Development and characterization of microsatellite molecular markers for the eye mask frog *Batrachyla taeniata* (Girard, 1855)

Ingrid Cisternas-Medina[1, 3*], Juan Carlos Ortiz[1] and Sandra Ferrada-Fuentes[2, 3, 4]

[1]Laboratorio de Sistemática y Conservación de Herpetozoos, Departamento de Zoología, Universidad de Concepción, Concepción, Chile.

[2]Laboratorio de Genética y Acuicultura, Departamento de Oceanografía, Universidad de Concepción, Concepción, Chile.

[3]Programa de Doctorado en Sistemática y Biodiversidad, Departamento de Zoología, Universidad de Concepción, Concepción, Chile.

[4]Instituto de Ciencias Naturales, Universidad de Las Américas, Campus El Boldal, Concepción, Chile.

Corresponding author: I. Cisternas-Medina

E-mail: ingridmarlciste@udec.cl

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ABSTRACT.

Batrachyla taeniata (Batrachylidae) is a small frog from Chile and Argentina with a distributional range that spans more than 1500 km. However, their populations are threatened due to the change in land use, deforestation, and human settlements. Through massive sequencing runs in ION Torrent PGM, was built DNA fragment library, from which we finally isolated and characterized 25 microsatellite loci from 40 individuals. The number of alleles per locus ranged from 2 to 23, allele sizes varied between 110 and 268 bp, observed heterozygosity ranged from 0.205 to 1.000 and the expected heterozygosity between 0.21 and 0.93. We found evidence of deviations of HWE for 10 microsatellite loci, linked to high inbreeding coefficients, indicating a loss of heterozygosity in those particular loci. There was no linkage disequilibrium found within the microsatellite loci tested through the sequential Bonferroni correction. These microsatellite markers can be

used to evaluate population problems such as low diversity and high genetic structure, with the purpose of optimize future conservation strategies for the populations of this species.

Keywords: *Batrachyla taeniata*; Microsatellites; ION Torrent; fragment analysis.

INTRODUCTION

Batrachyla taeniata (Anura: Batrachylidae) is a small frog species endemic to the temperate forests of Nothofagus that has a wide distribution in Chile and marginally in Argentina. In Chile, it extends from the Aconcagua province in the Valparaíso region to the Capitán Prat province in the Aysén region (Salaberry et al., 1981; Brieva and Formas 2001; Correa et al., 2014). In addition, it presents island distributions in at least three islands near the continent, Quiriquina, Mocha and Chiloé (Salaberry et al., 1981). This species is of particular interest from a biogeographical point of view, since it has been found in two different ecological regions: the Mediterranean and Oceanic regions (Di Castri, 1976) covering approximately more than 1,500 km from north to south. In its northern distribution is associated with hydrophilic coastal relict forests and mesophiles (ej: Zapallar y Quintero) surrounded by semi-arid vegetation and in the southern distribution with humid and shady forests in environments saturated with humidity (Cei, 1962; Formas, 1979). This species has been frequently reported from Concepción to the south, in contrast to the populations in central Chile where is less frequent and more geographically isolated (Correa et al., 2014). The populations of central Chile (Quintero) present high values of distance and genetic differentiation with respect to the populations of the southern distribution group (Brieva and Formas, 2001). The species has been categorized as "Least Concern" in the International Union for Conservation of Nature (IUCN) red list (2015), despite the fact that Chilean law considers it "Almost Threatened" by the Classification Regulation of Species (RCE) (2014) because northern populations are more affected by the change in land use, deforestation and human settlement. These modifications in the landscape can have a strong effect on the patterns of dispersion and genetic flow of the species, this due to that amphibians have a restricted dispersion capacity that tends to promote differentiation (Duellman, 1999; Funk et al., 2005). For this reason, they show a greater genetic structure in their populations compared to other groups of species (Semlitsch R and Skelly D, 2008).

The microsatellites are a type of molecular marker widely used in population genetics, conservation biology and evolutionary biology (Abdul-Muneer, 2014). These correspond to fragments of DNA sequence that consist of tandem repetitions of basic units that vary between 1 to 10 nucleotides in length and are repeated throughout the genome (Hamada et al., 1982; Vieira et al., 2016). The genetic variation of many microsatellite loci is characterized by high heterozygosity and polymorphisms (Litt and Luty, 1989; Ellegren, 2004), being a versatile tool for the identification of genetically deteriorated populations (Arif et al., 2011). Consequently, the use of this type of DNA markers can help to establish better management and protection plans for the conservation of biological populations. In the present study, we developed 25 microsatellite markers for *B. taeniata* with the purpose of using them in future population studies to investigate the genetic structure and diversity of their populations for conservation purposes.

