PRIMER NOTE

Polymorphic microsatellite loci in pheasant coucal (*Centropus phasianinus*)

G. MAURER,†* M. L. HALE,† M. H. VERDUIJN† and K. WOLFF†
*†School of Biology, University of Newcastle-upon-Tyne, Newcastle NE1 7RU, UK, *School of Botany and Zoology, Australian National University Canberra, ACT, 0200, Australia*

Abstract

Little is known about the effect of male parental care and behavioural sex-role reversal on the mating system of birds because genetic markers for species with these characteristics are lacking. We developed primers for nine polymorphic microsatellite loci in pheasant coucals (*Centropus phasianinus*). Eight of the primers were also polymorphic in African black coucals (*Centropus grillii*). Pheasant coucals are of particular interest in the study of evolutionary and behavioural ecology, because their sex-role reversal and extensive male parental care suggests low levels of extra-pair fertilizations, yet they have large testes indicating sperm competition.

Keywords: *Centropus phasianinus*, Cuculiformes, microsatellites, paternal care, sex-role reversal

Received 29 October 2004; revision accepted 7 January 2005

A thorough knowledge of the genetic mating systems of sex-role reversed species like the coucals (Centropodidae) is of particular importance for our understanding of sexual selection because it allows us to test its central hypotheses (Andersson 1994, 1995). For the genetic mating system of pheasant coucals (*Centropus phasianinus*), sexual selection theory provides two conflicting predictions: sex-role reversal, social monogamy, and extensive male parental care suggest low levels of extra-pair fertilization (EPF), while the male pheasant coucal's big testes size (Maurer & Wood unpublished) suggests sperm competition and high levels of EPF. To evaluate these predictions, we developed primers for nine polymorphic microsatellites in the pheasant coucal. We also tested these primers on DNA samples of four adult African black coucals (*Centropus grillii*) from the population described in Goymann & Wingfield (2004) to determine whether these primers may be used in other coucal species.

Blood and tissue samples were collected from four adults and 43 nestlings of a wild population of pheasant coucals (subspecies *melanurus*), near Darwin, Australia (12°26.411′S, 131°06.451′E). The samples were stored in 70% ethanol and DNA was extracted from the samples using the cetyltrimethyl ammonium bromide (CTAB) method (Weising *et al*. 1995). Then a DNA dinucleotide library, enriched for $[CA]_{15}$ and $[GA]_{15}$ repeats was created applying the method described in Hale *et al*. (2002). About 13 ng of this twice-enriched DNA was cloned into pUC 19 vector cut by *Bam*HI (Qbiogene) according to the manufacturer's instructions. Two microlitres of the vector were transformed into 100 μL QAComp-C01 competent *Escherichia coli* cells (Qbiogene) and plated out on Luria Bertani agar plates containing 50 μg/mL of ampicillin. Plates were incubated at 37 °C overnight, then colonies were screened for insert length by polymerase chain reaction (PCR) amplification in 10-μL reactions containing 1 × *Taq* buffer [16 mM of $(NH_4)_2SO_4$, 670 mM of Tris HCl, 0.1% Tween 20], 0.1 mM of each dNTP, 0.2 μM of the *M13* forward and reverse primer, respectively, 1.0 mM of $MgCl_2$, 0.5 U *Taq* (Bioline), and 0.5 μL of sample DNA (concentration *c.* 10 ng plasmid DNA/μL) using the following reaction cycle: 94 °C for 5 min, then 35 repeats of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. These and all the following reactions were performed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems). The products were run on 1.4% agarose gel and inserts between 250 and 600 bp were selected for sequencing. Plasmids containing inserts were extracted from 136 individual colonies using a QIAprep Spin Miniprep Kit (QIAGEN) and then sequenced

Correspondence: G. Maurer, Fax: 08-8922-0055; E-mail: golo.maurer@anu.edu.au

© 2005 Blackwell Publishing Ltd

Table 1 Primer sequences and polymorphism information for *Centropus phasianinus* microsatellite loci. H_E is the expected heterozygosity under Hardy–Weinberg equilibrium (HWE) and H_O is the observed heterozygosity. The two loci that did not deviate from HWE are shown in bold; all other loci deviated from HWE (after Bonferroni correction). Mean H_E across all loci is 0.638, mean H_O across all loci is 0.442. GenBank Accession nos A794013–A794022

