RESEARCH ARTICLE

Genetic variation in westslope cutthroat trout *Oncorhynchus clarkii lewisi*: implications for conservation

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Abstract Twenty-five populations of westslope cutthroat trout from throughout their native range were genotyped at 20 microsatellite loci to describe the genetic structure of westslope cutthroat trout. The most genetic diversity (heterozygosity, allelic richness, and private alleles) existed in populations from the Snake River drainage, while populations from the Missouri River drainage had the least. Neighbor-joining trees grouped populations according to major river drainages. A great amount of genetic differentiation was present among and within all drainages. Based on Nei's D_S, populations in the Snake River were the most differentiated, while populations in the Missouri River were the least. This pattern of differentiation is consistent with a history of sequential founding events through which westslope cutthroat trout may have experienced a genetic bottleneck as they colonized each river basin from the Snake to the Clark Fork to the Missouri river. These data should serve as a starting point for a discussion on management units and possible distinct population segments. Given the current threats to the persistence of westslope cutthroat trout, and the substantial genetic differentiation between populations, these topics warrant attention.

Keywords Westslope cutthroat trout · Population structure · Microsatellites · Conservation · Genetics

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Introduction

The westslope cutthroat trout *Oncorhynchus clarkii lewisi* is the most widely distributed subspecies of cutthroat trout, and despite its name, is found on both sides of the continental divide in the Northern Rockies (Allendorf and Leary 1988; Behnke 2002; Shepard et al. 2005). The subspecies inhabits small streams, rivers, and lakes, and is limited primarily by the requirement of cold, clean water. Historically, the range of westslope cutthroat trout included the Missouri, Columbia, and Saskatchewan river basins. They are believed to have diverged from coastal cutthroat trout *O. c. clarki* about 1.8 to 0.8 million years ago (Behnke 1992).

Populations of westslope cutthroat trout are in decline (Liknes and Graham 1988; Shepard et al. 1997, 2005). Habitat alterations, competition with and predation by nonnative fishes, overexploitation, and genetic introgression have all contributed to their loss (Allendorf and Leary 1988; Liknes and Graham 1988; Muhlfeld et al. 2009; Rieman and Apperson 1989; Shepard et al. 2005; Van Eimeren 1996). In 2008, populations were estimated to have lost a considerable amount of habitat, only occupying

58% of their historic range, with the majority of the remaining habitat found in Idaho (51%) (May 2009). Within this restricted range, genetic introgression has been extensive. In 2009, it was estimated that only about 44% of the current range supported genetically pure populations (25% of historical range), assessed by genetic testing or expert opinion (May 2009). The trend is similar in Canada (COSEWIC 2006).

Ongoing efforts to conserve cutthroat trout have included captive propagation, translocations, and isolation of populations from non-native species. Supplementation of small, potentially inbred populations has been discussed by managers. All of these efforts would benefit from a clear understanding of how genetic variation is distributed in westslope cutthroat trout. This includes identifying which populations have low levels of genetic diversity that might affect their persistence, and, perhaps more importantly, identifying the main evolutionary lineages within westslope cutthroat trout. These data would be useful for selecting stocks for translocations, ensuring that the main evolutionary lineages within the taxon are protected, and identifying locations for barriers to prevent the spread of non-native species (Fausch et al. 2009).

The goal of this investigation is to describe the distribution of neutral genetic diversity within westslope cutthroat trout throughout their range. This included estimating the amount of genetic diversity within populations and the amount of genetic differentiation among populations. To do this, we will describe genetic variation at 20 microsatellite loci from 25 populations of westslope cutthroat trout sampled from most of their range.

