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Novel microsatellite markers for an endangered freshwater snail, *Heleobia atacamensis* (Caenogastropoda: Cochliopidae), from the Atacama Saltpan

Alejandra A. Fabres [a], Moisés A. Valladares [a], Paola A. Sáez [a], Gonzalo A. Collado [b], Luis Pastenes [c] and Marco A. Méndez [a]

[a]Laboratorio de Genética y Evolución, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile. Las Palmeras 3425, Santiago, Chile; [b]Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bío-Bío. Av. Andrés Bello 720, Chillán, Chile; [c]Laboratorio de Genética y Adaptación a Ambientes Extremos, Departamento de Biología y Química, Facultad de Ciencias Básicas, Universidad Católica del Maule. Av. San Miguel #3605, Talca, Chile

ABSTRACT

The minute freshwater gastropod *Heleobia atacamensis* is only known from its type locality Tilopozo, located at the southern end of the Atacama Saltpan, Antofagasta Region, Chile. It is classified as Critically Endangered by the national agency, Ministerio del Medio Ambiente de Chile, due to its restricted distribution. For the first time, we have isolated and evaluated 65 microsatellite markers in 42 specimens of *H. atacamensis*, successfully characterising 11 as polymorphic. Cross-amplification testing was successful in three other species of the genus. As a result, the development and characterisation of these markers could be useful in future genetic studies of *H. atacamensis* and its congeners, regarding population structuring, dispersal patterns and recent demographic history. Furthermore, this information will also be significant to undertake conservation efforts in this endangered species, since its environment is highly threatened by lithium mining activities and a global water crisis.

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Conservation genetics; gastropod; lithium mining; high-throughput sequencing; repetitive DNA

Introduction

The Atacama Desert in northern Chile is one of the driest places known on earth. In this biome lies the biggest saltpan in Chile, the Atacama Saltpan, enclosed by the Andes mountains and the mountain range of Domeyko; it is located at 2300 m a.s.l and is 200 km long and 100 km wide. This saltpan is the principal source of lithium extraction in the continental brines of Chile, the second largest producer in the world, possessing more than half of the world's reserve of this mineral (U.S. Geological Survey 2020).

The Atacama Saltpan is an endorheic basin that receives a regular inflow of freshwater from the mountain ranges that surround it. The hydrological systems in the saltpan are of three types: freshwater and saline springs, saline lagoons, and lotic rivers in the north (Dorador et al. 2018). Only two gastropods have been described in this salt pan, the air-breathing land snail *Succinea labiosa* Philippi, 1860 (Stuardo & Vega 1985), which has not been studied after its first description, and the freshwater snail *Heleobia atacamensis* (Philippi, 1860), currently classified as 'Data Deficient' according to the criteria of the IUCN Red List of Threatened Species (Pastorino & Darrigan 2011), and as 'Critically Endangered' according to the 'Reglamento de Clasificación de Especies' of the 'Ministerio del Medio Ambiente de Chile' (RCE DS MMA) (Ministerio de Medio Ambiente 2014). This classification is based on the extent of its occurrence, which is estimated to be less than 1 km^2 (its actual estimated range extent is approximately 7 m^2); for only being known from its type locality, Tilopozo; and a decrease in the quality of its habitat, due to prolonged droughts and constant water extraction for mining activities. Tilopozo is a freshwater pond located in the southern end of the Atacama Saltpan. Recently, Collado et al. (2013), recorded populations of *Heleobia* sp. in Tilomonte, a small stream located at the middle edge of the Atacama Saltpan, and Peine, a stream located 20 km northeast of Tilopozo.

Species of *Heleobia* are characterised by their small size, typically around 2–8 mm in shell height. They inhabit fresh and brackish waters, and even though they have low vagility (Cazzaniga 2011), the genus is widely distributed in South America (Hershler & Thompson 1992). *Heleobia* is a speciose genus, with nearly 100 nominal species (Cazzaniga 2011; Hershler & Thompson 1992), of which 90 have been described for South America (Collado et al. 2016a; Martin & Díaz 2016). In Chile, there are 30 species of *Heleobia*, of which ten have been described for the Atacama Desert (Collado et al. 2011; Collado 2015; Collado et al. 2016b), although Collado et al. (2013, 2016a) have suggested that this number might be underestimated, due to the lack of malacological studies in the area.

