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PERMANENT GENETIC RESOURCES

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

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Abstract We isolated twelve microsatellite loci from westslope cutthroat trout (*Oncorhynchus clarkii lewisi*). These loci were genotyped in 30 individuals from a single population. The number of alleles ranged from 2 to 10, with an average of 5.7. The expected heterozygosity ranged from 0.36 to 0.86, with an average of 0.53. All loci conform to Hardy-Weinberg expectations. These highly polymorphic microsatellite loci can be used to describe population structure in westslope cutthroat trout.

The westslope cutthroat trout inhabits cold, clean waters of the Northern Rockies (Behnke 2002). It is the official state fish of Idaho and Montana, but has experienced substantial reductions in both abundance and distribution (e.g., Shepard et al. 2005) and has recently been considered for listing as an endangered species under the U.S. Endangered Species Act. The primary threats to westslope cutthroat trout have been habitat loss and non-native species. Conservation of westslope cutthroat is currently limited by a poor understanding of genetic relationship among populations of cutthroat trout living in different parts of the taxon's range. For example, state managers are considering moving fish from the Clark Fork River basin on the western side of the continental divide into the Missouri River basin on the eastern side of the continental divide and want to know if such translocations would admix gene pools that have been isolated for long periods of times. Federal managers want to know whether there are evolutionary lineages within westslope cutthroat trout that qualify for protection under the Endangered Species Act.

Microsatellite genetic data would be useful for informing these decisions. In a previous note (Vu and Kalinowski 2009) we described 12 tetranucleotide microsatellite

loci for westslope cutthroat trout. Analysis of population structure at these loci showed that populations were highly differentiated (S. Kalinowski, unpublished). Genetic distances such as F_{ST} or Nei's standard genetic distance (Nei 1978) have high sampling variances under these circumstances, and the only way to reduce this variance is to genotype more loci (Kalinowski 2002; 2005). Therefore, we developed another set of 12 loci, which we describe in this note.

For the development of the westslope cutthroat trout microsatellite library, we used a variation of the streptavidin/biotin hybridization method (Hamilton et al.1999). We combined the restriction of genomic DNA with the ligation of linkers into one reaction (DIG/LIG). The DIG/LIG reaction consisted of 2.0 µM double-stranded SNX linker, 6 µg BSA, 20 U restriction enzyme, 40 Unit *XmnI*, 800 U T4 DNA Ligase with rATP, 1X 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 (*HincII* and *PvuII*). The DIG/LIG thermoprofile consisted of 22 cycles (37 ℃ for 10 min and 16 ℃ for 30 min), and a final extension for 20 minutes at 65 ℃ 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 ℃ 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 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, and the DNA sequences were visualized using the 3100-*Avant* Genetic Analyzer (Applied Biosystems).

In equal proportion, we isolated and sequenced 900 DNA fragments from *HincII* and *PvuII* enriched libraries. Only 180 fragments contained microsatellite repeat motifs, and of these 105 fragments contained high quality sequences that were suitable for primer design. However, only 40 fragments were chosen for primer design and for subsequent testing, because they contained longer repeat motifs (≥11 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 40 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 30 individuals. We chose to perform PCR on these loci in multiplex format, with each multiplex PCR amplifying four loci. A typical multiplex PCR volume consisted of 1 µM of each primer, 5 µL 2X 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 ℃ for 15 min followed by 40 cycles (94 ℃ for 30s, T_a ℃ for 90 s and 72 ℃ for 60 s) (Table 1), and a final extension step at 72 ℃ for 30 min. We visualized PCR products using a 3100-*Avant* Genetic Analyzer (Applied Biosystems).

