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PERMANENT GENETIC RESOURCES

Characterization of eight microsatellite loci in the Galápagos endemic land snail *Bulimulus reibischi*, and their cross-species amplification

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Abstract

Variation in and amplification conditions for eight polymorphic microsatellite loci initially identified from *Bulimulus akamatus*, a pulmonate land snail from Galápagos, are described. Intraspecific polymorphism and heterozygosity of the eight markers were studied in 19 populations of *Bulimulus reibischi*, a closely related species of *B. akamatus*. Furthermore, the eight loci were also cross-amplified in six other closely related bulimulid species. The number of alleles across populations of *B. reibischi* at six loci is moderate (three to 10), but considerable for two other loci (19 and 20). There is no strong evidence for linkage among any of the loci examined.

Keywords: Bulimulidae, DNA markers, Galápagos, heterozygosity, microsatellites, Mollusca

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With more than 70 described species, Galápagos endemic land snails of the genus *Bulimulus* form the most species-rich genus on these islands. The species have colonized all of the major Galápagos Islands, and can be found in all vegetation zones except for the littoral zone which is mainly composed of sandy beaches and barren lava flows (Parent & Crespi 2006). Fifty-seven of the species are listed as Endangered or Threatened according to the International Union for the Conservation of Nature and Natural Resources (IUCN Redlist, Parent 2003), suffering from restricted distribution ranges and population declines principally due to habitat destruction by humans and introduced species. Genetic characterization of populations holds important implication for the understanding of the diversification of this group as well as its conservation. To further genetic population analysis of natural populations, we designed microsatellite primers for *Bulimulus akamatus* and tested their application in related species. We examined variation at eight microsatellite loci in 44 populations of eight bulimulid species from Galápagos Islands. This note describes the microsatellite loci and the amplification conditions used to assess their variation.

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Eight microsatellite loci and corresponding primer sites for their amplification (Table 1) were identified from a genomic library of *B. akamatus*, enriched for different microsatellite motifs (AAT, ATG and TACA) by Genetic Identification Services (GIS, www.genetic-id-services.com/). We optimized amplification reaction conditions for each locus. All reactions were performed in a total volume of 25 μL with the following components: 0.2 μM dNTP (GE Healthcare), 1× ThermoPol reaction buffer with 2 mM $MgCl_2$ (New England Biolabs), 0.1 μM IR-labelled forward primer (LI-COR Biosciences), 0.1 μM forward primer and 0.2 μM reverse primer (Invitrogen), 0.5 mM $MgCl_2$, 0.1 U Thermostable DNA polymerase (BIOTOOLS), and about 100 ng of template genomic DNA. All reactions were performed following a touchdown thermocycling protocol: denaturation at 94 °C for 3 min, 3 cycles of 92 °C for 40 s, 60 °C for 40 s, 72 °C for 35 s; 3 cycles of 92 °C for 40 s, 57 °C for 40 s, 72 °C for 35 s; 3 cycles of 92 °C for 35 s, 54 °C for 40 s, 72 °C for 35 s, 30 cycles of 92 °C for 40 s, 51 °C for 35 s, and a final extension at 72 °C for 4 min. Amplification products were separated in a 4% acrylamide: TBE gel using a LI-COR 4200 DNAnalyser, and analysed using GENE IMAGEIR 4.05 software (Scanalytics).

We genotyped a total of 338 *Bulimulus reibischi* specimens sampled across 19 populations on Santa Cruz Island, and 76 bulimulid specimens from seven other species, including

Table 1 Characteristics of eight polymorphic microsatellite loci amplified in *Bulimulus reibischi*