Methods

Tissue or DNA samples were obtained from 25 populations of westslope cutthroat trout distributed throughout the major drainages of their conterminous range (Fig. 1; Table 1). These samples included seven populations from the Missouri River basin, three populations from the Saint Mary River basin (Saskatchewan River basin), eight from the Clark Fork River basin, and seven from the Snake River basin. Samples were provided by Montana Fish, Wildlife and Parks, Idaho Department of Fish and Game, U.S. Fish and Wildlife Service (USFWS), University of Montana, or collected in the wild by the authors. Because samples were
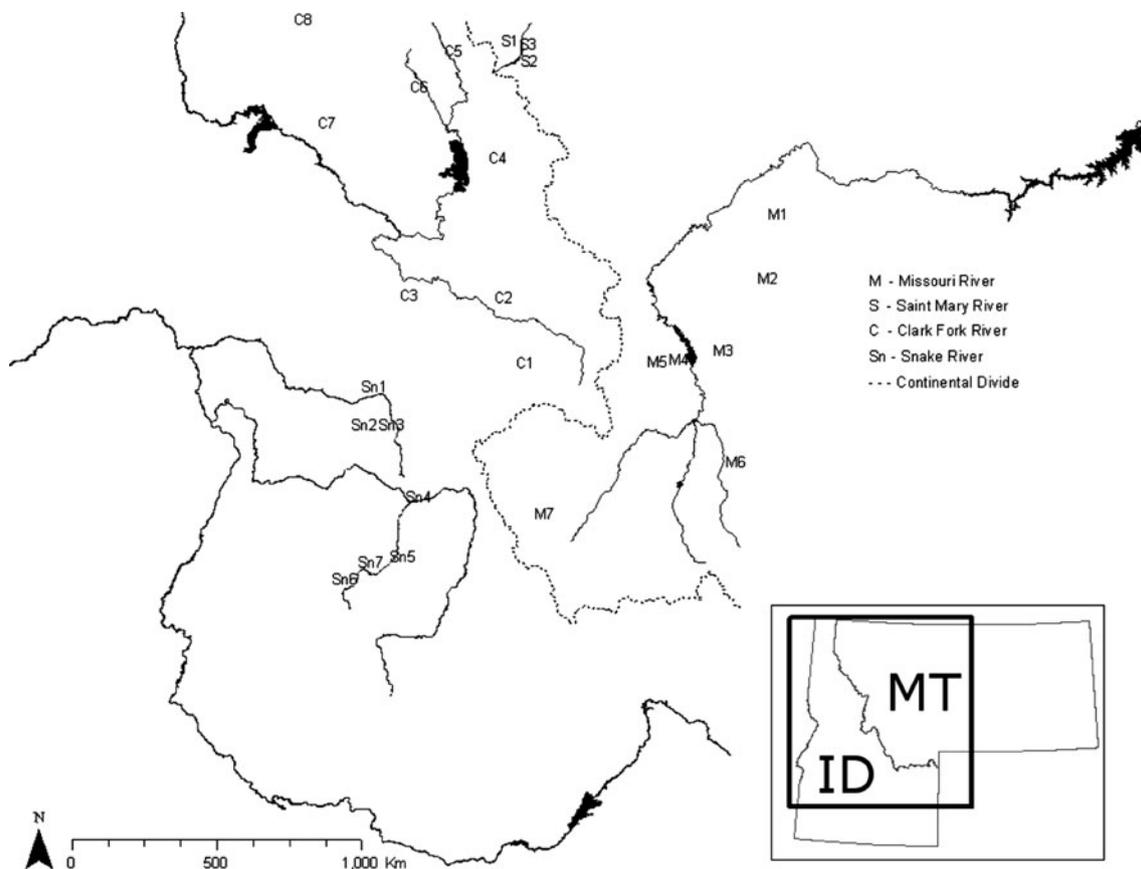
Fig. 1 Map of the westslope cutthroat trout populations sampled from Idaho and Montana, USA. See Table 1 for the names of each sampled location

