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TECHNICAL NOTE

The utility of existing passerine microsatellite markers for genetic studies in endangered species: as demonstrated for a critically endangered forest bird endemic to Réunion Island, the Réunion cuckooshrike (*Coracina newtoni*)

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Abstract Genetic data are increasingly recognized for their utility in conservation programs. However, many endangered species belong to families that have been understudied. Due to the urgency of their conservation status it is important to quickly identify polymorphic microsatellite loci from available resources. We show for the Réunion Cuckoo shrike *Coracina newtoni*, that this strategy can be very useful. Using 110 passerine microsatellite primer sets we identified eighteen polymorphic loci and tested them in 25 *C. newtoni* individuals. Following a Bonferroni correction one pair of loci displayed linkage disequilibrium (P-value < 0.0001).

Keywords Campephagidae · *Coracina newtoni* · Cross-species utility · Cuckoo shrike · Microsatellite · Passerine

The Réunion Cuckoo shrike, *Coracina newtoni*, a forest bird endemic to Réunion Island, is among the rarest and most threatened bird species in the world and was recently classified as critically endangered (IUCN 2009). The single remaining population is currently confined to a small area of 12 km^2 at altitudes ranging from 1,300 to 1,800 m and the total population size estimate is of 70–100 individuals (SEOR 2008). The causes of such a narrow distribution range are still debated and include introduced predators (rats and cats; Thiollay & Probst 1999; Salamolard et Ghestemme 2004; Ghestemme and Salamolard 2007).

Genetic approaches using neutral genetic markers have the potential to quickly improve our knowledge for endangered species (Allendorf and Luikart 2007) by describing diversity, inferring demographic history (e.g. Goossens et al. 2006; Johnson et al. 2009; Bourke et al. 2010). *C. newtoni* belongs to the Campephagidae subfamily, which contains eighty species currently listed by the IUCN, out of which 48 belong to the *Coracina* genus. Since no specific markers were available for Campephagidae we selected and screened a large set of microsatellite loci.

Blood sampling was conducted in the RNRE. Samples were taken from chicks just before they fledged each year between 2003 and 2009. Blood samples were kept on blotting paper and/or in a Longmire's buffer (Longmire et al. 1988). DNA was extracted by the method described by Medrano et al. (1990), and its concentration was assessed using a Nanodrop^{TM} spectrophotometer (Thermo Fisher Scientific Inc.).

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Table 1 List of polymorphic loci, accession number, species from which the primers were designed, primers, and related literature

