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PRIMER NOTE

Polymorphic microsatellite DNA markers for the white-breasted thrasher, *Ramphocinclus brachyurus*

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Abstract

We isolated and characterized six polymorphic microsatellite markers for the white-breasted thrasher from genomic libraries enriched for $(AC)_n$, $(GT)_n$, $(CAAA)_n$, $(TTTC)_n$, $(GAC)_n$, $(CT)_n$ and $(TTTG)_n$ microsatellites. The number of alleles per locus ranged from two to seven. Observed heterozygosity (H_O) ranged from 0.30 to 0.85.

Keywords: microsatellite, primer, *Ramphocinclus brachyurus*, white-breasted thrasher

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The white-breasted thrasher *Ramphocinclus brachyurus* is a passerine bird (family Mimidae) endemic to the islands of St Lucia and Martinique in the West Indies. With a global population of 1300–2800 breeding adults occupying a total area of about 13 km^2, it is classed as globally 'Endangered' (BirdLife International 2000, 2004; Temple 2005). Recent research has revealed that the species is a cooperative breeder (Temple 2005). The development of microsatellite primers for the white-breasted thrasher will facilitate research into the evolution of cooperative breeding in this species, as well as providing information that is of use for conservation management.

Microsatellite loci for the white-breasted thrasher were isolated using a modification of the enrichment protocol followed in Armour *et al*. (1994). Total genomic DNA was isolated from blood samples of six wild individuals using standard overnight proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation (Sambrook *et al*. 1989). Genomic DNA was digested by Sau3A1 (Roche), then ligated to linkers made by annealing equimolar amounts of Sau-L-A (5'GCGGTACCCGGGA-AGCTTGG-3') and 5-end phosphorylated Sau-L-B (5'-GATCCCAAGCTTCCCGGGTACCGC-3') oligonucleotides. Then DNA fragments in the range 200–800 base pairs (bp) were size selected and gel purified (QIAGEN QIAquick Gel Extraction Kit). This fraction was amplified in two 25 μL reaction volumes (in case only one fragment amplified) containing 1 μL (100 ng) DNA, 2.5 μL 10 × reaction buffer A (500 mM KCl, 100 mM Tris-HCl, pH 8.0, 0.1% Tween 20, 0.1% gelatine and 0.1% IGepal), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 μM of primer (Sau-L-A) and 1 U *Taq* DNA polymerase (MEB). Amplifications were performed in a Hybaid Express Cycler with hot start and consisted of an initial denaturation at 96 °C for 1 min, followed by 20 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 70 °C for 1 min, then mixing the polymerase chain reaction (PCR) products in two tubes together. This PCR product was enriched for AC, CAAA, CT, GT, TTTC, TTTG and GAC repeat motifs by overnight hybridization at 65 °C to Hybond-N membranes (Amersham Biosciences) to which poly(dA-dC)·(dG-dT) oligonucleotides (Amersham Biosciences), (AAAC-GTTT), (CT-AG), (TTTC-GAAA) and (GAC-GTC)s' PCR products had been bound. After stripping the enriched DNA from filters, the enriched fraction was made double-stranded by PCR (25 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 1 min; using 1 μM Sau-L-A as the PCR primer. PCR purification kits (Strategene® strataPrep™) were used to purify the PCR products. The purified products were ligated into p-GEM®-T Easy vector (Promega) at 4 °C overnight and were transformed into DH5α subcloning efficiency competent cells (Invitrogen). After plating cells on selective (carbenicillin and x-gal) media plates, about 143 white colonies were picked and transferred onto new reference Luria–Bertani agar plates for future use and also into 50 μL TE. To identify colonies containing AC, AAAC, CT, TTTC or GAC repeat motifs in