Table 1 Twenty-five sampled populations of westslope cutthroat trout

Figure ID	Population name	Latitude and longitude
Missouri River		
M1	NF Little Belt Cr.	47°25′27″ N, 110°38′47″ W
M2	Graveyard Gl.	46° 55′54″ N, 110°46′45″ W
M3	Ray Cr.	46°23′7″ N, 111°17′48″ W
M4	Hall Cr.	46°19′19″ N, 111°46′58″ W
M5	Muskrat Cr.	46°18′36″ N, 112°1′24″ W
M6	WF Wilson Cr.	45°31′40″ N, 111°11′4″ W
M7	Browns Cr.	45°8′38″ N, 113°15′33″ W
Saint Mary River		
S1	Boulder Cr.	48°47′19″ N, 113°32′40″ W
S2	Divide Cr.	48°40′57″ N, 113°25′34″ W
S3	Wild Cr.	48°45′36″ N, 113°26′43″ W
Clark Fork River		
C1	Sluice Gl.	46°18′21″ N, 113°27′7″ W
C2	Dirty Ike Cr.	46°48′36″ N, 113°42′10″ W
C3	Montana Cr.	46°48′33″ N, 114°45′23″ W
C4	NF Lost Cr.	47°53′11″ N, 113°46′57″ W
C5	Cyclone Lk.	48°42′17″ N, 114°17′47″ W
C6	Sanko Cr.	48°25′2″ N, 114°41′11″ W
C7	EF Bull R.	48°7′38″ N, 115°43′54″ W
C8	Canuck Cr.	48°54′50″ N, 116°2′39″ W
Snake River		
Sn1	Ballinger Cr.	46°6′0″ N, 115°9′13″W
Sn2	Lynx Cr.	45°48′36″ N, 114°57′47″W
Sn3	Meadow Cr.	45°48′36″ N, 115°7′12″W
Sn4	Roaring Cr.	45°15′33″ N, 114°38′42″W
Sn5	Cache Cr.	44°48′4″ N, 114°48′24″W
Sn6	Elkhorn Cr.	44°36′57″ N, 115°15′27″W
Sn7	Garden Cr.	44°44′38″ N, 115°8′21″W

collected by numerous agencies and individuals over a number of years, no strict sampling protocol was used. All populations were screened for evidence of hybridization with rainbow trout *O. mykiss* by Montana Fish, Wildlife and Parks, Idaho Department of Fish and Game, or the authors (Supplementary data). Populations were not generally screened for presence of Yellowstone cutthroat trout *O. c. bouvieri* alleles.

Eight individuals were sampled per population (except for one population, NF Little Belt Creek, which had a sample size of seven individuals). This is fewer than most studies of population structure, but this study design was intentional. When populations are highly genetically differentiated—as are westslope cutthroat trout (Allendorf and Leary 1988)—there is decreasing benefit to increasing the number of individuals sampled from each population (Kalinowski 2002a, b, 2005a). Simulations suggest that

when F_{ST} is greater than 0.2, there is little benefit from a sample size of more than eight individuals per populations (Kalinowski 2005a). In these circumstances, the accuracy of estimates of genetic distance can be improved by genotyping large numbers of loci, and/or by using loci that are as polymorphic as possible (Kalinowski 2002a, b). We followed both of these strategies by genotyping as many microsatellite loci as possible.

Twenty microsatellite loci were genotyped for all individuals sampled (Table 2). All of these loci have tetranucleotide repeat motifs and were originally developed from westslope cutthroat trout (Table 2). Amplification conditions and techniques are described by Vu and Kalinowski (2009).

Genotype counts were tested for agreement with Hardy–Weinberg expectations using the Markov chain Monte-Carlo exact test of Guo and Thompson (1992) implemented by GENEPOP (Version 4.0.10) to test for a deviance from heterozygote expectations (Raymond and Rousset 1995; Rousset 2008; Rousset and Raymond 1995). Linkage disequilibrium was tested using Fisher's method implemented by GENEPOP (Raymond and Rousset 1995; Rousset 2008). As reported below, there were widespread heterozygote deficiencies in the data. Null alleles likely contributed to this trend, but other factors may also have played a role (population substructure or non-random sampling) and could have introduced bias into the results. We estimated the frequency of putative null alleles using the maximum likelihood method of Kalinowski and Taper (2006).

The amount of genetic diversity within populations and river basins was quantified using expected heterozygosity (Nei 1978) and allelic richness (Kalinowski 2004). GENEPOP (Raymond and Rousset 1995; Rousset 2008) was used to calculate the expected heterozygosity, H_{exp}, at each locus in populations. Results were averaged across loci and populations to obtain an average H_{exp} for each watershed. Wilcoxon rank sum tests were performed among populations to examine differences in H_{exp}. A Bonferroni correction for multiple comparisons was made. HP-Rare (Kalinowski 2005b) was used to estimate the allelic richness for each population and watershed. Differences in sample size were taken into account.

The amount of genetic differentiation among populations was quantified with the number of private alleles per population, the standard genetic distance of Nei, D_S (Nei 1978), F_{ST}, estimated by θ (Weir and Cockerham 1984), and analysis of molecular variance (AMOVA). Estimates of private allelic richness were calculated using the computer program HP-Rare, after accounting for differences in sample size (Kalinowski 2005b). D_S and θ were calculated using a computer program in Visual Basic written by S. Kalinowski. AMOVA was estimated using GenAlEx 6.41 (Peakall and Smouse 2006).

