MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2012) doi: 10.1111/j.1755-0998.2012.03149.x

Discovery and characterization of a large number of diagnostic markers to discriminate *Oncorhynchus mykiss* and *O. clarkii*

V. L. PRITCHARD, A. ABADÍA-CARDOSO and J. C. GARZA
Southwest Fisheries Science Center, National Marine Fisheries Service and University of California, Santa Cruz, 110 Shaffer Road, Santa Cruz, CA 95060, USA

Abstract

Hybridization of cutthroat trout and steelhead/rainbow trout is ubiquitous where they are sympatric, either naturally or owing to introductions. The ability to detect hybridization and introgression between the two species would be greatly improved by the development of more diagnostic markers validated across the two species' many phylogenetic lineages. Here, we describe 81 novel genetic markers and associated assays for discriminating the genomes of these sister species. These diagnostic nucleotide polymorphisms were discovered by sequencing of rainbow trout expressed sequence tags (ESTs) in a diverse panel of both cutthroat trout and steelhead/rainbow trout. The resulting markers were validated in a large number of lineages of both species, including all extant subspecies of cutthroat trout and most of the lineages of rainbow trout that are found in natural sympatry with cutthroat trout or used in stocking practices. Most of these markers (79%) distinguish genomic regions for all lineages of the two species, but a small number do not reliably diagnose coastal, westslope and/or other subspecies of cutthroat trout. Surveys of natural populations and hatchery strains of trout and steelhead found rare occurrences of the alternative allele, which may be due to either previous introgression or shared polymorphism. The availability of a large number of genetic markers for distinguishing genomic regions originating in these sister species will allow the detection of both recent and more distant hybridization events, facilitate the study of the evolutionary dynamics of hybridization and provide a powerful set of tools for the conservation and management of both species.

Keywords: cutthroat trout, hybridization, introgression, rainbow trout, single nucleotide polymorphism, steelhead

Received 12 December 2011; revised received 29 February 2012; accepted 5 March 2012

Introduction

Hybridization is a key process in evolution, and the ability to detect it is critical for the conservation and management of natural populations. Hybridization can have a wide array of consequences for the hybridizing entities, including the *de facto* disappearance of populations and species (extinction through hybridization), as well as the creation of novel species (Wolf *et al.* 2001; Mavárez *et al.* 2006). Understanding the dynamics of hybridization and associated genetic introgression can provide insight into the processes of natural selection and, ultimately, speciation.

The cutthroat trout (*Oncorhynchus clarkii*) of North America comprises nine currently recognized extant subspecies, distributed along the Pacific coast and in numerous western river basins (Behnke 1992). All subspecies are capable of hybridizing with the closely related *O. mykiss* (steelhead, redband or rainbow trout) to produce viable offspring. Coastal cutthroat trout (*O. c. clarkii*) and some populations of westslope cutthroat trout (*O. c. lewisi*) are naturally sympatric with *O. mykiss* but remain distinct as a result of natural barriers to gene flow, which may involve spawning behaviour and selection against hybrids (Young *et al.* 2001; Bettles *et al.* 2005; Kozfkay *et al.* 2007; Moore *et al.* 2010). However, over the past two centuries, vast numbers of rainbow trout have been introduced into inland waters containing native cutthroat trout. Introductions have often initially led to hybridization, especially in areas where the two species were not historically sympatric, and over time can lead to hybrid swarms, with the functional extinction of the cutthroat trout population (e.g. Hitt *et al.* 2003). Hybridization with rainbow trout has been cited as a factor in the decline of all interior cutthroat trout subspecies and remains one of the primary threats to extant populations (Allendorf *et al.* 2001). Conservation efforts focus on identifying relatively nonhybridized cutthroat trout populations, protecting them from hybridization and competition with non-native trout through construction of fish movement barriers and reintroducing them to reclaimed habitat.

Correspondence: John Carlos Garza, Fax: 1-831-420-3977;
E-mail: carlos.garza@noaa.gov

Although the fitness consequences of introgressed genetic material in a population are variable and generally hard to predict (Edmands 2007), and the conservation value of hybridized cutthroat trout populations is debated (Allendorf *et al.* 2001; Peacock & Kirchoff 2004), most cutthroat trout conservation efforts focus on populations found to be free of introgression from *O. mykiss* (Campton & Kaeding 2005).

Conservation efforts and research on both the spread and fitness impacts of *O. mykiss* introgression into *O. clarkii*, and the nature of intrinsic barriers to gene flow, have been somewhat restricted by the relative paucity of genetic markers available to easily distinguish genomic regions from the two species. Most diagnostic genetic markers currently available were validated on a subset of *O. clarkii* subspecies and may perform poorly in other taxa. Many of these markers also suffer from other drawbacks, such as a requirement for lethal sampling (allozymes, e.g. Busack *et al.* 1980), uniparental inheritance (mtDNA, e.g. Young *et al.* 2001), lack of codominance (AFLPs, Young *et al.* 2001; PINEs, Kanda *et al.* 2002; dpPCR markers, Ostberg & Rodriguez 2002) and lack of fixed diagnostic differences between species (microsatellites, e.g. Pritchard *et al.* 2007). In recent years, the increasing ease of identification, decreasing cost of genotyping and high repeatability of single nucleotide polymorphisms (SNPs) and similar short sequence polymorphisms have made them the marker of choice for such purposes. Correspondingly, small numbers of SNP assays have recently been developed for the identification of *O. mykiss* introgression into, variously, coastal (*O. c. clarkii*), westslope (*O. c. lewisi*), Lahontan (*O. c. henshawi),* Paiute (*O. c. seleneris*) and Yellowstone (*O. c. bouvieri*) cutthroat trout (Finger *et al.* 2009; McGlauflin *et al.* 2010; Harwood & Phillips 2011; Kalinowski *et al.* 2011). Additionally, a Restriction site Associated DNA (RAD) sequencing approach has recently identified several thousand putative SNP sites that may be able to be developed into diagnostic assays between *O. mykiss* and westslope cutthroat trout (Hohenlohe *et al.* 2011).

In this paper, we present 81 novel SNP (single-base) and SNP-type (multi-base and insertion-deletion) assays that discriminate between genomic regions of *O. mykiss* and *O. clarkii*. In contrast to markers described previously, these assays have been explicitly designed for use with the broad array of phylogenetic lineages in the two species, including all extant cutthroat trout subspecies.

Methods

An ascertainment panel of 23 *O. clarkii* individuals (Table 1a) was selected to represent the range of phylogenetic variation known in the species and included all recognized extant subspecies except Paiute cutthroat trout, which is extremely closely related to Lahontan cutthroat trout (Nielsen & Sage 2002). Within each subspecies, populations were chosen to represent as many described genetic or geographical units as possible. One individual per population was included in the ascertainment panel, but limited quantities of DNA for some individuals did not allow the same individual to be used for all loci in all populations. Tissue samples were collected through a variety of methods, and purified DNA was generally obtained using DNeasy 96 Tissue kits (Qiagen Inc.) and kits from other manufacturers.

