TECHNICAL NOTE

Isolation and characterization of microsatellite loci for the endangered Midwife Betic toad *Alytes dickhilleni* (Discoglossidae)

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Abstract We have developed eleven polymorphic microsatellite loci for the amphibian *Alytes dickhilleni* using an enriched-library approach. We detected 98 alleles in 50 individuals genotyped (mean number of alleles per locus was 8.91) in two different populations in South East Spain. Expected heterozygosities ranged from 0.324 to 0.891 in one population (Arroyo Guadahornillos) and 0.424–0.909 in the other population (Cueva Paria). The levels of polymorphism of the developed markers render them readily applicable for population genetic studies of diversity, structure, and migration.

Keywords Alytes dickhilleni · Amphibian decline · Iberian Peninsula · SSR

Amphibians are among the groups of vertebrates pronest to extinction process upon habitat perturbation (Stuart et al. 2004). Among their main threats, the fragmentation of the habitat and the desiccation and contamination of the ponds were they breed are often highlighted. In the last decade, the emergence of a disease known as Chytridiomycosis, produced by the lethal fungus *Batrachochytrium dendrobatidis*, has killed thousands of individuals around the world, bringing amphibians in the focus of conservation programs. The Betic Midwife toad, *Alytes dickhilleni* (Arntzen and García-París 1995), is one of the Iberian amphibian species that have been seriously affected by the transformation of the habitat (García-París 2002; García-París et al. 2004). With a distribution restricted to the southeastern part of the Iberian Peninsula, the Betic Midwife toad lives in rocky mountain areas inhabited by farmers, where the competition for water with humans is really high. Adults usually breed in small streams or in cattle through used to assure fresh water provision to domestic animals (García-París 2002). In the last few years *A. dickhilleni* populations have rapidly declined in all their range, mainly because of the elimination, modification, or cleaning of cattle troughs by farmers (García-París et al. 2004). In order to investigate global and local patterns of genetic variation and estimate effective population size and migration rates between ponds, we have developed 11 highly variable microsatellite loci for the Betic Midwife toad.

We developed microsatellite libraries following Jones et al. (2002). We extracted approximately 100 μg of genomic DNA of one individual using the QIAGEN DNeasy Tissue Extraction kit. The DNA was partially restricted with seven blunt-end restriction enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I and *Eco*RV). Fragments (300–750 bp) were ligated with 20-bp oligonucleotides containing a *Hin*dIII site at the 5′ end, and subjected to magnetic bead capture.

Four libraries were prepared in parallel using Biotin-CA_{15}, Biotin-GA_{15}, Biotin-ATG_{12} and Biotin-AAC_{12} as capture molecules (CPG Inc.). Captured molecules were amplified and restricted with *Hin*dIII to remove the adapters, and the resulting fragments were ligated into the *Hin*dIII site of pUC19 plasmid and introduced into *Escherichia coli* DH5α by electroporation. One hundred recombinant clones were selected at random for sequencing and 73 of them contained a microsatellite sequence. Polymerase chain reaction (PCR) primer pairs were designed for 44 clones using DESIGNER pcr 1.03 (Research Genetics Inc.).

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Table 1 Characteristics of the 11 polymorphic microsatellite loci isolated from the species *Alytes dickhilleni* in two populations of Cazorla Natural Park: Arroyo Guadahornillos (AG) and Cueva Paria (CP), in the Province of Jaén, Spain

