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To cite this article: Gonzalo A. Collado , Carmen G. Fuentealba , Néstor J. Cazzaniga & Moisés A. Valladares (2020): Assessing biodiversity within the range of *Heleobia chimbaensis* (Caenogastropoda: Cochliopidae) on the Atacama Desert coast, Systematics and Biodiversity, DOI: [10.1080/14772000.2020.1783018](https://doi.org/10.1080/14772000.2020.1783018)

To link to this article: <https://doi.org/10.1080/14772000.2020.1783018>

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Research Article


Assessing biodiversity within the range of *Heleobia chimbaensis* (Caenogastropoda: Cochliopidae) on the Atacama Desert coast

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(Received 5 February 2020; accepted 28 May 2020)

Heleobia is the most specious genus in the family Cochliopidae, in which many species are morphologically similar. Here we used morphological, microstructural and phylogenetic criteria to investigate species delimitation within the *Heleobia chimbaensis* complex on the Atacama Desert coast. The range included subdivided populations of the species, the subspecies *Heleobia chimbaensis conica*, and the allopatric snails from Los Perales, first recognized as a population of *H. chimbaensis* and more recently considered a candidate species of the group. Traditional shell characters, morphometric analysis, operculum, and protoconch were either uninformative or ambiguous to separate taxa. However, the phylogenetic analysis identified *H. chimbaensis*, *H. chimbaensis conica* and four additional populations of the species as a monophyletic group while the population from Los Perales was recovered as a distantly evolutionary significant unit for which the name *Heleobia peralensis* sp. nov. is provided here. While the geographic isolation and limited dispersal seem to have influenced the diversification and current distribution of *H. chimbaensis* in Atacama, the origin of *Heleobia peralensis* sp. nov. remains unclear because of uncertain relationships. A conservation status assessment according to IUCN criteria and NatureServe methodology suggests that both *H. chimbaensis* and *Heleobia peralensis* sp. nov. face a high risk of extinction.

<http://www.zoobank.org/pub:91770DA4-B16F-46AD-A6D6-9638065ED899>

Key words: Allopatry, Chile, COI gene, cryptic species, freshwater snails, taxonomy, Truncatelloidea

Introduction

The species concept is an ongoing debate, and arguably no definition exists that leaves all communities satisfied. In fact, more than 20 species concepts have been proposed, which in turn contain different criteria for recognizing species and which are ultimately used, in operational terms, to delimit taxa (de Queiroz, 2007). Another problem is the existence of cryptic species, which also have different definitions. For instance, a cryptic species should be recently diverged, separable only with molecular analyses, occur in sympatry, or be reproductively isolated (Stebbins, 1950). Another

definition considers two or more species to be ‘cryptic’ if they are, or have been, classified as a single nominal species if they are at least superficially morphologically indistinguishable (Bickford et al., 2007). The particular characteristics of a group, such as its abundance, size, whether it is hyperdiverse or not, or the number of researchers dedicated to its study, among others, constitute additional potential problems to understand biodiversity, delimit taxa and recognize new species (Heller, 1984; Puillandre et al., 2012; Sites & Marshall, 2004; Valdovinos, 1999). Of the many problems that hinder our understanding of biodiversity and conservation, recent emphasis has been given to the identification of cryptic species (Delić et al., 2017; Pante et al., 2015).

The minute aquatic snails of the family Cochliopidae Tryon, 1866 constitute a diverse group of

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caenogastropods which inhabit a variety of freshwater and brackish ecosystems primarily in the New World (Hershler & Thompson, 1992). Within this family, *Heleobia* Stimpson, 1865 is the most species-rich genus, containing about 100 species (Cazzaniga, 2011; Hershler & Thompson, 1992; Martin & Díaz, 2016), many of which are morphologically similar. However, despite this high diversity, the amount of species is still probably underestimated considering the recognition of nominal species overlooked in the literature, the existence of cryptic species restricted to particular drainages and the finding of candidate species in recent molecular phylogenetic studies performed in the group (Collado *et al.*, 2011, 2013, 2016; Collado & Méndez, 2012; Kroll *et al.*, 2012).

In the Atacama Desert, northern Chile, 10 species and seven subspecies of *Heleobia* have been described. One of them is *Heleobia chimbaensis* (Biese, 1944), which was originally described based uniquely on external shell morphology by Walter Biese from Quebrada La Chimba, a ravine located north of the city of Antofagasta. Subsequently, using the same criterion, he described the subspecies *Heleobia chimbaensis conica* (Biese, 1947) with samples from Las Breas and Finca Tofala in Quebrada Taltal, and extended the distribution of *H. chimbaensis* to five other allopatric populations confined to small aquatic habitats that currently have hydric stress and pollution (Burgos, 2015; Collado, 2012) such as Quebrada Carrizo, Quebrada Cascabeles, Quebrada Huanillo and Aguada Galerce and Los Perales in Quebrada Paposo. Later, the species was found in Cueva de los Curas, a small spring located in the littoral of Antofagasta (Collado, 2012). A study of the type collection of *Heleobia* from Museo Nacional de Historia Natural de Santiago, Chile (MNHCL) yielded the finding of the lectotype of *H. chimbaensis* (catalogue number MNHCL: 200558) and the holotype of *Heleobia chimbaensis conica* (catalogue number MNHCL: 200564) (Collado *et al.*, 2011), both taxa initially assigned to the genus *Littoridina* Souleyet, 1852 (Biese, 1944, 1947).

A preliminary phylogenetic study performed in *Heleobia* populations from northern Chile using mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes (Collado *et al.*, 2013) recovered the snails from Quebrada Carrizo and Quebrada Cascabeles in a clade composed by topotype specimens of *H. chimbaensis* and *H. chimbaensis conica* while the snail population from Los Perales was recovered as an unrelated clade suggesting the presence of a candidate species of the genus. Regarding morphology, there is some evidence that the male copulatory organ of the snails from

Los Perales is different compared with that of *H. chimbaensis* (Collado *et al.*, 2011, 2013; Hubendick, 1955).

In the present study, we examine different characters under an integrative taxonomy framework to resolve the taxonomic status of subdivided populations of *H. chimbaensis* in the Atacama Desert, including *H. chimbaensis conica* and the snails from Los Perales. In this context, we also explored the utility of the morphological species concept *vs* the phylogenetic species concept (Donoghue, 1985) in *Heleobia* comparing topotype specimens of *H. chimbaensis* with snails from other localities.

