



Microgeographic differentiation among closely related species of *Biomphalaria* (Gastropoda: Planorbidae) from the Andean Altiplano

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Direct development and water dependence entail limited vagility in freshwater fauna. In these organisms, the population structure is probably linked to restrictions imposed by the habitat. In this study we investigate the relative contribution of processes stimulating the divergence of populations of *Biomphalaria costata* (Biese, 1951) and *Biomphalaria crequii* (Courty, 1907), two freshwater snails occurring in two contiguous and fragmented closed basins from the Andean Altiplano using mitochondrial DNA (cytochrome *c* oxidase subunit I) sequences, shell morphometric and radular morphology. In order to clarify the species boundaries, a third allopatric species was included: *Biomphalaria aymara* Valdovinos & Stuardo, 1991. Molecular analyses recovered two distinct clades: one composed of *B. aymara* from the Isluga swamps and *B. costata* from Spring 1 in Salar de Carcote, the single spring occupied by this species, and another integrated by snails from 12 springs spread across the Salar de Carcote and the Salar de Ascotán assigned to *B. crequii*, originally described from the Salar de Ascotán. Unlike shell morphometrics, radular morphology was informative for distinguishing these species. The division of the lineages occurred in the Late Pleistocene. A subclade that includes snails from the southernmost springs in Salar de Ascotán suggests fragmentation of the distribution of *B. crequii* associated with landscape discontinuities. In addition to microvicariance signals, the private haplotypes scattered around both salt spans show that close-range dispersal is a common biogeographic process in this species. As evolutionary units, the single isolated and restricted population of *B. costata* has a high priority for conservation.

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INTRODUCTION

The Neotropical Andean Altiplano is the second-highest plateau in the world after the Tibetan plateau in China (Gregory-Wodzicki, McIntosh & Velasquez, 1998; Sobolev & Babeyko, 2005). In the south-western region of the Altiplano the landscape includes a large number of closed basins, generally delineated by chains of volcanoes reaching 6500 m a.s.l., with the internal depressions occupied by a variety of freshwater wetlands (Fornari, Risacher & Féraud, 2001;

Risacher, Alonso & Salazar, 2003). The landscape has been shaped by strong volcanic activity lasting from the Miocene to the Holocene (Wörner *et al.*, 2000; Coutand *et al.*, 2001; Carrapa, Strecker & Sobel, 2006; Strecker *et al.*, 2007), and has also been affected by alternating episodes of expansion and regression of a succession of palaeolakes, commonly explained by climatic changes (Lavenú, Fornari & Sebrier, 1984; Clapperton, 1993; Lavenú, 1995; Fornari *et al.*, 2001; Risacher *et al.*, 2003; Placzek, Quade & Patchett, 2006; Blard *et al.*, 2011).

The endemic aquatic fauna of fishes and gastropods that inhabit the Altiplano has experienced species radiation in Lake Titicaca (Lauzanne, 1981; Parenti,

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1984a, b; Kroll *et al.*, 2012), and also vicariance in the fragmented surrounding area (Lüssen, Falk & Villwock, 2003; Vila, 2006; Collado, Vila & Méndez, 2011; Vila *et al.*, 2011; Kroll *et al.*, 2012). Comprehensive faunal searching carried out in the south-western Altiplano indicates a high number of endemic species in each basin, suggesting that allopatric speciation has stimulated differentiation of populations in the region (Vila, 2006; Vila *et al.*, 2011, 2013). For instance, the present distribution of the killifish *Orestias Valenciennes*, 1839 of the 'agassii complex' has been hypothesized to be a consequence of vicariance after extensive palaeolakes retracted during the Middle Pleistocene and Holocene (Lüssen *et al.*, 2003; Vila, 2006; Vila *et al.*, 2011, 2013). In similar desert isolated environments, freshwater organisms have also experienced structuring of populations and similar processes of diversification (Tang & Roopnarine, 2003; Moline *et al.*, 2004; Johnson, 2005; Guzik *et al.*, 2012; Murphy *et al.*, 2012). It has been suggested that habitat fragmentation probably enhances the population differentiation of such organisms, as suitable habitats are often surrounded by unsuitable terrestrial environments, and thus the gene flow among places will depend upon the dispersal capabilities of the species (Johnson, 2005).

Freshwater snails of the genus *Biomphalaria* Preston, 1910 are widely distributed in the south-western Altiplano (Courty, 1907; Biese, 1951; Valdovinos & Stuardo, 1991; Collado *et al.*, 2011; Collado & Méndez, 2012a, b). Phylogenetic analyses performed using ribosomal DNA sequences of the *ITS1* and *ITS2* nuclear regions and *16S* mitochondrial gene recovered the populations of *Biomphalaria* of the region in a strongly supported clade, together with the Neotropical species *Biomphalaria peregrina* (Orbigny, 1835) and *Biomphalaria oligoza* (Orbigny, 1835) (Collado & Méndez, 2012a). Phylogenetic analyses using the cytochrome *c* oxidase subunit I gene (*COI*) locus allowed us to infer a 'southern Altiplano species complex' of *Biomphalaria* whose sister group is *B. peregrina* (Collado *et al.*, 2011). In this region, almost all species of the genus have been described on the basis of shell morphology. This is the case of *Biomphalaria costata* (Biese, 1951), described from Cuchichá in Salar de Carcote (Biese, 1951; Ochsenius, 1974), and *Biomphalaria grangei* (Courty, 1907), *Biomphalaria crequii* (Courty, 1907), and the subspecies *Biomphalaria crequii junior* (Courty, 1907) from Salar de Ascotán (Courty, 1907), but details of the type localities within this fragmented salt pan are unknown (Collado & Méndez, 2012b). The exception is the species *Biomphalaria aymara* Valdovinos & Stuardo, 1991 from the Isluga swamps, the original description of which was also based on the anatomy of the repro-

ductive system and radular morphology (Valdovinos & Stuardo, 1991). From the taxonomic point of view, the species from Salar de Ascotán, assigned to *B. crequii* in our previous phylogenetic analyses, was synonymized [under the name *Biomphalaria thermala* (Biese, 1951)] with *Biomphalaria andecola* (Orbigny, 1835) by Malek (1985) and *B. costata* with *B. peregrina* by Paraense (1966), but these taxa actually represent different evolutionary entities (Collado *et al.*, 2011; Collado & Méndez, 2012a).

