

Development and evaluation of 200 novel SNP assays for population genetic studies of westslope cutthroat trout and genetic identification of related taxa

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Abstract

DNA sequence data were collected and screened for single nucleotide polymorphisms (SNPs) in westslope cutthroat trout (*Oncorhynchus clarki lewisi*) and also for substitutions that could be used to genetically discriminate rainbow trout (*O. mykiss*) and cutthroat trout, as well as several cutthroat trout subspecies. In total, 260 expressed sequence tag-derived loci were sequenced and allelic discrimination genotyping assays developed from 217 of the variable sites. Another 50 putative SNPs in westslope cutthroat trout were identified by restriction-site-associated DNA sequencing, and seven of these were developed into assays. Twelve *O. mykiss* SNP assays that were variable within westslope cutthroat trout and 12 previously published SNP assays were also included in downstream testing. A total of 241 assays were tested on six westslope cutthroat trout populations ($N = 32$ per population), as well as collections of four other cutthroat trout subspecies and a population of rainbow trout. All assays were evaluated for reliability and deviation from Hardy–Weinberg and linkage equilibria. Poorly performing and duplicate assays were removed from the data set, and the remaining 200 assays were used in tests of population differentiation. The remaining markers easily distinguished the various subspecies tested, as evidenced by mean G_{ST} of 0.74. A smaller subset of the markers ($N = 86$; average $G_{ST} = 0.40$) was useful for distinguishing the six populations of westslope cutthroat trout. This study increases by an order of magnitude the number of genetic markers available for the study of westslope cutthroat trout and closely related taxa and includes many markers in genes (developed from ESTs).

Keywords: admixture, hybridization, KASPar, *Oncorhynchus clarki*, population genomics, rainbow trout, SNP, Taqman, westslope cutthroat trout

Received 27 January 2012; revision received 9 April 2012; accepted 22 April 2012

Introduction

Cutthroat trout (*Oncorhynchus clarki*) are native to cold water environments in western North America from Alaska to New Mexico, USA. In the late Miocene and early Pliocene, cutthroat trout spread throughout portions of western North America via paleohydrological connections influenced by orogeny and climate (Smith *et al.* 2002). Subsequent isolation in major river basins led to the evolution of up to 14 subspecies that are geographically, phenotypically and genetically distinct (Behnke 2002). The coastal subspecies (*O. c. clarki*) inhabits lakes

and streams west of the Cascade crest and includes both resident and anadromous populations (Behnke 1992). The remaining three major lineages (westslope, Yellowstone group and Lahontan group) are found in streams and lakes of the Rocky Mountains on both sides of the continental divide (Behnke 1992; Wilson & Turner 2009).

All cutthroat trout are sensitive to changes in their environment, and human impact on water temperature and quality has caused the decline of many populations (Shepard *et al.* 2005). Introductions of non-native species to their habitat have also supplanted native cutthroat trout populations by way of competition for resources, predation and interbreeding (Griffith 1988; Young 1995; Ruzycki *et al.* 2003; Muhlfeld *et al.* 2009). These effects

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have led to the extinction of two cutthroat subspecies (*O. c. alvordensis* and *O. c. macdonaldi*) and another three subspecies, Lahontan (*O. c. henshawi*), Paiute (*O. c. seleniris*) and Greenback (*O. c. stomias*) cutthroat trout are listed as threatened under the US Endangered Species Act (ESA). Moreover, declining populations of westslope (*O. c. lewisi*), Yellowstone (*O. c. bouvieri*), Bonneville (*O. c. utah*), Colorado (*O. c. pleuriticus*) and Rio Grande (*O. c. virginalis*) cutthroat trout subspecies are of conservation concern although not currently protected under the ESA.

The most widely distributed of the cutthroat trout subspecies is the westslope cutthroat trout (Behnke 2002). Historically, this subspecies ranged from the Rocky Mountains of southern Canada in the north to central Idaho in the south (both east and west of the continental divide) with isolated populations in the eastern Cascade Range of British Columbia, Washington and Oregon (Behnke 1992). However, recent estimates have revealed that genetically pure westslope cutthroat trout now inhabit only 25% of their native range (COSEWIC 2006; May 2009). Despite these dismal statistics, many populations of this subspecies still persist across a wide geographical area suggesting that a great deal of genetic diversity still exists. Genetic tools to better understand the genetic relationships between westslope cutthroat trout populations and their habitats could be an important tool in their conservation and are the main focus of this study.