Materials and methods

Sampling

Adult snails were collected in 2011 from seven populations scattered within the range of *H. chimbaensis* in the coast of the Atacama Desert: Quebrada La Chimba (23°32'22.05"S, 70°21'36.40"W), Playa La Chimba (23°33'46.87"S, 70°24'01.47"W), Cueva de los Curas (23°34'47.46"S, 70°23'31.74"W), Quebrada Carrizo (= Quebrada La Negra) (23°41'56.72"S, 70°24'42.51"W), Quebrada Cascabeles (25°17'33.10"S, 70°26'45.40"W), Las Breas in Quebrada Taltal (25°30'10.10"S, 70°24'40.20"W), and Los Perales in Quebrada Paposo (25°01'45.60"S, 70°27'17.90"W). After sampling, the snails were anaesthetized with MgCl₂ and fixed in absolute ethanol.

Molecular analysis

Genomic DNA was isolated from the soft tissue of snails using the cetyltrimethyl ammonium bromide method (CTAB) (Winnepenninckx *et al.*, 1993). Partial DNA mitochondrial sequences of the COI gene were amplified using the primers HCO2198 and LCO1490 (Folmer *et al.*, 1994). The sequences were edited and aligned in BioEdit v.7.0.5.3 (Hall, 1999) together with sequences of related taxa downloaded from GenBank (Collado *et al.*, 2016; Falniowski *et al.*, 2008; Hershler *et al.*, 1999; Koch *et al.*, 2015; Kroll *et al.*, 2012) (Table S1). Phylogenetic analyses were conducted performing Bayesian inference (BI) in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003) previously selecting the best evolutionary model in jModeltest v.2.1.7 (Darriba *et al.*, 2012) under the Akaike information criterion. The analysis was run for 10 million generations sampling every 1000 generations using the GTR+I+G model with a burn-in period of 20%. Bayesian posterior probabilities (PP) were considered as clade credibility values. A

Maximum likelihood (ML) analysis was performed using the same model in RAxML v.7.2.6 (Stamatakis, 2014). BI and ML reconstructions were performed in the CIPRES cluster of the San Diego Supercomputer Center (Miller et al., 2010). Maximum parsimony (MP) analysis was implemented in PAUP v.4.0 (Swofford, 2003) using a heuristic search. An alternative Neighbour-joining method (Saitou & Nei, 1987) was also used to infer relationships. In both the ML and MP analyses the node support was assessed using a non-parametric bootstrap (BS) with 1000 replicates. The species *Heleobops docimus* Thompson, 1968 was used as an outgroup. The haplotype network of *Heleobia* species/populations was analysed using a median-joining algorithm (Bandelt et al., 1999) in PopART v.1.7 (Leigh & Bryant, 2015).

Morphological study

The shell of each snail was observed and photographed using a Motic SMZ-168 stereomicroscope with a Moticam 2000 integrated digital camera. The following six shell variables (mm) were measured (see Wolf et al., 1997): shell length (SL), shell width (SW), aperture length (AL), aperture width (AW), body whorl length (BWL), and spire length (SPL). Prior to statistical analysis, data were transformed to logarithm and examined for the assumptions of normality and homogeneity of variance. Because these assumptions were not fulfilled ($P < 0.05$), data were analysed using the Kruskal–Wallis non-parametric tests in STATISTICA v. 7.0 (StatSoft Inc., 2004). Variations of shell variables were also analysed performing canonical variate analysis (CVA) in PAST (Hammer et al., 2001). The protoconch, radula and operculum of snails were cleaned in a diluted sodium hypochlorite solution and then imaged using a scanning electron microscope (Hitachi SU3500). Type (and voucher) specimens were deposited in the Museo de Ciencias Naturales y Arqueología Profesor Pedro Ramírez Fuentes (MCNAPPRF–CC 158-1 to MCNAPPRF–CC 158-29), Chillán, Chile.

Conservation status

We performed a conservation status assessment of species under study according to IUCN (International Union for Conservation of Nature) criteria and the procedure proposed by NatureServe (Faber-Langendoen et al., 2012; Master et al., 2012). The main aspects considered by the IUCN to assess extinction risk of a species are geographic range, population size, and population decline (IUCN Standards & Petitions Committee, 2019). The species can be classified in the following threat categories: Extinct

(EX), Extinct in the wild (EW), Critically endangered (CR), Endangered (EN), Vulnerable (VU), Near threatened (NT), Least concern (LC), and Data deficient (DD). The NatureServe methodology contains the NatureServe Rank Calculator to facilitate the process of assigning status ranks to the species through automation after combining data on rarity (e.g., range extent, number of occurrences), threats (e.g., overall threat impact, vulnerability) and trends (e.g., short and long trends) to compute a global conservation status rank (G-rank), as follows: G1 = Critically Imperilled, G2 = Imperilled, G3 = Vulnerable, G4 = Apparently Secure, and G5 = Secure. It is important to know that the NatureServe methodology does not replace the system developed by the IUCN Red List of threatened species or that implemented in the *Reglamento para Clasificar Especies según Estado de Conservación (RCE) del Ministerio del Medio Ambiente de Chile (MMA)*. However, it facilitates, for example, direct comparisons regarding the conservation status of freshwater gastropods worldwide.

Results

Molecular analysis

We amplified 27 partial COI sequences of 589–639 nucleotides that were deposited in GenBank (MN921121–MN921148). All the molecular analyses (BI, ML, and MP) recovered the sequences of the populations from the coast of the Atacama Desert in two well-supported, distantly related clades (see Figure 1 for the ML analysis and Figures S1–S3 for the other analyses). One clade was composed by topotype specimens of *H. chimbaensis* from Quebrada La Chimba and *H. chimbaensis conica* from Las Breas together with snails from Playa La Chimba, Cueva de Los Curas, Quebrada Carrizo, and Quebrada Cascabeles (PP = 1.00; BS for ML, and MP = 100%). The second clade clustered exclusively snails from Los Perales (PP = 1.00; BS for ML, and MP = 100%), described here as the new cryptic species *Heleobia peralensis* sp. nov. (see below). Although the relationships among *Heleobia* clades were not completely resolved, *Heleobia peralensis* sp. nov. was recovered with moderate support in the ML analysis as a sister group of a clade formed by *H. chimbaensis* and populations/species from Argentina, the southern section of the Atacama Desert and the north central zone of Chile.