Molecular analyses recognized vicariance as the main process that has influenced the spatial distribution patterns of *Biomphalaria* in the south-western Altiplano (Collado *et al.*, 2011), and to a lesser extent dispersal, which has been reported in other species of the genus (Pointier, Paraense & Mazille, 1993; Campbell *et al.*, 2000; Mavárez *et al.*, 2002a, b). The predominance of vicariance in this orographically complex region is consistent with the limited vagility of these snails related to their aquatic lifespan and direct development. In the killifish *Orestias*, the isolation of Salar de Carcote and Salar de Ascotán, two contiguous closed basins from the south-western Altiplano, stimulated speciation and promoted the morphological and genetic diversification of populations within each system (Keller & Soto, 1998; Vila *et al.*, 2007, 2011, 2013; Morales, Vila & Poulin, 2011). Similarly, our previous phylogenetic analyses suggested two species of *Biomphalaria* inhabiting these systems, one at each basin (Collado *et al.*, 2011; Collado & Méndez, 2012a); however, these studies included only a few sampling sites, specifically one spring in Salar de Carcote (Spring 1) and two springs in Salar de Ascotán (Springs 2 and 6). In this study our main objectives were to investigate the relative contribution of processes stimulating population differentiation in *Biomphalaria* snails, with extensive sampling of springs in both basins. Under the vicariance model, it is expected that *Biomphalaria* species would be spatially circumscribed to each basin, and populations would be spatially ordered in relation to their springs of origin. If this were not the case, we would find dispersal signals, or a mixture of biogeographic processes. Additionally, we investigate the morphological and molecular species boundaries including a third congeneric species, *B. aymara*.

MATERIAL AND METHODS

SAMPLE COLLECTION AND STUDY SITES

The central Chilean fraction of the south-western Altiplano contains two contiguous, endorreic closed basins: Salar de Carcote (561 km²) and Salar de Ascotán (1757 km²) (Risacher, Alonso & Salazar,

1999). It is unknown whether these basins arose independently or were divided by the configuration of the Chigliapichina volcano (4483 m a.s.l.) during the Late Miocene and Pliocene (Ramírez & Huete, 1981), and whose cones currently separate both basins. At present, these systems constitute extreme high-evaporitic environments, in which the inflow of water is mostly provided by a series of thermal springs that are partially or totally isolated by terrestrial barriers. The Salar de Carcote basin, situated at 3690 m a.s.l., encloses Salar de Carcote itself (104–108 km²), a salt pan containing about 45 small springs isolated and partially isolated by an arid salt crust (Hurlbert *et al.*, 1976; Risacher *et al.*, 1999). The Salar de Ascotán basin, situated at 3716 m a.s.l., in the central depression is occupied by a homonymous salt pan (243 km²) that contains a score of isolated and partially isolated springs, the majority of which are located on the eastern side of the basin (Keller & Soto, 1998; Risacher *et al.*, 1999). Within this basin the water from Springs 2–6, which arise independently in the north-eastern part of the salt pan, flows downstream to the west and converges in the central depression of the system, originating a shallow central lagoon. Springs 7–11, located in a most southerly direction, are isolated by arid lands and salt crusts, and their waters apparently do not converge. In this salt pan there is no evidence of major changes in the water level in the last 12 000 years (Keller & Soto, 1998).

In Salar de Carcote, snails from three springs were obtained: Spring 1, the type locality of *B. costata* (Biese, 1951; Ochsenius, 1974), and Springs 2 and 3, two undescribed populations. Several other springs of this basin were searched, but *Biomphalaria* snails were not found. The Salar de Ascotán was extensively searched; we found snails in ten springs, specifically in Springs 2–11 (Fig. 1; Table 1). Snails from Springs 2 and 6 from this salt pan were assigned to *B. crequii* based on previous studies (Collado *et al.*, 2011; Collado & Méndez, 2012a). Sampling was carried out between 2006 and 2011, and snails were preserved in absolute ethanol for posterior morphometric and genetic analyses. Samples of *B. ayмара*, a species closely related to *B. costata*, were obtained from Isluga (3776 m a.s.l.), its type locality, a river-swamp system found in the homonymous basin, located approximately 220 km north of Salar de Carcote, also in the Chilean Altiplano (Fig. 1; Table 1).