In order to test for null alleles and other genotyping artifacts, we genotyped 30 fish from a population of westslope cutthroat trout in Hughes Creek, Montana (just south of Hamilton). The number of alleles in this sample ranged from 2 to 10 per locus, with an average of 5.7 (Table 1). We calculated observed and expected heterozygosities, and tested for Hardy-Weinberg proportions using HW-QuickCheck (Kalinowski 2006). The

expected heterozygosity ranged from 0.36 to 0.86, with an average of 0.54. All loci appear to conform to Hardy-Weinberg expectations. We also performed a pairwise test of genotypic equilibrium for each pair of loci using GenePop (http://genepop.curtin.edu.au/; Raymond & Rousset 1995). At the 0.001 level of significance, we discovered that there were significance associations of genotypes at three pairs of loci (OclMSU28 and OclMSU35, OclMSU26 and OclMSU33, OclMSU27 and OclMSU35). These results indicate that these loci should be useful for describing population structure of westslope cutthroat trout.

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Table 1 Summary information for 12 microsatellite loci, including diversity statistics for 30 individuals: locus name, repeat motif, primer sequence, annealing temperature (T_a), $MgCl_2$ concentration, number of alleles (N_a), size range (base pair), observed (H_o) and expected (H_e) heterozygosity, Hardy-Weinberg *P* value and GenBank accession number.

Locus	Repeat motif	Primer Sequence	T_a (ºC)	MgCl2 [mM]	N_a	Size range (bp)	*Ho/He*	P value	Accession no.
OclMSU26	(GA)23, (GA)43	F:CTGAACGTTACTGGGGGCTA R:AGCCAAGGCTGTCCAATCTA	60	3.0	6	257-297	0.43/0.38	0.17	HM153812
OclMSU27	(GTCT)11	F:GCCATCAAATCCTCAAATGG R:GTTACACAGCAGCCCACTCA	60	3.0	5	177-189	0.48/0.46	0.46	HM153813
OclMSU28	(GACA)26	F:GACTGCCAACCCAGAGAGAT R:CCGGTCTCACCACACATATC	60	3.0	2	251-329	0.45/0.42	0.55	HM153814
OclMSU29	(GTCT)60	F:TTCCAGCTATGATCTCCTCTCC R:CCATTCCAGAGCATAGCACA	60	3.0	5	300-320	0.63/0.54	0.05	HM153815
OclMSU30	(CT)22	F:GGTGGCTCCAGTGGATTTAG R:TATTGGGCTGGAGCAGAACT	64	3.0	5	222-283	0.40/0.38	0.34	HM153816
OclMSU31	(CT)21, (GT)4	F:CTGTTGGAATGGCGTCACTA R:CAGGAGACTTGCTTGCTGTG	64	3.0	5	222-234	0.63/0.61	0.28	HM153817
OclMSU32	(GTT)17	F:TTCGTGGCAAAATAACAGCTT R:TGGGGTCTCAGTGTTTCTCA	64	3.0	3	207-218	0.28/0.36	0.93	HM153818
OclMSU33	(GTCT)16	F:ACAGGGGATTTCTCCATGTG R:AGAGCAGTGGAATGCTACCC	64	3.0	10	185-234	0.83/0.86	0.66	HM153819
OclMSU34	(GACA)23	F:GGATGCCTGCTGATGAGTCT R:GGCCATGTGTGACGTTCTAA	64	3.0	8	242-339	0.43/0.49	0.89	HM153820
OclMSU35	(GACA)17	F:GTTGAGCCGTCTCTTGAACC R:TTTCTGGCTGTGTCCCATCT	64	3.0	5	326-342	0.37/0.46	0.87	HM153821
OclMSU36	(GACA)16, (BA)48	F:CCACAGCAGCAGATAAGCAA R:CACATGATCGCATGAGAGAGA	64	3.0	8	227-291	0.73/0.76	0.76	HM153822
OclMSU37	(GGCA)19, (GACA)6	F:TCCTTCGAATCCAGCATTTC R:TGGACCTACACAAGAACCACA	64	3.0	6	262-294	0.80/0.79	0.84	HM153823

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