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Hidden diversity in spring snails from the Andean Altiplano, the second highest plateau on Earth, and the Atacama Desert, the driest place in the world

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Abstract

Background: The Chilean Altiplano and the Atacama Desert in northern Chile harbor isolated hydrological systems or oases where it is possible to find minute aquatic snails of the genus *Heleobia* whose taxonomy is uncertain and where many populations remain unknown. Here, we obtained samples from 30 localities distributed in the region and used molecular (12S and 16S mitochondrial genes) and morphological (penis) characters to investigate diversity of this poorly known fauna.

Results: Molecular phylogenetic analysis consistently recovered five clades, one of which constitutes a cryptic species previously assigned to a species recognized in the area. Four other clades contained sequences of one nominal species consistent with its type locality and at least two additional candidate species, which were corroborated by a particular penis morphology. Furthermore, some morphological differences in penis morphology were observed in two Altiplano populations not resolved by the DNA sequences, providing support for two additional candidate species in the genus. A molecular clock analysis allowed tracing the origin of lineages back to the Early Pleistocene.

Conclusions: We found support for recognizing four nominal species, one undescribed species and at least other four candidate species of the genus *Heleobia* in northern Chile. We also suggest that the current level of species diversity of *Heleobia* in the region is underestimated by the use of conchological criteria to recognize species and by the limited sampling conducted to date.

Keywords: Cochliopidae; Distribution patterns; Semisalsinae; Spring snails; Taxonomy

Background

The practical matter of species delimitation is receiving increased attention considering the high rate of extinction of species along with the huge undiscovered biodiversity in a number of taxa (Sites and Marshall 2003, 2004; Bickford et al. 2007; Wiens 2007; Puillandre et al. 2011). In the Andean Altiplano, the second highest plateau on Earth (Babeyko and Sobolev 2005), the dynamic and complex geological history of the region has produced ecosystems with a high degree of endemism (Velooso and

Bustos-Obregón 1982; Dyer 2000; Vargas et al. 2004), probably related to the extensive terraces and restricted mountain ranges that have originated since the Miocene (Wörner et al. 2000; Risacher et al. 2003; Strecker et al. 2007). For example, the diversity of the killifish of the genus *Orestias* Valenciennes, 1839 and the lunged aquatic snails of the genus *Biomphalaria* Preston, 1910 is hypothesized to be principally a consequence of the fragmentation of populations during the Middle and Late Pleistocene after the regression of several paleolakes that existed in the area (Lüssen et al. 2003; Vila 2006; Vila et al. 2011, 2013; Collado et al. 2011a). West of this area, the Atacama Desert, the driest place in the world (McKay 2002; Vesilind 2003) with thousands of square miles of

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arid desert interrupted by occasional oases, is a hyper-arid system thought to be relatively simple ecologically and with low productivity (Noy-Meier 1973), but containing endemic species, for example, within the genus *Basilichthys* Girard, 1855 (Atheriniformes), that has allopatric distribution (Dyer 2000). Both regions harbor restricted and isolated hydrological systems ranging from springs on the banks of the Pacific coast along the Atacama Desert to watersheds situated at more than 4,000 m altitude in the Andes. In some of these watersheds, it is possible to find gilded snails of the genus *Heleobia* Stimpson, 1865 (Courty 1907; Biese 1944, 1947; Hershler and Thompson 1992; Collado et al. 2011b), subfamily Semisal-sinae Giusti and Pezzoli, 1980 (Bouchet and Rocroi 2005), sometimes living in amazing conditions of water stress and habitat degradation (Collado 2012). The group is characterized by small species that live in marine, brackish, and freshwater environments, and whose shells generally are less than 10 mm in length (Biese 1944, 1947; Hershler and Thompson 1992). The species have a wide range of ecological tolerances and reproductive strategies (Marcus and Marcus 1963, 1965; Cazzaniga 1982a; Martín 2002; Neves et al. 2010; Collado and Méndez 2011), and some of them serve as intermediate hosts in the life cycles of digenean trematodes (Etchegoin and Martorelli 1997; Simões et al. 2008, 2009, 2010; Alda et al. 2010; Merlo and Etchegoin 2011). Kroll et al. (2012) studied the phylogenetic relationships of species mainly distributed in the Altiplano region and its major internal Lake Titicaca, and this is the only phylogenetic study performed in the genus *Heleobia*.

The systematics of the Chilean Semisal-sinae fauna has been contentious at different taxonomic levels (De Francesco and Isla 2004; Cazzaniga 2011; Collado et al. 2011b). While some authors have included the species in the genus *Heleobia* Stimpson, 1865 (Davis et al. 1982; Hershler and Thompson 1992; Kabat and Hershler 1993), others have preferentially assigned them to the genus *Littoridina* Souleyet, 1852 (e.g., Pilsbry 1911; Preston 1915; Biese 1944, 1947; Haas 1955; Hubendick 1955; Stuardo 1961; Weyrauch 1963; Figueroa et al. 2003; Sielfeld 2001; Valdovinos 1999, 2006, 2008). At the species level, the knowledge of the group has not been clarified, so alpha taxonomic work is still needed (see Collado et al. 2011b).

In the Chilean Altiplano and the Atacama Desert, eight species and seven subspecies of *Heleobia* have been described based on conchological characters: *Heleobia atacamensis* (Philippi 1860), *Heleobia loaensis* (Biese 1947), *Heleobia opachensis* (Biese 1947), *Heleobia striata* (Biese 1944), *Heleobia transitoria* (Biese 1947), *Heleobia ascotanensis* (Courty 1907) with five subspecies, and *Heleobia chimbaensis* (Biese 1944) and *Heleobia copiapensis* (Biese 1944) with one subspecies (Hershler and Thompson 1992; Collado et al. 2011b). The taxonomic

status of these species and several previously unexamined populations distributed in the region has never been investigated. Additionally, the distribution ranges and relationships among the species have been addressed by few studies (Biese 1944, 1947; Kroll et al. 2012). Considering that these two geographical areas are difficult to access and concomitantly poorly explored, it is highly possible that the biodiversity of *Heleobia* is underestimated. In the present study, we use DNA sequence data of the large (16S) subunit and small subunit (12S) of ribosomal RNA mitochondrial genes and penis morphology to investigate diversity of *Heleobia*, using an extensive sampling covering the majority of the type localities and distribution range of the genus in the region and to test the hypotheses of nominal species proposed until now. We investigate penis morphology considering that the organ is discriminatory in *Heleobia* species (Gaillard and de Castellanos 1976; Cazzaniga 1980, 1982a, b; Hershler and Thompson 1992; Pons da Silva 1993; Collado et al. 2011b; Ovando and De Francesco 2011). Hubendick (1955) described the penis of *H. chimbaensis* from northern Chile, and Collado et al. (2011b) studied this organ in this and other species of the genus.

