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MICROSATELLITE LETTERS

Development of microsatellites for Southern Darwin's frog *Rhinoderma darwinii* (Duméril & Bibron, 1841)

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Abstract The Southern Darwin's frog *Rhinoderma darwinii* is a charismatic, mouth-brooding amphibian endemic to temperate forests of South America with a *Vulnerable* conservation status according to the IUCN Red List. We developed microsatellite markers from next generation sequence data that will aid genetic monitoring during and after re-introduction efforts. Using bioinformatics we characterized 3,521 perfect microsatellite repeats and designed primers for 35 of them. From these, 23 were polymorphic and amplified reliably. Number of alleles varied between 2 and 15, allele sizes varied between 84 and 299 bp, and observed heterozygosities varied between 0.105 and 0.904. These microsatellites represent a valuable resource to aid recovery of threatened Southern Darwin's frog populations.

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Populations of Southern Darwin's frog *Rhinoderma darwinii* (Amphibia: Rhinodermatidae), a charismatic, mouth-brooding amphibian endemic to temperate forests of South America, have decreased dramatically in recent decades, likely due to changes in land use, habitat fragmentation and chytridiomycosis (Ortiz and Heatwole 2010; Soto-Azat et al. 2013). Their current conservation status is set to *Vulnerable* according to the IUCN Red List (http://www.iucnredlist.org). Conservation measures and ex situ captive breeding initiatives are underway to aid re-introduction efforts, which can be assisted by the monitoring of genetic diversity. Here we report 23 polymorphic microsatellite DNA loci to implement genetic monitoring for *R. darwinii*.

Total genomic DNA was extracted from muscle tissue taken from a single individual (RdaCB10) preserved at Estación de Monitoreo y Reproducción Rana de Darwin (EMRRD), Universidad de Concepción. We used a salting-out protocol followed by phenol–chloroform purification and ethanol precipitation steps. Shotgun sequencing on a GS Junior System (Roche) was outsourced to OMICS SOLUTIONS (www.omics-solutions.cl) and generated 118,506 reads with an average length of 399 bp. QDD v 2.1 (Meglécz et al. 2010) was used to find 3,521 perfect microsatellites (mostly di-, tri-, and tetra-nucleotide repeats) containing suitable flanking regions and located outside regions with high prevalence of mobile and transposable elements. Optimal settings for primer design included: (1) 20–21 nucleotides in length, (2) similar G+C content (47–50 %) between each primer of the pair, and (3) melting temperature of 59 °C. We calculated Gibbs free energy in 150 randomly chosen primer pairs using the

Table 1 Characterization of microsatellites for *Rhinoderma darwinii*

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	*A*	Size (bp)	*N*	H_O	H_E	F_{IS}
Rda1	$(taga)_{11}$	F: CGGCAAAAGAAATGGAGAAG	59	15	187–299	15	0.600	0.904	0.367*
		R: ATGCAAAAGGAGTCCACAGC							
Rda2	$(gata)_9$	F: CCAAGACATATGCCCTCCTT	59	7	95 –120	16	0.313	0.771	0.615*
		R: TTGGTTTTGTCAGCCTTTTATG							
Rda3	$(gata)_{18}$	F: GAGTTGCTGCTCATTCTTTGG	57	8	183–207	20	0.200	0.585	0.672*
		R: GTAACAGGAAACCAGCGGAA							
Rda4	$(tatg)_{12}$	F: GGTTAGACATGGAAGGGACG	63	3	102–110	19	0.263	0.453	0.441
		R: GAAGGCTTTGGTGAGCTGTC							
Rda7	$(ggt)_6$	F: TTGTCTAAACTGGACAACCCC	59	2	94–97	20	0.500	0.480	−0.016
		R: TTTAAGTGCAAAGGTCAGACAC							
Rda9	$(taa)_{16}$	F: CTGTTCCAACAACAGATCCTACA	59	5	160–190	14	0.143	0.704	0.810*
		R: TTTAGTGATTCCTATATGATGTCTGAG							
Rda12	$(gt)_5$	F: CCGTTTAACCGTTTGTACCC	55	2	108–110	22	0.500	0.416	−0.179
		R: AATCGCTGTGAATTTGGGAG							
Rda14	$(ca)_{10}$	F: ATGGAGCCATTTGTGAGGAG	55	4	147–155	22	0.455	0.630	0.300
		R: GTCTGCGGTACGAGTGTTGA							
Rda16	$(tcc)_5$	F: CCTCATCCTCTGTGTGGTCA	59	7	118–148	11	0.545	0.764	0.330
		R: CAGTGGAAAATAAAGCGGGA							
Rda18	$(taa)_5$	F: CGCTACAGAACTTAATCTTGCTATG	62	9	210–237	22	0.864	0.845	0.001
		R: TCATGTGCATTGCTGTACCC							
Rda19	$(att)_6$	F: TTGTGTTGCCATAACAACAATTC	60	4	156–165	19	0.105	0.602	0.834*
		R: GAAGGTAAACGACTCATGGAGG							
Rda20	$(cat)_5$	F: CACCCAGGTTACTCGGTCTG	59	3	117–123	13	0.231	0.328	0.333
		R: ATAGGAGGGCGTCTGGTCTC							
Rda22	$(tta)_5$	F: ACTCTTCACCCGATGCACTT	62	4	182–191	16	0.125	0.527	0.776
		R: GCTGCCATGTCGTGAATAAA							
Rda23	$(agg)_5$	F: ACCAATAAAACCAAGTCCGC	62	3	130–136	15	0.467	0.56	0.200
		R: GTGGTCAAGCAGCCAGTTTT							
Rda24	$(att)_5$	F: TTAGTTGCAGTACCCCGTCA	55	4	87–96	15	0.400	0.420	0.082
		R: CGAATATGCGATGGGTGATA							
Rda25	$(aac)_5$	F: CAAAGACACCTTGGGACTTC	58	7	108–141	20	0.650	0.743	0.150
		R: TCCTCAAACATTTCCCTTGG							
Rda26	$(aca)_{11}$	F: ACCAGATTTTGCATTGGGTC	55	8	86–110	18	0.944	0.807	−0.142
		R: ATGCTACATTTGCCCTGGTC							
Rda27	$(ca)_8$	F: TCGCTATTGACCACAGCATT	58	4	114–122	19	0.579	0.663	0.154
		R: TGTGATGTTCGGAGCTGAAT							
Rda29	$(ac)_5$	F: TGAATGTGCCTGCTCTCATC	58	5	111–119	21	0.238	0.661	0.654*
		R: TTTTCGGACGTAATTCGAGG							
Rda32	$(tg)_5$	F: CATACAGTGATTGGGCTGGA	55	5	84–92	20	0.000	0.710	0.999*
		R: TCGGGTTATTGTTGGACATTT							
Rda33	$(ac)_6$	F: TCCTGGTGCCATTTCCTAAA	60	5	112–120	17	0.471	0.746	0.395
		R: GAACTGGACGATGGAGCAAT							
Rda34	$(ctat)_{16}$	F: CCAGAGGTGAGGGCAGATTA	60	13	109–185	18	0.778	0.898	0.162
		R: CAGCGGTCACTTTGACTGAA							
Rda35	$(tcta)_{20}$	F: CGCAAGCCACTGTGATAAAG	62	10	150–210	19	0.526	0.842	0.398*
		R: TGTGTTTTCCAAAGAAGAGCA							