MORPHOMETRIC ANALYSIS

This study includes 300 snails sampled from the two salt pans and Isluga. All snails were measured using an ocular micrometer accurate to 0.01 mm. Nine shell measurements were recorded in 10–32 specimens per site (Fig. S1). To reduce differences between means and variances among samples, all variables were transformed to logarithms (Sokal & Rohlf, 1981). To

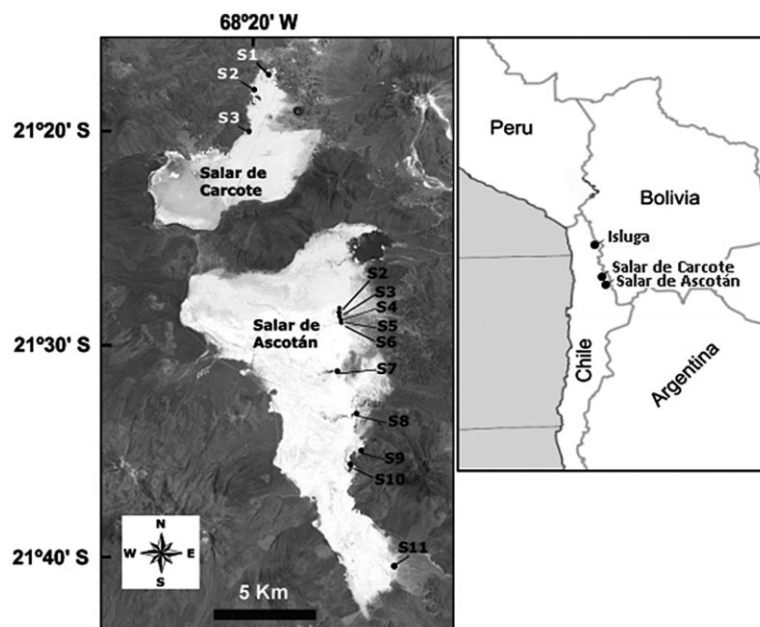


Figure 1. Sampling sites of the *Biomphalaria* snails considered in the present study. Salar de Carcote and Salar de Ascotán are magnified. The springs sampled in each salt pan are labelled S1, S2, . . . , etc.

Table 1. Sample localities, GenBank accession numbers, and number of specimens used in the present study

Salt pan/spring	Latitude/longitude	Number of specimens		
		Morphology	Sequences	Accession no.
Salar de Carcote				
Carcote S1*	21°16'59.10" S, 68°19'28.21" W	35	20	JX567510–JX567522+
Carcote S2	21°17'49.15" S, 68°20'08.35" W	27	16	JX567523–JX567538
Carcote S3	21°20'10.14" S, 68°20'15.80" W	28	18	JX567539–JX567556
Salar de Ascotán				
Ascotán S2†	21°29'21.20" S, 68°15'24.80" W	20	5	+
Ascotán S3	21°29'27.80" S, 68°15'25.60" W	20	3	JX567557–JX567559
Ascotán S4	21°29'39.80" S, 68°15'25.60" W	20	3	JX567560–JX567562
Ascotán S5	21°29'47.20" S, 68°15'24.50" W	20	5	JX567563–JX567567
Ascotán S6†	21°29'52.80" S, 68°15'24.40" W	20	3	+
Ascotán S7	21°32'01.10" S, 68°15'41.30" W	20	6	JX567568–JX567573
Ascotán S8	21°33'57.20" S, 68°14'38.70" W	20	8	JX567574–JX567581
Ascotán S9	21°36'10.40" S, 68°15'00.60" W	20	7	JX567582–JX567588
Ascotán S10	21°36'35.00" S, 68°15'00.70" W	20	6	JX567589–JX567594
Ascotán S11	21°41'13.90" S, 68°12'54.00" W	10	8	JX567595–JX567602
Isluga‡	19°15'10.00" S, 68°42'15.00" W	20	3	+
Total		280	111	93

*Snails assigned to *Biomphalaria costata*, based on the type locality.

†Snails assigned to *Biomphalaria crequii*, based on previous studies (Collado *et al.*, 2011; Collado & Méndez, 2012a, b).

‡Snails assigned to *Biomphalaria aymara*, based on the type locality.

Snails from the other springs were assigned to *Biomphalaria crequii* after the present study.

+, *COI* sequences obtained from GenBank (see text).

S1, Spring 1, etc.

evaluate the variability in shell morphology among sites we performed principal components analysis (PCA). To determine differences between sites we performed linear discriminant analyses (LDAs), which maximize the discrimination among groups using linear combinations of variables. For this analysis we use the scores of five variables obtained in the PCA. All analyses were performed in STATISTICA 6.0 (StatSoft, 2004).

RADULAR MORPHOLOGY

The radula has traditionally been documented as an important morphological criterion for taxonomic differentiation in molluscan species. In this study, adult specimens of *B. costata*, *B. crequii*, and *B. aymara* were dissected and the radular sac removed from the anterior end of the animal. The radular sac containing the radula was kept for 24 h in 10% sodium hydroxide to remove the organic material, and then washed in distilled water for final preservation in 70% ethanol. Images of radulae were taken at different magnifications using a Scanning Electron Microscope (SEM) JEOL T-300. For each species, the radular formula is given taking the sum of lateral

and marginal teeth around the rachidian or central tooth in six specimens per species, counting teeth in three transverse rows in the central part of the radula because the anterior end is convex, and is worn out by use, whereas the posterior end is the area of origin of the teeth. In this way we count the teeth in the widest and more homogeneous portion of the radula.

MOLECULAR ANALYSES

For the molecular analyses we used a subset of snails that were used in the morphological study. Genomic DNA was extracted from 93 snails using the cetyl trimethyl ammonium bromide (CTAB) method (Winnepennickx, Backeljau & De Wachter, 1993). Partial sequences of the *COI* gene were amplified by polymerase chain reaction (PCR) in 3–20 snails per spring from both salt pans (Table 1) using the primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATC A-3') and LCO1490 (5'-GGTCAACAAATCATAAAG ATATTGG-3') (Folmer *et al.*, 1994). The PCR conditions were performed following a previous study (Collado *et al.*, 2011). The PCR products were sequenced by the Macrogen Company, South Korea.