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the vector inserts, the plasmids were released from the bacteria transferred to the TE by boiling for 5 min, and were then insert amplified by PCR using M13 reverse and T7 primer (M13 reverse primer: 5′-GAGCGGATA ACAATTTCACACAGG-3′ and T7 primer: 5′-TAATAC-GACTCACTATAGGG). A 25-μL reaction volume contains 1 μL (100 ng) plasmid DNA, 2.5 μL 10 × reaction buffer A (500 mM KCl, 100 mM Tris-HCl, pH8.0, 0.1% Tween 20, 0.1% gelatine and 0.1% IGepal), 1.0 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 μmol of both primer (M13 reverse and T7 primer) and 1 U *Taq* DNA polymerase. Amplifications were performed in an MBS (Hybaid) PCR machine under the condition of 1 cycle of 5 min at 96 °C, followed 30–32 cycles of 45 s at 96 °C, 1 min at 55 °C, 2 min at 72 °C. PCR products were run on 1% agarose gels. PCR products were purified using Shrimp Alkaline Phosphatase (SAP, usb®) 0.4 μL, Exonuclease I (Biolab) 0.05 μL and Exonuclease I buffer 0.167 μL per μL of PCR product. Sequencing was carried out using a Big-Dye 3.1 Terminator Cycle sequencing Kit (Applied Biosystems) with T7 and M13 as the forward and reverse primers, respectively. Reactions were performed according to the manufacturer's instructions, with the exception that the amount of terminator was reduced from 8 μL to 1 μL. Products were visualized using an ABI PRISM 377 automatic sequencer. At least 16 sequences had sufficient flanking DNA around the AAAC and AC repeat regions to design primer pairs using PRIMER 3 (Rozen & Skaletsky 2000). Sequences were submitted to the GenBank database (Accession nos AY923150–AY923166).

The primers were tested for polymorphism on 20 wild individuals. Genomic DNA from those 20 individuals was extracted using standard overnight proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation (Sambrook *et al*. 1989). Approximately 5 ng of template DNA was amplified in 11-μL reaction volumes containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01%Tween 20, 0.01% gelatine, 0.01% Igepal, 0.2 mM each of dATP, dTTP and dGTP, 0.05 mM dCTP, 400 nM of each primer, 0.5 U *Taq* polymerase (MEB) and 0.1 μCi [α^{32}P]-dCTP. The PCR programme used was 4 min denaturing at 94 °C followed by 35 cycles of 45 s at 94 °C, 20 s at 60 °C and 30 s at 72 °C, ending with a 5-min final elongation stage at 72 °C. The PCR products were resolved on polyacrylamide gels, visualized by autoradiography and the alleles scored manually.

Six loci were found to be polymorphic (Table 1), while the other 10 primer pairs amplified loci that were either monomorphic, had a high null allele frequency or failed to amplify a consistent product. The expected heterozygosities (H_E) ranged from 0.38 to 0.75. Loci were tested for both Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using the software GENEPOP version 3.4 (http://wbiomed.curtin.edu.au/genepop). All the six loci were found in HWE ($P > 0.05$) and linkage disequilibrium was not identified between any sets of loci ($P > 0.05$), except one pairwise set (Th5 × Th6) ($P < 0.01$), which may indicate that these two loci are linked. These polymorphic loci are currently being used to study population structure and dispersal in populations of *R. brachyurus*.

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Table 1 Six microsatellite loci isolated from *Ramphocinclus brachyurus* and PCR primers that were employed to screen 20 unrelated individuals

Locus	Primer sequence (5′–3′)	Repeat motif in clone	Size range (bp)	No. of alleles	H_E	H_O	GenBank Accession no.
TH3	F: ACTGCACTGACAGCCTCAAG R: GACTGCCACAACAGCAACC	$(GTTT)_9$	136–156	6	0.71	0.85	AY923152
TH6	F: TCTAACACTTCTCCCTGTTTCG R: TCCTCAGACTCTGTTTCCTTCC	$A_5 (CAAA)_7 (CAGA)_4$	148–164	4	0.5	0.45	AY923155
TH12	F: CATCTCTACTGGGCACTGTTAAA R: GCTGTGTGGAGCACTTCAGG	$(CT)_6TT\ (CT)_9$	153–155	2	0.38	0.30	AY923161
TH15	F: GATCCCACATCCATTCACAC R: AGCTGCTCACCAGTCTCTCC	$(CA)_{13}$	151–155	3	0.48	0.60	AY923164
WB2	F: GATCAATTAATGGTTTTGGATGG R: CTGCCCTTATTTTGGCAGAC	$(AC)_{13}$	213–225	6	0.75	0.75	AY923165
WB5	F: GCAAGCTTCAGCATCTATCC R: CATCCTTGCTGAGGCAGAG	$(GT)_{15}$	170–202	7	0.72	0.70	AY923166

T_a, annealing temperature; H_E, expected heterozygosity; H_O, observed heterozygosity.

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