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PERMANENT GENETIC RESOURCES NOTE

Microsatellite loci characterized in three African crane species (Gruidae, Aves)

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Abstract

Forty-three microsatellite loci originally isolated in *Grus americana* and *G. japonensis* were tested for polymorphism in the blue crane (*G. paradisea*). Amplified products were sequenced in the blue crane to aid in the design of blue crane-specific primers. When characterized in 20 unrelated blue crane individuals from South Africa, 14 loci were polymorphic, with each locus displaying between 2 and 7 alleles. Eight polymorphic loci were characterized in the grey-crowned crane (*Balearica regulorum*) and ten in the wattled crane (*G. carunculatus*).

Keywords: Aves, blue crane, cross-species utility, Gruidae, *Grus*, microsatellite

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The cross-species utility of microsatellite loci is generally high between birds separated by small genetic distances (Primmer *et al*. 1996). Within the Gruidae, there are 15 crane species that are genetically very similar (mean $\Delta T_{50}H$ DNA–DNA hybridization distance of 0.7; Sibley & Ahlquist 1990). We therefore characterized loci originally isolated from the whooping crane (*Grus americana*; Glenn 1997; Glenn *et al*. 1997) and the red-crowned crane (*G. japonensis*; Hasegawa *et al*. 2000) in three southern African crane species: blue crane (*G. paradisea*), grey-crowned crane (*Balearica regulorum*) and wattled crane (*G. carunculatus*).

Genomic DNA was extracted from blood using an ammonium acetate protocol (Nicholls *et al*. 2000). Each 10-µL polymerase chain reaction (PCR) contained approximately 10 ng genomic DNA, 0.5 µm each primer, 0.2 mm each dNTP, 1.0–3.0 mm $MgCl_2$ (Tables 1 and 2) and 0.05 U *Taq* DNA polymerase (BioTaq, Bioline Ltd) in the manufacturer's buffer [final concentrations: 16 mm $(NH_4)_2SO_4$, 67 mm Tris-HCl (pH 8.8), 0.01% Tween-20]. PCR amplification was performed using either a DNA Engine Tetrad 2 thermal cycler (MJ Research) or a DNA Engine Tetrad PTC-225 Peltier thermal cycler (Bio-Rad). PCR amplification conditions were 94 °C for 3 min; then 35 cycles of 94 °C for 30 s, locus-specific annealing temperature (Table 1) for 30 s, 72 °C for 30 s; followed by one cycle of 72 °C for 10 min. PCR products were loaded on a 48-capillary ABI 3730 DNA Analyser using dye set DS-30, filter set D and ROX size standard and genotypes were assigned using GeneMapper software (Applied Biosystems).

Forty-three loci were tested in 10–20 unrelated blue crane individuals from the Karoo stronghold in South Africa (McCann *et al*. 2002): 12 loci failed to amplify a specific product, 17 loci were monomorphic and 14 loci were polymorphic (Table 1). The blue crane PCR products were sequenced to enable the design of blue crane-specific primers. For each amplifying locus, the blue crane PCR product was amplified, ligated into pGEM (pGEM-T Easy kit; Promega) and sequenced (EMBL Accession numbers are provided in Table 1). For five loci (*Gamµ7*, *Gamµ14*, *Gamµ101b*, *Gj-M15*, *Gj-M48b*), new primer sets were designed from sequence that was 100% homologous between the blue crane and the source crane species (whooping crane or red-crowned crane; Table 1). Primer 3 software was used (Rozen & Skaletsky 2000).

When tested in 20 unrelated blue cranes from the Karoo region, the number of alleles ranged from 2 to 7 (Table 1). Two loci (*Gamµ3* and *Gamµ14*) displayed significant

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Table 1 Characterization of *Grus* microsatellite loci in the blue crane (*Grus paradisea*).

