PRIMER NOTE

Isolation and identification of 21 microsatellite loci in the Copper redhorse (*Moxostoma hubbsi*; Catostomidae) and their variability in other catostomids

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Abstract

Catostomidae represent an important family of freshwater fishes mainly distributed in North America, but also found in Eurasia. This paper describes the development of microsatellite DNA markers for a highly threatened member of this family, the Copper redhorse (*Moxostoma hubbsi*), as well as cross-catostomids amplifications. 168 tetra-nucleotide loci were screened to develop 21 polymorphic markers, with an average number of 8.5 alleles per locus and observed heterozygosity ranging between 0.52 and 1.00. Successful amplification was obtained for 12 other members of the family at between seven to 19 loci, with between two to 18 loci being polymorphic per species.

Keywords: catostomidae, conservation, cross-species amplification, microsatellite development

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Catostomidae represent an important family of freshwater fishes comprising 61 species that are mainly distributed in North America but also found in Eurasia (Bernatchez & Giroux 2000). Many species of this family have suffered from increased anthropogenic effects that have resulted in their demographic decline. Among these, the Copper redhorse (*Moxostoma hubbsi*) has been listed as threatened in Canada under the Committee on the Status of Endangered Wildlife in Canada since 1987, as vulnerable under the IUCN (The World Conservation Union) Red List of Threatened Species since 1996 and as threatened under the Act respecting threatened and vulnerable species in Quebec since 1999. This status is mainly due to an extremely restricted and shrinking geographical distribution, now limited to the Richelieu River and a short section of the St. Lawrence River in Quebec, Canada. In order to prevent the extinction of the Copper redhorse, recovery efforts are focusing on a future supportive breeding program, with the intent of using genetic markers to avoid inbreeding and outbreeding depression. Thus, information on the population structure and individual relationships will be necessary in order to choose parents and appropriate locations for reintroduction. To further these objectives, the first step was the development of microsatellite genetic markers, described

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within this paper. Cross-species amplification and primer utility has been tested in 12 other catostomids: *Moxostoma valenciennesi*, *M. robustum*, *M. anisurum*, *M. macrolepidotum*, *M. carinatum*, *Scartomyzon congestus*, *Thoburnia rhothoeca*, *Hypentelium nigricans*, *Minytrema melanop*, *Erimyzon oblongus*, *Catostomus catostomus*, *C. commersonii*. As karyotypic analyses strongly suggests Catostomids are tetraploid (Uyeno & Smith 1972; Ferris & Whitt 1980), special attention was devoted to ascertaining if any primers amplified duplicate microsatellite loci during analyses.

Genomic DNA was extracted from liver samples of *M. hubbsi* using a Dneasy extraction kit (QUIAGEN). Microsatellite enrichment by biotin capture- di-nucleotide (CA_n and TC_n) and tetra-nucleotide ($GATA_n$) microsatellites from those samples was carried out by BC Research Inc. (Vancouver, BC, Canada). We obtained 384 bacterial plasmid clones of random genomic restriction fragments containing di- and tetra-nucleotide motifs. Purified plasmids were amplified directly in 50-µL reactions containing: (10.4 mM Tris-HCl, ph 9.0, 52 mM KCl, 1.56 mM MgCl$_2$, Triton X-100 0.1%), 62.5 µM of each dNTP's, 348.6 µM forward and reverse sequencing M13 universal primers, and 9 units of *Taq* DNA polymerase. Reaction mixtures were amplified by polymerase chain reaction (PCR) in a Biometra T-1 thermocycler with an initial denaturation time of 5 min at 94 °C followed by 35 cycles of 95 °C for 30 s, 40 s at 60 °C and 1 min at 72 °C, finalizing with a 10 min of elongation