Original sequences were edited in BioEdit (Hall, 1999) and aligned using ClustalW (implemented in BioEdit), together with eight sequences obtained from snails of Springs 2 and 6 from Salar de Ascotán (GenBank accession numbers GU168100–GU168107), seven from Spring 1 in Salar de Carcote (GU168093–GU168099), three of *B. aymara* (GU168090–GU168092), plus three sequences of the species *B. peregrina* from La Plata, Argentina (JN621901–JN621903), which was employed as the outgroup (Collado *et al.*, 2011). Phylogenetic relationships were examined using the maximum parsimony (MP) and maximum likelihood (ML) methods in PAUP* (Swofford, 2003) with two alternative matrices (see below). In the MP analysis we performed a heuristic search with the tree bisection and reconnection branch-swapping algorithm, the addition of random sequences, character states treated as unordered, and assuming equal weight. For the ML analysis, we selected the best evolutionary model under the Akaike information criterion using jModelTest 0.1.1 (Posada, 2008). The statistical confidence of the nodes was evaluated using 100 bootstrap pseudoreplicates (Felsenstein, 1985) in both analyses. Median-joining network analysis of the haplotypes based on the variable sites of the alignment using 111 sequences of the three species was performed using NETWORK 4.1.0.8 (Bandelt, Forster & Rohl, 1999). Nucleotide composition and percentage of *COI* sequence divergence for *B. crequii* and *B. costata* were obtained using MEGA 4 (Tamura *et al.*, 2007; for *B. aymara* data, see Collado *et al.*, 2011). Number of haplotypes (*h*), polymorphic sites (*S*), haplotype diversity (*Hd*), and nucleotide diversity (π) were identified with DnaSP 5.10.01 (Rozas *et al.*, 2003). Neutrality was analysed using Tajima's *D*- and Fu and Li's *F*-test statistics, which measure whether the data deviate from neutral expectations. To assess the number of populations and population spatial structure we performed further genetic analyses in GENELAND 1.0.7 (Guillot, Mortier & Estoup, 2005), which uses a Bayesian Markov chain Monte Carlo (MCMC) approach based on spatial statistical models using polymorphic sites and spatial coordinates of sampled individuals to cluster them into populations. We first identify the most probable number of classes (*K*) with distinctive genetic sequences in our data set, ranging from *K* = 1 to *K* = 13 (the total number of sites sampled for *B. crequii* and *B. costata*), performing five independent MCMC runs using 1×10^7 iterations and a thinning of 1000. To verify the consistency of the results, this analysis was subsequently performed with *K* values ranging from 3 to 6, 4 to 5, and other combinations. Finally, the MCMC was run three times with *K* fixed as either 4 or 5, for further comparison.

MOLECULAR CLOCK ESTIMATIONS

We performed molecular clock analyses in BEAST 1.5.4 (Drummond & Rambaut, 2007), which uses an MCMC algorithm. Previously, we generated input.xml files for BEAST in BEAUti 1.4.7 (Drummond & Rambaut, 2007) using a *COI* substitution rate of 2.0–4.0% per million years, which has been employed in previous *Biomphalaria* studies (Campbell *et al.*, 2000; Collado *et al.*, 2011). The substitution model HKY85 + G, obtained using jModelTest, was also used for input data. We performed analyses comparing the Yule model of speciation versus birth–death process of speciation and the three clock models (strict, uncorrelated lognormal, and exponential) using Bayes factors in TRACER 1.5 (Rambaut & Drummond, 2007), finding that the Yule model of speciation and the uncorrelated lognormal clock model were favoured. Convergence of posterior distributions for parameter estimates and effective sample sizes (obtained performing 50 million generations) were analysed in TRACER. The Bayesian tree obtained after removing burn-in (10%) with TreeAnnotator 1.5.3 was visualized in FigTree 1.3.1 (Rambaut, 2009).

RESULTS

SHELL MORPHOLOGY

In the PCA, the first principal components (PC1) had high loadings for shell length and accounted for 94.6% of the variance, whereas the second component (PC2) had high loadings for length of the peristome and explained 1.8% of the variance. Thus the two orthogonal axes capture 96.4% of the variance among sites. PCA showed that in the multivariate space there is separation of the samples of Springs 1 and 3 in Salar de Carcote, and a considerable overlap between the samples from Springs 1 and 2 in this salt pan (Fig. S2). Considerable overlap also remains between the samples from Salar de Carcote, Salar de Ascotán, and Isluga. The LDA showed significant differences between sites (Wilk's $\lambda = 0.01396$; $P < 0.0001$), and revealed that between 30.0 and 90.0% of individuals were correctly classified in its respective localities, with a mean of 66.3% (Table S1).

RADULAR MORPHOLOGY

The radulae of the three species of *Biomphalaria* studied here have a convex shape in the anterior end followed by a main columnar portion to the posterior end, with a number of teeth placed in longitudinal and transverse rows (Fig. 2). Each transverse row has a rachidian or central tooth, with marginal and lateral teeth arranged symmetrically on both sides. The central tooth is bicuspid or tricuspid, marginal

