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TECHNICAL NOTE

Characterization of small microsatellite loci for use in non invasive sampling studies of Gunnison Sage-grouse (*Centrocercus minimus*)

Sara J. Oyler-McCance · Judy St. John

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Abstract Primers for 10 microsatellite loci were developed specifically to amplify low quantity and quality DNA for Gunnison Sage-grouse (*Centrocercus minimus*), a species that has been petitioned for listing under the US Endangered Species Act. In a screen of 20 individuals from the largest population in the Gunnison Basin, Colorado, the 10 loci were found to have levels of variability ranging from two to seven alleles. No loci were found to be linked, although one locus revealed significant departures from Hardy–Weinberg equilibrium. These microsatellite loci will be applicable for population genetic analyses and for use in mark recapture studies that utilize DNA collected non invasively from feathers and fecal pellets, which will ultimately aid in management efforts.

Keywords *Centrocercus minimus* · Gunnison Sage-grouse · Microsatellite · Non invasive sampling

Gunnison Sage-grouse (*Centrocercus minimus)* have been severely impacted by loss and fragmentation of habitat and, consequently, populations are small and isolated (Oyler-McCance et al. 2001). The entire species range consists of one moderately sized population in the Gunnison Basin (~500 strutting males) that is surrounded by seven small satellite populations, most of which have fewer than 50 strutting males (Colorado Division of Wildlife, unpublished lek data). Management and recovery efforts are underway that range from the protection, improvement, and restoration of sagebrush (*Artemesia* spp.) steppe habitat to augmentation of genetic diversity and protection from additional human disturbances. Successful conservation efforts require an accurate method for inventorying and monitoring population sizes of Gunnison Sage-grouse. Traditionally, wildlife managers have used some form of lek counts as a way to monitor Sage-grouse population sizes under the assumption that this index to population size is representative of true abundance. However, many have criticized this use of lek counts, suggesting that it is unreliable for estimating population sizes and trends (Beck and Braun 1980; Applegate 2000; Anderson 2001). Traditional mark-recapture methods have been attempted and showed that lek counts significantly underestimated true population sizes (Walsh 2002). Further, these traditional methods require intensive capturing and marking of animals, which is particularly difficult with Gunnison Sage-grouse as they are widely dispersed and elusive for much of the year. Because fecal pellets and feathers are relatively easy to find throughout the year, they can potentially be used as a source of DNA to uniquely identify individuals for mark-recapture studies. In an attempt to determine whether population sizes could be estimated using molecular tagging methods, we isolated and developed primers for 10 microsatellite loci, specifically designing them so that amplified products were less than 200 base pairs in size. This makes them more likely to amplify when low quantity and quality DNA extracted from non invasively collected samples such as feathers or fecal pellets is used.

S. J. Oyler-McCance (✉) · J. St. John
Rocky Mountain Center for Conservation Genetics and Systematics, Department of Biological Sciences, University of Denver, Denver, CO 80208, USA
e-mail: sara_oyler-mccance@usgs.gov

S. J. Oyler-McCance
US Geological Survey, Fort Collins Science Center, 2150 Centre Ave, Building C, Fort Collins, CO 80526, USA

Blood samples were collected from 20 Gunnison Sage-grouse in the Gunnison Basin population (following Oyler-McCance et al. 2005) and were placed in EDTA-coated Eppendorf tubes (Brinkmann) and stored at −20°C. Fecal pellets from some of those individuals were collected during the trapping and blood collection procedure. Fecal pellets were placed in Ziploc bags and stored at −20°C. DNA was extracted from blood samples using the GenomicPrep Blood DNA Isolation Kit (GE Healthcare Life Sciences) using the manufacturer's instructions with modifications following Oyler-McCance et al. (2005). DNA extractions from fecal pellets were performed using the ZR fecal DNA kit (Zymo Research) following manufacturer's instructions.

Microsatellite isolation was performed at the Rocky Mountain Center for Conservation Genetics and Systematics applying the technique developed by St. John and Quinn (2008). Of the 10 loci isolated, four were from an isolation that utilized DNA pooled from three avian species (Gunnison Sage-grouse, lesser prairie-chicken [*Tympanuchus pallidicinctus*], and mountain plover [*Charadrius montanus*]) and were labeled MSP (referring to microsatellite pool) to reflect that. Six additional microsatellite loci were isolated specifically from Greater and Gunnison Sage-grouse. Briefly, genomic DNA was digested to yield fragment lengths of 300–1,200 bp using the endonucleases *Csp*6I and *Xma*I. Double-stranded DNA linkers specifically designed for this application were added into the ends of the DNA fragments. The MSP fragments were subsequently hybridized to an oligonucleotide probe that is bound to biotin containing the sequence $(CA)_{12}$. The Sage-grouse fragments were hybridized to a biotin containing $(GATA)_7$ oligonucleotide probe. These target fragments were captured using streptavidin magnetic beads that have been pretreated with a blocking buffer that prevents non-specific binding of the beads to nucleic acids. Captured fragments were amplified and cloned prior to sequencing. The isolation from the pooled avian DNA (MSP) yielded 21 clones that were subsequently sequenced. Additionally, during the isolation from Greater and Gunnison Sage-grouse DNA, 30 clones were sequenced. Primers were designed either by using the software PRIMER 3 (Rozen and Skaletsky 2000) or manually. The primers designed from all loci were optimized with Gunnison Sage-grouse DNA and were found to amplify both DNA extracted from blood and DNA extracted from corresponding fecal pellets.

