Molecular Ecology Notes (2006) **6**, 927–929 doi: 10.1111/j.1471-8286.2006.01402.x

PRIMER NOTE

A set of highly discriminating microsatellite loci for the Galápagos marine iguana *Amblyrhynchus cristatus*

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Abstract

We describe here the cloning of 12 (7 dinucleotide, 1 trinucleotide and 4 tetranucleotide) microsatellite loci for the Galápagos marine iguana *Amblyrhynchus cristatus*. When tested for individuals from five different island populations on the Galápagos archipelago, high genetic diversities (9–20 alleles per locus) and heterozygosities (0.200–0.944) were observed. All loci showed no obvious deviations from Hardy–Weinberg equilibrium. The new set of microsatellite loci was able to assign individuals reliably to their island of origin, thus being able to discriminate between residents and migrants between islands.

Keywords: *Amblyrhynchus cristatus*, Bayesian cluster analysis, Galápagos marine iguana, island differentiation, microsatellite loci

Received 3 March 2006; revision accepted 17 March 2006

Marine iguanas of the Galápagos archipelago (*Amblyrhynchus cristatus*) are the only aquatic iguanas worldwide and feed submerged mainly on green and red algal species along the rocky island shores of the 13 major islands of the archipelago. So far, patterns of gene flow based on three microsatellite loci and three minisatellite loci suggested substantial interpopulational gene exchange (Rassmann *et al*. 1997). Interestingly, this comparatively low genetical differentiation is contrasted by strong differences in coloration and morphology between island populations (Wikelski 2005). Here, we report the cloning procedure, amplification and the application of 12 newly and one published polymorphic microsatellite loci for *A. cristatus*.

Amblyrhynchus cristatus specific microsatellite loci were cloned by using an enrichment protocol that has proven to successfully isolate microsatellite loci from an amphibian species (*Salamandra salamandra*; Steinfartz *et al*. 2004) and from the Galápagos sea lion (*Zalophus californianus wollebaeki*; Wolf *et al*. 2005). The primary genomic DNA library was represented by a mix of different blood samples of individuals of *A. cristatus* collected from Isabela, San Cristobal and Caamaño in the year 1994. Genomic DNA was extracted using the SDS-proteinase K/phenol-chloroform extraction method, resuspended in 500 μL TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and subsequently digested with 1 μL RNAse A (10 mg/mL) for 1 h at room temperature. We used two tetranucleotide motifs ($GATA_{10}$, $AAGG_{10}$), one trinucleotide motif (AAC_{11}) and one dinucleotide motif (AC_{12}) for the enrichment of the sublibrary. The enrichment protocol followed the fast isolation by AFLP of sequences containing repeats (FIASCO) method (Zane *et al*. 2002) with the following modifications: (i) a 450–1000 bp fraction of the amplified fragment length polymorphism (AFLP) digestion–ligation reaction was cut from an agarose gel, and this fraction was rerun and isolated again; (ii) polymerase chain reaction (PCR) cycles to amplify *Mse*I adaptor-flanking fragments were reduced to 20 cycles; (iii) 500 ng DNA from this enrichment step was mixed with 100 pmol of a 5′-biotinylated $(AC)_{12}$ oligonucleotide probe in a total volume of 100 μL (4.2× SSC, 0.07% SDS) at 67 °C for 2 h; (iv) 200 μL of Dynabeads M-280 streptavidin beads solution (DYNAL) was washed with 1× PBS, 0.1% BSA, and subsequently resuspended at 67 °C in 400 μL (4.2 SSC, 0.07% SDS solution), and 200 μL of bead solution was added to the fragment-probe mix and incubated for 30 min at 67 °C; and (v) low stringency washes (5 min each) were performed four times with 1× SSC, 0.01% SDS at 38 °C. Four high stringency washes close to the dissociation equilibrium of the DNA fragment/probe complex were carried out in a 0.06× SSC and 0.01% SDS solution at 38 °C (10 min each). Further steps followed the

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Table 1 Characterization of 12 new microsatellite loci for the Galápagos marine iguana, *Amblyrhynchus cristatus*. Locus designation, GenBank accession numbers, forward and reverse primer sequences with the labelling dye used, repeat motif, size range, number of observed alleles and annealing temperature (T_a) of the amplification step (see text for remaining PCR details) are provided