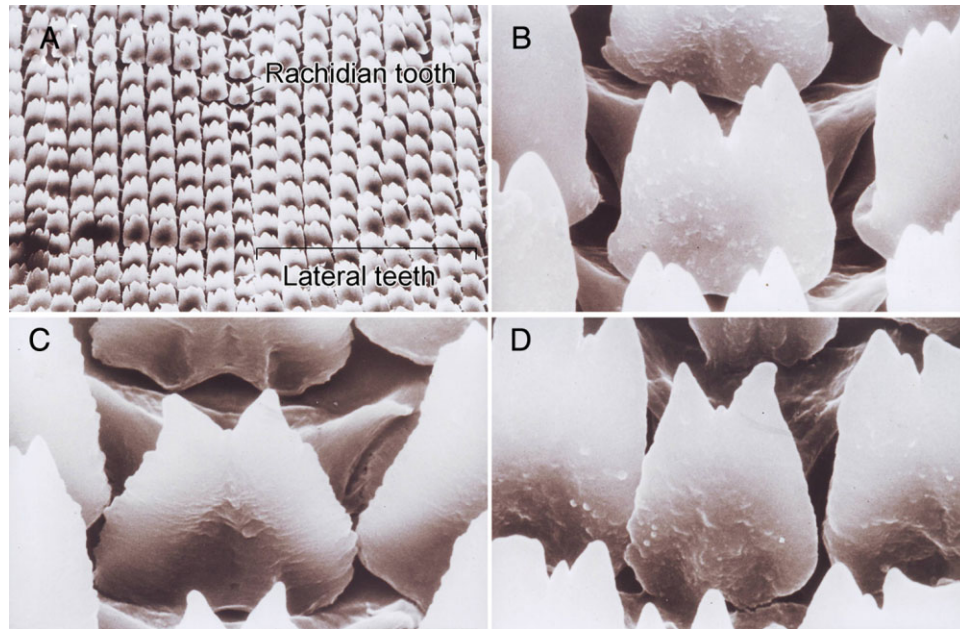


Figure 2. Radular morphology of *Biomphalaria* snails observed using scanning electron microscopy (SEM). A, general morphological appearance of the radula. B, C, and D, rachidian teeth of *Biomphalaria costata* (Salar de Carcote), *Biomphalaria crequii* (Salar de Ascotán), and *Biomphalaria aymara* (Isluga swamps), respectively.

teeth are tricuspid, and lateral teeth are multicuspid. When tricuspid, the cusps are called entocone, mesocone, and ectocone. The rachidian tooth is smaller than the lateral teeth. In *B. costata* the rachidian tooth is quadrangular, bicuspid, and with smooth edge; mesocone absent. Each cusp is divided into three minor cusps, growing in size and level of position from the inside out. The lateral teeth are tricuspid. The radular formula is 21 + 1 + 21 for three transverse rows of teeth in five specimens, and 22 + 1 + 22 in one specimen. In *B. crequii* the rachidian tooth is roughly globular in form, irregularly denticulate along the edges, tricuspid, with each cusp smooth; mesocone tiny. The lateral teeth are tricuspid, variable in shape and size. The radular formula is 18 + 1 + 18 in two specimens and 19 + 1 + 19 in four specimens. In *B. aymara* the rachidian tooth is tricuspid, with a small conic mesocone; the cusps are well developed and smooth. The radular formula is 25 + 1 + 25 in one specimen and 24 + 1 + 24 in five specimens. With the exception of the radular formula, the morphology of the radula is consistent with those data reported by Valdovinos & Stuardo (1991) in this species.

PHYLOGENETIC ANALYSES AND GENETIC VARIATION

Amplification of the *COI* gene produced a fragment of 663 nucleotides used as the final length of the alignment. Considering that preliminary MP and ML

analyses recovered two main clades with polytomies, some identical sequences were deleted from the initial matrix, and one final alignment of 43 sequences was subsequently used to reconstruct phylogenies using at least two sequences per spring and all the haplotypes found in the three basins. Nucleotide composition was 26.4% A, 13.4% C, 17.4% G, and 42.7% T. The number of informative characters for parsimony was 34. MP analyses retained a tree with a length of 38 steps (consistency index = 0.95; retention index = 0.99; rescaled consistency index = 0.94). This analysis recovered two distinct clades: one consisting of the sequences of the species *B. costata* from Spring 1 in Salar de Carcote and *B. aymara* from the Isluga swamps (Clade 1 in Fig. 3a); and another that included sequences of *B. crequii* from the ten springs in Salar de Ascotán together with all sequences from Springs 2 and 3 in Salar de Carcote (Clade 2 in Fig. 3a). The ML tree ($-\ln$ likelihood = 1076.7419) obtained under the HKY85 + G model of sequence evolution recovered the same topology as in the MP analyses, and similar bootstrap support values (Fig. 3a). The split into two clades was supported by a bootstrap value of 100% in both analyses, whereas each clade was supported by 100 and 96–98% bootstrap in the MP and the ML analyses, respectively. Within the *B. crequii* clade, we also inferred a subclade composed of a cluster of sequences corresponding to a unique haplotype obtained from Springs 9 and 10 in Salar de Ascotán (>96% bootstrap