Locus	Primer sequences (5′–3′)	Repeat	No. of alleles	Allelic size range (bp)	T_a (°C)	*n*	H_E	H_O
CP1	F: TCAGTGGAAGGGTTCATGACT R: GCTACTTTCCTGGTGGCCTA	$[GT]_{11}$	9	142–168	52	47	0.765	0.370
CP2	F: AAGGACAATGTAATTGGTGCTACA R: TCACAGAGCCATCAGGAATG	$[CA]_{10}$	10	152–176	52	46	0.891	0.522
CP3	F: CATTGCTGCTAGGCGAATTT R: TGACGTCATGAGCAACACAA	$[GT]_3CA[GT]_7$	3	206–212	50	47	0.390	0.468
CP4	F: GACCACCTAGAGCAGGTTGC R: TGGGCACTGACTGAAAGACA	$[TA]_3[GT]_7C[GT]_4$	9	176–206	52	47	0.716	0.596
CP5	F: AAAGGTCTGGAAGTGTTTTGC R: CAGCAGGTGGGCAGTATTTT	$[AC]_8$	4	126–134	48	47	0.631	0.064
CP6	F: CCAGCAGGGACATCAACTTT R: ATTCGTTCTGTGGGTGTTCC	$[AC]_7$	1	212	50	47	N/A	N/A
CP7	F: TGCCCATGACATTTAACCAA R: TTGGGCTTAGGCATGTAAGG	$[TC]_7T_3CTC_3$ $[TC]_2[CTT]_2[TC]_3$	3	224–228	50	47	0.523	0.574
CP9	F: CACAGGGCCACAAGAAGAAG R: AGACAGCTCCCTATCTGCTCA	$[TC]_{13}$	5	169–177	52	47	0.463	0.426
CP11	F: AAGACTCAAGTTTCAGTTGCAGTG R: TGGAGTTGCAAATGGAGAGA	$[AC]_8$	5	107–125	50	45	0.717	0.364
CP12	F: CCGCTGGAGTTCACCTTTAC R: CATGCTGACGATGGAGAAGA	$[TG]_9$	6	233–243	52	47	0.650	0.596

with BigDye Terminator Cycle Sequencing Chemistry (Applied Biosystems) on an ABI 310 Prism automated sequencer using *M13F* or *M13R* primers.

Twenty-four of the sequenced clones contained microsatellites with five or more repeats, 11 of which were interrupted. After discarding clones with the microsatellite region too close to the cloning site, 15 sequences were suitable to design primers. We used PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen & Skaletsky 2000) to design 12 primer pairs. To test them for amplification and polymorphism microsatellite fragments were labelled with fluorescent dCTPs (either R110 or R6G, Perkin-Elmer) and amplified in 15-μL reactions containing 1 × *Taq* buffer, 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.33 μM of each primer, 0.6 U *Taq* (Bioline) and 1 μM of [F]dCTP, with 0.5 μL of template DNA. The PCR cycle was 95 °C for 12 min, 10 cycles of 94 °C for 15 s, T_a for 15 s (see Tables 1 and 2), 72 °C for 15 s, followed by 20 cycles of 89 °C for 15 s, T_a (°C) for 15 s, 72 °C for 15 s, then a final extension of 72 °C for 10 min. Fluorescently labelled fragments were detected on an ABI 310 genetic analyser, with TAMRA 500 internal size standard, and analysed and sized using GENESCAN software (Applied Biosystems).

Amplification and polymorphism were initially assessed in six unrelated individuals from the pheasant coucal study population. Loci that showed polymorphism in this small subsample were amplified for all 47 individuals. Eleven of the 12 primer pairs amplified in the pheasant coucal and nine were polymorphic (Table 1). In the African black coucal, eight primer pairs amplified and were polymorphic, including one (*CP6*) that was not polymorphic in the pheasant coucal (Table 2).

