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Phylogenetic relationships and taxonomy of Altiplano populations of *Biomphalaria* (Gastropoda: Planorbidae): inference from a multilocus approach

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Isolated closed basins provide a natural laboratory to study the differentiation among wild populations. Here we examined the phylogenetic relationships of the Southern Altiplano populations of *Biomphalaria*, a genus with medical importance, using nuclear (ITS1, ITS2) and mitochondrial (16S) ribosomal gene markers and a species of *Helisoma* as outgroup. Phylogenetic trees based on separate and combined analyses show that these populations form a particular lineage within *Biomphalaria* along with *Biomphalaria peregrina* (d'Orbigny, 1835) and *Biomphalaria oligoza* Paraense, 1974. The origin of this clade was estimated to have occurred in the middle Pleistocene. Molecular analyses showed that the nominal species *Biomphalaria crequii* (Courty, 1907) from the Salar de Ascotán and *Biomphalaria costata* (Biese, 1951) from the Salar de Carcote, previously synonymized with *Biomphalaria andecola* (d'Orbigny, 1835) and *B. peregrina*, respectively, are distinct taxa. Molecular data did not resolve the relationship of *Biomphalaria aymara* Valdovinos & Stuardo, 1991 from the Isluga swamps to other *Biomphalaria* species, but confirm that the populations from the Lauca and Huasco basins may represent a distinct undescribed species of *Biomphalaria* from the Southern Altiplano. Snails examined for trematodes were found to be positive in some Altiplano localities.

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ADDITIONAL KEYWORDS: Chilean Altiplano – gastropods – freshwater snails – mitochondrial genes – nuclear ITS regions – systematics.

INTRODUCTION

The freshwater snails of the genus *Biomphalaria* Preston, 1910 are widely distributed in the Neotropical and African regions where some species are intermediate hosts of *Schistosoma mansoni* Sambon, 1907, a digenetic trematode which causes schistosomiasis (WHO, 1993, 1998). The taxonomy of *Biomphalaria* has been principally based on shell morphology and the anatomy of the reproductive system (Paraense, Fauran & Courmes, 1964; Paraense, 1966, 1981, 1984, 2003; Valdovinos & Stuardo, 1991), but the similarity of these characters among taxa has made classification difficult (Paraense, 1988; Estrada *et al*., 2006). In fact, convergent shell evolution among African *Biomphalaria* species has been detected, complicating species identification using conchological characters (Plam *et al*., 2008). The incorporation of molecular techniques, based mainly on restriction fragment length polymorphism (RFLP) analyses, has helped in the identification and recognition of species, although most of the studies have concentrated on Neotropical species (Caldeira *et al*., 1998, 2000; Vidigal *et al*., 1998, 2000a, b, 2001, 2002; Spatz *et al*., 1999, 2000; Velásquez *et al*., 2002). At the time of writing, 30–37 species are considered valid within *Biomphalaria* (DeJong *et al*., 2001; Jørgensen, Kristensen & Stothard, 2007; Caldeira, Teodoro & Carvalho, 2008).

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In the south-western Altiplano of South America there is a great diversity of hydrological systems inhabited by populations of *Biomphalaria*. The physical structure of the area has been shaped primarily by intense volcanic activity from the Miocene to the Holocene and a series of palaeolakes that have been explained by climatic changes (Wörner *et al*., 2000; Fornari, Risacher & Féraud, 2001; Risacher, Alonso & Salazar, 2003; Strecker *et al*., 2007). At present, the landscape comprises isolated closed basins generally delineated by high chains of volcanoes (Risacher *et al*., 2003). The hydrological systems restricted to the basins constitute enclosed evaporitic environments. Thus, this Altiplano region constitutes an ideal natural scenario to study the evolutionary diversification of the aquatic biota. From this area have been described *Biomphalaria costata* (Biese, 1951) from the Salar de Carcote, *Biomphalaria aymara* Valdovinos and Stuardo, 1991 from the Isluga swamps, and *Biomphalaria crequii* (Courty, 1907) [= *Biomphalaria grangei* (Courty, 1907), G. A. Collado & M. A. Mendez, unpubl. data] from the Salar de Ascotán. However, the taxonomy of these nominal species and the identity of other populations of the genus recently discovered in the region are not completely resolved. For example, Malek (1985) using morphological characters argued that the species from the Salar de Ascotán [considered by him to be the nominal species *Biomphalaria thermala* (Biese, 1951)] correspond to *Biomphalaria andecola* (d'Orbigny, 1835), a species from Peru and Bolivia, while for Paraense (1966) *B. costata* is a synonym of *Biomphalaria peregrina* (d'Orbigny, 1835), a species widely distributed in the Neotropical region. In a recent phylogenetic study, we utilized DNA sequences of the mitochondrial gene cytochrome oxidase subunit I (COI) to study the relationship among Altiplano populations of *Biomphalaria* (Collado, Vila & Méndez, 2011). In this study, sequences of *B. peregrina* from La Plata and Mendoza were recovered as sister to the Altiplano taxa, while individuals from the Salar de Ascotán, assigned to *B. crequii*, were recovered in a strongly supported clade. *Biomphalaria aymara* and *B. costata* formed a monophyletic group and could not be differentiated phylogenetically. This study also showed that the populations restricted to the Lauca and Huasco basins possibly comprise a distinct subgroup of *Biomphalaria*, as well as the populations restricted to the Caquena basin.

In the present study we obtained DNA sequences of the mitochondrial 16S rRNA gene and the ITS1 and ITS2 nuclear internal transcribed spacers regions of the rRNA gene complex from Altiplano populations and other species of *Biomphalaria* to examine its monophyly and systematic position among other species of *Biomphalaria* through an inclusive, multilocus analysis of the group. We also assess whether *B. crequii* is a synonym of *B. andecola*. Additionally, considering that Collado *et al*. (2011) assessed the synonymy of *B. costata* and *B. peregrina* postulated by Paraense (1966) using COI data, which was not supported, in the present study we further evaluate this hypothesis incorporating multilocus sequences (16S, ITS1, ITS2) of *B. peregrina* from a widespread range of the distribution of the species in South America and *B. costata* from Salar de Carcote for comparative purposes. Available data from GenBank for *B. peregrina* include sequences from Uruguay, Brazil (Minas Gerais, Rio Grande Do Sul, Ourinhos) and Argentina (Mendoza) (Table 1). We additionally obtained original sequences of this species from La Plata, Argentina, thanks to samples generously provided by Dr Alejandra Rumi. Finally, we investigate the possible existence of putative species among Altiplano populations of *Biomphalaria* and briefly discuss the taxonomy of the Neotropical *Biomphalaria schrammi* (Crosse, 1864).