Table 1 Characterization of 21 microsatellite loci in *Moxostoma hubbsi*

Locus ID/ Genbank	Repeat motif	Primer sequences (5′–3′)	T_a	n	Range	A	H_O	H_E	$T*$
Mohu-Lav194 AY633584	(GATA)$_{24}$	**F:** CTTTTTCTCCTGGCGAACG **R:** CACGCAGCGGAGGTATTATT	60	23	135–187	8	0.87	0.83	
Mohu-Lav198 AY633585	(TAGA)$_{15}$	**F:** TGTTTTATTTCCCAGCCCTAA **R:** TATAGTGCGCTGTGTGAATTGTG	60	23	175–203	4	0.78	0.66	
Mohu-Lav200 AY633586	(ATCT)$_{18}$	**F:** GCTTTTGTTTCCCTTTTTCTGA **R:** CCCCAAACTTTTGAACGGTA	63	23	134–186	10	0.83	0.78	
Mohu-Lav203 AY633587	(TGAG)$_{15}$(GATA)$_{10}$	**F:** TGTTTATTTTATTTTGGCTTTTGAA **R:** CACTCTACACTCACTCTGCCAAA	60	23	123–167	8	0.7	0.78	
Mohu-Lav211 AY633588	(ATCT)$_{18}$GTCT(ATCT)$_8$	**F:** CTGCAGTCTCAAACACATGG **R:** CGCTGCTGAGTATGTATGGA	63	20	135–183	11	0.8	0.83	*
Mohu-Lav212 AY633589	(GATA)$_{19}$	**F:** CGATAACAGCATGGGATCAA **R:** TCGATGAGTCACCCAACAAC	60	12	170–202	7	0.67	0.8	
Mohu-Lav213 AY633590	(ATCT)$_{17}$	**F:** GAAATGTTGCCAGGTCGTCT **R:** TCATCCTTGAAGCTGATGAAAA	60	22	137–169	7	0.91	0.8	
Mohu-Lav229 AY633591	(TAGA)$_{21}$	**F:** CGCCACTGTCATCCCCTAT **R:** CTCGGGTGTGCACTCATTC	63	23	157–245	15	0.87	0.8	*
Mohu-Lav237 AY633592	(GATA)$_{29}$	**F:** TGGGTTTTGAATGACATGAGAG **R:** TCGCCCCAAAATAAAAATCA	63	23	143–183	11	0.91	0.87	
Mohu-Lav268 AY633593	(GACA)$_5$(GGCA)$_2$GGTATA(TAGA)$_{23}$	**F:** CACAACAGCAGAATTAAGACAGG **R:** TCACCTTCAATCCATCATCAA	59	23	172–228	10	0.78	0.75	
Mohu-Lav270 AY633594	(ATCT)$_{17}$	**F:** CTGCAGCACATTGTTCCATT **R:** TGTAAGTCGTATGAACAATGACAAA	59	23	196–240	11	0.65	0.87	
Mohu-Lav277 AY633595	(TCTG)$_3$(TCTA)$_{20}$	**F:** GAAGGCCAGAGGGAAAGAAC **R:** AGTTTTGCAAGAGCAAGCAC	59	23	167–199	7	0.83	0.76	*
Mohu-Lav286 AY633596	(ATCT)$_{17}$	**F:** TGGGGTTCATCAATCAATCA **R:** GGATGACATGAGGGGGAAT	59	22	184–208	6	0.82	0.63	
Mohu-Lav294 AY633597	(GATA)$_{18}$	**F:** TGTCAGGTCGGGTTTAGAGC **R:** CGGAAATAGAAGCAATGACAGG	63	23	137–221	18	0.87	0.92	
Mohu-Lav296 AY633598	(ATCT)$_{21}$(TTCT)$_{11}$	**F:** TCCTGCTATCTTTGGCATATTTT **R:** TGCCCAACAGAGAAAGGAAC	60	23	145–221	16	1	0.91	
Mohu-Lav305 AY633599	(GATA)$_9$(GACA)$_3$GAGA(GACA)$_6$GATA	**F:** TGGAGAGTTATTTTTCTCACATCTAA **R:** AACTTGAATGTTTGATATTGCTTTT	60	22	183–191	3	0.59	0.61	
Mohu-Lav306 AY633600	(CAGA)$_7$(TAGA)$_5$(CAGA)$_7$(TAGA)$_{19}$	**F:** TCTTCTTCTATCATCCTACCTACTCAA **R:** TTCGGACTACTCTTGTCTATAATGTTT	59	21	222–246	7	0.52	0.8	
Mohu-Lav321 AY633601	(CTAT)$_2$CTGT(CTAT)$_{10}$CTGT(CTAT)$_{12}$ (CTGTCTAT)$_2$CTGT(CTAT)$_3$	**F:** TCAAGCTAATTAGAAAACGATTTATTG **R:** GTCATGAGGGGCCGAAAT	60	23	142–202	6	0.7	0.74	
Mohu-Lav329 AY633602	(TAGA)$_2$TATA(TAGA)$_{16}$	**F:** AGCTTGGTAAAGCCTTGTCG **R:** CCTTGTTCTGACTTCAAGTGCTA	60	23	171–211	6	0.7	0.68	
Mohu-Lav336 AY633603	(GATA)$_{15}$(GAGAGATA)$_3$	**F:** TCATAATTGATCAGTGTTTATGCAA **R:** GTTGGGAACCACTGTGTGTG	60	21	184–212	6	0.57	0.72	
Mohu-Lav347 AY633604	(GATA)$_{14}$	**F:** TGTTTTATTTCCCAGCCCTAA **R:** AGTGCGCTGTGTGAATTGTG	60	23	171–199	4	0.78	0.66	