Locus	Blue crane sequence EMBL Accession no.	Repeat motif observed in blue crane	New blue crane primer set‡	Primer sequence (5′–3′) with fluorolabel and 'pigtail' underlined†	T_a (°C)	$MgCl_2$ (mM)	*n*	*A*	Exp. allele size (bp)	Observed allele sizes (bp)†	H_O	H_E	Dev. from HWE	Est. null allele frequency¶	M.I. ††
Gamμ2	AM168501	(CA)$_{2}$	No	F: HEX-AACTTTTGCCCATACTTCTGTAGTAAT R: GTTCCCGCTTCTTTTGCTGTGT	60	2.0	19	2	174	173, 175	0.26	0.31	NS	0.07	NT
Gamμ3	AM282934	(GT)$_{9}$(GA)$_{10}$	No	F: HEX-ATCCCTGAAGCTAACAATAAACC R: CACATTGCCAGACTGTTGTAT	56	2.0	20	3	114	100–114	0	0.19	*(0.001)	0.91	NT
Gamμ5	AM168502	(CA)$_{10}$TT(GT)$_{5}$	No	F: FAM-CGGATGTGTACTTGGCTCAGAA R: TTTCCCTGTGGTTAGTTGTGTG	56	2.0	18	2	188	183, 188	0.11	011	NS	–0.01	NT
Gamμ6	AM168503	(CA)$_{10}$	No	F: HEX-CACCTTTTATTGCGTATGTATTTT R: GGATTATGTTTTGGTTTTGTTTTT	55	2.0	20	7	125	115–133	0.80	0.79	NS	–0.03	Yes
Gamμ7‡‡ (Z-linked)	AM168504	(CA)$_{13}$	Yes	F: HEX-TAAAGGAGTGGCTGCTGCTGTG R: GTTTCTT-CTGAGGCTCTGCTGTGGGAAAC	59	1.0	10M	3	143	144–150	0.80	0.68	NS	–0.12	NT
Gamμ11	AM168506	(CCT)$_{8}$	No	F: FAM-CAATGGAGCGCCGTCAC R: GTTTCTT-CAGTTTCTTGGCCGCTGTT	52	2.0	20	6	136	134–152	0.60	0.76	NS	0.10	Yes
Gamμ13	AM168508	(GT)$_{10}$	No	F: HEX-AATAAGTTTGGAAATGTTTCTCATA R: GTTTCTT-TTTTCTGGTCAATACTAAAGC	53	2.0	20	3	182	191–195	0.60	0.47	NS	–0.16	Yes
Gamμ14	AM168509	(GT)$_{18}$	Yes	F: FAM-CTTGTTCCACCGTTGTCAGC R: GTTTCTT-CAGAAGACTGCAATGAACTCCTG	56	1.0	17	4	117	118–124	0.41	0.68	*(0.046)	0.22	NT
Gamμ18	AM168511	(GT)$_{13}$	No	F: HEX-TAGCGAGGGTCTGAGGAGAACT R: GTTTCTT-ACACCGTTATATCCTTCCCACT	56	1.0	16	7	188	184–204	0.69	0.80	NS	–0.01	NT
Gamμ24	AM282937	(CA)$_{8}$(CCCA)$_{3}$	No	F: FAM-GCTGCCAGTACAGACCCTCTT R: GTTTCTT-TCTTCTTGCTAATCATCTTTCTAAC	53	1.0	18	6	243	251–275	0.72	0.74	NS	–0.02	NT
Gamμ25	AM282938	(CA)$_{8}$TA(CA)$_{2}$(TC)$_{3}$	No	F: FAM-TTAATAAAAATCCACAGTGAAT R: GTTTCTT-GTTCTAGACCAGGACTGTTAATA	53	1.0	20	1	111	114	0	0	—	—	NT
Gamμ101b	AM168516	(ATT)$_{6}$GTT(ATT)$_{4}$	Yes	F: FAM-CAGTATAAAAACAAACAGGTGAGA R: GTTTCTT-TGAAAAAAGTACAGGAGAACATAG	58	2.0	20	2	194	194, 200	005	0.05	ND	–0.01	NT
Gj-M15	AM168519	(GT)$_{11}$	Yes	F: FAM-TCTACCAGATATCATCAGAGCTTGC R: GTTTCTT-TGCGAATGAACAGATGGCCCCAAGA	59	2.0	20	6	111	106–120	0.90	0.81	NS	–0.06	Yes
Gj-M34	AM168520	(CA)$_{7}$	No	F: FAM-TGCTCAACATTCATCAGGATTTGGG R: TCCCTCTGGTGTTGGCTGAAAATAC	59	2.0	20	3	127	124–130	0.85	0.63	NS	–0.17	Yes
Gj-M48b	AM282940	(CA)$_{7}$	Yes	F: HEX-GGACCCTCCACCGAGAAG R: GTTTCTT-GATCCTGGGGGTTTTGTTG	58	1.0	12	7	198	206–218	0.75	0.73	NS	–0.03	NT

