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TECHNICAL NOTE

Isolation of 13 polymorphic microsatellite loci for slimy sculpin (*Cottus cognatus*)

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Abstract Our research on slimy sculpins (*Cottus cognatus*), a benthic, freshwater fish, requires highly polymorphic genetic markers, and microsatellite loci developed for other *Cottus* species were insufficient for our needs. We therefore developed 13 polymorphic microsatellite loci from *C. cognatus* libraries enriched for tri- and tetranucleotide repeats. These loci had 2–22 alleles and observed heterozygosities ranging from 0.36 to 0.86 in a sample of 47 individuals from one population. There was no evidence of linkage disequilibrium; however, one locus had a putative null allele. Twelve loci also worked for mottled sculpin (*Cottus bairdi*) but only eight were polymorphic in a sample of seven individuals.

Keywords Microsatellite · Cottus · Slimy sculpin · Enrichment

Slimy sculpins (*Cottus cognatus*) are small, freshwater, benthic fish widely distributed across Northern North America and Eastern Siberia. Their sedentary nature renders *Cottus* species ideal for population studies, and research on multiple species has revealed well-developed population structure (Meyer et al. 2008; Englbrecht et al. 2000). *Cottus* species have recently been indicated as ideal study organisms for investigating the early stages of speciation (Sweigart 2009) and there has been considerable interest in the behavior, ecology, phylogeography and conservation of these species (Adams and Schmetterling 2007). Current conservation efforts in Minnesota (USA) have included reintroducing *C. cognatus* into streams historically impacted by heavy agricultural land-use in order to rehabilitate the native fish fauna. Restoration activities have given researchers the opportunity to examine the consequences of local adaptation on the fitness of restored populations and aspects of genetic diversity indicators when multiple source populations are mixed. Currently available genetic markers are sufficient to differentiate *C. cognatus* populations used as sources (L. Miller, unpublished data); however, these microsatellite loci were developed for other *Cottus* species (Nolte et al. 2005; Fiumera et al. 2002; Englbrecht et al. 1999) and generally had low variation within *C. cognatus* populations. Our goal was to develop additional highly polymorphic loci to aid in parentage assignment for a study of factors that contribute to reproductive success at reintroduction sites.

Genomic DNA was extracted from *C. cognatus* organ tissue using a DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions. We constructed libraries enriched for microsatellite repeats using a protocol developed by Glenn and Schable (2005). To summarize, we digested the genomic DNA with *BstU I* and *Xmn I* (New England Biolabs) then ligated SuperSNX linkers (Forward: 5′-GTTTAAGGCCTAGCTAGCAGAATC; Reverse: 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA) to the digested fragments. We hybridized our DNA with a mixture of biotinylated oligonucleotide probes [$(AGAT)_8$; $(AAGT)_8$; $(ACAT)_8$; $(ACCT)_6$; $(ACAG)_6$; $(ATC)_8$; $(AAC)_6$; $(AAG)_8$; $(ACT)_{12}$; $(AGC)_6$] under high stringency conditions (52°C). Hybridized sequences were captured using Dynabeads® M-280 Streptavidin (Invitrogen), and then recovered through

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the polymerase chain reaction (PCR) with SuperSNX forward primer.

We used a pGEM®-T vector system (Promega) to clone the PCR-amplified enriched DNA pool. After plating and incubation on Ampicillin/X-Gal/IPTG LB agarose, putative transformants were identified by blue–white screening and 672 colonies were picked and PCR amplified. Amplified inserts were sized on 1% TBE agarose gels, and fragments larger than 550 base pairs (indicating inserts large enough to contain a repeat and flanking sequence for primer design) were identified. We submitted PCR products from 130 colonies to the BioMedical Genomics Center (University of Minnesota, St. Paul, MN) for sequencing using BigDye v3.1 chemistry and an ABI 3730xl DNA Analyzer (Applied Biosystems). Although a large percentage of sequences contained repeats, many were duplicated or had short or poor quality flanking sequence so they were unusable for primer design. We designed primer pairs from 19 sequences using the program Primer3 v. 0.4.0 (Rozen and Skaletsky 2000). Amplification with our primers yielded 13 unique, polymorphic microsatellite loci (Table 1). Two loci (*Cco04* and *Cco08*) were scorable through separation on 8% acrylamide gels followed by ethidium bromide staining and visualization on a UV light table. We obtained fluorescently labeled forward primers for six loci and attached M13 tails to the forward primer of the remaining five loci according to Boutin-Ganache et al. (2001).

All loci were characterized using 47 *C. cognatus* from a single population. We extracted DNA from fin clips fixed in ethanol by mixing each with 300 µl of 5% Chelex® (Sigma Chemical) solution, incubating samples overnight

Table 1 Characteristics of 13 microsatellite loci for *Cottus cognatus* (Beaver Creek, Minnesota, USA; n = 47)