MATERIALS AND METHODS

Samples

For the study DNA samples was obtained of specimens coming from Hualpén Botanic Park (36°47'S; 73°09'W), surroundings Hualpén (36°47'S; 73°05'W) Concepción, river headboard Tubul (37°12'S; 73°32'W) La Cal (37°12'S; 73°31'W) Arauco, Chile.

DNA preparation, sequencing and obtaining microsatellite markers

Through optimizations of Kit Promega Wizard Purification Systems, we extracted DNA of 27 individuals of *B. taeniata* from the sampled localities for use in the isolation of microsatellite loci. By quantifying DNA, we select

8 samples from different locations to perform the massive sequencing. The initial amount of DNA used was 100 ng, quantified spectrophotometrically (Nanodrop) and fluorimetrically (Qubit). DNA fragmentation at 400 bp was performed with the Covaris S220 sonicator. The DNA fragment library was constructed using the Ion Xpress Plus gDNA Fragment Library Kit (Thermo Scientific, manual MAN0009847, review C.0). For the preparation of library, emulsion and chip sequencing, the manufacturer's protocols were followed without modifications (Thermo Scientific, P.N. MAN0009847 revision D.0, MAN0010902 revision A.0 and MAN0009816 revision D.0). The readings in Fastq format were filtered using the Prinseq program (Schmieder and Edwards, 2011). The readings that passed the filters were transformed to Fasta format and were clustered at 90% identity by CD-HIT (Li et al., 2001). Subsequently, the MISA program (MISA webpage) was used for the identification and localization of dinucleotide microsatellites with at least 6 repetitions and tri /tetra-nucleotides with at least 5 repetitions. The identification of the primers was done with Primer3 (Rozen and Skaletsky, 2000) with a range in amplicon size between 100-500 bp and an optimum of 350 bp, length of the primer of 19-25 bp with an optimum of 21 bp, Melting Temperature (TM) between 53-57°C with an optimum of 55°C and a GC percentage between 30-80%.

Microsatellite Genotyping

Of the 221 repetitive motifs obtained, 60 pairs of primers were selected, and these were tested for amplification using DNA from 10 individuals. PCR amplifications were performed in a total volume of 8 uL containing 10X Buffer Taq (Thermo Scientific), 25 mM $MgCl_2$ (Thermo Scientific), 2.5 mM dNTPs (Promega), 10 uM of each primer (Macrogen), 0.5 U uL-1 Taq DNA Polymerase (Thermo Scientific) and ca. 100 ng uL-1 DNA. The thermocycler conditions for all the loci was with an initial denaturation of 5 min at 95°C, followed by 20 cycles of 30 s 95°C, 30 s at the optimum annealing temperature of 65-55°C and a final extension of 30 s at 72°C. In each cycle, the temperature was decreased in 0.5°C and the following conditions were continued: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, 30 s of extension at 72°C and a final extension of 5 min at 72°C.

The PCR products were separated by 2.5% electrophoresis in agarose gels (BM-0120 Winkler), which ran between 60 min and 90 min. A first approximation of the microsatellite loci was estimated with the molecular weight marker Bench Top 100 pb DNA ladder (Promega).

For fragment analysis, 30 pairs of primers were selected, which were marked with four fluorophores (6FAM, PET, VIC, and NED). The selected microsatellites were amplified again using DNA from 40 individuals from the locality of Hualpén with the same initial conditions. The PCR products were run on an Applied BioSystems 3730 DNA Analyzer sequencer in Advanced Analysis Center, University of Guelph, Canada.

Statistical Analyses

Alleles were scored using the PeakScanner version 1.0 (Applied Biosystems). The number of alleles (A), Size the range of observed alleles in base pairs (bp), number of individual genotyped (N), observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding coefficient (FIS) were tested for by using GenALEx 6.5 (Peakall and Smouse, 2006). Tests for deviations from the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were made in GENEPOP v 4.5 (Rousset, 2008). We also compare the structure of two populations separated by urbanization in the locality of Concepción in the Structure v 2.3.4 software (Pritchard et al., 2010) with the 11 most polymorphic microsatellites developed in this work.