Methods

The snails were collected from macrophyta or sediment using a sieve, from 2010 to 2012 from 30 localities situated in the Chilean Altiplano and the Atacama Desert (Figure 1, Table 1). The snails were preserved in 70% to 100% ethanol. The material included specimens collected from Quebrada La Chimba, the type locality of *H. chimbaensis*, a ravine near Antofagasta city, Vertiente Opache, the type locality of *H. opachensis* in San Salvador River, and Las Cascadas, the type locality of *H. loaensis* in Loa River. The last two localities are situated near the city of Calama in Loa Basin. The snails were also obtained from Tilopozo in the Salar de Atacama, Chilean Altiplano, the type locality of *H. atacamensis*, and from Quebrada Cachina, the type locality of *H. transitoria*, a ravine located in the Pacific coast in the Atacama Desert. *Heleobia copiapensis*, *H. copiapensis costata*, and *H. striata* were described from the Copiapó River and from its tributary stream Ojancos, the type locality of the species (Biese 1944, 1947); we sampled the Copiapó River at four sites located over the majority of its length. We also obtained snails from two isolated springs from the Salar de Ascotán, the type locality of *H. ascotanensis* (see Courty 1907; Collado and Méndez 2012a). Because we assigned snails to *H. ascotanensis* in a previous study with samples of Spring 2 from this salt pan, we have kept this name for a set of snails obtained from this site which formed a monophyletic group. Additional internal subclades formed with samples of this site and from Spring 11 from the Salar de Ascotán were treated as *Heleobia* sp. Voucher specimens were deposited in the Laboratorio de Genética y Evolución (GEVOL), Facultad

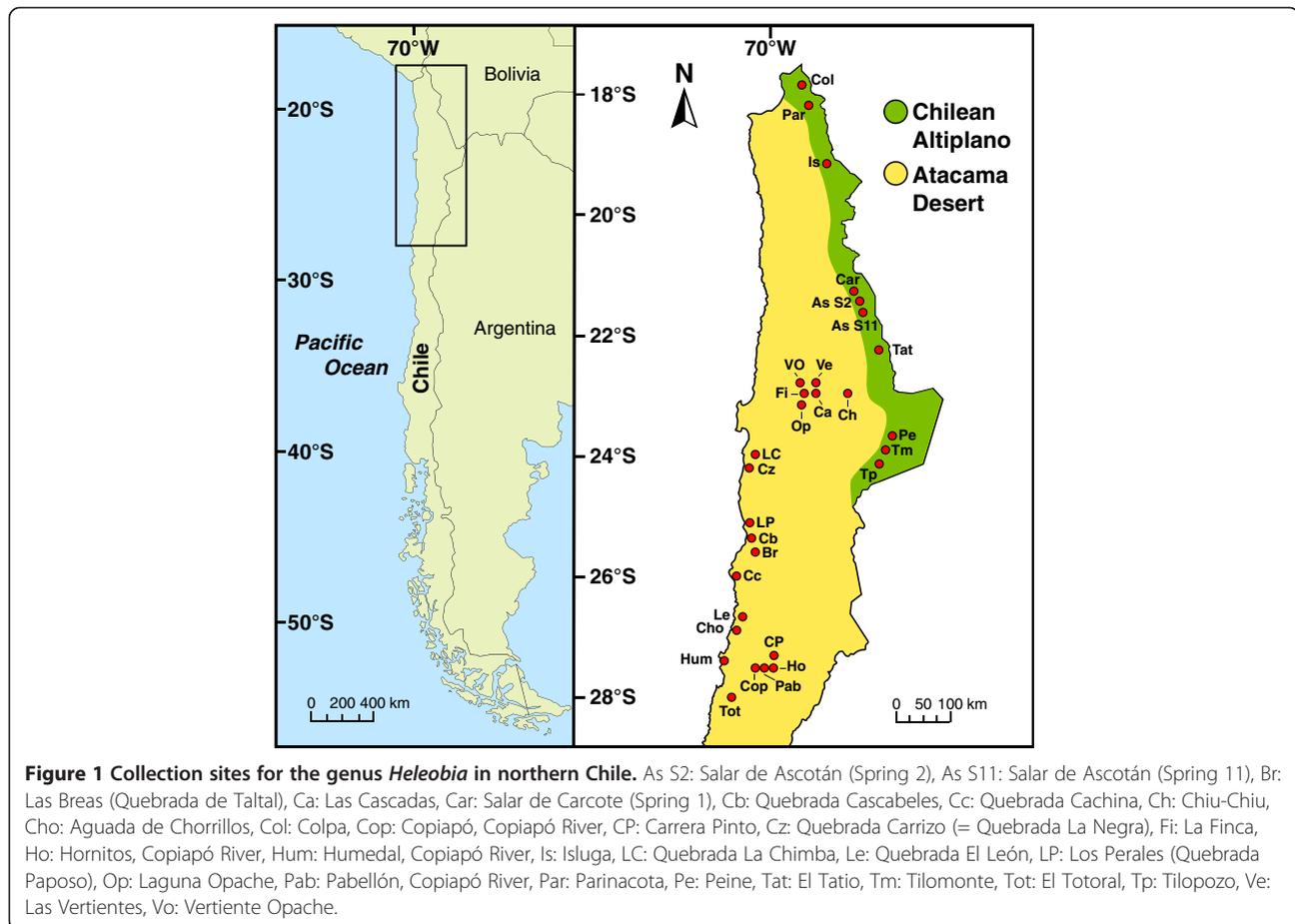


Figure 1 Collection sites for the genus *Heleobia* in northern Chile. As S2: Salar de Ascotán (Spring 2), As S11: Salar de Ascotán (Spring 11), Br: Las Breas (Quebrada de Taltal), Ca: Las Cascadas, Car: Salar de Carcote (Spring 1), Cb: Quebrada Cascabeles, Cc: Quebrada Cachina, Ch: Chiu-Chiu, Cho: Aguada de Chorrillos, Col: Colpa, Cop: Copiapó, Copiapó River, CP: Carrera Pinto, Cz: Quebrada Carrizo (= Quebrada La Negra), Fi: La Finca, Ho: Hornitos, Copiapó River, Hum: Humedal, Copiapó River, Is: Isluga, LC: Quebrada La Chimba, Le: Quebrada El León, LP: Los Perales (Quebrada Paposo), Op: Laguna Opache, Pab: Pabellón, Copiapó River, Par: Parinacota, Pe: Peine, Tat: El Tatio, Tm: Tilomonte, Tot: El Totoral, Tp: Tilopocho, Ve: Las Vertientes, Vo: Vertiente Opache.

de Ciencias, Universidad de Chile and the malacology section of the Museo Nacional de Historia Natural, Santiago, Chile (MNHNCL).

