Molecular Ecology Resources (2009) **9**, 759–763 doi: 10.1111/j.1755-0998.2009.02532.x

MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Six diagnostic single nucleotide polymorphism markers for detecting introgression between cutthroat and rainbow trouts

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Abstract

Ten primer pairs were screened to develop single nucleotide polymorphism (SNP) *Taq*Man assays that will distinguish California golden trout and some rainbow trouts (*Oncorhynchus mykiss sspp., O. m. aguabonita*) from the Paiute and Lahontan cutthroat trouts (*Oncorhynchus clarkii seleniris, O. c. henshawi*). From these 10 primer pairs, one mitochondrial and five nuclear fixed SNP differences were discovered and developed into *Taq*Man assays. These six assays will be useful for characterizing and monitoring hybridization between these groups. Additional *Oncorhynchus clarkii sspp.* and *Oncorhynchus mykiss sspp.* were assayed to determine if these assays are useful in closely related species.

Keywords: hybridization, *Oncorhynchus clarkii*, *Oncorhynchus mykiss*, single nucleotide polymorphism, *Taq*Man assay

Received 22 August 2008; revision accepted 9 November 2008

Molecular markers are useful tools for assessing levels of introgression between native and closely related introduced or domesticated species (e.g. Randi 2008). Such markers have been useful in genetic monitoring studies of hybridization between populations of rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*Oncorhynchus clarkii*) in particular (Campbell *et al*. 2002; Gunnell *et al*. 2008). Examples include microsatellites (Cegelski *et al*. 2006), mtDNA restriction fragment length polymorphisms (Dowling & Childs 1992), allozymes (Dowling & Childs 1992), amplified fragment length polymorphisms (Young *et al*. 2001), and PINEs (Kanda *et al*. 2002). However, use of these markers can be costly, time-consuming, and difficult to standardize across laboratories. These marker types may also lack species-specific diagnostic alleles.

Single nucleotide polymorphisms (SNP) have successfully been employed to efficiently detect hybridization between salmonids (Stephens 2007a,b). Here we present six SNP markers that distinguish Lahontan and Paiute cutthroat trout (*Oncorhynchus clarkii henshawi* and *O. c. seleniris*) from four subspecies of rainbow trout (collectively referred to hereafter as *O. m. sspp*., following Stearley & Smith 1993; see below). For a discussion of the genus *Oncorhynchus*, see Bagley & Gall (1998) and Stearley & Smith (1993). In the past, managers have relied on morphology alone to determine introgression levels in populations of Lahontan and Paiute cutthroat trout, but this can lead to an overestimation of the number of populations that are 'pure' (Allendorf & Leary 1988). These six SNP markers will provide managers with a quick, inexpensive and effective method for characterizing some *O. m. sspp*.-introgressed Lahontan and Paiute cutthroat trout populations.

The Paiute cutthroat trout and Lahontan cutthroat trout are both listed as Threatened species under the US federal Endangered Species Act and are geographically restricted to small portions of their historic native ranges (Coffin & Cowan 1995). A lack or near-lack of body spotting and a yellowish body hue differentiate the former subspecies from the latter (Moyle 2002). Numerous conservation efforts over the past several decades have been directed at eradicating established populations of introduced non-native fishes and restoring both the Lahontan and the Paiute cutthroat trouts to their native ranges through restocking (Coffin 1995; USFWS 2004). Diagnostic SNP markers will facilitate identification of *O. m. sspp*.-introgressed Lahontan and Paiute cutthroat populations and the selection of non-introgressed populations suitable for use as donor stocks.

