POLYMORPHIC MICROSATELLITE LOCI FOR STUDIES OF THE OZARK HELLBENDER (*CRYPTOBRANCHUS ALLEGANIENSIS BISHOPI*)

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Word count: 997

Keywords: amphibian, *Cryptobranchus*, hellbender, salamander, population genetics, microsatellites

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ABSTRACT

The hellbender is the only North American member of the aquatic salamander family Cryptobranchidae and is a species of conservation concern across its range. We developed eight polymorphic microsatellite loci for hellbenders using a magnetic bead enrichment protocol and a PCR-based detection technique. Allelic diversity averaged 4.0 (± 1.8 SD) per locus and heterozygosity averaged 0.56 (± 0. 30 SD). The hellbender is rare and difficult to study due to its cryptic life history. These loci will provide a valuable resource for population studies, which could inform future conservation and management decisions.

The hellbender, *Cryptobranchus alleganiensis*, is a large, permanently aquatic salamander that inhabits swift-flowing, rocky streams of the eastern United States. It is the only North American member of the family Cryptobranchidae, and is comprised of two subspecies (*C. a. alleganiensis* and *C. a. bishopi*) that are of conservation concern across their discontinuous ranges (Phillips and Humphries 2005). While many factors have likely contributed to hellbender declines (e.g., introduced fishes, human recreation, illegal and scientific collections, diseases), habitat loss or alteration is likely the most important threat (Mayasich et al. 2003). Previous genetic investigations of hellbenders have revealed low levels of variation at allozyme and mitochondrial loci (Merkle et al. 1977, Routman 1993), and highly polymorphic markers are needed for landscape and population genetic studies that are crucial to the implementation of conservation strategies.

Genomic DNA was extracted from blood samples of three Ozark hellbenders using the DNeasy Blood and Tissue Kit (Qiagen). Using the methods of Kongrit et al. (2008), we digested DNA with *Nhe*I and *Xmn*I, selected fragments of 200-1000 bp in an agarose gel, and ligated those fragments to double stranded SNX linkers. Microsatellite containing fragments were hybridized to $(GT)_{15}$ and $(GATA)_8$ biotinylated oligos and extracted using streptavidin-coated Dynabeads (Invitrogen). We amplified the microsatellite-enriched DNA using SNX linkers as primers, ligated the products into pBluescript SKII(+/-), and transformed them into *E. coli* XL1-Blue supercompetent cells (Stratagene). We selected 960 colonies and isolated vector-insert hybrid molecules by incubation at 100 °C for 10 min in T.E buffer. Colonies containing microsatellite inserts were selected using the detection protocol of

Kongrit et al. (2008). Forty microsatellite-containing colonies were sequenced in both directions.

We designed primers for 35 loci using Primer3 v.0.4.0 (Rozen and Skaletsky 2000) and screened them for polymorphism on a set of eight individuals. The remaining five microsatellite-containing colonies had insufficient flanking sequence for primer design. Of the 35 loci, 13 amplified multiple products, 13 were monomorphic, two were found to be identical, and eight were polymorphic and amplified a single product of the expected size. These eight loci (Table 1) were labeled with 5' fluorescent dyes and screened on a set of 20 Ozark hellbenders (15 from the North Fork of the White River [NF], Ozark Co., MO, and 5 from Bryant Creek [BC], Ozark Co., MO). Amplifications were performed in 15.0 μL volumes containing 15-20 ng of template DNA, 1X PCR Gold buffer (Applied Biosystems), 0.4 μM fluorescently labeled forward primer, 0.4 μM reverse primer, 0.2 mM each dNTP, 2.0 mM $MgCl_2$ and 0.5 U *Taq*Gold DNA polymerase (Applied Biosystems). The PCR profile consisted of a 10 min denaturation at 95 °C, followed by 35 cycles of 45 sec denaturation at 95 °C, 45 sec primer annealing at 58 °C, 1 min primer extension at 72°C, and a final extension of 10 min at 72°C. Fragment analysis was performed in an ABI 3730 DNA Analyzer using Big Dye Terminator cycle sequencing chemistry and Liz 600 size standards (Applied Biosystems) at the University of Missouri DNA Core Facility. Alleles were scored using Gene Marker (Gene Codes, Inc.).

We used GenePop (Raymond and Rousset 1995) to test for deviations from expectations under Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium. Only locus CRAL13 was found to deviate from HWE due to an excess of

heterozygotes (Table 1). There was no evidence of linkage between any of the loci. Testing in Micro-Checker version 2.2.3 (van Oosterhout et al. 2004) found no evidence of null alleles.