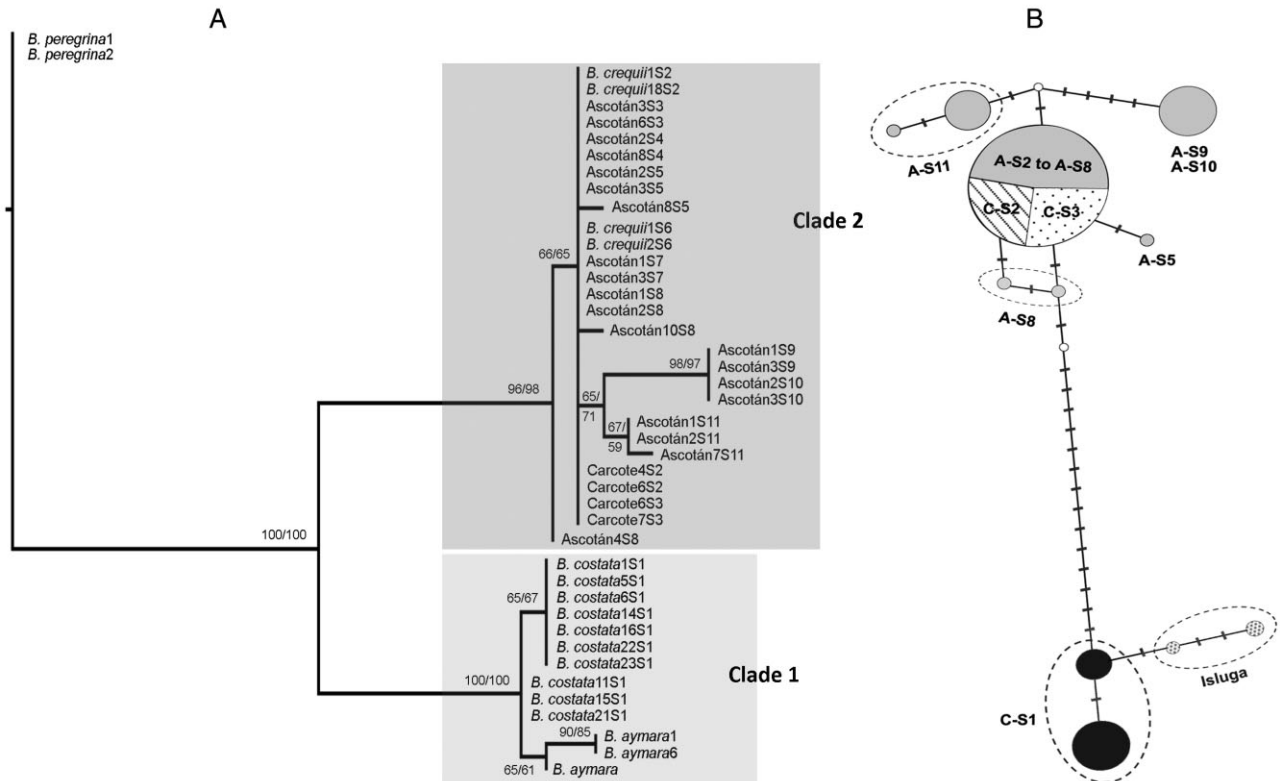


Figure 3. Molecular analyses among *Biomphalaria* populations (springs sampled in Salar de Carcote and Salar de Ascotán are numbered S1, etc.). A, tree obtained by maximum-likelihood (ML) analysis using mtDNA *COI* sequences. Identical topology was recovered in the maximum parsimony (MP) analysis. Numbers above the nodes indicate bootstrap values obtained under ML and MP analyses, respectively. The first number after the salt pan and species name indicates the specimen sampled. B, median-joining network of haplotypes obtained from *Biomphalaria* snails sampled in Salar de Carcote (C), Salar de Ascotán (A), and Isluga. The circle sizes are proportional to the number of observations of each haplotype. White circles represent missing haplotypes. The clades recovered by the phylogenetic analyses are also indicated.

in both analyses), and a second cluster composed of sequences from Spring 11 of this salt pan (59 and 67% bootstrap, respectively), which groups two private haplotypes. Nucleotide variation of the sequences from snails within the springs was low, and in several cases it was not detected. Sequences from Springs 5 and 8 from Salar de Ascotán and Spring 1 from Salar de Carcote had 0.1% variation. The *COI* percentage sequence divergence among *B. costata* from Spring 1 in Salar de Carcote and the other springs was estimated between 2.5 and 3.2% (Table 2). The mean divergence time estimated between Clades 1 and 2 was of 0.74–0.64 Mya. Intraspecific lineage divergence within *B. costata* began to occur 0.49–0.41 Mya. The mean divergence between sequences of *B. costata* and *B. aymara* was 0.36–0.27 Mya. The mean divergence of the subclade composed of *B. crequii* snails from Springs 9 and 10 versus snails from Spring 11 within Salar de Ascotán occurred 0.33–0.24 Mya.

HAPLOTYPE NETWORKS

Figure 3b shows the network obtained for the *COI* haplotypes identified in *Biomphalaria* snails from the three basins studied. Two haplogroups separated by 15 mutational steps were identified. A haplogroup includes haplotypes of *B. costata* and *B. aymara* (Clade 1 in the phylogenetic analyses), with two private haplotypes in each species. The second haplogroup, corresponding to *B. crequii* (Clade 2), includes seven haplotypes. In *B. costata*, the diversity indexes were $h = 2$, $S = 1$, $Hd = 0.395$, and $\pi = 0.00060$; in *B. crequii*, the diversity indexes were $h = 7$, $S = 10$, $Hd = 0.45$, and $\pi = 0.00248$. The hypothesis of selective neutrality was not rejected by Tajima's D and Fu or Li's F statistics for Clade 1 ($D = 0.72261$, $P > 0.10$; $F = 0.76517$, $P > 0.10$). Neither test rejected the neutrality expectations in *B. crequii* ($D = -0.44212$, $P > 0.10$; $F = -1.24677$, $P > 0.10$). One

Table 2. Percentage mean divergence among mtDNA *COI* sequences of *Biomphalaria costata* and *Biomphalaria crequii* using the Kimura two-parameter model (Kimura, 1980)

Site	CS1	CS2	CS3	AS2	AS3	AS4	AS5	AS6	AS7	AS8	AS9	AS10	AS11
Carcote S1*													
Carcote S2	2.6												
Carcote S3	2.6	0.0											
Ascotán S2†	2.6	0.0	0.0										
Ascotán S3	2.6	0.0	0.0	0.0									
Ascotán S4	2.6	0.0	0.0	0.0	0.0								
Ascotán S5	2.6	0.0	0.0	0.0	0.0	0.0							
Ascotán S6†	2.6	0.0	0.0	0.0	0.0	0.0	0.0						
Ascotán S7	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0					
Ascotán S8	2.5	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0				
Ascotán S9	3.2	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8			
Ascotán S10	3.2	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.0		
Ascotán S11	2.6	2.6	2.6	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.7	0.3	

*Snails assigned to *Biomphalaria costata*, based on the type locality.

