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PRIMER NOTE

Cloning and characterization of 29 tetranucleotide and two dinucleotide polymorphic microsatellite loci from the endangered marbled murrelet (*Brachyramphus marmoratus*)

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Abstract

We developed 31 novel, polymorphic microsatellite loci in the marbled murrelet (*Brachyramphus marmoratus*), a critically endangered seabird. Variability was tested on 15 individuals from the Santa Cruz, California population, with each locus characterized by two to 12 alleles. Observed levels of heterozygosity ranged from 0.13 to 0.93. These loci provide a valuable means of assessing the population structure and demographic parameters of this species, which may be critical to its conservation across a fragmented habitat.

Keywords: *Brachyramphus marmoratus*, marbled murrelet, microsatellite, parentage

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The marbled murrelet (*Brachyramphus marmoratus*) is an endangered seabird found throughout coastal areas in the Pacific Northwest, Canada and Alaska, associated with old growth forests (USFWS 1997). Understanding the population structure and demographic parameters (e.g. by parentage analysis) of this species is critical to its continued conservation. One approach to gaining inference about fine-scale structuring is from multiple variable STR (short tandem repeat) loci. To this end, we have developed and characterized 31 STR loci from a marbled murrelet genomic library enriched for di-, tri- and tetranucleotide STRs.

A genomic library of marbled murrelet DNA was generated following the protocol of Glenn & Schable (2005). Blood samples were collected from individual marbled murrelets and stored in either 95% ethanol or Longmire's solution at −20 °C. Genomic DNA was extracted from blood using DNEasy Extraction Kit (QIAGEN) following the manufacturers instructions. Four replicates of genomic DNA were digested with *Rsa*I restriction enzyme (New England Biolabs). Digested DNA was ligated to universal SNX linkers SuperSNX24F and SuperSNX24+4P. The four restriction ligation reactions were each double enriched by hybridization with a cocktail of the following biotinylated oligonucleotide probes: replicate 1: $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, $(ATCC)_5$, $(AC)_{13}$; replicate 2: $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AAC)_8$, $(AAT)_8$, $(ACT)_{12}$; replicate 3: $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACCT)_6$, $(ACAG)_6$, $(ACTC)_6$, $(ACTG)_6$; and replicate 4: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$. The hybridized DNA was captured by isolation with Dynabeads (Dynal) (Glenn & Schable 2005).

We compiled a library of 372 serially enriched clones and generated sequences for 224 of these. Individual bacterial colonies were lysed by boiling, and the cloned inserts were amplified by polymerase chain reaction (PCR, Mullis & Faloona 1987) using T7 and M13 oligonucleotide primers, under the following reaction conditions. Approximately 10 ng of genomic DNA was amplified in a 20-μL reaction containing 67 mm Tris-HCl (pH 8.8), 2 mm $MgCl_2$, 16.6 mm $(NH_4)_2SO_4$, 10 mm β-mercaptoethanol, 0.2 mm of each dNTP, 0.4 U *Taq* DNA polymerase (New England Biolabs) and 1 μm of each primer. The PCR consisted of a denaturing step for 2 min at 94 °C; 28 cycles of 1 min at 94 °C, 1 min at 54 °C and 4 min at 72 °C. PCR amplifications took place on a Dyad thermocycler (MJ Research). PCR products were sequenced with T7 and M13 oligonucleotide primers using standard ddNTPs-based cycle sequencing (BigDye

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version 3.1, Applied Biosystems) according to the manufacturer's instructions. Cycle sequence products were resolved on an ABI 3730 automated sequencer (Applied Biosystems). Out of 224 clones, 151 sequences contained a microsatellite. We therefore obtained 67% enrichment of inserts containing marbled murrelet microsatellite DNA.