Table 2 Twenty loci were investigated in westslope cutthroat trout

Locus	Accession no.	T_A (°C)	$MgCl_2$ (mM)	Primer sequence
OclMSU14	GQ249043	61	3.0	F:AGGCTGCATGCTTTCAAAAT
				R:TCCCTTGTGCTGATTGACAG
				(Vu and Kalinowski 2009)
OclMSU16	GQ249042	61	3.0	F:TGCCCTGGAGAGAGAGAAAG
				R:TCAGAGTATTAGGGCTACCAGGA
				(Vu and Kalinowski 2009)
OclMSU17	GQ249044	61	3.0	F:GCCCTGTTTTGGTTTACGTT
				R:GGGAGGGAGAGAAAAGGAGA
				(Vu and Kalinowski 2009)
OclMSU18	GQ249045	54[a]	3.0	F:TGGGTATCGGCCTAATTCTG
				R:GGCCCATATGAATGTTCCAC
				(Vu and Kalinowski 2009)
OclMSU21	GQ249048	59	3.0	F:TCCTGTCCTTTGCAGCAGTA
				R:TCCTCTCCTCTCGCTCTCTG
				(Vu and Kalinowski 2009)
OclMSU23	GQ249050	63	3.0	F:ACTTTGTGTATGTAAACTTCTGACC
				R:CAATCTTAGCCAAACCTGAA
				(Vu and Kalinowski 2009)
OclMSU24	GQ249051	63	3.0	F:TCCCTCCATGTCTCCTTGTC
				R:GAAGATCCGCACCACAGTCT
				(Vu and Kalinowski 2009)
OclMSU25	GQ249052	63	3.0	F:CTGAGGGATGAGGACACCAC
				R:TCCCTTTGCTAATAAAGCCATT
				(Vu and Kalinowski 2009)
OclMSU26	HM153812	60	3.0	F:CTGAACGTTACTGGGGGCTA
				R:AGCCAAGGCTGTCCAATCTA
				(Vu and Kalinowski unpublished)
OclMSU27	HM153813	60	3.0	F:GCCATCAAATCCTCAAATGG
				R:GTTACACAGCAGCCCACTCA
				(Vu and Kalinowski unpublished)
OclMSU28	HM153814	60	3.0	F:GACTGCCAACCCAGAGAGAT
				R:CCGGTCTCACCACACATATC
				(Vu and Kalinowski unpublished)
OclMSU29	HM153815	60	3.0	F:TTCCAGCTATGATCTCCTCTCC
				R:CCATTCCAGAGCATAGCACA
				(Vu and Kalinowski unpublished)
OclMSU30	HM153816	64	3.0	F:GGTGGCTCCAGTGGATTTAG
				R:TATTGGGCTGGAGCAGAACT
				(Vu and Kalinowski unpublished)
OclMSU31	HM153817	64	3.0	F:CTGTTGGAATGGCGTCACTA
				R:CAGGAGACTTGCTTGCTGTG
				(Vu and Kalinowski unpublished)
OclMSU32	HM153818	64		F:TTCGTGGCAAAATAACAGCTT
				R:TGGGGTCTCAGTGTTTCTCA
				(Vu and Kalinowski unpublished)
OclMSU33	HM153819	64	3.0	F:ACAGGGGATTTCTCCATGTG
				R:AGAGCAGTGGAATGCTACCC
				(Vu and Kalinowski unpublished)

Table 2 continued

Locus	Accession no.	T_A (°C)	$MgCl_2$ (mM)	Primer sequence
OclMSU34	HM153820	64	3.0	F:GGATGCCTGCTGATGAGTCT
				R:GGCCATGTGTGACGTTCTAA
				(Vu and Kalinowski unpublished)
OclMSU35	HM153821	64	3.0	F:GTTGAGCCGTCTCTTGAACC
				R:TTTCTGGCTGTGTCCCATCT
				(Vu and Kalinowski unpublished)
OclMSU36	HM153822	64	3.0	F:CCACAGCAGCAGATAAGCAA
				R:CACATGATCGCATGAGAGAGA
				(Vu and Kalinowski unpublished)
OclMSU37	HM153823	64	3.0	F:TCCTTCGAATCCAGCATTTC
				R:TGGACCTACACAAGAACCACA
				(Vu and Kalinowski unpublished)

Accession no. Genebank ID, T_A annealing temperature

[a] Temperature different than published specification

Genetic relationships among populations were summarized with neighbor-joining trees based on D_S and θ (Saitou and Nei 1987). The degree to which the trees fit the genetic distance matrices was quantified using R^2 (Kalinowski 2009) which was calculated using the computer program TreeFit (Kalinowski 2009). The robustness of the tree to sampling error was measured by bootstrapping across loci (Felsenstein 1985). The program TreeFit was also used to do this.