Initial PCR was performed using 225 primer pairs that had previously been used to generate DNA sequence data for SNP discovery in *O. mykiss* (Abadía-Cardoso *et al.* 2011). These primers were originally designed from randomly chosen nuclear tentative consensus (TC) sequences generated from *O. mykiss* expressed sequence tags compiled in the Harvard DFCI Gene Index project (http://compbio.dfci.harvard.edu/tgi/tgipage.html; accessed on December 8, 2006). As DNA was limited and of variable quality, all samples were subjected to a preamplification step as follows: 5 μL multiplex master mix (Qiagen Inc.), 7 μL deionized water, 3 μL primer mix containing up to 44 primer pairs at a concentration of 0.05 μM per primer, and 3 μL of template DNA was incubated at 95 °C for 15 min, followed by 14 cycles of: 30 s at 94 °C, 90 s initially at 48 °C and increasing by 1 °C per cycle, 90 s at 72 °C, with a final extension step of 72 °C for 15 min. Preamplification product was then diluted 1:10 with deionized water. Subsequent PCR amplifications were performed in 15-μL reactions containing: 6.55 μL deionized water, 1.5 μL 10 × PCR buffer (Applied Biosystems Inc.), 1.0 μL dNTPs (2.5 mM each), 0.9 μL $MgCl_2$ (25 mM), 0.5 μL each primer (5 mM), 0.041 U Ampli*Taq* DNA polymerase (Applied Biosystems) and 4 μL diluted preamplified template. Thermal cycling conditions employed a 'touchdown' protocol and were as follows: an initial denaturation step of 95 °C for 4 min, then 60 °C for 2 min and 72 °C for 1 min; followed by 12 cycles of: 30 s at 94 °C, 30 s initially at 59°C then decreasing 1 °C per cycle, 1 min at 72 °C; followed by 11 cycles of: 95 °C for 30 s, 48 °C for 30 s, 72 °C initially for 1 min then increasing by 10 s per cycle; and a final extension step of 5 min at 72 °C.

PCR products were separated by gel electrophoresis in 2% agarose and visualized by ethidium bromide staining. Loci that produced a single robust PCR product band in at least 50% of ascertainment panel samples were purified using an Exo-SAP protocol: 5 μL of PCR product, 0.15 mL of Exonuclease I (20 U/mL), 1 μL of shrimp alkaline phosphatase (1 U/mL), 0.5 μL of 10× buffer and 3.36 μL of deionized water were incubated at 37 °C for 60 min and then 80 °C for 20 min. Forward and reverse

Table 1 (a) *Oncorhynchus clarkii* and (b) *Oncorhynchus mykiss* ascertainment panels

Taxon	Common name	Drainage	Population
(a)			
O. c. bouvieri	Yellowstone cutthroat trout	Yellowstone Lake, WY	Hatchery Creek
O. c. bouvieri	Yellowstone cutthroat trout	Yellowstone River, MT	Upper Deer East Fork
O. c. bouvieri	Yellowstone (Snake River form)	Snake River, WY	Salt River
O. c. clarkii	Coastal cutthroat trout	Kuiu Island, AK	Slippery Lake
O. c. clarkii	Coastal cutthroat trout	Columbia River, WA	Mill Creek South Fork
O. c. clarkii	Coastal cutthroat trout	Columbia River, WA	Abernathy Creek
O. c. henshawi	Lahontan cutthroat trout	Quinn River, NV	Three Mile Creek
O. c. henshawi	Lahontan cutthroat trout	Pilot Peak, UT	Pilot Peak
O. c. henshawi	Lahontan cutthroat trout	Independence Lake, CA	Independence Lake
O. c. henshawi	Lahontan (Humboldt form)	Humboldt River, NV	Humboldt North Fork
O. c. lewisi	Westslope cutthroat trout	Clark Fork River, MT	Granite Creek
O. c. lewisi	Westslope cutthroat trout	Columbia River, MT	Copper Creek
O. c. lewisi	Westslope cutthroat trout	Missouri River, MT	Bear Creek
O. c. lewisi	Westslope cutthroat trout	Missouri River, MT	McClellan Creek
O. c. pleuriticus	Colorado River cutthroat trout	Colorado River, CO	Lake Nanita
O. c. stomias	Greenback cutthroat trout	Gunnison River, CO	West Antelope Creek
O. c. stomias	Greenback cutthroat trout	Arkansas River, CO	Severy Creek
O. c. stomias	Greenback (Bear Creek form)	Arkansas River, CO	Bear Creek
O. c. utah	Bonneville cutthroat trout	Deep Creek, UT	Birch Creek
O. c. utah	Bonneville (Bear River form)	Bear River, UT	McKenzie Creek
O. c. virginalis	Rio Grande cutthroat trout	Rio Grande, NM	Columbine Creek
O. c. virginalis	Rio Grande cutthroat trout	Rio Grande, NM	Comanche Creek
O. c. virginalis	Rio Grande cutthroat trout	Canadian River, NM	McCrystal Creek

Taxon	Common name	Drainage	Population	*n*
(b)				
O. mykiss	Steelhead	Scott Creek, CA	Scott Creek	10
O. mykiss	Steelhead	Eel River, CA	Middle Fork, Summer run	4
O. mykiss	Rainbow trout	Hatchery	Whitney Strain	3
O. mykiss	Rainbow trout	Hatchery	Wyoming/Virginia Strain	3
O. m. newberri	Great Basin redband trout	Goose Lake, OR	Bauers Creek	2
O. c. clarkii	Coastal cutthroat trout	Little River, CA	Little River	2

sequences were generated from the purified products using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), with reagent proportions and cycling conditions following the manufacturer's protocol. Sequencing reaction products were purified using 6% Sephadex columns, and the sequence was read by capillary electrophoresis on a 3730 DNA Analyzer (Applied Biosystems).

Forward and reverse sequences for each locus were pruned, aligned, assembled into contigs and edited using Sequencher 4.10.1 (Gene Codes Corporation) and without knowledge of the identity of the individual fish. When a polymorphism was suggested, the chromatograms were visually examined for confirmation. Cutthroat trout sequences were then aligned with the previously edited matching sequences from a panel of 22 *O. mykiss* (Abadía-Cardoso *et al.* 2011) and two coastal cutthroat trout (Table 1b). With these two coastal cutthroat trout, the total number of individuals for which sequence was obtained increased to 25. Tables summarizing sequence variation were generated for each locus and examined for the presence of fixed polymorphisms (single base, multi-base or indel) distinguishing *O. mykiss* from at least six subspecies of *O. clarkii*.

To minimize linkage disequilibrium between markers, a single polymorphic site in each locus was chosen for design of 5′ nuclease (TaqMan) SNP genotyping assays (Applied Biosystems). When an assay could not be designed for a selected site, either an alternative diagnostic site at the same locus was chosen or flanking sites that were rarely variable were recoded with the most common nucleotide to provide more options for primer and probe locations.

Putative function for each sequenced locus was investigated by running a Blastx search on the NCBI nonredundant protein sequence database with the TC

sequence from which primers were designed. Protein-coding regions (CDS) and untranslated regions (UTR) were identified in TC sequences by aligning them with the best-matching annotated nuclear sequence returned from the search and by verifying the reading frame in Sequencher. Sequences that did not return a Blastx match were subject to an additional Blastn search. Intronic regions and gene features in the sequences generated for SNP discovery were identified by aligning them with the annotated TC sequences associated with that EST.

Species diagnostic assays were validated with 120 *O. mykiss*, 178 *O. clarkii* and 10 known F1 *mykiss-clarkii* hybrids (Table 2). The *O. clarkii* samples comprised all of the nine extant described subspecies and included 17 geographical units previously recognized as 'genetically distinct' (Johnson *et al.* 1999; Metcalf *et al.* 2007; Pritchard *et al.* 2008; Peacock *et al.* 2010; Drinan *et al.* 2011). The *O. mykiss* samples were comprised of (i) commonly stocked hatchery rainbow trout strains and one population originating from a known stocking event; (ii) southern steelhead, from populations not sympatric with coastal cutthroat trout; (iii) northern steelhead, from populations sympatric with coastal cutthroat trout; (iv) Klamath Basin redband trout (*O. m. newberri*); and (v) Columbia River redband trout (*O. m. gairdneri*), from populations historically sympatric with westslope cutthroat trout. SNP genotyping was carried out in 96.96 Dynamic Genotyping Arrays on an EP1 Genotyping System (Fluidigm Corporation), with a preamplification step, following the manufacturer's protocol. SNP calls were determined using the Fluidigm SNP Genotyping Analysis software (v 3.0.2), with confidence threshold set to 85%. As evidence suggested that DNA quality affected both genotype call rate and call accuracy, only samples successfully called for at least 86 of the 96 loci on an array were retained in subsequent analyses. Multi-locus heterozygosity within the validation groups was calculated using Genetix (Belkhir *et al.* 1996–2004).

