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TECHNICAL NOTE

Development of microsatellite markers in *Myotis sodalis* and cross-species amplification in *M. gricescens*, *M. leibii*, *M. lucifugus*, and *M. septentrionalis*

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Abstract The Indiana bat (*Myotis sodalis*) is a highly endangered vespertilionid bat whose distribution is associated with limestone caves in the eastern United States. We present nine new polymorphic nuclear microsatellite markers for *Myotis sodalis* developed using an enriched library method. A total of 62 *M. sodalis* from two populations were used to estimate genetic diversity parameters. In *M. sodalis*, the number of alleles observed for each locus ranged from 17 to 48 alleles and expected heterozygosity ranged from 0.894 to 0.973. The 9 microsatellite markers were also tested on *M. gricescens*, *M. leibii*, *M. lucifugus*, and *M. septentrionalis*. These polymorphic microsatellite markers provide a valuable tool for investigating the population genetics of these species and will provide important genetic data useful for the conservation and recovery of the endangered Indiana bat.

Keywords Microsatellite markers · *Myotis gricescens* · *Myotis leibii* · *Myotis lucifugus* · *Myotis septentrionalis* · *Myotis sodalis*

The Indiana bat (*Myotis sodalis*) was initially listed under the Endangered Species Preservation Act of 1966 and is currently listed as endangered under the amended Endangered Species Act of 1973. Critical habitat was designated across six states in 1976 including major hibernacula consisting of 11 caves and 2 mines (USFWS 2007). Continued decline of the species has been documented through estimates of hibernating Indiana bats across their range. In 1965, a total estimate of hibernating Indiana bats was 883,300 (USFWS 2007) compared to a 2007 estimate of 468,184 (King 2008). Population structure of the Indiana bat has been investigated using mitochondrial DNA from Indiana bats sampled at 13 hibernacula with the discovery of four separate population groups: Midwest, Appalachia, Northeast 1, and Northeast 2 (USFWS 2007). Knowledge on the spatial organization of genetic variation, gene flow, and any relationships with fragmentation and/or isolation is needed to develop long term conservation and recovery strategies for this endangered species. Therefore, the aim of this study was to develop a set of polymorphic microsatellite (simple sequence repeats—SSR) markers that will be useful in describing the population genetic structure of *Myotis sodalis*.

Total DNA was extracted following established protocols (Maniatis et al. 1982) from a female Indiana bat that had been submitted to the Missouri Department of Health and Senior Services—Rabies Lab for rabies testing by a member of the public. A genomic library enriched for SSR was constructed following the protocols of Glenn and Schable (2005). Genomic DNA was digested with restriction enzymes and fragmented into ~500 base pair (bp) fragments using Rsa I and Xmn I. Double stranded linkers were ligated onto the ends of the DNA fragments followed by enrichment of the genomic library for SSR containing fragments using biotinylated oligonucleotide sequences. Genomic DNA fragments were enriched for di-, tri-, and tetra-nucleotide SSR containing fragments. Microsatellite enriched fragments were ligated into pCR®2.1-TOPO vectors (Invitrogen, Carlsbad, CA) as described by the supplier and used to transform TOP10 *E. coli* competent cells (Invitrogen) following the suppliers protocol with selection by growing colonies overnight on imMedia™

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Table 1 Characterization of 9 polymorphic microsatellite loci for *Myotis sodalis* including locus name, primer sequences, repeat type (interrupted microsatellites are indicated with (…) between repeat units), number of alleles (*A*), allele size range (bp), observed (H_O) and expected (H_E) heterozygosity for each population, and test for departure from Hardy–Weinberg equilibrium (HWE)