One representative individual was selected from each of three Paiute cutthroat trout populations (Fly Valley Creek,

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FVC; Four Mile Creek, FMC; and North Fork Cottonwood Creek, NFC) determined by previous genetic research to be nonintrogressed with coastal rainbow trout (*Oncorhynchus mykiss irideus*) (Cordes *et al*. 2004) and three nonintrogressed Lahontan cutthroat trout populations (East Carson River/above falls, ECRAF; Poison Flat Creek, PFC; and Murray Canyon Creek, MCC). DNA from each individual was amplified by polymerase chain reaction (PCR) with 10 primer pairs at the following loci: BAC B9, BAC F5, HOXD, RAG1175-1700, URO, CHITUTR, ID1C (Sprowles *et al*. 2006), and OmyP9-B1 (Bagley *et al*. 1997; Sprowles *et al*. 2006), CTDL1 and F17a (Bagley & Gall 1998) using conditions described in each respective reference. PCR products were cleaned using QIAquick PCR purification kit (QIAGEN) and sequenced in the forward direction on an ABI 3730 Capillary Electrophoresis Genetic Analyser (Applied Biosystems) at the UCDNA Sequencing Facility, University of California, Davis. Individuals were also sequenced in the reverse direction for the locus RAG1175-1700.

Sequences were evaluated by eye for quality submitted to GenBank (Appendix), and aligned with sequences from Volcano Creek California golden trout (*Oncorhynchus mykiss aguabonita*), North Fork American River coastal rainbow trout (*O. m. irideus*), and Mount Shasta strain hatchery rainbow trout (*O. m. sspp*.) individuals (Sprowles *et al*. 2006) using Sequencher 4.7 software (Gene Codes Corporation). Five nuclear sequences and one mtDNA sequence containing diagnostic SNPs were submitted to Applied Biosystems' Assays-by-Design service for SNP *Taq*Man assay development. This generated six 5′ exonuclease assays (Holland *et al*. 1991) with 6-FAM and VIC allele-specific probes for genotyping SNPs using real-time PCR (Table 1). Genotypes were submitted to dbSNP (accession names and numbers in Table 1).

Genotyping assays were performed on the populations listed in Table 2 using the Chromo4 Real-Time PCR Detector (MJ Research/Bio-Rad Laboratories, Inc.) in 96-well reaction plates with a general thermal-cycling protocol as follows: an initial step of 95 °C for 10 min followed by 40 cycles of 92 °C for 15 s and a primer-specific annealing and extension temperature (Table 1) for 1 min. Reaction volumes totalled 5 μL, containing 2× *Taq*Man Universal Master Mix (Applied Biosystems), 540 nM each primer, 120 nM each probe, and 10 ng template DNA. On each 96-well plate, four wells were set aside for positive and negative controls: one each with template from a known homozygote for each allele, one with template from a known heterozygote, and one negative control. MJ Opticon Monitor 3.1.32 (MJ Research/Bio-Rad Laboratories, Inc.) software was used to determine individual genotypes by creating a scatter plot depicting the endpoint fluorescence of each allele-specific probe in each well. Baseline average fluorescence over the 10–30 cycle range was subtracted to reduce noise, and endpoint fluorescence clusters were identified by comparing fluorescence with positive and negative controls.

To further assess if the six discovered SNPs are indeed fixed for species-specific alleles, additional Paiute cutthroat trout from a restored population above a fish barrier (Llewellyn Falls) in Silver King Creek, Lahontan cutthroat trout from Frazer Creek, Forman Creek and Carson River, California golden trout from Volcano Creek, Mount Shasta strain hatchery rainbow trout, Little Kern golden trout (*Oncorhynchus mykiss whitei*) from Upper Soda Spring Creek, and McCloud river redband trout (*O. m.* ssp.) from Sheepheaven Creek were assayed. A few individuals of Bonneville cutthroat (*Oncorhynchus clarkii utah*), Westslope cutthroat (*Oncorhynchus clarkii lewisi*) and Yellowstone cutthroat (*Oncorhynchus clarkii bouvieri*) were also assayed (Table 2). To test the utility of these markers in detecting hybridization, a population of fish ($N = 250$) from Silver King Creek below Llewellyn Falls was assayed (Table 2). This section of Silver King Creek is a native Paiute cutthroat

Table 1 *Taq*Man assay name, dbSNP accession names and numbers primer and probe sequences and annealing temperatures (T_a) for each SNP locus

