CHARACTERIZATION AND ISOLATION OF FIVE
MICROSATELLITE LOCI IN NORTHERN FLYING SQUIRRELS,
GLAUCOMYS SABRINUS (SCIURIDAE, RODENTIA)

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ABSTRACT.—Northern flying squirrels (*Glaucomys sabrinus*) are found in boreal forests of northern and northwestern North America, but a small population is isolated to the Black Hills of South Dakota and Wyoming. Few microsatellite primers have been developed for this species, though they are needed to examine the genetic structure of these populations. Thus, we isolated and characterized 5 microsatellite loci in northern flying squirrels through a series of steps involving microbiology, molecular biology, and genetic techniques. Data analyses with these primers indicated that the northern flying squirrel population found in the Black Hills may have low heterozygosity and significant departure from Hardy–Weinberg equilibrium. The development of these primers not only provides additional data for analyzing a small, disjunct population but also serves as a mechanism for understanding population dynamics and assisting with overall management and conservation of unique populations.

RESUMEN.—Las ardillas voladoras del norte (*Glaucomys sabrinus*) se encuentran en los bosques boreales del norte y noroeste de Norteamérica, pero existe una pequeña población aislada en las Colinas Negras de Dakota del Sur y Wyoming. Pocos cebadores microsatélites se han desarrollado para esta especie, a pesar de que se necesitan para examinar la estructura genética de estas poblaciones. Por consiguiente, aislamos y marcamos cinco loci microsatélites en ardillas voladoras del norte utilizando varios pasos de microbiología, biología molecular y técnicas de genética. Los análisis de datos con estos cebadores indicaron que la población de ardillas voladoras del norte encontradas en las Colinas Negras podría tener baja heterocigosidad y una desviación significativa del equilibrio de Hardy–Weinberg. El desarrollo de estos cebadores no solo brinda datos adicionales para analizar una pequeña población disyunta, sino que también sirve de mecanismo para entender la dinámica poblacional y auxiliar en el manejo y conservación general de poblaciones únicas.

Northern flying squirrels, *Glaucomys sabrinus*, are distributed in boreal forests of northern and northwestern North America (Wells-Gosling and Heaney 1984). Populations of northern flying squirrels found in the Black Hills of South Dakota and Wyoming are small, isolated, and disjunct from other populations within their range (King 1951, Wells-Gosling and Heaney 1984). The northern flying squirrel is one of 62 species in this region that have differentiated into distinct subspecies as a result of local selection pressures (Turner 1974). As part of an ongoing project to understand the genetics of these populations, we developed a set of microsatellite primers to determine the genetic variability and structure of the Black Hills populations and their relatedness to nearby populations. Moreover, few microsatellite primers have been developed for North American flying squirrels (Zittau et al. 2000, Fokidis et al. 2003, Winterowd et al. 2005); thus, isolating and characterizing microsatellite loci for northern flying

squirrels can help with local conservation efforts and with general research activities.

Genomic DNA was extracted from muscle tissue of 2 individuals following manufacturer's protocols for the DNeasy blood and tissue extraction kit (QIAGEN). Tandem repeat regions were isolated using the subtractive hybridization method (Hamilton et al. 1999).

Digested DNA was enriched for 7 oligonucleotide repeats: (CA)₁₅, (GT)₁₅, (CCT)₁₀, (AAT)₁₀, (GCG)₁₀, (GCA)₁₀, and (GCT)₁₀. Enriched polymerase chain reaction (PCR) products were cloned using pBluescript II SK (+) and XL1-Blue MRF' Supercompetent cells (Stratagene). Colonies were screened using X-gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and IPTG (isopropyl-beta-D-thiogalactopyranoside; Sigma-Aldrich). Approximately 500 color-positive (white) colonies were suspended separately in 100 mL T.E. buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0), and 2 mL of each colony solution was

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TABLE 1. Characterization of microsatellite loci in 79 samples of northern flying squirrel, *Glaucomys sabrinus*. Given for each locus are the annealing temperature (T_a), number of alleles observed (A), allelic size range (in base pairs [bp]), and observed heterozygosity (H_O) and expected heterozygosity (H_E). PET, VIC, and FAM refer to the fluorescent label used on each primer.