The median joining haplotype network of the Atacama populations inferred seven haplotypes. The analysis also recovered two distinct haplogroups separated by 20 mutation steps, one including topotype specimens of *H. chimbaensis* and the subspecies *H. chimbaensis conica* together with the four other Atacama populations and a second one clustering only

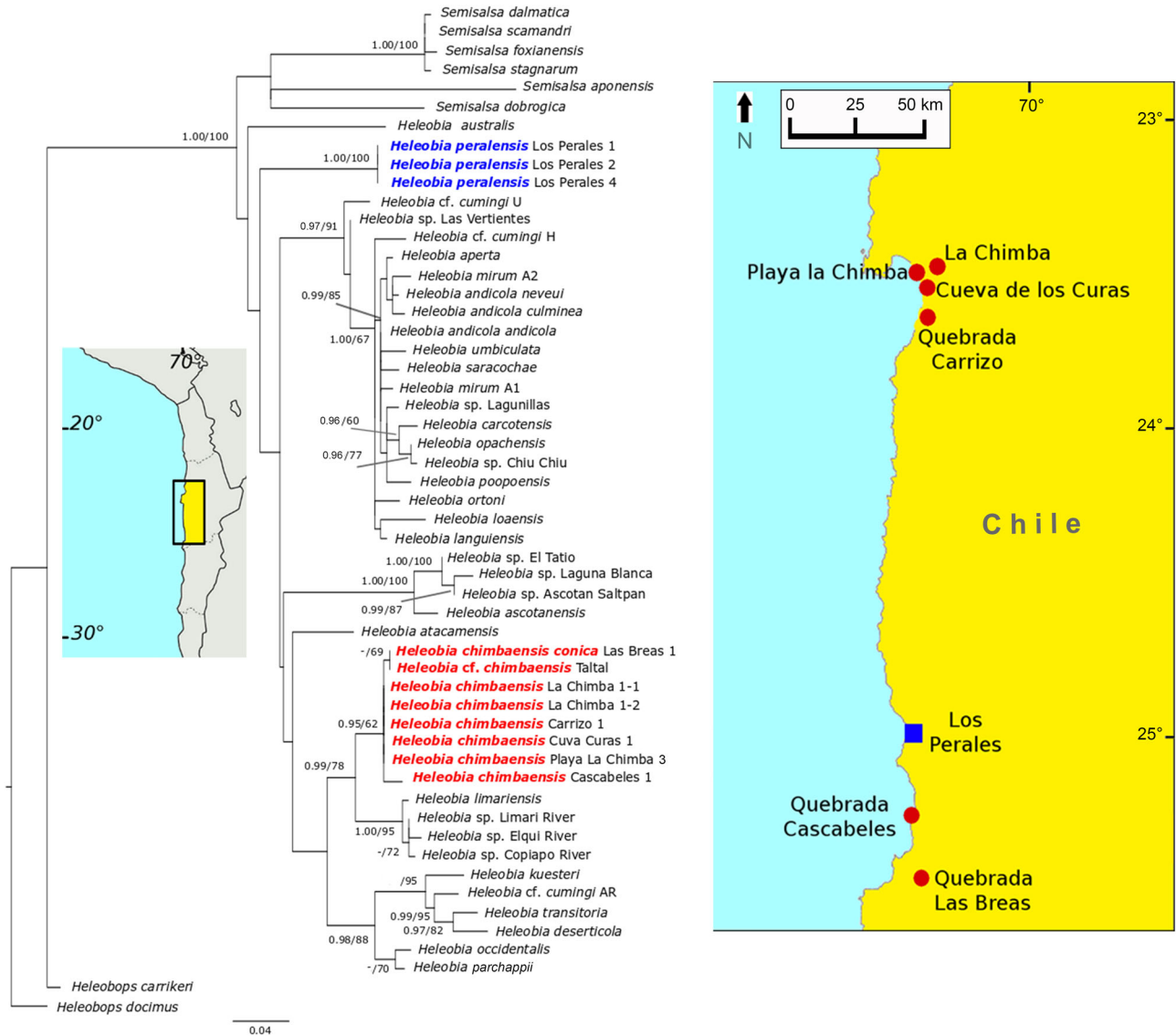


Fig. 1. Tree obtained by the ML analysis of COI marker and geographic distribution of *Heleobia* spp. on the Atacama Desert coast. Node support represents Bayesian posterior probabilities (>0.94) obtained in BI analysis following by bootstrap values obtained in the ML analysis (>50%).

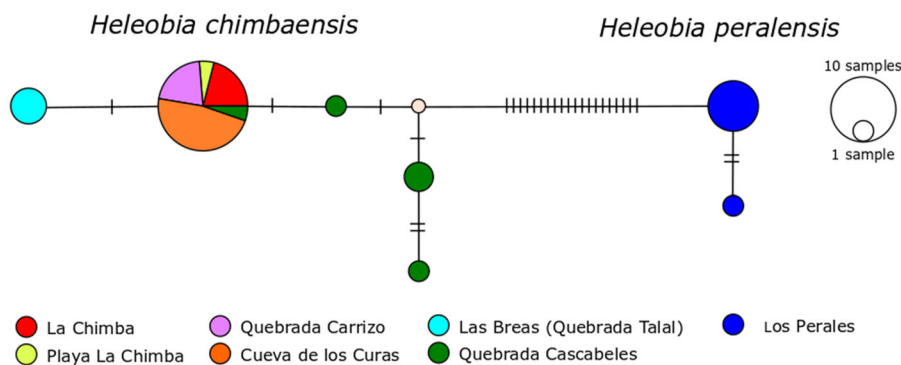


Fig. 2. Median-joining haplotype network using COI sequences of *Heleobia* spp. on the Atacama Desert coast. Small perpendicular lines indicate mutation steps among haplotypes.

Table 1. Mean shell dimensions (mm) ± standard deviation from five populations measured in this study.