A forward primer dye-labeled with Beckman Coulter dyes D2, D3 or D4 (Proligo) and an unlabeled reverse primer (Sigma-Genosys) were used in PCRs for six loci (SG MS6.2, SG MS6.4, SG MS6.6, SG MS6.8, MSP7, and MSP18; Table 1). PCRs were performed as in St. John et al. (2005) [125 μM each dNTP, 1× *Taq* buffer (67 mM Tris–HCl pH 8.0, 6.7 mM $MgSO_4$, 16.6 mM NH_4SO_4 10 mM β-mercaptoethanol; Kahn et al. 1998), 0.5 μM each primer and 0.625U *Taq* polymerase (Promega)] except using half-reactions (12.5 μl). The thermal profile was as follows: preheat at 94°C for 2 min, denature at 94°C for 1 min, anneal at varying temperatures (Table 1) for 1 min, and extend at 72°C for 1 min. Each PCR had 35 amplification cycles (MJ Research PTC-200, Bio-Rad) and concluded with a 10 min 72°C extension. For the remaining four loci, PCRs were performed using a M13-tailed forward primer as described by Boutin-Ganache et al. (2001). Each 12.5 μl reaction contained 125 μM each dNTP, 1× *Taq* buffer (Kahn et al. 1998), 0.034 μM M13-tailed forward primer, 0.5 μM non-tailed reverse primer, 0.5 μM M13 dye-labeled primer with Beckman Coulter dyes D2, D3 or D4 (Proligo), and 0.31U *Taq* polymerase (Promega). The thermal profile for both the forward dye-labeled and the M13 dye-labeled reactions was as follows with the appropriate annealing temperature given in Table 1: preheat at 94°C for 1 min, denature at 94°C for 1 min, anneal for 1 min, and extend at 72°C for 1 min. Each PCR had 35 amplification cycles (MJ Research PTC-200, Bio-Rad). The PCR products were diluted and run on the CEQ8000 XL DNA Analysis System (Beckman Coulter). All loci were run with the S400 size standard (Beckman Coulter) and analyzed using the Frag 3 default method.

The 10 loci had low to moderate levels of polymorphism, consistent with a species shown to have reduced genetic variability (Oyler-McCance et al. 1999), with the number of alleles ranging from two to seven (Table 1). Hardy–Weinberg expectations were determined using the computer program ARLEQUIN version 2.000 (Guo and Thompson 1992; Schneider et al. 2000) and linkage disequilibrium was tested using GENEPOP (Raymond and Rousset 1995). One locus (MSP18) was out of Hardy–Weinberg equilibrium thus suggesting the possibility of null alleles (Table 1). Analysis of the genotype frequencies (GENEPOP, Raymond and Rousset 1995) revealed that no pair of loci was in linkage disequilibrium using a P value of 0.005 that corrected for multiple comparisons. Each primer pair was tested on multiple DNA samples extracted from fecal pellets and was shown to amplify in multiple fecal samples. These microsatellite loci will be applicable for population genetic analyses and for use in mark recapture studies that utilize DNA collected non invasively from feathers and fecal pellets, which will ultimately aid in management efforts by providing better estimates of population size and/or survival rates.

Table 1 Microsatellite primer sequence and locus information

Locus	GenBank Accession No.	Primer sequence (5′–3′)	Anneal temp°C	Repeat of cloned allele	No. of alleles	Allele size range	H_O	H_E
SG MS06.1	GQ902764	F: **M13**-TCAGCTATTGATTTAGAGACGTCAG R: GATACAATTCATCCACCCTCC	55	$(CAT)_7$	2	228–231	0.556	0.500
SG MS06.2	GQ902765	F*: TATACCCCTGCATAAGCCCTG R: CCAGCCTGCACTTTTGTCC	55	$(GATA)_7(GACA)_4(GATA)_2$	2	123–136	0.238	0.210
SG MS06.3	GQ902766	F: **M13**-GGCGTTCAACACAGAAGCC R: CTTTGCAGTTGAGCATGGC	55	$(GATA)_6$	3	158–166	0.667	0.542
SG MS06.4	GQ902767	F*: CCTGGAGCAACTTGAGG R: GTGACATTTCCCCCCAC	58	$(GATA)_2(GGTA)_6(GATA)_5$	7	138–174	0.650	0.675
SG MS06.6	GQ902768	F*: CAAACAACTGTCTTCCAGTAAGAC R: AGAGCCTTCATTTCTGGCAG	58	$(CAT)_{16}$	3	132–146	0.550	0.521
SG MS06.8	GQ902769	F*: GCAAAATCAATAGAAGTAGAGAGG R: CAGTAGCAGCTTTGTTTGG	52	$(GATA)_{17}$	5	140–156	0.762	0.620
MSP7	GQ902770	F*: GTGCCTGGAAGAGGTAGG R: TCACAAAGGTGACCTGGTC	55	$(CA)_8$	5	139–148	0.667	0.716
MSP8	GQ902771	F: **M13**-CAGTGGCTGAGACCAGAAC R: ACATAGGATTTTGCCCGAG	55	$(CA)_4N_6(CA)_7$	2	131–133	0.250	0.430
MSP11	GQ902772	F: **M13**-CACACCTAGATGGTGGTG R: CATTGTCAGCTTGCAGAC	52	$(CA)_{22}$	4	231–247	0.267	0.242
MSP18	GQ902773	F*: CAATGACAGTATTTCCCAGATTA R: GAATGGTAATATACTAAGCACAGG	55	$(CA)_{14}$	5	97–107	**0.529**	**0.754**

H_O and H_E are observed and expected heterozygosities, respectively. *Bold* indicates significant deviations from Hardy–Weinberg equilibrium

*Forward primer directly dye-labeled with Beckman Coulter dyes D2, D3, or D4

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