Locus name	dbSNP Accession name	dbSNP Accession no.	Primer oligonucleotide sequences (5′–3′)	Probe oligonucleotide sequences (5′–3′)	T_a (°C)
B9_228	GVL_B9_228	ss94323488	F: ACATTTTCATGCACAGAACACAGC R: GTGCCTCGACTCTTTTCTGTTG	VIC-TCAAGGCAAC**T**ATTTGTA FAM-AAGGCAAC**G**ATTTGTA	60
CTDL1_243	GVL_CTDL1_243	ss94323450	F: GAGTAGTACATTATATGTATTATCAACATACGGTGATTT R: GTAAAGACGGAGCCCGTGTTA	VIC-TTATTATCACGTGTTT**G**GCTT FAM-TTATTATCACGTGTTT**T**GCTT	58
F5_136	GVL_F5_136	ss94323468	F: GCACGCCAGAGGACAGTAG R: GTGATACCATTCATTAGCATTGCATTCTG	VIC-ACCTGAT**G**AAAACC FAM-ACCTGAT**C**AAAACC	58
HOXD_287	GVL_HOXD_287	ss94323437	F: CACAGGAAGAGCAGCTTTAAAGAC R: AGGTATAAAGAACCGAAGGATGAAGTG	VIC-TCCTGCAG**C**TTCCCA FAM-CCTGCAG**A**TTCCCA	58
RAG11_280	GVL_RAG1175_280	ss94323420	F: ACATCACGTTTTACATGACGGTATGT R: ACCGCTGATATGAGATTCACACAAA	VIC-CATCCTTCATG**A**AGCAAA FAM-CATCCTTCATG**T**AGCAAA	60
URO_302	GVL_URO_301	ss94323503	F: CCATTGAGTTTGTCATAGTCTCGTACA R: TTCAGCAGCATCTGTTTACAGATGT	VIC-ACTATTCTGCT**A**ACTTGTC FAM-ATTCTGCT**G**ACTTGTC	58

Table 2 Fish assayed for each SNP, grouped by subspecies and sampling location. Alleles displayed at each of the six SNP loci are given

			SNP locus					
Subpecies/group	Location	*N*	B9_228	CTDL1_243	F5_136	HOXD_287	RAG11_280	URO_302
Paiute cutthroat trout (*Oncorhynchus clarkii seleniris*)	Connell's Cow Camp + Upper Fish Valley	38	GG	A	CC	AA	AA	GG
Lahontan cutthroat trout (*Oncorhynchus clarkii henshawi*)	Carson River	10	GG	A	CC	AA	AA	GG
	Forman Creek	10	GG	A	CC	AA	AA	GG
	Frazer Creek	10	GG	A	CC	AA	AA	GG
Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*)	Cannuck Creek	5	TT	C	CC	AA	AA	GG
	Garden Creek	5	TT	A,C	CC	AA	AA	GG
	Geode Creek	5	TT	C	CC	AA	AA	GG
Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*)*	1	10	TT	A	CC	AA	AA	GG
Bonneville cutthroat trout (*Oncorhynchus clarkii utah*)†	2	9	TT	A	CC	AA	AA	GG
Rainbow trout (*Oncorhynchus mykiss sspp.*)	Mount Shasta Strain	24	TT	C	GG	CC	TT	AA
Golden trout (*Oncorhynchus mykiss aguabonita*)	Volcano Creek	40	TT	C	GG	CC	TT	AA
Redband trout (*O. m. ssp.*)	Sheepheaven Creek	22	TT	C	GG	CC	TT	AA
Little Kern golden trout (*Oncorhynchus mykiss whitei*)	Upper Soda Spring Creek	21	TT	C	GG	CC	TT	AA
3 known hybrid fishes *O. m. sspp.* + *O. c. sspp.*	Silver King Creek/Canyon Site	1	TT	C	GG	CC	TA	AA
	Silver King Creek/Canyon Site	1	TG	C	GG	CC	TT	AA
	Silver King Creek/Canyon Site	1	TT	C	GG	CA	TT	AA

*Two fish from Yellowstone Lake, Idaho, three from Upper Snake River/Henry's Fork, Idaho and five from Upper Snake River/Blackfoot, Idaho were assayed. †Four Southern Bonneville form fish from the Glenwood Hatchery, Sevier River, Utah, and five Bear River form fish from Bear Lake, Idaho/Utah were assayed.

trout stream, but has been stocked with hatchery rainbow trout, California golden trout and Lahontan cutthroat trout. Fish in the stream resemble rainbow-golden trout hybrids (William Somer, personal communication).