T_a, annealing temperature used in polymerase chain reaction; n, sample size of Copper redhorse used to assess variability; range, allelic range (bp); a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; T, tetraploidy (*: duplicated loci with one locus polymorphic; **: Duplicated loci with both loci polymorphic).

phase at 72 °C. Among 168 clones tested containing tetra-nucleotide motifs, 76 have successfully been amplified. Approximately 5 μL of the PCR products was loaded onto a 1.2% agarose gel stained with ethidium bromide in order to ensure sufficient product for sequencing, which was carried out on an ABI™ Prism 3100 sequencer (Perkin Elmer). Microsatellite primers were developed for 36 sequences using PRIMER3 software (Rozen & Skaletsky 1998) and AMPLIFY version 1.2 (Engels 1993).

Locus variability in the Copper redhorse and other catostomids was tested with total genomic DNA extracted from fin tissue, using the Dneasy kit (QUIAGEN). PCRs were carried out in a 11-μL reaction containing: 1 μL (25–50 ng) of total genomic DNA, 1.2 μL of reaction buffer (10.9 mM Tris-HCl, ph 9.0, 54.5 mM KCl, 1.6 mM MgCl$_2$, Triton X-100 0.1%), 56.8 μM of dGTP, dCTP and dATP, 28.4 μM of dTTP, 0.5 pmol of forward and reverse primers, 1.7 μM of dUTP TAMRA (Molecular Probe) fluorescent incorporation labelling and 2 units of *Taq* DNA polymerase. For *Mohu-Lav200* and *Mohu-Lav212*, MgCl$_2$ concentration was reduced to 1.2 mM. The PCR reactions were performed using a Biometra T-1 thermocycler with identical thermal cycling conditions as previously explained except for the annealing temperature, which was specific to each locus (Table 1). An 8% denaturing polyacrylamide gel was used to separate PCR products by electrophoresis. Bands were visualized on an FMBIO II scanner (Hitachi) and scored using the GENESCAN-500 size standard (Applied Biosystems,

Inc.). Locus variability in the Copper redhorse was successfully analysed for 20–23 (depending on locus) individuals sampled from the St-Lawrence River (Table 1). The software GENETIX 4.03 (Belkhir *et al.* 2000) was used to calculate the observed and expected heterozygosity. If more than two alleles were detected for some individuals, the loci were considered as being duplicated (Table 1 and Table 2).

Of the 33 primer pairs developed from the genomic bank, 21 amplified polymorphic loci for the Copper redhorse (Table 1). The number of alleles ranged from three to