Species/population	n	SL	SW	AL	AW	BWL	SPL
<i>Heleobia chimbaensis</i> La Chimba	31	3.6 (0.5)	2.0 (0.3)	1.6 (0.2)	1.3 (0.2)	2.5 (0.3)	1.1 (0.2)
<i>Heleobia peralensis</i> Los Perales	31	3.4 (0.2)	1.9 (0.1)	1.6 (0.1)	1.2 (0.1)	2.4 (0.2)	1.1 (0.1)
<i>Heleobia chimbaensis</i> Quebrada Carrizo	20	3.1 (0.4)	1.7 (0.1)	1.3 (0.1)	0.9 (0.1)	2.0 (0.2)	1.1 (0.2)
<i>Heleobia chimbaensis</i> Quebrada Cascabeles	20	4.4 (0.6)	2.3 (0.3)	1.8 (0.2)	1.2 (0.2)	2.7 (0.3)	1.6 (0.4)
<i>Heleobia chimbaensis conica</i> Las Breas	27	4.2 (0.5)	2.1 (0.2)	1.8 (0.2)	1.2 (0.2)	2.5 (0.3)	1.7 (0.2)

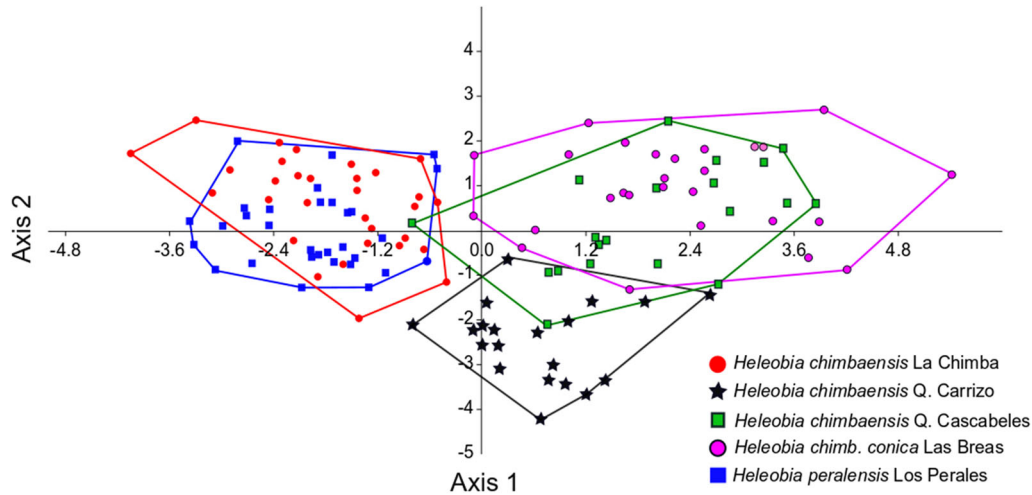


Fig. 3. Canonical variable analysis of the shell dimensions of *Heleobia chimbaensis* populations and *Heleobia peralensis* sp. nov.

specimens from Los Perales (Fig. 2). Of all the seven haplotypes, five belonged to the haplogroup that clusters *H. chimbaensis* populations. The main haplotype of this species included 19 specimens from the six populations of the species. Quebrada Cascabeles was the most diverse population, accounting for four haplotypes; *H. peralensis* sp. nov. was represented by two haplotypes.

Morphometry

The populations of *H. chimbaensis* from Quebrada Cascabeles and Quebrada Carrizo were the largest and smallest snails, respectively (Table 1). The results of the Kruskal–Wallis test (Tables S2–S7) showed significant differences between topotype specimens of *H. chimbaensis* and *H. chimbaensis conica* in three variables (SL, AL, SPL) whereas none of the shell measurements distinguished this last population from that of Quebrada Cascabeles. In addition, significant differences between topotype specimens of *H. chimbaensis* and snails from Los Perales were not found. The snails from Quebrada Carrizo were statistically different from all other populations except Los Perales in three shell variables (SL, SW, SPL). The CVA plot depicted three morphological groups, as follows: (1) topotype specimens of *H. chimbaensis* and *Heleobia peralensis* sp. nov. from Los

Perales, (2) Quebrada Carrizo, and (3) *H. chimbaensis conica* from Las Breas and snails from Quebrada Cascabeles (Fig. 3).

Conservation status assessment

Based on IUCN criteria, and using the NatureServe Rank Calculator, both *H. chimbaensis* and *Heleobia peralensis* sp. nov. face a high risk of extinction. The evaluation made in the NatureServe framework for both species regarding the area of occupancy was <1 km², the lowest available values, since both species inhabit small springs and water wells. The data used for *H. chimbaensis* regarding the range extent, number of occurrences, overall threat impact and intrinsic vulnerability were 250–1000 km², 6–20, medium and moderately vulnerable, respectively. For *Heleobia peralensis* sp. nov. these data were <100 km², 1–5, very high and highly vulnerable, respectively.

Taxonomic account

Here we provide a redescription of *Heleobia chimbaensis* and a full description of a new species of *Heleobia*.

Superfamily Truncatelloidea Gray, 1840

Family Cochliopidae Tryon, 1866

Genus *Heleobia* Stimpson, 1865. The diagnosis of the genus is provided in Hershler and Thompson (1992).

Type species: *Paludestrina culminea* d'Orbigny, 1840; SD, Pilsbry, 1911.

Heleobia chimbaensis (Biese, 1944)

The general shell shape, aperture and colour pattern in all populations of *H. chimbaensis* (Fig. 4A–F) is highly similar to that of topotype specimens of the species, although there is some intrapopulation variability. However, conspicuous differences in external features appropriate to qualitatively separate morphospecies were not found. Morphological data provided here were obtained using topotype specimens (Fig. 4A, G; 4H–S).

Material examined. Fifty-five specimens from Quebrada La Chimba. Voucher specimens: MCNAPPRF 158-17, MCNAPPRF 158-20, MCNAPPRF 158-21, MCNAPPRF 158-23, MCNAPPRF 158-25, MCNAPPRF 158-28, and MCNAPPRF 158-29.

Type locality. Quebrada La Chimba, a ravine found about 5 km north-east of Antofagasta, Chile.

