Conservation Genet Resour (2010) 2:243–245
DOI 10.1007/s12686-010-9253-y

TECHNICAL NOTE

# Nineteen polymorphic microsatellite loci isolated from the Eastern Massasauga Rattlesnake, *Sistrurus c. catenatus*

**Christine S. Anderson · H. Lisle Gibbs · James Chiucchi**

Received: 10 May 2010 / Accepted: 14 May 2010 / Published online: 30 May 2010
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**Abstract** Conservation genetic analyses can aid in the management of the eastern massasauga rattlesnake, *Sistrurus c. catenatus,* which is currently in decline throughout its range. To this end, we designed primers that amplify 19 novel microsatellite loci that are highly variable. Based on genotypes from 27 adults from a single population in Ohio, the number of alleles per locus ranged from two to 13 and observed and expected heterozygosities varied from 0.370 to 0.963 and 0.425 to 0.883, respectively. These loci will be useful for conservation and population genetic analyses in this endangered snake.

**Keywords** Crotalidae · Microsatellite DNA loci · PCR primers · Population genetic analyses · Endangered venomous snakes

DNA-based genetic analyses can yield valuable information for assessing levels of variation and population genetic structure in endangered species (Avise 1994). The eastern massasauga rattlesnake (*Sistrurus catenatus catenatus*) is currently found in remnant populations throughout eastern North America and is in decline primarily due to habitat fragmentation and destruction (Greene and Campbell 1992). This has led the United States Fish and Wildlife Service to list it as a candidate subspecies for endangered status in the United States (Szymanski 1998). Previous population genetic analyses of this snake were constrained by small numbers of loci to analyze limited population sites (Gibbs et al. 1997). Here, we describe an additional 19 novel highly polymorphic microsatellite loci, which we are using to conduct detailed studies of population structure and genetic diversity in this threatened rattlesnake.

Genomic DNA was extracted from a blood sample collected from an adult eastern massasauga rattlesnake in Spring Valley, Ohio, USA using phenol–chloroform. DNA was then enriched for microsatellites using three probe mixes [Mix2 = (AG)12, (TG)12, (AAC)6, (AAG)8, (AAT)12, (ACT)12, (ATC)8; Mix3 = (AAAC)6, (AAAG)6, (AATC)6, (AATG)6, (ACAG)6, (ACCT)6, ACTC)6, ACTG)6; Mix4 = (AAAT)8, (AACT)8, (AAGT)8, (ACAT)8, (AGAT)8] following the protocol of Glenn and Schable (2005). Briefly, *Rsa*I restriction fragments were subjected to microsatellite enrichment and then ligated into a pCR 2.1-TOPO vector. Bidirectional sequence was determined for 192 plasmids containing inserts from this enrichment using an ABI 3130xl Genetic Analyzer.

Candidate loci were selected by identifying sequences with six or more uninterrupted repeats and flanking regions with nonrepetitive sequences. Primer pairs were designed using MSATCOMMANDER 0.8.1 (Faircloth 2008) and OLIGO 6.71 (Molecular Biology Insights). Primers were labeled at the 5′ end with one of three fluorescent dyes (FAM, HEX, or NED). In total, primers were designed for 42 potential loci with tetranucleotide, trinucleotide, and dinucleotide repeats. We assessed locus-specific variation using DNA from 27 adult eastern massasauga rattlesnakes from a population in Killdeer Plains Wildlife Area, Ohio, USA. Blood samples were collected and DNA was extracted

C. S. Anderson · H. L. Gibbs · J. Chiucchi
Department of Evolution, Ecology and Organismal Biology, 300 Aronoff Laboratory, The Ohio State University, 318 W. 12th Avenue, Columbus, OH 43210, USA

C. S. Anderson (✉)
Department of Biological and Environmental Sciences, Capital University, 1 College and Main, Columbus, OH 43209, USA
e-mail: canders2@capital.edu