For morphological data, the shell of adult specimens was cleaned in 1% sodium hydroxide for 10 h to remove adhered sediment, washed in distilled water, and finally preserved in 70% ethanol. The snails were photographed at the same magnification with a Motic SMZ-168 Stereo Microscope with a Moticom 2000 (British Columbia, Canada) integrated digital camera. For the study of penis morphology, we followed the method implemented by Collado et al. (2011b), dissecting three male specimens for each population surveyed when possible. The penis was photographed using the same equipment. Considering the similarity of the characters of the shell and penis between some populations of the same species (see for instance Collado 2012), we do not show these data for all locations, but only those representatives of geographic areas or clades, or when it is required to emphasize the possibility of finding candidate species. For the molecular analyses, genomic DNA was isolated from the gill and mantle tissue of the snails fixed in 100% ethanol using the cetyltrimethyl ammonium bromide method (CTAB) (Winnepeknickx et al. 1993). The mitochondrial 16S rRNA gene was amplified by polymerase

chain reaction (PCR) using the primers 16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr-H (5'-CCGGTCTGAACTCAGATCACGT-3') (see Palumbi 1996). PCR conditions were described in Collado and Méndez (2012b) for this molecular marker. The mitochondrial 12S rRNA gene was amplified by PCR using the primers L1091 (5'-AAAAAGCTTCAAACCTGGGATTAGATACCCCACTAT-3') and H1478 (5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3') (Kocher et al. 1989). The PCR reaction cycle was 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 45°C for 45 s, and 72°C for 60 s. Nucleotide sequences were obtained from the Macrogen Company (South Korea), edited in BioEdit (Hall 2001), and aligned in Clustal X (Thompson et al. 1997), with final visual inspection. Phylogenetic analyses were performed using maximum parsimony (MP) and Bayesian inference (BI) for the separate and combined analyses. Alternative analyses were performed using the neighbor-joining (NJ) method in Mega 5 (Tempe, AZ, USA) (Tamura et al. 2011). The MP analysis was performed with PAUP* 4.0 (MA, USA) (Swofford 2003) using a heuristic search with the TBR algorithm and the addition of random sequences. Characters not informative were excluded from the analyses. The statistical confidence of the nodes was evaluated

Table 1 Characteristics of water systems and identification of snails sampled used in the present study

Locality	System, altitude (m)	Latitude (S), longitude (W)	Voucher		GenBank accession number		
			GEVOL	MNHNCL	12S rDNA	16S rDNA	
Caquena Basin							
Colpa	Stream-swamp, 4,384	18°03'29.00", 69°13'52.00"	2	5900	KF285868	KF285827	
Lauca River Basin							
Parinacota	Stream-swamp, 4,399	18°12'51.00", 69°18'05.00"	2	5901	KF285869	KF285828	
Isluga Basin							
Isluga	River-swamp, 3,776	19°15'10.00", 68°42'15.00"	1	5902	KF285870	-	
			2	5903	KF285871	-	
Salar de Carcote Basin							
Salar de Carcote	Spring 1, 3,688	21°16'04.00", 68°19'21.00"	9 V1	5904	KF285872	KF285829	
			11 V1	5905	KF285873	KF285830	
Salar de Ascotán Basin							
Salar de Ascotán	Spring 2, 3,716	21°29'07.00", 68°15'21.00"	67 MG	5906	KF285874	KF285831	
			68 MG	5907	KF285875	KF285832	
			70 MG	5908	KF285876	-	
			84 MP	5909	KF285877	KF285833	
			86 MP	5910	KF285878	-	
Salar de Ascotán	Spring 11, 3,734	21°41'13.90", 68°12'54.00"	1 V11	5911	KF285879	-	
			6 V11	5912	KF285880	-	
			7 V11	5913	KF285881	-	
Loa River Basin							
El Tatio	Stream-geysers, 4,264	22°20'10.00", 68°00'59.00"	8	5914	KF285882	-	
			17	5915	KF285883	-	
Las Cascadas	River, 2,260	22°29'54.00", 68°58'18.00"	2	5916	KF285889	KF285838	
			5	5917	-	KF285839	
			8	5918	-	KF285840	
			11	5919	-	KF285841	
Las Vertientes	Spring, 2,181	22°13'15.20", 68°58'20.00"	1	5920	KF285890	KF285845	
			2	5921	KF285891	KF285846	
La Finca	River, 2,099	22°30'34.62", 68°59'27.90"	1	5922	-	KF285842	
			2	5923	KF285892	KF285843	
Chiu Chiu	River, 2,470	22°20'02.00", 68°38'57.00"	13	5924	KF285893	-	
			14	5925	KF285894	-	
Vertiente Opache	Spring-river, 2,184	22°29'02.50", 69°00'08.00"	1	5926	KF285895	-	
			2	5927	-	KF285847	
			3	5928	KF285896	KF285848	
Laguna Opache	Lagoon, 2,100	22°30'20.50", 68°59'43.30"	1	5929	-	KF285844	
Pacific coastal basins							
Quebrada La Chimba	Spring, 500	23°32'22.05", 70°21'36.40"	1-1	5930	KF285897	KF285849	
			2-2	5931	KF285898	KF285850	
Quebrada Carrizo	Spring-stream, 72	25°41'56.72", 70°24'42.51"	1	5932	KF285899	KF285851	
			2	5933	KF285900	KF285852	
Quebrada Cascabeles	Spring, 42	25°17'33.10", 70°26'45.40"	1	5934	-	KF285853	
			2	5935	KF285901	-	
Quebrada El León	Spring, 243	26°57'34.70", 70°44'15.00"	1	5936	KF285902	KF285854	
			2	5937	-	KF285855	

Table 1 Characteristics of water systems and identification of snails sampled used in the present study (Continued)

