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PERMANENT GENETIC RESOURCES

Characterization of 15 tetranucleotide microsatellite markers in the ringtail (*Bassariscus astutus*)

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Abstract

We characterized 15 polymorphic tetranucleotide microsatellite markers for the ringtail, *Bassariscus astutus*. We tested these loci in 21 individuals captured in Arizona and Texas and found six to 19 alleles per locus. Observed and expected heterozygosities ranged from 0.381 to 1.000 and from 0.381 to 0.941, respectively. All loci were in Hardy–Weinberg equilibrium, and none were in linkage disequilibrium. These markers may be used to investigate population genetics and mating patterns in this species.

Keywords: *Bassariscus astutus*, genomic library, microsatellite, ringtail

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The raccoon family Procyonidae consists of 17 species of mostly New World carnivores including the ringtail (*Bassariscus astutus*) (Nowak 2005; Koepfli *et al*. 2007). Ringtails are relatively small, nocturnal omnivores with a diet of insects, fruits, and small animals (Toweill & Teer 1977). The ringtail range extends from Mexico into the western and southwestern USA, including three islands in the Sea of Cortez. There are 14 named subspecies (Poglayen-Neuwall & Toweill 1988).

Ringtails breed from mid-April to mid-May with parturition occurring 51–54 days after mating; litter sizes vary from one to five kits (Poglayen-Neuwall & Toweill 1988). The distribution of home ranges and degree of overlap suggest that ringtails are not monogamous (Trapp 1978), but mating systems have not been studied using modern genetics and there have been no genetic studies of their population or phylogeographical structure. Here we describe 15 microsatellite loci characterized for the ringtail for use in mating system and population studies.

We enriched a genomic library for tetranucleotide microsatellites based on the protocol of Bardeleben *et al*. (2004). We selected three motifs isolated in the raccoon — GATA, GATC and AAAG (Cullingham *et al*. 2006; Fike *et al*. 2007). Blood and tissue samples were collected from ringtails captured in the Chiricahua Mountains, AZ (CHIR), and in Guadalupe Mountains National Park, TX (GUMO). Genomic DNA (gDNA) was extracted using a QiaAmp DNA Mini-Kit (QIAGEN).

gDNA from four individuals was pooled and ~3 μg was digested separately with Sau3A1 or BstY1 enzymes (New England Biolabs). Oligo A and B adaptors (Refseth *et al*. 1997) were generated and ligated to gDNA. Each ligated gDNA was size-fractionated on a 1% agarose/TAE gel. DNA in the 0.5–1.5 kb range was excised from the gel and purified by a Zymoclean kit (Zymogen). All hybridizations were carried out using ~1 μg of DNA with 50 nM biotin-labelled probe [5′-(Motif)$_7$GTGA(Biotinyl-T)C-3′], as previously described (Bardeleben *et al*. 2004). Enriched DNA was eluted from the magnetic beads using water preheated to 99 °C, then amplified using polymerase chain reaction (PCR) with Oligo A as a primer. A second round of hybridization was carried out using 25 μL of this PCR product and identical conditions to the first round selection. Enriched gDNA was cloned using a TOPO TA pCR 2.1 Cloning Kit (Invitrogen), following the manufacturer's protocol. White colonies were picked and grown overnight at 37 °C in Terrific Broth medium with 0.06 μg/μL kanamycin. A total of 126 bacterial plasmids were isolated using a MiniPrep Kit (QIAGEN) then sequenced using an Applied Biosystems (ABI) BigDye Terminator version 3.1 Cycle Sequencing Kit, and the M13 (–20) forward primer.

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Table 1 Characterization of 15 novel loci tested in two populations of *Bassariscus astutus*. ASR, approximate size range in base pairs (bp)