Primer set	Accession number	Species from which the primers were designed	Primer sequence 5′–3′	Fluorescent label	N	Alelle size range	A	He	Ho	HWE	Reference for sequence isolation and primer set design
Ase18	AJ276375	*Acrocephalus sechellensis*	F:ATCCAGTCTTCGCAAAAGCC R:TGCCCCAGAGGGAAGAAG	6FAM	25	118–120	2	0.50	0.60	0.42	Richardson et al. (2000)
Asu15-ZEST	AY172993	*Ammodramus savannarum*, *Taeniopygia guttata*	F:AATAGATTCAGGTGCTTTTTCC R:GGTTTTTGAGAAAATTATACTTTCAG	6FAM	21	145–151	4	0.63	0.65	0.33	Bulgin et al. (2003); Dawson et al. (unpublished)
CK.1B6G	AF026333	*Corvus kubaryi*	F:ATGGAGTGGAGGGAGAGATG R:AGTACTACCAGTTCACCTGC	6FAM	25	276–292	5	0.69	0.68	0.38	Tarr and Fleischer (1998)
CK.5A5F	AF026338	*Corvus kubaryi*	F:GTGGTTATACCAGAGGTCCT R:TTTTGTTCTCTCAAGACACC	HEX	25	328–348	6	0.74	0.68	0.47	Tarr and Fleischer (1998)
CmeH2	AY330729	*Corcorax melanorhamphos*	F:AAGCTTTGGGTCATATGTGT R:CTGCAGCTTGAGTGAAAGTT	6FAM	25	253–261	5	0.54	0.52	0.11	Beck et al. (2003)
MSLP4-ZEST	AB031376	*Locustella pryeri*, *Taeniopygia guttata*	F:CAGTGCAAGCCTCTACTCCTCTGC R:CCTTGGCTCTGCCRCACCTC	HEX	25	212–214	2	0.25	0.20	0.38	Ishibashi et al. (2000); Dawson et al. (unpublished)
Pca3	AJ279805	*Cyanistes caeruleus* (*Parus caeruleus*)	F:GGTGTTTGTGAGCCGGGG R:GGTGTTTGTGAGCCGGGG	HEX	25	127–143	7	0.70	0.68	0.75	Dawson et al. (2000)
PmaTGAn42	AY260540	*Parus major*	F:ACTTCCACATGCCAGTTTCC R:TGTTAAGGCAGAGAGGTGGG	6FAM	25	189–195	4	0.63	0.68	0.29	Saladin et al. (2003)
Ppi008	FM865709	*Pica pica*	F:ATCAGCCAGAACCCCTTTTT R:TGAGGGAAGAGTCAAACTCCA	HEX	25	138–140	2	0.33	0.32	1.00	Martín-Gálvez et al. (2009)
Ppi012	FM865713	*Pica pica*	F:GCAGAGGCTGCTCCCTAAG R:GTGACAGTTCCTCGTTTCTGC	HEX	25	123–125	2	0.43	0.52	0.37	Martín-Gálvez et al. (2009)
Ppi016	FM865717	*Pica pica*	F:AAAGCTTTCTGGAGAACAGTGC R:CGTTGCATCTATGAGAGCTGAG	6FAM	25	140–172	8	0.81	0.80	0.19	Martín-Gálvez et al. (2009)
Ppi018	FM865719	*Pica pica*	F:GATTGTTCGAGTGCTCTCAATG R:TGGATTTATGCACTTCCAAGC	6FAM	25	161–179	3	0.08	0.08	1.00	Martín-Gálvez et al. (unpublished)
SAP47-ZEST	AY823673	*Eastern phoebe* (*Sayornis phoebe*), *Taeniopygia guttata*	F:CAGTGCAAGCCTCTACTCCTCTGC R:CTTGGCTCTGCCGCACCTC	HEX	22	383–391	2	0.51	0.50	1.00	Beheler et al. (2007); Dawson et al. (unpublished)
TG02-088	DV579347	*Taeniopygia guttata*, *Gallus gallus*	F:TGTGTGTTGACAGTATTCTCTTGC R:TTTAAACCTAATAAACGTCACACAGTC	6FAM	25	258–264	2	0.25	0.28	1.00	Wada et al. (2006); Dawson et al. (2010)
TG04-004	DV946288	*Taeniopygia guttata*, *Gallus gallus*	F:CTGGAGCAGTATTTATATTGATCTTCC R:GAAGATGTGTTTCACAGCATAACTG	HEX	25	170–172	2	0.48	0.52	1.00	Replogle et al. (2008); Dawson et al. (2010)
Tgu07	DV948303	*Taeniopygia guttata*	F:CTTCCTGCTATAAGGCACAGG R:AAGTGATCACATTTATTTGAATAT	HEX	25	103–107	5	0.65	0.80	0.43	Replogle et al. (2008); Slate et al. (2007)

Table 1 continued

Primer set	Accession number	Species from which the primers were designed	Primer sequence 5′–3′	Fluorescent label	N	Alelle size range	A	He	Ho	HWE	Reference for sequence isolation and primer set design
TGZ-037	DV945670	*Taeniopygia guttata*, *Gallus gallus*	F:AAAACACCTTGTAATTTAAAACTGG R:CATAGATACATATCAATACAGCACATTC	6FAM	19*	109–162	2	0.14	0.14	1.00	Replogle et al. (2008); Dawson (2007)
ZEST09-005	DV954446	*Taeniopygia guttata*	F:AACCCAACCAACAAAATTGG R:CCAACTATCAGTTTTACAAGGCATAC	6FAM	25	160–162	2	0.50	0.44	0.69	Dawson et al. (unpublished), see Ball et al. (2010)

Number of alleles (*N*), unbiased expected Heterozygosity (*He*), observed Heterozygosity (*Ho*) and *P* value for departure from Hardy-Weinberg equilibrium test (*HWE*)