Of the 151 sequences containing tandem repeats, 94 were simple repeats of dinucleotide ($n = 40$), trinucleotide ($n = 1$) or tetranucleotide ($n = 53$) motifs. The other 57 loci were comprised of either compound or interrupted repeats. Locus names signify the repeat motif (the dominant motif when two or more were present). The number of tandem repeats among the clones for which primers were designed ranged from five (BmaGACA340) to 53 (BmaGATA465).

Locus-specific oligonucleotide primers were designed for 50 loci using PRIMER 3 software (Rozen & Shaletsky 2000), and to the 5′-end of each forward oligonucleotide primer, a universal M13 (5′-TGTAAAACGACGGCCAGT-3′) tail was added. Oligonucleotide primers were obtained (Integrated DNA Technologies), and experimental conditions optimized. Genotyping was carried out in a single PCR with the addition of a 6FAM-labelled M13 oligonucleotide primer, thereby adding a fluorescent label to the PCR amplification products (Schuelke 2000). PCRs were carried out in 10 μL volumes under the following conditions: approximately 10 ng of genomic DNA were amplified in a cocktail including 67 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 0.2 mM of each dNTP, 0.4 U *Taq* DNA polymerase (New England Biolabs), 800 nM of reverse primer, 800 nM of M13F FAM primer and 200 nM of forward primer. The PCR consisted of a denaturing step for 2 min at 94 °C; 22–24 cycles of 30 s at 94 °C, 30 s at 59 °C (see exceptions in Table 1) and 30 s at 72 °C (step 1); 8–12 cycles of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C (step 2); and a final extension for 10 min at 72 °C. Extension times were increased to 60 s for BmaGACA56 and BmaGATA464. Amplifications took place on a Dyad thermocycler (MJ Research), and PCR products were visualized on 1.7% agarose gels stained in ethidium bromide.

Of the 50 loci tested, 37 produced distinct bands on agarose, and the amplification products were sized by electrophoresis on an ABI 3730 sequencer using LIZ 500 size standard (Applied Biosystems) to screen for variability. Variability was tested using 15 individual marbled murrelet samples from the Santa Cruz, California population. Alleles were scored using GENOTYPER 3.7 software (Applied Biosystems).

Out of the 37 loci screened, 2 were monomorphic and 35 were polymorphic (2–12 alleles), but 4 polymorphic loci were unable to be scored and were excluded from the analyses. For the 31 remaining loci, the mean number of alleles was 6.16.

Observed (H_O) and expected (H_E) heterozygosities were estimated using an unpublished ANSI-C program written by PJP. H_E was estimated as the expected proportion of heterozygote individuals given the observed allele frequencies for the target locus. H_O was calculated as the observed number of heterozygote individuals at the target locus. The probability of obtaining H_O by chance, given the observed population allele frequencies, was estimated by randomizing alleles among individuals and estimating H_O. The probability of H_O was estimated as the fraction of 10 000 simulations that yielded a similar or more extreme value of H_O. After correcting for multiple tests (Rice 1989), three loci still exhibited statistically lower H_O than expected under random mating (Table 1). H_O ranged from 0.13 to 0.93, and the mean H_O was estimated at 0.60 for all loci and at 0.77 for the 20 most variable loci. The probability of identity (Paetkau *et al*. 1995) for each locus is listed in Table 1; values of I ranged from 0.028 to 0.62, with the mean estimated at 0.19. For all loci combined, I was estimated at 4.6×10^{-29}. We tested for linkage disequilibrium using GENEPOP (Raymond & Rousset 1995) and found no evidence of significant linkage among the 31 loci tested.

The 31 STR loci presented here yield unambiguous data with high levels of variation, making them suitable to estimate genetic divergence among populations as well as individuals.

