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TECHNICAL NOTE

Isolation and characterization of microsatellites in the kakerori (*Pomarea dimidiata*) using feathers as source of DNA

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Abstract The kakerori (*Pomarea dimidiata*) is an endangered forest bird in the Cook Islands, South Pacific. We have developed 10 microsatellite markers using kakerori feathers as the DNA source. Seven of these loci were found to be polymorphic in 42 individuals examined. The number of alleles per locus in the polymorphic loci varied from 3 to 5. Observed and expected heterozygosity ranges were 0.57–0.74 and 0.50–0.74, respectively. All loci isolated conformed to Hardy–Weinberg equilibrium expectations. We believe these loci will be useful in studying kakerori conservation genetics, and our success in developing microsatellite markers from feather samples will encourage the use of less invasive sample sources in microsatellite isolation studies.

Keywords Microsatellite · Kakerori · Rarotonga flycatcher · Cook Islands · Feathers

The kakerori (*Pomarea dimidiata*; Monarchinae), also known as Rarotonga flycatcher or Rarotonga monarch, is a small forest passerine endemic to Rarotonga in the southern Cook Islands. The population has suffered a major decline in the late 1980's with an estimate of 29 surviving birds in 1989 (McCormack and Künzle 1990; Tiraa and Saul 1998). Subsequent management efforts, including the set up of the Takitumu Conservation Area Project by local landowners, has helped the kakerori population recover to about 250 birds at present, with a small insurance population on the island of Atiu (Robertson et al. 1994; Robertson and Saul 2006; Robertson et al. 2006). In this study, we isolated microsatellite DNA markers for assessing genetic variation and population structure in the recovering kakerori population.

When isolating microsatellites from avian species, the most commonly used sources of DNA are blood and muscle tissue. Because kakerori seem to suffer from stress when they are bled, we used feathers as a DNA source for our study. We constructed an enriched library using nylon membranes following Armour et al. (1994) as modified by Berry et al. (2003) and Chan et al. (2005) with a number of further modifications to the protocol as described in Berry et al. (2003) and Chan et al. (2005). Genomic DNA was extracted from the quills of 10 individuals' feather samples (6 breast feathers from each bird) using High Pure PCR Template Preparation Kit (Roche). About 250 ng of DNA from each individual was pooled and digested with *Sau*3A or *Taq*I restriction endonucleases (Roche), and the products were size fractionated on a 1% LE agarose gel (Roche). Although there were insufficient quantities of digested DNA on the gel to be visualized, we excised gel fragments corresponding to the 0.5–1.5 kb size range and recovered the DNA using High Pure PCR Product Purification Kit (Roche). The *Sau*3A digested DNA was ligated to SAU linkers (Royle et al. 1992), and *Taq*I digested DNA was ligated to TAQ linkers, a modification to the recognition sites of SAU linkers, made by combining oligonucleotides TAQLA (5′-GCGGTA CCCGGGAAGCTTGT-3′) and TAQLB (5′-GCACAAG CTTCCCGGGTACCGC-3′). Pre-enrichment PCR was

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performed in 25 µl reactions using 0.5 µM SAULA primer (5′-GCGGTACCCGGGAAGCTTGG-3′) or TAQLA primer, 1× *Ex Taq* buffer (Takara), 3 mM $MgCl_2$, 0.8 mM dNTPs, 0.625 U *Ex Taq* polymerase (Takara), and 5 µl template DNA. Reactions were cycled through 94°C—3 min, 30 × (94°C—30 s, 58°C—30 s, 72°C—90 s), 72°C—7 min on an Eppendorf Mastercycler ep thermocycler. We checked the PCR products by agarose gel electrophoresis and confirmed that fragments within the expected size range had been amplified. Nylon membranes (Roche), cut into 3 mm^2 squares, saturated with $(CA/GT)_n$, $(GA/CT)_n$, $(CAT/GTA)_n$, $(GAT/CTA)_n$, $(AAAG/TTTC)_n$, $(AAAC/TTTG)_n$, $(CATA/GTAT)_n$, $(GATA/CTAT)_n$, $(GACA/CTGT)_n$, or $(GTCA/CAGT)_n$ solutions were used to select for microsatellite containing fragments as described in Berry et al. (2003).