MATERIAL AND METHODS

Dataset

We compiled DNA sequences of the 16S rRNA gene and ITS1 and ITS2 region available in GenBank from several sources (Table 1) and obtained original sequences of these three molecular loci from Altiplano populations of *Biomphalaria* and other species of the genus (see below). The sequences used in this study were sourced from across a large fraction of the generic range and thus we can estimate the systematic position of the populations of *Biomphalaria* restricted to the Neotropical Southern Altiplano. We used *Helisoma trivolvis* as the outgroup for molecular analyses based on previous phylogenetic studies (Bandoni, Mulvey & Loker, 1995; DeJong *et al*., 2001; Morgan *et al*., 2002); its sequences were obtained by DeJong *et al*. (2001).

Sampling and DNA extraction

We sampled snails of populations of *Biomphalaria* distributed in the southern Altiplano (18–22°S), which were fixed in absolute alcohol for DNA extraction. Some living specimens were dissected using a stereoscopic microscope to investigate the presence of trematodes. Snails were sampled using a small sieve from macrophytes in localities belonging to six closed basins located at more than 3500 m elevation (Fig. 1). The Caquena basin included five localities (Visviri, 4048 m altitude; Umaqui, 4132 m; Vioco, 4383 m; Colpa, 4384 m; Caquena, 4398 m), and the Lauca basin seven (Parinacota, 4399 m altitude; Chuviri,

Table 1. Geographical origin of the samples of *Biomphalaria* and GenBank accession numbers of the DNA sequences used in the phylogenetic analyses

Species/population	Accession number			Origin
	16S	ITS1	ITS2	
Biomphalaria alexandrina (Ehrenberg, 1831)[a]	AY030203	AY030371	AY030371	Egypt (1)
	AY030204	AY030372	AY030372	Egypt (2)
Biomphalaria amazonica Paraense, 1966[a]	AY030216	AY030384	AY030384	Brazil (1)
	AY030217	AY030385	AY030385	Brazil (2)
Biomphalaria angulosa Mandahl-Barth, 1957[b]	DQ084848	DQ084889	–	Tanzania
Biomphalaria camerunensis (Boettger, 1941)[a]	AY030198	AY030366	AY030366	Cameroon (1)
	AY030199	AY030367	AY030367	Cameroon (2)
Biomphalaria choanomphala (Martens, 1879)[a]	AY030202	AY030370	AY030370	Tanzania
Biomphalaria edisoni Estrada et al. (2006)[c]	–	AY364446	AY364450	Colombia (1)
	–	AY364448	AY364451	Colombia (2)
	–	AY364449	AY364452	Colombia (3)
Biomphalaria glabrata (Say, 1818)[a]	AY030206	AY030374	AY030374	Dominican Republic
	AY030207	AY030375	AY030375	Puerto Rico
	AY030208	AY030376	AY030376	Brazil
	AY030209	AY030377	AY030377	Venezuela
Biomphalaria helophila (D'Orbigny, 1835)[a]	AY030230	AY030399	AY030399	Cuba
Biomphalaria intermedia (Paraense & Deslandes, 1962)[a]	AY030215	AY030383	AY030383	Paraguay
Biomphalaria kuhniana (Clessin, 1883)[a]	AY030210	AY030378	AY030378	Dominican Republic
	AY030211	AY030379	AY030379	Colombia
	AY030212	AY030380	AY030380	Venezuela
Biomphalaria obstructa (Morelet, 1849)[a]	AY030228	AY030397	AY030397	USA
	AY030229	AY030398	AY030398	Mexico
Biomphalaria occidentalis Paraense, 1981[a]	AY030221	AY030389	AY030389	Brazil
Biomphalaria oligoza Paraense, 1974[d]	–	–	AF198679	Brazil (1)
	–	–	AF198680	Brazil (2)
Biomphalaria peregrina (d'Orbigny, 1835)[a]	AY030231	AY030400	AY030400	Uruguay
	AY030232	AY030401	AY030401	Brazil (Minas Geraís1)
Biomphalaria peregrina (d'Orbigny, 1835)[d]	–	–	AF198676	Brazil (Minas Geraís2)
	–	–	AF198677	Brazil (RS)
Biomphalaria peregrina (d'Orbigny, 1835)[e]	GU168591	–	–	Argentina (Mendoza1)
	GU168592	–	–	Argentina (Mendoza2)
Biomphalaria peregrina (d'Orbigny, 1835)[f]	–	–	DQ143961	Brazil (Ourinhos1)
	–	–	DQ143962	Brazil (Ourinhos2)
Biomphalaria peregrina (d'Orbigny, 1835)[g]	JF309030	–	–	Argentina (La Plata1)
Biomphalaria peregrina (d'Orbigny, 1835)[g]	JF309031	JF308974	JF309001	Argentina (La Plata2)
Biomphalaria peregrina (d'Orbigny, 1835)[g]	JF309032	–	–	Argentina (La Plata3)
Biomphalaria pfeifferi (Krauss, 1848)[a]	AY030193	AY030361	AY030361	Senegal
	AY030194	AY030362	AY030362	Cameroon
	AY030195	AY030363	AY030363	Sudan
	AY030196	AY030364	AY030364	Madagascar
Biomphalaria prona (Martens, 1873)[a]	AY030222	AY030390	AY030390	Venezuela (1)
	AY030223	AY030391	AY030391	Venezuela (2)
Biomphalaria schrammi (Crosse, 1864)[a]	AY030233	AY030402	AY030402	Brazil (Minas Geraís1)
Biomphalaria schrammi (Crosse, 1864)[d]	–	AY425743	AF198681	Brazil (Minas Geraís2)
	–	AY425744	AF198682	Brazil (Minas Geraís3)
Biomphalaria smithi Preston, 1910[a]	AY030205	AY030373	AY030373	Uganda
Biomphalaria sp. Bolivia[a]	AY030218	AY030386	AY030386	Bolivia
Biomphalaria sp. Cuba[a]	AY030224	AY030393	AY030393	Cuba (1)
Biomphalaria sp. Cuba[a]	AY030225	AY030394	AY030394	Cuba (2)
Biomphalaria sp. Puerto Rico[a]	AY030226	AY030226	AY030226	Puerto Rico
Biomphalaria stanleyi (Smith, 1888)[a]	AY030197	AY030365	AY030365	Uganda
Biomphalaria straminea (Dunker, 1848)[a]	AY030213	AY030381	AY030381	Brazil (1)
	AY030214	AY030382	AY030382	Brazil (2)
Biomphalaria sudanica (Martens, 1873)[a]	AY030200	AY030368	AY030368	Kenya
	AY030201	AY030369	AY030369	Tanzania
Biomphalaria temascalensis Rangel-Ruiz, 1987[a]	AY030227	AY030396	AY030396	Mexico
Biomphalaria tenagophila (d'Orbigny,1835)[a]	AY030219	AY030387	AY030387	Paraguay
	AY030220	AY030388	AY030388	Brazil
Biomphalaria sp. Umaqui[g]	GU168043	JF308977	JF309004	Umaqui (1)*
Biomphalaria sp. Vioco[g]	GU168044	–	JF309005	Vioco (1)*
Biomphalaria sp. Visviri[g]	GU168045	JF308978	JF309006	Visviri (1)*
Biomphalaria sp. Lauca Sur[g]	GU168046	JF308984	–	Lauca Sur (1)*
Biomphalaria sp. Piacota[g]	GU168049	JF308987	JF309015	Piacota (1)*
	JF309036	JF308988	JF309016	Piacota (3)*
Biomphalaria sp. Lauca[g]	GU168050	JF308983	JF309012	Lauca (1)*
Biomphalaria sp. Caquena[g]	GU168052	JF308975	JF309002	Caquena (1)*
Biomphalaria sp. Chungará[g]	GU168053	–	JF309007	Chungará (3)*
Biomphalaria sp. Cotacotani[g]	GU168054	JF308981	JF309008	Cotacotani (2)*
Biomphalaria sp. Chuviri[g]	GU168055	JF308980	JF309010	Chuviri (1)*
Biomphalaria sp. Colpa[g]	JF309033	JF308976	JF309003	Colpa (2)*
Biomphalaria sp. Parinacota[g]	JF309035	JF308985	JF309013	Parinacota (1)*
	GU168058	JF308986	JF309014	Parinacota (3)*
Biomphalaria sp. Salar de Huasco[g]	GU168059	JF308982	JF309011	Salar de Huasco (1)*
Biomphalaria sp. Chibatambo[g]	JF309034	JF308979	JF309009	Chibatambo (1)*
Biomphalaria aymara Valdovinos & Stuardo, 1991 and Stuardo, 1991[g]	GU168056	JF308989	–	Isluga (1)*
	GU168057	JF308990	JF309017	Isluga (2–6)*
	JF309041	–	JF309018	Isluga (3)*
	JF309042	–	JF309019	Isluga (4)*
Biomphalaria costata (Biese, 1951)[g]	JF309037	JF308991	JF309020	Salar de Carcote (1V1)*
	GU168048	JF308992	JF309021	Salar de Carcote (6V1)*
	JF309038	JF308993	JF309022	Salar de Carcote (11V1)*
	JF309039	JF308994	JF309023	Salar de Carcote (14V1)*
	JF309040	JF308995	JF309024	Salar de Carcote (16V1)*
Biomphalaria crequii (Courty, 1907)[g]	GU168047	JF308996	JF309025	Salar de Ascotán (1V2)*
	GU168051	JF308997	JF309026	Salar de Ascotán (6V2)*
	JF309043	JF308998	JF309027	Salar de Ascotán (18V2)*
	JF309044	JF308999	JF309028 JF309025	Salar de Ascotán (21V2)*
	JF309045	JF309000	JF309029	Salar de Ascotán (23V2)*

