

## PRIMER NOTE

# Polymorphic microsatellite DNA markers for the grey fantail, *Rhipidura albiscapa*

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## Abstract

We isolated and characterized five polymorphic microsatellite markers for the grey fantail *Rhipidura albiscapa* from genomic libraries enriched for (AC)<sub>n</sub> and (GT)<sub>n</sub> microsatellites. In 34 adult individuals, the number of alleles per locus ranged from eight to 17. Observed and expected heterozygosities ranged from 0.65 to 0.94 and 0.83 to 0.94, respectively. These markers will be useful for analysing extra-pair paternity in fantails.

*Keywords:* fantail, microsatellite, primer, *Rhipidura albiscapa*

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The grey fantail *Rhipidura albiscapa* is a small, grey, monochromatic passerine in the family Dicruridae. Its range extends from New Caledonia, Vanuatu, Lifu, Banks Island, south Solomon Island and San Cristobal through Australia including Tasmania, but excluding coastal mangrove and the arid interior (Schodde & Mason 1999). With a high degree of paternal investment and male and female song, the grey fantail was previously thought to be socially and genetically monogamous. However, observations of southeast Australian grey fantails suggest that there may be significant levels of extra-pair copulations (EPCs) in this species. Males have pronounced cloacal protuberances suggestive of high levels of sperm competition. Territory intrusions, chases and EPCs have also been observed. It is therefore a useful model for investigations of various questions relating to the resolution of sexual conflict between individuals. For this reason, markers were developed to measure the levels of extra-pair paternity in clutches of fantails.

Microsatellite loci were isolated using a modification of the enrichment protocol of Armour *et al.* (1994). Total genomic DNA was isolated from blood samples collected from two wild individuals using standard overnight proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. Genomic DNA was digested by *Sau3AI* (Roche) then ligated to linkers made by annealing equimolar amounts of SAU-L-A (5'-GCGGTACCCG-GGAAGCTTGG-3') and 5'-end phosphorylated SAU-L-B (5'-GATCCCAAGCTTCCCGGTACCGC-3') oligonucle-

otides. DNA fragments in the range of 300–800 bp were then size-selected and gel-purified (QIAGEN QIAquick Gel Extraction Kit). This fraction was amplified in two 25 µL reaction volumes, each 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% gelatin and 0.1% Igepal), 1.5 mM MgCL<sub>2</sub>, 0.2 mM of each dNTPs, 1 µmol of primer (SAU-L-A) and 1 U *Taq* DNA polymerase. Amplifications were performed in a Hybaid PCR Express thermocycler with a 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 and extension at 70 °C for 1 min. Polymerase chain reaction (PCR) products from the two tubes were then mixed together. The resulting PCR products were then enriched for AC repeat motifs by overnight hybridization at 65 °C to Hybond-N membranes (Amersham Biosciences) to which poly (dA-dC)-(dG-dT) oligonucleotides (Amersham Biosciences) had been bound. After stripping the enriched DNA from filters, the enriched fraction was made double-stranded by PCR using SAU-L-A primer (25 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 1 min). A PCR purification kit (Stratagene StrataPrep) was used to purify the PCR products. Purified products were ligated into pGEM-T Easy Vector (Promega) at 4 °C overnight and transformed into DH5α subcloning efficiency competent cells (Invitrogen). After plating the cells on selective (carbenicillin and X-gal) media plates, approximately 100 white colonies were picked and grown overnight in LB media with carbenicillin. Plasmid DNA was extracted using a standard mini-prep

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**Table 1** Five microsatellite loci isolated from *Rhipidura albiscapa*, and PCR primers that were employed to screen 34 adults

Locus	Primer sequence (5'–3')	Repeat motif in clone	Allelic size range (bp)	$T_a$ (°C)	No. of alleles	$H_E$	$H_O$	HWE probability	GenBank Accession no.
FT1.10	F: CATGACTGGCCCTATGTTTG R: ACAGGGCTGAGATGGACTTG	(CA) <sub>16</sub>	213–247	60	14	0.94	0.85	0.160	AY789917
FT1.6	F: GCAGCTACACAGAAACTAGATAAGG R: GTTGTGTTGTCATGCGGTTG	(GT) <sub>12</sub>	162–202	60	17	0.91	0.91	0.152	AY789919
FT2.18	F: ACAGTGTTTTTAAAGCAGAGATGG R: CAAATGCCCAAGAGAAGCAG	(GT) <sub>13</sub>	140–154	53	8	0.83	0.65	0.010	AY789920
FT2.32	F: CTGCGTACCCAGCAGAGG R: AAGCCACGAACCATTCAAAG	(AC) <sub>13</sub>	194–238	60	15	0.92	0.94	0.549	AY789921
FT2.5	F: AATTCATTAAACAGGAACCTCCACAG R: GCTGCAGAGGGTGACAGTG	(AC) <sub>16</sub>	197–233	60	16	0.92	0.91	0.378	AY789922

(Sambrook *et al.* 1989). The plasmid DNA was dot-blotted onto Hybond-N membranes (Amersham Biosciences) and screened for inserts using a  $\gamma$ -<sup>32</sup>P labelled (CA)<sub>n</sub> probe (Cunningham *et al.* 1999). Forty-eight colonies were identified to have inserts. M13 forward and reverse primers (forward primer: 5'-CGCCAGGGTTTTCCCAGTCAC GAC-3'; reverse primer: 5'-GAGCGGATAACAATTTTCACACAGG-3') were used to sequence those positive plasmids. Sequencing was performed using a BigDye version 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems). Most of the reactions were performed according to the manufacturer's instruction except that the amount of terminator was reduced from 8 to 1  $\mu$ L. The result was analysed on an ABI PRISM 377 automatic sequencer. Twenty-one sequences had sufficient flanking DNA around the AC repeat region to design primer pairs using PRIMER 3 (Rozen & Skaletsky 2000).

Thirty-four adult individuals were used to determine locus polymorphism characteristics. Approximately 5 ng of template DNA, extracted as described above, was amplified in 11- $\mu$ L reaction volumes containing 10 mM Tris-HCL, pH 8.0, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 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  $\mu$ Ci [ $\alpha$ <sup>32</sup>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 the annealing temperature (see Table 1) 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 and visualized by autoradiography. Alleles were scored manually relative to standards of known size. Tests for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were implemented using GENEPOP program (Raymond & Rousset 1995).

Five loci were found to be polymorphic (Table 1). These sequences have been submitted to the GenBank database (Accession nos AY789917 and AY789919–AY789922). Sixteen additional loci were either monomorphic, failed to

amplify a consistent product, exhibited a high null allele frequency or were difficult to score. All polymorphic loci exhibited heterozygosity greater than 65% and had eight or more alleles. The expected heterozygosities ( $H_E$ ) range from 0.83 to 0.94. Four of the five loci were observed to be in HWE (Table 1). After Bonferroni correction for multiple tests, no pairs of loci were found to be in LD. These polymorphic loci are currently being used to study extra-pair paternity in *Rhipidura albiscapa*.

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