All but two loci (*CP3*, *CP7*) departed significantly from Hardy–Weinberg equilibrium (HWE) after Bonferroni correction for multiple comparisons, calculated using ARLEQUIN version 2.000 (Schneider *et al*. 2000). All had a deficit of heterozygotes (Table 1). This deficit is probably caused by the structure of our sample. Most of the individuals tested were related nestlings from broods of four. This composition is also likely to explain the high levels of linkage disequilibrium found in the population. Linkage disequilibrium was detected in four pairs (*CP1-CP5*, *CP2-CP5*, *CP2-CP11*, and *CP4-CP11*) after Bonferroni correction.

Although our samples are from closely related animals and the repeat length of the microsatellites is small, the number of alleles per locus is surprisingly high with up to 10 alleles for *CP2*. Interestingly this locus did not amplify in the African black coucal. The microsatellite loci found in this study will be sufficient to determine parentage in the pheasant coucal and might prove useful for population studies and parentage analysis in other coucal species.

Table 2 Primer sequences and polymorphism information for African black coucal *Centropus grillii* microsatellite loci

Locus	Primer sequences (5′–3′)	Repeat	No. of alleles	Allelic size range (bp)	*n*	T_a (°C)
CP1	F: TCAGTGGAAGGGTTCATGACT R: GCTACTTTCCTGGTGGCCTA	$[GT]_{11}$	3	144–148	4	52
CP3	F: CATTGCTGCTAGGCGAATTT R: TGACGTCATGAGCAACACAA	$[GT]_3CA[GT]_7$	3	206–210	4	50
CP4	F: GACCACCTAGAGCAGGTTGC R: TGGGCACTGACTGAAAGACA	$[TA]_3[GT]_7C[GT]_4$	3	176–184	3	52
CP5	F: AAAGGTCTGGAAGTGTTTTGC R: CAGCAGGTGGGCAGTATTTT	$[AC]_8$	3	126–138	3	48
CP6	F: CCAGCAGGGACATCAACTTT R: ATTCGTTCTGTGGGTGTTCC	$[AC]_7$	2	212–218	3	50
CP7	F: TGCCCATGACATTTAACCAA R: TTGGGCTTAGGCATGTAAGG	$[TC]_7T_3CTC_3$ $[TC]_2[CTT]_2[TC]_3$	2	222–224	3	50
CP9	F: CACAGGGCCACAAGAAGAAG R: AGACAGCTCCCTATCTGCTCA	$[TC]_{13}$	3	169–173	4	52
CP11	F: AAGACTCAAGTTTCAGTTGCAGTG R: TGGAGTTGCAAATGGAGAGA	$[AC]_8$	6	112–124	4	50
CP12	F: CCGCTGGAGTTCACCTTTAC R: CATGCTGACGATGGAGAAGA	$[TG]_9$	3	236–244	4	52

Acknowledgements

The study was conducted under ethics permits of the Animal Ethics committees of the Australian National University (F.BTZ.56.03) and the Charles Darwin University (A04015) and a permit from the Parks and Wildlife Commission of the Northern Territory (#16937) and was supported by a Marie Curie Fellowship of the European community programme Human Potential under contract number: HPMT-CT-2001–00272 and a Stuart Leslie bird research award, a National Institute of Bioscience Grant to G.M. We want to thank Wolfgang Goymann for providing us with samples of *C. grillii* DNA and Greg Adcock for advice.

References

Andersson M (1994) *Sexual Selection*. Princeton University Press, Princeton, New Jersey.

Andersson M (1995) Evolution of reversed sex roles, sexual size dimorphism, and mating system in coucals (Centropodidae, Aves). *Biological Journal of the Linnean Society*, **54**, 173–181.

Goymann W, Wingfield JC (2004) Competing females and caring males. Sex steroids in African black coucals, *Centropus grillii*. *Animal Behaviour*, **68**, 733–740.

Hale ML, Squirrell J, Borland AM, Wolff K (2002) Isolation of polymorphic microsatellite loci in the genus *Clusia* (Clusiaceae). *Molecular Ecological Notes*, **2**, 506–508.

Rozen S, Skaletsky H (2000) PRIMER3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN, *Version 2.000: a software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Weising K, Wolff K, Nybom H, Meyer W (1995) *DNA Fingerprinting in Plants and Fungi*. CRC Press, Boca Raton, Florida.