†Snails assigned to *Biomphalaria crequii*, based on previous studies (Collado *et al.*, 2011; Collado & Méndez, 2012a, b). The numbers following S denote the numbers of the springs sampled in Salar de Carcote (C) or Salar de Ascotán (A).

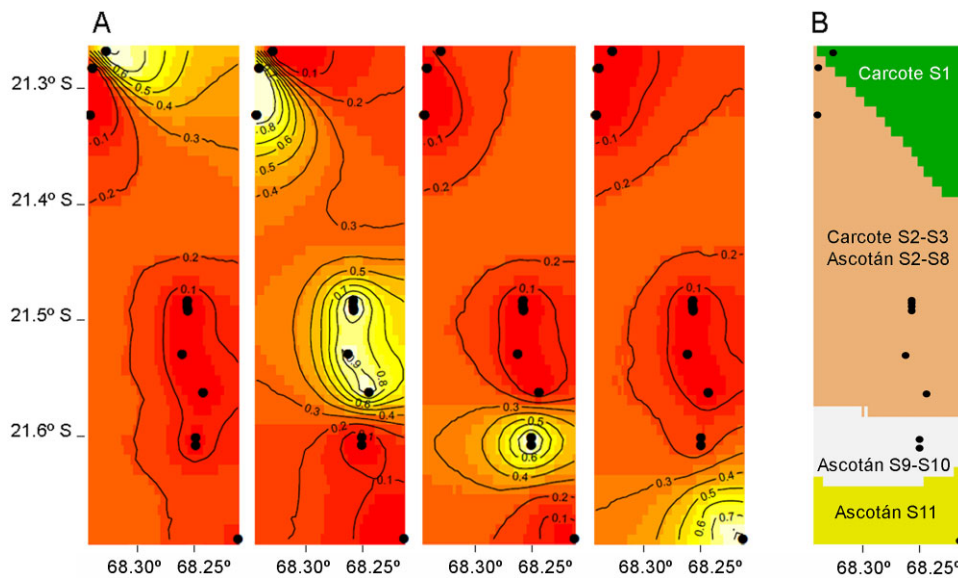


Figure 4. Spatial genetic structure obtained in GENELAND (setting $K = 4$) for *Biomphalaria* snails (the springs sampled in Salar de Carcote and Salar de Ascotán are numbered S1, etc.). A, posterior probabilities of population membership in clusters 1–4 (from left to right, respectively). Lighter shading indicates higher probabilities of population membership. B, summary of estimated cluster membership of the four clusters and their respective localities. The black dots correspond to the localities analysed in this study.

of the private haplotypes of *B. crequii* from Springs 2–8 in Salar de Ascotán was also found in Springs 2 and 3 in Salar de Carcote, which confirms the results of the phylogenetic analyses.

SPATIAL GENETIC STRUCTURE

The Bayesian approach performed in GENELAND identified four genetic clusters as the most likely

number of populations occurring in Salar de Carcote and Salar de Ascotán (Fig. 4) (for this analysis the distant *B. aymara* was excluded). One cluster was represented by snail sequences of *B. costata* collected from Spring 1 in Salar de Carcote, whereas a second cluster included snail sequences of *B. crequii* collected from Springs 2 and 3 in this salt pan and Springs 2–11 in Salar de Ascotán. Within this clade,

two subclades were represented by sequences of this species from Springs 9 and 10 and from Spring 11 in this salt pan, respectively. These subclades occupy the southernmost springs of Salar de Ascotán.

DISCUSSION

Biomphalaria costata was described based on shell morphology from Cuchichá, a thermal northern spring (Spring 1) in Salar de Carcote in 1951 by Walter Biese, and since then nothing has been said about the *Biomphalaria* populations inhabiting Springs 2 and 3 from this salt pan. As these springs are located within the same closed basin where a single species of *Orestias* occurs (Morales *et al.*, 2011; Vila *et al.*, 2011, 2013), at the beginning of the study the most parsimonious interpretation for the presence of populations of *Biomphalaria* in Salar de Carcote was that they belonged to *B. costata*. However, independently of the method used to reconstruct phylogenies, the trees show that the *Biomphalaria* snails sampled from Spring 1 of this salt pan constitute a monophyletic group separate from the snails of Springs 2 and 3 whose sequences clustered in the *B. crequii* clade. The percentage sequence divergence between these groups, based on 663 bp of the mitochondrial *COI* gene, was estimated to be 2.5–3.2%, which is in the range of values estimated to delimit species of *Biomphalaria* (Jørgensen, Kristensen & Stothard, 2007; Collado *et al.*, 2011) and other freshwater gastropod taxa (Hershler, Liu & Thompson, 2003; Liu, Hershler & Clift, 2003; Hershler *et al.*, 2006; Bichain *et al.*, 2007). Based on the same argument, there is no reason to recognize additional nominal *Biomphalaria* taxa in Salar de Ascotán, and we propose the name *B. grangei* as synonyms of the species *B. crequii*. In agreement with the phylogenetic analyses, haplotype networks showed two clearly differentiated phylogroups. The timing of evolutionary branching events of these two lineages would be congruent with the final development phase and regression of Lake Ballivián during the Middle and Late Pleistocene (Servant & Fontes, 1978; Argollo & Mourguiart, 1995; Lavenú, 1995), but it should be considered that the age of this lacustrine episode is controversial (Clapperton, 1993; Servant-Vildary & Mello e Sousa, 1993; Wirrman & Mourguiart, 1995; Fornari *et al.*, 2001).