CONTACT Marco A. Méndez mmendez@uchile.cl Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Región Metropolitana, 7800003, Chile
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The taxonomy and systematics of *Heleobia* has been studied mainly using morphological features and, to a minor extent, genetic data (e.g. Biese 1944, 1947; Haas 1955; Weyrauch 1963; Collado et al. 2011, 2013, 2016a, 2019; Kroll et al. 2012). Only a few studies have addressed a phylogeographic scenario for populations of this genus (Collado et al. 2016; Kroll et al. 2012; Valladares et al. 2018). All of these studies have used only mitochondrial DNA markers, revealing deeper evolutionary events. Therefore, it is crucial to develop molecular markers, such as microsatellites, that reflect contemporary evolutionary events, in order to infer genetic flow patterns or fine-scale population structuring in species of *Heleobia*. The aim of the present study is to develop and characterise novel microsatellite markers for *H. atacamensis*, providing a tool for future research in conservation genetics and population analysis of species of *Heleobia* from the Altiplano. In this context, we also performed cross-amplifications in populations belonging to different lineages than *H. atacamensis*, such as *Heleobia* sp. El Tatio, and two populations inhabiting the Atacama Saltpan (Tebenquiche and La Punta lagoons), treated here as *Heleobia* spp.

Materials and methods

Sampling and DNA extraction

Specimens of *H. atacamensis* were sampled in the freshwater pond of Tilopozo (23°47′2.06″S, 68°14′57.95″W), Atacama Saltpan, from sediments and macrophytes using a sieve with a 1 mm mesh diameter. The snails were later fixed in 99% ethanol for future molecular analysis. A piece of mantle with gills was dissected from each snail and used for the DNA extraction performed with the cetyl trimethylammonium bromide (CTAB) method, modified from Winnepenninckx et al. (1993), for all samples. Cross-amplification was done with individuals of *Heleobia* sp. El Tatio (22°20′10″S, 68°00′59″W), a population retrieved as a sister lineage of *H. atacamensis* (Collado et al. 2013, 2016a). Additionally, we analysed populations from two localities inside the Atacama Saltpan, Tebenquiche lagoon (23°08′04.68″ S, 68° 15′31.38″ W), located at the north edge of the Atacama Saltpan, and La Punta lagoon (23° 43′18.96″S, 68°14′17.93″W), located 5 km north of Tilopozo. The *Heleobia* sp. present in the localities of Tebenquiche and La Punta have been ascribed by our group to two new putative species of *Heleobia* (Valladares et al. in preparation).

Library preparation and high-throughput sequencing

The genomic DNA was physically fragmented to 400 bp with a Covaris S220 ultrasonicator. The fragments were later emulsified and used to prepare and sequence an enriched library using the Ion Xpress™ Plus gDNA Fragment Library Kit, with the Ion 314™ chip kit v2 BC in an ION Torrent Personal Genome Machine® (PGM™) system, according to manufacturer's recommendation in OMICS–Solutions' facility (http://omics-solutions.cl/). Sequences were analysed with the programme Prinseq (Schmieder & Edwards 2011) that is used to filter, reformat and trim next-generation sequence data. The software MISA was used to identify and localise target microsatellites, and the identification of primers was done with Primer3 (Rozen & Skaletsky 2000).

Selection of microsatellite markers

Selection of the microsatellite markers was done in silico based on the optimal amplification characteristics, their behaviour and properties using the Oligo Analyzer tool from Integrated DNA Technologies company. A total of 65 pairs of primers was selected and tested on eight individuals of *H. atacamensis* by polymerase chain reactions (PCR). The PCR mixture had a total volume of 15 µl, and contained 1X PCR buffer (200 mM Tris-HCL pH 8.4, 500 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.13 µM of each forward and reverse primer, 0.3 U *Taq* DNA polymerase (Invitrogen™), and 15 ng of template DNA. Thermocycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, the specific annealing temperature of each primer (Table 1) for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min. After verifying amplification by electrophoresis in 2% agarose gels, a total of 30 primers were selected.