Aguada de Chorrillos	Spring, 5	27°12'32.40", 70°57'03.30"	1	5938	KF285907	KF285858
			6	5939	KF285908	KF285859
El Totoral	Stream, 200	27°53'50.70", 70°54'01.50"	3	5940	KF285922	KF285863
			4	5941	KF285923	KF285864
Los Perales, Quebrada Paposó	Spring-stream, 332	25°01'45.60", 70°27'17.90"	1	5942	KF285924	KF285865
			4	5943	KF285925	KF285866
			6	5944	KF285926	KF285867
Salar de Atacama Basin						
Tilomonte	Stream, 2,365	23°47'24.40", 68°06'34.20"	1-4	5945	KF285884	KF285834
Tilopozo	Pool, 2,313	23°47'05.00", 68°14'12.30"	21	5946	KF285885	KF285835
			22	5947	KF285886	KF285836
Peine	Stream, 2,440	23°41'00.00", 68°03'31.00"	1	5948	KF285887	-
			2	5949	KF285888	KF285837
Taltal Basin						
Las Breas, Quebrada de Taltal	Spring-pool, 588	25°30'10.10", 70°24'40.20"	1	5950	KF285903	-
			2	5951	KF285904	-
Pan de Azúcar Basin						
Quebrada Cachina	Spring, 321	25°54'03.40", 70°36'47.90"	1	5952	KF285905	KF285856
			2	5953	KF285906	KF285857
Copiapó River Basin						
Hornitos, Copiapó River	River, 826	27°46'04.40", 70°09'43.60"	8	5954	KF285909	-
			10	5955	KF285910	-
			12	5956	KF285911	KF285860
Copiapó, Copiapó River	River, 465	27°26'25.90", 70°16'02.60"	19	5957	KF285912	-
			20	5958	KF285913	-
			21	5959	KF285914	-
Pabellón, Copiapó River	River, 693	27°39'54.90", 70°13'55.80"	13	5960	KF285915	-
			15	5961	KF285916	-
			18	5962	KF285917	-
Humedal, Copiapó River	River-swamp, 17	27°19'14.80", 70°55'08.90"	2	5963	KF285918	-
			3	5964	KF285919	-
Carrera Pinto	Spring, 1,565	27°06'52.50", 69°53'52.00"	1	5965	KF285920	KF285861
			2	5966	KF285921	KF285862

GEVOL: specimen voucher number deposited in the Laboratorio de Genética y Evolución; MNHNCL: sample of tissue (or DNA) deposited in the Museo Nacional de Historia Natural, Santiago, Chile.

using 100 bootstrap pseudoreplicates (Felsenstein 1985). The BI was performed in the MrBayes v. 3.1.2 program (Ronquist and Huelsenbeck 2003), selecting the best evolutionary model with jModelTest (Posada 2008) for each data partition based on the Bayesian information criterion. This analysis was run three times for three million generations for the combined analyses. Trees were sampled every 1,000 generations, and posterior probabilities were obtained after a burn-in period of 10%.

Divergence times were estimated using the BEAST program (version 1.5.3, GNU Lesser General Public License, Boston, MA, USA) (Drummond and Rambaut 2007). We used a nucleotide substitution rate of 1.9% per million years for the 16S gene, the average divergence rate of this

gene estimated for invertebrates (Cunningham et al. 1992; Patarnello et al. 1996) and previously used in gastropods (DeJong et al. 2001; Collado and Méndez 2012b). The analysis was performed using a lognormal molecular clock, the general time reversible (GTR) substitution model plus the gamma distribution and the Yule process of speciation. Convergence of posterior distributions was analyzed in Tracer (version 1.5, GNU Lesser General Public License) (Rambaut and Drummond 2007), and the Bayesian tree was obtained after removing burn-in (10%) with TreeAnnotator (version 1.5.3, GNU Lesser General Public License).

Original 16S rRNA and 12S rRNA sequences obtained in this study were deposited in the National Center for

Biotechnology Information database [GenBank: KF285827 to KF285867 for 16S; KF285868 to KF285926 for 12S] (Table 1). The 12S rRNA and 16S rRNA sequences of the Lithoglyphidae *Lithoglyphus naticoides* (Pfeiffer, 1828) [GenBank: AF445341 and AF445351, respectively] and the Pyrgulidae *Pyrgula annulata* (Linnaeus, 1758) [GenBank: AF445340 and AF445350, respectively] were used as outgroup in the molecular analyses (Hausdorf et al. 2003). The matrix used in the combined analysis included taxa for which we did not have sequences for some of the two loci; in this case, the taxa were coded as “missing data”, which has shown not to unduly influence the phylogenetic resolution (Wiens and Reeder 1995; Wiens 1998; Collado and Méndez 2012b).

Results

Morphology

With few exceptions, the morphology of the penis showed differences between populations and species of *Heleobia* from northern Chile (Figure 2). Conversely, the shell of the snails presented little differentiation (Figure 3), with a size always greater than 3.5 mm (and less than 8 mm), so they belong to the large-sized species group proposed in the genus (see Biese 1944, 1947).

12S rRNA gene

We amplified 369 bases of the 12S rRNA gene from 59 *Heleobia* specimens, the final length of the alignment including the two outgroup sequences. The base composition was A = 0.41, C = 0.14, G = 0.16, and T = 0.29. For MP, 48 characters were informative, and 321 were excluded from the analysis. This analysis recovered 100

trees (not shown) with a length of 65 steps, consistency index = 0.83, retention index = 0.93, and rescaled consistency index = 0.78. For the BI (tree not shown), the best model of evolution for this data set was the general time reversible model (Rodríguez et al. 1990) and gamma distribution rate heterogeneity (GTR + G). The mean genetic divergence among all the populations and species of *Heleobia* from northern Chile was 1.20% using the two-parameter model of Kimura (1980) (K2P). The greatest distance among the nominal species occurred between *H. ascotanensis* and *H. atacamensis* (1.95%), and between the latter species and *H. chimbaensis* (1.95%, data not shown).

16S rRNA gene

We amplified 497 to 499 bases of the 16S rRNA gene from 41 *Heleobia* specimens. The alignment was 499 nucleotide sites in length considering the two outgroup sequences. The base composition was A = 0.34, C = 0.14, G = 0.19, and T = 0.33. For MP, 54 characters were informative, and 445 were excluded from the analysis. This analysis recovered two trees with the same topology (not shown) and a length of 88 steps, consistency index = 0.76, retention index = 0.89, and rescaled consistency index = 0.68. For the BI, the best evolutionary model was GTR + G. The mean genetic divergence among all populations and species of *Heleobia* was 1.30% using the K2P model. Genetic distance analysis was performed between *Heleobia* species included in the present study plus 16S sequences of the Semisalsinae taxa, *Semisalsa dalmatica* Radoman, 1974 [GenBank: AY676119] (Wilke 2005) and *Semisalsa stagnorum* Gmelin, 1791 [GenBank: JX970535] (Wilke et al.