Table 1 STR loci for *Brachyramphus marmoratus*, including primer sequences, PCR conditions (annealing temperatures, number of cycles), number of individuals genotyped (N), number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), P value for the probability of H_O, probability of identity (I) and GenBank Accession nos for the sequences of the clones from which the markers are derived

Locus	Primer sequences	T_a	Cycles	N	No. of alleles	Size range (bp)	H_O	H_E	P value	I	GenBank Accession no.
BmaAAAC336	F: CCTTTTCCCTGTTGTTGTGTT R: ATGCTGTGTGTAGTCCGTGAA	59/53	22/8	15	5	176–196	0.13	0.62	**< 0.0001**	0.20	DQ173162
BmaAAAG043	F: GGCAAAATTCTCAGTTTTACCAA R: CTGGGATTTAAGTTGTCTGAAGAA	59/53	22/8	15	2	209–213	0.33	0.28	< 0.9999	0.56	DQ173163
BmaAAAG433	F: TCAGAAGATCCTTCTCCCTCA R: CCAAAGGCCAAAGAATGATTA	59/53	22/8	15	7	165–203	0.87	0.79	< 0.8109	0.077	DQ173164
BmaACCT555	F: GACAGAATATAAATGGAGACATGG R: AGGCAGAGATGAGAAGGCTAA	59/53	22/8	15	9	132–194	0.93	0.87	< 0.8153	0.032	DQ173165