Enriched DNA fragments were PCR amplified under the same conditions as for the pre-enrichment PCR except the annealing temperature was increased to 67°C. Linkers were cleared by digestion with *Sau*3A or *Taq*I restriction endonucleases (Roche), and products were recovered with High Pure PCR Product Purification Kit (Roche). The digested fragments were ligated to pHSG396 plasmids (Takara; Takeshita et al. 1987) and transformed into competent *Escherichia coli* strain DH5α cells (Takara) which were plated on L-agar plates containing chloramphenicol, IPTG and X-gal (Bioline). White colonies were selected and transferred onto nylon membranes (Roche). $(CA/GT)_n$, $(GA/CT)_n$, $(CAT/GTA)_n$, $(GAT/CTA)_n$, $(AAAG/TTTC)_n$, $(AAAC/TTTG)_n$, $(CATA/GTAT)_n$, $(GATA/CTAT)_n$, $(GACA/CTGT)_n$, and $(GTCA/CAGT)_n$ probes were radioactively labelled with ^{32}P using High Prime DNA labelling kit (Roche), and were used to screen colonies on nylon membranes as described in Berry et al. (2003). After washing, the membranes were dried and exposed on Fuji Super RX film (Fujifilm). Candidate colonies were identified from autoradiographs. Plasmids were isolated from 353 positive colonies (219 from *Sau*3A digest and 134 from *Taq*I digest) using QIAprep Miniprep kit (Qiagen), their avian DNA inserts were sequenced on an Applied Biosystems 3730 Genetic Analyzer.

We designed 30 sets of PCR primers based on recovered DNA sequences using the OLIGO software (version 4.0 for Macintosh; Molecular Biology Insights, http://www.oligo.net). Sequences with sufficient flanking region (>30 bp on each end) to the microsatellites (18 from *Sau*3A digest, 12 from *Taq*I digest) were selected. We were able to optimize amplification conditions for 10 loci (Table 1). Candidate loci were amplified in 10 µl reactions containing 1× *Ex Taq* buffer (Takara), 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM of each primer, 0.25 U *Ex Taq* polymerase (Takara), and about 10 ng of DNA templates. Reactions were run in an Eppendorf Mastercycler ep thermocycler for 94°C—3 min, 30 × (94°C—15 s, T_a—15 s, 72°C—30 s), 72°C—7 min (T_a values are given in Table 1). The loci were checked for allele size polymorphisms in 42 kakerori individuals by incorporating 1 µM of fluorescein-12-dUTP (Roche) into PCR products and genotyping them on an Applied Biosystems ABI Prism 377 DNA sequencer. Seven loci were found to be polymorphic, and the forward primers for amplifying these loci were fluorescently labelled (Table 1) for genotypic analyses on an Applied Biosystems 3730 Genetic Analyzer. At least one example of each allele was sequenced to confirm that all size variation occurred due to changes in number of repeat units. In our genotyping and sequencing data, we encountered unexpected observations at 3 of the loci (marked with * in Table 1). In *Pdim2021*, *Pdim3031* and *Pdim5657*, the alleles isolated from the clones were not found in the kakerori population samples screened, and in *Pdim3031*, the repeat motif in the cloned allele $(CTAT)_{12}$CCATC-TAT$(CCAT)_{11}$ was not observed when PCR products from genomic DNA amplifications were sequenced. All examples of genomic *Pdim3031* alleles we have sequenced showed the $(CTAT)_n(CCAT)_n$ repeat motif. It is possible that these are artefacts generated in the enrichment process (Koblízková et al. 1998), but regardless, these should not affect the use of the loci in subsequent population genetic studies. However, these observations do highlight the importance of using DNA sequencing to characterize ambiguous alleles or to detect potential size homoplasy as recommended by Chambers and MacAvoy (2000).