T_a, annealing temperature; *n*, number of individuals in which a product was amplified; *A*, number of alleles; Exp. allele size, expected allele size based on the blue crane sequence; H_O, observed heterozygosity; H_E, expected heterozygosity; M, male (data provided only for males); Dev. from HWE, deviation from Hardy–Weinberg equilibrium calculated with GenePop version 3.4, Raymond & Rousset (1995); NS, nonsignificant calculated with GenePop version 3.4, Raymond & Rousset (1995); *, significant at the 0.01% probability level; ND, not done (GenePop software did not perform HWE analysis presumably due to the low level of heterozygosity observed); †, estimated null allele frequencies were calculated with Cervus version 2.0 Marshall *et al.* (1998); NT, no individuals belonging to a known family tested; ‡, alternative primer set used to that published in Glenn (1997) or Hasegawa *et al.* (2000): these were redesigned to allow for successful PCR amplification in blue crane; §, the 5′ end of one primer in each pair was labelled with a fluorescent phosphoramidite and, where stated, the 'pigtail' sequence GTTTCTT was added to the other primer, to reduce noise from variable adenylation during the PCR (Brownstein *et al.* 1996); ¶, the seven bases of the pigtail are included in the observed allele size; ††, M.I. indicates Mendelian inheritance, as observed in a single blue crane family (mother, father and two chicks genotyped); ‡‡, Z-linked locus: observed heterozygosity was calculated using male (ZZ) genotypes only.

Loci that failed to amplify in blue crane (with Accession number from source species sequences where available): *AA-1* (Glenn 1997), *ATC-1* (Glenn 1997), *AAC-1* (Glenn 1997), *Gamμ1* (Glenn 1997), *Gamμ8* (AM084719), *Gamμ9* (AM084720), *Gamμ16* (AM084727), *Gamμ20* (AM084731), *Gamμ23* (AM084734), *Gamμ102* (AM084739), *SHC-AG-1* (AM084741), *WC-AG-1* (AM084711).

Loci identified as monomorphic in blue crane, *Grus paradisea* (with Accession number from blue crane sequence): *AA/GC* (AM168499), *AT/AC-1* (AM168500), *Gamμ4* (AM282935), *Gamμ10* (AM168505), *Gamμ12* (AM168507), *Gamμ15* (AM168510), *Gamμ17* (AM282936), *Gamμ19* (AM168512), *Gamμ21* (AM168515), *Gamμ22* (AM168513), *Gamμ103* (AM168517), *Gj-M08* (AM168518), *Gj-M11a* (AB041859), *Gj-M13* (AB041860), *Gj-M40* (AM168521), *SHC-AG-2* (AM168522).

Note: the *Gamμ* loci were originally isolated from whooping crane, *G. americana*, (Glenn 1997), and the *Gj-M* loci were originally isolated from red-crowned crane, *G. japonensis*, (Hasegawa *et al.* 2000).

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Table 2 Characterization of *Grus* microsatellite loci in the grey-crowned crane (*Balearica regulorum*) and the wattled crane (*Grus carunculatus*)