Distribution and habitat (Fig. 1). Playa La Chimba, Cueva de los Curas, and Quebrada Carrizo on the littoral of Antofagasta city, in addition to its type locality. It is also found in Quebrada Cascabeles and Las Breas in Quebrada Taltal, further south of Antofagasta. *Heleobia chimbaensis* was not found in Quebrada Huanillo and Aguada Galerce, localities reported previously for this species (Biese, 1947). The site of Finca Tofala, where it was first recorded (Biese, 1947), was not located. *Heleobia chimbaensis* inhabit small springs and wells.

Redescription

SHELL (Fig. 4A, G): Elongate-conic (mean = 3.6 mm), 5.9 whorls. Teleoconch with five shell whorls and fine axial striae. Umbilicus small, slightly implied. Aperture ovate; outer lip thin. According to Biese (1944), the shell of *H. chimbaensis* is thin, turritiform and pointed, of greenish-white colour, with fine lines of growth and up to 6³/₄ whorls. Aperture oval, with full edge, blunt and a brown colour list.

Protoconch (Fig. 4H). *c.* 0.9 whorls.

Operculum (Fig. 4I). Paucispiral, ovate, thin, translucent, nucleus eccentric with a small attachment scar near the centre.

Radula (Fig. 4J–M). Taenioglossan. Rachidian (central) tooth (Fig. 4K) reaching 25.8 (± 0.3) and 41.1 (± 2.7)

µm wide in its upper margin and central (measured at the height of the beginning of the tongue) section, respectively; basal tongue U-shaped; central cusp elongate, pointed distally, with 5–7 lateral cusps on each side; one basal cusp. Lateral teeth with 3–4 conic cusps around an elongated-conic central cusp. Inner marginal teeth (Fig. 4L) with 20–22 cusps; outer marginal teeth with 28–32 cusps (Fig. 4M).

Soft body (Fig. 4N–S). Mantle with irregular greyish-black spots on a light brown background, narrow grey margin. Foot dark brown-black, propodium white; head light brown (Fig. 4O–Q), snout brown, distal lips white. Tentacles light brown with a greyish band in the base and a greyish tint at the tip. Penis (Fig. 4R–S) elongated conical, distally pointed, grey in colour, with light brown tones along its length; 4–6 grey apocrine glands in the convex margin having black spot at the base; distal portion tapered with a minute terminal papilla and a small lobe or subterminal protuberance in the concave margin. Hubendick (1955) partially drew the copulatory organ of this species, with four apocrine glands, which was subsequently described by Collado *et al.* (2011) and Collado (2012).

Reproductive biology. *Heleobia chimbaensis* is a gonochoric species, with direct development (Collado & Méndez, 2011). The life cycle of the species is unknown.

Conservation rank. It is suggested that *Heleobia chimbaensis* should be assessed as Endangered (EN) under IUCN criteria (IUCN 2019) and G1 (Critically Imperilled) using NatureServe methodology. The species is currently restricted to six localities, the number of known populations has decreased and presents severe threats such as landslides, habitat alteration, pollution, droughts, and water extraction (Collado, 2012; present study). Besides, the realized Area of Occupancy (AOO), adding the six small and fragile aquatic systems, is much less than 1 km².

Remarks. *Heleobia chimbaensis* is a species of large size among congeners, reaching up to 5.9 mm length by 2.7 mm wide (Biese, 1947). Biese (1944, 1947) included *H. chimbaensis* in the group of *Heleobia parchapii* (d'Orbigny, 1835), which contains large-sized species of *Heleobia* of a long-spire and 5–8 whorls.

Heleobia peralensis sp. nov.

Holotype. Adult specimen sampled from the spring Los Perales, Quebrada Paposos, Región de Antofagasta, Chile (25°01'45.60"S, 70°27'17.90"W) (Fig. 5A, B). Collector: G. Collado *et al.*, 2011. Specimen (shell) housed at MCNAPPRF 158-1. Shell measurements (mm): SL = 3.0, SW = 1.6, AL = 1.3, AW = 1.0, BWL =