Locus	Repeat motif	Primer sequence (5′–3′)	N_{ind}	N_{pop}	N_a	A_{range}	H_O	H_E	HWE
A114	$(TAT)_{12}$	F: TCCCTAGAAACCACCAACTG	213	14	20	180–237	0.5399	0.7985	*
		R: AGAGGAGGAGGGGAGGAT	12	—	10	183–219	0.6667	0.8841	NS
			18	—	5	195–207	0.6667	0.6397	NS
			19	—	7	192–234	0.6316	0.7425	NS
			19	—	5	186–213	0.3684	0.3300	NS
A115	$(AAT)_{15}$	F: CCAATGTGCCGTCACTTA	77	4	3	161–167	0.2857	0.3345	*
		R: ACCCCATAGGAGGAGAGC	20	—	2	161–167	0.1500	0.1423	NS
			18	—	3	161–167	0.2778	0.2556	NS
			20	—	2	161–167	0.3500	0.2962	NS
			19	—	3	161–167	0.3684	0.5704	NS
A116	$(TAT)_{12}$	F: TGGTTTGAATGTTATGTTCGA	65	4	19	243–312	0.6769	0.9261	NS
		R: ATCTTTCGCTTGTTTTTACAGG	19	—	12	243–282	0.6842	0.9147	NS
			18	—	9	243–270	0.6667	0.8603	NS
			19	—	12	243–273	0.5789	0.8848	NS
			9	—	12	255–312	0.8889	0.9608	NS
B6	$(ATG)_9$	F: TAGACGACGGCAGAGAATAA	330	19	10	183–210	0.7030	0.7790	*
		R: TTGGATGCTGGTGTTTTG	20	—	7	183–204	0.6500	0.8205	NS
			18	—	5	183–204	0.6111	0.6587	NS
			20	—	6	186–204	0.6500	0.7872	NS
			19	—	7	183–204	0.7895	0.7340	NS
B107	$(ATG)_{14}$	F: GTCGGAGACTTGGTAAGAGG	94	6	7	126–144	0.4255	0.6674	*
		R: TCATTTTGCGGTGTTGAG	15	—	3	132–138	0.5333	0.6414	NS
			13	—	3	132–138	0.3846	0.6000	NS
			17	—	5	129–144	0.3529	0.4795	NS
			17	—	4	132–141	0.3529	0.7326	*
B110	$(ATG)_9$	F: CGACGGTATCAGGCTAAGTA	333	19	10	108–156	0.5676	0.6493	NS
		R: CCTCCAAAAAAGCATAACAG	20	—	4	117–126	0.7000	0.6910	NS
			18	—	5	117–141	0.7778	0.7333	NS
			20	—	5	117–129	0.6500	0.6244	NS
			19	—	6	117–156	0.5263	0.5917	NS
B118	$(ATG)_9$	F: TGAAAGCAAAACATCACAGAAG	218	16	8	165–204	0.3716	0.5675	*
		R: CTCCTCCAAAAGAGCATAACAG	19	—	3	165–204	0.5263	0.5704	NS
			15	—	4	165–204	0. 2000	0.6138	*
			15	—	6	165–204	0.4000	0.6092	NS
			18	—	4	168–204	0.6111	0.6508	NS
B123	$(ATG)_9$	F: CACGTTTTAACTACTGCAATGA	65	5	7	126–150	0.2462	0.5249	*
		R: AAAAGAGCATAACAGCTCGTAG	14	—	4	126–147	0.2143	0.6534	*
			14	—	4	138–150	0.4286	0.6032	NS
			11	—	3	135–144	0. 1818	0.3247	NS
			11	—	3	138–147	0.2727	0.4978	NS

For each, locus name, repetitive sequence, primer sequences (the forward primers were labelled), number of individuals (N_{ind}) and number of populations (N_{pop}) genotyped are indicated. The resulting number of alleles (N_a), allele size range (A_{range}), observed (H_O) and expected (H_E) heterozygosity, and results of exact *P* values for Hardy-Weinberg equilibrium (HWE) for all *Bulimulus reibischi* individuals genotyped, and for the four most polymorphic populations of *B. reibischi* are also indicated (NS, nonsignificant; $*P < 0.05$).