Locus	Repeat motif in clone (5'-3')	Primer sequences (5'-3')	T_a (°C)	A	Allele sizes (bp)	H_O	H_E	Accession No.
GLSA 12	(GT) ₅ A(TG) ₃ TTT(GT) ₅	F: PET-AGCATATCGAACCCTCATATCAG R: GGCAAGATTGTATCCTTGG	54.8	2	154-170	0.00	0.03	FJ755452
GLSA 22	(CA) ₁₅	F: VIC-CCTGAAATGATCCATCTGG R: AGACTAGGCTGTCTTTGAGG	54.8	7	168-188	0.19	0.18	FJ755453
GLSA 48	(CA) ₁₀ (CC) ₆ (CA) ₆	F: PET-CATCCATTTCATCCTCGTA R: GAGTGGCTCTCAGGTTGAC	52.5	8	304-320	0.47	0.64	FJ755454
GLSA 52	(CA) ₁₆	F: FAM-TCCATCCACAGTGTGTGAGC R: CCTGCAGTCCACTCAAGCAT	57.5	6	210-254	0.61	0.52	FJ755455
GLSA 65	(GT) ₁₇	F: FAM-TTTGGGAATTGAGGCTATGG R: TTCACAGTGACAGCAGGTGAC	52.5	6	170-210	0.22	0.23	FJ755456
MEAN				6		0.27	0.32	

used to screen for microsatellite regions by hybridization with oligonucleotides (Glenn and Schable unpublished data) and the Phototype detection system (New England Biolabs).

Subsequently, 50 positive clones were picked and further screened for insert size by PCR. Reactions were conducted in 25 μ L volumes that contained 1 μ L of 50 ng template DNA, 0.8 mM each of primers T3 and T7 (Integrated DNA Technologies), 1X ThermoPol Reaction Buffer (10 mM KCl; 10 mM (NH₄)₂SO₄; 20 mM Tris-HCl, pH 8.8; 2 mM MgSO₄; 0.1% Triton X-100; New England Biolabs), 200 mM of each dNTP, and 0.2 units of Vent-Exo DNA polymerase (New England Biolabs). The PCR protocol had an initial denaturing step at 96 °C for 5 minutes followed by 30 cycles of 96 °C for 45 seconds, 51 °C for 1 minute, and 72 °C for 2 minutes. PCR products were run electrophoretically in a 1.5% agarose 1XTBE gel and visualized by SYBR® green and UV light.

Twenty clones that exhibited a single amplified band of 500–1000 base pairs were cleaned with 16 units of Exonuclease I and 3 units of Antarctic Phosphatase (New England Biolabs), followed by ethanol precipitation. Cleaned PCR products were sequenced using the T3 primer in DYEnamic ET Terminator Cycle Sequencing Kit (General Electric) 1/8th volume reactions. Sequences were electrophoresed on an ABI Avant 3100 Genetic Analyzer.

Sequenced clones were edited and analyzed with DNASTar® (Lasergene). Eighteen clones contained a repeat unit. Sequences with 95% or greater homology were placed in contigs to avoid primer design of paralogous sequences. Paralogous sequences were removed from further development. Eight sequences were used to design and test primers. Primer3 (<http://frodo.wi.mit.edu/primer3/>) was used to design 8 primer pairs, and IDT SciTools (<http://www.idtdna.com/SciTools/SciTools.aspx>) was used to estimate annealing temperatures and levels of self-priming. Amplification performance was optimized for each primer pair using a 48–64 °C annealing temperature gradient. Five primer pairs produced reproducible polymorphic products (Table 1).

