Molecular Ecology Notes (2003) **3**, 517–519 doi: 10.1046/j.1471-8286.2003.00497.x

PRIMER NOTE

Novel polymorphic microsatellite markers for paternity analysis in the red-capped robin (*Petroica goodenovii*: Aves)

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Abstract

Seven microsatellite loci were isolated and characterized from the red-capped robin *Petroica goodenovii*, using nonradioactive polymerase chain reaction (PCR)-based techniques to screen an enriched genomic library. Five loci showed no evidence of null alleles and were variable [mean heterozygosity (H_E) = 0.440, mean number of alleles = 8]. Cross-amplification using primers for microsatellites in *Phylloscopus occipitalis* and *Emberiza schoeniclus* yielded another two polymorphic loci. The combined set of five red-capped robin and two cross-amplified loci are suitable for paternity assignment (exclusion probability for seven unlinked loci = 0.9760).

Keywords: microsatellite, parentage, *Petroica goodenovii*, red-capped robin

Received 15 May 2003; revision received 29 June 2003; accepted 29 June 2003

During the past decade, genetic markers have been widely applied to resolve parentage in populations of wild animals. These studies have revealed unexpected variation in reproductive success among members of socially monogamous species. In birds, this variation occurs because paired males obtain extra-pair fertilizations (EPFs; Birkhead & Møller 1992), thereby enhancing their own reproductive success at the expense of the cuckolded individual.

Across bird species, variation in the frequency of extra-pair paternity is associated with the expression of male secondary sexual characters. Strong sexual dichromatism in plumage (where males have more brightly coloured plumage than females) is correlated with high rates of extra-pair paternity (Møller 1997; Owens & Hartley 1998). Therefore, objective measurement of reproductive success using molecular tools is essential for studies that seek to examine whether a potential ornament is maintained through sexual selection.

The red-capped robin (*Petroica goodenovii*, Petroicidae) is a socially monogamous but strikingly sexually dichromatic bird, in which adult males possess bright red forehead and breast patches, and females are grey-brown in colour (Boles 1988). Here, we describe a microsatellite-based genotyping system that allows unambiguous assignment of parentage in this species.

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We extracted DNA from blood samples taken from red-capped robins in Terrick Terrick National Park (36°10′ S, 144°13′ E) in northern Victoria, Australia, using a salting-out procedure (Bruford *et al*. 1992). Genomic DNA was enriched for GAAA and GT repeat-containing fragments in separate polymerase chain reactions (PCRs) with the same conditions, using the method described by Gardner *et al*. (1999) with modifications as detailed in Adcock & Mulder (2002). Positive clones were amplified in 50 μL reactions and the DNA extracted using the Qiaquick PCR purification kit (QIAGEN) following the manufacturer's instructions. PCR products were sequenced commercially (SUPAMAC, Sydney, Australia).

From 369 colonies screened in the GAAA-enriched genomic library, 16 positive clones were sequenced. Primers were designed (Life Technologies) for seven of these that contained five repeats or more and had suitable flanking sequence. From 48 colonies screened in the GT-enriched genomic library, nine positive clones were sequenced. Of these, primers were developed for three loci. Primer pairs that gave consistent, specific products were tested for polymorphism. One primer in each pair was manufactured with a 5′-M13 (5′CACGACGTTGTAAAACGAC) tail for use in the universal dye-labelling method described by Boutin-Ganache *et al*. (2001). Primer sequences, optimum annealing temperatures and $MgCl_2$ concentrations are listed in Table 1. We assessed polymorphism by typing at least 16 putatively unrelated individuals at each microsatellite

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Table 1 Characterization of microsatellite loci in the red-capped robin *Petroica goodenovii*. The final annealing temperature (T_M) is in °C and the $MgCl_2$ concentration in mM. The number of individuals tested (n), number of alleles (N_A), allele size range in base pairs (bp), and observed (H_O) and expected (H_E) heterozygosity and the probability of excluding a father (Ex) are listed for each locus. The M13 prefix in the primer sequence denotes a 5′ M13 tail (CACGACGTTGTAAAACGAC), attached to the primer sequence. Cloned sequences have been deposited with GenBank under Accession numbers AY289550–AY289556. Cross-amplification using primers for microsatellites in *Phylloscopus occipitalis*[1] and *Emberiza schoeniclus*[2] yielded two more polymorphic loci in *Petroica goodenovii*