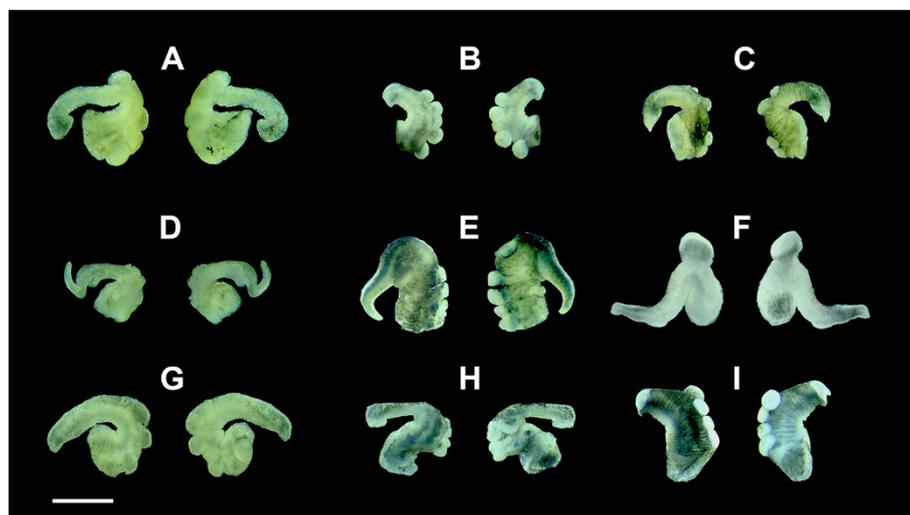
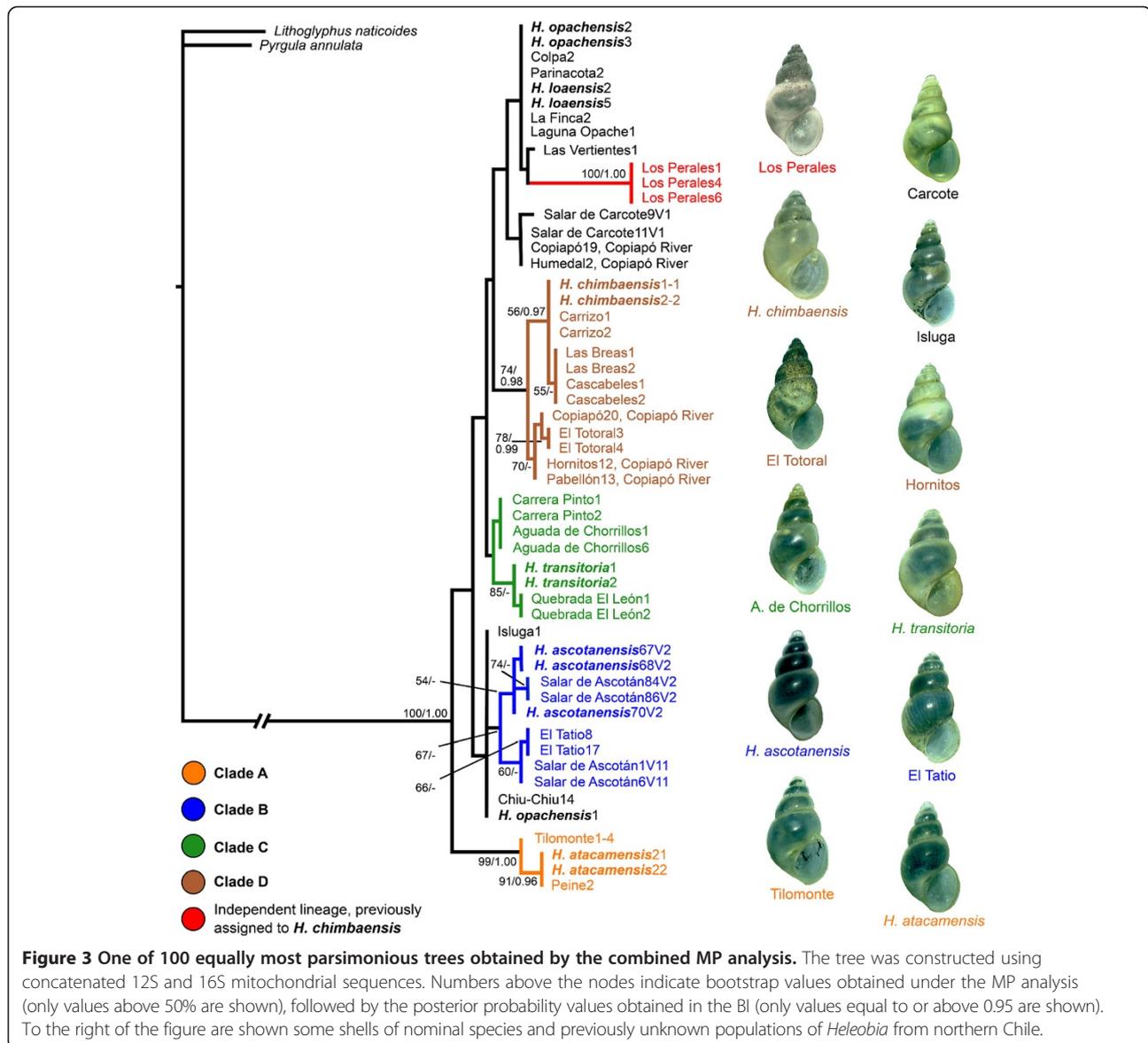


Figure 2 Penis morphology of species and populations of *Heleobia* from northern Chile. (A) Los Perales, (B) Salar de Carcote, (C) Isluga, (D) Aguada de Chorrillos, (E) *Heleobia transitoria*, (F) *Heleobia ascotanensis*, (G) El Tatio, (H) Tilomonte, (I) *Heleobia atacamensis*. Left, dorsal view; right, ventral view. Scale bar = 0.5 mm.