Locus	Sequence (5'-3')	Repeat structure	Size (bp)	AG				CP			
				K	Ho	He	HW	K	Ho	He	HW
AlyA115	F:TCCAGTGTAACGGAGGTACTCA R:AAGAGGTCGGAGAGGGTGA	$(TG)_{11}$	73–173	4	0.64	0.555	0.101	4	0.32	0.527	0.001
AlyA103	F:TGTTGCTGCCATGATGATCT R:GTGTTGCTGCTGTCAAGGACCAG	$(GTGC)_5(GT)_{13}$	95–180	8	0.76	0.741	0.219	6	0.44	0.635	0.091
AlyC1	F:GGTGGCATGTATCCGTAGATAG R:CAGAAAGATGGAACTGGTATGC	$(TGT)_{10}$	105–210	5	0.56	0.561	0.532	4	0.8	0.709	0.655
AlyB108	F:AATGCTATCATTTCTCCACCAG R:ACGAGTGGCGTCACTGAC	$(TC)_{20}$	241–300	7	0.44	0.475	0.395	5	0.56	0.682	0.189
AlyC119	F:TTTTATCTGTAAAAACAGTCCCCTAAA R:TGGACTTGGTGTTCATTACTAGTTG	$(TTG)_8(ATA)_5(TTA)_3$	160–262	5	0.32	0.324	0.575	5	0.72	0.583	0.934
AlyB116	F:TTAGGCGAGAGAGGCTACC R:ACGAGTGGCGTCAATGAC	$(CT)_8T(CT)_{11}$	181–237	8	0.92	0.775	0.954	11	0.92	0.891	0.288
AlyB103	F:CAGCAACCCTCCTACCAA R:GCCACATAATCCATCTGTTTG	$(TC)_{21}$	191–290	12	1	0.891	1	9	0.88	0.82	0.742
AlyD2	F:ATGCCTTGTATGCCATTAAAG R:AACCACTGCTTACGTTACAGC	$(GAT)_{29^*}$	262–400	4	0.56	0.665	0.254	3	0.48	0.549	0.013
AlyB107	F:GAAGCAAGGAGGTGCAGAAG R:AGGGCTGGAAATGTGTATGC	$(CA)_{12}$	290–390	6	0.76	0.745	0.702	5	0.68	0.728	0.434
AlyC132	F: CTGTGACTCTCTGGGAACTCAG R:TGGATTACCATAACCAGTGGAT	$(ACA)_4$	240–340	14	0.92	0.864	0.906	15	0.92	0.909	0.515
AlyC12	F:TTGTGAGACACCTTTGAACAC R:GTTGGCTTTGAGACTGATTG	$(TAA)_3(CAA)_6TA(ACA)_9$	251–360	7	0.541	0.749	0.072	4	0.125	0.425	0

k number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, *HW* nominal *P*-values for the test of deviations from Hardy–Weinberg equilibrium. PCR products were indirectly labelled using FAM, VIC, NED or PET (Applied Biosystems) dyes on an additional 19-bp M13 primer (5'-CACGACGTTGTAAAACGAC-3') following Boutin-Ganache et al. (2001). A sequence tail (5'-GTGTCTT-3') was added to the 5' end of the reverse primer to improve adenylation and facilitate genotyping

* This marker was interrupted by five occurrences composed of three nucleotides ATY. GenBank accession numbers HQ213788-HQ213798

For primer testing, we isolated DNA from toe clips from adults and tail tips from tadpoles in 25 individuals collected in two populations: Cueva Paria (CP) and Arroyo Guadahornillos (AG), both in the Natural Park of Cazorla, Segura y las Villas, Jaén Province, Spain, using a standard phenol/chloroform extraction DNA protocol (Sambrook et al. 1989). Polymerase chain reactions were performed in final volume of 20 μL containing $1\times$ buffer [67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20], 2.5 mM $MgCl_2$, 0.01% BSA, 0.25 mM dNTP, 0.40 μM dye-labelled M13 primer, 0.40 μM tailed-reverse primer, 0.04 μM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 50 ng of genomic DNA. Samples were amplified with a 'touchdown' PCR in a BIO-RAD DNA Engine Peltier Thermal Cycler, with an initial denaturation step of 2 min at 94°C; 17 cycles at 92°C for 30 s, annealing at 60–44°C for 30 s (1°C decrease in each cycle) and extension at 72°C for 30 s; 25 cycles at 92°C for 30 s, 44°C for 30 s and 72°C for 30 s with a final extension for 5 min at 72°C. Amplified fragments were analyzed on an ABI 3130xl Genetic Analyser and scored using GeneMapper 4.0 (Applied Biosystems) and LIZ 500 size standard.

We tested a total of 44 primer pairs from which we finally retained 11 polymorphic markers after inspecting their observed and expected heterozygosities (Cervus 3.0; Kalinowski et al. 2007) and testing for deviations from Hardy–Weinberg equilibrium, gametic disequilibrium (GenePop 4.0; Rousset 2007), and the presence of null alleles (Micro-Checker 2.2.3; Oosterhout et al. 2004). We used Bonferroni corrected *P*-values to assess the significance of multiple tests.

From the 11 loci selected, we detected a total of 80 alleles in AG and 71 alleles in CP, with individual locus values ranging from 4–14 to 3–15 alleles, respectively. The expected heterozygosity ranged from 0.324 to 0.891 in AG and from 0.424 to 0.909 in CP (Table 1). Two loci showed a significant deviation of the Hardy–Weinberg equilibrium in one population after Bonferroni corrrection (AlyC12 and AlyA115 in population CP). The presence of null alleles was suggested by Micro-Checker as a likely cause for the observed heterozygote deficit for these two loci and AlyA103 in CP and for AlyC12 in AG.

The microsatellite markers described in this study provide a valuable tool for the analysis of the demographic and genetic consequences of habitat fragmentation and human pressure on populations of the Betic Midwife toad.

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