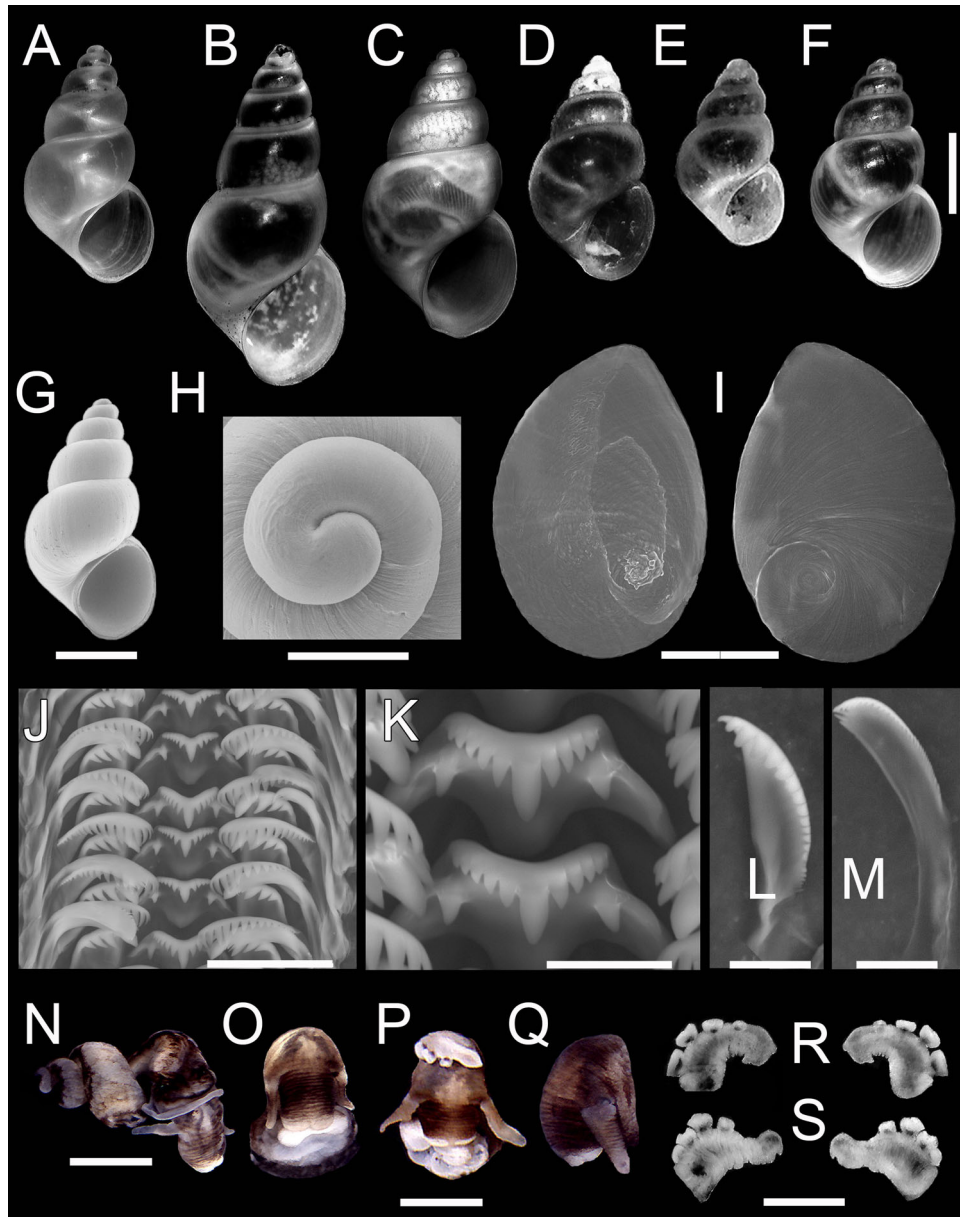


Fig. 4. *Heleobia chimbaensis*. A. Topotype specimens from Quebrada La Chimba observed using stereomicroscope (MCNAPPRF 158-17). B–F. Representative specimens from Las Breas (B), Quebrada Cascadeles (C), Cueva de Los Curas (D), Playa La Chimba (E), and Quebrada Carrizo (F). G. Topotype specimen from Quebrada La Chimba observed using SEM (same specimen shown in A) (MCNAPPRF 158-17). H. Protoconch (MCNAPPRF 158-20). I. Operculum, outer and inner sides (MCNAPPRF 158-21). J–M. Radula. J. Section of the radular ribbon (MCNAPPRF 158-23). K. Two rachidian (central) teeth (MCNAPPRF 158-24). L. Inner marginal tooth (MCNAPPRF 158-23). M. Outer marginal tooth (MCNAPPRF 158-23). N. Soft body (MCNAPPRF 158-25). O. Head-foot of a female (MCNAPPRF 158-26). P. Head-foot-penis of a male (MCNAPPRF 158-27). Q. Head of this male in lateral view with the penis dissected. R–S. Penis of two specimens (ventral and dorsal view) (MCNAPPRF 158-28 and MCNAPPRF 158-29). Scale bar: A–F = 1 mm; G = 1 mm; H = 200 μ m, I = 400 μ m, J = 25 μ m; K–M = 10 μ m; N = 1 mm; O–Q = 1 mm; R, S = 500 μ m.

0.9, SPL= 0.8. Protoconch *c.* 0.9 whorls and 351.6 μ m in length in its largest diameter (Fig. 5E).

Paratypes. Ten adult specimens from the spring Los Perales. Paratypes 1–4 housed at MCNAPPRF (158–2 to 158–5). Paratypes 5–10 housed at

Laboratorio de Malacología y Sistemática Molecular, Universidad del Bío-Bío. Shell length (mm): Paratype 1 = 3.3, Paratype 2 = 3.7, Paratype 3 = 3.2, Paratype 4 = 3.2, Paratype 5 = 3.1, Paratype 6 = 2.9, Paratype 7 = 3.1, Paratype 8 = 3.4, Paratype 9 = 3.3, Paratype 10 = 3.5.