The previously selected primers were tested for polymorphism on eight individuals of *H. atacamensis*. For this, we modified the forward primer, attaching the M13 universal primer sequence (5′-TGT AAA ACG ACG GCC AGT-3′), and used a fluorescent dye label (PET, FAM, VIC and NED) attached to the same M13 universal primer sequence (Schuelke 2000). The PCR mixture used had a total volume of 15 µl, and contained the same reagent concentrations as before, but we added 0.05 µM of the forward primer with the M13 tail, 0.13 µM of the reverse primer, and 0.13 µM of the M13 tail labelled with fluorescent dye. Thermocycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, the specific annealing temperature of each primer (Table 1) for 30 s, 72°C for 45 s, with a second stage of 8 cycles with an initial denaturation of 94°C for 30 s, the specific annealing temperature of the M13 label primer (53°C) for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min (Schuelke 2000). After verifying amplification by electrophoresis in 2% agarose gel, PCR products were sent to the Sequencing

Table 1. Characteristics of the 11 microsatellites developed for *Heleobia atacamensis* (N = 42).

Locus	Primer sequence (5′-3′)	Repeat motif	Ta(°C)	Na	Ho/He	Size Range	PIC	Dye Label
Hel_45	F: AAGAACATGTCTCCTACAG R: GTCTGTGACTTAGATTATGG	(CA)6	56	3	0.211/0.196	242–248	0.184	PET
Hel_32	F: CTTCAGGAAGTTGATAGAC R: CTGTGTTTAAAGAGAGTGTC	(CA)13	58	5	0.394/0.558	224–232	0.487	6-FAM
Hel_80	F: TATAGAGAGAGAGAGCGTG R: CTGTCCATCTGATAAGTTG	(TTC)7	58	2	0/0.089	180–183	0.083	NED
Hel_09	F: CGTACTTCTATGAGAATCTG R: TCATCATAGTCATCACTGTC	(GAT)6	56	4	0.162/0.179	224–239	0.171	VIC
Hel_73	F: CACAACTTCTTACTCATCC R: GTGTTCTCTTTCTTACTGTG	(ATT)6	56	2	0.143/0.416	149–161	0.325	NED
Hel_08	F: CTACAATAGCATGGAGTAAC R: TCACTCTTGGTATGAGATG	(CAT)5	56	2	0/0.428	171–189	0.329	PET
Hel_11	F: ATACGTCTCGATAGTCATAC R: AGTCCACTACACTGATTTG	(TTC)5	58	2	0.03/0.03	201–204	0.029	6-FAM
Hel_86	F: ACCGTAACCTGTACTAAAC R: GACATAAAACAGACAGAGC	(ATC)6	56	2	0.184/0.212	151–154	0.187	PET
Hel_94	F: ACAGAAAAGCTACCTCTAC R: GTCGTCTTCATATGTAGATG	(AAGA)5	56	2	0.447/0.504	222–226	0.373	6-FAM
Hel_92	F: ATTATGTCTGTCTGTGTGAC R: GACTAGATGGAAAAGACTG	(CTGT)5	58	3	0.342/0.319	160–168	0.288	VIC
Hel_87	F: CTGTGTTTTGAGAGGTATAC R: CTTGTCATGAACTACCATC	(GTCC)5	56	3	0.842/0.505	164–176	0.387*	VIC

Note: Ta is the annealing temperature, Na the number of alleles, Ho/He the ratio of observed and expected heterozygosity, PIC the polymorphic information content, Dye Label specifies which fluorescent dye was used to label the microsatellite.
*Indicates significant deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.001$)

Center at the Pontificia Universidad Católica de Chile to be genotyped and analysed on an ABI PRISM 310 Genetic Analyzer using GeneScan500 LIZ as the internal size standard (Applied Biosystems). Allele scoring was done using Geneious v6.1.8 software (https://www.geneious.com), resulting in a total of 11 polymorphic microsatellite markers for *H. atacamensis*, which were later amplified with 42 individuals of the species.

