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TECHNICAL NOTE

Isolation and characterization of microsatellite loci in the redside dace, *Clinostomus elongatus*

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Abstract We isolated and characterized eight polymorphic microsatellite loci for redside dace (*Clinostomus elongatus*), a colorful North American cyprinid that is threatened or endangered throughout most of its range. The number of alleles per locus ranged from three to eighteen, with observed heterozygosity ranging from 0.31–0.92. Cross-amplification revealed that these markers will also be useful for examining closely related and more distantly related species, including the rosyside dace (*Clinostomus funduloides*), Lahontan redside shiner (*Richardsonius egregius*), hornyhead chub (*Nocomis biguttatus*), and central stoneroller (*Campostoma anomalum*). These microsatellite loci will provide a valuable set of tools for examining fine and coarse scale population structure, exploring reproductive success, and testing outcomes of proposed conservation initiatives (e.g. captive breeding and translocation of wild individuals) for redside dace.

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Redside dace (*Clinostomus elongatus*) is broadly distributed in the Laurentian Great Lakes basin, and historically occurred in streams of all five Great Lakes, the upper Mississippi drainages, the upper Susquehanna River, and the Ohio River (Page and Burr 1991; COSEWIC 2007). Redside dace are visual surface feeders with a diet primarily consisting of terrestrial insects (Daniels and Wisniewski 1994; Scott and Crossman 1998). This specialized feeding ecology requires redside dace to live in clear, cool flowing water with overhanging vegetation (Novinger and Coon 2000), making it particularly sensitive to environmental perturbations. Due to habitat alteration, likely arising from deforestation, agriculture, urban development and pollution (Trautman 1981; Lyons et al. 2000; COSEWIC 2007), redside dace is threatened or endangered throughout most of their range. For example, in Canada, redside dace was initially listed as Vulnerable by COSEWIC (Committee on the Status of Endangered Wildlife in Canada) in 1987 (Parker et al. 1988) but is now listed as Endangered (COSEWIC 2007).

There is no published research investigating genetic population differentiation in redside dace, although allozyme analyses of several populations have been initiated (Redside dace Recovery Strategy 2005). As most populations are small and isolated, genetic diversity may prove important in the conservation of this species throughout its range. We developed and characterized eight microsatellite loci for redside dace, which can be used to determine genetic diversity and population structure among the remaining populations of this imperiled species.

Genomic DNA for library construction was extracted from fin clips stored in 95% ethanol using a phenol–chloroform method with ethanol precipitation. Extracted DNA was enriched for microsatellites according to a protocol adapted from Hamilton et al. (1999). Genomic DNA was simultaneously digested with *Rsa*I and the resulting blunt-ended fragments were ligated to SNX adapter-primer complexes consisting of a 22-mer (5′ CTAAGGCCTT GCTAGCAGAAGC 3′) and a phosphorylated 26-mer (5′ P-GCTTCTGCTAGCAAGGCCTTAGAAAA 3′). The resulting segments were hybridized with pooled biotinylated oligo ($GACA_4$) and (CA_7) probes, and captured with streptavidin-coated beads (Roche, Indianapolis, USA). Enriched fragments were cloned into TOPO vectors and then used to transform One Shot® competent *Escherichia coli* cells (Invitrogen, Burlington, Canada).

One hundred and ninety-two recombinant clone inserts were amplified using M13 forward and reverse primers and sequenced using an ABI 3100xl DNA analyzer (Applied Biosystems, Foster City, USA). Primers were designed for 16 clones that contained >8 uninterrupted di- or tetra-nucleotide repeats using PRIMER 3 (Rozen and Skaletsky 2000) and NetPrimer (Premier Biosoft International) software.

Preliminary screening for amplification and polymorphism was performed in 25 μL PCR reactions with an Eppendorf epgradient S Mastercycler (Brinkmann Instruments, Inc.). Each reaction included approximately 50 ng template DNA, 32 μM dye-labelled forward primer, 0.5 μM reverse primer, 200 μM of each dNTP, 2.5 mM $MgCl_2$, and 0.5 U *Taq* DNA polymerase (Applied Biosystems, Foster City, USA) in a 1× PCR buffer. Primers were tested on 64 *C. elongatus* individuals from one putative population, Rathburn Run (Wayne County, Ohio, USA; N 40° 48.658′ W 082° 01.400′), using the following thermocycling protocol: 2 min at 95°C; 30 cycles of 15 s at 95°C, 15 s at 55°C, 30 s at 72°C, followed by a 2 min extension at 72°C and a 4°C hold. Optimal annealing temperatures and suitability of loci were determined using a LiCor 4300 DNA Analyzer and the software Gene ImagIR 4.05 (Scanlytics, Inc.) to score the genotypes. All loci were examined for genotyping errors, allelic dropout and null alleles using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). Tests for genotypic disequilibrium were performed using GENEPOP 4.0.7 (with the following Markov-chain parameters: 10,000 dememorizations, 100 batched, 5,000 iterations per batch; Bonferroni corrected for multiple tests) Rousset (2007) and for Hardy–Weinberg equilibrium (HWE) using ARLEQUIN 3.0 (Schneider et al. 2000).