Locus	Repeat motif	Primer sequences (5′–3′)	Size range (bp)	T_A (°C)	N_A	H_o	H_e	Genbank accession no.
Cco01	$(AAG)_{13}(AAC)_2(AAG)_8AAC(AAG)_7$	F: AAAGTTGTGAAAAGACATAATGG R: CCAAGGGGATTAATAAAGTATACC	243–327	50	13	0.77	0.77	GQ341572
Cco02	$(AAG)_{14}$	F: TTCTTGTTCTCCGTCTTGAGC R: CCCATCTTCTCCTCCTGTCC	263–278[a]	50	5	0.68	0.66	GQ341573
Cco04	$(AAG)_{15}$	F: GGGTTGAATTTGCAATCTGG R: TTAATTTCAGGCAGGATCACG	79–160	46	4	0.72	0.70	GQ341574
Cco07	$(CT)_{33}$	F: ATTCTCTTCGGTATACACGG R: AATCAACCAAACCCTGCTGG	204–260[a]	55	12	0.67	0.86[b]	GQ341575
Cco08	$(AAG)_7$	F: TTGCAAACTTCAGACAGTAAAGC R: GCTGAGAATCCAGGAAGGAG	98–110	55	3	0.59	0.60	GQ341576
Cco09	$(AAG)_{24}$	F: GGAGAAGAACACAGTAAACAAATTC R: TCTTCGTTGGCGGTTTTAAG	252–330	55	22	0.80	0.82	GQ341577
Cco10	$(AAG)_{18}$	F: GACCCTTGCCCTGAATCG R: AGCTCTGAACCGCCACAC	122–185	55	9	0.68	0.78	GQ341578
Cco11	$(TG)_{16}$	F: GCAGGAGGAACACGAAGATG R: CTCAAGGAACTACACACACATGC	220–232[a]	55	2	0.41	0.36	GQ341579
Cco13	$(ATG)_{11}$	F: CCTGGAATTTCACCAAGGTC R: TCACAACAAAGCCAGAGGAC	246–258[a]	55	5	0.48	0.47	GQ341580
Cco14	$(ACAG)_7$	F: CATAAAACCTGTGGCTTTGG R: GACGCTCTGCTGGAGAGATG	167–191	55	6	0.72	0.78	GQ341581
Cco15	$(AAG)_{15}$	F: TTGGCACATTGTGGAGACTG R: TGGAAAAGTTGTTCTTGTAACG	164–227	55	11	0.83	0.86	GQ341582
Cco16	$(AC)_9n14(ACACACAT)_5n4(ACAT)_{16}$	F: GTTGCTTCCTCTTTGTGGTTG R: TCACACAAAAGACCTACAAGGAC	220–260[a]	50	8	0.72	0.79	GQ341583
Cco17	$(AAG)_{30}$	F: TCGTCTTGGAAATGGAAAGC R: CATGTCAGCAGGATATCACGTC	108–126	55	6	0.79	0.81	GQ341584

Shown are repeat motifs, primers, annealing temperatures (T_A), number of alleles per locus (N_A), observed and expected heterozygosities (H_o and H_e, respectively), and accession numbers for clone sequences

[a] Loci amplified with an M13-tailed primer. Product size is reduced by approximately 18 base pairs if fluorescently labeled primers are used

[b] Deviates from Hardy–Weinberg Equilibrium

in a water bath (56°C) then boiling for 8 min. Amplifications for *Cco04* and *Cco08* as well as the fluorescently labeled loci were performed in 15 µl reaction volumes consisting of 5 µl DNA template, 5X GoTaq® DNA polymerase buffer with 1.5 mM $MgCl_2$ (Promega), 0.2 mM each dNTP, 0.33 µM of each primer, 0.5 U GoTaq® DNA polymerase, and water to the final reaction volume. In some cases additional $MgCl_2$ was needed up to a final concentration of 2.5 mM. Amplifications using the M13 tailed primers were carried out in 15 µl reactions according to Boutin-Ganache et al. (2001) with the exception that the molar ratio between the M13 labeled primer and the M13-tailed forward primer was 4:1 instead of 15:1. Primer concentrations were as follows: 0.4 µM M13 labeled primer, 0.4 µM reverse primer, and 0.10 µM M13-tailed forward primer. Concentrations of all other reagents agreed with those conditions used for the other eight loci. Thermocycler conditions for amplification were a 3 min denaturation at 95°C, 35 cycles of 95°C for 30 s, primer annealing temperature (Table 1) for 30 s, and an extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products from the labeled and M13-tailed loci were resolved on an ABI 3730xl DNA Analyzer, and alleles sized using Genotyper 2.5 and LIZ 500 size standards (Applied Biosystems).

Our 13 microsatellite loci had 2–22 alleles and observed heterozygosities ranging from 0.36 to 0.86 (Table 1). We used the program HW-Quick Check (Kalinowski 2006) to calculate observed and expected heterozygosities and to detect deviations from Hardy–Weinberg Equilibrium (HWE). One locus (*Cco07*) deviated from HWE, with evidence for a null allele according to the program MicroChecker (Oosterhout et al. 2004). We found no evidence of linkage disequilibrium after Bonferroni correction using GENEPOP v4 (Raymond and Rousset 1995).

We also amplified all loci using seven individuals from two populations of mottled sculpin (*Cottus bairdi*), the only other *Cottus* species found in our study region. We used the same PCR conditions applied to *C. cognatus* except for an annealing temperature of 50°C for all loci. Eight of the loci were variable for *C. bairdi*, four loci appeared fixed, and one did not amplify (Table 2). Several of the loci have non-overlapping allele size ranges, at least for these few samples, and may prove useful for genetically confirming the identity of these morphologically similar species.

These new polymorphic microsatellite loci will be useful for investigations of *C. cognatus* population structure and processes. The amplification success of these markers on another species demonstrates that they may also be useful for research on other *Cottus* species.

Table 2 Amplification of 13 *Cottus cognatus* microsatellite loci for seven *Cottus bairdi*

Locus	Amplified	Product size range
Cco01	Yes	270–300
Cco02	Yes	160–166
Cco04	No	na
Cco07	Yes	172–224
Cco08	Yes	80
Cco09	Yes	210–249
Cco10	Yes	120
Cco11	Yes	204
Cco13	Yes	255–264
Cco14	Yes	264
Cco15	Yes	171–180
Cco16	Yes	260–300
Cco17	Yes	99–132

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