Locus	Primer sequences (5′–3′)	Repeat motif	ASR (bp)	N	N_A	H_O	H_E	T_{a1}, T_{a2} (°C)	GenBank Accession no.
BAAS_GATA 5	F: CGGTCCTGGGATAGAGTCCT R: CCTTTGCCTACCTGTTTATTTTC	$(GATA)_2GAT(GATA)_{11}$	213–279	20	8	0.45	0.655	55, 50	EU915250
BAAS_GATA 47	F: AAGGCATAAAAACAAAGTAAAACCA R: GGTGACCCTATATCTGTCAGCTT	$(GATA)_2GAT(GATA)_{11}$ ACA$(GATA)_2$	182–218	21	9	0.714	0.852	59, 53	EU915251
BAAS_GATA 105	F: CCAAATCAGGCTCCAAGTTC R: GCTAATCAATTGAGCGTTGAAA	$(GATA)_{14}$	231–255	21	6	0.381	0.381	59, 53	EU915253
BAAS_AAAG 2	F: TCTCATGCTTCTTGCCAATG R: TACTGCTCCCCCTGCTTATG	$(CTTT)_{19}$	265–347	21	18	0.857	0.911	57.7, 53.5	EU915254
BAAS_AAAG 3	F: CCTACTCCCTCTGCTTGTGG R: TCTCAAGAAACAATTTTCCAACC	$(AAAG)_{18}AG(AAAG)_{15}$ $AG(AAAG)_{18}A(AAAG)_2$	213–341	21	14	0.714	0.878	55, 50	EU915255
BAAS_AAAG 20	F: TCTCTTGGTTATAAATACTCTTGGTTG R: AAGCCTGAAGTCCACCCTCT	$(AAAG)_{20}$	144–214	21	11	1	0.87	59, 53	EU915256
BAAS_AAAG 22	F: GCCATTGGCCTTTCAATAAA R: TCAAGGAGCTTACATAGAAATTAGGA	$(AAAG)_{13}AACA(AAAG)$	174–213	21	9	0.905	0.854	59, 53	EU915257
BAAS_AAAG 28	F: GAAGACGAATTCTGCCCGTA R: GTCAAAACTTGACCCCCTGA	$(CTTT)_9CCTT(CTTT)_{23}$	161–289	21	11	0.571	0.64	59, 55	EU915258
BAAS_AAAG 30	F: CGAGGGAGGCAATAAAGTTG R: CCACAATATTTATTCAGTGGTTTCA	$(CTTT)_{21}CT(CTTT)_{19}$	254–478	21	19	0.81	0.941	57.7, 53.5	EU915259
BAAS_AAAG 36	F: TGCTTCTCCCTCTCCATCTG R: TGACCTTGATTTAGGCAAAGG	$(AAAG)_8GAAG(AAAG)_{13}AA(AAAG)_2$	186–222	21	10	0.81	0.882	59, 53	EU915260
BAAS_AAAG 45	F: TTGCACGCTAATATGTGAATCAT R: CACTCGGGTGTTCCTCTTTT	$(AAAG)_{18}GAAG(AAAG)_2$	277–335	21	14	0.905	0.901	59, 53	EU915261
BAAS_GATA 73	F: TCATGCGTGTGTGTGTATGC R: TCCCTCTCAGTGGATAGAAATAGG	$(TATC)_3ATC(TATC)_{11}$	198–234	21	10	0.952	0.898	57.7, 53.5	EU915252
BAAS_AAAG 81	F: TCCTCTCTCTGCCTACTTGTGA R: CTATGCTCCTTCACCCCAAA	$(AAAG)_{15}GAAG(AAAG)_{10}$	222–308	21	18	0.81	0.922	59, 53	EU915262
BAAS_AAAG 84	F: TACATGTGCCTTCCAGCTTC R: TGTGGCAGGCATTTATCAGT	$(CTTT)_{19}$	222–256	21	8	0.714	0.81	55, 50	EU915263
BAAS_AAAG 89	F: ATGCCTTTGTGAAGGGCTTA R: TCTTCAGAACCGAACCATCA	$(AAAG)_5AAAA(AAAG)_3AG(AAAG)AA(AAAG)AGAGAG(AAAG)$ $AGAG(AAAG)AAACTAAG(AAAG)_{18}$	274–342	21	16	0.905	0.926	59, 53	EU915264

N, number of individuals successfully genotyped out of 21; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; T_{a1}, T_{a2}, first and second annealing temperatures (in °C), respectively, of the primer pair during step-down PCR cycle. Note that the M13 (-20) forward sequence was added to the 5′ end of each forward primer.

Clean PCR products were sequenced on an ABI 3730xl sequencer.

Sixty-nine sequences were unique and contained tetranucleotide repeats. For those sequences containing sufficient flanking sequence and no complex repeats, primers were designed using Primer 3 (http://frodo.wi.mit.edu/). An M13-hybrid primer process was used, and thus, the M13F (–20) sequence was added to the 5′ end of each forward primer (Boutin-Ganache *et al*. 2001). Fifty-three primer sets were tested on a panel of 21 individuals. The PCR mixtures contained the following reagents in a 10 μL volume: ~45 ng of DNA, 2× Multiplex Mix (QIAGEN), 0.4 mg/mL BSA, 0.1 μM forward primer, 0.1 μM 6-FAM dye-labelled M13 primer (ABI), and 2 μM reverse primer. The following step-down PCR cycle was used initially for each locus: 95 °C for 15 min, 25 cycles of 94 °C for 30 s, 59 °C for 1.5 min, and 72 °C for 1 minute, followed by 20 cycles of 94 °C for 30 s, 53 °C for 1.5 min, and 72 °C for 1 min, with a final extension of 60 °C for 30 min. Annealing temperatures for some loci were optimized to improve amplification (Table 1). PCR amplifications were performed on either a Primus 96Plus (MWG Biotech), a PTC-100, or a PTC-200 (both MJ Research). Amplified PCR products were mixed with LIZ500 size standard, characterized on a 3730XL sequencer, and scored using GeneMapper 4.0 (all ABI).

Fifteen tetranucleotide microsatellite markers were selected for evaluation in the panel of ringtails. Loci were typed in 20–21 individuals (10 from CHIR and 11 from GUMO) with six to 19 alleles each. GenePop version 3.4 was used to calculate observed heterozygote excess, heterozygote deficiency, and linkage disequilibrium across all loci (Raymond & Russet 1995). We used a Bonferroni correction (Rice 1989) for multiple comparisons. All loci were found to be in Hardy–Weinberg equilibrium and none were found to be in linkage disequilibrium. Cervus version 3.0 (Kalinowski *et al*. 2007) was used to determine the expected and observed heterozygosity values for the CHIR and GUMO populations, which ranged from 0.381 to 1.000 and from 0.381 to 0.941, respectively. Given that little is known about the population structure of the ringtail, these genetic markers will provide useful tools for insights into the population biology and social structure of this species.

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