Taxa sequences incorporated in this study: a: DeJong *et al*., 2001, b: Jørgensen *et al*., 2007, c: Estrada *et al*., 2006, d: Vidigal *et al*., 2000c, e: Standley *et al*., (unpublished); f: Tuan, R. & dos Santos, P. (unpublished); g: Present study; *: Chilean Altiplano localities.

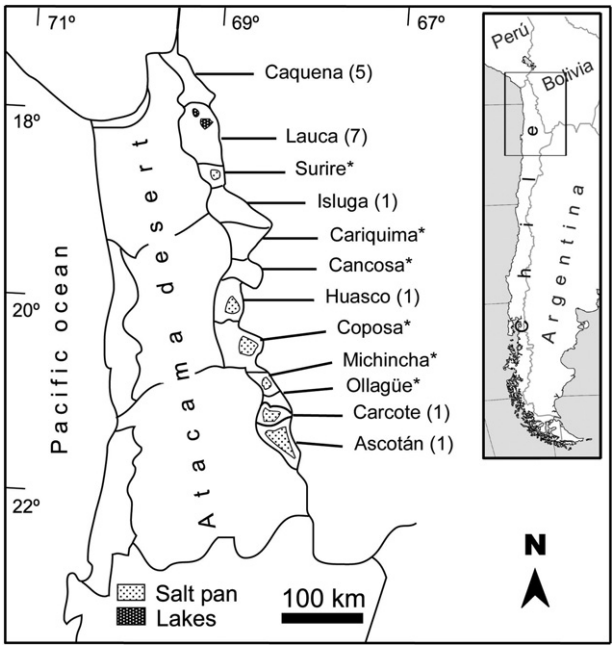

Figure 1. Sampling localities of populations of *Biomphalaria* of the southern Altiplano. The number of localities sampled by basin is shown in parentheses. Asterisks indicate basins where *Biomphalaria* snails were not found.

4374 m; Piacota, 4427 m; Chibatambo, 4232; Lauca, 4395 m; Lauca Sur, 4232 m; Chungará lake, 4583 m). The basin of the Salar de Huasco (3805 m altitude), Isluga swamps (3776 m), Salar de Carcote (3688 m; spring 1) and Salar de Ascotán (3716 m; spring 2) had one locality each.

Genomic DNA was extracted using the CTAB method (cetyltrimethyl ammonium bromide) (Winnepennickx, Backeljau & De Wachter, 1993). We separated the shell of the animals from the soft tissues, and a small part of the foot muscle tissue was dissected and placed in a tube with 600 µL of 100 mM Tris, 1.4 M $NaCl_2$, 20 mM EDTA, 2% CTAB, 0.2% β-mercaptoethanol and 0.01 mg proteinase K. Samples were macerated and incubated at 65 °C for 40 min with agitation every 15 min. Proteins and lipids were removed using 600 µL chloroform by centrifuging at 16 500 g for 10 min. DNA was precipitated in cold isopropanol for 30 min at 4 °C and then centrifuged at 16 500 g for 15 min. Finally, DNA was washed in 70% ethanol, dried and resuspended in 50 µL distilled water.

DNA amplification

We obtained partial sequences from 1–5 specimens per basin and locus. To amplify the 16S mitochondrial gene we used the primers 16Sar (5′-CGCCTGTTT ATCAAAAACAT-3′) and 16Sbr (5′-CCGGTCTGAAC TCAGATCACGT-3′) (Palumbi, 1996). We amplified the ITS1 nuclear region using primers ETTS2 (5-TAA CAAGGTTTCCGTAGGTGAA-3) (Kane & Rollinson, 1994) and ITS1R (5-ACGAGCGAGTGATCCACCGC-3) (Vidigal *et al*., 2004). The ITS2 nuclear region was amplified using primers ETTS1 (5′-TGCTTAAGTTCA GCGGGT-3′) (Kane & Rollinson, 1994) and ITS2F (5-CGTCCGTCTGAGGGTCGGTTTGC-3) (Vidigal *et al*., 2000c). PCR amplifications were performed in a total reaction volume of 50 µL with 0.8 U *Taq* DNA polymerase, 15 mM $MgCl_2$, 200 µM dNTPs and 1 µM of each primer. The PCR programme for 16S included an initial denaturation at 94 °C for 60 s, 35 cycles of 94 °C for 30 s, 55 °C for 50 s and 72 °C for 60 s, and a final extension at 72° for 10 min. The PCR programme for the nuclear regions was an initial denaturation at 95 °C for 60 s, 34 cycles of 94 °C for 45 s, 54 °C for 60 s and 72 °C for 2 min, and a final extension at 72° for 5 min. Amplified products were sequenced by Macrogen Company, South Korea. We incorporated 33 original sequences for 16S (GenBank accession numbers GU168043–GU168059 and JF309030–JF309045), 27 for ITS1 (GenBank accession numbers JF308974–JF309000), and 29 for ITS2 (GenBank accession numbers JF309001–JF309029) (see Table 1).