**Table 1** Characteristics of 19 microsatellite loci in *Sistrurus catenatus catenatus* based on 27 individuals from Killdeer Plains Wildlife Area, Ohio, USA

| Locus | GenBank accession no. | Repeat motif | Primer sequences (5′–3′) | Dye | $T_a$ (°C) | $N_a$ | Size range (bp) | $H_O$ | $H_E$ | *P*-HWE |
|---|---|---|---|---|---|---|---|---|---|---|
| Scu200[a] | HM179510 | ATCT^20 | F: TAATGTGTGCCATTGTGCC<br>R: CAATGTATCTCATCTCCAACCCC | HEX | TD65-62 | 13 | 224–266 | 0.704 | 0.883 | 0.004* |
| Scu201[a] | HM179511 | CTTT^15 | F: TGCAGCTAAAACAATAGTATGACCC<br>R: TGCAATTGGAAATCTTGTGACTG | FAM | TD65-62 | 8 | 210–250 | 0.741 | 0.850 | 0.001* |
| Scu202[b] | HM179512 | AC^22 | F: TCCAGTCCCAGTGAGCACG<br>R: GGCTCGCTTTTCCTTTAGGC | HEX | 65 | 5 | 175–187 | 0.667 | 0.621 | 0.933 |
| Scu203[b] | HM179513 | CTGT^11 | F: TTTTAGGAACAACGAAGAAGAGG<br>R: TGGATGGATGGATGGGTGG | FAM | 65 | 4 | 231–255 | 0.741 | 0.735 | 0.527 |
| Scu204[b] | HM179514 | AATG^17 | F: TCCAATTGCCAAAGACCAATAAG<br>R: TGGGCATCACTCCAAGAGG | NED | 65 | 4 | 147–167 | 0.852 | 0.736 | 0.454 |
| Scu205[c] | HM179515 | ATCT^21 | F: AGGCTCCATGAAACATTTGC<br>R: GAAGCGATGGTGATGGGTG | FAM | TD65-60 | 8 | 190–222 | 0.889 | 0.781 | 0.559 |
| Scu206[c] | HM179516 | GT^6 | F: GGTTGCTAAACGGGGTAGTC<br>R: TCAGCCCTAGGAAGGAGGG | NED | TD65-60 | 2 | 188–194 | 0.370 | 0.425 | 0.648 |
| Scu207[c] | HM179517 | ACT^10 | F: TAACCTCCAAGGTCCCTTCC<br>R: CCTTCTTTGAGGAAGATGGGC | HEX | TD65-60 | 7 | 254–278 | 0.815 | 0.772 | 0.114 |
| Scu208[d] | HM179518 | ACCT^11 | F: AGCAAGCAGTAGGTTGGGG<br>R: TTTCACGGAATGGGCAACC | HEX | 65 | 6 | 160–192 | 0.815 | 0.771 | 0.944 |
| Scu209[d] | HM179519 | AC^13 | F: GAAGCTGCCAGGAATGACC<br>R: AGCAGAAAACAACTCTGTGC | NED | 65 | 2 | 179–181 | 0.556 | 0.507 | 0.707 |
| Scu210[e] | HM179520 | (AGT)^13 | F: CCTGAATATTACTCCAAAAACG<br>R: ATATTGTTGCATCTGCTATCCA | FAM | 60 | 10 | 164–218 | 0.704 | 0.763 | 0.206 |
| Scu211[e] | HM179521 | (ATCT)^16 | F: TCCCTTTTCTAGAATAAGTCTC<br>R: TACCTTTGAAGATCATTTGGAG | FAM | 60 | 9 | 294–326 | 0.852 | 0.780 | 0.415 |
| Scu212[e] | HM179522 | (AGAT)^23 | F: ATGGGTGGTCTAGAAACATGAT<br>R: AAATTAGCTTCTGGGTCTGTCA | HEX | 60 | 10 | 335–375 | 0.926 | 0.866 | 0.207 |
| Scu213[e] | HM179523 | (ATCT)^24(ATCC)^4 | F: GAAAGGGAGGACCACAATA<br>R: ATATGCCACCTAGAATCACT | HEX | 60 | 9 | 202–246 | 0.963 | 0.862 | 0.002* |
| Scu214[f] | HM179524 | (AGAT)^25 | F: CTTCAAATTACAATAACCCACA<br>R: ACACTTTGAGAATAAGCAACTA | FAM | 55 | 9 | 158–202 | 0.963 | 0.832 | 0.528 |
| Scu215[f] | HM179525 | (AG)^15 | F: CCCCAGAAATTACCCTAAAG<br>R: GTGTCCCGCTTGAGA | HEX | 55 | 8 | 112–158 | 0.556 | 0.783 | 0.008* |
| Scu216[f] | HM179526 | (ATCT)^22(ACCT)^15 | F: AGGGAAAACTGTCAGTTCAC<br>R: CAGGCAAAGATCAATCAG | HEX | 55 | 13 | 205–285 | 0.815 | 0.867 | 0.015 |
| Scu217 [g] | HM179527 | (AC)^22 | F: AGTCCCAGTGAGCACGTGTG<br>R: CGCTTTTCCTTTAGGC | FAM | 55 | 5 | 167–179 | 0.667 | 0.621 | 0.937 |
| Scu218 [g] | HM179528 | (GAGT)^10 | F: CCCCAAAAACTTAACTCG<br>R: GAACCACACCCACAAGTT | FAM | 55 | 6 | 274–302 | 0.852 | 0.791 | 0.772 |

