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TECHNICAL NOTE

Isolation and characterisation of microsatellite markers from the South Island robin (*Petroica australis*)

Sheena M. Townsend · Tania M. King · Ian G. Jamieson

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Abstract We identified 20 novel polymorphic microsatellite markers for use in South Island robins (*Petroica australis*). Markers were isolated from clones enriched for microsatellite repeats, and using 454 pyro-sequencing. South Island robins, like many New Zealand endemic species, have relatively low microsatellite diversity. Alleles per locus ranged from 2 to 7 (mean 3.00, SE 0.299). Mean expected heterozygosity was 0.436 (SE 0.047). Markers were amplified using cost-effective universal fluorescent labeling in multiplex PCR and will be useful in conservation genetics research involving this locally threatened species.

Keywords New Zealand robin · *Petroica australis* · Multiplex PCR · Microsatellites · 454 GS-FLX · Genetic diversity

The South Island robin (*Petroica australis*) is a small forest passerine native to New Zealand and includes the subspecies *P. a. rakiura* on Stewart Island (Higgins and Peter 2002; Miller and Lambert 2006). Although classified as a species of least concern (IUCN 2011), robins are locally threatened by introduced predators, and the Stewart Island subspecies is listed as 'Nationally Vulnerable' (Higgins and Peter 2002; Miskelly et al. 2008). On Stewart Island, population declines prompted the translocation of 25 individuals in 2000 to Ulva Island, an open sanctuary established by the Department of Conservation and Ulva Island Trust. This population is closely monitored and represents an opportunity to study a small, wild bird population for which a high quality pedigree has been constructed (Jamieson 2010). An informative set of neutral markers will complement the pedigree for use in conservation genetics studies of South Island robins aimed at better understanding the genetic consequences of bottlenecks, small population size and inbreeding (Jamieson 2010).

Ten microsatellite loci, originally developed for use in other species, were previously identified as polymorphic in South Island robins, including those on Ulva Island (Boessenkool et al. 2007). Increasing the number of markers will offset low genetic diversity typical of this and other New Zealand native birds.

We prepared a library enriched for dinucleotide STRs $(GA)_{12}$ and $(GT)_{12}$ using DNA extracted from two South Island robins following the methods of Glenn and Schable (2005). We sequenced positive clones using an ABI 3730 Genetic Analyser (Applied Biosystems) and edited sequences using SEQUENCHER v3.7. We also isolated microsatellites using shotgun sequencing scaled to 1/8th of a run on the Roche 454 GS-FLX System (Roche, Penzberg, Germany) following the methods of Abdelkrim et al. (2009). Reads were screened for di-, tri and tetranucleotide microsatellite repeats using MSATCOMMANDER v0.8.1 (Faircloth 2008). Primers were design for sequences obtained from both methods using PRIMER3PLUS (Untergasser et al. 2007). We appended forward primers with M13 sequence (5′-TGTA AAACGACGGCCAGT-3′) at the 5′ end to facilitate the use of universal dye labeled primers as described in

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S. M. Townsend (✉) · T. M. King · I. G. Jamieson
Department of Zoology, Allan Wilson Centre for Molecular Ecology and Evolution, University of Otago, 340 Great King Street, Dunedin 9016, New Zealand
e-mail: townsend.sheena@gmail.com

Schuelke (2000) and added PIG-tails (5′-GTTTCTT-3′) to the 5′ end of reverse primers to improve scoring (Brownstein et al. 1996). Additional information about clone screening and read yields from 454-sequencing can be found in the supplementary material (Online Resource ESM_1).

Table 1 Characterisation of 20 microsatellite loci developed for use in South Island robins

