

PRIMER NOTE

Polymorphic microsatellite loci for studies of bronze-cuckoo species (Genus *Chalcites*: Aves)

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Abstract

A set of 17 microsatellite loci was shown to provide at least seven that were polymorphic in each of three bronze-cuckoo species (*Chalcites basalis*, *C. lucidus* and *C. minutillus*) representing the taxonomic range of this genus. This set includes nine newly isolated loci from genomic libraries constructed from *C. basalis* and *C. lucidus*. For these three species, each had seven or more polymorphic loci that showed no significant linkage or Hardy–Weinberg disequilibrium with more than five alleles (mean 7.6–12.1) and expected heterozygosity greater than 0.5 (mean 0.78–0.85).

Keywords: bronze-cuckoo, *Chalcites*, microsatellite, polymorphism

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There are six or more species and numerous subspecies of the brood-parasitic bronze-cuckoos (Genus: *Chalcites*), found in Australia and southern Asia (Payne 2005). Molecular markers are likely to be particularly useful for illuminating aspects of their breeding biology, which cannot easily be determined by field observations because the cuckoo's crypsis is a key adaptation for brood parasitism (Davies 2000). A further use for molecular markers is to examine the pattern of genetic variation that results from the host-parasite evolutionary arms race that each cuckoo species is undertaking with one or more hosts. Here we report the development of new markers to add to those previously reported (Adcock *et al.* 2005) that we believe will be useful for population and behavioural studies in all *Chalcites* species.

Cuckoo DNA was extracted using a salting-out procedure (Bruford *et al.* 1992). For all species, samples that were presumed to be unrelated were chosen to assess variation in each locus. Horsfield's bronze-cuckoo (*Chalcites basalis*) samples are described in Adcock *et al.* (2005) with additional tissue samples ($n = 10$) from eastern Australia from the Australian National Wildlife Collection (ANWC).

The ANWC also supplied samples of shining bronze-cuckoos (*Chalcites lucidus*, $n = 8$) from eastern Australia and little bronze-cuckoos (*Chalcites minutillus*, $n = 22$) from northern and eastern Australia, in addition to samples we collected, respectively, for each species from Canberra ($n = 8$) and Darwin ($n = 2$).

Microsatellite loci were isolated from two libraries enriched for clones containing GA repeats. A new library was constructed using DNA from a single *C. lucidus* individual following the methods reported in Gardner *et al.* (1999) with modifications (Adcock & Mulder 2002). The second library was previously constructed from *C. basalis* DNA (Adcock *et al.* 2005). For both, repeat-containing clones were identified with a polymerase chain reaction (PCR)-based method and sequenced on both strands using published methods (Adcock & Mulder 2002). For the *C. lucidus* library, 264 clones were screened and 40 were sequenced and for *C. basalis* 48 screened and seven sequenced. Primers were manufactured (Proligo) for the 14 clones from *C. lucidus* and the four from *C. basalis* that contained seven repeats or more and flanking sequence suitable for primer design. One primer in each pair had a 5'-M13 (TGTAACGACGGCCAGT) tail for use in the universal dye-labelling method described by Schuelke (2000). Initial testing for amplification with these primers

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Table 1 New microsatellite loci isolated from Horsfield's (*Chalcites basalis*) and shining (*Chalcites lucidus*) bronze-cuckoos. The asterisk (*) in the primer sequence denotes a 5' tail (TGTAACGACGCGCCAGT), attached to the primer sequence

Locus	GenBank	Repeat motif in clone	Primer sequence (5'-3')
Clones from <i>C. basalis</i>			
Cba08	DQ914553	(CT) ₁₅ T(TC) ₆	AACACCAACCTGTTCAGAGC *TCCTGAGAAACTGTCACTGC
Cba09	DQ914554	(AG) ₁₃ (TG) ₂ GCA(TG) ₆ (AG) ₅	TGTAATAGCTCTTCACTTGACTGC *AGCTTTGGTGTGCGTGTACT
Cba10	DQ914555	(GA) ₇ GG(GA) ₆ ... (TTTG) ₆	GACAGAAAGAGCCGAGACG *CTCCTGGCTGAGTGTGTGC
Clones from <i>C. lucidus</i>			
Clu01	DQ914556	(CA) ₇ (GA) ₁₁	CATCATGAATGGAGTAATTTGG *GCCACAACAGTAGGAGAAGG
Clu02	DQ914557	(GATG) ₃ GAT(AG) ₃ G(GA) ₇	GGCATCTTCATGTGAGATAGG *GCTTTTATTTAGCATTGATTTGG
Clu03	DQ914558	(CG) ₆ (TC) ₂ (TG) ₉ A(GT) ₅	CTGTACACTGCCTGCCTTGG *CTGTGCTCCTGAATTTCC
Clu04	DQ914559	(GA) ₈	CTGAGATGGCTTCTGTCAAC *AAAGGAGCACTTGCTGTCC
Clu05	DQ914560	(CT) ₁₀ T ₁₀	TTGAATAGAAACATTTCAGAGAGC *CACAACCACTGTGTGTGAGC
Clu06	DQ914561	(AG) ₃ T(GA) ₁₁	AATATTTCTGCCCTAGCAAGC *CTTTGGTTGCATAAACAGC