Locus	Fluorescence labelling, primer sequences (5′–3′)	Repeat motif	Size range (bp)	No. of alleles	T_a (°C)
MIG-E2	F: HEX-GTGTGAGTGACATTTCTGCA	$(GAAA)_4(GA)_2$	229–265	10	60
DQ376111	R: TGAAAGTATGCTTTGCTCCCTTTGC	$(GAAA)_{10}(GA)_2$			
MIG-E3	F: FAM-CCGTTTATATGACTAGATGGTTTGG	$(GT)_{21}$	137–159	11	57
DQ376112	R: AACCTGATCCACATTGGGACT				
MIG-E4	F: HEX-TTGAGCTAAGTGGGAAAAGAAGAC	$(GT)_{22}$	223–271	20	57
DQ376113	R: AAAGTCTTCCCAGGAGATCACAC				
MIG-E6	F: HEX-ACGTCACTGGAGCTGACACA	$(TG)_{20}$	150–172	12	57
DQ376115	R: GAACAGTATCTAGGCACTCTCCAAA				
MIG-E8	F: FAM-ACCAAGCAAATGGTTTCCAG	$(AAGG)_{14}$	152–184	11	57
DQ376116	R: TTGTTCCAAATAGCATAAAATATCA				
MIG-E10	F: FAM-CCTTATAAATGCTGATCTGGAGCTGT	$(AAGG)_{14}$	194–250	13	57
DQ376117	R: CTTTTGCAGTGTTTACTTTTTCCAT				
MIG-E11	F: HEX-CAGTCCATTCTGCTTCCTCA	$(GT)_{19}$	163–183	11	57
DQ376118	R: CCTCAAACTCTGCCCTCTTG				
MIG-E12	F: HEX-GGAAGACACTTCAGGCAGCACTTTG	$(GT)_{20}$	169–195	13	57
DQ376119	R: TTAGTCAAACCTTTACTCCGACCTG				
MIG-E13	F: HEX-GAGGATGAACAGATGGTAAGTCAAT	$(CA)_{18}$	265–277	9	57
DQ376120	R: AGAACTCTGAGGTATGGAGGAAGAT				
MIG-E14	F: FAM-AAATTTTCTGCAGTTCTGTTGTCAT	$(AAGG)_{20}$	237–305	19	57
DQ376121	R: AGAATCATAGAAGTGGAAGGGACTC				
MIG-E15	F: FAM-AGACAGGACTGATGTCCTCTAAGAA	$(TG)_{19}$	140–160	11	57
DQ376122	R: GGTTGACAACTTATAAGCCTGAAGA				
MIG-E16	F: HEX-ACTAGCATAATCAGAGGTCATCCTG	$(GTT)_{13}$	222–240	9	57
DQ376123	R: ACCAGAGTTCGATTCTCCATTTAG				

standard FIASCO protocol (Zane *et al*. 2002). Fragments enriched for the different microsatellite motifs were cloned using the TOPO TA Cloning Kit from Invitrogen. Ninety-six clones were randomly picked from an agar plate and sequenced with the T3 primer (5′-ATTAACCCTCACT-AAAGGGA-3′) using an ABI 3100 automated DNA sequencer following the manufacturer's protocols.

Out of these 96 clones, we were able to find primer sequences for 16 microsatellite clones in the flanking region of the microsatellite by using the program PRIMER 3 (Rozen & Skaletsky 2000). Twelve primer combinations (4 tetranucleotide, 1 trinucleotide and 7 dinucleotide microsatellite loci) and the published primers Am(GT)4 (Rassmann *et al*. 1997) amplified consistently polymorphic alleles (Table 1). We tested the usefulness of the 13 microsatellite loci in multiple individuals of *A. cristatus* populations from Genovesa (00.31065°N, 89.97349°W), Marchena (0.29429°N, 90.50726°W), Pinta (00.54505°N, 90.73612°W), Santa Fé (Miedo) and San Cristobal (00.92256°N, 89.61793°W). All PCRs were performed in a 20 μL scale: template concentration ranged from 50 to 100 ng of genomic DNA, 1× Ampli*Taq* Gold PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer (fluorescently labelled with FAM, HEX or TET from Operon) and 1 U of Ampli*Taq* Gold DNA polymerase. Except for the annealing temperature (see Table 2), the PCR profile was the same for all loci: (i) initiation of the hot start polymerase activity at 95 °C for 10 min; (ii) 95 °C for 0.30 min; (iii) annealing at the respective temperature for 0.30 min (see Table 1); (iv) 72 °C for 0.45 min; (v) 30 repeats of steps ii–iv; and (vi) 72 °C for 30 min. We analysed all PCR products on an ABI 3100 automated DNA sequencer using the GENESCAN 500-Rox size standard (Aplied Biosystems) and following the manufacturer's protocols. Alleles were scored using the GENEMAPPER version 3.7 program from Applied Biosystems.