Finally, a subset of these markers (n = 72) were evaluated in a sample of 84 putative *O. mykiss* from Little River and Ryan Creek in northern California, suspected from the results of previous population genetic analyses (Garza *et al.* 2004) to contain individuals introgressed by coastal cutthroat trout.

Results

Of the 225 primer pairs initially tested, 201 produced PCR products that were considered amenable to sequencing. Of these, 173 produced sequence of sufficient quality, length and subspecies coverage to be considered for SNP discovery. Total consensus sequence length for *O. clarkii* was 88 475 bp, with a mean of 23.5 individuals yielding sequence per locus (Table 3). We observed fixed polymorphisms (single base, multi-base or insertion/deletion) between *O. mykiss* and at least six subspecies of *O. clarkii* in 127 of the 173 loci. Polymorphisms in 83 of these loci were successfully converted into TaqMan assays. Polymorphic sites in the remaining loci were not amenable to assay design because (i) the polymorphism was a length difference in a repetitive region; (ii) the polymorphism was larger than 5 bp; (iii) surrounding variable sites precluded design of amplification primers or TaqMan probes; or (iv) the sequence obtained did not extend sufficiently far beyond the polymorphism in both directions to allow primer design. Sixty-five of these 83 assays targeted sites diagnostic between *O. mykiss* and all cutthroat trout subspecies in the ascertainment panel (Table 4). Others targeted sites with fixed differences between *O. mykiss* and all but coastal cutthroat trout (n = 8), westslope cutthroat trout (n = 5), Lahontan cutthroat trout (n = 1, owing to a third fixed alternative base in this subpecies) or all but Lahontan and westslope cutthroat trout (n = 1). We also designed two assays for sites that were diagnostic only between *O. mykiss* and Lahontan or coastal cutthroat trout, and one assay diagnostic only for Lahontan, coastal and westslope cutthroat trout, again owing to a third base that was fixed in the other subspecies. Two assays were rejected following testing, as they failed to distinguish heterozygotes from one of the homozygote classes. The final panel of 81 assayed polymorphisms comprised 75 single-base differences, one 2-bp difference and five indels (Table 5). Eighty per cent of these polymorphisms were in loci for which a putative function was identified from Blast searches (Supplementary Table S1).

Genotyping results for the validation panel are provided in Table 4 and in Supplementary files. As results did not differ between the closely related Yellowstone, Bonneville, greenback, Colorado River and Rio Grande cutthroat trout, we discuss these subspecies collectively as the 'Yellowstone group'. Similarly, we discuss Lahontan and Paiute cutthroat trout collectively as the 'Lahontan group'. The genotype call rate was very high, with the exception of two assays expected *a priori* to fail in specific taxa owing to a third alternative base present at the SNP site (Ocl_97077D and Ocl_102567D) and a third assay (Ocl_125998D) which amplified poorly in some populations. Forty-five (55.6%) of the loci were completely fixed between the taxa that they were designed to discriminate. An additional 17 (21%) exhibited one or two allele calls from the alternative group in the validation panel. One locus (Ocl_117742D) was polymorphic in all four *O. mykiss* groups. Over all loci, we observed the greatest amount of variation within coastal cutthroat trout (H_e = 0.045) followed by Columbia River redband trout (H_e = 0.032), Great

Table 2 *Oncorhynchus mykiss* and *Oncorhynchus clarkii* validation panels. (S) indicates a population known to be naturally sympatric with the alternative species

Taxon	Drainage/Location	Population	*n*
Hatchery rainbow trout	American River Hatchery	Eagle Lake Strain	6
O. mykiss	Fillmore Hatchery	Coleman strain	4
	Fillmore Hatchery	Shasta Strain	4
	Fillmore Hatchery	Virginia Strain	4
	Fillmore Hatchery	Whitney Strain	4
	Fillmore Hatchery	Wyoming Strain	4
	Hot Creek Hatchery	Kamloops Strain	4
	New Zealand	Lake Taupo	4
Steelhead (Northern)	Columbia River	Clackamas River (S)	3
O. mykiss	Columbia River	Columbia River (S)	3
	Columbia River	Lemhi River (S)	3
	Columbia River	Washougal River (S)	4
	Eel River	MF Eel River (S)	7
	Klamath River	SF Trinity River (S)	4
	Mad River	Mad River (S)	4
	Russian River	Dry Creek (S)	4
	Russian River	EF Russian River (S)	4
Steelhead (Southern)	Big Creek	Big Creek	6
O. mykiss	Feather River Hatchery	Hatchery stock	5
	Guadalupe River	Guadalupe Creek	4
	Sacramento River	Yuba River	4
	Santa Ynez River	Hilton Creek	5
	Scott Creek	Big Creek	4
	Soquel Creek	Soquel Creek	4
Great Basin redband trout	Chewaucan Basin	Chewaucan River	3
O. m. newberri	Goose Lake Basin	Drews Creek	3
	Upper Klamath Lake	Leonard Creek	2
	Upper Klamath Lake	NF Sprague River	3
Columbia River redband trout	Kootenai River	Yahk River	2
O. m. gairdneri	Kootenai River	Kilbrennan Lake (S)	2
	Salmon River	Wilson Creek (S)	3
Coastal cutthroat trout	Columbia River	Abernathy Creek (S)	4
O. clarkii clarkii	Columbia River	SF Mill Creek (S)	4
	Humboldt Bay	Jolly Giant Creek (S)	4
	Little River	Carson Creek (S)	4
	Mad River	Maple Creek (S)	3
	Margaret Lake, AK	Margaret Lake (S)	1
	Redwood Creek	Prairie Creek (S)	7
Westslope cutthroat trout	Blackfoot River	McCabe Creek	6
O. c. lewisi	Clark Fork River	Flat Creek	4
	Clark Fork River	Granite Creek	2
	Clark Fork River	Schwartz Creek	3
	Kootenai River	McGuire Creek	4
	Kootenai River	Trout Creek	4
	Salmon River	Cache Creek	4
	Salmon River	Wilson Creek (S)	2
Lahontan cutthroat trout	Humboldt River	Foreman Creek	4
O. c. henshawi	Humboldt River	Frazer Creek	2
	Humboldt River	North Fork	2
	Humboldt River	West Mary River	3
	Independence Lake, CA	Independence Lake	7
	Lahontan NF Hatchery	Hatchery Stock	4
	Pilot Peak, UT	Pilot Peak	3
	Pyramid Lake, NV	Pyramid Lake	5
	Quinn River	Line Creek	2
	Quinn River	Washburn Creek	3

Table 2 (Continued)

Taxon	Drainage/Location	Population	*n*
	Summit Lake, NV	Summit Lake	3
Paiute cutthroat trout	Cottonwood Creek	NF Cottonwood Ck	6
O. c. seleneris	San Joaquin River	Stairway Creek	6
Bonneville cutthroat trout	Glenwood Hatchery	Hatchery stock	9
O. c. utah	Bear River	Smith's Fork	1
Yellowstone cutthroat trout	LeHardy Rapids Hatchery	Hatchery stock	4
O. c. bouvieri	Snake River	Salt River	1
	Yellowstone Lake	Hatchery Creek	2
	Yellowstone River	McBride Lake	1
	Snake River	Barnes Creek	6
	Portneuf River	Gibson Jack Creek	4
Colorado River cutthroat trout	Colorado River	SF Parachute Creek	2
O. c. pleuriticus	Colorado River	SF Slater Creek	4
	Lake Nanita, CO	Lake Nanita	2
	San Juan River	Navajo Creek	1
	South Platte River	Hunter's Creek	3
	Williamson Lake, CA	Williamson Lake	3
Greenback cutthroat trout	Arkansas River	Bear Creek	2
O. c. stomias	Arkansas River	Severy Creek	3
	Colorado River	Bobtail Creek	1
	Colorado River	Cunningham Creek	4
	Gunnison River	W Antelope Creek	2
Rio Grande cutthroat trout	Canadian River	McCrystal Creek	3
O. c. virginalis	Rio Grande	Columbine Creek	3
	Rio Grande	El Rito Creek	4
	Rio Grande	Osier Creek	3
	Rio Grande	U Comanche Creek	2
	Rio Mora	Pecos River	2
F1 *O. mykiss* x *O. c. henshawi*			5
F1 *O. mykiss* x *O. c. clarkii*			5
Total			308

