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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Diagnostic single nucleotide polymorphisms for identifying westslope cutthroat trout (*Oncorhynchus clarki lewisi*), Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) and rainbow trout (*Oncorhynchus mykiss*)

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Abstract

We describe 12 diagnostic single nucleotide polymorphism (SNP) assays for use in species identification among rainbow and cutthroat trout: five of these loci have alleles unique to rainbow trout (*Oncorhynchus mykiss*), three unique to westslope cutthroat trout (*O. clarkii lewisi*) and four unique to Yellowstone cutthroat trout (*O. clarkii bouvieri*). These diagnostic assays were identified using a total of 489 individuals from 26 populations and five fish hatchery strains.

Keywords: species identification, SNP, diagnostic, cutthroat trout, rainbow trout

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The westslope cutthroat trout (*Oncorhynchus clarki lewisi*) is the most widely distributed subspecies of cutthroat trout, and despite its name, it is found on both sides of the continental divide in the Northern Rockies (Allendorf & Leary 1988; Behnke 2002). The westslope cutthroat is a Montana icon and the official state fish, but has experienced great reductions in both abundance and distribution (e.g. Shepard *et al.* 2005) and is now considered a 'Species of Special Concern' by the state of Montana. One of the primary threats to the persistence of westslope cutthroat is hybridization with non-native rainbow trout (*Oncorhynchus mykiss*). Westslope cutthroat readily hybridize with rainbow trout, and, as F1-hybrids are viable, populations of westslope cutthroat trout frequently become 'hybrid swarms'—populations with all individuals having mixed ancestry (Allendorf *et al.* 2001). Two-thirds of extant westslope cutthroat populations now contain non-native ancestry (Shepard *et al.* 2005). The effects of hybridization upon cutthroat trout are poorly understood, but it is clear that even modest amount of hybridization can dramatically decrease the reproductive success of hybrid individuals (Muhlfeld *et al.* 2009).

One of the challenges of managing westslope cutthroat trout is accurately identifying hybrid individuals. Nonhybridized cutthroat trout can usually be discriminated from nonhybridized rainbow trout using morphology, but individuals of mixed ancestry are difficult to classify, especially if they have a small proportion of non-native genes (Allendorf *et al.* 2004 and references within). The problem is compounded in many parts of Montana by hybridization with Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*). In the first half of the 20th century, hundreds of millions of Yellowstone cutthroat trout were collected from Yellowstone Lake, WY, and transplanted throughout western North America (Behnke 2002). Many of these fish ended up in the historic range of westslope cutthroat trout, and now some populations of trout in Montana are hybrid swarms of three taxa: westslope cutthroat, Yellowstone cutthroat and rainbow trout.

Molecular markers offer the only practical methods for accurately identifying westslope cutthroat trout (WCT), Yellowstone cutthroat trout (YCT) and rainbow trout (RBT). Several types of diagnostic markers have been developed; these include: allozymes, interdispersed nuclear elements, insertion-deletions and single nucleotide polymorphisms (SNPs) (Smithwick 2000; Kanda *et al.* 2002; Ostberg & Rodriguez 2004). Of these marker types, SNPs are particularly attractive. They are increasingly cost-effective to genotype, have promise for high-throughput applications and produce data that are readily comparable between laboratories (e.g. Sprowles *et al.* 2006; Finger *et al.* 2009; Stephens *et al.* 2009). The most significant challenge to using SNPs to identify trout species is finding loci that are diagnostic—that is fixed for alternative alleles in different taxa.

We began a search for diagnostic SNP loci by utilizing the available gene index of rainbow trout (Rexroad *et al.*

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2003). We selected and designed primers for 113 expressed sequence tags and genes using Primer3 (http://frodo.wi.mit.edu/primer3/; Rozen & Skaletsky 2000). We used PCR (polymerase chain reaction) to amplify these loci in seven to 23 individuals from populations that fisheries managers believe have not been affected by hybridization. These individuals included, on average five RBT, four WCT and four YCT. Of the 113 loci for which sequencing was attempted, 65 loci (58%) yielded PCR products that were suitable for sequencing. The remaining 48 loci either produced multiple PCR products or failed to amplify. Thirty-four loci of 65 (52%) produced readable sequences that could be aligned. The majority of these alignments contained SNPs (29 of 34), but only 17 loci contained potentially diagnostic SNPs. In approximately equal proportion, we designed Taqman Assays (File Builder version 1.0; Applied Biosystems): five RBT assays, six WCT assays and six YCT assays.