Cross-species amplification was done for 28 individuals of *Heleobia* sp. El Tatio, 28 individuals of *Heleobia* sp. Tebenquiche, and 40 individuals of *Heleobia* sp. La Punta.

Data analysis

The allele matrices for all *Heleobia* spp. were scored and built using Geneious v6.1.8, and 18 bases were subtracted to the total length of each allele, in reference to the M13 tail added during PCR conditions (Schuelke 2000). We used the software MicroChecker v2.2.3 (van Oosterhout et al. 2004) to identify potential scoring errors due to stuttering, large allele dropouts and presence of null alleles in the matrix. The observed (H_O) and expected (H_E) heterozygosity, the number of alleles (N_a), deviations from Hardy-Weinberg equilibrium (HWE) and the polymorphic information content (PIC) were estimated with Cervus v3.0 (Kalinowski et al. 2007). Linkage disequilibrium (LD) was tested in FSTAT v 2.9.4 (Goudet 2003) using 1000 permutations.

Results

A total of 400,000 reads were obtained from the high-throughput sequencing, from which 2,083 pairs of primers were designed with an average length of 191 bp. From these NGS data, 65 microsatellite primers were selected as the most promising (3%), of which 30 amplified, and 11 proved to be polymorphic for *H. atacamensis* with well scorable peaks. Null alleles were identified at four loci (Hel_32, Hel_80, Hel_73 and Hel_08), and one locus (Hel_87) presented a deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.001$) (Table 1). The mean observed heterozygosity was 0.25 and the mean expected heterozygosity was 0.31. There was no significant linkage disequilibrium found.

The cross-amplification test was successful, all 11 loci amplified. In the case of *Heleobia* sp. El Tatio seven loci were polymorphic. Null alleles were identified at two loci (Hel_09 and Hel_08), and none presented a deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.001$). For *Heleobia* sp. Tebenquiche nine loci were polymorphic. No null alleles were identified, and no loci showed a deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.001$). For *Heleobia* sp. La Punta ten loci were polymorphic. Null alleles were identified at two loci (Hel_73 and Hel_86), and one locus (Hel_73) presented a deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.001$). There was no significant linkage disequilibrium found in any of the analysis. From this cross-amplification only five microsatellite markers (Hel_32, Hel_09, Hel_86, Hel_94 and Hel_87) were polymorphic for all *Heleobia* spp. tested (Table 2).

Discussion

The development of microsatellites has improved during the last decade, although their isolation in

Table 2. Characteristics of the 11 microsatellites after the cross-amplification test for *Heleobia* sp. El Tatio, *Heleobia* sp. Tebenquiche and *Heleobia* sp. La Punta.

	Heleobia sp. El Tatio N = 28				*Heleobia* sp. Tebenquiche N = 28				*Heleobia* sp. La Punta N = 40			
Locus	Na	Ho/He	Size Range (bp)	PIC	Na	Ho/He	Size Range (bp)	PIC	Na	Ho/He	Size Range (bp)	PIC
Hel_45					2	0.071/0.07	242–244	0.067	3	0.375/0.359	242–246	0.3
Hel_32	5	0.385/0.501	210–222	0.462	3	0.32/0.61	218–226	0.512	3	0.425/0.423	224–228	0.376
Hel_80					2	0.107/0.166	183–189	0.149	2	0.05/0.049	183–186	0.048
Hel_09	2	0.045/0.274	215–221	0.232	2	0.042/0.042	221–233	0.04	2	0.05/0.049	233–239	0.048
Hel_73									3	0.15/0.505	149–161	0.386*
Hel_08	6	0.286/0.659	150–168	0.606	3	0.36/0.313	171–189	0.278				
Hel_11	2	0.231/0.212	177–180	0.183					2	0.3/0.425	201–204	0.332
Hel_86	2	0.577/0.491	121–127	0.366	2	0.08/0.078	142–154	0.074	4	0.15/0.313	139–154	0.295
Hel_94	2	0.036/0.036	220–224	0.034	2	0.296/0.257	222–226	0.221	2	0.275/0.339	222–226	0.279
Hel_92					3	0.227/0.479	160–168	0.405	4	0.8/0.658	156–168	0.59
Hel_87	5	0.786/0.719	168–184	0.654	3	0.741/0.488	164–184	0.381	3	0.425/0.348	164–184	0.3