Table 1 *Continued*

Locus	Primer sequences	T_a	Cycles	N	No. of alleles	Size range (bp)	H_O	H_E	P value	I	GenBank Accession no.
BmaAGGT503	F: CTCAGCAAAACCAGGAAAATA R: TTTAAGTCTAATATTGGTCTCTCAGC	59/53	22/8	15	4	218–260	0.47	0.55	< 0.2141	0.25	DQ173166
BmaATAC370	F: CCTGATGACCTTTGATGGCTCT R: ACCTGTGCCTGCGTTGGT	55/53	24/8	15	6	186–204	0.87	0.74	< 0.9127	0.11	DQ173167
BmaATTT351	F: TGGGAATATCTTTTGGTTTGG R: TCCAGCCTTTCCTTGTCTCTA	59/53	22/8	15	4	165–207	0.53	0.73	< 0.0450	0.12	DQ173168
BmaCCAT301	F: AGATCTATCCCTTGGCTGGA R: TATCTGCCAAAATCTGCTGAA	59/53	22/8	15	6	152–172	0.87	0.78	< 0.8229	0.079	DQ173169
BmaCCAT443	F: TGCCAGGCCATCTACTTTAAT R: GCTTATCTTTCCCTCCATCCT	59/53	22/8	15	9	178–214	0.93	0.85	< 0.8630	0.037	DQ173170
BmaGACA340	F: GGCCATCTGAGTTGGATAAAA R: GTTGGGTGGATCATGGTTTAG	59/53	22/8	15	2	136–140	0.40	0.32	< 0.9999	0.51	DQ173171
BmaGACA456	F: ACTGGTCTCTTTGCTTGATGG R: GGAAGAGCACACCTTTACCAG	59/53	23/12	14	4	395–407	0.64	0.68	< 0.3909	0.16	DQ173172
BmaGATA365	F: GCTTTATCTGTGGCAACACTG R: GCTGTAGGGAGGATATGATGC	59/53	22/8	15	7	225–253	0.80	0.73	< 0.7765	0.10	DQ173173
BmaGATA439	F: GAGGGGAGGGTGTATCTTTTC R: ATGTCACTCTGGTGGAGAACC	59/53	22/8	15	9	315–351	0.80	0.78	< 0.5944	0.068	DQ173174
BmaGATA464	F: GCACCATGCTCAGATCACTAA R: ATCTGTGCTTGAGGGAGAGAA	59/53	23/12	15	6	414–438	0.47	0.66	< 0.0272	0.14	DQ173175
BmaGATA465	F: TCAGAGGGGGAAACAACATAG R: GGGAATTTGCATTCAGTCTGT	59/53	22/8	15	12	245–303	0.47	0.88	**< 0.0001**	0.028	DQ173176
BmaGATA553	F: TTGTGAGAGGGTCACTTATCAAAT R: CATCTCTCTTTCAGAAGAGCAGTC	59/53	22/8	15	8	136–165	0.73	0.78	< 0.2850	0.069	DQ173177
BmaGGAT313	F: CTCTAAAGGTCCCTTCCAACC R: TGACTTCACAGTTCCTCATGC	59/53	22/8	15	5	235–251	0.73	0.77	< 0.3592	0.088	DQ173178
BmaGGAT368	F: AATCACCAAGGATAAAGGATGATA R: AGGGGACCTGCCCATATATTA	59/53	22/8	15	11	212–293	0.93	0.87	< 0.7955	0.029	DQ173179
BmaGTTT332	F: TCTCCAAATCCAGAAAAATGG R: ATAATCCTGTGAGGGGTTTCC	52/53	22/8	15	4	171–197	0.27	0.60	**< 0.0011**	0.22	DQ173180
BmaGTTT428	F: GCATGTAACAAGTCCATTTGC R: CAGGGGCAGCTTAAGTAAAGT	52/53	22/8	15	2	143–147	0.13	0.39	< 0.0188	0.45	DQ173181
BmaGTTT515	F: CAGAATCACGTCTTCCCTTGT R: CCTTGGTTCTTTACCAGCAAC	52/53	22/8	15	3	220–228	0.27	0.45	< 0.0377	0.36	DQ173182
BmaGTTT534	F: TGAACGACAACAACAGTGAGA R: CCCATGGCTTTATATGGAATC	58/53	24/8	15	2	172–176	0.33	0.28	< 0.9999	0.56	DQ173183
BmaTATC353	F: TGTGGTATGCTCTGGACTGAC R: ATATAGCCCATTCCCACTTCC	59/53	22/8	15	2	245–249	0.33	0.46	< 0.2429	0.39	DQ173184
BmaTATC356	F: GTCCACTGAGTTTAGCAGCAA R: TGCAGCTCACTATACCAAGGA	58/53	24/8	15	9	140–182	0.87	0.84	< 0.5919	0.042	DQ173185
BmaTATC371	F: GTCCCCTTTCTAACAGGCACT R: GTAAAGGTGGGGGAGCATATT	59/53	22/8	15	9	259–299	0.93	0.81	< 0.9347	0.060	DQ173186
BmaTATC444	F: CAAAAAGTTGGGGAAAGTTTG R: CCCGCATTTCTAAGCTGTATT	59/53	22/8	15	2	369–373	0.13	0.23	< 0. 1983	0.62	DQ173187
BmaTATC453	F: TCCTCCACATGTTTTGCAGTA R: CAGGAGCACCATGTATGTTTG	59/53	22/8	15	12	250–302	0.87	0.86	< 0.5284	0.034	DQ173188
BmaTATC462	F: GAGAGGGCATGAAAATTCAGA R: CAAAGATCTCACCCTCTGCTC	59/53	22/8	15	9	271–311	0.53	0.81	< 0.0031	0.060	DQ173189
BmaTGAA523	F: TGAATCCAGTGGAACAAAACA R: AATGAACTAATGAGGGCGATG	59/53	22/8	15	9	125–157	0.73	0.74	< 0.4747	0.085	DQ173191
BmaCA382	F: AAGGGATGCTTAATCGTGATG R: AGCTCTTCCCAATGACTGCT	59/53	22/8	15	5	142–174	0.60	0.58	< 0.6035	0.21	DQ173192
BmaCA561	F: GCAAAACAGGTGGGATACCTA R: TGTCCTCCAGAGGGACAATAC	59/53	22/8	13	7	191–219	0.77	0.75	< 0.5892	0.10	DQ173193

P values in boldface indicate loci with significantly lower H_O than expected, assuming random mating. Annealing temperatures (T_a) for nested PCR steps 1 and 2 (as referred to in the text) are given in °C, and numbers of PCR cycles likewise correspond to steps 1 and 2.

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