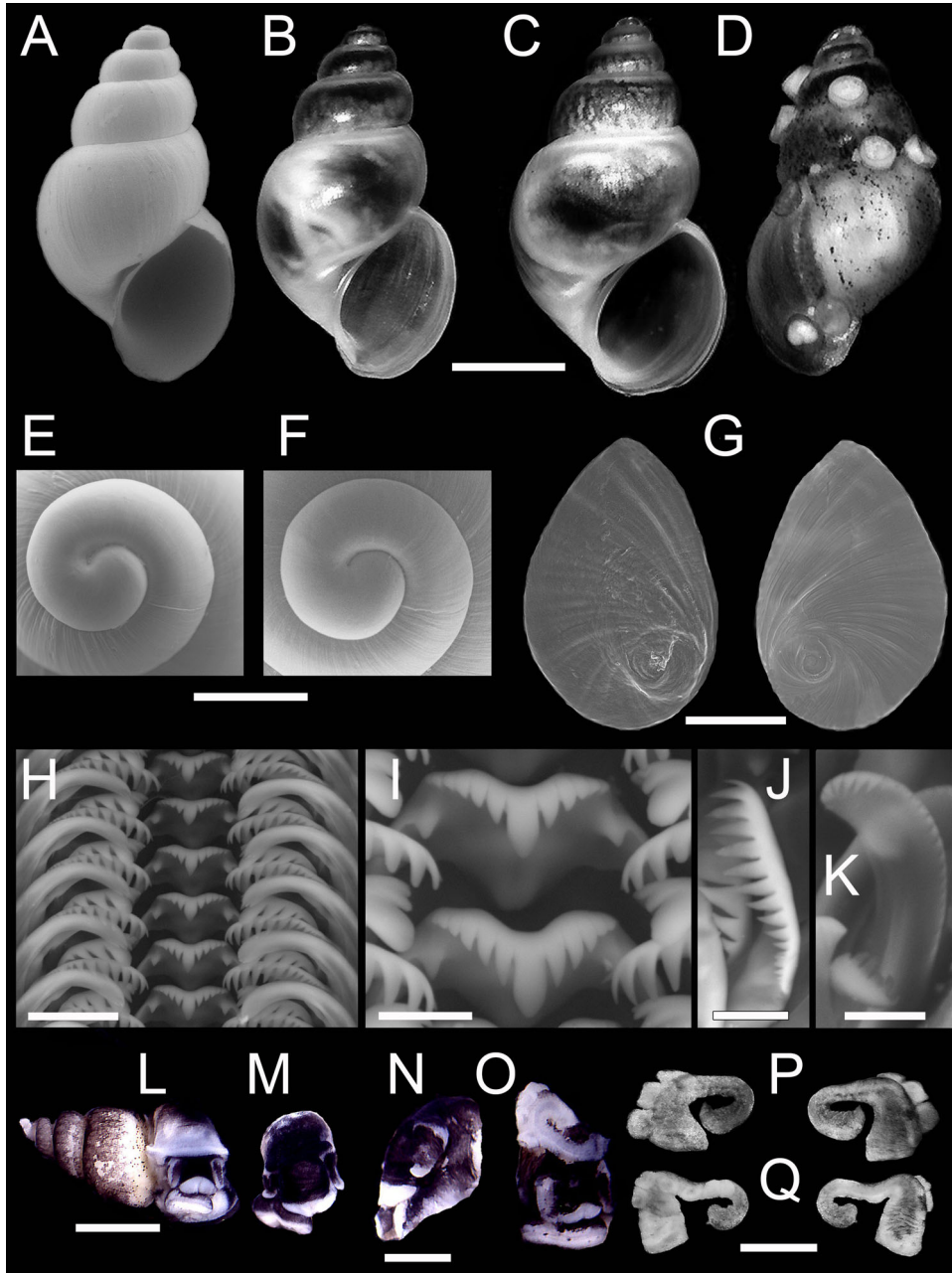


Fig. 5. *Heleobia peralensis* sp. nov. A, B. Shell of the holotype observed under SEM and stereomicroscope, respectively (MCNAPPRF 158-1). C. Shell of the paratype 1 (MCNAPPRF 158-2). D. A topotype specimen with egg capsules attached to the shell (MCNAPPRF 158-6). E. Protoconch of the holotype (MCNAPPRF 158-1). F. Protoconch of a topotype specimen (MCNAPPRF 158-7). G. Operculum, outer and inner sides (MCNAPPRF 158-8). H–K. Radula. H. Section of the radular ribbon (MCNAPPRF 158-10). I. Rachidian (central) teeth (MCNAPPRF 158-10). J. Outer marginal tooth (MCNAPPRF 158-11). K. Inner marginal tooth (MCNAPPRF 158-11). L. Soft body of a topotype specimen (MCNAPPRF-158-12). M, N. Head-foot of a female (MCNAPPRF 158-13). O. Head-foot of a male showing the penis (MCNAPPRF 158-14). P, Q. Penises of two topotype specimens (ventral and dorsal view) (MCNAPPRF 158-15 and MCNAPPRF 158-16). Scale bar: A–D = 1 mm; E–F = 200 μ m; G = 400 μ m; H = 25 μ m; I–K = 10 μ m; L = 1 mm; M–O = 1 mm; P, Q = 500 μ m.

Other studied material. Thirty-one topotype specimens used in the morphometric and CVA analyses.

Type locality. Los Perales (Fig. 1), a spring located in Quebrada Paposo about 2.5 km in a straight line from Paposo town on the Atacama Desert coast,

Antofagasta Region, Chile. This is the only place of occurrence of this species. The snails were collected from freshwater macrophytes and smooth sediment of the spring.

Etymology. The specific name is a Latinised demonym from the type locality (spring Los Perales, Chile).

Reproductive biology. *Heleobia peralensis* sp. nov. is a gonochoric species, with direct development. Several adult individuals with single-egg capsules attached to the shells with a juvenile inside were sampled (Fig. 5D). Additional data of the life cycle of the species are unknown.

Conservation rank. It is suggested that *Heleobia peralensis* sp. nov. should be assessed as Critically Endangered (CR) under IUCN criteria (IUCN 2019) and G1 (Critically Imperilled) using NaturServe methodology. The species is restricted only to the type locality, which also presents the same threats as those affecting *H. chimbaensis*.

Description

Shell (Fig. 5A–D). Elongate-conic, 5.9 whorls. Teleoconch with five shell whorls having very fine axial striae. Mean measurements of 31 topotypes are shown in Table 1. Umbilicus small, slightly implied. Aperture ovate, incomplete, adapically narrow and abapically widened; outer lip thin.

Protoconch (Fig. 5E, F). *c.* 0.9 whorls and mean 338.9 (± 10.8) μm in its largest diameter (range: 325–356 μm , $n = 16$).

Operculum (Fig. 5G). Paucispiral, ovate, thin, translucent, nucleus eccentric with a small attachment scar near the centre.

Radula (Fig. 5H–K). Taenioglossan. Rachidian (central) tooth (Fig. 5I) reaching 19.6 (± 1.1) and 31.6 (± 1.2) μm wide in its upper margin and central (measured at the height of the beginning of the tongue) section, respectively; basal tongue U-shaped; central cusp elongate, pointed distally, with five (rarely six) lateral cusps on each side; one basal cusp. Lateral teeth with three conic cusps around an elongated-conic central cusp. Inner marginal teeth (Fig. 5J) with 15–18 cusps; outer marginal teeth with 21–23 cusps (Fig. 5K).

Soft body (Fig. 5L–Q). Mantle black with a wide grey margin (Fig. 5L). Foot black, propodium white; head

black (Fig. 5M–O), snout black, lips white. Tentacles black with a grey band in the base and a grey tint at the tip. Penis grey (Fig. 5P–Q), with wide columnar base that occupies about one third of the organ and another one elongated which occupies the remaining two-thirds of the organ, 4–6 apocrine glands, with two or three of them located closest to the base in the convex margin and the remainder located in the inner (ventral) surface; distal portion globose with a minute terminal papilla and a small lobe in the concave margin.

Remarks. *Heleobia peralensis* sp. nov. is ascribed to *Heleobia* based on 12S, 16S, and COI DNA mitochondrial sequences (Collado et al., 2013; present study) as well as penis morphology following the diagnosis of the organ performed by Hershler and Thompson (1992) for the genus. *Heleobia peralensis* sp. nov. is indistinguishable from *H. chimbaensis* through the external shell morphology and morphometry. However, it differs from *H. chimbaensis* in COI sequences (nucleotide site 48, 156, 162, 165, 174, 180, 187, 216, 240, 243, 252, 261, 267, 277, 300, 318, 349, 384, 393, 459, 477, 498, 534, 609 and 624 of the alignment), the groundplan of the penis (elongated conic, distal portion tapered in *H. chimbaensis*, bipartite in *H. peralensis* sp. nov., with distal portion globose), as well as the number of cups of radular marginal teeth, being more numerous in *H. chimbaensis*.