Locus	Primer sequences	Repeat motif	*A*	Size range	Pilot knob ($N = 30$) H_O/H_E	Great scott ($N = 32$) H_O/H_E
MS3D02	F: CTAAGACCCTTTCCAGCTCTCA[a]	$(AC)_{16}$	17	197–229	0.900/0.916	0.875/0.894
	R: GATACCATCACTCTTTCCCCTG					
MS3D09	F: GACATTTCTCACTGTTCCTCCTCC	$(TGTA)_4$…$(ATCT)_3$…$(ATCT)_{10}(ATC)_2(ATCT)_3$	30	186–258	0.967/0.942	0.969/0.950
	R: TAATCTGTGTTGCATTTCTGGC[a]					
MS4C09	F: CCTAAGACCAACTTATCTCCC[a]	$(ATAG)_3$…$(GATA)_3(GAT)_4(GATA)_{13}$…$(GATA)_3$	48	176–271	0.867/0.973	0.750/0.968*
	R: GAACATAACCTCCTTGGATTGCT					
MS4C05	F: CAGCCTCCCTGGTTAGAAGAAT[a]	$(GC)_5T(CA)_{17}$	20	227–271	0.833/0.933	0.531/0.914*
	R: GACACCAAAACTACACCCTGCTT					
MS1B11	F: GGCTGTCTGTGTGTGTGTCTATTTT	$(GA)_{17}$	19	286–332	0.700/0.925*	0.781/0.911
	R: GTTTCCTTTATGCACCCTTCTT[a]					
MS3F05	F: GTTTTGGTCTTTTGCTTTGTGG	$(AC)_{18}$	20	291–331	0.833/0.933	0.531/0.914*
	R: ACGATTAGGCAGAGGTTGTGAT[a]					
MS1C01	F: TCAGAGGTCACAGCTCATGTCT[a]	$(TG)_{20}$	21	327–377	0.833/0.923	0.781/0.937
	R: GATTTATATGGGGCTAAAGGGC					
MS3E10	F: CATTTTCCTGGGGATGGTATAA[a]	$(TATC)_8$	40	331–453	0.667/0.965*	0.781/0.969*
	R: GTGAGTCTGTATGTTCTTGATGGG					
MS3E02	F: GCCAATAAGAGCCCAGACATAC[a]	$(AC)_{22}$	21	369–409	0.900/0.914	0.906/0.932
	R: GGGGATTAGGGATAGGTTAGCA					

GenBank accession numbers for nucleotide sequences of clones containing the isolated microsatellites are FJ744416–FJ744424

* $P < 0.05$ after sequential Bonferroni correction for Hardy–Weinberg equilibrium

[a] indicates fluorescently labeled primer

Amp Agar plates (Invitrogen) spread with IPTG and X-gal. Recombinant colonies were incubated in LB broth at 37°C for 40 h. Inserts were amplified using PCR followed by sequencing using BigDye v3.1 dye terminator technology sequenced on an ABI 3730*xl* DNA Analyzer (Applied Biosystems, Foster City, CA) at the University of Missouri DNA Core Facility. Sequences were trimmed of linker and vector sequence in Sequencher v4.8 (Gene Codes Corporation, Ann Arbor, MI) and screened for SSR using WebSat (http://wsmartins.net/websat/; Martins et al. 2009) which utilizes Primer3 (Rozen and Skaletsky 2000) for designing PCR primers. Primers were designed in the flanking region for 9 isolated SSR with one primer from each of the 9 primer pairs fluorescently labeled at the 5′ end (6-FAM, VIC, NED, PET).

Isolated SSR were characterized using 62 individuals from two populations of *M. sodalis*, 20 individuals from one population of *M. gricescens*, 10 individuals each from one population of *M. lucifugus* and *M. septentrionalis*, and four individuals from one population of *M. leibii*. Genomic DNA was extracted from a 2 mm wing biopsy using a DNeasy Blood and Tissue Kit (QIAGEN Inc., Valencia, CA) following the extraction protocol for animal tissues with elution of DNA in 50 µl AE buffer. The optimized PCR reaction was 25 µl in total volume and consisted of the following: 10–25 ng genomic DNA, 1.5 mM $MgCl_2$, 2.5 µl 10× PCR Buffer B (Fisher Scientific, Pittsburgh, PA; 500 mM KCl, 100 mM Tris–HCl, pH 9.0), 2.5 µl 10 mM dNTP mix (2.5 mM dATP, dTTP, dCTP, dGTP, in 0.1 M Tris–HCl, pH 7.9), 1 µl of a 10 mM solution of each primer, 1.0 Unit of *Taq* DNA Polymerase (Fisher Scientific), and brought to volume with de-ionized water. Cycling parameters consisted of 3 min denaturation at 95°C followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 10 min. Genotyping was performed on an ABI 3730*xl* DNA Analyzer (Applied Biosystems) at the University of Missouri DNA Core Facility and the raw data were analyzed with GeneMarker® v1.8 (SoftGenetics LLC, State College, PA) using the LIZ 600 size standard (Applied Biosystems).