Genetic analyses within cutthroat trout subspecies using microsatellite markers have revealed that populations are often highly genetically distinct from one another, even within the same drainage (e.g. Taylor *et al.* 2003; Wofford *et al.* 2005; Drinan *et al.* 2011). These substantial genetic differences are attributable to physical barriers to fish passage that reduce gene flow, and relatively small breeding populations with high rates of genetic drift (Waples *et al.* 2001; Fausch *et al.* 2009; Young 2011). Human impacts on fish passage and water quality have likely led directly to the isolation of wild cutthroat to headwater streams (Fausch *et al.* 2009) and, in many streams, introduction of rainbow trout and subsequent hybridization has further isolated pure cutthroat trout to small tributary streams.

Rainbow trout and cutthroat trout are sister species, and hybridization between them is common when they share habitat. Hybridization with introduced rainbow trout has been described as the primary threat to the survival of many cutthroat trout subspecies because of diminished fitness and reproductive success of hybrids and their offspring (Leary *et al.* 1985; Allendorf & Leary 1988; Muhlfeld *et al.* 2009). Mechanisms for reduced fitness may include genetic incompatibilities such as chromosomal rearrangements, possible differences in the

numbers of chromosomes (rainbow trout: $N = 58-64$; cutthroat trout: $N = 64-68$) and the disruption of co-adapted gene complexes, which contribute to outbreeding depression (Leary *et al.* 1985). Despite these potentially deleterious effects, rainbow trout DNA can spread rapidly through a cutthroat trout population because of the relatively high survival and reproductive success of first generation (F1) hybrids (Muhlfeld *et al.* 2009). Owing to the difficulty in the phenotypic identification of hybrids, molecular and genetic markers such as allozymes, AFLPs and microsatellites have been used to study hybridization (Allendorf & Leary 1988; Hitt *et al.* 2003; Pritchard *et al.* 2007). More recently, however, diagnostic single nucleotide polymorphisms (SNP) loci have become available for detection of invasive rainbow trout DNA (Kalinowski *et al.* 2010; Hohenlohe *et al.* 2011; Pritchard *et al.* 2012).

In this study, we identified variation in cutthroat trout DNA sequence data and developed assays for SNPs in westslope cutthroat trout and for substitutions that distinguish westslope cutthroat trout from other cutthroat trout subspecies and from rainbow trout. Variable sites were identified by sequence alignment and assays were developed for the most polymorphic loci, focusing on SNPs that were variable within westslope cutthroat trout or substitutions diagnostic between taxa. Another seven SNP loci were developed based on restriction-site-associated DNA (RAD) sequence data generated using rainbow and cutthroat trout samples (Hohenlohe *et al.* 2011). All assays were evaluated by genotyping a set of cutthroat trout samples from 11 populations and a single rainbow trout population. Assays with poor plot quality (i.e. genotype resolution) and significant deviations from Hardy-Weinberg proportions were removed from the data set. The remaining assays were evaluated for their ability to distinguish populations of westslope cutthroat trout and identify cutthroat trout subspecies and hybrids with rainbow trout.

Methods

SNP discovery: ascertainment panel 1

Salmonid DNA sequences, mostly from mRNA transcripts, were collected from online database entries in TIGR and NCBI. Sequences containing 300+ bases of 3-prime un-translated region (UTR) were chosen preferentially as these tend to contain more variation than coding sequence. Primers were designed using either PRIMER3 (<http://frodo.wi.mit.edu/primer3/>) or the embedded primer design tool in NCBI's database to target gene regions 400–800 bp in length. All primer pairs ($N = 163$) were initially tested for amplification of a single product using DNA from two westslope cutthroat

trout. PCR conditions were 1× multiplex master mix (Qiagen, Valencia, CA, USA), 250 nM primers and 1–2 µL of extract DNA in a 10 µL reaction. Thermal cycling conditions were [95 °C for 15 min (95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, repeated 40 cycles) and 4 °C hold]. Amplified products were size separated on a 1% agarose gel and stained using SYBR-safe reagent (Life Technologies, Grand Island, NY, USA). Primer pairs that produced clean PCR products ($N = 134$) were used to screen an ascertainment panel of 63 individuals (Table S3).

