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PRIMER NOTE

Characterization of tetranucleotide microsatellites for Rio Grande cutthroat trout and rainbow trout, and their cross-amplification in other cutthroat trout subspecies

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Abstract

We describe the isolation and characterization of 12 tetranucleotide microsatellites for Rio Grande cutthroat trout (*Oncorhynchus clarkii virginalis*) and rainbow trout (*Oncorhynchus mykiss*), and subsequently investigate their performance in Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*), greenback cutthroat trout (*Oncorhynchus clarkii stomias*) and Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri*). All 12 loci are polymorphic in all subspecies of *O. clarkii* examined.

Keywords: Colorado River cutthroat trout; greenback cutthroat trout; microsatellite; *Oncorhynchus clarkii*; *Oncorhynchus mykiss*; Rio Grande cutthroat trout

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The cutthroat trout (*Oncorhynchus clarkii*) of western North America have suffered major declines over the past 130 years, and all subspecies are currently the focus of conservation efforts (Behnke 2002). Threats include population fragmentation and hybridization with introduced non-native taxa, in particular rainbow trout (*Oncorhynchus mykiss*) and Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*). In this study, we describe 12 tetranucleotide microsatellite loci isolated from Rio Grande cutthroat trout (*Oncorhynchus clarkii virginalis*) and rainbow trout. These loci were developed as a tool to examine non-native introgression and population genetic structure within *O. c. virginalis*, a trout native to the Canadian, Pecos and Rio Grande drainages of the southwestern USA. We subsequently examined the performance of the loci in four closely related cutthroat trout subspecies.

DNA was extracted from rainbow trout or Rio Grande cutthroat trout tissue using the PureGene DNA Extraction Kit (Gentra Systems), and partially restricted with cocktails of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV). Fragments in the size range 300–750 bp were adapted by ligation to 26-bp oligonucleotides containing a *Hin*DIII site at the 5′-end (Integrated DNA Technologies, Inc.). Adapted fragments were subjected to magnetic bead capture (CPG, Inc.), using biotinylated capture molecules. Libraries were prepared in parallel for the two taxa using Biotin-TACA(8), Biotin-TAGA(8), Biotin-CAGA(8) and Biotin-CATA(8) (Integrated DNA Technologies) in a protocol provided by the bead manufacturer. Resulting libraries were designated G, H, J and K, respectively. Captured molecules were polymerase chain reaction (PCR) amplified in 25-μL reactions containing 4 μL gel-extracted DNA, 0.2 mM primer complementary to the adaptor, 0.2 mM each premixed dNTPs, 12 U TaKaRa Ex *Taq* and 1× TaKaRa Ex *Taq* buffer (2 mM Tris–HCl, pH 8.0; 2 mM $MgCl_2$; 10 mM KCl_2; 0.01 mM EDTA; 0.1 mM DTT; 0.05% Tween 20; 0.05% Nonidet P-40; 5% glycerol, TaKaRa Mirus Bio, Otsu, Japan). We conducted 25 PCR cycles of 94 °C (45 s), 57 °C (45 s) and 72 °C (60 s), followed by a final extension at 72 °C (180 s). Resulting fragments were restricted with *Hin*DIII to remove the adapters and ligated into the *Hin*DIII site of pUC19. Recombinant molecules were electroporated into *Escherichia coli* DH5α. Each library produced 10 000–15 000 recombinant clones, of which approximately 75% contained microsatellites. Randomly selected clones were sequenced on an ABI PRISM 377 Genetic Analyser (Applied Biosystems, Inc.),

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Table 1 GenBank Accession no., taxon from which locus was isolated (V, *Oncorhynchus clarkii virginalis*, M, *Oncorhynchus mykiss*) and forward (F) and reverse (R) primer sequences for each locus. Indices of genetic diversity were assessed in a broodstock line of *O. c. virginalis* maintained by Colorado Division of Wildlife ($n = 30$) and in the Shasta hatchery strain of *O. mykiss* ($n = 30$). Number of individuals successfully amplifying for each locus (n), number of alleles (a) and observed (H_O) and expected (H_E) and heterozygosity are shown. Following Bonferroni correction for multiple tests, no significant deviations from Hardy–Weinberg equilibrium were observed