* Due to Z-linkage of TGZ-037 loci, He, Ho and HWE calculation have been performed using only one allele for female and both alleles for males

We tested a total of 110 microsatellite primer pairs (corresponding to 108 loci) for amplification (Supplementary Table 1) including 54 marker sets developed to be of high-utility in multiple species (Dawson 2007; Dawson et al. 2010; Dawson et al. unpublished data), 29 microsatellite loci developed from related corvid species (Martín-Gálvez et al. 2009) and several other loci which were known or suspected to be of high-utility based on their testing in multiple species in Galbusera et al. (2000), Lillandt et al. (2002) and from the BIRDMARKER database provided by the Molecular Ecology Laboratory of the University of Sheffield (http://www.shef.ac.uk/nbaf-s/databases-birdmarker.html; Dawson et al. unpublished).

All 110 microsatellite primer sets were first tested on one individual for amplification using the following PCR protocol. The cycling conditions for amplification included 5 min at 95°C for polymerase activation followed by 35 cycles of 30 s at 94°C, 30 s at 50, 55 or 60°C (i.e. three hybridization temperature were always separately tested), and 30 s at 72°C and a final elongation step consisting of 10 min at 72°C. Reactions (10 µl) contained Gotaq reagents (Promega) with 1.5 mM of $MgCl_2$, 0.4 units of Taq, 0.8 mM of dNTPs, 0.1 µM (each) of forward and reverse primers, and 10 ng of template DNA. PCR amplification and size of the product were assessed by using 5% high resolution MetaPhor® agarose gel electrophoresis of PCR products. Both clearly and weakly amplifying products from labeled primers (all except 17, see Supplementary Table 1) were subsequently tested on ABIprism XI3130 Genotyper (Applied Biosystems). As Supplementary Table 1 indicates, 93 of the 110 primer pairs amplified, and 77 (corresponding to 75 loci) of them displayed clear amplification products close in size to that expected.

The 75 loci were tested for polymorphism using six unrelated individualsby multiplex amplifications performed using 2–6 fluoro-labeled primers pairs in a 15 µl reaction using 7.5 µl 2× Qiagen Multiplex buffer with 0.1 µM (each) of forward and reverse primers, and 10 ng of template DNA. The primers were labeled with either 6FAM or HEX (Supplementary Table 1). PCR cycling conditions for amplification included 5 min at 95°C for polymerase activation followed by of 38 cycles of 30 s at 94°C, 90 s at 58°C and 60 s at 72°C and a final elongation step consisting of 30 min at 60°C as recommended by the supplier. Obtained profiles were analyzed with the Peak Scanner™ Software Version 1.0 (Applied Biosystems). Based on the typing of six individuals, 43 loci were monomorphic and 10 were hard to score, the remaining 22 appeared polymorphic and were selected (Supplementary Table 1) to genotype 25 unrelated individuals.

Out of the 22 loci one appeared to be actually monomorphic (TCIIB4E), and three loci (Pdo23; Ppi011; TG01-147) were removed due to difficulty in peak interpretation.

After sequential Bonferroni correction (Rice 1989), linkage disequilibrium was detected using GENETIX 4.05.2 (Belkhir et al. 2004) between one pair of loci (SAP47 and CmeH2, P-value < 0.0001). None of the eighteen polymorphic scorable loci (Table 1) departed from Hardy–Weinberg equilibrium using a Markov-chain method implemented in GENEPOP v.4.0 (Rousset 2008). No null alleles or other amplification errors were detected using MICRO-CHECKER (van Oosterhout et al. 2004), in any of the 18 loci (Table 1). One locus (TGZ-037), known to be sex-Z linked in various passerine species (Dawson 2007; Dawson et al. unpublished data) was also found to be Z-linked in *C. newtoni*.

Our study indicates that from starting with 108 microsatellite loci we were able to identify 18 polymorphic loci in a highly endangered bird with a very low population size. This proportion (ca. 17%) suggests that even if it is necessary to test many microsatellite loci to identify polymorphic ones, there is currently a large enough database to identify a reasonably large set of usable microsatellites for many endangered passerine species. We believe that the microsatellite markers we have successfully identified will be crucial to study genetic diversity in the numerous endangered Campephagidae species of the Indian Ocean and elsewhere.

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