Table 3 Summary of *O. clarkii* sequencing effort

	Total	Mean (range) per locus
EST loci sequenced	173	
Base pairs sequenced	2 079 418	
Length of consensus sequence	88 475	511 (220–1261)
Individuals sequenced		23.5 (10–25)
Observed substitutions (bp)	1860	10.75 (0–43)
Sites with 3 alternative bases	12	
Transitions (C-T or A-G)	995	
Transversions (C-A, C-G, T-A or T-G)	877	
Insertion/deletion (bp)	1751	10.12 (1–187)
Insertion/deletion (*n*)	231	1.34 (0–9)
Short tandem repeat (*n*)	10	0.06 (0–2)
Fixed differences between *O. clarkii* and *O. mykiss*	286	1.65 (0–8)

Basin redband trout (H_e = 0.021), westslope cutthroat trout (H_e = 0.015), northern steelhead (H_e = 0.009), hatchery rainbow trout (H_e = 0.008), southern steelhead (H_e = 0.006), the Lahontan group (H_e = 0.002) and the Yellowstone group (H_e = 0.002). In some cases, polymorphism was largely owing to alternative genotypes at high frequencies in specific populations. For example, the '*O. clarkii*' (C) diagnostic alleles observed at Ocl_110201D and Ocl_117370D in the rainbow trout populations were completely restricted

Table 4 Summary genotyping results for validation panel

		Coastal cutthroat		West slope cutthroat		Lahontan group		Yellowstone group		Hatchery rainbow		Southern steelhead		Northern steelhead		Columbia River RB		Great Basin RB		
Locus	*A priori* not diagnostic	*n*	C%	*n*	C%	*n*	C%	*n*	C%	*n*	C%	*n*	C%	*n*	C%	n	C%	*n*	C%	NC%
Ocl 96127D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	1.0
Ocl 96222D	WCT	27	**100**	29	0	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 96500D		27	98.1	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 96899D		27	**100**	29	**100**	52	99.0	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 97077D	YSG	27	**100**	29	**100**	52	**100**	73	F	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	1.0
Ocl 97954D		27	**100**	29	69.0	52	**100**	73	**100**	34	**0**	32	**0**	36	1.4	7	14.3	11	31.8	0.0
Ocl 98409D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 98683D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 100771D		27	**100**	29	**100**	52	**100**	73	95.8	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 100884D	CCT	27	81.5	29	93.1	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 100974D	WCT/YSG	27	**100**	29	0	52	**100**	73	0	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 101506D		27	**100**	29	**100**	52	**100**	73	99.3	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 101704D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 102195D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	9.1	0.0
Ocl 102267D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 102414D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 102420D		27	98.1	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 102505D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 102567D	LCT	26	**100**	29	**100**	52	F	73	99.3	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 103122D	WCT/YSG	27	**100**	29	0	52	**100**	73	0	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.7
Ocl 103350D	WCT	27	**100**	29	0	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 104216D		27	**100**	29	**100**	52	**100**	73	96.6	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 104519D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	1.7
Ocl 105115D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	28.6	11	**0**	0.3
Ocl 105714D	WCT	27	**100**	29	0	52	99.0	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 105768D	WCT/LCT	27	**100**	29	0	52	0	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 106313D		27	96.3	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 106419D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 106457D	CCT	20	50.0	29	79.3	52	**100**	68	**100**	32	**0**	22	**0**	35	**0**	7	**0**	9	**0**	0.0
Ocl 106479D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 106747D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 107031D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 107074D	WCT	27	**100**	29	0	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 107336D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 107607D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 108007D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 108505D		27	**100**	29	**100**	52	99.0	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 108820D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 109568D	CCT	27	66.7	29	98.2	52	**100**	73	**100**	34	**0**	32	6.5	36	6.9	7	28.6	11	**0**	1.0
Ocl 109693D		20	**100**	28	**100**	37	98.6	43	**100**	34	**0**	22	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 109874D		27	**100**	29	**100**	52	99.0	73	**100**	34	**0**	32	7.8	36	**0**	7	**0**	11	**0**	0.7
Ocl 110201D		27	**100**	29	**100**	52	**100**	73	99.3	34	9.7	32	**0**	36	2.8	7	**0**	11	5.0	2.1
Ocl 110362D		27	98.1	29	98.3	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 110495D	WCT	27	**100**	29	0	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 110571D		27	**100**	29	**100**	52	99.0	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 111084D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	1.4	7	**0**	11	**0**	0.0
Ocl 111312D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 111383D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	1.6	36	13.9	7	33.3	11	22.7	0.3
Ocl 111681D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 112208D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	1.4
Ocl 112419D		26	55.0	29	**100**	52	**100**	68	**100**	32	**0**	22	**0**	35	2.9	7	**0**	9	**0**	0.0

Table 4 (Continued)

Locus	*A priori* not diagnostic	Coastal cutthroat		West slope cutthroat		Lahontan group		Yellowstone group		Hatchery rainbow		Southern steelhead		Northern steelhead		Columbia River RB		Great Basin RB		
		n	C%	*n*	C%	*n*	C%	*n*	C%	*n*	C%	*n*	C%	*n*	C%	n	C%	*n*	C%	NC%
Ocl 112669D		27	98.1	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	1.0
Ocl 112876D	CCT	20	45.0	29	**100**	52	**100**	68	**100**	32	**0**	22	**0**	35	**0**	7	**0**	9	**0**	0.4
Ocl 113979D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 114250D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 114448D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 117144D		27	**100**	29	**100**	52	**100**	73	99.3	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 117370D		27	**100**	29	**100**	52	**100**	73	**100**	34	16.2	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 117432D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	13.6	0.0
Ocl 117742D		27	**100**	29	**100**	37	**100**	73	**100**	34	13.6	22	6.8	36	6.9	7	25.0	11	27.3	1.9
Ocl 117815D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 118175D		27	98.1	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	2.8	7	**0**	11	**0**	0.0
Ocl 118654D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 118938D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	4.7	36	**0**	7	16.7	11	**0**	0.3
Ocl 119108D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 123044D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 123048D		27	98.1	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 123470D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	1.4
Ocl 125998D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	15.2
Ocl 126160D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 127510D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 127556D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.3
Ocl 128693D	CCT	20	17.5	29	84	52	**100**	68	**100**	32	**0**	22	**0**	35	**0**	7	**0**	9	**0**	1.5
Ocl 129144D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 129458D		27	96.3	29	**100**	52	**100**	73	**100**	34	**0**	32		36	**0**	7	**0**	11	**0**	0.7
Ocl 129870D	CCT	20	82.4	29	**100**	52	**100**	68	**100**	32	**0**	22	**0**	35	**0**	7	28.6	9	**0**	1.1
Ocl 130295D	CCT	20	55.0	29	**100**	52	**100**	68	**100**	32	**0**	22	**0**	35	**0**	7	**0**	9	**0**	0.0
Ocl 130720D		26	**100**	28	**100**	32	**100**	43	**100**	34	**0**	22	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 131460D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0
Ocl 131785D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	2.4
Ocl 131802D		27	**100**	29	**100**	52	**100**	73	**100**	34	**0**	32	**0**	36	**0**	7	**0**	11	**0**	0.0

N, sample size; C%, percentage of called alleles that are 'cutthroat diagnostic'; NC%, total percentage of individuals whose genotypes were not called, excluding loci with a known, third nonassayed base in specific groups (F). '*A priori* not diagnostic' indicates taxa for which loci were not expected to be diagnostic based on sequence information from the ascertainment panel. Sample sizes for some loci were reduced owing to failure of an assay in an entire Fluidigm plate for reasons unconnected with the overall performance of the marker.