was carried out with four individuals from each species using 10- μ L reactions containing the following: 40–100 ng of genomic DNA, 2.5 mM MgCl₂, reaction buffer (concentration in reaction: 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100) and dNTPs (200 μ M) supplied by Promega. Cycling conditions were: 1 cycle of 94 °C for 90 s, 2 cycles of 94 °C for 20 s, 58 °C (annealing) for 20 s and 73 °C for 90 s, then 2 cycles with annealing temperature of 56 °C, then 34 cycles with annealing temperature of 54 °C, finishing with 5 min at 73 °C. About 5 μ L of product was visualized on agarose gels using standard methods (Sambrook *et al.* 1989). Primers that failed to amplify were amplified with stringency as low as 48 °C final annealing temperature and 3 mM MgCl₂. Where multiple bands or smeared products were observed, stringency was raised as high as 60 °C annealing temperature and 1.5 mM MgCl₂. A minimum of eight individuals were tested for polymorphism for each species. The reaction conditions differed from the screening reactions as they contained an M13 primer (200 nM), 5'-labelled with an ABI dye (VIC, FAM or NED), and the locus-specific tailed (15 nM) and untaild primer (200 nM). All primer pairs that gave consistent specific products were able to be amplified with final annealing temperature 52 °C and 2.5 mM MgCl₂. PCR products were electrophoresed on an ABI 3100 automated sequencer together with a LIZ-500 size standard (ABI) according to the manufacturer's instructions. Fragment sizes were estimated using the ABI GENESCAN software.

Table 1 describes details of all new polymorphic loci and Table 2 summarizes the variation observed for each species

in these loci and those reported previously (Adcock *et al.* 2005). We tested for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium using the tests implemented in GENEPOP version 3.1b (Raymond & Rousset 1995) in those loci where the level of variation warranted the testing of 16 or more individuals. No loci appear to be linked, however significant ($P < 0.05$) deviations from HWE after Bonferroni correction for multiple tests, presumably caused by null alleles, were observed for at least one locus for each species (Table 2). In this sample for *C. basalis*, Cba7 is in HWE although the probability was low ($P = 0.07$). In a smaller data set ($n = 17$), Adcock *et al.* (2005) reported that this locus was not be in HWE ($P = 0.03$), thus null alleles might also be present in this locus. Table 2 shows that a set of at least seven loci in HWE are highly variable in each species (mean number of alleles 7.6–12.1 and mean expected heterozygosity 0.76–0.78). An indication of the power of these loci for behavioural studies is that the general probability of excluding a parent when the other is not typed (equation 2a in Jamieson & Taylor 1997) is greater than 0.999 for each species.

Phylogenetic evidence suggests that species radiation in this group is recent (Payne 2005) and therefore that a relatively small amount of genetic divergence has occurred among species. This closeness and the fact that the three species reported here are not sister taxa (i.e. they are more closely related to other species) suggests that it is reasonable to expect that these microsatellite loci will be useful for studies involving all other *Chalcites* species.

Table 2 Characterization of microsatellite loci in three bronze-cuckoo species. Loci were amplified in N individuals using the same PCR conditions (see text). Listed for each locus is the number of alleles (N_a), allele size range in base pairs, observed (H_O) and expected (H_E) heterozygosities. Loci that failed to amplify are labelled 'No Amp' in the Range column. Underlined values of H_E indicate that the locus is not in Hardy–Weinberg equilibrium (HWE, $P < 0.05$). The last row gives the average values for loci (marked with *) in HWE with five or more alleles and $H_E > 0.5$

	<i>Chalcites basalis</i>					<i>Chalcites minutillus</i>					<i>Chalcites lucidus</i>				
	N	Range	N_a	H_O	H_E	N	Range	N_a	H_O	H_E	N	Range	N_a	H_O	H_E
Cba01	27	147–543	22	0.78	<u>0.92</u>	10	113	1	0.00	0.00	16	105–115	4	0.38	0.53
Cba02	27	260–272	*10	0.56	0.75	16	266–274	8	0.49	<u>0.61</u>	16	258–270	*6	0.85	0.69
Cba03	27	206–214	*7	0.56	0.65	10	199–205	2	0.11	0.10	8	198–199	2	0.08	0.08
Cba04	27	143–435	*17	0.89	0.88	10	124	1	0.86	0.78	16	124–262	9	0.59	<u>0.78</u>
Cba05	27	180–212	*10	0.85	0.79	24	163–380	*19	0.96	0.92	16	186–213	*10	0.93	0.85
Cba06	27	113–133	*9	0.73	0.75	10	No Amp	0	0.00	0.00	8	117–121	2	0.25	0.22
Cba07	27	225–547	*36	0.93	0.97	10	No Amp	0	0.00	0.00	8	No Amp	0	0.00	0.00
Tmm6	27	129–166	*8	0.64	0.78	10	124–130	3	0.75	0.53	16	128–133	*5	0.50	0.67
New to this study (Table 1)															
Cba08	27	190–213	*12	1.00	0.85	10	178–192	2	0.67	0.44	16	172–232	8	0.43	<u>0.83</u>
Cba09	27	162–178	*9	0.78	0.82	24	167–201	*11	0.84	0.82	16	167–194	*16	1.00	0.93
Cba10	8	252–260	3	0.22	0.20	24	246–259	*5	0.50	0.57	16	250–255	*5	0.73	0.73
Clu01	8	168–174	3	0.57	0.44	24	184–217	*10	0.76	0.84	16	176–182	*5	0.64	0.50
Clu02	27	254–329	*19	0.93	0.92	24	240–260	*7	0.74	0.76	16	240–248	3	0.36	0.31
Clu03	27	239–260	*8	0.78	0.72	24	251–344	*16	0.83	0.80	16	249–263	*13	0.71	0.87
Clu04	8	213–216	2	0.25	0.22	10	211–213	2	0.58	0.48	16	214–215	2	0.25	0.22
Clu05	27	138–145	*6	0.71	0.61	24	142–154	*6	0.64	0.75	16	137–146	*8	1.00	0.84
Clu06	27	256–280	*7	0.72	0.68	10	254–273	3	0.50	0.47	8	254–256	2	0.38	0.33
Average*			12.1		0.78			10.6		0.78			7.6		0.76

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