RESULTS

Twenty-five of the pairs of primers tested amplified high-quality PCR products that exhibited polymorphisms. The characteristics of the loci are reported in Table 1. Most of the microsatellite primers contained dinucleotide and tetranucleotide repeat units. The number of alleles (A) varied between 2 and 23 (mean, 9.52). The sizes of the alleles varied between 110 to 268 bp, the heterozygosity observed (Ho) varied between 0.21 and 1.00 (mean, 0.71) and the expected heterozygosity (He) between 0.21 and 0.93 (mean, 0.68).

Table 1: Characterization of microsatellites for *Batrachyla taeniata*.

Locus	Repeat motif	Primer sequences (5'-3')	A	Size (bp)	N	H_o	H_e	F IS		GenBank Accession No
LocBat1	(AT)7	F: TTTCTTCCTATAGACCAGTG R: GGTATTGAGGCTACATCTTA	13	162-180	39	0.410	0.801	0.488	**	MK910322
LocBat4	(CA)9	F: GATACCAGGTAATTGTTGC R: ACCTGCTACTTCCTCTATTT	10	110-130	29	0.414	0.587	0.296	**	MK910323
LocBat12	(GA)14	F: GAATAAAGCCAGTGTGAAC R: GTAAAGGACCCTTAGAAAAG	21	123-164	40	0.900	0.919	0.021		MK910324
LocBat17	(AT)11	F: GGGGACACTTTATAATCAG R: AATGACAGACGTCAGTAGAC	10	142-161	34	0.676	0.854	0.208	**	MK910325
LocBat9	(TA)8	F: CTAATAACGTAAGTGCAACC R: GTTGTAACGAGTAAGTCCAT	8	171-220	34	0.441	0.510	0.136	**	MK910326
LocBat14	(AT)9	F: ATGTGCTCACAGAAGAAAG R: GATATACACCCTTGGTCCT	5	135-144	39	0.923	0.625	-0.477		MK910327
LocBat27	(TGC)8	F: GGACTGAATGTTCTTGTAGT R: ATTAAACTGTTCTAGGAGGC	22	132-169	39	0.846	0.922	0.083		MK910328
LocBat18	(TAA)8	F: GATTACTTCAGTGCTAGTCAG R: TAGAGCTATTTGATCAGAGG	15	200-235	38	0.737	0.869	0.152		MK910329
LocBat19	(TAC)7	F: TACAGTTACCAAGAGTCCC R: GATAGCAAGGTTAGTGGTAA	8	150-166	39	0.641	0.757	0.153	**	MK910330
LocBat20	(CTG)7	F: GTTGAGACCACAAATCAAG R: TTAGTTGGAGTCTCTAATGC	2	188-191	40	1.000	0.500	-1.000		MK910331
LocBat7	(AC)9	F: CAATACATCAGACTTTCTCC R: TATTTGGTCCTAGTGAAGAG	9	154-167	34	0.765	0.726	-0.054		MK910332
LocBat22	(TCC)7	F: CTGTGTACATTTTCTTGGG R: GAGCAGATGCTTGTATCTAT	2	129-135	40	1.000	0.500	-1.000		MK910333
LocBat25	(CAT)9	F: CTAACATAGTGGTCATGGAT R: CATATTCACTAAAGCAGAGC	13	137-165	36	0.667	0.815	0.182	**	MK910334
LocBat36	(TAGA)9	F: AGAGATAAGGAAAGTTGGG R: CCATCTATCTACCTAACTCCTA	6	116-140	40	0.625	0.494	-0.266		MK910335
LocBat43	(AT)8	F: TGTACCTCACCTAAGAAAGA R: GGAAGGGAATAATACTTCTG	17	164-180	38	0.395	0.897	0.560	**	MK910336
LocBat31	(AGTT)10	F: TAGATCTCAACAGGAACAAG R: AGGTTAGACTTTTAAGCCAC	13	129-188	32	0.563	0.855	0.342	**	MK910337
LocBat37	(GTCT)6	F: AGATAGGAGCTTTAGGATTC R: GTACTCTGAAGTTGTGGTAAC	3	132-137	40	1.000	0.620	-0.613		MK910338
LocBat34	(ATAG)6	F: ATACAGACTCAAAGTGTCGT R: CCACCAATGTTAGTTTACC	23	177-268	40	0.975	0.933	-0.045		MK910339
LocBat52	(CTG)5	F: TAAATACCCATGTAGTCCAG R: AATTTCTTACCCGCTACTC	2	137-144	39	0.974	0.500	-0.950		MK910340
LocBat51	(GA)7	F: TCCAGTTAGATAAGCGTTAC R: ACATATAGCACTTCTACCCTC	6	172-179	39	0.205	0.214	0.040		MK910341
LocBat53	(CTG)6	F: CCTTTCTGATGCTACACTAC R: TAGTTTGGAGTCTCTAATGC	3	248-251	36	1.000	0.514	-0.947		MK910342
LocBat54	(ATA)6	F: GGCTCTATTGATGACAATC R: CTCTGTAAGTCTGCTTTTGT	14	126-159	38	0.921	0.881	-0.046		MK910343
LocBat55	(ATT)6	F: TACCTACATGGGATGATTAC R: GTCAATCCAAGTTAGTTGC	4	122-135	29	0.207	0.599	0.655	**	MK910344
LocBat 56	(AGG)6	F: CTACTGTGTCAAGCTGATAAC R: TAGTGTAGCTGCTGTGTGTAT	6	142-158	30	0.600	0.734	0.182	**	MK910345
LocBat59	(GTCT)6	F: AGATAGGAGCTTTAGGATTC R: CTCTGAGTTGTGGTAACCT	3	129-133	39	1.000	0.500	-1.000		MK910346