Sequence edition and phylogenetic analysis

DNA sequences were edited with the BioEdit program (Hall, 1999) and aligned with Clustal X (Thompson *et al*., 1997), with a final adjustment by visual inspection. For phylogenetic reconstructions we used the maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian analysis (BA) methods. MP analysis was performed with PAUP* (Swofford, 2003), using a heuristic search with the TBR algorithm with the addition of random sequences and retaining 100 trees in each analysis. Characters not informative for parsimony were excluded from the analyses. The statistical confidence of the nodes was evaluated using 100 bootstrap pseudoreplicates (Felsenstein, 1985). ML analysis was also performed with PAUP*, selecting the best evolutionary model with the jModelTest 0.1.1 program (Posada, 2008) under the Akaike Information Criterion. BA was performed using the program MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003), selecting the best evolutionary model with jModelTest under the Bayesian Information Criterion. This analysis was only applied to the groups of the combined data set and was run three times for 5 million generations. Trees were sampled every 1000 generations and posterior probabilities were based on 4501 trees obtained after the burn-in period (10%). With the original sequences determined in this study and those obtained from GenBank we constructed data matrices with 78 sequences for the 16S gene, 75

sequences for ITS1 and 83 sequences for ITS2, which were analysed separately and using total evidence, which allows data to be combined (Kluge, 1989). The combined analysis was performed using two strategies. One approach was to construct a data matrix which included all the taxa for which sequences were available for the three loci we used. The second included taxa for which we did not have sequences for some loci; in this case, entries for the incomplete taxa included 'missing data'. This has been shown to increase the phylogenetic resolution without producing ambiguous results (Wiens & Reeder, 1995; Wiens, 1998). Using this procedure we obtained a matrix of 67 concatenated sequences in the first case and a matrix of 90 sequences with the second strategy.

Divergence times

To estimate divergence times of the clades and lineages we constructed a tree based on Bayesian inference using the program BEAST (version 1.5.3; Drummond & Rambaut, 2007), which employs a Bayesian Markov chain Monte Carlo (MCMC) algorithm. With this program the distance of the extant terminal taxa and the hypothetical ancestors in the node may be converted to divergence times if there is a reference date, such as a fossil record or geological event, or a known mutation rate. We used a nucleotide substitution rate of 1.6–2.2% per million years for the 16S gene, as estimated for invertebrates (Cunningham, Blackstone & Buss, 1992; Patarnello *et al*., 1996). In this analysis we use only one sequence by species and population of *Biomphalaria*. BEAST analysis was performed using a strict molecular clock, the general time-reversible (GTR) substitution model and the Yule process of speciation. We combine output files from independent runs of BEAST using LogCombiner (version 1.5.3). Convergence of posterior distributions for parameter estimates was analysed in Tracer (version 1.5; Rambaut & Drummond, 2007), and the Bayesian tree was obtained after removing burn-in (10%) with TreeAnnotator (version 1.5.3).

RESULTS

Separate analyses of the three datasets (Table 2) yielded trees with well-supported clades that in general were congruent among data partitions. Under the MP analysis we constructed a majority-consensus tree (not shown) of 100 equally parsimonious trees for each dataset (see Table 2 for statistics). The sequences of *B. schrammi* were located outside of *Biomphalaria* with 100% bootstrap support in all separate analyses performed. The MP 16S analysis recovered a diverse clade (79% bootstrap support) composed of several subclades including the North American and Caribbean species, the majority of the Neotropical species and all the African species, and its sister group composed of *B. peregrina* and the Altiplano populations of *Biomphalaria* (100%). Within this clade were recovered four subclades corresponding to sequences from Lauca and Salar de Huasco basins (85%), Salar de Ascotán (54%), Salar de Carcote (69%), and *B. peregrina* from Argentina (67%). The remainder of the Altiplano sequences and those of *B. peregrina* from Uruguay and Brazil were not resolved by this gene. The ML 16S analysis (GTR + G; $-\ln L = 2284.154$) produced three trees which recovered the same main clades and subclades inferred by MP analysis. The MP ITS1 analysis resolved three main clades, the largest composed by the African species and its sister *B. glabrata* (100%), and a subclade rich in Neotropical species (70%). A second clade included the species from Mexico and the Caribbean (100%); the third clade contained *B. peregrina* and the Altiplano populations of *Biomphalaria* (100%). These clades did not show resolution among themselves or with respect to *B. helophila*, which formed an independent branch in the tree. Within the *B. peregrina*/Altipano clade we found resolution of the sequences from the Salar de Ascotán (72%), Lauca and Salar de Huasco (52%), and *B. peregrina* (71%). The ML ITS1 analysis (GTR + G; $-\ln L = 3090.3933$) produced a tree which recovered the same clades inferred by MP, but with the North American and the

Table 2. Summary of the amplified sequences of the three locus and tree statistics obtained under maximum parsimony (MP) analysis

	Nucleotide composition							MP index			
Locus	A	C	G	T	N	F	L	I	TL	CI	RI
16S	36.4	10.5	15.7	37.4	33	412–439	457	108	307	0.49	0.88
ITS1	22.4	25.8	29.3	22.4	27	385–395	494	222	460	0.73	0.97
ITS2	16.8	26.8	27.7	28.7	29	418–471	528	183	594	0.58	0.89

CI, consistency index; F, length of the fragment amplified; I, number of parsimony-informative characters; L, final length of the alignment; N, number of individuals sequenced; RI, retention index; TL, tree length.

Caribbean species and *B. helophila* as consecutive sister groups of the rest of the species. Within the *B. peregrina*/Altipano clade the sequences from the Lauca and Salar de Huasco and *B. peregrina* were rseolved. The MP ITS2 analysis recovered five clades, including the *B. glabrata* and the African species clade (100%) and the clade comprising *B. peregrina* and the Altiplano taxa, which also included *B. oligoza* (100%) whose sequences were available for this locus. Within this clade were resolved the sequences from the Salar de Carcote (56%) and *B. peregrina* together with *B. oligoza* (71%). The remainder of the Neotropical species formed three well-supported but not resolved clades. As in the 16S and ITS1 analyses, the position of *B. helophila* was not resolved. The ML ITS2 analysis (TVM + G; $-\ln L = 3877.094$) produced a topology similar to MP analysis, including a cluster comprising *B. peregrina*, *B. oligoza*, and the Altiplano taxa.