					O. c. virginalis				*O. mykiss*			
Locus	GenBank	Taxon	Repeat unit in clone	Primer sequence (5′–3′)	n	a	H_E	H_O	n	a	H_E	H_O
J3	DQ095864	V	$(CAGA)_{11}$	F: AGCACTGCCTGGACTTAGT R: AGAAGGTGTTCATCACATTTG	29	3	0.53	0.55	29	2	0.16	0.17
J14	DQ095865	V	$(CAGA)_{13}$	F: AGAGCTGCCAGAGCTACAG R: GCCAAAGACAGACAGACAAG	29	7	0.68	0.52	30	4	0.19	0.20
J103	DQ095866	V	$[(GACA)(GACCA)]_4$ $(GACA)_{11}$	F: CATGGATTTGGGAAAAGT R: TTCCAACTCACCCTTACC	30	13	0.86	0.87	0	NA	NA	NA
J132	DQ095867	V	$(GACA)_{10}$	F: GGGCAAGAAGACAACTTTCA R: GCACAGTGGATGTAGTGTATGG	30	6	0.78	0.90	0	NA	NA	NA
K216	DQ095968	V	$(CATA)_2(CGTA)$ $(CATA)_5(CGTA)$	F: CAAGCATTTCGCTAAACTCG R: GCACGGACTCGTCATCAG	30	11	0.88	0.83	30	3	0.49	0.60
K222	DQ095869	V	$(CATA)_{10}(TATA)$ $(CATA)_8(TATA)(CATA)_{24}$	F: CGACGAGAAAACTTGAATAGAC R: GCTGAAATAGCCGAATCC	30	5	0.61	0.63	30	4	0.44	0.43
H18	DQ106406	M	$(TAGA)_{25}$	F: CAAACAATGGCTGTCTGTTAC R: CCATCCCTGTTAAATTGCTAC	30	6	0.72	0.77	30	7	0.72	0.63
H114	DQ106405	M	$(TAGA)_{26}(TAGC)$ $(TAGA)_6$	F: GAATGGGGGGCTAAAACTC R: AGGCAGCCACTTCAGTCAG	30	4	0.57	0.63	30	7	0.83	0.90
H118	DQ106407	M	$(TAGA)_{16}(TACA)$ $(TAGA)_2$	F: GTGTCCAATGTTGTTAGTTGTG R: GGCACTCCTTATGTAAAGACG	29	3	0.40	0.48	28	8	0.82	0.89
H126	DQ106408	M	$(GATA)_{21}$	F: CAGCCCCTCGTTCATTTC R: ACCCCACCTCCACAGTCA	29	7	0.78	0.59	30	5	0.76	0.73
H220[1]	DQ106410	M	$(TAGA)_{12}$	F: GGAGGAGAGGAGAAGAAGG R: GAGAAGGGTTCATCACTACTTG	30	6	0.81	0.77	30	6	0.73	0.70

[1]Independently isolated from *O. mykiss* by Rexroad CE III, Palti Y, Coleman RL, Fincham RM, Hershberger WK (unpublished data), as OMM1231, GenBank Accession no. AF470011.

using ABI PRISM *Taq* dye terminator cycle sequencing methodology. Primer pairs were designed for 18 loci using DESIGNER PCR 1.03 (Research Genetics, Inc.). Each primer pair (Integrated DNA Technologies) was tested for its ability to amplify the microsatellite locus in its corresponding clone. Following characterization in reference samples of rainbow trout and cutthroat trout, 12 loci were selected for further use. One of these loci (H12) was found to have previously been isolated from *O. mykiss* by Rexroad *et al*. (2002), as OMM1036. Primer sequences and GenBank Accession nos for the remaining 11 loci are provided in Table 1.

For genotyping, PCR amplification included an M13 labelling procedure (D. Broderick, personal communication). A 23-base M13 sequence (5′-GGGTTTTCCCAGTCACGACGTT, Integrated DNA Technologies) was added to the 5′-end of the forward primer. The reaction mixture contained the free M13 oligonucleotide, 5′-labelled with 6-FAM, HEX, or NED for fluorescent detection. As the PCR product developed, the labelled M13 oligonucleotide primed off the anti-M13 sequence at the 3′-end of the product of a previous round of amplification. The reaction yielded a labelled product 23-bp longer than the amplicon that would be produced by nontailed forward and reverse primers. Microsatellites were amplified in 20-μL reactions in the following reaction mix: 1 μL template DNA, 2 ng/mL; 0.2 mM each reverse and M13-modified forward primers; 0.1 mM M13-labelled oligo; 0.2 mM each premixed dNTPs; 1.5 mM $MgCl_2$; 0.25 U Bio*taq* DNA polymerase (Bioline USA, Inc.); and 1× Bio*taq* buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% Tween-20]. PCR was conducted using an MJ Research PTC-100 96 V thermocycler with the following conditions: initial denaturation 95 °C (5 min), followed by 10 cycles of 94 °C (30 s), 57 °C (60 s), and 72 °C (30 s), followed by 22 cycles of 94 °C (30 s), 55 °C (60 s), and 72 °C (30 s), and terminating with a final extension at 72 °C for 10 min. Amplification products were mixed 1:1 with 98% formamide loading dye, denatured for 3 min at 95 °C, and then cooled on ice before running on 5% denaturing acrylamide gels at 35 W for 70 min. Products were detected using the ABI PRISM 377 and sized using GENOTYPER 2.0 software and ROX-500 size markers (Applied Biosystems).