Observed and expected heterozygosity values were calculated using the Microsatellite Analyser software (version M3.15; Dieringer and Schlötterer 2002; Table 1). Deviations from Hardy–Weinberg equilibrium (HWE) were tested by the Markov chain method (Guo and Thompson 1992) as implemented in GENEPOP (version 3.4; Raymond and Rousset 1995). None of the loci showed significant deviations from HWE ($P > 0.05$) while *Pdim2021* showed significant heterozygote excess ($P = 0.01$). Linkage disequilibrium was also tested using the GENEPOP software, and no significant linkage disequilibrium was detected. Sequential Bonferroni corrections (Rice 1989) were applied to all multiple tests.

We expect that the loci isolated here will be useful in studying population genetics in the kakerori, and the success in developing microsatellite markers from feathers will encourage less invasive sampling strategies in conservation genetics studies.

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Table 1 Microsatellite loci isolated from the kakerori

Locus	Primer sequences 5′–3′	Repeat motif as first isolated	*N*	Allele sizes	T_a	H_O	H_E	Enzyme
Pdim2021	F: *NED*-TGGAGAGAAGAAATGAGA R: AAATGAGGTTAGATGTTGAT	$(CTTC)_5(CTTT)_9TTT(CTTT)_{13}TT(CTTT)_4[(CT)_2(CTTT)_2]_2$ $(CT)_2CTTT(CCTT)_3CTTT[(CCTT)_3(CTTT)_2]_2$	4	354–388 (384*)	53	0.74	0.58	*Sau*3A
Pdim2829	F: *6FAM*-GATTCCCTCCCCACCCACTCA R: CATTTGGGGCTGGTGCTGCTGTA	$(CTGT)_5(CTAT)_8$	4	348–372 (348)	63	0.74	0.72	*Sau*3A
Pdim3031	F: *VIC*-GATTCCCCACTGACAGGCACTTC R: GAATCCCGGGGAAACGCTCTG	$(CTAT)_{12}CCATCTAT(CCAT)_{11}$*	4	347–375 (343*)	51	0.67	0.66	*Sau*3A
Pdim3233	F: *6FAM*-CCAGACACCAGGGGCTACA R: TTGGAGGCAGCATGGGGAAGA	$CTATCTGT(CTAT)_6CTAC(CTAT)_9$	3	234–242 (238)	60	0.69	0.63	*Sau*3A
Pdim3435	F: *VIC*-ATTTGCTTAGGTGCCATTC R: GAAGCACTATTTTACCCCACAGA	$(GATA)_{16}$	5	197–221 (217)	54	0.64	0.61	*Sau*3A
Pdim3637	F: *6FAM*-GGCTTAATGGCAGCACTC R: ATTCCTTAAATCTCCCGGTGT	$CTATCCAT(CTAT)_9$	3	144–160 (160)	51	0.57	0.50	*Sau*3A
Pdim5657	F: *VIC*-CTGGTACTCCCTGGGATGCTGAG R: GGAGAAAGGCTGGCACCACATA	$(CAT)_{20}$	5	138–159 (153*)	61	0.60	0.74	*Sau*3A
Pdim0203	F: TTGAGGCTCAGGCTCTCT R: GCATCCTGCTGATAAATTTG	$(GTTT)_6$	1	249 (249)	51	–	–	*Sau*3A
Pdim1011	F: CCTCGGCTCGGTCTCCAA R: ACGGTCCCTCGCAGCAACTT	$(CT)_7$	1	382 (382)	51	–	–	*Taq*I
Pdim5051	F: GGCAGTGACCAAAGTGGGCTATG R: TCCGAGTCCTGCTTATAG	$(GAT)_2GT(GAT)_5GAC(GAT)_5$	1	164 (164)	51	–	–	*Sau*3A

N = number of alleles, T_a = annealing temperature of PCR in °C, H_O = observed heterozygosity, H_E = expected heterozygosity. The allele sizes column shows the range of allele sizes in base pairs observed in 42 individuals, with the sizes of the cloned alleles in brackets. The restriction endonuclease which was used initially to digest genomic DNA is indicated in the Enzyme column. DNA sequences of microsatellite loci isolated in this study have been deposited in DDBJ under Accession Nos. AB364309–AB364318

sample collection, also Ian Karika (Takitumu Conservation Area, Cook Islands) for arranging field work logistics. The Takitumu Conservation Area was established in Rarotonga in 1996 by local landowners to protect native animals and plants in the Cook Islands.

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