The combined analysis with the matrix of 90 sequences recovered the same main clades and produced nodal support values similar to those obtained in the combined analysis of 67 sequences and for this reason we focus on the first analysis. The analysis with the matrix of 90 sequences included 1479 characters, of which 513 were parsimony-informative. Excluding *B. schrammi*, which was located outside *Biomphalaria*, the majority consensus tree (Fig. 2) of 100 equally parsimonious trees (length = 1402, consistency index = 0.59, retention index = 0.92) recovered a main clade formed by *B. helophila*, species from North America and the Caribbean, a conglomerate of mainly South American species, and a subclade that includes *B. glabrata* and the African species (100% bootstrap support). Recovered as sister to this group was a clade comprising *B. peregrina*, *B. oligoza*, and the Altiplano populations of *Biomphalaria*. Within this group, sequences of the first two taxa were recovered as a monophyletic group (65%), as well as the sequences from the Salar de Carcote (93%) and the Salar de Ascotán (96%). The ML analysis with the matrix of 90 sequences recovered the North American and Caribbean species being the sister group of the rest of the species of *Biomphalaria* (100%) (excluding *B. schrammi*) (Fig. 3), and the same two groups recovered by the MP analysis. BA recovered a consensus tree (Fig. 4) similar to that of the ML analysis. The North American and the Caribbean species also appear as the sister group of the rest of the species of *Biomphalaria* (excluding *B. schrammi*), but the conglomerate of mainly South American species was recovered as the sister group of the clade composed of *B. glabrata* and the African species. The clade comprising *B. peregrina*, *B. oligoza*, and the Altiplano taxa was recovered as the sister group of this last main clade in the ML analysis, but without bootstrap support.

Divergence times

The divergence times between the *Biomphalaria* clade and *B. schrammi* and the outgroup clade was estimated at 7.76–6.77 Mya. The origin of the *Biomphalaria* clade (excluding *B. schrammi*) occurred in the Early Pliocene (≈ 5.33–4.59 Mya). Divergence between *B. glabrata* and the African species was estimated to about 2.55–2.20 Mya, in agreement with previous estimates (Woodruff & Mulvey, 1997; Campbell *et al*., 2000; DeJong *et al*., 2001). Divergence within the new Neotropical clade occurred in the middle and late Pleistocene (Fig. 5).

DISCUSSION

The majority consensus tree produced by the combined MP analysis revealed that the genus *Biomphalaria* (excluding the clade formed by the sequences of *B. schrammi*) is divided phylogenetically into four subclades. One subclade includes the majority of the Neotropical species, a second includes *B. glabrata* and all the African species, and other the North American and Caribbean species of the genus. *Biomphalaria helophila* was recovered as sister group to these three main subclades. A fourth subclade comprised *B. peregrina*, *B. oligoza*, and the Altiplano taxa, which are sister to the remaining species. The ML tree and the consensus tree produced by BA using the combined matrix of 90 sequences show some incongruence with the MP analysis in the sense that the first two analyses recovered the species from North American and Caribbean as the sister group of the rest of the species of *Biomphalaria* (excluding *B. schrammi*), although both analyses recovered the clade composed of *B. peregrina*, *B. oligoza*, and the Altiplano taxa with 100% bootstrap support and a posterior probability of 1.00, respectively. This monophyletic group had 100% bootstrap support in the MP separate analyses of the three dataset. The shortest fragment of ITS1 reported by DeJong *et al*. (2001) in *B. peregrina* compared with other species of the genus, probably indicative of deletion events according to these authors, was also found for the Altiplano populations, suggesting a common ancestor. Molecular analyses also revealed that the clade *B. peregrina*/*B. oligoza*/Altiplano taxa is separated from the other clades by a long branch, probably indicative of the differences in evolutionary rates among species and supporting the designation of a subgenus within *Biomphalaria* for this group. In fact, Collado *et al*. (2011) postulated a 'Southern Altiplano species complex' of *Biomphalaria* for the Altiplano taxa. In general, within the *B. peregrina*/*B. oligoza*/Altiplano taxa the sequences of *B. costata* and *B. crequii* were well resolved with high node support values; they

