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TECHNICAL NOTE

Twelve tetranucleotide microsatellite loci for westslope cutthroat trout *Oncorhynchus clarki lewisi* (Salmonidae)

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Abstract We isolated 12 tetra-nucleotide microsatellite loci from westslope cutthroat trout (*Oncorhynchus clarki lewisi*). These loci were tested against 58 individuals from a single creek for polymorphism. The number of alleles ranged from 2 to 8, with an average of 4.3. The expected heterozygosity ranged from 0.12 to 0.79, with an average of 0.52. Ten of the twelve loci conformed to Hardy–Weinberg expectations. These microsatellite loci will be useful for describing population structure in westslope cutthroat trout.

Keywords *Oncorhynchus clarki* · Westslope cutthroat trout · Microsatellite loci · Population genetics · Montana

The westslope cutthroat trout is the most widely distributed subspecies of cutthroat trout, and despite its name, is found on both sides of the continental divide in the Rocky Mountains of North America (Allendorf and Leary 1988; Behnke 1992). The subspecies inhabits diverse habitats, limited primarily by the requirement of cold, clean water (Benke 2002). The westslope cutthroat trout has recently experienced severe reductions in abundance and distribution (Hanzel 1959; Liknes and Graham 1988; Shepard et al. 1997; Shepard et al. 2003) and is now considered a "Species of Special Concern" in the state of Montana. The primary threats to westslope cutthroat trout populations have been hybridization and competition with non-native rainbow trout and habitat loss (Behnke 1992; Liknes and Graham 1988). Genetic data could improve the management of surviving populations of westslope cutthroat trout—for example, by identifying populations with low levels of genetic diversity or by describing population structure—but, to date, the only genetic markers available for such analyses have been allozymes loci (Allendorf and Leary 1988) or microsatellites developed in other species (e.g., Muhlfeld et al. 2009). Here we describe 12 new microsatellite loci developed specifically for westslope cutthroat trout.

Genomic DNA was isolated from a small piece of fin tissue preserved in ethanol. We used a variation of the streptavidin biotin hybridization method of Hamilton et al. (1999) to develop a westslope cutthroat trout microsatellite library. We combined the restriction of genomic DNA with the ligation of universal DNA linker into one reaction (DIG/LIG). The DIG/LIG reaction consisted of 2.0 μM double-stranded SNX linker, 6 μg BSA, 20 Unit restriction enzyme, 40 Unit *Xmn*I, 800 Unit T4 DNA Ligase with rATP, 1× NEBuffer2, ~250 ng genomic DNA, and enough water for a final volume of 60 μl. To increase the diversity of DNA fragments, we performed two separate DIG/LIG reactions, each with a different restriction enzyme (*Hinc*II and *Pvu*II). The DIG/LIG thermoprofile consisted of 22 cycles (37°C for 10 min and 16°C for 30 min), and a final extension for 20 min at 65°C to denature all enzymes. This reaction produced DNA fragments with size ranging from 300 to 1500 base pairs. We performed the subtractive hybridization procedure at 48°C using $(GATA)_4$ and $(GACA)_4$ biotinylated tetra-nucleotide probes. To isolate DNA fragments from our enriched libraries, we cloned each library using the TOPO TA cloning method (Invitrogen). Approximately 1800 colonies were isolated, and each was then amplified using the Templiphi reaction (GE Healthcare). The Templiphi

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method was used for ease of scale, and because the product can be directly sequenced. We sequenced these Templiphi reactions using Big Dye Terminator v3.1 Kit (Applied Biosystems). Sequencing products were cleaned using the Ethanol/EDTA/Sodium Acetate Precipitation method, and they were visualized using a 3100-Avant Genetic Analyzer (Applied Biosystems).

In equal proportion, we isolated and sequenced 1795 DNA fragments from *HincII* and *PvuII* enriched libraries. Only 365 fragments contained microsatellite repeat motifs, and of these, 217 fragments contained high quality sequences that were suitable for primer design. However, only 80 fragments were chosen for primer design and for subsequent testing, because they contained longer repeat motifs (≥15 repeats). To test for variability of our candidate loci, we performed PCR on 7 westslope cutthroat trout individuals, and visualized the PCR products via agarose gel electrophoresis. Of the 80 loci tested, only 12 loci appeared to have informative bands. We fluorescently labeled the 5' end of the forward primer for these 12 loci, and we expanded our test samples to 58 individuals. We chose to perform PCR on these loci in multiplex format, with each multiplex PCR amplifying 3 to 5 loci. A typical multiplex PCR volume consisted of 1 μM of each primer, 5 μl 2× Qiagen Multiplex PCR Kit, ~50 ng DNA, and enough water for a final volume of 10 μl (Qiagen). The thermoprofile consisted of one activation step at 95°C for 15 min followed by 40 cycles (94°C for 30 s, 60°C for 90 s and 72°C for 60 s), and a final extension step at 72°C for 30 min. We visualized PCR products using a 3100-*Avant* Genetic Analyzer (Applied Biosystems).