Ta Annealing temperature, Na number of alleles, Ho/He observed and expected heterozygosity, PIC polymorphic information content
*Indicates significant deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.001$)

certain taxa, such as Mollusca, remains problematic (McInerney et al. 2011). The main problem is caused by cryptic repetitive DNA in flanking regions of microsatellite-containing sequences. This phenomenon has also been observed in plants, insects and crustaceans (Bailie et al. 2010; Meglécz et al. 2007; Tero et al. 2006). From the primers designed for *H. atacamensis*, 3% (65) were selected as the most promising, avoiding those with flanking regions having repetitive DNA. If such primers had been selected, later PCR would have had interference from the repeats, causing PCR failure (null alleles) or difficulties in amplification.

This study retrieved 11 polymorphic loci, which we consider adequate, considering that *Heleobia* is a non-model species, and the low number of primer pairs retrieved by the high-throughput sequencing. In general, our results found one locus with a high allelic diversity (Hel_32), while most loci had two alleles, with at least one heterozygous individual (Hel_80, Hel_73, Hel_08, Hel_11, Hel_86 and Hel_94). The exceptions were Hel_80 and Hel_08, these had multiple types of homozygous individuals, but no heterozygotes. Despite this lack of heterozygotes we have included these loci, because they do not present a deviation from HWE after the Bonferroni correction and they have a higher Ho in other populations of *Heleobia* tested. This low Ho could be explained by allelic dropouts, due to the stochastic failure of an allele not amplifying, or to the presence of null alleles, related to flanking regions (McInerney et al. 2011).

The present study considers the populations of *Heleobia* sp. El Tatio and *Heleobia* spp. from Tebenquiche and La Punta as species distinct to *H. atacamensis*. Our microsatellite data shows that the cross-amplification was successful. Of the seven polymorphic loci identified for *Heleobia* sp. El Tatio, two (Hel_94 and Hel_87) had the same range of allele sizes as *H. atacamensis*. Similarly, of the nine polymorphic loci identified for *Heleobia* sp. Tebenquiche, six (Hel_45, Hel_32, Hel_09, Hel_08, Hel_92 and Hel_94) had the same allele size range as *H. atacamensis*, and of the ten polymorphic loci identified for *Heleobia* sp. La Punta, six (Hel_45, Hel_32, Hel_09, Hel_73, Hel_11 and Hel_94) had the same size range as *H. atacamensis*.

Although we provide novel microsatellite markers for three populations of *H. atacamensis* from the Atacama Saltpan, more extended research must be carried out involving other *Heleobia* populations from the Altiplano in order to confirm the low heterozygosity pattern found in the population of Tilopozo. The description of these microsatellites for this endemic freshwater snail will be useful for future population genetics studies of the genus *Heleobia* from this saltpan and probably other closed basins from the Altiplano. Facilitating studies on population analysis to microgeographic scales will be useful for conservation planning of the critically endangered *H. atacamensis* in the Atacama Saltpan, a place with major lithium mining activity.

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No potential conflict of interest was reported by the author(s).

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ORCID

Alejandra A. Fabres http://orcid.org/0000-0002-8888-9320
Moisés A. Valladares http://orcid.org/0000-0002-0294-174X
Paola A. Sáez http://orcid.org/0000-0002-9794-6737
Gonzalo A. Collado http://orcid.org/0000-0001-9076-4255
Luis Pastenes http://orcid.org/0000-0001-7543-0898
Marco A. Méndez http://orcid.org/0000-0001-6041-4617

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