Westslope cutthroat trout were the best represented subspecies in the ascertainment panel, and samples were chosen from 48 collections from 27 different drainages in Idaho, Montana and British Columbia. Another two samples each of Yellowstone, Bonneville and coastal cutthroat trout were included in the ascertainment panel as well as one doubled haploid rainbow trout (Table S3). Prior to PCR, all DNA extracts were quantified and individuals from the same drainage/collection were duplexed (two individual extracts combined into one aliquot) to increase the number of screened genomes while keeping sequencing costs low.

Ascertainment samples were then PCR amplified using multiplexed primers such that each reaction produced four products and with the same PCR and cycling conditions as in the initial amplification test. PCR products were cleaned by treatment with Exonuclease 1 and shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA, USA) to remove unincorporated dNTPs and primers. Sequencing reactions were performed in one direction using the Big Dye v.1.1 sequencing kit (Life Technologies) and with 2 µL of each product (as the sequencing primers are different for each amplicon, there is no need to separate them prior to sequencing). The reaction products were purified by ethanol-EDTA-NaAcetate precipitation prior to sequencing by capillary electrophoresis using a 3730 automated DNA Analyzer instrument (Life Technologies). Chromatogram data were analysed with Sequencing Analysis v.5.4 (Life Technologies), and Sequencher v.4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) was used to align and edit the data. Observed SNPs and small indels were recorded and minor allele frequencies calculated for each. Sequences containing sites chosen for assay design were submitted to dbSNP (Tables S1 and S2).

SNP discovery: ascertainment panel 2

A second Sanger sequencing SNP discovery effort using a distinct ascertainment panel (Table S3) focused on variation within westslope cutthroat trout and between cutthroat trout subspecies. An expanded version of this same panel was used previously for identification of sites diagnostic between rainbow and cutthroat trout

(Pritchard *et al.* 2012). Initial PCR was performed using 242 primer pairs, designed from randomly chosen nuclear tentative consensus sequences generated from *O. mykiss* expressed sequence tags reported to the Harvard/DFCI Gene Index project (<http://compbio.dfci.harvard.edu/tgi/tgipage.html>). PCR, subsequent visualization, product purification and sequencing were as described in Pritchard *et al.* 2012. Variable sites were identified by visual examination of sequence data aligned using Sequencher v4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences containing sites chosen for assay design were submitted to dbSNP (Tables S1 and S2). It is noted there is no overlap between the Pritchard *et al.* assays and the assays reported here as that study focuses strictly on diagnostic markers between *O. mykiss* and *O. clarki*. Assays reported here are either variable within westslope cutthroat trout or diagnostic between cutthroat subspecies.

SNP discovery: ascertainment panel 3

Restriction-site-associated DNA sequence data generated on an Illumina GAI instrument for identification of diagnostic SNPs between rainbow and cutthroat trout (Hohenlohe *et al.* 2011) had also identified SNPs within westslope cutthroat trout. RAD-tagged sequences from 11 westslope cutthroat trout samples included fish from wild populations from both sides of the continental divide in Montana (Table S3). Strict filtering based on observed heterozygosity and deviations from Hardy-Weinberg (HW) proportions was applied to remove homologs (described in Hohenlohe *et al.* 2011). Fifty of the loci with highest minor allele frequency that were sequenced in at least ten of the samples were identified for assay design. A search with these 48-bp sequences against a draft of the *O. mykiss* genome (M. Miller personal communication – miller.michael.ryan@gmail.com) was used to generate enough flanking sequence for designing assays.