Instances where a locus exhibited no variation in a taxon are indicated in bold.

CCT, coastal cutthroat trout; WCT, westslope cutthroat trout; LCT, Lahontan cutthroat trout; YSG, Yellowstone group.

to the Eagle Lake population, while the only C alleles in the *O. mykiss* sample at Ocl_105115D and Ocl_129870D occurred in Columbia River redband trout from the Yahk River. Similarly, the '*O. mykiss*' (M) diagnostic alleles in the Yellowstone group at Ocl_100771D and Ocl_104216D were found only in Cunningham Creek and McCrystal Creek, respectively, populations that exhibited C alleles at all other loci. Alternative alleles in the validation samples were distributed across multiple individuals. All F1 *O. mykiss* x *O. c. henshawi* individuals had the expected heterozygote genotypes; occurrence of homozygotes in F1 *O. mykiss* x *O. c. clarkii* individuals appeared to be owing to polymorphism in the parents (Supplementary Table S2).

For the Ryan Creek and Little River test samples, we omitted eight SNPs that exhibited high levels of polymorphism in coastal cutthroat trout (Supplementary Table S2). With the remaining results, we classified 77 individuals as pure *O. mykiss*; 46 of these contained no C allele copies while the remainder exhibited one (26) or two copies (5; Fig. 1). The remaining individuals

Table 5 Name, NCBI dbSNP submission number (ss#), polymorphism (C: *Oncorhynchus clarkii*/M: *Oncorhynchus mykiss*), forward and reverse amplification primers and labelled TaqMan probes for the 81 markers described in this study

Assay	NCBI_ss#	Target (C/M)	Primers (5′-3′)	Probes (5′-3′)
Ocl96127D	469275265	T/G	F: AGTGGTAATCAGTGAATGTGTTGCT	VIC: AGGCCTTGGTAGAGATA
			R: GGCTGCCTACCACTTAGGG	FAM: AGGCCTTGGTAGAGCTA
Ocl96222D	469275268	*/GAA	F: CAGCCTGAAATGAATGGTGAACAAA	VIC: TGTGGTAACCTATTAAGCC
			R: TCAAGAACATGATCACAGGGACATC	FAM: TGGTAACCTATTCTTAAGCC
Ocl96500D	469275270	A/G	F: GCATCATAAAAACATCTTTCTGTACATCGT	VIC: TCGCCCATAGTCCTGTGGT
			R: GACTCCAGAAAGCAGTAGAAGAAAATAAAT	FAM: CGCCCATAGTCCTGCGGT
Ocl96899D	469275272	A/T	F: CACCTCTACACCAGTCTGAATGTTT	VIC: TCCCGAAGTATCTTCTTCCTCAGA
			R: CCCACACCACATAGTCATGAAGT	FAM: TCCCGAAGTATCATCTTCCTCAGA
Ocl97077D	469275274	G/A	F: CACCATTGCCACTTCTCACAAAT	VIC: CATAACTGGAGCGTTAAG
			R: ATCTTGGAAATGTATGGGTACCTGAAC	FAM: ATCATAACTGGAGTGTTAAG
Ocl97954D	469275276	G/A	F: TGGTCCTCAGGCCTCAGA	VIC: CCGCCACCCCACCGG
			R: CGGCACCTGCCTGGA	FAM: CCGCCACCCTACCGG
Ocl98409D	469275278	T/G	F: GTCGAAGCACGCCAATGAG	VIC: AGCATCAGCTTTACTGCGTA
			R: TGACTACATAATTCTACTCTGTGATGAAAGA	FAM: AGCATCAGCTTTCCTGCGTA
Ocl98683D	469275280	G/A	F: CCACCTGCTGGAGGATACG	VIC: ATCCCCGTCCCCAGCAA
			R: CACCTCAGCTTTCATTAGAGCACTA	FAM: ATCCCTGTCCCCAGCAA
Ocl100771D	469275282	T/C	F: CTGTTTTAAGAACCAAACGGAGCAA	VIC: TGTTGCAAAACATTTCTTA
			R: GGGTGTATTCATTCCGCCAATTC	FAM: TGTTGCAAAACGTTTCTTA
Ocl100884D	469275284	T/G	F: TCCCTTTAATTCTCTTGGACAATGCT	VIC: ATTGTCATGATCTGTCAGTCAGTTTG
			R: TCTCCCCAACGTAAGTGAAGGA	FAM: ATTGTCATGATCTGTCAGTC CGTTTG
Ocl100974D	469275286	C/T	F: TGAACGGAGATCCTGCAACAC	VIC: CATTTTTCTGCCATACTGTAAT
			R: CATGTAGTGCAAGGCCTCTCA	FAM: TTTCTGCCATGCTGTAAT
Ocl101506D	469275288	C/G	F: GCAGTGCAAGTTCTCAAATGGT	VIC: ATTGTATTAACAGACACTTTT
			R: GATAGAACATCACTATTTAAAACTTTCA GGGATACA	FAM: TTGTATTAACACACACTTTT
Ocl101704D	469275290	A/G	F: GTGTGGTCAGCGGTGAGA	VIC: ACCCCGCCTCATCAT
			R: CTAGTGGAGGAGATCAAGAGAAGGA	FAM: ACCCCGCCTCACCAT
Ocl102195D	469275292	T/C	F: GCCCACCGGAGACATTGTTAT	VIC: CAGGCTCCAAGCTGT
			R: CTCCCTTTCCCTGTAGCTTCTG	FAM: CAGGCTCCGAGCTGT
Ocl102267D	469275294	T/C	F: TGGTAGAGCATGGTGCTTACAAC	VIC: TTCGTACTGATCCCCC
			R: CCGCTAGCACAAGTTACTTTTTCC	FAM: TCGTACTGGTCCCCC
Ocl102414D	469275296	A/G	F: TGTGCACACAGTGTAGCTCTACTA	VIC: TGTCCATTACTGTCATACAT
			R: ACTCAGGTGTGTAACTAAGATTGCATTT	FAM: TCCATTACTGCCATACAT
Ocl102420D	469275298	C/A	F: CAATGGCATCAGGTAACACGTTT	VIC: CACACTGTTGACTTGAAAT
			R: CATCATGATGTAGCCCTGTTTGC	FAM: ACACTGTTGACTTTAAAT
Ocl102505D	469275300	T/C	F: GTTCTGTTACTCTAAGCTCTGCCA	VIC: ATAGCCTCAGAAGTATCT
			R: GCATCTTTTTGCTACCATGTCAGTT	FAM: TAGCCTCAGAGGTATCT
Ocl102567D	469275302	C/T	F: TGCAACAATCAAGGATATCTGAGCTT	VIC: ACAATCACGGATGTTGAG
			R: TTTCTTCGAAACTGACTTGCCTAGTT	FAM: CAATCACGGATGTTAAG
Ocl103122D	469275304	G/T	F: CCTCATTACTTGGTACTGTTTTATTTTATT GTTGT	VIC: TTCCTTTACAACAATTACA
			R: ACAATCTGCAGAAGAATTGAGTCGTA	FAM: TTTTCCTTTACAACCATTACA
Ocl103350D	469275306	A/T	F: AGAACCTGTATGCAATAATCTAATGAGCAA	VIC: CCACCTTGTGCAAGTC
			R: GCAATAGGCAGATTATGGCCCATTA	FAM: CCACCTTGAGCAAGTC
Ocl104216D	469275308	C/A	F: TGCCTTCCATCAAGAATGCCATT	VIC: ACAACCCAATACTTTCT
			R: GGTCTCGCCATGTTTGTAGAAC	FAM: AACCCAAGACTTTCT
Ocl104519D	469275310	A/G	F: TGGAGAGCAGGTAAAGGGTCT	VIC: TGTAAACGTGTGAGTTTGCGGTAA
			R: TGTTTGTCCTGTAATTGTGTATTCCA	FAM: TGTAAACGTGTGAGTTTGCAGTAA
Ocl105115D	469275312	ATCGA/*	F: GTACGATAATTATGAATGTCAGCGTTGAG	VIC: CATGTAGCTAGCTATTCAT
			R: AACCTTTAAGGTATTTCTATGAGCGTGTA	FAM: TGACATGTAGCATTCAT
Ocl105714D	469275314	T/A	F: CCAGTGTGGGCACCAAGAG	VIC: CCTGCCACAGAGGAG
			R: GAGTGGAGTCCTCTGTTTTCCTT	FAM: CCTGCCTCAGAGGAG
Ocl105768D	469275316	T/A	F: GTACAGGACTAGACCTCAGAGACA	VIC: ATGCTGAGTTGTAACACTA
			R: ATTGCAGAGGTTAACTATACAGTAGCT	FAM: ATGCTGAGTTGTTACACTA