A = Number of alleles, Size the range of observed alleles in base pairs (bp), N = Number of individual genotyped, H_o = Observed heterozygosity, He = Expected heterozygosity, FIS = Inbreeding coefficient

** Significant evidence ($p < 0.01$) against Hardy-Weinberg equilibrium model

We found evidence of deviations of HWE for 10 microsatellite loci, linked to high inbreeding coefficients (FIS), indicating a loss of heterozygosity in those particular loci. The inbreeding coefficient values were significantly different from zero ($p < 0.05$) with negative FIS value detected for the loci LocBat 14, LocBat 20, LocBat7, LocBat22, LocBat36, LocBat37, LocBat34, LocBat52, LocBat53, LocBat54, and LocBat59, indicating that the population possess a greater number of heterozygotes in those particular loci, supported by the observed heterozygosity values (Ho).

There was no linkage disequilibrium found within the microsatellite loci tested through the sequential Bonferroni correction. Nevertheless, significant deviations from the HWE expectations in the population reflected the occurrence of heterozygote deficiency.

Genetic summary statistics of eleven microsatellite loci from for two population of *B. taeniata* (Table 2) show that in both populations the Ho was less than He (average Ho = 0.699: He = 0.832 and Ho = 0.627: He = 0.807). The values of the inbreeding coefficient were low for both populations, however, the population of the city of Hualpén shows a higher number of loci with high FIS (average FIS = 0.239).

Table 2: Genetic summary statistics of eleven microsatellite loci from two locations of *Batrachyla taeniata* in Concepción

Locus	**Hualpén botanic park** (N=30)				**Hualpén city** (N=10)			
	A	H_o	H_e	FIS	A	H_o	H_e	FIS
LocBat1	11	0.414	0.761	0.456	7	0.400	0.780	0.487
LocBat4	8	0.435	0.542	0.197	6	0.333	0.694	0.520
LocBat12	18	0.867	0.901	0.038	13	1.000	0.905	-0.105
LocBat17	8	0.640	0.811	0.211	7	0.778	0.827	0.060
LocBat27	18	0.931	0.903	-0.031	8	0.600	0.840	0.286
LocBat18	15	0.793	0.866	0.084	8	0.556	0.809	0.313
LocBat25	12	0.643	0.798	0.195	8	0.750	0.797	0.059
LocBat43	16	0.464	0.893	0.480	8	0.200	0.825	0.758
LocBat31	13	0.640	0.864	0.259	5	0.286	0.673	0.576
LocBat34	23	0.967	0.935	-0.034	11	1.000	0.890	-0.124
LocBat54	13	0.900	0.878	-0.025	8	1.000	0.836	-0.196
Average	*14*	*0.699*	*0.832*	*0.167*	*8*	*0.627*	*0.807*	*0.239*

A = Number of alleles, H_o = Observed heterozygosity, H_e = Expected heterozygosity, FIS = Inbreeding coefficient N = Sample size

The genetic distance value of Nei among the population of Hualpén botanic park and Hualpén city was from 0.416. However, the estimates in STRUCTURE indicate that the best K value for both previously separated groups is 1 (Table 3), indicating the presence of a single population (likelihood values k = 1: -2194.5, k = 2: -2276.2, k = 3: -3081.4).