Assay design and genotyping

Suitable SNP and diagnostic sites were used to design either TaqmanTM (Life Technologies) or KASPar (KBioscience, Herts, England, UK) genotyping assays, which use different chemistry, by submitting sequence data to each company. Both types of assay were designed for some of the SNP sites, but only one for most sites. These assays, along with another eight previously published diagnostic assays (Harwood & Phillips 2011), were used to genotype 12 collections of approximately 30 fish each representing each of the following species/subspecies: rainbow trout (one collection), westslope (six collections), Yellowstone (two collections), Bonneville

(one collection), coastal (one collection) and Lahontan cutthroat trout (one collection). All genotyping was performed using the EP1 system with 96.96 Dynamic Genotyping Arrays (Fluidigm). Taqman™ assay genotyping was modified from the manufacturers' suggested protocols to include a sample preamplification step and an increase to 50 cycles of PCR. The sample preamplification used 1× Qiagen multiplex master mix, 50 nM pooled primers and 2 µL of extract DNA in a 7 µL reaction, [thermal cycler conditions: 95 °C–15 min (95 °C–15 s, 60 °C–4 min, 14 cycles) and 4 °C–hold]. KASPar assay genotyping used reagents and thermal cycling conditions as recommended by the manufacturer and also included a preamplification step.

Data analysis

The program GENEPOP (Raymond & Rousset 1995) was used to evaluate deviations from HW Proportions and test for statistically significant linkage disequilibrium (LD), after corrections for multiple tests with B-Y false discovery rate (Narum 2006). Assays that produced poor quality genotyping plots or significant deviations from HW proportions in multiple collections were removed from further testing. The program GENETIX (Belkhir *et al.* 2004) was used to calculate G_{ST} and evaluate genetic differentiation by factorial correspondence analysis (FCA). Individual assignment tests were performed using the program GENECLASS2 (Piry *et al.* 2004) to assess the ability of this set of assays to assign individuals back to their collection of origin. Program settings used a Bayesian method (Rannala & Mountain 1997) for individual assignment and a score threshold of 0.05.

Results

Of the 335 primer pairs producing clean PCR fragments, 260 also generated adequate sequence data for SNP detection. Another 98 190 RAD-tagged DNA fragments (Hohenlohe *et al.* 2011) were examined for identification of SNPs within westslope cutthroat trout. There were 246 sites chosen for assay design with either variation among westslope cutthroat trout samples or that were putatively diagnostic between westslope cutthroat trout and either rainbow trout or other cutthroat trout subspecies. Of these, a total of 220 were successfully converted into genotyping assays (Taqman™ = 126; KASPar = 94; both = 22). Assay primer/probe sequences are listed in Supplemental Tables S1 and S2.

The designed assays were then evaluated by genotyping samples from 11 cutthroat trout collections and a single rainbow trout collection (Table 1). These samples were also genotyped using 12 rainbow trout assays and 12 previously published diagnostic assays (Supplemental

Table 1 Assignment test among collections of cutthroat trout subspecies and rainbow trout using GENECLASS2

Collection	Drainage	N	% Correct assignment	Ave. Confidence (%)
Wilson Cr. (<i>O. mykiss</i>)	Salmon R.	27	100.0	100.0
Dog Cr. (<i>O. c. clarki</i>)	Hood R.	22	100.0	100.0
Glenwood Hat. (<i>O. c. utah</i>)	Hatchery stock	31	100.0	100.0
L. Lenore (<i>O. c. heshawi</i>)	Hatchery stock	31	100.0	100.0
Barnes Cr. (<i>O. c. bouvieri</i>)	South Fork Snake R.	31	100.0	100.0
Gibson Cr. (<i>O. c. bouvieri</i>)	Portneuf R.	24	100.0	99.9
Trout Cr. (<i>O. c. lewisi</i>)	Kootenai R.	30	100.0	100.0
McGuire Cr. (<i>O. c. lewisi</i>)	Kootenai R.	29	100.0	100.0
Schwartz Cr. (<i>O. c. lewisi</i>)	Upper Clark R.	24	100.0	100.0
McCabe Cr. (<i>O. c. lewisi</i>)	Blackfoot R.	30	100.0	100.0
Flat Cr. (<i>O. c. lewisi</i>)	Lower Clark R.	30	100.0	100.0
Cache Cr. (<i>O. c. lewisi</i>)	Salmon R.	30	100.0	100.0

N, number of individuals; Ave. confidence, Average of the likelihood score for correct assignment calculated for each individual within a population by GENECLASS2.

Tables S1 and S2). Assays with poor quality genotyping plots, low genotyping success and duplicated loci were removed from further analysis ($N = 35$). Mitochondrial SNPs were collapsed into haplotypes, and the 10 unique haplotypes observed were coded into single genotypes. Significant deviations from HWE within multiple populations were observed in eight SNP loci. Two of these were sex-linked SNPs and were retained, because the observed heterozygote deficits were expected, and the other six were removed from further analysis.

