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TECHNICAL NOTE

Isolation and characterization of 14 polymorphic microsatellite DNA loci for the endangered Whooping Crane (*Grus americana*) and their applicability to other crane species

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Abstract Fourteen microsatellite DNA loci were isolated from the endangered Whooping Crane (*Grus americana*) and genetic variability assessed from 45 captive reared individuals. Allele numbers detected at each locus ranged from 2 to 6, the highest seen for this species. Mean observed heterozygosity varied from 0.04 to 0.79. These markers were then successfully amplified for two non-migratory populations of Sandhill Crane [Florida (*Grus canadensis pratensis*) and Missisippi (*Grus canadensis pulla*)], underscoring their utility for the conservation of threatened crane species.

Keywords Cranes · *Grus americana* · *Grus canadensis* · Microsatellite DNA · PCR primers

The family of Cranes (*Gruidae*) is one of the most endangered families of birds in the world, with 13 of its 15 species in peril (Crane Conservation Act 2008). Of the 15 extant species, only two, the Whooping Crane (*Grus americana*) and the Sandhill Crane (*Grus canadensis*), are found in North America. As of October 2009, the extant Whooping Crane population stands at 534 birds, all of which descend from a 1941 population which consisted of only 21 birds (US Fish and Wildlife Service 2007). In 1967, a captive breeding program was established for the conservation of Whooping Cranes (Meine and Archibald 1996). Previous microsatellite DNA markers were developed (Glenn et al. 1997) and used for the genetic conservation of Whooping Cranes (Jones and Nicolich 2001; Jones et al. 2002) and other crane species (Jones et al. 2005a, b, 2006; Hayes et al. 2006; Meares et al. 2008), however, these loci were predominately di-nucleotide repeats which exhibited little variation in Whooping Cranes (1–3 alleles per locus, Jones et al. 2002) and were difficult to score. With the advent of new enrichment procedures, we ventured to obtain more highly variable loci to add to the complement of loci available for this species. Here we describe 14 variable tri- & tetra-nucleotide microsatellite loci developed for the Whooping Crane. Similar to previously published loci, researchers should find these new loci applicable to both individual and population level analyses for all crane species.

Samples from 45 captive birds were available from a previous study (Jones et al. 2002). DNA from several individuals were pooled and serially enriched twice for microsatellites using three probe mixes [Mix 2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$; Mix 3 = $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$; Mix 4 = $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$] following the methods of Glenn and Schable (2005). DNA was digested with two restriction enzymes (*RsaI* and *BstUI*, New England Biolabs) in separate reactions, pooled, and then ligated to

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double-stranded SuperSNX linkers (SuperSNX24 Forward 5′-GTTTAAGGCCTAGCTAGCAGCAGAATC and Super-SNX24 Reverse 5′-GATTCTGCTAGCTAGGCCTTAAA CAAAA). Linker-ligated DNA was then denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, which were subsequently captured on magnetic streptavidin beads (Dynal). Unhybridized DNA was washed away and remaining DNA was eluted from the beads, amplified in polymerase chain reactions (PCR) using the forward SuperSNX24 as a primer, and cloned with TOPO-TA Cloning Kits (Invitrogen). Inserts from a total of 192 clones were PCR amplified and sequenced with M13 forward and reverse primers using the BigDye Terminators v3.1 (Applied Biosystems) and ABI-3130xl capillary sequencer. Sequences from both strands were assembled and edited in Sequencer 4.6 (Genecodes). Microsatellites were identified using MsatCommander version 0.8.1 (Faircloth 2008) and primers designed with Primer3. One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAG TCGGGCGTCATCA-3′) to enable use of a third primer in the PCR identical to the CAG tag that was fluorescently labeled for detection.