2013) for comparative purposes (Table 2). The greatest distance occurred between *H. atacamensis* and *H. transitoria* (2.63%), followed by *H. atacamensis* and *H. chimbaensis* (2.40%). The percentage of genetic divergence between *Semisalsa dalmatica* and *Semisalsa stagnorum* was 0.43%, much lower than several pair-wise comparison values obtained in the present study (Table 2). The BEAST analyses showed that the origin of the lineages occurred in the Early Pleistocene, with an average of 2.1 million years to the most ancestral node of *Heleobia*.

Combined analysis

For the combined phylogenetic analyses, 5 sequences were left as missing data within the 12S rRNA and 16 within the 16S rRNA data partition for which these sequences were unavailable (the alignment data are available from

the corresponding author). The data matrix included 55 *Heleobia* sequences and 868 characters, of which 102 were parsimony informative. This analysis recovered 100 trees (one of them shown in Figure 3) with a length of 148 steps, consistency index = 0.82, retention index = 0.92, and rescaled consistency index = 0.75. These indexes were slightly lower than those obtained with the 12S gene but considerably higher than those obtained with the 16S gene. Base frequencies were A = 0.38, C = 0.13, G = 0.17, and T = 0.31. Most of the major clades retained in the separate analyses were recovered in the combined MP analysis, with similar support values for most nodes. We used the GTR + G model to perform the combined BI analyses (tree not shown).

MP analysis of the combined data set and the BI recovered five major clades, four of which were composed

Table 2 Percentage sequence divergence (K2P) between pairs of mtDNA 16S sequences of *Heleobia* populations from northern Chile

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
1. <i>S. stagnorum</i>																									
2. <i>S. dalmatica</i>	0.43																								
3. <i>H. ascotanensis</i>	2.20	2.20																							
4. <i>H. atacamensis</i>	2.67	2.67	2.21																						
5. <i>H. chimbaensis</i>	2.41	1.97	1.52	2.24																					
6. <i>H. loaensis</i>	1.97	1.52	1.52	1.97	1.30																				
7. <i>H. opachensis</i>	1.97	1.52	1.52	1.97	1.30	0.00																			
8. <i>H. transitoria</i>	1.97	1.52	1.30	2.67	1.52	1.08	1.08																		
9. Ag. de Chorrillos	1.75	1.31	0.09	2.20	1.08	0.65	0.65	0.43																	
10. Ascotán (S2)	2.20	2.20	0.00	2.21	1.52	1.52	1.52	1.30	0.86																
11. Carrera Pinto	1.75	1.31	0.09	2.20	1.08	0.65	0.65	0.43	0.00	0.86															
12. Carcote (S1)	1.97	1.52	1.52	1.97	1.30	0.00	0.00	1.08	0.65	1.52	0.65														
13. Carrizo	2.41	1.97	1.52	2.42	0.00	1.30	1.30	1.52	1.08	1.52	1.08	1.30													
14. Cascabeles	2.41	1.97	1.52	2.42	0.00	1.30	1.30	1.52	1.08	1.52	1.08	1.30	0.00												
15. Colpa	1.97	1.52	1.52	1.97	1.30	0.00	0.00	1.08	0.65	1.52	0.65	0.00	1.30	1.30											
16. El León	2.19	1.75	1.52	2.88	1.74	1.30	1.30	2.15	0.65	1.52	0.65	1.30	1.74	1.74	1.30										
17. Hornitos	1.97	1.52	1.52	2.42	0.43	0.86	0.86	1.08	0.65	1.52	0.65	0.86	0.43	0.43	0.86	1.30									
18. La Finca1	1.74	1.31	1.74	1.75	1.52	0.21	2.14	1.30	0.86	1.74	0.86	0.21	1.52	1.52	0.21	1.52	1.08								
19. Laguna Opache	1.97	1.52	1.52	1.97	1.30	0.00	0.00	1.08	0.65	1.52	0.65	0.00	1.30	1.30	0.00	1.30	0.86	0.21							
20. Las Vertientes	1.97	1.52	1.52	1.97	1.30	0.00	0.00	1.08	0.65	1.52	0.65	0.00	1.30	1.30	0.00	1.30	0.86	0.21	0.00						
21. Los Perales	2.43	1.98	1.75	1.99	1.52	1.08	1.08	1.74	1.30	1.75	1.30	1.08	1.52	1.52	1.08	1.97	1.52	1.08	1.08	1.08					
22. Parinacota	1.97	1.52	1.52	1.97	1.30	0.00	0.00	1.08	0.65	1.52	0.65	0.00	1.30	1.30	0.00	1.30	0.86	0.21	0.00	0.00	1.08				
23. Peine	2.67	2.67	2.20	0.00	2.43	1.97	1.97	2.65	2.20	2.21	2.20	1.97	2.42	2.42	1.97	2.88	2.42	1.76	1.97	1.97	1.99	1.97			
24. Tilomonte	2.89	2.89	1.98	0.21	2.65	2.20	2.20	2.42	1.97	1.98	1.97	2.20	2.65	2.65	2.20	2.66	2.65	1.97	2.20	2.20	2.21	2.20	2.14		
25. El Totoral	2.19	1.75	1.74	2.66	0.65	1.08	1.08	1.30	0.86	1.74	0.86	1.08	0.65	0.65	1.08	1.52	2.15	1.30	1.08	1.08	1.75	1.08	2.66	2.88	

exclusively of one each of the nominal species recognized in the region: *H. ascotanensis*, *H. chimbaensis*, *H. transitoria*, and *H. atacamensis*. The first clade (clade A, Figure 3) was integrated by *H. atacamensis* from Tilopozo and the allopatric snails from Peine and Tilomonte. This clade was inferred as the sister group of all the rest of the species and populations of *Heleobia* sequenced (100% bootstrap, 1.00 posterior probability). However, the sister group position of all *Heleobia* snails from northern Chile was occupied by a different taxon or lineage in the 12S and 16S analyses performed, suggesting that further work is needed to resolve this issue. Likewise, relationships among the remaining species were not resolved. The monophyly of the sequences of *H. atacamensis* and those from Peine was supported by 91% bootstrap and 0.96 posterior probability. Within this clade, the snails from Tilomonte were recovered as the sister group of these sequences with high support values (99% bootstrap, 1.00 posterior probability). The sequences of the snails from Salar de Ascotán formed a monophyletic group (clade B in Figure 3) with the sequences from the allopatric locality of El Tatio with moderate bootstrap values (67%). Within this clade, two subclades were inferred, one containing snails from Spring 2 from this salt pan (assigned to *H. ascotanensis*) plus a group of snails from this same spring, which was supported by 54% bootstrap, and another composed of snails from Spring 11 from Salar de Ascotán and those from El Tatio, with moderate bootstrap values (60%).