25 distinct populations (on average three populations and 10 individuals per species) from Santa Cruz, Santiago and Fernandina islands, Galápagos, Ecuador. The failure rate of cross-species amplification has been shown to be associated with the evolutionary distance between the species for which the microsatellite loci have been developed and the species that are used for amplification (Primmer *et al*. 2005; references therein). We tested for such a correlation using the proportion of individuals that failed to amplify at any one locus for each species included in this study and phylogenetic distance of each species to *B. akamatus* (obtained from the best maximum-likelihood tree presented elsewhere; Parent & Crespi 2006). Although the correlation between phylogenetic distance and amplification failure is not significant ($r = 0.364$, $P > 0.05$, $n = 7$), it is in the predicted direction.

Table 2 Cross-amplification data of seven Galápagos bulimulid species using the primers developed for *Bulimulus akamatus*

			Locus															
			A141		A115		A116		B6		B107		B110		B118		B123	
Species	N_{ind}	N_{pop}	N_a	A_{range}	N_a	A_{range}	N_a	A_{range}	N_a	A_{range}	N_a	A_{range}	N_a	A_{range}	N_a	A_{range}	N_a	A_{range}
B. akamatus	14	4	9	195–228	—	—	11	246–312	5	189–195	2	132–135	7	108–129	—	—	4	139–151
B. ochsneri	10	1	10	186–225	2	162–168	6	252–288	—	—	2	132–135	6	108–129	3	195–204	4	136–148
B. indefatigabilis	23	7	9	192–216	—		5	261–285	7	186–210	—	—	8	108–135	—	—	9	127–151
B. olla	6	2	4	189–201	—	—	2	261–267	3	186–198	—	—	3	108–126	—	—	3	142–148
B. darwini	3	2	2	201–204	—	—	3	264–273	3	186–198	—	—	4	108–132	—	—	2	142–148
B. sculpturatus	12	5	9	189–216	—	—	9	252–288	3	192–198	—	—	4	108–129	—	—	6	127–151
B. perrus	8	4	—	—	—	—	1	252	3	192–198	—	—	2	117–120	—	—	3	136–148

Number of individuals (N_{ind}), number of populations (N_{pop}) for each species, as well as number of alleles genotyped (N_a), and the allele size range (A_{range}) for each marker and each species are reported; —, not amplified.

Number of alleles per locus varied. If we consider allelic diversity for *B. reibischi* populations alone, we find a range of five to 10 different alleles for the 'B' loci and the A115 locus, and 19 and 20 distinct alleles for A116 and A114, respectively (Table 1). Similar allelic diversity is found when all other species genotyped are considered. All loci were polymorphic across all specimens genotyped, except for one species that was monomorphic at one locus (Table 2). All the loci genotyped across *B. reibischi* populations were polymorphic (Table 1). We did a BLAST search for the clone sequences against the GenBank nucleotide database to determine if loci with relatively low allelic richness are impoverished because of selection on a gene of well-known function. We found no significant similarity between our clone sequences and any sequence from the GenBank database.

Observed and expected heterozygosities for loci over all *B. reibischi* genotyped individuals and for four of the more polymorphic populations were calculated using the software POPGENE version 1.32 (Yeh *et al*. 1997) (Table 1). Hardy–Weinberg equilibrium was tested across all loci including all *B. reibischi* individuals genotyped, and also for the four most polymorphic populations using exact *P* values from the GENEPOP software version 3.4 (Raymond & Rousset 1995) (Table 1). There is marked differentiation among several of the populations of *B. reibischi* studied, inflating the difference between expected and observed heterozygosities calculated over all individuals. Deviations from Hardy–Weinberg equilibrium in direction of heterozygote deficiency for some populations for some loci might have resulted from genetic drift, Wahlund effect, or other effects. However, for none of the loci was there significant deviation from Hardy–Weinberg equilibrium for all populations, indicating that the presence of null alleles is unlikely. A test for genotypic disequilibrium in each population performed in POPGENE did indicate significant association of genotypes for a few pairs of loci: A114 and B110 in three populations, A114 and B118 in one population, and A114 and B123 in one population out of the 48 populations tested. However, since no locus association was consistent across all or even several populations, the few significant associations observed might be the result of small sample size for some of the populations genotyped.

The eight microsatellite loci described here are expected to be useful markers for elucidation of population structure and differentiation in this unique taxonomic group of endangered land snails.

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