Statistically significant LD was detected in 190 of 35 532 pairwise tests for one or more of the six westslope cutthroat trout collections (P -value adjusted for multiple tests <0.004). Seven of these locus pairs, involving 11 total SNPs, were in significant LD in at least two of the collections (Supplemental Table S4). Assays were excluded based on significant LD only for FCA and assignment testing (exclusion of four assays resolved all seven significant pairs). Only westslope cutthroat trout were used for this analysis because of the lack of variation within the other subspecies collections. Several pairs of loci known to be from the same sequence were not in significant LD

because of the diagnostic nature (near fixation between taxa) of one or both of the sites. Likewise, lack of heterozygosity within the westslope cutthroat trout collections may have masked LD for many other pairs of loci.

The remaining genotypes were used to evaluate the utility of these assays to differentiate populations of westslope cutthroat trout as well as distinguish cutthroat trout subspecies from one another and from rainbow trout. Analysis of the G_{ST} values for each locus reveals that more than half of the assays are informative only for differentiation between the tested species/subspecies (Fig. 1 and Supplemental Tables S1 and S2). This leaves 87 markers with significant differentiation between the six included westslope cutthroat trout collections ($G_{ST} > 0.10$), with the average G_{ST} of 0.41, suggesting a high degree of differentiation between these six westslope cutthroat trout collections. Average heterozygosity within these collections for that subset of SNPs ranged from 0.096 to 0.333 indicating that heterozygosity within individual westslope cutthroat trout populations is quite variable. The population with the lowest observed heterozygosity was McGuire Cr. in the upper Kootenai River drainage. The Trout Cr. population from the lower Kootenai River, however, showed much higher heterozygosity ($H_{OBS} = 0.27$) implying isolation of the McGuire Cr. population from the lower Kootenai River gene pool.

The genotyped collections separated extremely well using this set of markers as evidenced by the FCA plots (Fig. 2) and assignment tests (Table 1). The large number of markers that were informative for differentiation between both the different cutthroat subspecies and between cutthroat/rainbow trout allowed the major lineages to be easily distinguished from one another (Fig. 2a). Five fish from the coastal cutthroat collection were identified as rainbow trout, and four fish were identified as rainbow/cutthroat trout hybrids by their intermediate positions on the FCA graph. The misidentified and hybrid fish were then removed from the data set for FCA and assignment testing of westslope cutthroat trout collections.

Separation of westslope cutthroat trout collections on the FCA plot was less defined (Fig. 2b), as it relied on variation in a much smaller subset of SNPs in more genetically similar groups. The first two axes of the westslope FCA, however, only explained 26.1% of the total variation observed with these markers. Subsequent assignment testing showed that every individual fish could be correctly assigned back to their collection of origin using only genetic data from these markers (Table 1). Moreover, the confidence of the assignments was high and no misassignments were observed even between populations from the same lineage, including the two populations of Yellowstone cutthroat trout for which differences in allele frequency of 20% or more occur in only 11 of the markers. Supplemental Tables S1 and S2 contain minor allele frequencies and population differentiation information that describes the utility of each marker.

Discussion

This study used multiple methods for SNP discovery that were influenced by different ascertainment biases. For example, one of the Sanger sequencing efforts used an ascertainment panel directed at finding SNPs within the westslope cutthroat trout subspecies while the other included roughly equal representation of all extant cutthroat trout subspecies. As each panel contained individuals from several subspecies, it was possible to obtain both putatively diagnostic markers and SNPs that were variable within westslope cutthroat trout from both efforts. However, there was significant ascertainment bias for variation in a small number of westslope cutthroat trout populations when the second panel was used, because of the limited number and origin of westslope cutthroat trout samples included in discovery (Pritchard *et al.* 2012). A total of 36 SNP sites variable within westslope cutthroat trout were chosen from this sequencing panel. Likewise, limited representation of the cutthroat trout subspecies in all three panels made the identification of truly diagnostic markers from sequence