Quantitative PCR conditions were the same for all of the assays. A 10-μL reaction mix contained 5.0 μL of 2× Taqman Universal Master Mix (Applied Biosystems), 0.25 μL of 40× Assay (Applied Biosystems), 10–50 ng of gDNA and enough water to fill. The reaction and data acquisition were performed using the Rotor-Gene 6000 (Corbett Robotics). The thermoprofile consisted of one denature step at 95 °C 10 min, followed by 45 cycles of 95 °C 15 s and 60 °C 1 min (data acquiring step). Genotypes were visualized and scored using Rotor-Gene 6000 Series Software (Corbett Robotics).

To confirm that our assays were diagnostic, we tested the assays on a baseline of 489 individuals from 31 populations believed by state agencies to be unaffected by

Table 1 Baseline populations used to confirm that SNP assays were diagnostic. The number of individuals genotyped is shown by *N*

Population	Taxon	*N*	Major watershed	Minor watershed	State	County	Latitude	Longitude
Eagle Lake Redband rainbow (Pine Creek)	RBT	24	n/a	n/a	CA	Lassen	40.5744	−121.0880
Jocko River Trout Hatchery (Arlee, MT)	RBT	24	n/a	n/a	MT	Lake	47.1690	−114.0820
Fish Lake Strain (FLS)	RBT	24	n/a	n/a	MT	Gallatin	45.2047	−111.7951
Browns Creek	WCT	15	Missouri	Beaverhead	MT	Beaverhead	45.1438	−113.2591
Bull River, East Fork	WCT	16	Columbia	Clark Fork	MT	Sanders	48.1271	−115.7316
Cabin Creek, Middle Fork	WCT	8	Missouri	Madison	MT	Gallatin	44.8866	−111.3343
Canuck Creek	WCT	10	Columbia	Kootenai	ID	Boundary	48.9317	−116.0558
Chamberlain Creek	WCT	16	Columbia	Blackfoot	MT	Cascade	46.9872	−113.2515
Cottonwood Creek	WCT	16	Missouri	Beaverhead	MT	Beaverhead	44.9358	−112.4298
Dirty Ike Creek	WCT	16	Columbia	Clark Fork	MT	Missoula	46.8100	−113.7027
Garden Creek	WCT	10	Columbia	MF Salmon	ID	Challis	44.4935	−114.2972
Graveyard Gulch	WCT	12	Missouri	Smith River	MT	Meagher	46.9317	−110.7793
Hall Creek	WCT	16	Missouri	Crow Creek	MT	Jefferson	46.7846	−111.7846
Little Belt, North Fork	WCT	12	Missouri	Willow Creek	MT	Cascade	47.4245	−110.6500
Main Cabin Creek	WCT	8	Missouri	Madison	MT	Gallatin	44.8974	−111.3141
''McClure Creek'' (Unofficial Name)	WCT	15	Missouri	Smith River	MT	Meagher	46.8000	−111.2945
Muskrat Creek	WCT	4	Missouri	Boulder River	MT	Jefferson	46.3100	−112.0234
North Fork Lost Creek	WCT	16	Columbia	Flathead	MT	Lake	47.8863	−113.7825
Ray Creek	WCT	20	Missouri	Missouri	MT	Broadwater	46.3896	−111.3798
Rock Creek	WCT	17	Columbia	Clark Fork	MT	Missoula	47.0746	−114.3710
Washoe Park Fish Hatchery (MO12)	WCT	32	n/a	n/a	MT	Anaconda	46.1340	−112.9600
Wallace Creek	WCT	26	Columbia	Clark Fork	MT	Missoula	46.7816	−113.6855
Wilson Creek, West Fork	WCT	25	Missouri	Gallatin River	MT	Gallatin	45.5278	−111.1844
Willson Creek 2006	WCT	8	Missouri	Gallatin River	MT	Gallatin	45.5278	−111.1844
Henry Creek	WCT	16	Columbia	Clark Fork	MT	Glacier	47.4671	−114.7506
Sun Ranch Fish Hatchery	WCT	8	Missouri	Madison	MT	Gallatin	44.9713	−111.5941
Bear Lake	YCT	10	Snake River	Snake	ID	Bear Lake	42.0299	−111.3322
Blackfoot River, ID	YCT	10	Snake River	Snake	ID	Caribou	42.8647	−111.5890
Goose Lake	YCT	31	Missouri	Stillwater	MT	Park	45.1141	−109.9140
McBride Creek	YCT	15	Missouri	Yellowstone	WY	Park	44.9629	−110.2540
Yellowstone Lake	YCT	9	Missouri	Yellowstone	WY	Park	44.4708	−110.3560