Among the populations assayed, SNPs F5–136, HOXD_287, RAG11–280 and URO_302 showed fixed allelic differences between all assayed *Oncorhynchus clarkii* ssp. and all assayed *O. m. sspp*. B9–228 distinguished Lahontan and Paiute cutthroat trouts from all other subspecies assayed. Locus CTDL1–243 is fixed for all *O. m. sspp.* assayed, and fixed for the opposite allele in all *Oncorhynchus clarkii sspp.* assayed, except for the Westslope cutthroat trout; additional Westslope cutthroat trout individuals should be assayed to determine if CTDL1–243 is useful for detecting introgression in this subspecies (Table 2). However, this locus does appear to be useful for detecting rainbow trout hybridization in other cutthroat subspecies. It is recommended that more Bonneville, Westslope and Yellowstone cutthroat trout be assayed at all loci, as only five to 10 fish of each subspecies were analysed.

The diagnostic SNP markers described here will aid managers in determining if some cutthroat trout subspecies are introgressed with populations of some rainbow trout subspecies and vice versa. This information will be invaluable for restoring and monitoring some of North America's threatened native trout species.

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Appendix

Appendix 1. Sequence names submitted to GenBank for each primer set (locus) with associated GenBank accession numbers. Prefix was designed to reflect the population from which the individual came (see text for abbreviations), and the suffix indicates the locus (ex: FVC_A1A8 is a sequence for an individual from Fly Valley Creek at locus A1A8).

Locus	GenBank Accession number
A1A8	
ECRAF_A1A8a	EU423086
FVC_A1A8a	EU423087
NFC_A1A8a	EU423088
B9	
ECRAF_B9	EU423089
FMC_B9	EU423090
FVC_B9	EU423091
MCC_B9	EU423092
NFC_B9	EU423093
CHIT	
ECRAF_CHIT	EU423094
FMC_CHIT	EU423095
FVC_CHIT	EU423096
MCC_CHIT	EU423097
NFC_CHIT	EU423098
PFC_CHIT	EU423099
CTDL1	
ECRAF_CTDL1	EU423124
FMC_CTDL1	EU423125
FVC_CTDL1	EU423126
MCC_CTDL1	EU423127
NFC_CTDL1	EU423128
PFC_CTDL1	EU423129
F5	
ECRAF_F5	EU423100
FVC_F5	EU423102
MCC_F5	EU423103
NFC_F5	EU423104
PFC_F5	EU423105
HOXD	
FVC_HOXD	EU423106
PFC_HOXD	EU423107
ID1C	
ECRAF_ID1C	EU423108
FMC_ID1C	EU423109
FVC_ID1C	EU423110
MCC_ID1C	EU423111
NFC_ID1C	EU423112
OmyP9B	
ECRAF_OmyP9B	EU423113
FMC_OmyP9B	EU423114
FVC_OmyP9B	EU423115
MCC_OmyP9B	EU423116
NFC_OmyP9B	EU423117
PFC_OmyP9B	EU423118
RAG1175-1700	
NFC_RAG1175	EU423119
FMC_RAG1175	EU423120
ECRAF_RAG1175	EU423121
MCC_RAG1175	EU423122
PFC_RAG1175	EU423123
URO	
NFC_URO	EU442397
ECR/AF_URO	EU442398
FVC_URO	EU442399
FMC_URO	EU442400
PFC_URO	EU442401

Appendix 2. TaqMan Assay names and dbSNP accession numbers. Assay names were created by preceding the locus name and SNP location (ex: B9_228) with GVL (Genomic Variation Lab).

dbSNP Accession Name	dbSNP Accession number
GVL_B9_228	ss94323488
GVL_CTDL1_243	ss94323450
GVL_F5_136	ss94323468
GVL_HOXD_287	ss94323437
GVL_RAG1175_280	ss94323420
GVL_URO_301	ss94323503