Forty-eight primer pairs were tested for amplification and polymorphism on eight individual Whooping Cranes. PCR conditions were then optimized using a larger subset of individuals from the Whooping Crane population, and carried out in 13 µl volumes (10 mM Tris pH 8.4, 50 mM KCl, 0.4 µM unlabeled primer, 0.04 µM tag labeled primer, 0.36 µM universal dye-labeled primer, 1.2 mM MgCl2, 0.8 mM dNTPs, 0.5 U Taq DNA Polymerase (Promega), and 20 ng DNA template) using a Bio-Rad

Table 1 Characteristics of 14 microsatellite loci isolated from the Whooping Crane

Locus	Primer sequence (5′ ⇒ 3′)	Repeat	Size (bp)	TD	n	A	H_o	H_e
Gram 6	CAGTCGGGCGTCATCATGGCAGTTTAAGCACTTCAC GGCTGTTATTTCCACAAAGATG	$(AGGT)_{10}(AGAT)_{12}$	247–267	65	45	5	0.69	0.64
Gram 8	CAGTCGGGCGTCATCATCTGCTGGTTCCTGGTTCTC GGCTCCTACTACCATCCCAC	$(AGAT)_7 \ldots (AGAT)_{10}$	405–413	65	44	3	0.38*	0.64
Gram 11	CAGTCGGGCGTCATCATTGGTCTACTGAAGTCTCTGGC GCAGGGTTGTTAGTCACAGTTC	$(AGAT)_{13}$	260–288	65	43	5	0.79	0.75
Gram 17	CAGTCGGGCGTCATCACAGGCCAAGAAACTGTAGC ATTTCAACCGCGACAACAC	$(CTTT)_{12}$	403–427	58	42	6	0.67	0.69
Gram 20	CAGTCGGGCGTCATCAGGAGACATTTGTCCTAGATTTGC CCCTGGGTGCTTTGACTTAC	$(AAAG)_{11}$	378–430	65	43	6	0.63	0.76
Gram 22	CAGTCGGGCGTCATCACCATTGGCACAATCCCTC AACCTATTTGCTGTTCCTATTACTC	$(AAAC)_9$	158–170	65	43	3	0.49	0.48
Gram 24	CAGTCGGGCGTCATCACCATTGGCACAATCCCTC AACCTATTTGCTGTTCCTATTACTC	$(AAAC)_9$	357–377	65	44	5	0.36	0.35
Gram 25	CAGTCGGGCGTCATCAGCAAAGAGGAGGGAAGAATG TGAACATAGCAAGATCGTGGAG	$(GTTT)_8$	166–178	58	45	4	0.51	0.53
Gram 30	CAGTCGGGCGTCATCATGTAAAGCTCCTTGGGCTG ATGAAGGGTGACAACGTAAAC	$(AAGG)_7$	158–166	65	43	2	0.33	0.28
Gram 31	CAGTCGGGCGTCATCAACACCAACATACTTGTCTCACC CATGAACAGCCATACACATACAAG	$(GTTT)_7$	259–263	58	45	2	0.29	0.31
Gram 32a	CAGTCGGGCGTCATCACAAATGTGCTGTTCACTTTC CCTCATGAGCCTAAGGATTGAG	$(CAAT)_7$	235–259	58	43	5	0.60	0.72
Gram 41	CAGTCGGGCGTCATCATTGGGCTTGATTCTGAGAGC GGATTTGTTTACTTTACCCAGGAAG	$(AAC)_8$	257–269	65	45	3	0.51	0.48
Gram 42	CAGTCGGGCGTCATCAGCATAAGGCATTTCGAGCAC AAGTGTTGGTTCTCATCCCG	$(AAC)_7$	165–168	58	45	2	0.08	0.09
Gram 45	CAGTCGGGCGTCATCAGTATCCTTGCCAGAGACATGC CCAGTAAAGCACCACACGAG	$(AAG)_7$	261–270	58	45	2	0.04	0.04

The observed allele range (bp), touchdown thermocycling protocol (TD), number of individuals (n), number of alleles (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) are provided for each locus

* Significant deviations from Hardy–Weinberg expectations at $P < 0.001$

iCycler. Touchdown thermal cycling (Don et al. 1991) encompassing a 9°C span of annealing temperatures ranging between 65–57°C (TD65) or 58–50°C (TD58) were used all loci (Table 1). Each reaction was run with an initial denaturation of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, TD65 or TD58 for 30 s, 72° for 40 s, with a final extension step of 72°C for 5 min. PCR products were pooled together and run against Genescan™ 500 ROX™ (red) internal size standard on an ABI 3760 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Samples were genotyped using GeneMapper v. 4.0 (Applied Biosystems, Inc., Foster City, CA). The number of alleles per locus, observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated with GENEPOP (Raymond and Rousset 1995), while FSTAT (Goudet 2001) was used to test for deviations from Hardy–Weinberg equilibrium and to evaluate loci for linkage disequilibrium. A sequential Bonferroni test (Rice 1989) was then used to compensate for multiple comparisons.