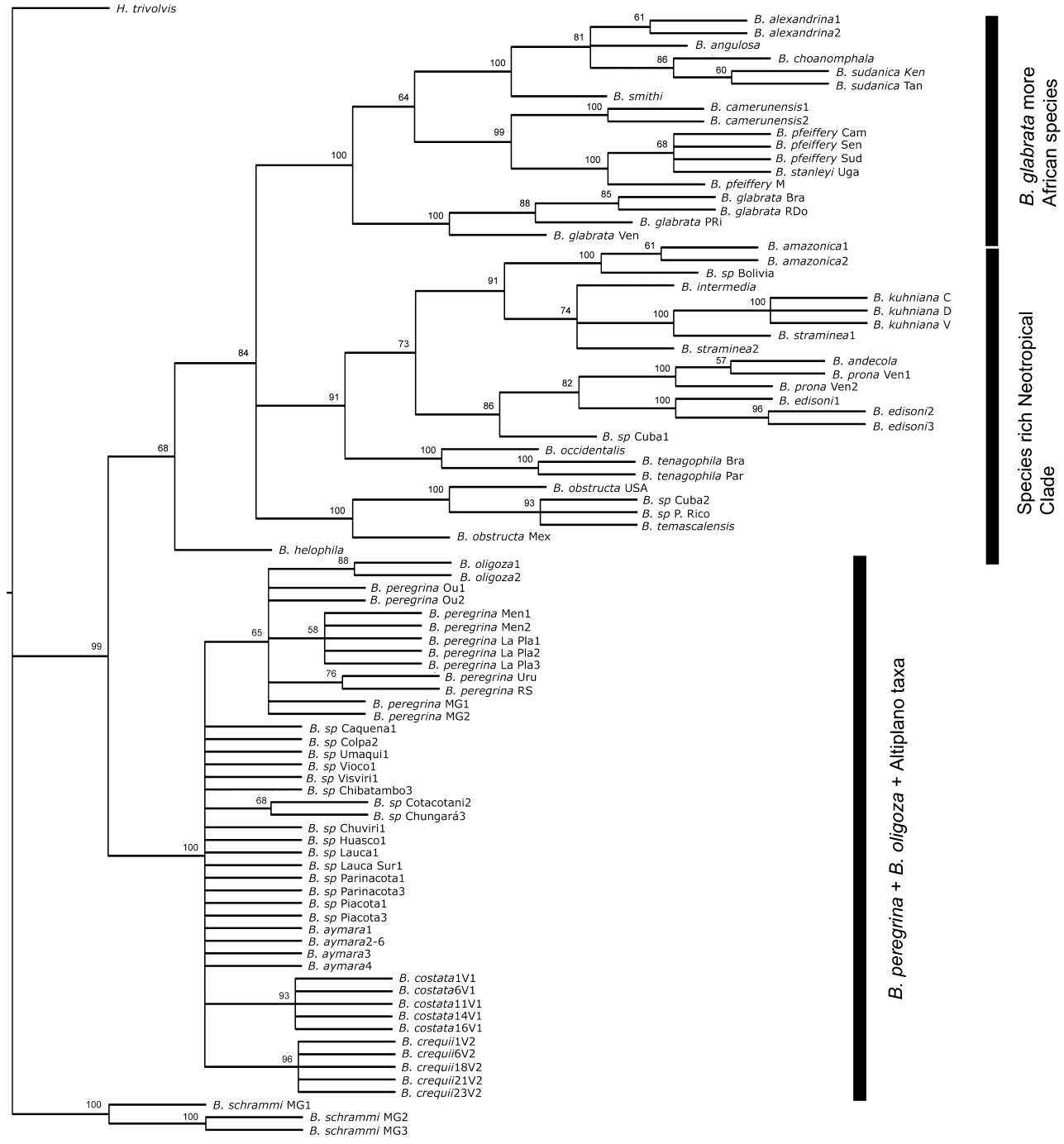

Figure 2. Majority consensus tree obtained from the maximum-parsimony analysis using the combined matrix of 90 sequences. Numbers at nodes indicate bootstrap support values (only those above 50% are given). The origin of the sequences is shown using numbers or initials (see Table 1).

and others in this clade are closely related taxa. *Biomphalaria peregrina* and *B. oligoza* were also recovered as closely related species in previous phylogenetic analyses using the ITS2 locus (Vidigal *et al*., 2000c) and RFLP profiles (Spatz *et al*., 2000).

The phylogenetic hypothesis postulated by DeJong *et al*. (2001), one of 234 equally parsimonious trees, topologically congruent with the ML tree, recovered *B. peregrina* as the sister group to the rest of the species of the genus (DeJong *et al*., 2001). The MP

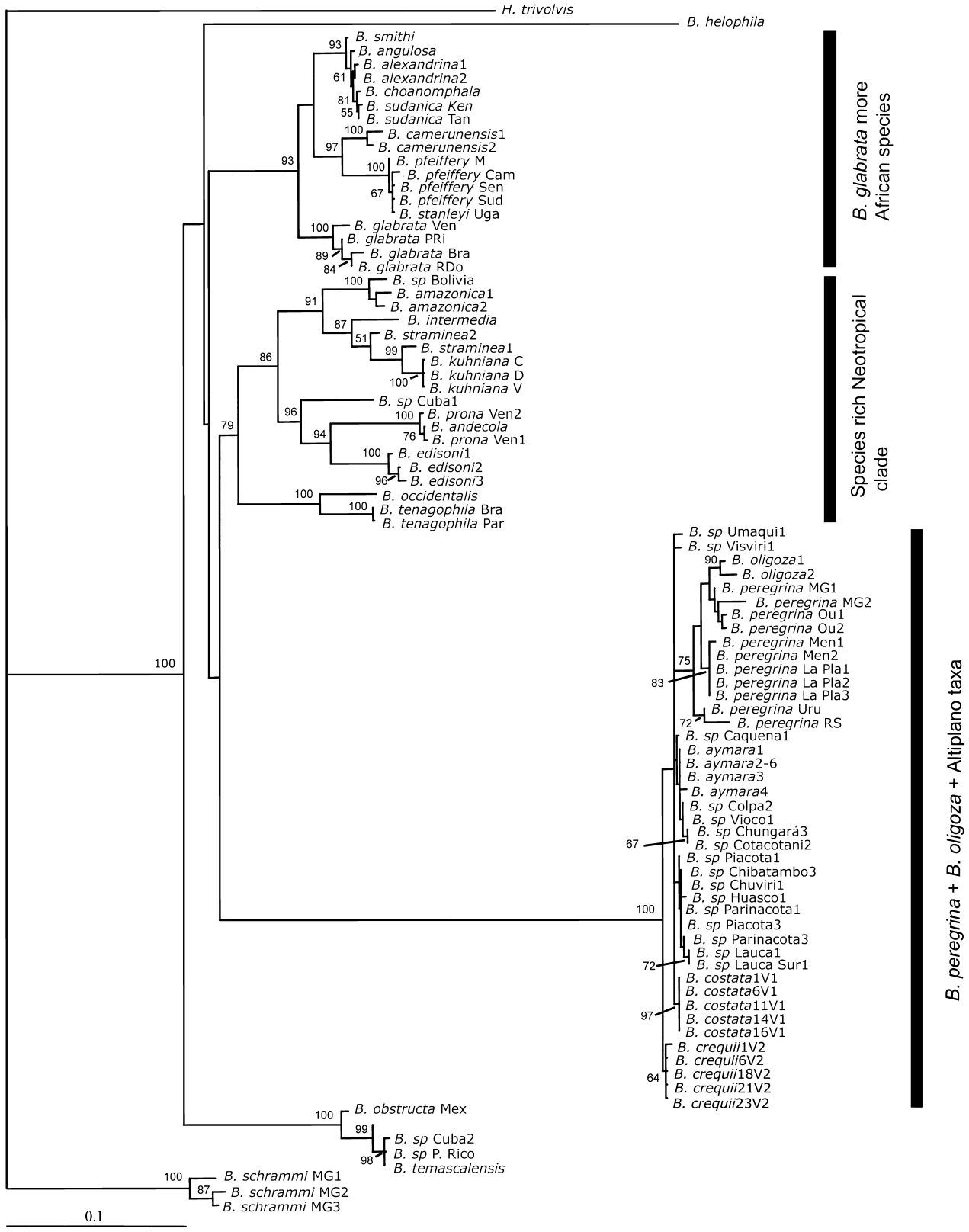

Figure 3. Tree obtained from the maximum-likelihood analysis using the combined matrix of 90 sequences. Numbers at nodes indicate bootstrap support values (only those above 50% are given). The origin of the sequences is shown using numbers or initials (see Table 1).