In the present study we could not differentiate unambiguously among species using shell morphometrics. Although *B. costata* snails from Spring 1 in Salar de Carcote are well separated from Spring 3 in multivariate space, in agreement with the phylogenetic analyses, there is substantial morphological overlap with snails sampled from Spring 2, which belong to the *B. crequii* lineage. The lack of

morphological differentiation between Springs 1 and 2 may result from either phenotypic plasticity or the development of ecotypes. On the other hand, *B. crequii* has been well differentiated from *B. costata* and other Altiplano lineages by means of molecular approaches (Collado *et al.*, 2011; Collado & Méndez, 2012a). This is not the case for *B. costata*, as it could not be distinguished unambiguously from the disjunct Altiplano *B. aymara* using *COI* sequences (Collado *et al.*, 2011; present study). However, these species were differentiated through anatomical characters of the female reproductive system (Valdovinos & Stuardo, 1991), and a multilocus molecular study showed that they do not form a monophyletic group (Collado & Méndez, 2012a). Consistent with this, the rachidian tooth of the radula shows clear differences between the two species. Additionally, there is no overlap in the sum of lateral and marginal teeth around the rachidian tooth among the three species studied here. Considering that *B. crequii* and *B. aymara* are distinct lineages, the slight similarity in the morphology of the rachidian tooth may result from convergence or morphological stasis. The discrepancy between the radular formula of *B. aymara* given by Valdovinos & Stuardo (1991) with our data could be explained because we measure the rows of teeth in the central portion of the organ.

In Salar de Ascotán, structuring of the populations of *Orestias ascotanensis* Parenti, 1984 has been reported, probably stimulated by habitat fragmentation since the Late Pleistocene to the beginning of the Holocene (Morales *et al.*, 2011). Similarly, we also detected fine-scale genetic structure in *B. crequii* in this basin, revealed by three genetically divergent populations, two of which correspond to the subclades of snails that inhabit the southernmost springs of this salt pan. This suggests that in this area gene flow is partly blocked by topographical barriers, and therefore populations are prone to drift and selection. Considering the definition by Mayr (1963) of a subspecies as an aggregate of local populations of a species inhabiting a geographic subdivision of the range of the species, the southern subclade in Salar de Ascotán may represent the subspecies *B. crequii junior* described by Courty (1907). The divergence of the southern lineages of Salar de Ascotán from snails inhabiting northern springs occurred during the Late Pleistocene, before the start of the last glacial period. This time frame differs from that reported in *Orestias*, possibly because of different rates of substitution and the molecular markers used. In particular, we detected low haplotype diversity and very low nucleotide diversity in *B. costata* during this time in Salar de Carcote, suggesting that the species suffered a bottleneck or a founder event that reduced the genetic variation. It has been recognized that

populations of tropical freshwater snails experience regular fluctuations in size and bottlenecks caused by recurring drought and flooding (Wethington *et al.*, 2007). Considering that Salar de Ascotán is situated at a higher altitude than Salar de Carcote, the most parsimonious interpretation for the presence of *B. crequii* in the springs from this salt pan is through colonization post-speciation starting from Salar de Ascotán. This would have happened after the development and regression of the Tauca palaeolake (18 000–14 5000 years ago), which flooded Salar de Carcote (but not Salar de Ascotán) to a depth of several tens of metres (Blard *et al.*, 2011), and would probably have erased any trace of differentiation detected in the present study. The *COI* haplotypes of *B. crequii* found in Springs 2 and 3 in Salar de Carcote correspond to the more common haplotype found at Salar de Ascotán. Colonization could have been promoted by passive dispersal at close range through drift of downstream surface waters during possible unrecorded flooding, subterranean water connections, as well as through birds, a phenomenon documented in gastropods (Malone, 1965; Rees, 1965; Wesselingh, Cadée & Renema, 1999; Wada, Kawakami & Chiba, 2012). For a hermaphroditic, self-compatible species like *Biomphalaria*, in extreme cases the propagule necessary to establish a peripheral isolate may consist of a single individual (Frey, 1993; Campbell *et al.*, 2000).

The elucidation of the distribution range and taxonomic status of *B. costata* and *B. crequii* presented here has noteworthy implications related to the biodiversity of species in this geological and dynamically complex area, which is composed of fragile environments potentially prone to disturbances by mining activities and water extraction (Keller & Soto, 1998; Vila, 2006; Vila *et al.*, 2007; Morales *et al.*, 2011). On the basis of its restricted distribution, *B. costata* should have a high priority for conservation.

CONCLUSIONS

Molecular phylogenetic analyses show that *B. costata* and *B. crequii* are distinct evolutionary lineages, with allopatric distribution. These species were also differentiated using radular morphology, as well as *B. aymara*. Whereas *B. costata* inhabits only its type locality, a northern spring in Salar de Carcote, *B. crequii* is widespread over Salar de Ascotán and this system. Molecular analyses suggest that microvicariance processes have stimulated the differentiation of populations of this species in the southern area of Salar de Ascotán. Apart from *B. crequii*, there is no reason to recognize additional *Biomphalaria* species in Salar de Ascotán as previously suggested. It is highly recommended to preserve

the particular habitat of *B. costata* in Salar de Carcote.

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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:

Figure S1. Shell morphological variables measured in *Biomphalaria* snails.

Figure S2. Plot of the principal components (PC1 and PC2) of shell measurements.

Table S1. Classification matrix of *Biomphalaria* snails obtained by linear discriminant analysis (LDA).