Table 1 summarizes the amount of genetic variation at these 12 loci. The number of alleles observed per locus ranged from 2 to 8, with an average of 4.3. We calculated expected heterozygosity using HW-QuickCheck (Kalinowski, 2006), and it ranged from 0.12 to 0.79, with an average of 0.52. HW-QuickCheck was also used to test for agreement with Hardy–Weinberg proportions, using the exact test of Guo and Thompson (1992). Two loci (OclMSU20 and OclMSU23) showed significant heterozygote deficiencies

Table 1 Characterization of 12 microsatellite loci in Westslope cutthroat trout

Locus	Repeat motif	Primer sequences	T_a (°C)	$MgCl_2$ (mM)	N_a	Size range (bp)	H_o/H_e	Accession no.
OclMSU14	$(GACG)_8$, … $(GACA)_{15}$	F:AGGCTGCATGCTTTCAAAAT R:TCCCTTGTGCTGATTGACAG	61	3.0	7	124–182	0.71/0.66	GQ249043
OclMSU15	$(GATA)_{25}$	F:GCCAAACCTGAAAGCAACTT R:AACTTTGTGTATGTAAACTTCTGACC	61	3.0	4	168–280	0.47/0.43	GQ249041
OclMSU16	$(GACA)_{26}$	F:TGCCCTGGAGAGAGAGAAAG R:TCAGAGTATTAGGGCTACCAGGA	61	3.0	3	216–290	0.69/0.79	GQ249042
OclMSU17	$(GTCT)_{15}$	F:GCCCTGTTTTGGTTTACGTT R:GGGAGGGAGAGAAAAGGAGA	61	3.0	2	258–262	0.11/0.12	GQ249044
OclMSU18	$(CT)_{25}$, $(GTCT)_{25}$	F:TGGGTATCGGCCTAATTCTG R:GGCCCATATGAATGTTCCAC	61	3.0	5	250–277	0.69/0.77	GQ249045
OclMSU19	$(GATA)_{37}$	F:GGGTTCAGGGTCAAACAGAA R:TTTCTCCAACTAATAGAGGGTACAG	61	3.0	3	195–219	0.51/0.67	GQ249046
OclMSU20	$(GGCA)_4$, $(GACA)_{12}$	F:TTCAAGGGTCATTGTGTGGA R:TGCTATTGGTCGTGTTCTGC	59	3.0	4	219–231	0.67/0.60*	GQ249047
OclMSU21	$(GATA)_{18}$	F:TCCTGTCCTTTGCAGCAGTA R:TCCTCTCCTCTCGCTCTCTG	59	3.0	4	175–191	0.48/0.46	GQ249048
OclMSU22	$(GGCA)_{17}$, $(GACA)_{17}$	F:TGGGACAGAGAGCTGTGATG R:TCTGGTATGGAAAGTCAGTCTCA	59	3.0	4	241–305	0.48/0.43	GQ249049
OclMSU23	$(TATC)_{23}$	F:ACTTTGTGTATGTAAACTTCTGACC R:CAATCTTAGCCAAACCTGAA	63	3.0	4	183–296	0.48/0.41*	GQ249050
OclMSU24	$(GTCT)_4$, … $(GTCT)_3$, … $(GTCT)_4$, … $(GTCT)_{13}$	F:TCCCTCCATGTCTCCTTGTC R:GAAGATCCGCACCACAGTCT	63	3.0	3	220–241	0.15/0.12	GQ249051
OclMSU25	$(GTCT)_{21}$, … $(CT)_{22}$	F:CTGAGGGATGAGGACACCAC R:TCCCTTTGCTAATAAAGCCATT	63	3.0	8	223–256	0.71/0.77	GQ249052

T_a Annealing temperature, N_a number of alleles, H_e expected heterozygosity

* Statistical significant deviation from Hardy–Weinberg expectations ($P < 0.05$ after adjusting for multiple comparisons)

at the 0.05 level of significance. We also performed a pairwise test of each locus for genotypic equilibrium using GENEPOP (Raymond and Rousset 1995). At the 0.001 level of significance, we discovered that there were significance associations of genotypes between OclMSU18 and OclMSU22, OclMUS17 and OclMSU25, OclMSU15 and OclMSU23, OclMSU14 and OclMSU24.

We were encouraged to learn that a high level of polymorphism exists in our geographically isolated test population. Consequently, we believe that these loci can be useful in assessing population structure across a wider geographic range.

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