Isolation by distance was examined using Mantel tests in the program R (R Development Core Team 2010). Range wide Euclidean distance was compared to genetic distance, and within river basins, stream distances were compared to genetic distances. Some streams were isolated within their drainage. To counter this, streams were manually connected to their closest downstream neighbor so as to create the shortest path possible. Undoubtedly, this created bias in the results and caution must be used when drawing conclusions. Distances were calculated using ArcGIS (Environmental Systems Research Institute, Inc., Redlands, CA). Map layers were supplied by Montana Department of Fish, Wildlife and Parks and Idaho Department of Water Resources. Layers were accurate to a 1:100,000 scale.

Results

We will organize much of our results by river basin. Because we have only three samples from the Saint Mary River basin (Saskatchewan River basin), and these samples were not distributed widely within the drainage, we will focus our presentation on the Missouri, Clark Fork, and Snake river drainages, and discuss the Saint Mary River populations at the end of this section.

Considerable genetic variation was present at all 20 microsatellite loci genotyped. On average, slightly more than 17 alleles were detected per locus, with a maximum of 40 and a minimum of seven. No evidence of linkage disequilibrium was found (Supplementary data). The PCR failure rate was notably high, with an overall failure rate of 7% of samples. Our measure of genetic diversity in the Snake River was most affected by amplification failure (12%). It was less of an issue with the Missouri (2%) and Clark Fork (6%) river estimates. Hardy–Weinberg tests showed widespread heterozygote deficiencies in the data (Supplementary data). The Clark Fork River populations had the greatest amount of heterozygote deficient loci at 23%. This was followed by the Snake (16%) and Missouri (6%) river populations.

Null alleles were estimated to have an average frequency of 0.09 per locus. The Snake and Clark Fork river populations had the greatest estimated frequency at 0.12 for both. The Missouri River appeared to be less influenced by null alleles with an average estimated frequency of 0.05.

Because of the relatively high rate of PCR failure, and the apparently high frequency of null alleles, two analyses were performed; (i) using the original data set and (ii) removing loci from populations with less than 50% amplification success. Both analyses produced biologically similar conclusions (Supplementary data). The following results are based on the analysis using the original data set.

We found less genetic diversity within populations in the Missouri River than in the other drainages (Table 3). Average H_{exp} in the Missouri, Clark Fork, and Snake rivers were 0.27, 0.58, and 0.62 respectively. The mean H_{exp} of Missouri River populations was significantly less than the mean H_{exp} of the Clark Fork and Snake river basin populations, after a Bonferroni correction for multiple

Table 3 Genetic characteristics of the populations of westslope cutthroat trout in three major river basins

Basin	H_{exp}	A	A_p	θ	θ'	Avg. D_S
Missouri R.	0.27	2.35	0.56	0.36	0.49	0.23
Clark Fork R.	0.58	4.78	2.16	0.19	0.45	0.41
Snake R.	0.62	5.56	3.51	0.25	0.69	1.10

H_{exp} Average expected heterozygosity within populations, A average allelic richness within populations, A_p average private allelic richness within populations, θ basin-wide estimate of Weir and Cockerham's (1984) θ, θ' basin-wide estimate of standardized θ (Hedrick 2005), Avg. D_S average pairwise value of Nei's standard genetic distance within drainage

comparisons (Both pairwise comparisons had a *P*-value less than 0.01.). Allelic richness was lowest in the Missouri River at 2.35 alleles per locus and highest in the Snake River (5.56). The Clark Fork and Snake river basins had similar heterozygosities and allelic richnesses.

Snake River populations had the greatest estimated number of private alleles (3.51 alleles per locus). Missouri River populations had the fewest estimated private alleles: 0.56 alleles per locus, or about one-seventh the private alleles in the Snake River (Table 3). The Clark Fork River

populations had an intermediate number of private alleles: 2.16 per locus.