Fourteen of the tested primer pairs amplified well and exhibited polymorphism (Table 1). No significant linkage was detected after correcting for multiple comparisons. Allele numbers detected at each locus ranged from 2 to 6, with an average of 3.8 alleles per locus. Mean observed heterozygosity varied from 0.04 to 0.79. One locus deviated significantly from Hardy–Weinberg expectations (HWE, Table 1), however, a comparison of samples of known parents and their offspring indicates the disequilibrium is more indicative of a small N_e than of null alleles. We did not, therefore, remove this locus or compensate for null alleles.

To determine their utility across other crane species, we amplified the 14 Whooping Crane primers across 40 Florida and 94 Mississippi Sandhill Cranes available from another study (J. Henkel, unpublished data). All 14 primers successfully amplified for both the Florida and Mississippi Sandhill Cranes, however, two of the loci were monomorphic for this species (Table 2). For the remaining 12 loci, allele numbers detected at each locus ranged from 2 to 13, with an average of 5.3 alleles per locus. Mean observed heterozygosity varied from 0.05 to 0.98. All loci but one were in linkage equilibrium after correcting for multiple comparisons. Deviations from HWE were seen in only one locus, and in only one population (Table 2). Interestingly, this was the same locus that deviated in the Whooping Crane population. As we could not verify the Mendelian transmission of alleles in the Florida Sandhill population, Gram 8 should be used with the assumption that null alleles are possible.

Given the successful amplification across these two species, these microsatellite loci can now be utilized for the estimation of genetic diversity, genetic structure and gene flow, as well as determinations of inter-individual relatedness in these and other crane species. As both the Whooping Crane and the Mississippi Sandhill Crane have captive breeding programs, population managers can now use these more highly polymorphic loci to incorporate genetic relatedness and parentage analysis with pedigree knowledge, resulting in more educated breeding decisions.

Table 2 Cross-species amplification results for all fourteen loci

Locus	Florida Sandhill Crane (*G. c. pratensis*)				Mississippi Sandhill Crane (*G. c. pulla*)			
	N	*A*	H_o	H_e	*n*	*A*	H_o	H_e
Gram 6	40	10	0.88	0.89	94	5	0.83	0.80
Gram 8	38	9	0.47	0.80*	90	4	0.33	0.52
Gram 11†	40	13	0.70	0.90	93	8	0.79	0.70
Gram 17	35	6	0.80	0.75	84	6	0.80	0.75
Gram 20	39	12	0.74	0.86	88	4	0.55	0.51
Gram 22	39	4	0.54	0.53	92	3	0.51	0.52
Gram 24	31	1	0.00	0.00	93	1	0.00	0.00
Gram 25	30	1	0.00	0.00	94	1	0.00	0.00
Gram 30	34	8	0.85	0.81	90	7	0.98	0.78
Gram 31	40	2	0.32	0.28	92	2	0.52	0.48
Gram 32a	40	5	0.72	0.65	94	3	0.28	0.27
Gram 41	33	4	0.67	0.53	93	3	0.38	0.34
Gram 42	40	4	0.60	0.57	93	2	0.40	0.33
Gram 45	40	2	0.05	0.05	94	2	0.22	0.20

The number of individuals (*n*), number of alleles (*A*), observed heterozygosity (H_o) and expected heterozygosity (H_e) are given for each locus

* Significant deviations from Hardy–Weinberg proportions at $P < 0.001$

† Signifigant linkage with locus Gram 17 in MSC, and Gram 6, 17, 20, and 42 in FSC at a corrected $P = 0.05$

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