$T_a$ annealing temperature for optimization amplification, $N_a$ number of alleles, $H_O$ observed heterozygosity, $H_E$ expected heterozygosity, *P*-HWE *P* value for exact test of Hardy–Weinberg equilibrium with * indicating significance after adjustment for multiple tests

[a–g] Loci multiplexed together

using phenol–chloroform. Nineteen of these primer pairs successfully amplified scoreable polymorphic products using the conditions described below and in Table 1.

We set up 10-µl multiplex reactions containing 5-µl of Qiagen multiplex PCR Master Mix, 1-µl of primer mix (containing 2 µM of each primer), and 3.5-µl of RNase-free $H_2O$. Thermal cycling was carried out in an MJ PTC-200 thermal cycler (Bio-Rad); conditions included an initial hot start at 95°C for 15 min, a cycle of 94°C for 30 s, annealing temperature for 90 s, and extension at 72°C for 60 s repeated 34 times or alternatively a touchdown program combined with 21 cycles at the lowest annealing temperature, and a final extension step at 60°C for 30 min (Table 1). Products were run on an ABI 3100 Genetic Analyzer. Fragments were sized with the NAUROX size standard described in DeWoody et al. (2004) and alleles were binned using GENEMAPPER 3.7 software.

We calculated observed and expected heterozygosity and the number of alleles per locus in GENEPOP 3.4 (Raymond and Rousset 1995). Loci were tested for departures from Hardy–Weinberg equilibrium with the Markov chain method in GENEPOP and $P$ values were corrected for multiple tests with the Benjamini-Yekutieli (B-Y) method (Narum 2006). Frequencies of possible null alleles were estimated using equation 4 from Brookfield (1996). Observed and expected heterozygosities ranged from 0.370 to 0.963 and 0.425 to 0.883, respectively, and the mean number of alleles per locus was 7.3 (2–13 alleles; Table 1). Scu 200, Scu 201, Scu 213 and Scu 215 deviated from Hardy–Weinberg equilibrium but only locus Scu 201 showed a significant deficiency of heterozygotes (Table 1). The majority of loci had null allele frequencies estimated at less than 0.05 except for Scu 201 (0.059), Scu 200 (0.095), and Scu 215 (0.127). Finally, we tested for linkage disequilibrium using Fisher's method with GENEPOP 3.4 and found that 42 pairs of loci out of 171 had significant disequilibria after B-Y adjustment (Narum 2006). All 19 loci were found in 2–9 significant pairwise comparisons. The novel polymorphic microsatellite loci described here will be useful for detailed population genetic analyses in eastern massasauga rattlesnakes and closely related species.

**Acknowledgments** This work was supported by a grant from the Ohio Department of Natural Resources (ODNR) Division of Wildlife. We are grateful to T.C. Glenn for isolating microsatellite fragments, J. Diaz and A. (Poesel) Nelson for discussion in the laboratory, C. Caldwell for long term support of this research and the ODNR Division of Wildlife for permission to collect samples at Killdeer Plains Wildlife Area and Spring Valley in Ohio, USA.

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