Locus	Repeat motif in clone	Primer sequence (5′–3′)	n	T_M	[$MgCl_2$]	N_A	bp	H_O	H_E	Ex
Pgm1	$(CTTT)_3T(CTTT)_{20}$	F: TTTACTTGCTTAGCAGAAATGG R: M13-TTTCACAATTTTTGTGCATAGG	25	55	1.5	20	207–277	0.88	0.93	0.82
Pgm2	$(TC)_5(CTTT)_6CTGT(CTTT)_2$ CCTTCTTTCTCTTT	F: TCCTGTTACAAAACACTAATGAGG R: M13-TGTCTCACCACACCTTTATGC	29	52	2.5	8	223–240	0.38	0.47	0.29
Pgm3	$(TC)_4T(CTTT)_5$	F: M13-CACTGGGATGAAAAGACCTG R: TCTCCAGAGCTGGCTATAAAC	23	52	2.5	5	185–202	0.26	0.24	0.13
Pgm4	$(CTTT)_5$	F: AGGCTCATAGCCTGAAAGC R: M13-TGCTCATTTTTCATTTATCAGG	16	52	2.5	2	244–248	0.19	0.18	0.08
Pgm5	$(GT)_2TT(GT)_{16}$	F: AGTGCTGAACTGGGAGACC R: M13-AACCTGTCCTGCTTCTCTCC	30	54	2	5	201–289	0.47	0.39	0.2
Pgm6	$(CTTT)_6$	F: TGGGTAAGGAAAAGCTGACC R: M13-CAAATTTGTGAAGGAACAAGG	21	52	2.5	6	183–215	0.33	0.63	
Pgm7	$(CTTT)_5CTTA(CTTT)_3$	F: AGAGGCTGAGGACTAGTTGC R: M13-TCAACCCTTTGACAGTTTGC	28	52	2.5	12	220–260	0.46	0.91	
Pocc6[1]	—	F: TCACCCTCAAAAACACACACA R: M13-ACTTCTCTCTGAAAAGGGGAGC	16	52	2.5	6	196–206	0.75	0.75	0.50
Escmu6[2]	—	F: M13-CATAGTGATGCCCTGCTAGG R: GCAAGTGCTCCTTAATATTTGG	25	52	2.5	6	120–132	0.56	0.67	0.43

[1]Bensch *et al*. (1997); [2]Hanotte *et al*. (1994).

locus. All PCRs used *Taq* polymerase (0.25 units/10 μL), $MgCl_2$ (see Table 1 for concentrations), and a reaction buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100) and dNTPs (200 μM) supplied by Promega. Reactions were run in 0.2 mL microtitre plate (Greiner) wells layered with a drop of mineral oil (Sigma) on a Corbett Research PC-960C thermocycler. Reactions (10 μL) contained an M13 primer (200 nM) 5′-labelled with a Beckman Coulter dye (D2, D3 or D4), and the locus-specific tailed (15 nM) and untailed primer (200 nM), 40 ng of genomic DNA and the optimal concentration of $MgCl_2$. A total of 40 cycles of amplification were run: one cycle of 90 s at 94 °C followed by 40 cycles of 94 °C for 20 s, the optimal annealing temperature for 20 s and 73 °C for 90 s. PCR products (0.25 μL) were electrophoresed on a Beckman Coulter 8000XL automated sequencer using the CEQ 8000XL fragment analysis kit (Beckman Coulter) according to the manufacturer's instructions. Fragment sizes were estimated using the Beckman Coulter 8000XL fragment analysis software.

Seven loci were polymorphic. Table 1 shows the number of alleles found and the observed and expected heterozygosity for each locus. *Pgm4* had low levels of variability ($N_A = 2$, $H_E = 0.18$). The remaining loci were more variable ($N_A = 5–20$, $H_E = 0.24–0.93$). *Pgm6* and *Pgm7* showed heterozygote deficiency, probably indicating the presence of null alleles, and analysis using GENEPOP 3.3 (Raymond & Rousset 1995) indicated both loci deviated significantly from Hardy–Weinberg equilibrium. To increase the general exclusion probability (Equation 1a in Jamieson & Taylor 1997) for identifying extra-pair young, we used two further microsatellites isolated by cross-amplification using the *Pocc6* and *Escmu6* primers (Table 1). Using tests implemented in GENEPOP 3.3 (Raymond & Rousset 1995), no other loci showed significant deviation from Hardy–Weinberg equilibrium, and there was no linkage disequilibrium among loci ($P > 0.05$). The general exclusion probability, in the case where the mother is known, of seven variable, unlinked loci (*Pgm1*, *2*, *3*, *4*, *5*, *Pocc6*, *Escmu6*) is 0.9760. Offspring are rarely recruited close to their natal territories (DK Dowling, unpublished data). Thus, assigning parentage is straightforward because potential parents are not closely related.

Acknowledgements

This research was supported by the Australian Research Council, Birds Australia, the Department of Zoology, University of Melbourne, and the Holsworth Wildlife Research Fund. Blood samples were collected under Animal Experimentation and Ethics Register 99127 from the University of Melbourne and permits from the Department of Sustainability and Environment, Victoria.

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