Table 5 (Continued)

Assay	NCBI_ss#	Target (C/M)	Primers (5′-3′)	Probes (5′-3′)
Ocl106313D	469275318	A/C	F: GTCCCTGACATACGTTACTTACCAA	VIC: CCATTGGTTTGATTTTTCCAAA
			R: ATGTGGTGTTCTGTGTCTGAAGT	FAM: CATTGGTTTGAGTTTTCCAAA
Ocl106419D	469275320	C/T	F: TCCGTCAGCCGTGTGATT	VIC: CCAGCAAATGCC
			R: GGCAATACGGAGCTCTATGCT	FAM: CCAGCGAATGCC
Ocl106457D	469275322	C/T	F: TGAGAGAGAGAGGGATGTTACTTGAC	VIC: TGCTTCGAGAAGAGCA
			R: CCTCATGGCCCTGACAGAGA	FAM: TGCTTCGAAAAGAGCA
Ocl106479D	469275324	A/G	F: GCCGTTTGGGAGCTTTGTC	VIC: TCCGAACTCTGACTTTCGTGCTA
			R: CAAACACTACATGGATGCATCGAAA	FAM: TCCGAACTCTGACTTCCGTGCTA
Ocl106747D	469275326	C/A	F: CAGCACCAAAGGGAGGTACT	VIC: TAGGTTTTCAGAAGAATTAA
			R: TGCTCCTGCCTGCAAGAC	FAM: TAGGTTTTCAGAATAATTAA
Ocl107031D	469275328	T/C	F: CCACACAGGAGCAGAAAGACATTT	VIC: AGTTCACATCCAATGCC
			R: TGCCACAGTTCTCATTGAAACAGTA	FAM: TCACGTCCAATGCC
Ocl107074D	469275330	T/C	F: CTGCTGACAGGCCTGAGA	VIC: TTGACCCCAAACCACGC
			R: CCGGGCTGTCATGTGACT	FAM: TGACCCCAGACCACGC
Ocl107336D	469275332	C/G	F: CGGCTAGTGATGGGTGTTGT	VIC: CTGGAGCTAACGGAGCTA
			R: CTCTCACTCATGACATCAACTTCTG	FAM: CTGGAGCTAACCGAGCTA
Ocl107607D	469275334	A/G	F: TGAGACAACCCAAAGCTTTAAGGAA	VIC: CTATCAGATCACATTTAGGAGC
			R: CTCCAGCGATGTAGGCTACTC	FAM: TCAGATCACATCTAGGAGC
Ocl108007D	469275336	T/C	F: GGCACATGATGGCAAATGCTTTT	VIC: TTCCTTGCATCAGTCC
			R: CTAGGCACCAAACGGAAGAAAAC	FAM: TCCTTGCGTCAGTCC
Ocl108505D	469275338	C/T	F: CCACAGGTGAAGCCAGATGT	VIC: AGGCCGGTTGGACGTA
			R: AGCTGTTGGCAGAAAATTCAATGTT	FAM: AGGCCGGTTAGACGTA
Ocl108820D	469275340	C/A	F: CCTTAAAATATTCTAAGATGGGCAATTA TTCCAAAA	VIC: AAACTGTATCAAGGATAAAA
			R: TTTTGTTTGGATATTTGTATTTCTTTGGTTGG	FAM: AAACTGTATCAATGATAAAA
Ocl109568D	469275342	A/C	F: CATATAATTCAGTGATATTGACAGGAA	VIC: AGTCACTATTAAAGGAACACT
			R: TCACTGGACCAGGGTTCGA	FAM: AAGTCACTATTAAAGTAACACT
Ocl109693D	469275344	C/T	F: GGACTCTCTGACAACAACAGTTCTT	VIC: CAGATACCGAAGGGAC
			R: CATGGGAATGAGGGAGAGTTGTC	FAM: CAGATACCAAAGGGAC
Ocl109874D	469275346	/CATC	F: TTACAGAAATAGAATGGGTAGCTAAAC	VIC: ATGAATAGGCATTAATACAGGTAT
			R: CCCCTGACACTAACTTCTAATCCTAAATAC	FAM: TAGGCATTAATACAGGTAGGTAT
Ocl110201D	469275348	G/T	F: TCTGACTATTTTGATTGTTTGGCTATTGAA	VIC: TGGATCTATGTTGTTATTTACA
			R: TCCCGTTGCCCATGGC	FAM: TGGATCTATGTTGTTCTTTACA
Ocl110362D	469275350	C/T	F: AGGTCGAAATGTGATTAAGGTTGATCTT	VIC: CCCTATGTGGCTCTGAC
			R: GTGCCACCCTATACCCCATC	FAM: CCCTATGTGACTCTGAC
Ocl110495D	469275352	A/T	F: CTGAAAAGGTAAGTCATTATGCAACTGT	VIC: ACATGTAGTGCACACATAA
			R: CTTGCTGTGTCTGCAAATCAGAA	FAM: ATGTAGTGCACACAAAA
Ocl110571D	469275354	G/A	F: ACTATGAAGGAGGTGGCTCTGA	VIC: TTCCCTCAACTCCTCC
			R: CTGGTCGCCTCCTGAGTT	FAM: TCCCTCAATTCCTCC
Ocl111084D	469275356	AT/*	F: CCACGTCCTGGGAACCAA	VIC: ATCCAGGTAAGATAACTTTA
			R: AAGAAGAAAGGAAACCTGTCACGAT	FAM: TCCAGGTAAGAACTTTA
Ocl111312D	469275358	T/C	F: CCACTGGTTCTCATCCAATCAGA	VIC: CTTGAACCTGACATCCG
			R: GGCCATTACAGATGAGCTGGAG	FAM: TTGAACCTGGCATCCG
Ocl111383D	469275360	A/G	F: GCTGCAAGCTCCAAAATCTAGAGA	VIC: TGCCTAAAGACCTTTATCCA
			R: AATTCCCCTGACACGCTTGT	FAM: TGCCTAAAGACCTCTATCCA
Ocl111681D	469275362	A/G	F: GGCGTCCATCCCAGCAA	VIC: CCCGGCCATGTTC
			R: TGGAAACTACATTGTAATGGTGCATGA	FAM: TCCCGGTCATGTTC
Ocl112208D	469275364	G/T	F: ATCATTAATATAACGAGTTCTACTGACAT	VIC: CCAACTGTTGCCATCTT
			R: CACTGTCAGTCAGCATCTACGT	FAM: CAACTGTTGACATCTT
Ocl112419D	469275366	A/C	F: CCCTTCTACTGTGAGAGTCAATGTAAGT	VIC: CTCCTTACTAGCTCATGAAT
			R: TCGAAAGTCTGCATGAGTT	FAM: CTTACTAGCGCATGAAT
Ocl112669D	469275368	T/C	F: GGCCAGTTTTCAAAAGCATCTAAGT	VIC: TGTTTTAAACGACAATGTC
			R: CTGTCTGACATCACCATTTGAATCCT	FAM: TTTAAACGGCAATGTC
Ocl112876D	469275370	T/G	F: GGAGAAGAATCTTATGGATGTAGTTTCACT	VIC: CCTAATTTCTTACATACCCTCTC
			R: TGGGTTCATTGACTCACCTGTAAAA	FAM: AATTTCTTACATCCCCTCTC
Ocl113979D	469275372	A/G	F: AAACTGACCAGCACACTACTATACAC	VIC: CCTTCAGACAATGGAAAA