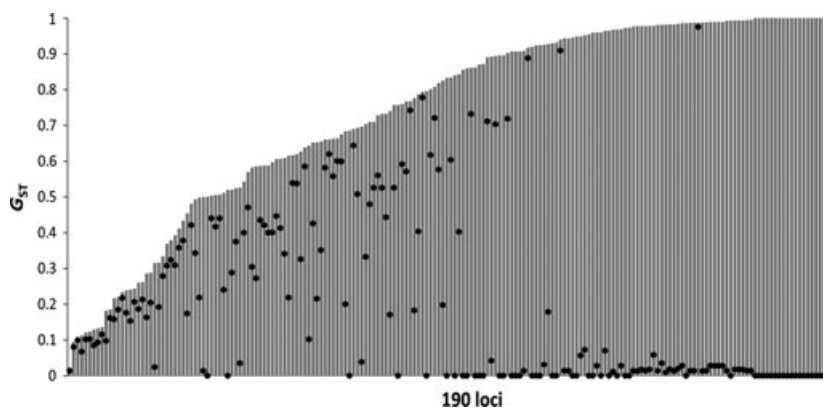


Fig. 1 Graph of G_{ST} for each of the 190 single nucleotide polymorphism loci. Grey bars indicate G_{ST} when all collections are included and black dots indicate G_{ST} within only the westslope cutthroat trout populations.

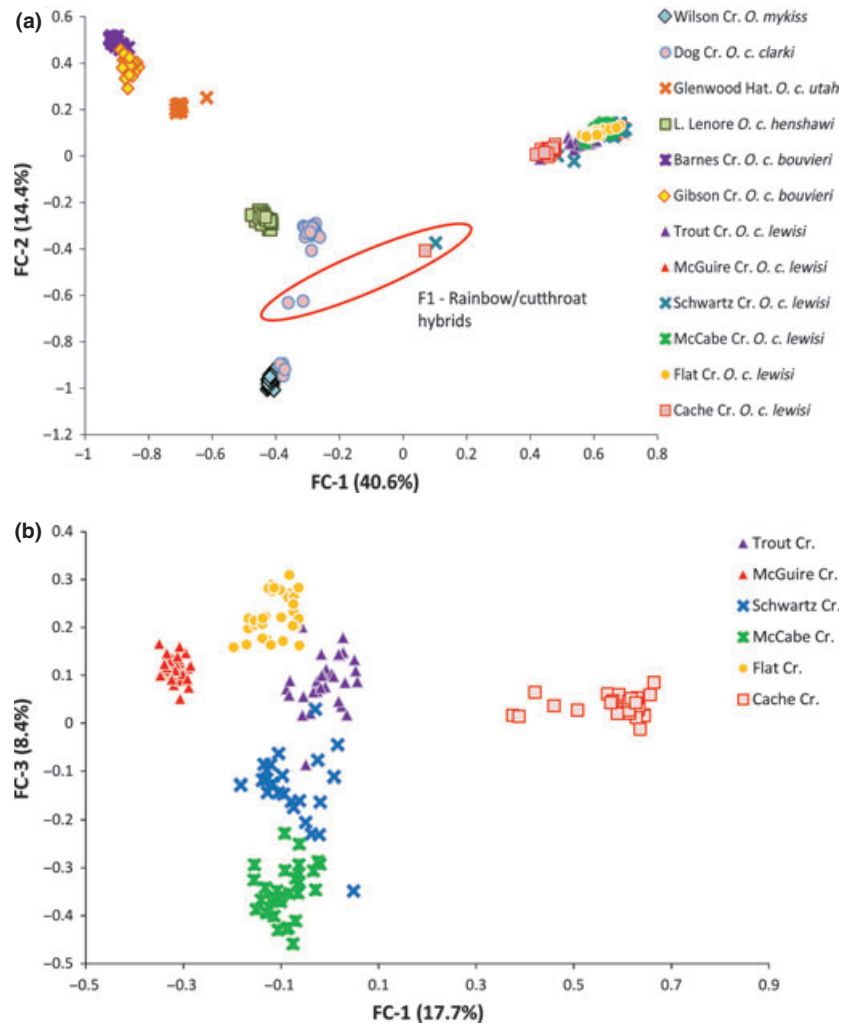


Fig. 2 Factorial correspondence analysis plots generated using the program GENETIX (190 markers). (a) Individuals from all species/subspecies collections. F1 rainbow/cutthroat trout hybrids are indicated in the red ellipse. (b) Individuals in the six westslope cutthroat trout populations.