T_a annealing temperature, *A* number of alleles, *Size* the range of observed alleles in base pairs, *N* number of individuals genotyped, H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient

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

Oligo Analyzer program (http://www.idtdna.com). This allowed identifying the most stable and less likely to interact during PCR through self-dimers, hairpin-loops and hetero-dimers. We finally selected 35 primer pairs to be synthesized at Macrogen, Inc. (South Korea).

Primer standardization was performed on DNA isolated from other 21 deceased juveniles donated by EMRRD. PCR reactions were performed in a final volume of 25 μL which contained 0.5 U μL^{-1} Taq DNA Polymerase (Thermo Scientific), 1X PCR buffer 2.5 μL (Thermo Scientific), 0.2 mM dNTPs 2 μL (Promega), 2.5 mM $MgCl_2$ 2.5 μL (Thermo Scientific), and ca. 5.7 ng μL^{-1} DNA. Thermocycler conditions for all loci were an initial denaturation step of 3 min at 94 °C, followed by 34 cycles of 30 s at 94° C, 30 s at the optimum annealing temperature of 55 to 63 °C (obtained experimentally using a thermal gradient) and 30 s at 72 °C, and a final extension of 5 min at 72 °C in an Optimax Cat TC9610-G-230V thermocycler (Labnet).

PCR products were separated by electrophoresis in 6 % polyacrylamide gels that were run between 5 and 6 h, followed by traditional silver staining procedures. For scoring allele sizes we used a 10 bp DNA ladder (Invitrogen, USA) and the software LabWork™ v 4.6.00.0 (UVP®, Inc.). Twenty-three (out of 35) loci were polymorphic and showed reliable amplification (Table 1), whereas five microsatellites were monomorphic and seven microsatellites showed multiple bands that could not be optimized. Number of alleles (2–15), observed (H_O: 0.105–0.944) and expected heterozygosities (H_E: 0.328–0.904) were calculated in GENALEX v 6.5 (Peakall and Smouse 2006). Using Genepop v 4.2 (Rousset 2008) we found evidence for deviations from Hardy–Weinberg equilibrium linked to high inbreeding coefficients in eight microsatellites, consistent with differences between H_O and H_E and homozygote excess at these loci. We attribute this phenomenon to small effective population sizes (Soto-Azat et al. 2013), family-biased sampling as many juveniles genotyped in the study were half-sibs, or both. These microsatellite loci represent a valuable molecular resource that promise to aid recovery of threatened Darwin's frog populations through ex situ captive breeding initiatives, followed by appropriate re-introduction efforts.

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References

Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinformatics 26:403–404

Ortiz JC, Heatwole H (2010) Status of conservation and decline of the amphibians of Chile. In: Heatwole H, Barrio-Amorós CL, Wilkinson JW (eds) Amphibian biology, vol 9., Status of decline of amphibians: Western HemisphereSurrey Beatty & Sons, Chipping Norton, pp 20–27

Peakall R, Smouse P (2006) GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Res 8:103–106

Soto-Azat C, Valenzuela A, Clarke B, Busse K, Ortiz JC, Barrientos C, Cunningham A (2013) Is chytridiomycosis driving Darwin's frogs to extinction? PLoS ONE 8(11):e79862. doi:10.1371/journal.pone.0079862