Number of alleles ranged from nine (MIG-E13, MIG-E14) to 20 alleles (MIG-E4) with observed heterozygosities ranging from 0.200 (MIG-E12 on Pinta) to 0.944 (MIG-E12 on Marchena) (Table 2). Significant deviations from Hardy–Weinberg equilibrium were observed only in four cases (Table 2). When using the Bayesian-based approach of individual assignment implemented in the program BAPS version 3.2 (Corander *et al*. 2004), out of a total of 187 individuals, 183 were assigned to their island of origin thus demonstrating the discriminating power of this new set of loci.

Acknowledgements

This work has been supported thanks to funds to AC from a National Geographic Society grant (NGS 7589–04), and the Yale

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Table 2 Deviation from Hardy–Weinberg equilibrium and linkage equilibrium for 13 *Amblyrhynchus cristatus* microsatellite loci ($k = 13$) for populations on Pinta, Genovesa, Marchena, San Cristobal and Santa Fe. For each population and each locus, the number of individuals analysed (N), the observed (H_O) and expected (H_E) heterozygosities are listed. Significant deviations from Hardy–Weinberg equilibrium (Bonferroni correction: $\alpha = 0.05$, $k = 13$) are marked with an asterisk. Significant associations of alleles between loci are marked by shared alphabetical superscript (Bonferroni correction: $\alpha = 0.05$, $k = 13$)

Locus	Population	N	H_O	H_E	Locus	Population	N	H_O	H_E
MIG-E2	Pinta	29	0.793	0.728	MIG-E11	Pinta	30	0.600	0.597
	Genovesa	25	0.600^{A}	0.753		Genovesa	27	0.890	0.809
	Marchena	35	0.800	0.774		Marchena	36	0.917	0.863
	San Cristobal	52	0.827	0.786		San Cristobal	50	0.860	0.760
	Santa Fe	42	0.762	0.734		Santa Fe	42	0.690	0.706
MIG-E3	Pinta	29	0.690	0.639	MIG-E12	Pinta	30	0.200	0.219
	Genovesa	25	0.720*	0.830		Genovesa	27	0.741	0.704
	Marchena	35	0.743	0.754		Marchena	36	0.944	0.793
	San Cristobal	52	0.558	0.699		San Cristobal	52	0.731	0.802
	Santa Fe	42	0.881	0.857		Santa Fe	42	0.881	0.851
MIG-E4	Pinta	30	0.800	0.794	MIG-E13	Pinta	29	0.690	0.745
	Genovesa	26	0.654^{B}	0.704		Genovesa	27	0.667	0.661
	Marchena	36	0.861	0.862		Marchena	36	0.778	0.733
	San Cristobal	51	0.765	0.858		San Cristobal	52	0.750	0.731
	Santa Fe	41	0.805	0.851		Santa Fe	42	0.643	0.656
MIG-E5	Pinta	29	0.690^{A}	0.693	MIG-E14	Pinta	30	0.633	0.620
	Genovesa	25	0.600^{AB}	0.608		Genovesa	27	0.370	0.594
	Marchena	35	0.829^{A}	0.822		Marchena	36	0.583*	0.851
	San Cristobal	52	0.712^{A}	0.799		San Cristobal	52	0.923	0.835
	Santa Fe	42	0.833^{A}	0.890		Santa Fe	42	0.810	0.908
MIG-E6	Pinta	30	0.800	0.781	MIG-E15	Pinta	30	0.670	0.675
	Genovesa	27	0.593	0.624		Genovesa	27	0.704	0.652
	Marchena	31	0.839	0.809		Marchena	36	0.667	0.697
	San Cristobal	50	0.760^{CD}	0.689		San Cristobal	52	0.462	0.521
	Santa Fe	41	0.829	0.835		Santa Fe	42	0.571	0.582
MIG-E8	Pinta	28	0.714	0.751	MIG-E16	Pinta	30	0.467	0.442
	Genovesa	25	0.800^{B}	0.857		Genovesa	27	0.926	0.766
	Marchena	32	0.875	0.879		Marchena	36	0.889	0.739
	San Cristobal	52	0.596*	0.710		San Cristobal	50	0.520^{CE}	0.567
	Santa Fe	41	0.805	0.790		Santa Fe	42	0.524	0.569
MIG-E10	Pinta	29	0.724	0.710	Am(GT)4	Pinta	30	0.733	0.688
	Genovesa	26	0.577^{A}	0.590		Genovesa	27	0.667	0.748
	Marchena	36	0.833	0.825		Marchena	36	0.528	0.674
	San Cristobal	51	0.725	0.801		San Cristobal	50	0.700^{DE}	0.668
	Santa Fe	42	0.810	0.886		Santa Fe	42	0.786	0.853

Institute for Biospheric studies. Sebastian Steinfartz has been supported by a fellowship (STE 1130/2–1) of the German Research Community (DFG).

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