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TECHNICAL NOTE

Development of twenty-five polymorphic microsatellite markers for the endangered red-cockaded woodpecker (*Picoides borealis*)

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Abstract Twenty-five polymorphic microsatellite markers were developed for the endangered red-cockaded woodpecker (*Picoides borealis*). The number of alleles ranged from two to five and observed heterozygosities ranged from 0.036 to 0.750. These loci should be useful tools for conducting research towards the management and conservation of this species.

Keywords Microsatellite · Red-cockaded woodpecker · *Picoides borealis*

Red-cockaded woodpeckers (RCWs), *Picoides borealis*, are endangered, non-migratory, cavity-nesting cooperative breeders that inhabit old-growth pine forests of the south-eastern United States (Haig et al. 1994). The harvesting of old-growth pine forests throughout the southeast has led to an overall decline in their abundance, leading to extensive population fragmentation (Jackson 1971). A few genetic studies have examined population structure of these birds using allozymes and RAPDs (Stangel et al. 1992; Haig et al. 1994; Haig et al. 1996; Daniels and Walters 2000); however, to our knowledge no microsatellite markers exist for this species. The availability of microsatellite markers for the RCW should help researchers gain a deeper understanding of genetic, behavioral, reproductive, and movement parameters needed for the recovery of this endangered species.

We prepared two genomic libraries enriched for either tetranucleotide [$(GATA)_7$, $(GATC)_7$, and $(GACA)_7$] or dinucleotide [$(GT)_{12}$ and $(CT)_{12}$] repeats using a microsatellite cloning protocol based on Hamilton et al. (1999) and Hauswaldt and Glenn (2003), modified as reported earlier (Beheler et al. 2004). To construct the library, genomic DNA was extracted from one RCW muscle sample (incidental take from Fort Polk, Louisiana) using a standard Phenol–Chloroform extraction protocol. Five hundred and thirty six colonies were screened and recombinant clones ($n = 384$) with polymerase chain reaction (PCR) amplification products greater than 300 bp were sequenced. We imported sequence data into Sequencher 4.1 (Gene Codes Corporation) for analysis.

We selected a subset ($n = 53$) of microsatellite-containing sequences and designed primers for PCR amplification of these microsatellites using the software program PRIMER3 (Rozen and Skaletsky 2000). We amplified these 53 microsatellite loci in 10 ml PCRs using a Master-cycler ep gradient (Eppendorf) and 20 ng of template DNA, 0.2 mM of each dNTP, 0.25 mM of each primer, 1 U of *Taq* DNA polymerase (NEB) and 1X reaction buffer (10 mM Tris-HCl,, 50 mM KCl, 0.05 mg/ml BSA). The amplification conditions were as follows: 94°C for 2 min, then 94°C for 30 s, annealing temperature (Table 1) for 15 s, 72°C for 15 s for 30 cycles, then 72°C for 10 min and a final extension at 60°C for 45 min. PCR products were initially screened on 2% agarose gels stained with ethidium bromide to verify amplification. Forty-nine

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Table 1 Characterization of 25 polymorphic microsatellite loci developed for the RCW