Locus	GenBank number	Primer sequence (5′–3′)	Dye label	N_A	Allelic size range (bp)	H_o/H_e	N
Pau1	JQ013708	F: TTAGAAGTGAAAGGCTTG	6-FAM	2	123–125	0.454/0.466	273
		R: GAGGAATAAAAACAATGC[a]					
Pau2	JQ013709	F: TTGCCAGAAACCTCACACTG	6-FAM	3	267–270	0.491/0.458	271
		R: CTCTTCCACCACAAAGCACA					
Pau4	JQ013710	F: TCCCTTGCAATCCCTTCATA	VIC	5	273–298	0.612/0.598	273
		R: GTGGCCTCATTCAAAACCAC					
Pau6	JQ013711	F: CTGAGGTTCAAAGTTTCC	6-FAM	3	124–129	0.390/0.367	272
		R: ACCAGCCATCCTTATGC					
Pau7	JQ013712	F: TTCCTTGACTGAAGTGGGTTG	PET	3	271–276	0.655/0.642	264
		R: TGTGTTCTCCATGCTTCTGC					
Pau8	JQ013713	F: AATAAATGCACCCAGCAACC	VIC	2	364–368	0.155/0.143	252
		R: GCAGGATTGCTTTTTGTCCT					
Pau9	JQ013714	F: ATGATGTAGTCAGAGTCG	6-FAM	7	134–158	0.824/0.805[b]	272
		R: TATTTTGCAACCTTCTTG					
Pau16	JQ013715	F: GTTGGGACTGATGGAGTGGT	PET	2	285–287	0.500/0.496	266
		R: TGGCAGCTTTATTGCTGATG					
Pau17	JQ013716	F: CTTGGTAAGAAAATGATC	VIC	3	139–146	0.517/0.496	263
		R: TCAAGATGAGTGTTACTG					
Pau24	JQ013717	F: ATACCATTCAGGCAGAGATGC	VIC	3	167–175	0.376/0.399	271
		R: TCTGAATACATTACACCGAGAGGA					
Pau25	JQ013718	F: TGGCAAAGTCAGTTCCATGT	NED	3	194–198	0.631/0.618	255
		R: TCTGAGGTGAACTGGTGGTAGA					
Pau26	JQ013719	F: AAATTACTACAGTGTTACGGTGAAAA	6-FAM	4	186–198	0.663/0.633	273
		R: GGGACCACCAAGAACTTCAA[a]					
Pau28	JQ013720	F: GCATCACGCTGCAGTAGAAG	6-FAM	3	164–167	0.121/0.115	272
		R: GCTCCTAAGGAAGCCTCTGG					
Pau39	JQ013721	F: CAGTTTTCAGGCAGCACAAG	NED	2	175–177	0.213/0.231	267
		R: CTCCTGATCCTCCAGCTCTC[a]					
Pau63	JQ013722	F: TCTGGTGAAAGCAAGGAACC	VIC	5	152–176	0.781/0.758	270
		R: CCCATAAGAACCTGCCACTG					
Pau66	JQ013723	F: TGGGCCAGTTTATACCCTCT	PET	2	124–132	0.261/0.227	268
		R: ATGAAAGGGTTCCATGATGC[a]					
Pau67	JQ013724	F: CCAGGAAAGGTGCTCAGAGT	6-FAM	2	198–209	0.495/0.471	273
		R: TGTCTGTGTTGGCCTGATCT[a]					
Pau77	JQ013725	F: TCAATCTTGTTGGAGCTTTGC	PET	2	175–178	0.576/0.500	269
		R: TCTGGTACTTGAAAGCTTGTTGA					
Pau81	JQ013726	F: ACCTCCTGTGAGGCTGACC	NED	2	151–154	0.246/0.227	268
		R: TGCAGATGTGTGAGCAACAG					
Pau82	JQ013727	F: CCTGCTTTTGTAGGGGTGAA	6-FAM	2	166–168	0.084/0.080	274
		R: AGCTTCTGCCATCATCATCC					

F Forward primer, *R* reverse primer, N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, *N* number genotyped. Allelic size range is based on the total amplified fragment length

[a] Reverse primer without PIG-tail

[b] Deviates from Hardy–Weinberg equilibrium after Bonferroni correction

We screened loci for polymorphism initially using simplex PCR in 12 to 24 individuals, assigning polymorphic loci to multiplex groups with the aid of MULTIPLEX MANAGER v1.1 (Holleley and Geerts 2009). Online supplemental material contains details on multiplex grouping, repeat motifs and the assignment of these loci to homologous locations in the zebra finch genome following Olano-Marin et al. (2010) (Online Resource ESM_1). For PCR amplification, Multiplex PCR was carried out in 384-well plates containing 10 ng of DNA dried into wells at room temperature prior to PCR set-up. Each 2 µl reaction contained 1 µl Type-it Master Mix (QIAGEN), 1 µl primer mix containing forward (0.04 µM) and reverse (0.16 µM) locus-specific primers and M13-tagged fluorescent dyes (6-FAM, VIC, NED, PET) (0.16 µM × number of loci) (Schuelke 2000).

Thermocycling conditions were 95°C for 15 min, followed by a touchdown sequence comprising 94°C for 30 s, annealing for 90 s and extension at 72°C for 60 s. Annealing temperature started at 60°C and reduced 1 degree per cycle for 8 cycles. This was followed by 25 cycles at 94°C for 30 s, 52°C for 90 s and 72°C for 60 s with a final 30 min hold at 60°C.

Following PCR amplification, fragment sizes were resolved on an ABI 3730 Genetic Analyser (Applied Biosystems) with GeneScan 500 (LIZ)^{tm} size standard and scored using GENEMAPPER v4.1 (Applied Biosystems). GENEMAPPER scores were visually confirmed and ambiguous scores omitted. Expected and observed heterozygosities were calculated using the GENALEX v6.41 (Peakall and Smouse 2006) extension for Microsoft Excel 2011. Deviation from Hardy–Weinberg equilibrium was calculated using GENEPOP v4.0.10 (Raymond and Rousset 1995; Rousset 2008). Data were checked for evidence of null alleles and scoring errors with MICRO-CHECKER v2.2.3 (van Oosterhout et al. 2004) and for linkage between pairs of loci using genotyping data from 2007 (n=31), 2008 (n=30) and 2009 (n=45) separately with GENEPOP (Raymond and Rousset 1995; Rousset 2008).

Of the 69 loci screened, 20 were used to genotype 275 birds from Ulva Island (Table 1). Alleles per locus ranged from 2 to 7 (mean 3.00, SE 0.299) and mean expected heterozygosity was 0.436 (SE 0.047). After sequential Bonferroni correction (Holm 1979), Pau09 showed deviations from Hardy–Weinberg equilibrium, however, we found no evidence of null-alleles. No locus pairs showed evidence of significant linkage disequilibrium across the 3 years examined. Two additional polymorphic loci, Pau20 (GenBank accession number JQ289044) and Pau23 (JQ289045), failed to amplify well in our multiplex groups using M13 primers but sequence information is available online (http://www.ncbi.nlm.nih.gov/genbank/).

The markers described here will be used in conservation genetics studies of South Island robins and would be candidates for screening in other New Zealand passerines.

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