Table 1 provides genetic diversity indices for the 12 loci, assessed in an *O. c. virginalis* broodstock containing genetic material from several wild populations, and in one hatchery

Table 2 Number of individuals successfully genotyped (*n*), product size in base pairs, total number of alleles observed (*A*) and allelic richness based on the minimum sample size of 41 genotyped individuals (*R*), for five different *Oncorhynchus* taxa

	O. c. virginalis (*n* = 190)				*O. mykiss* (*n* = 133)				*O. c. bouvieri* (*n* = 45)				*O. c. pleuriticus* (*n* = 75)				*O. c. stomias* (*n* = 100)			
Locus	*n*	Size (bp)	*A*	*R*	*n*	Size (bp)	*A*	*R*	*n*	Size (bp)	*A*	*R*	*n*	Size (bp)	*A*	*R*	*n*	Size (bp)	*A*	*R*
J3	189	213–237	4	4.0	128	221–261	3	2.3	45	225–241	5	5.0	67	221–241	5	4.1	100	217–221	2	2.0
J14	186	198–238	10	8.3	114	186–250	12	10.9	41	206–262	10	10.0	74	198–262	11	8.8	93	206–238	6	5.7
J103	190	296–356	14	12.2	0	0	0	0	41	320–428	24	24.0	61	316–416	13	12.2	81	288–356	13	11.7
J132	180	177–205	8	6.8	0	0	0	0	45	189–205	5	5.0	74	193–205	4	3.8	100	185–205	6	5.3
K216	188	193–265	16	12.0	87	169–233	8	6.3	45	197–289	8	7.9	74	197–241	7	6.1	96	197–241	7	6.1
K222	188	124–152	7	6.1	115	128–220	15	13.2	45	136–152	5	4.9	70	136–152	5	4.8	97	132–196	9	8.8
H18	188	181–205	6	5.1	112	201–311	21	16.1	45	193–237	11	10.7	71	193–213	5	5.0	99	185–205	5	4.8
H114	189	231–255	6	5.2	131	243–311	17	14.4	42	339–411	18	17.9	73	239–263	3	2.6	96	215–307	6	5.3
H118	187	162–178	4	3.7	128	198–264	15	12.6	44	194–218	7	7.0	74	162–202	4	3.8	98	162–174	4	3.3
H126	185	216–252	10	7.7	131	232–312	11	10.7	44	220–280	15	14.8	55	220–268	4	3.9	89	212–224	3	2.5
H220	189	203–275	13	10.7	121	191–271	15	12.8	42	223–243	5	5.0	75	231–263	6	4.7	98	223–259	5	4.6
H12[2]	187	237–269	9	7.8	130	253–333	15	12.1	42	249–325	15	14.9	73	245–265	6	5.9	98	221–293	8	7.1

[2]Independently isolated from *O. mykiss* by Rexroad *et al*. (2002), as OMM1036, GenBank Accession no. AF346686.

line of *O. mykiss*. We further assessed microsatellite polymorphism using samples from six wild *O. c. virginalis* populations within the Rio Grande and Canadian drainages (*n* = 160); five additional *O. mykiss* strains (*n* = 103); one hatchery line each of morphologically typical *O. c. bouvieri* and its fine-spotted Snake River form (*n* = 45); four populations of greenback cutthroat trout (*Oncorhynchus clarkii stomias*) from the Arkansas and South Platte River drainages in Colorado (*n* = 100); and three populations of Colorado River cutthroat trout (*Oncorhynchus clarkii pleuriticus*) from the San Juan and White River drainages in Colorado (*n* = 75) (Table 2). All indices of genetic diversity were calculated using FSTAT 2.9.3.2, and deviations from Hardy–Weinberg equilibrium assessed using a permutation test (Goudet 2001). Linkage disequilibrium for each pair of loci in each sample was examined using an exact test implemented in GENEPOP 3.4 (Raymond & Rousset 1995). The significance of linkage disequilibrium between each pair over all samples was examined using the binomial likelihood function (Chapman *et al*. 1999). The null hypothesis of overall linkage equilibrium was rejected where $L < 0.05$.

All loci were polymorphic in all taxa in which they amplified successfully. Loci J103 and J132 failed to amplify for most *O. mykiss* individuals, and we also observed poor amplification of K216 in this taxon. We observed significant heterozygote deficiencies and multiple nonamplifying individuals at loci J103 and H126 for several *O. c. pleuriticus* and *O. c. stomias* samples, and at loci H18 and K222 for several *O. mykiss* samples, suggesting the presence of null alleles at these loci in these samples. We observed no significant deviation from linkage equilibrium for any locus pair. A supplementary table, providing indices of genetic diversity, F_{IS}, and significance of deviations from Hardy–Weinberg equilibrium for all samples, is available from the authors on request.

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