PERMANENT GENETIC RESOURCES

Isolation and characterization of polymorphic microsatellite markers in *Iberolacerta monticola*, and cross-species amplification in *Iberolacerta galani* and *Zootoca vivipara*

N. REMÓN,* M. VILA,* P. GALÁN† and H. NAVEIRA*
*Departamento de Bioloxía Celular e Molecular, Universidade da Coruña, Campus A Zapateira, E-15008, A Coruña, Spain
†Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía, Universidade da Coruña, Campus A. Zapateira, E-15008, A Coruña, Spain

Abstract

Fourteen polymorphic microsatellite loci are described for the Iberian rock lizard, *Iberolacerta monticola*. Genetic variation in a sample of 20 individuals from Piornedo (northwestern Spain) was quantified both by the number of alleles per locus, which ranged from six to 13, and by the expected frequency of heterozygotes under random mating (heterozygosity), which ranged from 0.761 to 0.902. Single locus and global exclusion probabilities were also computed, and indicate a high power of these markers for paternity assignments and mating system studies of *I. monticola*. All the analysed loci were also polymorphic in *Iberolacerta galani*, but only seven in *Zootoca vivipara*.

Keywords: *Iberolacerta monticola*, microsatellites, molecular markers, polymorphism, rock lizards

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Iberolacerta monticola Boulenger (1905) is an endemic lizard species living in the northwest of the Iberian Peninsula, mostly at middle-high altitude sites (Carranza *et al*. 2004). However, several populations have been found at low altitude, associated to Atlantic forests growing at riversides, or rocky elevations near the seaside (Galán 1999). Microsatellite markers were developed with the aim of studying population structure, mating strategies and determinants of individual reproductive success in this species.

Genomic DNA was extracted from tissue samples of tail tips using a modified salt-extraction method (Aljanabi & Martinez 1997). Four enriched microsatellite libraries were made by Genetic Identification Services (GIS, http://www.genetic-id-services.com/) from size-selected genomic DNA ligated into the *Hin*dIII site of pUC19, using magnetic bead capture molecules for dinucleotide (CA), trinucleotide (AAC) and tetranucleotide (CATC and TAGA) repeats (Jones *et al*. 2002), yielding so far, after plasmid sequencing, 85 different microsatellite-containing clones. Primers were designed using DESIGNERPCR 1.03 (Research Genetics) for 71 microsatellite loci, and a subset of them (24 loci) was tested for polymorphism using five individuals of *I. monticola*.

Fourteen microsatellite loci deemed polymorphic in the former assay were subjected to additional analyses. After confirming by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/.f) that none of these loci had been previously identified, we assayed their variation among 20 individuals caught at Piornedo, a middle-high altitude site at the Ancares mountain range (northwestern Spain). Genomic DNA was extracted as described above. Polymerase chain reaction (PCR) amplifications were performed in a 20 μL reaction volume, containing 1× reaction buffer with 1.5 mM $MgCl_2$ (Roche), 0.2 mM of each dNTP, 0.6 μM of each primer (Table 1; forward primer fluorescently labelled), 1 U *Taq* DNA polymerase (Roche) and 0.1–1.5 ng/μL genomic DNA. Reactions were carried out in an Eppendorf Mastercycler using the following profile: 3 min at 95 °C; 35 cycles of 40 s at 95 °C, 40 s at the locus-specific annealing temperature (Table 1) and 1 min at 72 °C; ending by a final extension of 7 min at 72 °C. Genotyping of fluorescent fragments was performed with a 3130xl automated sequencer of Applied Biosystems (ABI), using GeneScan-500 (–250) ROX size standard (ABI). Allele size was called with the software GeneMapper version 3.7 (ABI). The number of alleles per locus, observed and expected heterozygosities, probabilities

Correspondence: N. Remón, Fax: +34981167065;
E-mail: nremon@udc.es

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Table 1 Characterization of 14 microsatellite loci from *Iberolacerta monticola* (20 individuals genotyped). F, forward primer; R, reverse primer; labels used are given in bold; T_a, optimal annealing temperature; n_a, number of alleles; H_E, expected frequency of heterozygotes under random mating; H_O, observed frequency of heterozygotes; F_{IS}, inbreeding coefficient (** significant departure at the 1% level of observed from expected heterozygotes, after Bonferroni correction); PEP, paternity exclusion probability

