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TECHNICAL NOTE

Isolation and development of 12 polymorphic tetranucleotide microsatellite markers for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*)

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Abstract In order to analyze population genetic structure at multiple spatial scales, 12 polymorphic microsatellite loci were developed for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). The number of alleles per locus ranged from 6 to 12 (mean of 9.25 per locus) and the observed heterozygosity ranged from 0.645 to 0.935 among 31 individuals. Two loci exhibited significant deviations from Hardy–Weinberg equilibrium. No evidence for linkage disequilibrium was detected between pairs of loci. These markers will be used to examine population genetic structure across the geographic range of this declining species.

Keywords Hellbender Genetics Genetic diversity

Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are large paedomorphic salamanders that historically occurred throughout much of the central and eastern United States. Hellbender populations, like many amphibian populations worldwide, are declining at alarming rates across their range (Stuart et al. 2004). Purported reasons for these declines include illegal collection, disease, and habitat degradation (Mayasich et al. 2003; Nickerson et al. 2002). Current hellbender populations are often small, isolated, and composed of older age classes with little or no evidence of population recruitment (Wheeler et al. 2003). A greater understanding of the genetic ramifications of these declines is essential if we are to develop adequate management strategies for this species (Jehle and Arntzen 2002). Here, we report the isolation and characterization of 12 highly polymorphic tetranucleotide microsatellite markers for *C. a. alleganiensis*, which can be used to examine fine-scale genetic structure for future conservation and management efforts for this species.

Genomic DNA was extracted from blood samples collected from two southern Indiana hellbenders using an ammonium acetate protocol (modified from the PUREGENE kit; Gentra Systems). To construct the library, we enriched for tetranucleotides [$(GATA)_7$, $(GATC)_7$, and $(GACA)_7$] and dinucleotides [$(GT)_{12}$ and $(CT)_{12}$], following the microsatellite cloning protocol of Hamilton et al. (1999), with modifications as described in Beheler et al. (2004) and Williams and DeWoody (2004). Over 427 recombinant clones were initially sequenced and analyzed using Sequencer 4.1 (Gene Codes Corporation). We selected 56 recombinant clones that produced microsatellite sequences with adequate flanking regions, contained the highest number of repeats, and which successfully sequenced in both directions. We then designed primers for 34 loci using PRIMER 3 software (Rozen and Skaletsky 2000). Of those 34 putative loci, 22 consistently worked in both forward and reverse directions and produced bands of the expected size during 10 μl PCR reactions that contained 20 ng of template DNA, 0.25 mM of each primer, 1U of *Taq* polymerase (NEB), 1X reaction buffer (10 mM Tris–HCL, 50 mM KCL, 0.05 mg/ml BSA), 1 mM $MgCl_2$, and 0.2 mM of each dNTP. PCRs were conducted on a Master-cycler ep gradient (Eppendorf) using the following thermal profile: 94 C for 2 min, 94 C for 30 s, primer specific annealing temperature (Table1) for 30 s, and 72 C for 30 s for 30 cycles, 72 C for 10 min, followed by 60 C for 45 min.

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We screened for levels of polymorphism across the 22 loci using a panel of 31 individuals from a single Indiana population. The protocol for this screening is the same as that given above, except for each reaction the concentration of dTTP was reduced to 0.15 and 0.05 mM chromatide rhodamine green 5 dUTP (Molecular Probes) was added. PCR products were genotyped on ABI 3730 automated sequencer (Applied Biosystems) and a ROX-labeled size standard was used for scoring genotypes in each sample (Genemapper version 3.7). We selected 12 of the most polymorphic loci, ordered labeled primers (forward) from Integrated DNA Technologies, and genotyped the screening panel of 31 individuals at all 12 loci.

We calculated allelic richness, observed and expected heterozygosities using Genepop version 4.0.10 (Rousset 2008). Tests for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium also were calculated using Genepop version 4.0.10. The frequency of null alleles was calculated using Micro-Checker version 2.2.3 (Oosterhout et al. 2004). Additional statistics included calculating the probability of identity using API-Calc 1.0 (Ayres and Overall 2004) and exclusion probabilities using Cervus 3.0.0 (Kalinowski et al. 2007).