data unreliable. Of the 86 sites chosen from sequence data as putatively diagnostic, only 17 had fixed genotypes in all subsequently genotyped taxa. However, although not strictly diagnostic, many of these markers were nearly so, given the low frequency of alternate alleles, and were still powerful for population identification. The occurrence of alternate diagnostic alleles may also be due to limited introgression within some of the tested populations. Moreover, as only a single population of rainbow trout was genotyped, the representation of *O. mykiss* diversity was far from exhaustive.

Previous studies using microsatellite loci have shown marked genetic distinctions among westslope cutthroat trout populations (Young *et al.* 2004; Drinan *et al.* 2011). These genetic differences, often even within the same watershed, have led to recommendations for stream-level

management (Young *et al.* 2004). Although population coverage is limited, our results also reveal strong genetic differentiation among westslope cutthroat trout populations. We also observed low heterozygosity within some populations, suggesting a risk of inbreeding depression, but it is unclear the extent to which this is owing to ascertainment bias. The average heterozygosity of westslope cutthroat trout informative SNPs ($H_{OBS} = 0.209$) is somewhat lower than that observed in similar SNP panels for populations of anadromous, less genetically isolated sockeye and coho salmon ($H_{OBS} = 0.267$ and 0.260 ; Campbell & Narum 2011). This is likely a result of limited migration and gene flow in relatively isolated populations of westslope cutthroat trout.

The development of informative genetic markers for the study of nonmodel organisms is the most costly

segment of most population genetics studies. Once a set of markers has been developed and validated, the costs associated with population genetic analysis decreases significantly. Here, we describe a set of 216 SNP and diagnostic marker genotyping assays and their relative utility in differentiation of populations of westslope cutthroat trout and diagnosis for several cutthroat trout subspecies and rainbow trout. The SNP assays evaluated in this study will be useful for a broad range of research questions, including westslope cutthroat trout population structure, detection of other cutthroat trout subspecies and identification of introgression and hybrids between related taxa.

Acknowledgements

We would like to thank Matt Campbell, Denise Hawkins, Jessica Metcalf, Kirk Patten, Mary Peacock, Dennis Shiozawa, Christian Smith, Chris Rossel, Robb Leary and Montana Fish Wildlife and parks for supplying cutthroat trout samples for genotyping. Thanks also to Sally Painter and Megan Moore for help with DNA extraction and genotyping. Funding for this study was provided by the Bonneville Power Administration, the Rocky Mountain Research Station, the US Fish and Wildlife Service (Lahontan National Fish Hatchery and Abernathy Fish Technology Center) and the National Oceanic and Atmospheric Administration (Southwest Fisheries Science Center).

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Study design, sample collection, and oversight by KS McKelvey, MK Young, MK Schwartz, JC Garza, G Luikart and SR Narum. SNP identification using Sanger sequencing was performed by NR Campbell and VL Pritchard. SNPs were identified within RAD sequencing data by SJ Amish and G Luikart. Assay design, genotyping, and assay evaluation was completed by NR Campbell, SJ Amish, and VL Pritchard.

Data accessibility

SNP loci: dbSNP accessions ss494474925 – ss494475112. The accessions are matched with reported assay names in supplemental data tables.

SNP detection assays: Tables with primer/probe sequences uploaded as online supplemental material.

Genotype Data: Genotypes at all tested loci for individuals in all 12 collections, GenePop files used for analysis, and related raw data available in the Dryad database (doi:10.5061/dryad.2m3h2m5 p).

Supporting information

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

Table S1 Describes each of the Taqman™ assays, the panel used for SNP discovery, their minor allele frequencies for each population tested, and their informativeness for distinguishing populations (G_{ST}).

Table S2 Describes each of the KASPar assays, the panel used for SNP discovery, their minor allele frequencies for each population tested, and their informativeness for distinguishing populations (G_{ST}).

Table S3 Describes the 3 ascertainment panels used for SNP discovery.

Table S4 Statistically significant linkage disequilibrium (LD) between marker pairs within 2 or more populations.

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