Table 5 (Continued)

Assay	NCBI_ss#	Target (C/M)	Primers (5′-3′)	Probes (5′-3′)
			R: GGGTCTTTGGCCATCGGTTT	FAM: CTTCAGACAACGGAAAA
Ocl114250D	469275374	C/A	F: GTAGGAGAGAAACCTGACAGTCATT	VIC: CTCCTTTGCATGTAGATAT
			R: TTCATTGCAATCTGACAAGTTGGTT	FAM: CTCCTTTGCATTTAGATAT
Ocl114448D	469275376	C/G	F: TGAAGCTTTTCTCTGGTCTTTGTCTT	VIC: TTGAGGATGAGGAACCT
			R: GCATTACACACCAATAGAGGGTACAC	FAM: TTGAGGATGACGAACCT
Ocl117144D	469275378	GA/CG	F: CCCACTGAGCAGCAGCAA	VIC: CCATGCCCCCCTCCT
			R: ACAATGCTGGCTGTAGTACATAGC	FAM: CCCATCTCCCCCTCCT
Ocl117370D	469275380	G/T	F: CGAGAAGGGAATTATGCAGAAGGTA	VIC: CATTGCTTCCAAATTG
			R: GCAAGTACGGAACAAATAAGCCATT	FAM: CATTGCTTACAAATTG
Ocl117432D	469275382	A/G	F: ACTCAACGCTGTGATCAACGA	VIC: AGGCGTTCTCCTTATAC
			R: CTGATGGGCCTGTCATGGT	FAM: AGGCGTTCTCCCTATAC
Ocl117742D	469275384	T/G	F: CCCTGACGTTCATTACACTGAAGTTAA	VIC: CTGAAAACAGATAAAAGTAC
			R: CACTAAGTGTGCAGAGAGTGCAA	FAM: CTGAAAACAGATACAAGTAC
Ocl117815D	469275386	C/T	F: ATGATGCAATGGTAGGACTTCTTGT	VIC: AGTACACTGCAGGTATAT
			R: AGCAAATGGTTAAGCTACATCAGGAT	FAM: AGTACACTGCAAGTATAT
Ocl118175D	469275388	C/T	F: TGTTCACCTGGACAAAGCATAGG	VIC: ACATGTGACATTGTCAAAA
			R: TCTGCAAGAGAGTGCATGTGT	FAM: ACATGTGACATTATCAAAA
Ocl118654D	469275390	G/T	F: GCCTCCCGGTCCTCAAC	VIC: AAGCTGGGACTGACTG
			R: CTTGAAGCTCATGTCCACATTGAC	FAM: AGCTGGGCCTGACTG
Ocl118938D	469275392	C/G	F: AGTGGAGTTTTTCACCAATGATAAATGC	VIC: ACATGTTAAATATTAAAATTGTCTC
			R: ATCTGTACAATATTCCATGGAAACCAAC	FAM: CATGTTAAATATTAAAATTCTCTC
Ocl119108D	469275394	T/A	F: CTGACCTGCCGCCTGTAT	VIC: CTGCTGGTTAACCTTC
			R: GGTTGCCTTTTTTACTGGAGAGACT	FAM: CTGCTGGTTTACCTTC
Ocl123044D	469275395	C/T	F: CCAGGTCGTCGGACACC	VIC: CAGCCTGGGAAGCT
			R: CCCACAGTGGCCTCCTT	FAM: AGCCTGGAAAGCT
Ocl123048D	469275397	A/T	F: CTGTGAGGTGCCATTGATCTGA	VIC: TTGTCTCCCTTCTAGCATAT
			R: ACAGTAGTGCAGCTTCACATACAG	FAM: TCTCCCTTCAAGCATAT
Ocl123470D	469275399	T/C	F: TGGATTTGTGCCCATGTCTCAT	VIC: TTGAGTGAACGAAAAGT
			R: CATTAAGGAGTTGATGATATATTAGCATGCTC	FAM: TTGAGTGAACGGAAAGT
Ocl125998D	469275401	G/T	F: AGGCCTAATCTGTCCACC	VIC: CAATTGATCTACTGACCTTC
			R: CTTGAAAGTACATTGCTTTTATAACTAGCTTATGT	FAM: TTGATCTACTGAACTTC
Ocl126160D	469275403	T/A	F: GTGTTGGTGAGCAAGATAATTGTGT	VIC: ACATGCATGAGGTTAAT
			R: AAAACACCTTTTGGTTTCTACTGTAAACAG	FAM: CATGCATGTGGTTAAT
Ocl127510D	469275405	T/G	F: TGATATTGTCAAAGGTAAACAACTTATTTCCCT	VIC: ATGCAGTTCTAAATATTGTAC
			R: GGTATCCTCTGTCTTTTTAACTTCCCCTAT	FAM: ATGCAGTTCTAAATCTTGTAC
Ocl127556D	469275407	G/C	F: TGAAATATGCTTTGGTGGTGAATGT	VIC: TCATGCAAATCCC
			R: ACCATCTCAATGTCATACCCTATCCT	FAM: TCATGGAAATCCC
Ocl128693D	469275409	A/G	F: GCCCTGGAGGAACACAACAA	VIC: CTTCATTCGGTTGGCCAA
			R: GTGCCTCCCGTTTCTCCTT	FAM: TCATTCGGCTGGCCAA
Ocl129144D	469275411	C/A	F: CACCCAGCCTGGCATCA	VIC: CCCACCTGGGTGATGT
			R: GGTTTAGTCCCGGCTTCGT	FAM: CCCACCTGTGTGATGT
Ocl129458D	469275413	A/C	F: CCTGCTCATGTCAACAAACTGATG	VIC: TGGAATTAACACTAGTTGATG
			R: GGCTTTCTGTTGACACCTGGAATAA	FAM: TGGAATTAACACTATTTGATG
Ocl129870D	469275415	G/T	F: AGATACTGTACACTGTATTAGCCTCAGTT	VIC: ACCATATCAACTGAGTATC
			R: CAGCCTGTCTCCCTTGTGT	FAM: ACCATATCAAATGAGTATC
Ocl130295D	469275417	G/A	F: TTGTTCATACTGTATGTCTTATGCCTTTTCT	VIC: CCTGCAAGCACTTAGTTT
			R: TGGACAGAATGTTCTACAAGTTGCA	FAM: CCTGCAAGCATTTAGTTT
Ocl130720D	469275419	*/ATT	F: GGAACTCCAGTACTAGGGAATAGGT	VIC: CCTCATTATAAACAAAACAAT
			R: ACCTGCATTGTGTGGTGGG	FAM: CTCATTATAAACAATAAAACAAT
Ocl131460D	469275421	T/C	F: CAAAATAGCCAGGGATGTAGAAGGA	VIC: CCTTGCTCAATTGTTC
			R: GAATGTAAGAATAGCAGTAACACACAGATTAT	FAM: CTTGCTCGATTGTTC
Ocl131785D	469275423	T/G	F: AGCCTTTTCCTTCAAGTATATGCC	VIC: AGATCTGTTATGCTTGAAAG
			R: TGAAGCATGTATAATGTGTCCTCTTTAGC	FAM: AGATCTGTTATGATTGAAAG
Ocl131802D	469275425	C/G	F: AGCCATGTTTTGTTCAGTTGTTGTG	VIC: TTGGAACTTAGTGTAACATT
			R: AGGAGTGCCTAGGGTGTAAGG	FAM: TTGGAACTTAGTCTAACATT

*indicates nucleotides absent.