Allelic richness ranged from 6 to 12 per locus and averaged 9.25 alleles per locus (Table 1). Observed and expected heterozygosities ranged from 0.645 to 0.935 and from 0.763 to 0.903, respectively (Table 1). We tested 66 pairwise comparisons and found no evidence of linkage disequilibrium between pairs of loci after a Bonferroni correction ($P < 0.00076$). Two loci (Call 352 and Call 261) were found to deviate from Hardy–Weinberg equilibrium after a Bonferroni correction ($P < 0.0042$), one of which (Call 261) also exhibited evidence for the presence of null alleles at a frequency of 7.5% (Table 1). Total average probability of identity was 2.91×10^{-15}. Exclusion probabilities with either one or both parents known ranged from 99.98 to 99.99%, respectively. These highly variable polymorphic markers should prove valuable for investigating genetic diversity and fine scale structure of threatened hellbender populations.

Table 1 Characterization of 12 polymorphic microsatellite loci developed for *Cryptobranchus aleganiensis alleganiensis*

Locus	Genbank accession no.	Primer sequence	Motif	PCR product	A	T_A	H_e	H_0
Call171	Pending	F: GGTGAGCGCTCTACAAGC R: TCCAGCCTTATGTGTCAGACC	$(GATA)_{13}$	113–141	7	57	0.781	0.645
Call127	Pending	F: TGAACTGTGGAGTTGGATCG R: CACATATGGGTAGTAACTGCATGG	$(TATC)_{14}$	326–362	9	57	0.879	0.871
Call351	Pending	F: ACATTAATTCTCCTTCCTGTCACC R: CAATCCTAAGGAGGAATTGAAGC	$(TATC)_{20}$	227–255	8	60	0.857	0.710[a]
Call204	Pending	F: TTCGCGAGCATTACTCTACG R: ACGGTCCAGACATTGTTGC	$(GATA)_{21}$	212–252	10	60	0.872	0.871
Call205	Pending	F: TTTGAGCTCTCTTGGCTTATG R: TGGACTCCTTCCCTTTCTCC	$(GATA)_{20}$	159–191	9	64	0.846	0.710
Call232	Pending	F: CGTATGCCTGGCACATAACC R: CCACCATAAGATTCACACTGC	$(TGTC)_{18}$	162–202	10	64	0.855	0.871
Call347	Pending	F: ACCAGCAGCAACCTTATCTGG R: ACCATGCAGCCGGTAAGC	$(GATA)_{20}$	182–226	11	64	0.886	0.935
Call282	Pending	F: ACCCGAAAGGGTGGTTTATAG R: TAATGAGCCGTTAGCCCTTG	$(GATA)_{15}$	195–235	9	64	0.763	0.774
Call341	Pending	F: GCAAGAAGGTGAGCAAGAGG R: CCATCTGAATATACCTGCAATCTG	$(GATA)_{15}$	232–252	6	64	0.775	0.871
Call261	Pending	F: TTCTAGCGCTGCGAGACC R: TGTAGATTCCGCTACCTTCTACG	$(TATC)_{18}$	144–188	12	66	0.889	0.742[ab]
Call26	Pending	F: CATAATGGTAATAGCTGCATGG R: CCTTGTTCCAGATTCACACC	$(GATA)_{18}$	174–218	12	60	0.903	0.935
Call266	Pending	F: TCTGCAAGCCACTAAATAGCC R: AACATTGGGAGGCTGGTATG	$(GATA)_{11}$	210–238	8	66	0.840	0.871

The primer pair, repeat motif, size range of alleles (bp), number of alleles (A), annealing temperature in C (T_A), expected (H_e) and observed (H_0) heterozygosities based on 31 individuals are reported

[a] significant heterozygote deficiency ($\alpha = 0.0042$)

[b] frequency of null alleles >0.05

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