Eight polymorphic loci were found (GenBank accession numbers GQ150754-GQ150761). The number of alleles ranged from 3 to 18 per locus (mean +/− s.e.: 8.4 +/− 2.1) and the observed and expected heterozygosity ranged from 0.31 to 0.92 (mean +/− s.e.: 0.59 +/− 0.08) and 0.31–0.90 (mean +/− s.e.: 0.65 +/− 0.08), respectively (Table 1). Three of the eight loci, RSD42A, RSD2-91 and RSD142, were determined to be out of HWE (see Table 1). None of the loci appear to be in linkage disequilibrium. The PCR products from cross-species amplification tests using the

Table 1 Primer sequences and characteristics of eight redside dace (*Clinostomus elongatus*) microsatellite loci

Locus	Primer sequence (5′–3′)	Repeat motif	N_A	Size range (bp)	T_a (°C)	GenBank accession number	H_O	H_E
RSD42A	F: AACTGCAGACAGGGATCTGG	$(TC)_{14}(AC)_7$	5	188–196	60	GQ150754	0.33	0.37*
	R: TATCTGTGCCTGCTGGTGAG							
RSD53	F: TTGCTGCAGGACAGTGTTTT	$(AC)_{13}(AT)_4$	8	179–193	54	GQ150755	0.75	0.77
	R: TGTGTCAGGTTTTGCTCACTTC							
RSD2-58	F: TGAAATCAAAATGGTCAGTCCTT	$(CA)_{13}(TA)_6$	5	196–208	57	GQ150756	0.31	0.31
	R: TGCGCTAAACGTCATCAGAG							
RSD70	F: TGCAGTGGTTTGCAATCTAAG	$(GT)_{14}$	3	242–252	53	GQ150757	0.59	0.56
	R: CCGACGACCCCTTTAAGAAT							
RSD86	F: CACAAAAACGGGATGAATTG	$(TG)_{20}$	6	209–223	53	GQ150758	0.44	0.64
	R: GCGAACTGCAGCACTTACAG							
RSD2-91	F: ACAGCCACTATACCTGAAATCAA	$(TCTA)_{21}$	18	233–309	60	GQ150759	0.92	0.90*
	R: CGCAAATAAAGGTGACTTGAC							
RSD142	F: CACCCTGCTGTTTCTGTTCA	$(TATC)_{20}$	17	200–264	55	GQ150760	0.76	0.90*
	R: ATTGCTTTCCCTGTGAATCG							
RSD179	F: GCTAGTCAAACTGGTCTCTTTCC	$(AT)_2GTCT(GT)_{16}$	5	210–213	55	GQ150761	0.66	0.72
	R: GGCTGCCAGCAAATATTAGAA							

For each microsatellite developed, forward (F) and reverse (R) primer sequences, repeat motif, number of alleles (N_A), size range in base pairs (bp), annealing temperature (T_a), GenBank accession number, observed heterozygosity (H_O), and expected heterozygosity (H_E; * denotes significant HWE departures ($P < 0.05$, corrected for multiple tests))

Table 2 Cross-amplification of microsatellite loci in rosyside dace (*Clinostomus funduloides*), hornyhead chub (*Nocomis biguttatus*), Lahontan redside shiner (*Richardsonius egregius*) and central stoneroller (*Campostoma anomalum*) using the eight primers developed for redside dace (*C. elongatus*; cf. Table 1)

Locus	*Clinostomus funduloides*	*Nocomis biguttatus*	*Richardsonius egregius*	*Campostoma anomalum*
RSD42A	−	−	−	−
RSD53	+	+	−	+
RSD2-58	−	+	+	−
RSD70	+	−	+	−
RSD86	+	+	+	+
RSD2-91	+	+	+	+
RSD142	+	−	+	−
RSD179	+	−	+	+

Cross-species amplification tests; (+) successful amplification and (−) no amplification

same cycling conditions produced fragments of expected sizes for most loci, for all four of the related species examined (Table 2).

These newly characterized loci can be used to provide much needed genetic information for the imperiled redside dace. First, these markers will allow for the assessment of genetic variation and gene flow in natural populations to determine how much habitat degradation is affecting their population viability. Second, these markers will be useful for helping decide whether or not to initiate proposed conservation actions, such as captive breeding and translocation of wild individuals. Third, these loci will allow for a detailed assessment of natural and sexual selection in the redside, by allowing for the assignment of paternity in order to assess patterns of reproductive success among sexes and individuals in this promiscuous, non-resource based mating system (see Koster 1939, Pitcher et al. 2009). Finally, the present work will also contribute to molecular ecology studies of other minnow species by providing a set of new microsatellite primers successfully tested for species related to the redside dace and threatened in parts of their range, rosyside dace (*Clinostomus funduloides*) and Lahontan redside shiner (*Richardsonius egregius)*, and two other species, hornyhead chub (*Nocomis biguttatus)* and central stoneroller (*Campostoma anomalum*).

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