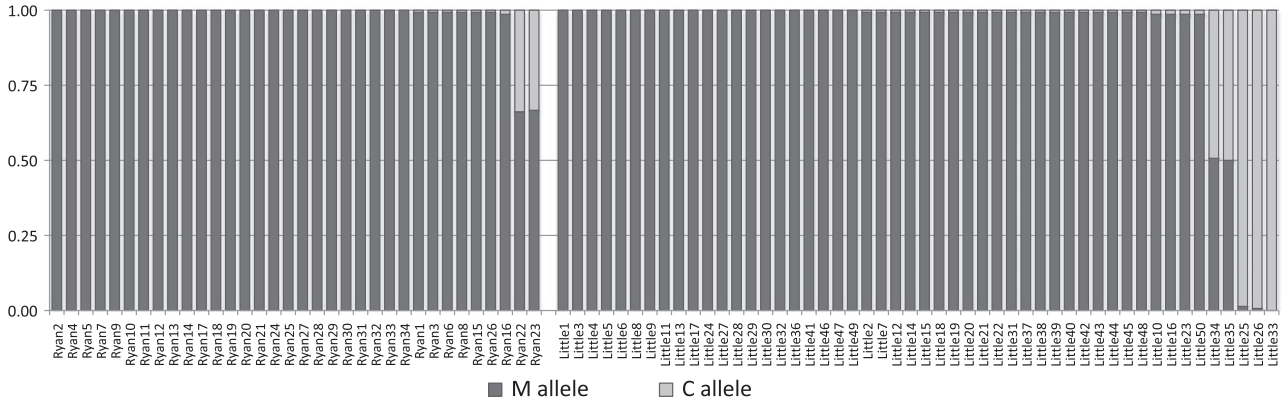

Fig. 1 Proportions of '*Oncorhynchus clarkii* diagnostic' (C) and '*O. mykiss* diagnostic' (M) alleles observed in samples of putative *O. mykiss* from Little River and Ryan Creek.

comprised three pure *O. clarkii* (two or fewer *M* alleles), two F1 hybrids and two later generation crosses that contained more C alleles than would be expected from a simple backcross.

Discussion

In this paper, we present 81 novel SNP and SNP-type assays to discriminate between *O. mykiss* and *O. clarkii*. These assays were designed and tested on a wider range of taxa than were those described previously, including all nine extant, recognized cutthroat subspecies. Our design approach, with no more than a single assay for each sequenced locus, will minimize linkage disequilibrium between the markers. Although the nature of the data set precluded statistical testing, 84% of the new markers are in genes for which *O. mykiss* or *O. tshawytscha* SNP assays have also been developed and are known to be in linkage equilibrium (Abadía-Cardoso *et al.* 2011; Clemento *et al.* 2011). Blast searches enabled identification of the codon (or noncoding) position of 65 of the novel polymorphisms; six were inferred to be substitutions that altered the amino acid composition of the protein produced. This information may aid in the interpretation of differential introgression patterns observed for these SNPs in hybrid zones between *O. mykiss* and *O. clarkii*.

Over half of the novel markers were completely fixed within the taxa between which they were designed to discriminate, with most of the others fixed except for one or two copies of the alternative allele. If we limit our analysis to the inland cutthroat trout subspecies and hatchery rainbow trout, the number of completely diagnostic loci increases to 61, with an additional 11 exhibiting just a single copy of the alternative allele. The observation of limited intra-taxon variability in these otherwise diagnostic markers is not surprising. The highest levels of polymorphism were observed in taxa where there is sympatry between *O. clarkii* and *O. mykiss* – coastal cutthroat, westslope cutthroat, interior redband and northern steelhead – and there is evidence for both historic and ongoing gene flow between them (Brown *et al.* 2004; Bettles *et al.* 2005; Kozfkay *et al.* 2007). In addition, the widely distributed coastal cutthroat trout may have maintained a larger effective population size over its evolutionary history than the interior cutthroat subspecies, allowing the retention of more ancestral polymorphism shared with *O. mykiss*. While the lower level of polymorphism observed in northern steelhead compared with coastal cutthroat trout may suggest asymmetric introgression, it could also be the result of ascertainment bias owing to the much larger steelhead sample in the ascertainment panels. Within the hatchery rainbow trout sample, most polymorphism in the markers was found in the Eagle Lake strain, which is believed to be related to redband trout from the Great Basin and may also contain genetic material from Lahontan cutthroat trout (Busack *et al.* 1980). Other domestic rainbow trout strains were found to be fixed for '*O. mykiss*' alleles at all but one locus, despite possible *O. clarkii* contributions to some of these strains (Busack & Gall 1980). Rare alternative ('*O. mykiss*') alleles appearing in Lahontan and Yellowstone group cutthroat trout validation populations may be remnants of historical introgression with introduced rainbow trout or reflect ancestral polymorphism or *de novo* mutation. A number of the populations included in the validation panel have a known history of transplantation or stocking with *O. mykiss* or other cutthroat trout subspecies (e.g. Metcalf *et al.* 2007). Although genotyping error may also have contributed to the appearance of rare alternative alleles, duplicate genotyping experiments (results not shown) found all SNP calls to be repeatable.

The 81 novel markers described here increase threefold the number of validated SNP and SNP-type assays currently available to discriminate the *O. mykiss* and *O. clarkii* genomes. These assays will provide a valuable tool for accurately assessing levels of introgression between cutthroat trout and steelhead/rainbow trout populations and for understanding the dynamics and evolutionary consequences of this introgression in both natural and artificially created hybrid zones between the two species. In addition, the large amount of sequence data generated as part of this project will facilitate the development of additional genetic markers to, for example, investigate hybridization within and between different *O. clarkii* subspecies and for population genetic investigations within cutthroat trout populations.

Acknowledgements

We are grateful to the following for providing DNA samples: Nate Campbell and Shawn Narum, Columbia River Inter-Tribal Fish Commission; Denise Hawkins and Catherine Sykes, US Fish and Wildlife Service; Andrew Kinziger, Humboldt State University; Gordon Luikart, University of Montana; Jessica Metcalf and Andrew Martin, University of Colorado; Kirk Patten, New Mexico Department of Game and Fish; Mary Peacock, University of Nevada, Reno; and Dennis Shiozawa, Brigham Young University. We also thank members of the Molecular Ecology and Genetic Analysis Team of the Southwest Fisheries Science Center, Lisa Heki, Mary Peacock, Tommy Williams and Christian Smith for valuable discussion and other assistance with this project. The US Fish and Wildlife Service (Lahontan National Fish Hatchery and Abernathy Fish Technology Center) and National Oceanic and Atmospheric Administration (Southwest Fisheries Science Center) provided funding for this work.

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This work is part of a larger effort to discover, develop and apply SNP markers for use in population and ecological genetic investigations of anadromous and marine fishes present in the northeastern Pacific Ocean and its tributaries. VLP led the cutthroat trout component of this work and AA-C led the steelhead/rainbow trout component. JCG initiated and supervised these efforts. VLP, AAC and JCG performed the research and wrote the paper.

Data accessibility

DNA sequences: NCBI dbSNP accessions between ss469275265-ss469275425 (nonconsecutive see Table 5). Genotype data: Supplemental Table S2. Dryad identifier for the data is: doi:10.5061/dryad.3v8c8j49.

Supporting information

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

Fig. S1 Proportions of □ *O. clarkii* diagnostic and ■ *O. mykiss* diagnostic alleles observed in validation samples. Bars not summing to one indicate 'no call' individuals.

Table S1 Putative functions of loci containing SNPs and SNP location with respect to gene features. Superscripts indicate species in which the best Blast match was found: [1]*O. mykiss*; [2]*Salmo salar*; [3]*Danio rerio*; [4]other. Only matches with an E-value ≤1e-[15] are shown. Location abbreviations refer to the following: UTR: untranslated region; CDS: protein-coding sequence; ss: synonymous polymorphism; ns: nonsynonymous polymorphism. Amino acid codes show changes generated by nonsynonymous substitutions.

Table S2 SNP calls for all samples. C: homozygous *O. clarkii* diagnostic; M: homozygous *O. mykiss* diagnostic; H: heterozygote; NC: no call; nr: assay not run or failed over entire Fluidigm plate.

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