Variation at the 5 loci was quantified using a sample of 79 northern flying squirrels from the Black Hills. One of the primers for each locus was labeled at the 5' end with one of 3 fluorescent dyes, 6-FAM, VIC, or PET (Applied Biosystems; Table 1). PCR was performed with

15 μ L reactions containing 50 ng genomic DNA, 10 μ M of each primer (forward and reverse), 400 μ M of each dNTP (dATP, dGTP, dCTP, and dTTP), 0.50 units of Taq polymerase in buffer (Promega), and 3 mM of $MgCl_2$. PCR started with initial denaturing at 95 °C for 4 minutes followed by 35 cycles of 95 °C for 45 seconds, annealing at a temperature between 52.5 °C and 57.5 °C for 45 seconds (Table 1), extension at 72 °C for 1 minute 30 seconds, and a final elongation step of 72 °C for 7 minutes.

Confirmation of PCR amplification was conducted by running a sample on a 1.5% agarose 1X TBE gel using SYBR® Green (Invitrogen) as a nucleic acid stain in each sample and a water blank to test for contamination of products in every PCR run. PCR products were diluted, pooled together using all 5 primers per sample, and mixed with Hi-Di formamide and the LIZ 500 size standard before electrophoresis on an Avant 3100 genetic analyzer (Applied Biosystems). Cross-species amplification was conducted with 12 southern flying squirrel specimens from Wisconsin, and GLSA12, GLSA22, GLSA48, and GLSA65 primers showed some success of cross amplification (Viall et al. 2009).

Alleles were scored using GeneMapper software (Applied Biosystems) and input into POPGENE (Yeh and Boyle 1997) for analysis of observed and expected heterozygosity and allelic variation. GENEPOP 1.2 (Raymond and Rousset 1995) was used to test for linkage disequilibrium, and MicroChecker (Oosterhout et al. 2004) was used to test for the presence of null alleles.

For the 5 polymorphic loci, the number of alleles in 79 individuals ranged from 2 to 8 (Table 1). Tests indicate that loci GLSA12 ($H_o = 0.00$), GLSA48 ($H_o = 0.47$), and GLSA 65 ($H_o = 0.22$) show a significant departure from Hardy–Weinberg Equilibrium (HWE), but overall heterozygosity is similar to that found in other studies ($H_o = 0.27$). Northern flying squirrel heterozygosity levels ranged from 0.13 to 0.44 in an Alaskan population (Bidlack and Cook 2002) and was 0.36 in eastern North American populations (Arbogast et al. 2005). None of the loci show significant linkage disequilibrium ($P > 0.05$). However, 2 loci (GLSA12 and GLSA48) show evidence of null alleles. Null alleles may affect heterozygosity estimates, thereby possibly leading to a departure from HWE.

Locus GLSA12 has 2 alleles which are restricted to 2 different regions in the Black Hills: the north central region ($n = 52$) and the south central region ($n = 27$). The allele found at 170 bp is expressed in individuals from the north central region, whereas the allele found at 154 bp is expressed in individuals from the south central region. Regionalized alleles and low levels of heterozygosity shown at several loci are likely the result of isolation of the northern flying squirrel population in the Black Hills.

A population with allelic variation at all loci is considered to be in HWE (Platz and Grudzien 2003). Low levels of heterozygosity can cause a departure from HWE, and small populations tend to show a departure from HWE. Genotypes in subpopulations in the Black Hills do not adhere to Hardy–Weinberg expectations, thereby causing a significant departure from HWE. A departure from HWE in the Black Hills could be explained by low heterozygosity and nonamplifying (null) alleles, possibly as a result of past events that caused their isolation.

Because this squirrel has been isolated to the Black Hills, varied conservation measures may be required. The northern flying squirrel is classified as imperiled by the South Dakota Natural Heritage Program because of rarity or other factors that may cause extinction (SDGFP 2006). Because this species is found in an isolated ecosystem, we found a unique opportunity to examine and compare genetic variation within and among populations found in the Black Hills. According to our data, these squirrels are likely not panmictic and have low levels of variation. These primers will assist in the ongoing research, conservation, and management of this unique population.

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