In *M. sodalis*, each locus was polymorphic with 17–48 alleles (Table 1). The observed (H_O; 0.531–0.969) and expected (H_E; 0.894–0.973) heterozygosity were calculated in Arlequin v3.11 (Excoffier et al. 2005). All loci except MS1B11 and MS3E10 in the Pilot Knob samples and MS4C05, MS3F05, and MS3E10 in the Great Scott samples conformed to Hardy–Weinberg expectations after sequential Bonferroni corrections were applied (Rice 1989). Linkage disequilibrium in all pair-wise combinations of the loci were tested using GENEPOP version 3.4 (Raymond and Rousset 1995) across both populations. Significant linkage disequilibrium was detected between MS3D09 and MS3E02 ($P = 0.05$ level). Cross-species amplifications of the nine microsatellite loci were successful for seven of the nine loci in all species tested except for *M. leibii* where only six loci resulted in an amplification product (Table 2).

The microsatellite markers described here will be useful in studying the population genetics of *M. sodalis*, in investigating the link between summer foraging and maternity roosts to winter hibernacula, and will provide

Table 2 Cross-species amplification of 9 polymorphic microsatellite loci isolated in *Myotis sodalis* and tested on *M. gricescens*, *M. leibii*, *M. lucifugus*, and *M. septentrionalis* including number of alleles (*A*), allele size range (bp), observed (H_O) and expected (H_E) heterozygosity for each species, and test for departure from Hardy–Weinberg equilibrium (HWE)

Locus	*M. gricescens* ($N = 20$)			*M. leibii* ($N = 4$)			*M. lucifugus* ($N = 10$)			*M. septentrionalis* ($N = 10$)		
	A	Size range	H_O/H_E	*A*	Size range	H_O/H_E	*A*	Size range	H_O/H_E	*A*	Size range	H_O/H_E
MS3D02	9	203–327	0.850/0.841	5	201–327	0.500/0.893	13	197–241	0.800/0.942	10	189–327	0.500/0.926
MS3D09	8	190–234	0.450/0.740*	6	175–285	1.000/0.923	12	187–230	0.500/0.937*	10	195–371	0.700/0.905
MS4C09	9	157–235	0.700/0.772	3	185–193	0.500/0.464	4	209–221	0.000/0.747*	12	158–257	0.556/0.948*
MS4C05	8	229–265	0.389/0.478	[a]			5	227–243	0.300/0.742*	7	223–251	0.400/0.737*
MS1B11	[a]			[a]			[a]			[a]		
MS3F05	11	303–327	0.842/0.896	5	313–321		9	295–327	1.000/0.905	13	295–331	1.000/0.963
MS1C01	4	325–355	0.050/0.191*	2	325–327	0.750/0.857	2	326–328	0.300/0.268	5	325–339	0.600/0.668
MS3E10	[a]			[a]			[a]			[a]		
MS3E02	8	375–401	0.789/0.765	5	377–395	0.500/0.857	12	371–487	0.500/0.942*	10	377–399	0.600/0.932

Primer sequences and repeat motifs are specified in Table 1. PCR reaction conditions and annealing temperatures are as described in text for *M. sodalis*

* $P < 0.05$ after sequential Bonferroni correction for Hardy-Weinberg equilibrium

[a] Loci that did not amplify

genetic data useful for the conservation and recovery of this endangered species. In addition, these microsatellite markers will be useful in investigating the population genetics of *M. gricescens*, *M. leibii*, *M. lucifugus*, and *M. septentrionalis.*

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