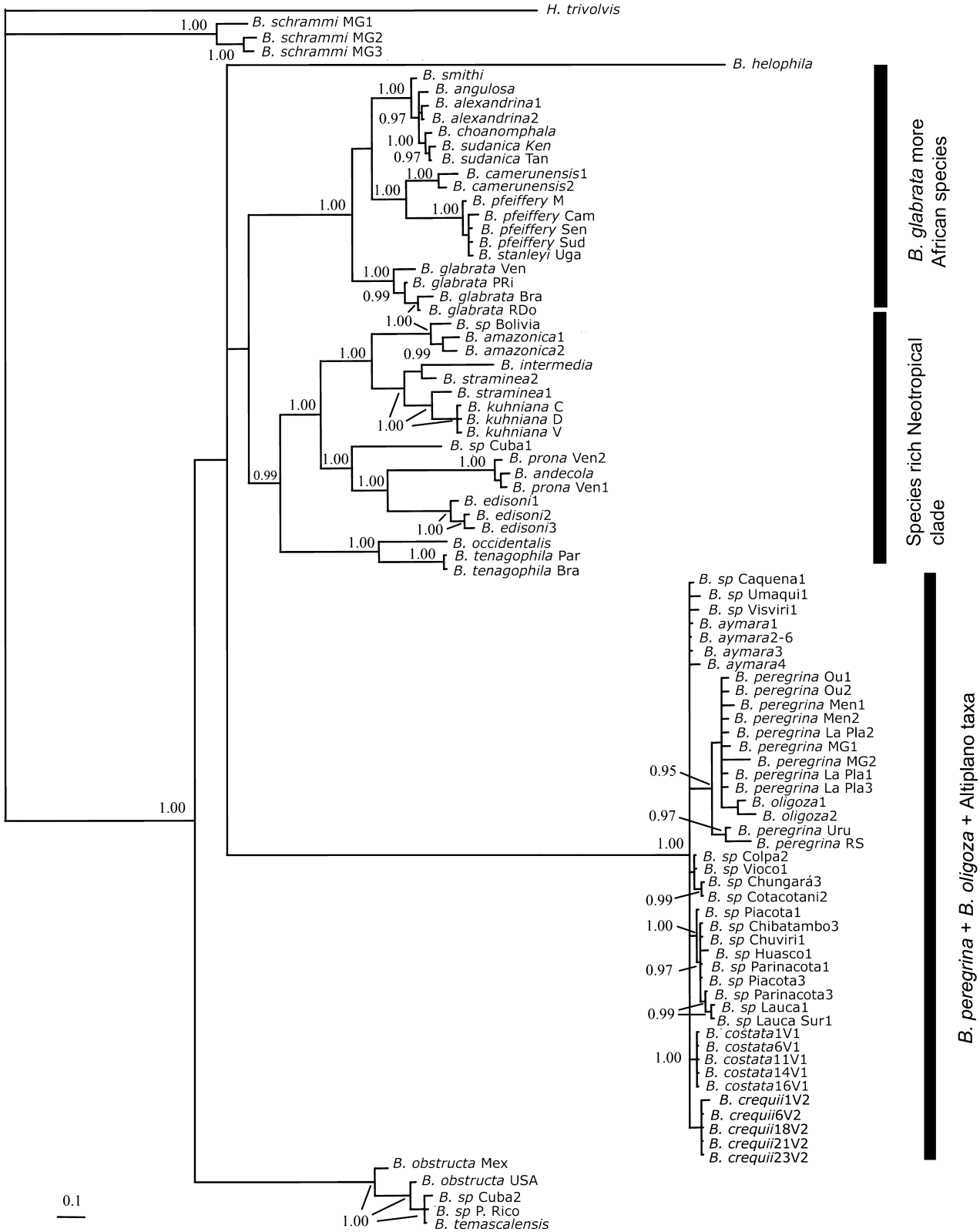

Figure 4. Consensus tree obtained from Bayesian analysis using the matrix of 90 sequences. Numbers at nodes indicate posterior probability values (only those above 0.94 are given). The origin of the sequences is shown using numbers or initials (see Table 1).

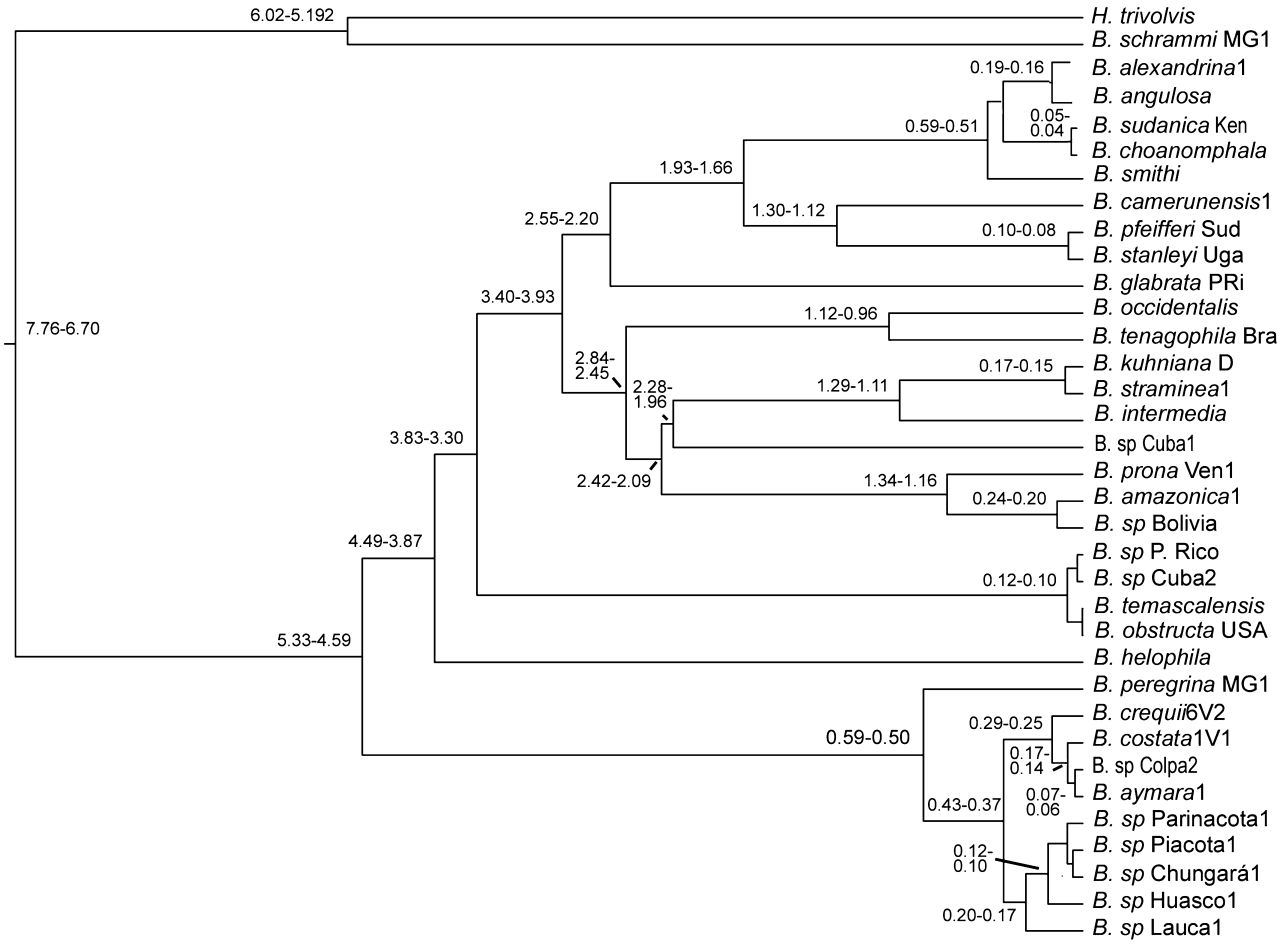

Figure 5. Reconstruction of the divergence times of lineages of *Biomphalaria* estimated using a substitution rate of 1.6–2.2% per million years for the 16S locus. We used one sequence per population/species to generate a Bayesian tree. Numbers at nodes represent millions of years. The origin of the sequences is shown using numbers or initials (see Table 1).