Total allelic diversity was similar for both NF (x=3.4 ± 1.8 SD) and BC (x=2.6 ± 1.4 alleles/locus) populations. Overall, allelic diversity averaged 4.0 (± 1.8 SD) alleles/locus and heterozygosity averaged 0.556 (± 0.302 SD). These loci will contribute to the conservation and management of this species, which is a candidate for listing under the Endangered Species Act, by providing a novel genetic resource that can be used to elucidate patterns of gene flow, delineate population structure, and assess effective population sizes.

REFERENCES

Kongrit C, Siripunkaw C, Brockelman W, Eggert LS (2008) Isolation and characterization of dinucleotide microsatellite loci in the Asian elephant (*Elephas maximus*). Mol Ecol Res 8:175-177.

Mayasich J, Granmaison D, Phillips C (2003) Eastern hellbender status assessment report. Natural Resources Research Institute, Technical Report 9:1-41.

Merkle DA, Guttman SI, Nickerson MA (1977) Genetic uniformity throughout the range of the hellbender, *Cryptobranchus alleganiensis*. *Copeia*, **1977**, 549-553.

Phillips CA, Humphries WJ (2005) *Cryptobranchus alleganiensis* (Daudin, 1803). In: Amphibian declines: The conservation status of United States species (ed. Lanoo M), pp. 648-651, University of California Press, Berkeley, CA.

Raymond M, Rousset F (1995) GENEPOP version 1.2: Population genetics software for exact tests and ecumenicism. J Hered 8:248.

Routman E (1993) Mitochondrial DNA variation in *Cryptobranchus alleganiensis*, a salamander with extremely low allozyme diversity. Copeia 1993:407-416.

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology (eds. Krawetz S, Misener S), pp 365-386, Humana Press, Totowa, NJ.

van Oosterhout C, Hutchinson, WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535-538.

ACKNOWLEDGEMENTS

We thank C. Phillips and E. Routman for providing samples, and W. Savage and I. Wang for helpful comments and suggestions. Funding was provided to LSE by the University of Missouri Division of Biological Sciences. Research was conducted in compliance with animal care guidelines and scientific collection permits issued by Missouri Department of Conservation.

Table 1. Locus name, repeat motif, primer sequences, annealing temperature (T_A), and locus characteristics for eight microsatellite loci characterized in the hellbender (*Cryptobranchus alleganiensis*). Listed for each locus is the observed heterozygosity (H_O), expected heterozygosity (H_E) under HWE, and the probability that the locus conforms to expectations under HWE (P_{HWE}).

Locus	Repeat motif	Primer Sequence (5'-3')	T_A (°C)	No. of alleles	Size range	H_O	H_E	P_{HWE}	Accession no.
Cral02	$(CA)_{18}$	F: VIC-CGTAGAATTGAATGGATTGCTTT R: CCACATCGAATGTGTCTGCT	57	6	182-196	0.650	0.659	0.218	TBD
Cral04	$(CA)_{10}$	F: NED-AGGGCACCACACAAACAAA R: AAACCCAGAGACATGCTTCC	60	2	160-162	0.150	0.296	0.070	TBD
Cral08	$(CA)_{18}CGC(CA)_4$	F: NED-TCAGAGGGAAGTCTGTGTAGCA R: AAAGTGGGGAAAAACCCATC	56	6	194-210	0.650	0.628	0.675	TBD
Cral09	$(CA)_{12}$	F: 6FAM-CCCACCCCTAGAGAAGAAGG R: AAGGGACTGTGTGTGTACCTTAGA	60	3	120-126	0.200	0.273	0.163	TBD
Cral10	$(AC)_{14}$	F: NED-GCTCGGATGACAGAGGTTTC R: TGGCAAATTTCATTCTGCTTC	58	3	237-241	0.600	0.512	0.780	TBD
Cral13	$(GT)_{12}$	F: 6FAM-TCAACGTATAAAGTAACATAAAACCAA R: GGCTCAGAATGTCTAGGTGGTC	60	4	125-133	1.000	0.584	0.000	TBD
Cral15	$(CA)_3TG(CA)_8$	F: 6FAM-TGGGGTTTCATACAGGCTTC R: GGTTGAGATTGTGCATGGTG	60	2	114-118	0.350	0.296	1.000	TBD
Cral17	$(CTAT)_{24}$	F: VIC-ATTCCAAGGGGGCTGAATAC R: CGCCTTGATGTAGCTTTTGG	53	6	137-157	0.850	0.731	0.834	TBD