The monophyly of the sequences of *H. transitoria* (clade C in Figure 3) from its type locality Quebrada Cachina plus snails from Quebrada El León was supported by high bootstrap values (85%), but low posterior probability. The snails from Carrera Pinto and Aguada de Chorrillos were recovered as the sister group to this subclade but without node support.

The sequences of *H. chimbaensis* from the type locality Quebrada La Chimba nested in a clade together with snails from the Atacama Desert (74% bootstrap, 0.98 posterior probability) (clade D in Figure 3). Within this clade, the sequences from El Totoral formed a monophyletic group with a 78% bootstrap and high posterior probability values (0.99). The sequences of snails from Los Perales (clade red in Figure 3) formed a monophyletic group supported by 100% bootstrap and 1.00 posterior probability.

The sequences of snails from the Salar de Carcote in the Chilean Altiplano nested in a clade together with sequences from the Copiapó River but without support. The penis morphology of these snails, however, showed similarities with those of the species *H. loaensis*, *H. opachensis*, and *H. atacamensis*, in the latter case suggesting convergence of characters (Figure 2).

The sequences of snails from Las Cascadas, the type locality of *H. loaensis* and Vertiente Opache, the type

locality of *H. opachensis*, were not resolved by the combined analysis, as well as the sequences of the snails from Chiu-Chiu, La Finca, Laguna Opache, and Las Vertientes in the Loa basin, and those from Colpa, Parinacota and Isluga in the Chilean Altiplano. However, we found the penis morphology of snails from Isluga to be different from those of other localities (Figure 2).

Discussion

The combined and separate phylogenetic analyses recovered the populations of *Heleobia* from Los Perales as a monophyletic group with high support values. These snails were originally assigned to *H. chimbaensis* by Biese (1947) based on external shell morphology, but our results show that they constitute an independent lineage phylogenetically separated from this species, and the male copulatory organ (Figure 2) is markedly different (see Hubendick 1955; Collado et al. 2011a; Collado 2012); it is apparent that a description and a new scientific name is required for this cryptic species. Apart from this clade, our molecular analyses show that the populations of *Heleobia* from northern Chile form four other generally well supported clades. The division of the clades suggests that a relatively long amount of time elapsed to produce genetic lineage diversification in the region, which is not reflected in the morphology of the shell (see Figure 3). This is not infrequent in rissooidean snails, a taxon with a large number of genera, many of which include species morphologically similar but genetically divergent (e.g., Liu et al. 2003; Hershler et al. 1999; Hershler et al. 2003; Bichain et al. 2007; Hershler et al. 2007; Falniowski et al. 2012). The percentage sequence divergence between the *Heleobia* species observed in the present study was generally greater than those found in other rissooidean taxa. For example, there is no variation between 12S sequences of the species of the family Tateidae *Tatea huonensis* (Tenison-Woods, 1876) and *Tatea rufilabris* (A. Adams, 1862) [GenBank: FJ619852 and FJ619856, respectively] (see Colgan and da Costa 2009), while divergence between the Lithoglyphidae *Benedictia baicalensis* (Gerstfeld) and *Benedictia maxima* (W. Dybowski) [GenBank: AF445349 and AF445348, respectively] (Hausdorf et al. 2003) is 0.83% (K2P), and the divergence between *Potamopyrgus antipodarum* (Gray, 1843) and *Potamopyrgus estuarinus* (Winterbourn, 1971) [GenBank: HQ875146 and GQ996415, respectively] (Neiman et al. 2010) is 2.25%. In the case of 16S sequences, the divergence between the Hydrobiidae *Sulawesiidrobia bonnei* (Abbott, 1945) and *Sulawesiidrobia botak* Haase and Bouchet, 2006 [GenBank: HM587413 and HM587411, respectively] (Zielske et al. 2011) is 0.19%, while the divergence between the Tateidae *Potamopyrgus antipodarum* and *Potamopyrgus oppidanus* Haase, 2008 [GenBank: AY634104 and AY634090, respectively] (Haase 2005) is 0.21%.

In the genus *Heleobia*, several studies, including original descriptions of species, show overlapping characters in the external morphology of the shell (e.g., Bavay 1904; Courty 1907; Pilsbry 1911, 1924; Biese 1944, 1947; Haas 1955; Preston 1915; Weyrauch 1963). In fact, the *Heleobia* fauna from Lake Titicaca and closely related taxa from the surrounding areas form a species flock, in some cases including morphologically parallel species (Kroll et al. 2012). The clades inferred by our molecular analyses are concordant with the distribution patterns, and some of them include candidate species or new lineages within the genus not detected previously and whose copulatory organs show a particular morphology.

The Salar de Atacama contains snails from Tilopozo, Peine, and Tilomonte (clade A, Figure 3). Philippi (1860) described *H. atacamensis* from Tilopozo, and since then, it has not been addressed in the literature, with the exception of checklists of the species (Stuardo 1961; Sielfeld 2001; Valdovinos 1999, 2006, 2008) or the description of its penis (Collado et al. 2011a). The monophyly of the sequences of *H. atacamensis* from Tilopozo and Peine was well supported in the MP and BI analyses, while the sequence from Tilomonte appears as the sister to this group in all analyses performed. Likewise, the penis morphology shows differences between *H. atacamensis* and snails from Tilomonte (Figure 2), suggesting that this population represents a candidate species of the genus. These three localities are located on the east bank of the Salar de Atacama in the homonymous basin. Tilopozo is separated from Peine by about 20 km, while Tilomonte is located in the middle, at a distance of about 13 km from each of these locations. The Tilomonte oasis includes a small stream that dries at a distance of approximately 6.5 km of the shore of the Salar de Atacama, while the oasis of Peine is formed by a stream with a greater flow of water that flows at irregular intervals into the salt pan. It is apparent that seasonal flooding could connect Tilopozo and Peine, producing dispersal of the snails between these locations, while the partial isolation of Tilomonte could explain the divergence of these populations as a product of vicariance.