Discussion

Heleobia is a hyperdiverse group, containing dozens of species, many of them morphologically similar (Cazzaniga, 2011; Collado et al., 2011; De Francesco & Isla, 2004). Using the morphospecies concept, Biese (1947) found minor differences in the size of the snails and aperture shape between the typical *H. chimbaensis* and the snails from Los Perales, which probably led him to classify these samples as the same species. Similarly, in the present study we were unable to distinguish between these samples based on the qualitative observation of the external shell morphology, morphometry and CVA, results that maybe justify the early allocation of snails from Los Perales to *H. chimbaensis* (Biese, 1947). Conversely, the COI phylogenetic tree showed that these snails are distantly related, revealing the presence of a *Heleobia* cryptic lineage in Atacama. A previous molecular analysis based on mitochondrial 12S and 16S sequences also inferred the snails from Los Perales positioning in a different clade from *H. chimbaensis* (Collado et al., 2013). However, it is important to note that both species would be cryptic considering several overlapping characters, but not cryptic species in a

stricter sense, since there are stable morphological differences regarding penis and radula. Thus, *H. peralensis* sp. nov. is a different species from *H. chimbaensis* not only under the phylogenetic species concept, which recognizes a species as a lineage (Baum, 1992), but under morphological species concepts as well.

The results of the network analysis agreed with the molecular phylogenetic analysis that identified *H. peralensis* sp. nov. within the distribution area of *H. chimbaensis*. Out of the seven haplotypes, five belonged to the haplogroup clustering populations of *H. chimbaensis*. The other two haplotypes are private of *H. peralensis* sp. nov. In the first haplogroup, the subspecies *H. chimbaensis conica* was inferred separated of the main haplotype of *H. chimbaensis* by one mutation step. Despite the significant differences in shell dimensions between these snails, the low genetic divergence recovered leads us again to conclude there is not enough evidence to consider *H. chimbaensis conica* as a subspecies. However, it is worth noting that we analysed only a few individuals (27 specimens), and a larger sampling could reveal a wider variation.

Based on the COI gene, the phylogenetic relationships of *H. chimbaensis* with the species of the southern section of the Atacama Desert were well supported in the present study. This was not the case for *H. peralensis* sp. nov. whose systematic position varied according to the method used to infer relationships. In the ML analysis it positioned without bootstrap support as sister of all *Heleobia* species except *Heleobia australis* (d'Orbigny, 1835) while in the NJ analysis it was recovered as a sister of a major clade composed by *H. chimbaensis* and *Heleobia* spp. from northern and central Chile, including species from the Atacama Desert and Argentina. Perhaps another molecular marker, such as the 12S or 16S gene, which evolves at a lower rate regarding the COI gene, could help solve this problem.

Geographic isolation involves physical barriers dividing populations. The network analysis indicates that the populations of *H. chimbaensis* from La Chimba, Playa La Chimba, Cueva de los Curas, and Quebrada Carrizo are not genetically differentiated, which could be related to the proximity of the oases they inhabit, scattered in an area of near 30 km from La Chimba in the north to Quebrada Carrizo in the south. Located southernmost, the populations from Las Breas and Quebrada Cascabeles showed a greater genetic variability, suggesting some geographic effect on the population structure. Thus, geographic isolation and dispersal seems to have influenced the diversification and current distribution of *H. chimbaensis* in the coast of the Atacama Desert. Recently, Valladares *et al.* (2018) pointed out the importance of habitat fragmentation in the genetic

structuring of the *Heleobia* populations of the Salar de Ascotán, Chilean Altiplano, a system with isolated and semi-isolated water systems. They also found that the geographic distance as a unique factor is not enough to predict the degree of divergence between the studied populations.

Biese (1947) noted some similarities between the shell aperture of *H. chimbaensis* and *Heleobia transitoria* (Biese, 1947), and between that of *H. chimbaensis conica* and *Heleobia copiapoensis* (Biese, 1944). Biese (1947) also recognized the similarity of *Heleobia chimbaensis* and *Heleobia kuesteri* (Strobel, 1874) from Argentina regarding the measures and proportions of the shell. In both cases, this should be explained as a consequence of the conservative form of the shell since these taxa are not closely related species.

Regarding the conservation status, *H. chimbaensis* is classified as 'Vulnerable' after the criteria stated by the Ministerio del Medio Ambiente de Chile (Collado, 2014). Until now, this species has been recorded from nine small and fragile isolated ecosystems spanning about 265 km of coastline in the Atacama Desert: La Chimba, Playa La Chimba, Cueva de Los Curas, Quebrada Carrizo, Finca Tofala, Aguada Galerce and Los Perales in Quebrada Paposo, Las Breas in Quebrada Taltal, Quebrada Cascabeles and Quebrada Guanillo (Biese, 1944, Biese, 1947; Collado, 2012). The present study raises two important differences from the previous data. The first one is that, as aforementioned, the intensive sampling performed in 2011 was negative for specimens of *H. chimbaensis* in Aguada Galerce and Quebrada Guanillo, several localities are polluted and disturbed, and the locality of Finca Tofala could not be found (Collado, 2012). Additionally, the population from Los Perales does not belong to *H. chimbaensis* entailing range reduction. Thus, we believe that the category of Vulnerable assigned to this species should be modified. In fact, the conservation status assessment performed in the present study categorized this species as Endangered under IUCN criteria (IUCN 2019) and Critically Imperilled using the NatureServe methodology. Burgos (2015) reported that in the last decades the risk of floods and drought have caused the conditions for an imminent loss of habitat and biodiversity in the Región de Antofagasta, which could force a change in the assigned categories of most species in the area.

Acknowledgements

We acknowledge Cristian Suárez for support during SEM observations, the curator of the MCNAPPRF, Chillán, Chile, and referees for improving the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data

Supplemental data for this article can be accessed here <https://dx.doi.org/10.1080/14772000.2020.1783018>.

Funding

This work was partially supported by the CONICYT-FONDECYT under Grant 11130697 to G.A.C.

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Associate Editor: Barna Pall-Gergely