Locus	GenBank no.	Primer sequence	Motif	PCR product (bp)	T_A	A	H_E	H_O
RCW01	EU817853	F: AATGAAACCTTCAAGCTTACTCG R: CCTGCTCCAGACAGTAGCC	$(AC)_{10}$	177–179	64	2	0.399	0.393
RCW03	EU817854	F: CCTGCAAGGTAGACCACACC R: CTCTTCAGAACTCTTCAGGTAATGC	$(TG)_8$	220–236	64	3	0.105	0.107
RCW05	EU817855	F: TCAGGGTAAGTTTGCACAGC R: TTCTTGGTCTGTTTAGGATCACC	$(GT)_{15}$	326–344	64	5	0.677	0.571[a, b]
RCW06	EU817856	F: CGTTGTAGAGACCATCTTCATCC R: GGAGGAATTTGAGAAAGAGAACC	$(AC)_{10}$	150–152	64	2	0.223	0.250
RCW10	EU817857	F: TTTGCCATGTGTGGTTGG R: CTCTCTTCACCACCAGTAAGACC	$(GT)_{10}$	180–184	64	3	0.586	0.643
RCW12	EU817858	F: TCACTTCTTGTTATCGGGAGAG R: CCATGTGCTCAGTAGTGTTGG	$(GT)_8$	201–203	64	2	0.321	0.321
RCW14	EU817859	F: GCTTTAAATATGCTGCGTTGG R: TCCCTGTGTGAGAAGTGAGC	$(AC)_9$	221–227	64	3	0.623	0.536[b]
RCW16	EU817860	F: TTCACCTTATGTTTCCATACTACAGC R: TCCTGTTTCCATTGAACTTCC	$(GT)_9$	332–342	64	4	0.482	0.500
RCW17	EU817861	F: AGCTGAGAATCTGGCTCTGC R: TCTCCAGCAGGAGTGTAGCC	$(AC)_{10}$	291–299	64	3	0.641	0.536[b]
RCW18	EU817862	F: GTTTCAGAATCTTCCCCTCATC R: CCACGGATATTATATGCCATAACTC	$(AG)_{11}$	190–192	64	2	0.499	0.429[b]
RCW20	EU817863	F: AAACGGTGATGCCAAAGC R: AGGAAGAGTGACTGTCAGGTAGC	$(AC)_{10}$	294–300	64	4	0.655	0.679
RCW22	EU817864	F: AATGTGTGACCTCCTAGAACACC R: AAACAGAGACCAACCCAAGC	$(AC)_8$	262–264	64	2	0.493	0.536
RCW26	EU817865	F: AGTCAGCAGCAGTGTCAGTAGC R: GGTAAGCAGGTGAGACAGACG	$(AG)_{10}$	234–240	64	3	0.441	0.429
RCW28	EU817866	F: ACTGTCTTTAATCAAACTCATGATCC R: GTACCTCTGTGCCGACAGC	$(CT)_7$	281–287	64	4	0.566	0.607
RCW34	EU817867	F: GTTTCCTTCCCTCCTCTTCC R: TTTCCCATCCATCCTTGTTC	$(TA)_6(TG)_8$	123–137	65	3	0.521	0.393[b]
RCW36	EU817868	F: TCAGCAGCAGTGTCAGTAGC R: GTATACATGCTGTATCTAGTGGATCTG	$(AG)_{10}$	108–114	65	3	0.441	0.429
RCW38	EU817869	F: TTGGTATGTTCCTGTTGCTACC R: CTTGACTGTGGTCACTGTTCC	$(GT)_8$	215–219	65	3	0.464	0.143[a, b]
RCW40	EU817870	F: CATTCTGACCTTTGAAGCATCC R: CATCCACTTCCTCCAAGTGC	$(AC)_8$	225–237	65	3	0.637	0.750
RCW42	EU817871	F: GGACTGGCTTATATCACATCAGG R: TGTTGCAATCAAGCAGTTGG	$(AC)_6$	274–282	65	3	0.071	0.071
RCW44	EU817872	F: GTGGACAGTTTATTATTCTCACTGG R: TGTACAATGGAGATGCTTTGC	$(AC)_6$	209–211	64	2	0.456	0.393[b]
RCW45	EU817873	F: CTTCAGGGAGCAGGCACAC R: CTATCAGCCTCCAGCAGCAC	$(AC)_8$	171–173	65	2	0.486	0.500
RCW46	EU817874	F: TTCTGCAGGAGAGCACAGC R: CCTAATCATGAGTCAGGCAAGC	$(GT)_8$	241–243	65	2	0.444	0.357[b]
RCW47	EU817875	F: CGCATAGGTAGTGGACTAAA R: AAATTAAGAGGAACAGAATTGA	$(GT)_8$	150–156	54	4	0.202	0.214
RCW50	EU817876	F: TCTGCCAGGCTGTAGTGG R: CACAACTGGCTTCTAAATAATGC	$(AC)_8$	204–208	65	2	0.036	0.036

Table 1 continued

Locus	GenBank no.	Primer sequence	Motif	PCR product (bp)	T_A	A	H_E	H_O
RCW52	EU817877	F: TTAACACAGGTGCCCTTTCC R: CCACCTCCTGTGCATTTACC	$(AG)_6$	228–246	65	2	0.036	0.036

Annealing temperature (T_A), number of alleles (A), expected (H_E) and observed (H_O) heterozygosities are reported. Loci were run on 28 individuals

[a] Significant heterozygote deficiency ($\alpha = 0.05$)

[b] Frequency of null alleles >0.05

of the 53 loci consistently produced products of the expected size and subsequently were screened for polymorphism using a panel of 28 RCWs sampled from 17 breeding territories located on the Avon Park Air Force Range in Florida in 1994. The protocols for this polymorphism screening were the same as those given above, except that (1) in each reaction, the concentration of dTTP was reduced to 0.15 mM and 0.05 mM of chromatide rhodamine green 5dUTP (Molecular Probes) was added, and (2) PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems) and genotypes were assigned using GeneMapper v3.7 (Applied Biosystems). Of the 49 primer sets screened, 25 exhibited polymorphism.

For each polymorphic locus, we calculated observed heterozygosity (H_O), expected heterozygosity (H_E) and null allele frequencies using CERVUS 1.0 (Marshall et al. 1998). GENEPOP version 3.4 (Raymond and Rousset 2000) was used to test for evidence of linkage disequilibrium and deviations from Hardy–Weinberg equilibrium. The number of alleles per locus ranged from two to five, and single locus heterozygosities ranged from 0.036 to 0.750 (Table 1). The test for Hardy–Weinberg equilibrium was not performed for two loci (RCW50 and RCW52) because almost no heterozygotes were detected in the screening set population. Significant heterozygote deficiencies were detected at two loci and high frequencies of null alleles were found at eight loci (Table 1). We tested 300 pairwise comparisons for linkage disequilibrium and found evidence for genotypic linkage disequilibrium between one set of paired loci (RCW26–RCW36) after a sequential Bonferroni correction was applied ($P < 0.00003$).

Although the screening set of 28 birds came from 17 different breeding territories, the RCW population on the Avon Park Air Force Range in Florida is relatively small (<30 breeding groups) and isolated (>20 km from the nearest population) and, like most RCW populations, has also likely declined from historical levels (Jackson 1971). These population patterns may have limited the number of detectable polymorphism at our microsatellite loci. Further screening of these loci in RCWs from additional geographically distinct populations should resolve this question.

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