of certain lineages and reducing genetic structure levels.

Although one of the reasons that may account for patterns of absence of genetic structure is the retention of ancestral polymorphism (Funk and Omland 2003), our coalescent simulations indicated that the low genetic structure found in *T. marmoratus* was unlikely to be due to incomplete lineage sorting. Our results indicate that only the models incorporating migration account for the observed patterns of low structure. While expected cycles of greater connectivity in the Altiplano after ice melting during postglacial phases (e.g., post Last Glacial Maximum) are a plausible explanation for the high historical gene flow in *T. marmoratus*, our coalescent simulations suggest that high connectivity events should have also occurred during more recent periods. Models suggest that high levels of gene flow in *T. marmoratus* might have occurred within the last 1000 years. Several paleoenvironmental evidence sources about the climate patterns in the Altiplano show that wet levels have been very unstable, with contrasting rainfall cycles, and with an important role of El Niño Southern Oscillation (ENSO), after the Puna Glaciation (Maldonado and Rozas 2008). Even during the Holocene, climatic fluctuations were common, and, for example, Lake Titicaca reached its current size only 3500 ybp (De La Riva et al. 2010). Bräuning (2009) provides an overview of what can be derived about changes of temperature and moisture conditions in the humid and arid parts of the Andes. He suggests that a progressive increase of humidity is evidenced during the late Pleistocene in humid Andes, since approximately 1000 ybp. Studies based on paleohydrological reconstructions in the central-southern Altiplano (18°–26 °S) show abrupt paleohydrological and paleoclimatic changes synchronous with the termination of the Little Ice Age from approximately 500 ybp (Valero-Garcés et al. 2003). Latorre et al. (2003), based on midden records from central Andes in arid prepuna (22–23°S), identify conditions wetter

than today during several periods including up 1.2 ka, and Rech et al. (2003), studying mid-Holocene deposits, suggest that local ground-water levels rise and wetland deposits aggrade in deep canyon systems, such as in the Río Puripica (approximately 23°S), which is connected to the west Altiplano. In their analysis they show evidence for many depositional environments, which indicate that it formed during a period of higher regional ground-water levels that were sustained by enhanced precipitation and recharge in the High Andes as recently as 500 ybp. According to the above, although we do not discard the role of increased connectivity during postglacial cycles, our results and previous paleoclimatic evidence suggest that population connectivity in *T. marmoratus* should have lasted until very recently (a few centuries ago), which is consistent with paleoclimatic evicence of increased humidity during the late Holocene.

Biodiversity, in simple terms, refers to all of the different life forms on our planet, and includes both species diversity and genetic diversity (Freeland 2005). Genetic erosion is assumed to decrease the mean fitness of populations (Reed and Frankham, 2003), and population genetic diversity (alpha and beta spatial scales) is believed to be essential to ensure species viability (Berthier et al. 2005). The most effective way of preserving biodiversity is by maintaining self–sustaining populations of native species in their natural ecosystems (Rodrigues and Gaston, 2002). This often requires the designation of nature reserves, areas where the conservation of biodiversity is a priority over other forms of land use (Pérez-Lozada and Crandall 2003). In this sense, biodiversity conservation strategies adopt a form of risk analysis that involves estimating patterns of variation, and then trying to conserve as much of that estimated variation as possible as a way to retain "options" (possible values) for the future (Faith and Baker 2006). The current system of protected areas in Chile does not adequately represent terrestrial vertebrate diversity. Chilean protected areas increase in frequency and size toward the south and have a strong bias towards temperate forest ecosystems (Tognelli et al. 2008). This partly justifies an urgent assessment of the geographic distribution of intraspecific conservation values based on phylogenetic diversity, mainly regarding the biodiversity components of central and northern Chile.

One difficulty we can foresee is that limited resources for conservation may impose practical limitations on the conservation of these units of diversity (the so-called 'resources' problem, McNeely et al. 1990). In order to optimize the prioritization of conservation units (areas), PD was proposed by Faith (1992b) as a measure of biodiversity option value. The evolutionary value of populations within a species should be one of the key components of any system that assigns conservation priorities. In reference to the proportion of PD of the *T. marmoratus* complex that is currently protected in Chile, our results show that all lineages are adequately represented within areas from the SNASPE. Of the unprotected areas (Figure 1), those that would contribute the most in raising the PD are the populations from the sites of Caquena and Quebe, from Arica-Parinacota and Tarapacá, Chile, respectively. The importance of protecting the Caquena population stems from its status as an independent evolutionary lineage relative to close populations, and that its closest sister-lineage within Chile is 150 km south, in the Isluga site. On the other hand, the Quebe population stands out because of its high phylogenetic diversity, as this population included haplotypes from different lineages, adding to a higher value of branch length.

Studies focusing on intraespecific diversity in these localities are scarce and the taxonomic and systematic knowledge in this area of the Altiplano is currently under development. Therefore, phylogenetic diversity for other taxa distributed in Caquena and Quebe had not been explored, so we do not know whether these sites also possess a high conservation value for multiple species. Correa et al (2010), in regards to populations of amphibian *Rhinella spinulosa*, detected a clear northern lineage associated to small endorheic drainage systems that included the Caquena and Quebe sites. Additionally, Collado et al (2011) working with snails from genus *Biomphalaria*, described 2 clustered monophyletic groups restricted to several aquatic systems within the Caquena and Lauca basins that may represent separate candidate species. More studies are needed in order to determinate priority areas in these systems, considering information from co-distributed taxa in those populations (including Caquena and Quebe). On the other hand, current legislation in Chile regarding protected areas is disperse, disarticulated, and incomplete, weakening strategies that could be potentially adopted in order to protect and conserve biodiversity. For this reason, the creation of a legislative bill that aims to create the Service of Biodiversity and Protected Areas is currently in process, whose objectives are to improve the representativeness of inland water ecosystems. Although the present criteria for prioritizing conservation areas are mainly based in neutrally evolving DNA, we are aware that this should be complemented with ecological and adaptive criteria (Crandall et al. 2000). However, our results are a good starting point for improving proposals for designing protected areas to preserve intraspecific variants of the Chilean biota.

The present study is the first that focuses on phylogeographic patterns for *Telmatobius* species occurring in Chile's highlands, as previous publications concentrated on relationships at the supraspecific level and were centered on species from Bolivia and Peru (Benavides et al. 2002; Benavides 2005; Aguilar et al. 2012). Additionally, this work constitutes the first approach in a *Telmatobius* species complex that quantifies evolutionary intraspecific diversity including protected areas in Chile. This type of approach has been used previously in vertebrates, but evaluating the protected PD at the multispecies level (Zupan et al. 2014). Our results highlight the importance of inferring historical processes that explain the current geographic distribution of lineages and considering this information in the optimization of conservation strategies at an intraspecific level.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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