Accession no./ locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)	n_a	H_E	H_O	F_{IS}	PEP
EU233459 A5	$(CW)_{17}AA(CA)_5$	F: **FAM**-ATTTAAGAGGAAAGGCCAGATC R: GTGGAGTAGCTTGCACTGTTC	53	200–224	12	0.872	0.950	–0.063	0.744
EU233460 A8	$(CA)_{21}$	F: **HEX**-TGCCCTATCACTGTCTTCTTG R: GTGGAATGCTCTCCTCAGG	54	226–248	9	0.761	0.350	0.558**	0.578
EU233461 B107	$(GYT)_{17}$	F: **FAM**-AAGCAATCCATTTGGTGAAC R: TGCCATTTTACTCTTCCAGG	52	164–188	9	0.822	0.850	–0.008	0.651
EU233462 B114	$(CAA)_7(CA)_{10}$	F: **NED**-TACCTTCCTGATCCTCTATGTC R: AAACCACTTTGTAGTTTTGAGC	52	178–202	8	0.817	0.850	–0.014	0.644
EU233464 B135	$(CAA)_{10}$	F: **FAM**-GCCTGTCTTAGGATCTTTGG R: CTGCTGACAAGGCTGATTAG	53	205–229	9	0.845	0.750	0.138	0.695
EU233465 C5	$(SCAT)_{14}$	F: **FAM**-GCACATTCAGTAGGATGTTGTG R: TGACCAAGGGATAGAAAATCAC	52	146–178	9	0.845	0.900	–0.040	0.688
EU233466 C9	$(GSAT)_{13}$	F: **NED**-TGACCAAGGGATAGAAAATCAC R: ATTAGGCCCAGTAAAGGTTAGC	52	213–241	10	0.854	0.900	–0.029	0.706
EU233467 C103	$(GGAT)_{11}$	F: **HEX**-ATGCCCTAATTCACATGCCTA R: TGTAGCCATCACTGCAACTTC	54	239–263	6	0.775	0.450	0.230	0.526
EU233468 C113	$(GGAT)_{11}$	F: **NED**-CCCTGACCCTGCTGTATT R: CCAATGTCTCAATCACCTTG	55	218–262	7	0.775	0.450	0.440**	0.571
EU233469 C118	$(CCAT)_{10}$	F: **FAM**-CCTCCAAAAGGATGAGAGTG R: ATGCTCCCAGGTAAATGGTTA	53	150–178	8	0.792	0.900	–0110	0.610
EU233470 D101	$(GATA)_{14}CAGTAGGTA$ $(GATA)_{11}$	F: **HEX**-GTGGTTGGGTTTTTTTTGTG R: GGAGCCTCTCAGTCAGTGG	54	161–269	13	0.902	0.850	0.084	0.802
EU233471 D109	$(GAYA)_{17}CC(GA\ TA)_{13}$ $(YAGA)_{13}$	F: **HEX**-CAAATATCTCTACGGTGACTG R: GCACCTGATGAAAATTCC	50	199–267	10	0.864	0.555	0.382**	0.726
EU233472 D115	$(GATA)_{15}T(GATA)_2$	F: **HEX**-CAGGAGCAAAGTCTTGTCAG R: AAGTGGGCACATACAGAGTAAG	55	220–272	9	0.787	0.700	0.136	0.605
EU233473 D119	$(GATA)_{11}GAGAT(GATA)_4$ $GAT(GAAA)_{13}$	F: **NED**-GGCTCTACCCAAGCAGTG R: CTCCACAAACCAGTATGAAGTC	53	234–286	11	0.879	0.700	0.228	0.756
Mean over all loci (combined PEP)					9.3	0.830	0.723	0.149	0.999

of genetic identity and paternity exclusion (PEP), were estimated using Identity 4 (Wagner & Sefc 1999). Randomization-based tests for departure from Hardy–Weinberg (HW) equilibrium (1500 randomizations) and linkage equilibrium (2100 permutations) were carried out with the aid of FSTAT version 2.9.3.2 (Goudet 2001). MICRO-CHECKER (Van Oosterhout *et al*. 2004) was used to find signals of null alleles, and to obtain the corresponding adjusted allele and genotype frequencies for the affected loci. The markers were also used to screen two clutches of known maternity, so that they could aid in the detection of putatively segregating null alleles.

As shown in Table 1, the number of observed alleles per locus ranged from six (locus C103) to 13 (D101), with an average of 9.3, whereas heterozygosity (expected frequency of heterozygotes under random mating) varied from 0.761 (A8) to 0.902 (D101), with an average of 0.830. Total paternity exclusion probability of this set of 14 markers was estimated in 0.999, which together with their extremely low combined probability of genetic identity (2.365×10^{-20}), will make them very useful tools for parentage studies in this species. Three loci, namely A8, C113, and D109 showed a significant excess of homozygotes over HW expectations (adjusted P value for 5% significance following Bonferroni corrections = 0.00357), which, in principle might be due to segregating null alleles. Relative frequencies of these putative alleles would be 0.2334, 0.1831 and 0.1655, respectively, estimated as in Brookfield (1996) with the aid of MICRO-CHECKER. Genotype frequencies obtained for each suspect locus after taking into account the segregation of null alleles do not depart significantly from HW expectations. Partial confirmation of the existence of such null alleles at the loci C113 and D109 also follows from clutch analyses, based on the distribution of failed PCRs and the detection of

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Table 2 Cross-species amplification using 14 microsatellite primer pairs designed for *Iberolacerta monticola*. For each species, allele size range (bp) based on five individuals is indicated; 0 means no amplification was obtained. Loci followed by asterisk (*) need PCR optimization, since unspecific bands were also observed. Monomorphic loci are listed in bold

Locus	*I.galani*	*Z.vivipara*
A5	199–217	**179**
A8	238–246	222–224
B107	158–191	125–128
B114	175–184	160–172
B135	205–223	214–217*
C5	146–158	**188**
C9	213–225	**254**
C103	234–259	179–195
C113	242–262	**222**
C118	150–178	**132**
D101	277–221	133–176
D109	203–239*	120–140
D115	232–280	**166**
D119	262–322	0

'homozygotes' in the offspring for alleles not present in the corresponding mother). No evidence of linkage disequilibrium was found for any pair of loci (adjusted *P* value for 5% significance following Bonferroni corrections = 0.000476).

Results of amplification of these markers in the closely related species *Iberolacerta galani* and the distantly related *Zootoca vivipara* are shown in Table 2. All the markers were polymorphic in *I. galani*, and presumably will be applicable with no or slight refinements in this species. Half of the markers proved polymorphic in *Z. vivipara*, but in this case, only four of them displayed a sufficiently large range of allele sizes to grant their utility.

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