Table 2 Assay name, locus name, database identification, primer and probe oligonucleotide sequences, and genotypes in rainbow trout (RBT), westslope cutthroat trout (WCT) and Yellowstone cutthroat trout (YCT) for each SNP locus. Genbank accession numbers and other information relating to the DNA sequences used to develop these assays are available in a supplementary table

Assay name	Locus name	dbSNP	Primer and probe oligonucleotide sequences (5′-3′)	RBT	WCT	YCT
CytB-143	Cytochrome B	ss177926242	F:CGAATGAGTCAGCCGTAACTAACAT VIC-CAGATGTGGCAGACAGA 6FAM-AGATGTGGCAAACAGA	A	G	G
Try-517	Trypsin III precursor	ss177926237	F:GTCAGGGTAGTTGCCTAGAGATG R:ACCACTCAGAACCAAAGAGATAATGAC VIC-AGAGACTAATCATTTTCTG 6FAM-AGAGACTAATCCTTTTCTG F:TGTCAGGGTAGATGCCTAGAGATG* R:GACCACTCAGAACCAAAGAGATAATGA* VIC-AGAGACTAATCATTTTCTG* 6FAM-AGAGACTAATCCTTTTCTG*	AA	AA	GG
VIM-337	Vimentin	ss177926255	F:GCCCCGGCTCTCGT R:GGCTTTGAACTCCGAGTTGATG VIC-AAGTCCACAGTATCCG 6FAM-AGTCCACCGTATCCG	TT	GG	GG
RAG1-233	Recombination activation gene-3′ UTR	ss177926250	F:GTCCCCCACACCAGCA R:TGCACAAAATTATCATTATCTGGCTAATAATTGG VIC-AAACATTTTCCCTTGAGGCC 6FAM-CATTTTCCCCTGAGGCC	TT	CC	CC
CBR1-407	Carbonyl reductase	ss177926278	F:TGTGCCAGCCAGCTCTTAATT R:GGAGAGGTTAGGTCATTTACACACA VIC-TTGCGAGTTAAACTTAATAGA 6FAM-CGAGTTAAACTGAATAGA	CC	AA	CC
MT1B-251	Metallothionein B	ss177926274	F:ACGGTGCTTCGCCAAAGA R:CGCGCTTCTTTAGTCAAATGAAGTATTT VIC-AGTTGTAAGCTTTAAATTTT 6FAM-TGTAAGCTTTGAATTTT F:AAACGGTGCTTCGCCAAAG* R:GCGCTTCTTTAGTCAAATGAAGTATTTCA* VIC-TTGTAAGCTTTAAATTTCT* 6FAM-AAGCTTTGAATTTCT*	AA	AA	GG
Thymo-320	Thymosin beta	ss177926246	F:TGATATGACACATGAATACTATATTTTAAACTTAA TTAGCATTTT R:CATTGAAATGACGTGGAATCAACGT VIC-TGTGCCCAGTGGGTTA 6FAM-TGCCCAGCGGGTTA	AA	GG	GG
P53-307	tumour supressor p53 gene	ss177926265	F:CCCTGGTCCTGGGTTGAC R:GAGAGGAAGTTCCCATCACTGTVIC-CCAACTAATG TCTTGTTATAGTA 6FAM-CAACTAATGTCTTGTCATAGTA	CC	TT	CC
Cal-155	Calreticulin	ss177926260	F:TGGGTGCATATCAGTTGTAGTTTTGT R:TGTAACGAGGGAATAAATTAAAAGGATGTTGA VIC-CAGTTGTATGCTGTACTGTA 6FAM-CAGTTGTATGCAGTACTGTA	TT	AA	AA
INV-1106	invariant chain S25-7	ss177926285	F:TGCATGGTTTAAAATGTAACACACATCTT R:GGACAGTATAAATGCTGTGAACAATATTAACAG VIC-TCAACACAGCTTTGTAAAG 6FAM-CAACACAGCTATGTAAAG	TT	TT	AA
PrL2-2483	Prolactin 2	ss177926281	F:GTGAGGAGAGCACAGAGCTTTAC R:CTCCAGACACCCAAGGACAA VIC-ACTCTAAGCACTTGCTCC 6FAM-CTAAGCGCTTGCTCC	GG	GG	AA