Clade B (Figure 3) clusters populations from Springs 2 and 11 in Salar de Ascotán and the allopatric snails from the locality of El Tatio. *Heleobia ascotanensis* was described by Courty (1907) (under the genus *Paludestrina* d'Orbigny, 1840) from the Salar de Ascotán together with five subspecies and two species of the genus *Bythinella* Moquin-Tandon, 1856 based on shell morphology (see Collado and Méndez 2012a). The monophyly of the sequences and support values suggest that two distinct lineages co-occur in Spring 2 from this salt pan, but we have no evidence of reproductive isolation between them. Additionally, the snail sequences from Spring 11 form a subclade together with the snails from El Tatio. In the 12S

analysis, the separation of this subclade from the sympatric snails from Spring 2 was supported by a moderate bootstrap value in the MP analysis (66%) but with a high posterior probability value in the BI (0.97). Furthermore, the penis morphology of *H. ascotanensis* (Collado et al. 2011b; present study) is different from those of the snails of El Tatio (Figure 2), suggesting the occurrence of an additional candidate species in our data set. At present, we cannot establish if the snails from Spring 11 from Salar de Ascotán constitute a different species from those of El Tatio, although the support values for their monophyly recovered in the 12S separate analysis seem to indicate this. There is no evidence of subterranean or surface water connection between Spring 2 in the north of the Salar de Ascotán and Spring 11 in the south; these two springs are separated by a distance of approximately 21.5 km. Structuring of populations of the killifish *Orestias ascotanensis* Parenti, 1984 in Salar de Ascotán was recently reported, including those fish located in Spring 11, a process probably stimulated by habitat fragmentation occurring from the Late Pleistocene to the beginning of the Holocene (Morales et al. 2011). Keller and Soto (1998) suggested that in this salt pan, there is no evidence of major changes in the water level in the last 12,000 years. Recently, we also detected microvicariance processes structuring the populations of the aquatic gastropod *Biomphalaria crequii* (Courty 1907) inhabiting the southernmost springs of the system (Collado and Méndez 2013). There is no evidence either of subterranean or surface water connection between Spring 11 and El Tatio, a geyser field located 75 km to the south in a different basin.

Heleobia transitoria integrates a clade (clade C, Figure 3) with allopatric Atacama populations that are separated by arid desert. After its original description, this species has not been addressed in the literature, with the exception of local checklists. The type locality of the species, a small spring in Quebrada Cachina, is separated by approximately 151 km from the Carrera Pinto oasis, another small southwestern spring, 117 km from a small southern spring in Quebrada El León and 147 km from the southernmost spring Aguada de Chorrillos. Within this clade, the monophyly of *H. transitoria* and the snails from Quebrada El León was well supported in the MP 16S and the combined analyses; they are probably conspecific. The snails from Aguada de Chorrillos (and Carrera Pinto) never formed a monophyletic group with *H. transitoria* in the analyses performed, and the penis morphology shows conspicuous differences between them (Figure 2).

Clade D (Figure 3) clusters the sequences of *H. chimbaensis* from Quebrada La Chimba and snails from other Atacama localities, almost all of them with allopatric distribution. The monophyly of snails from Las Breas in Quebrada de Taltal and Quebrada Cascabeles was weakly supported in the combined analysis, but was recovered

with relatively high support values in the 12S analysis (65% bootstrap, 0.97 posterior probability), suggesting the presence of a single species within this assemblage. The monophyly of the sequences from El Totoral and the Atacama populations from Copiapó River was supported by 70% bootstrap, suggesting some phylogenetic structure. This is consistent with the location of El Totoral, an intermittent stream in the Atacama Desert 63 km south of the Copiapó River. The snails disseminated within the Copiapó River (Humedal, Copiapó, Hornitos, and Pabellón) never clustered together within the subclade integrated by *H. chimbaensis* or another described species of the genus. The lack of monophyly suggests the presence of more than one taxon in this hydrological system, for which the names *H. copiapensis*, *H. copiapensis costata*, and *H. striata* are available (Biese 1944, 1947).

From the taxonomic point of view, Biese (1947) considered *H. loaensis* and *H. opachensis* as different species based on external shell morphology. The sequences of these species were not resolved by any analyses performed in the present study, with the exception of the NJ analysis (tree not shown), which recovered these taxa in a moderately well-supported clade (65% bootstrap). Considering that these taxa have similar shell and penis morphology (Biese 1944, 1947; Collado et al. 2011a), their validity needs to be tested using additional morphological characters or a distinct, faster-evolving DNA locus. Apart from snails from Salar de Carcote and Isluga, which probably represent other candidate species judging from penis morphology, the identity and systematic position of snails from Colpa and Parinacota in the Chilean Altiplano and La Finca, Laguna Opache, Chiu-Chiu, and Las Vertientes from the Loa basin in the Atacama Desert need to be clarified.

Heleobia carinata (Biese), a name that does not meet the criteria of availability according to the Code (International Commission on Zoological Nomenclature ICZN 1999) (see Collado et al. 2011b), was found in Pleistocene lacustrine facies in northern Chile (Biese 1961; Ochsenius 1974). Regardless of the availability of the name, these *Heleobia* record fossil data agree with our molecular calibration that places the origin of the *Heleobia* in the region in the Early Pleistocene, implying a recent origin of the group. Kroll et al. (2012) also found Pleistocene divergence using the mitochondrial cytochrome c oxidase subunit 1 (COI) gene among the *Heleobia* populations distributed in northern Chile and the Altiplano region.

According to our results, the speciation of *Heleobia* fauna in the Chilean Altiplano and the Atacama Desert is suggested as being allopatric from the Early Pleistocene. We consider the diversity of *Heleobia* at the species level in the region to be underestimated. We suggest that this is a consequence of the use of conchological criteria to delineate

species boundaries and the limited sampling conducted to date. The discovery of a cryptic lineage in Los Perales and other candidate species in different localities in the region supports this statement.

Conclusions

The present results support the conclusion that the snails from Tilomonte, El Tatio, Aguada de Chorrillos, Isluga, and Salar de Carcote (Spring 1) represent candidate species of the genus *Heleobia*. The molecular analysis provides support for recognizing the nominal species *H. chimbaensis*, *H. atacamensis*, *H. transitoria*, and *H. ascotanensis* as valid taxa, while the specific status of *H. opachensis* and *H. loaensis* could not be resolved. The phylogenetic analysis and penis morphology revealed that the snails from Los Perales constitute a cryptic species of the genus. We also suggest that vicariance may largely explain the distribution patterns of *Heleobia* populations disseminated in the Chilean Altiplano and the Atacama Desert. A more intensive effort will be necessary to test the cryptic species boundaries thoroughly in *Heleobia*.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GAC participated in the field trips for sampling, obtained the sequences, performed the analyses, and wrote the manuscript. MAV performed the morphological study, made the figures and reviewed the manuscript. MAM made intellectual contributions, reviewed the manuscript and provided technical and financial support. All authors read and approved the final manuscript.

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