Table 3: Estimate of the best value of K in STRUCTURE software

K	Ln prob. of data	Mean value of Ln likelihood	Variance of Ln likelihood
1	-2194.5	-2161.5	66.0
2	-2276.2	-2090.6	371.2
3	-3081.4	-2039.2	2084.5

K= Number of populations, Ln=Likelihood.

DISCUSSION

The advances in the massive sequencing have allowed great advantages for the development of molecular markers of microsatellite type (Sung-Yin et al., 2018; Zhang et al., 2018). These markers are one of the most widely explored genetic markers in several research areas due to their robustness and high polymorphism (Senan et

al., 2014; Zhuang et al., 2017) and are an excellent tool to know the structure of natural populations and help the conservation of the species (Mu-Yeong et al., 2017; Nakahama et al., 2018).

A good model for investigating the genetics structure of wild animal populations areamphibians (Beebee, 2005), because are highly dependent on their habitat (Duellman and Trueb, 1994) with relatively low dispersal capacities (Jehley and Arntzen, 2002). In addition, their populations show a greater genetic structure with respect to other groups (Johns and Avise, 1998), giving rise to populations that can represent unique genetic entities, between relatively short geographic distances (Jehle and Artzen, 2002; Beebee, 2005).

The genetic variability of a natural population is important for its continuity over time (Lacy, 1997), like its genetic structure, therefore, the combination of ecological and genetic data can help identify the factors that limit the size of the population and determine the potential conservation of them (Liu et al., 2013). However, these studies are not yet represented in the conservation efforts for Chilean amphibians' populations. Despite the advantages of microsatellite markers for population analysis, only a few species count on their development and characterization, including *Pleurodema thaul* (D'elía et al., 2014), *Rhinoderma darwinii* (Fuentes et al., 2014), *Telmatobufo bullocki* (Moreno-Puig et al., 2014), *Telmatobius chusmisensis* (Fabres et al., 2018).

In this study, we developed for the first time a set of twenty-five polymorphic microsatellites for B. taeniata. As seen in the results, the number of alleles (A) varied between 2 and 23 (mean, 9.52), finding highly polymorphic alleles such as those reported in *P. thaul* where the number of alleles per locus varied between 7 and 22 (D'elía et al., 2014). As mentioned, the heterozygosity observed (Ho) varied between 0.21 and 1.00 (mean, 0.71), with higher values per locus than reported in *P. thaul* (D'elía et al., 2014), *R. darwinii* (Fuentes et al., 2014), *T. bullocki* (Moreno-Puig et al., 2014) and *T. chusmisensis* (Fabres et al., 2018).

In this work was no linkage disequilibrium found within the microsatellite loci tested through the sequential Bonferroni correction. Nevertheless, significant deviations from the HWE expectations in the population reflected the occurrence of heterozygote deficiency, which can be given because of high inbreeding within the population, a scenario that has been demonstrated in other Chilean amphibians (Fuentes et al., 2014).

As mentioned, the genetic structure of populations is not always reflected in the geographical proximity of individuals (Evanno et al., 2005). However, in the analysis carried out for individuals of *B. taeniata* from two locations separated by high human urbanization, they show that they still correspond to the same population (the best k = 1). This indicates that individuals still share ancestry of the same ancestral population, perhaps due to a recent temporary separation of the analyzed localities.

CONCLUSION

It is important to advance in the development of obtaining genetic information from this species to improve strategies and prioritize the conservation of their populations, especially the northern populations that are more affected by the change in land use, deforestation and human settlement. In summary, we report twenty-five microsatellite markers in *B. taeniata* identified by massive sequencing. These microsatellite markers represent a valuable resource for future population analysis for conservation purposes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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