Locus	Touchdown PCR temp. range (°C)	Exp. allele size (bp)	Grey-crowned crane, *Balearica regulorum* (from KwaZulu-Natal, Eastern Cape and Mpumalanga provinces)					Wattled crane, *Grus carunculatus* (from KwaZulu-Natal province)				
			n	A	Observed allele sizes (bp)‡	H_O	H_E	n	A	Observed allele sizes (bp)‡	H_O	H_E
Gamμ2	63–57	174	7	1	171	0	0	8	2	167, 175	0.13	0.13
Gamμ3	59–53	114	7	4	109–119	§	§	8	1	111	0	0
Gamμ5	59–53	188	7	1	177	0	0	8	3	179–183	0.38	0.34
Gamμ6	57–52	125	7	2	119, 125	0.14	0.14	8	1	123	0	0
Gamμ7† (*Z*-linked)	62–56	143	6 (1 F, 5 unk)	2	148, 150	§	§	8 (6 F, 2 unk)	4	142–150	§	§
Gamμ11	55–49	136	7	3	137–143	0.42	0.39	8	2	131, 137	0.50	0.40
Gamμ13	56–50	182	7	—	No amplification	NA	NA	8	–	No amplification	NA	NA
Gamμ14†	59–53	117	1	1	119	0	0	5	3	123–143	0.40	0.73
Gamμ18	56–50	188	7	1	182	0	0	8	1	190	0	0
Gamμ24	59–53	243	7	2	239, 255	0.14	0.14	8	2	251, 255	0.13	0.13
Gamμ25	56–50	111	7	2	109, 182	0.43	0.50	5	3	117–190	0.14	0.67
Gamμ101b†	61–55	194	7	3	186–200	0.57	0.58	8	3	198–206	0.38	0.51
Gj-M15†	62–56	111	7	2	113, 117	0.14	0.14	8	2	111, 113	0.25	0.23
Gj-M34	62–56	127	7	1	115	0	0	8	1	123	0	0
Gj-M48b†	61–55	198	7	1	120	0	0	8	4	130–138	0.63	0.60

Exp. allele size, expected allele size based on the blue crane (*Grus paradisea*) sequence; n, number of individuals in which a product was amplified; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F, female; unk, unknown sex; NA, not available; †, a new primer set was used which was designed from blue crane sequence (see Table 1); ‡, the 5′ end of one primer in each pair was labelled with a fluorescent phosphoramidite and, where stated, the 'pigtail' sequence GTTTCTT was added to the other primer, to reduce noise from variable adenylation during the PCR (Brownstein *et al.* 1996). The seven bases of the pigtail are included in the observed allele size; §, Z-linked locus: observed heterozygosity was not calculated for grey-crowned crane and wattled crane since few male individuals were available.
Note: the *Gamμ* loci were originally isolated in whooping crane (*Grus americana*, Glenn 1997) and the *Gj-M* loci were originally isolated in the red-crowned crane (*Grus japonensis*, Hasegawa *et al.* 2000).

deviation from Hardy–Weinberg equilibrium and a high predicted null allele frequency (GenePop version 3.4, Raymond & Rousset 1995; Cervus version 2.0, Marshall *et al*. 1998; Table 1). Pairwise tests for linkage disequilibrium (GenePop version 3.4), identified no pairs of loci with significant deviations. This was not unexpected, since the sample sizes were small.

The 14 polymorphic loci were checked for sex-linkage by genotyping known-sex individuals from three crane species. One locus (*Gamμ7*) was confirmed to be Z-linked (in agreement with Jones & Glenn 1999).

Fifteen loci were tested for cross-species utility in seven grey-crowned cranes and eight wattled cranes. The following touchdown PCR programme was used to amplify these individuals: 94 °C for 3 min; then 30 cycles of 94 °C for 30 s, annealing temperature (Table 2) for 30 s, 72 °C for 30 s, and finally, one cycle of 72 °C for 4 min. The first five cycles started with the highest annealing temperature (Table 2), dropping 1 °C per cycle until the lowest annealing temperature was reached (Table 2), which was then used for the remaining 25 cycles. PCR products were loaded on a 16-capillary ABI 3130XL DNA Analyser using dye set DS-30, filter set D and ROX size standard, and allele sizes were assigned using GeneMapper software (Applied Biosystems). Eight loci were polymorphic in grey-crowned crane and ten in the wattled crane (Table 2). Five loci were polymorphic in all four crane species tested (including their source species) and may be of utility in a high proportion of other crane species: *Gamμ7* (Z-linked), *Gamμ11*, *Gamμ24*, *Gamμ101b* and *Gj-M15* (Tables 1 and 2).

Five non-Gruidae avian species were tested for amplification with the 15 blue crane loci (Meares 2007). PCR amplification was performed as for the blue crane and products visualized on a 2% agarose gel. Locus *Gamμ11* amplified in four of the five non-Gruidae avian species tested.

We have successfully characterized polymorphic microsatellite markers in the blue crane, grey-crowned crane and wattled crane. A selection of these loci will have potential utility in a range of population and conservation applications in various crane species.

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