analysis confirms this inference, but now with a clade of *B. peregrina* and its close relatives that is widespread across South America. ML and BA recovered the North American and Caribbean species of *Biomphalaria* as the sister group of the remaining species of the genus (excluding *B. schrammi*). It has been suggested that *Biomphalaria* could have originated in North America (DeJong *et al.*, 2001; Morgan *et al.*, 2002), and both topologies agree with this hypothesis.

Taxonomic implications

The molecular phylogenetic analyses showed that the synonymy of the nominal species described for the Chilean Altiplano, i.e. *B. crequii* and *B. costata* with *B. andecola* and *B. peregrina*, respectively, is not appropriate. In the first case we could only compare the ITS2 region as only this sequence is available for *B. andecola*; however, we also included this taxon in the combined analysis of 67 and 90 sequences. All these analyses showed that *B. andecola* forms part of the species-rich Neotropical clade (Dejong *et al.*, 2001) within which it forms a monophyletic group with *B. prona*. The sequences of *B. crequii* from the Salar de Ascotán, its type locality (see Courty, 1907), in general were recovered as a strongly supported group within the clade *B. peregrina* and its close relatives. Thus, both species belong to different clades and represent distinct lineages. For *B. peregrina* and *B. costata* we were able to compare sequences of the three loci. The separate and combined analysis indicated that the sequences of *B. costata* from the Salar de Carcote (spring 1), its type locality (see Biese, 1951), never conform to a monophyletic group compared with those of *B. peregrina*, which groups with *B. oligoza*. In the 16S ML analysis the three sequences of *B. peregrina* from La Plata amplified in the present

study formed a monophyletic group with two sequences of Mendoza and two from Brazil and Uruguay reported for this species (DeJong *et al*., 2001; Estrada *et al*., 2006); the molecular evidence does not support the synonymy established for these taxa. The COI data obtained by Collado *et al*. (2011) also show that *B. costata* from the Salar de Carcote is a distinct lineage of *B. peregrina*. Radular morphology (G. A. Collado & M. A. Mendez, unpubl. data) confirms the separation of the two taxa. We cannot be conclusive with respect to the Altiplano species *B. aymara* due to the scarce resolution obtained within its clade, although its sequences are never sister to those of *B. costata* and *B. crequii*. The populations from the Lauca and Huasco basins were recovered as a strongly supported group in the 16S MP analysis (85% bootstrap support), confirming previous studies using COI sequence data that suggested that these populations could represent a candidate species of *Biomphalaria* from the Southern Altiplano (Collado *et al*., 2011). The ML analysis also recovered this monophyletic group. The populations restricted to the Caquena basin were not resolved in the present study, as compared with previous results (Collado *et al*., 2011).

Although a number of phylogenetic studies of *Biomphalaria* have included *B. schrammi*, its systematic position within the genus has been placed in doubt. Vidigal *et al*. (2000c) suggested that *B. schrammi* is a taxon 'intermediate' between *H. trivolvis* and other species of *Biomphalaria*. DeJong *et al*. (2001) suggested that *B. schrammi* is equally distant from other *Biomphalaria* and from *Helisoma* from the genetic point of view, and may represent either a separate or an intermediate lineage. The 5.8S sequences obtained by DeJong *et al*. (2001) were identical for all the *Biomphalaria* species studied and for *H. trivolvis*, with the exception of *B. schrammi*, which had two nucleotide differences. The phylogenetic study of Morgan *et al*. (2002) using ribosomal 28S RNA and actin exon placed *B. schrammi* within a clade formed by eight species of *Biomphalaria*, which supports the inclusion of this species within the genus. However, this result may be due to the small number of species considered and/or the more evolutionarily conserved nature of this molecular locus. The present study, the most inclusive in terms of number of *Biomphalaria* taxa utilized, in all analyses showed *B. schrammi* forming a lineage independent of the rest of the species of the genus.

Trematode infection

Within the clade *B. peregrina*/*B. oligoza*/Altiplano taxa the species *B. peregrina* is considered a potential intermediate host of *S. mansoni* (Paraense & Corrêa, 1973) while *B. oligoza* is resistant to this trematode

Figure 6. Trematodes parasitizing *Biomphalaria aymara* from Isluga, Chilean Altiplano. S, shell; T, trematodes in the soft body.

(see Spatz *et al*., 2000). We have found some snails of the species *B. aymara* collected from the Isluga swamps infected with adult trematodes (Fig. 6). Although there are no records of local cases of schistosomiasis in Chile (OMS, 2008), Paré & Black (1999) reported schistosomiasis in the Chilean flamingo *Phoenicopterus chilensis* Molina, 1782 caused by trematodes tentatively assigned to the genus *Dendritobilharzia* Sktjabin & Zakharow (1920). Given that several species of trematodes infect *Biomphalaria*, the poorly characterized digenean fauna in South America (DeJong *et al*., 2001), and that the ability of these snails to act as an intermediate host for trematodes appears to have evolved following several separate acquisition events during its evolutionary history (Vidigal *et al*., 2000c; DeJong *et al*., 2001), the species and the definitive hosts of the helminthes discovered in the present study need to be elucidated.

CONCLUSIONS

Molecular phylogenetic analyses support the monophyly of the clade formed by the Altiplano taxa, *B. peregrina*, and *B. oligoza* with strong branch support. MP analysis recovered this clade as sister to the remaining species of *Biomphalaria* (excluding *B. schrammi*). Given that ML and BA recovered the clade formed by the North American and Caribbean species as sister to remaining species of *Biomphalaria* (excluding *B. schrammi*), further work is needed to clarify the relationships in basal nodes. Taxonomically the present study shows (1) that the nominal species *B. crequii* is phylogenetically differentiated from *B. andecola*; they fall in different clades. (2) The synonymy of *B. costata* and *B. peregrina* was not supported; the results suggest that they represent distinct lineages within the same clade. (3) The specific status of *B. aymara* could not be resolved in this study. (4) Snails from some Altiplano localities are infected with trematodes. (5) *Biomphalaria schrammi*

in all the analyses performed appears outside of *Biomphalaria*, suggesting that it does not belong to this lineage.

Finally, our results suggest that species diversity is possibly underestimated at the Chilean Altiplano and additional sampling is required.

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