Table 2 Continued

Assay name	Locus name	dbSNP	Primer and probe oligonucleotide sequences (5′-3′)	RBT	WCT	YCT
Tnsf-387	Transferrin intron 6	ss177926270	F:CAACTGTAAAATGCTAACTCTGGATCTAGA R:GGTGACGTGTGAGTGGTTTGA VIC-ACATTTACACTAAACATTAAC 6FAM-ATTTACACTAAGCATTAAC VIC-ACATTTACACTTAACATTAAC* 6FAM-ATTTACACTTAGCATTAAC*	CC	TT	CC

*Extra assay was designed and used together with primary assay because of nontargeted SNPs located in priming and/or probe sites.

hybridization. This baseline included 21 wild WCT populations, two WCT hatchery populations, five YCT populations and three RBT hatchery strains cultured in Montana (Table 1). To be diagnostic, an assay must be fixed for alterative alleles in each taxon. Because SNPs have two alleles, and we compared three taxa, each assay had to have an allele unique to one of the three taxa (see Kalinowski 2010 for a discussion of how to analyse such data). For example, our mitochondrial assay, *CytB-143*, had an *A* in RBT and a *G* in WCT and YCT.

Finding diagnostic SNPs to distinguish between rainbow and cutthroat trout proved straightforward. The five assays we identified from our initial sequencing were also diagnostic in our baseline of 489 individuals (Table 2).

Finding diagnostic SNPs to distinguish between WCT and YCT was more difficult. Five assays showed polymorphism within WCT and YCT, and we dropped them from further consideration. The remaining seven assays were diagnostic (Table 2) in the complete baseline—or nearly so. Four of the seven assays possessed a small amount of polymorphism in some populations. *P53-307* had two heterozygous individuals, *Tnsf-387* had eight heterozygous individuals and one homozygote, *PrL2-2483* had six heterozygous individuals and the mitochondrial assay, *CytB-143*, had one conflicting haplotype. Such rare polymorphisms are typical for these subspecies (Robb Leary, Montana Fish Wildlife and Parks, personal communication) and may be almost inevitable if a sufficient number of populations are examined.

Low levels of polymorphism at loci that are otherwise diagnostic can originate in several possible ways. These include: anthropogenic hybridization, homoplasy (convergent mutation), ancestral polymorphism and natural hybridization. Fisheries managers sometimes cull entire populations that are believed to have non-native ancestry, so differentiating between anthropogenic hybridization and other sources of nondiagnostic alleles can be important. This can be difficult if putative non-native alleles are present at low frequencies. Because of the possibility of homoplasy, ancient polymorphism and natural hybridization, it may be prudent to avoid classifying a population as containing non-native alleles unless non-native alleles are found at more than one locus. If this precaution is taken, all of the loci described here should be useful for species identification.

The power of these loci for detecting hybridization depends on the number of individuals genotyped and the history of genetic admixture in the population. For example, if a population has become a hybrid swarm that is 1% RBT and 99% YCT, the set of nuclear loci described here would have a 96% probability of detecting a RBT allele. Increasing the sample size to 30 would increase this probability to over 99%. These probabilities are reassuringly high, but apply only to hybrid swarms. If a population is not a hybrid swarm, the probability of detecting non-native genes depends on the specific distribution of genotypes in the population. Furthermore, these high probabilities apply only to samples of individuals collected from a population. The probability of detecting hybridization in a single individual is much lower (and depends on the distribution of genotypes within the individual). Therefore, these loci will probably be the most useful for looking for hybridization within populations or when used in conjunction with other loci for detecting hybridization within individuals.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. The table below includes information for the DNA sequences used in SNP discovery (RBT, Rainbow trout, WCT, westslope cutthroat trout, YCT, Yellowstone cutthroat trout).

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