Table 2 Cross-amplification data of 12 additionnal taxa of catostomids

Locus	*Moxostoma robustum*	*Moxostoma macrolepidotum*	*Moxostoma anisurum*	*Moxostoma carinatum*	*Moxostoma valenciennes*	*Scartomyzon congestus*
n	3	5	5	5	5	5
Mohu-Lav194	+	+	+	+	+	+
Mohu-Lav198	•	+	+	+	•	+
Mohu-Lav200	−	−	−	−	−	−
Mohu-Lav203	+	+	+	+	−	+
Mohu-Lav211	+	+	+	+	+	+
Mohu-Lav212	+	+	+	+	+	+
Mohu-Lav213	+	+	+	+	+	•
Mohu-Lav229	+*	+*	+*	+*	+*	+*
Mohu-Lav237	+	+	−	+	+	+
Mohu-Lav268	+	+	+**	+	+	+
Mohu-Lav270	•	+	•	•	+	−
Mohu-Lav277	•*	+*	+*	+*	+*	+*
Mohu-Lav286	+	+	+	-	+	+
Mohu-Lav294	+	+	+	+	+	+
Mohu-Lav296	+	+	+	+	+	+
Mohu-Lav305	+	+	+	+	+	+
Mohu-Lav306	+	+	+	+	+	+
Mohu-Lav321	+	−	+	+	+	•
Mchu-Lav329	+	+	+	+	+	+
Mehu-Lav336	+	+	+	+	+	+
Mohu-Lav347	•	+	+	+	•	+
Total Amp	20	19	19	19	19	19
Total Poly	16	19	18	18	17	17

Locus	*Thoburnla rhothoeca*	*Hypentelium nigricans*	*Minytrema melanops*	*Erimyzon oblongus*	*Catostomus catostomus*	*Catostomus commersoonii*
n	5	5	3	2	2	5
Mohu-Lav194	•	•	•	−	−	−
Mohu-Lav198	+	+	•	+	•	•
Mohu-Lav200	−	−	−	−	−	−
Mohu-Lav203	−	+	+	•	−	−
Mohu-Lav211	+	+	•	•	−	•
Mohu-Lav212	•	•	•	•	•	•
Mohu-Lav213	•	+	•	+	•	•
Mohu-Lav229	+*	+*	•	•	+*	+*
Mohu-Lav237	−	−	−	−	−	−
Mohu-Lav268	+	+	•	−	+	+
Mohu-Lav270	+	−	•	−	−	•
Mahu-Lav277	+*	•*	•*	+*	+*	+*
Mohu-Lav286	+*	+	−	•	−	−
Mohu-Lav294	•	+	+	•	+	+
Mohu-Lav296	+	+	−	•	•	−
Mohu-Lav305	+	•	•	•	•	+
Mohu-Lav306	•	+	+	−	−	−
Mohu-Lav321		−	−	−	−	−
Mohu-Lav329	+	+	•	−	−	−
Mohu-Lav336	+	+	+	+	−	−
Mohu-Lav347	+	+	•	+	•	•
Total Amp	17	17	16	13	10	10
Total Poly	12	13	4	5	4	5

−, multiple bands, smear of no amplification; • amplification but insufficient data to determine polymorphism; +, amplification and polymorphism; *, duplicated loci with non overlapping range; **, duplicated loci with overlapping ranges.
Total Amp, total number of amplified (+ or •) loci within species.
Total Poly, total polymorphic loci within species.

18 with an overall average of 8.5 alleles per locus, while observed heterozygosities ranged from 0.52 to 1.00. We tested for Hardy–Weinberg equilibrium using the Markov chain method in the GENEPOP package version 3.4 (Raymond & Rousset 1995) in order to estimate heterozygote deficiency or excess for each locus (Guo & Thompson 1992). No departure from HWE was detected following Bonferonni corrections for multiple tests ($\alpha = 0.05$, k = 21) (Rice 1989). A preliminary Fisher's test of linkage disequilibrium implemented in GENEPOP version 3.4 over 210 locus pair tests indicated one locus pair (*Mohu-Lav198* and *Mohu-Lav347*) in linkage disequilibrium at the table-wide significance level of 0.05 ($P < 0.0002$). Three primer pairs amplified duplicate loci of tetraploid origin, one for which the two loci were polymorphic (*Mohu-Lav211*), whereas the others amplified only one polymorphic and one monomorphic locus (*Mohu-Lav229* and *Mohu-Lav270*). Between two to five individuals per species were tested for cross-specific amplification (Table 2). Amplifications worked well for most species using identical reagent and thermal cycling conditions, especially for other members of the genus *Moxostoma* but also for the *Scartomyzon* species. Overall, these results illustrated the utility of these 21 loci towards assessing patterns of genetic diversity and parentage in the Copper redhorse as well as in other species of the genus *Moxostoma.* A subset of these markers should also be applicable to any other member of the catostomid family.

Acknowledgements

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