Neighbor-joining trees grouped populations from each drainage together (Figs. 2 and 3). The tree based on D_S grouped the Missouri River populations in a cohesive unit at one end of the unrooted tree, and the Snake River populations loosely at the other end of the tree. The Clark Fork populations fell in the middle of the tree, not quite as cohesive as the Missouri River populations, but not as distinct from each other as the Snake River populations.
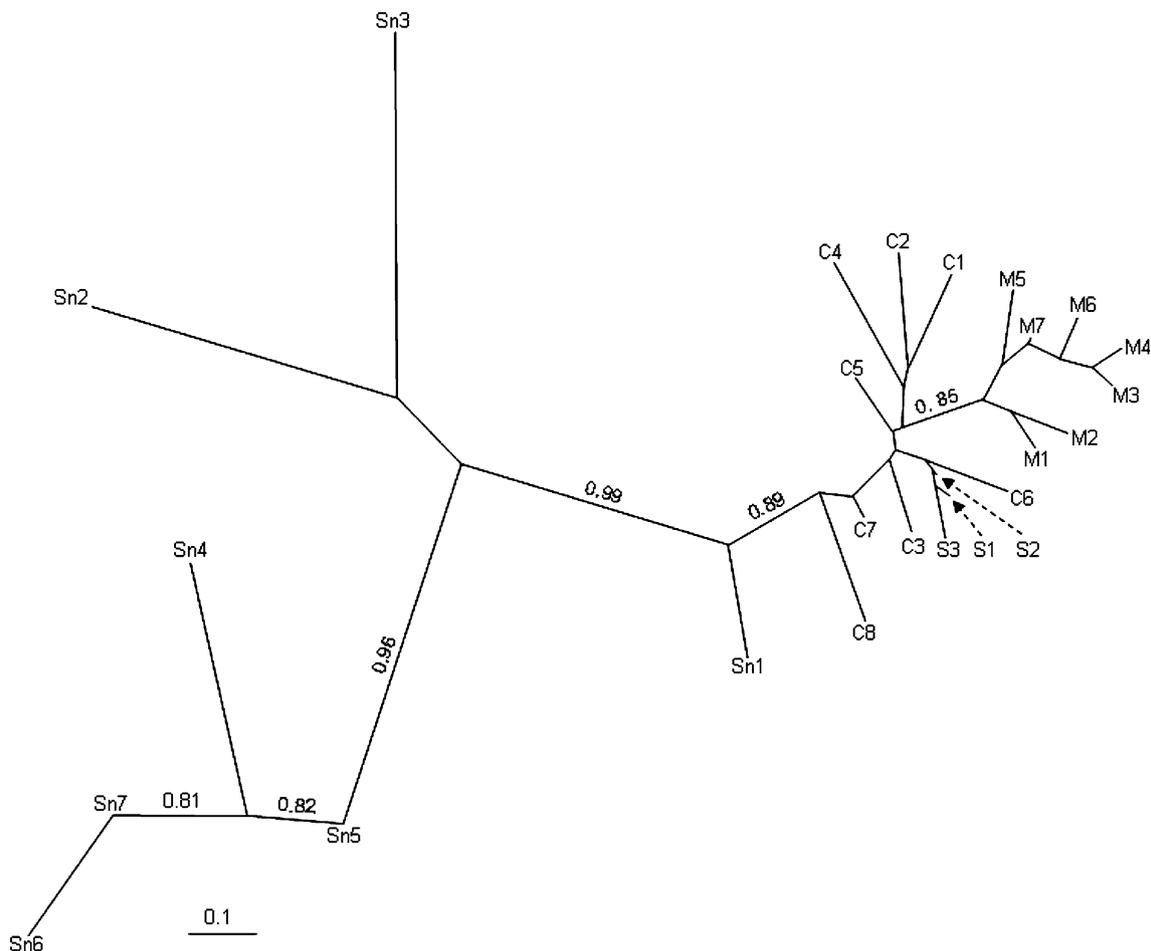
Fig. 2 A neighbor-joining tree of Missouri, Clark Fork, Saint Mary, and Snake river drainage populations. Distances based on Nei's (1978) genetic distance. *Numbers* indicate bootstrap support (results are shown only for *branches* with ≥80% support)
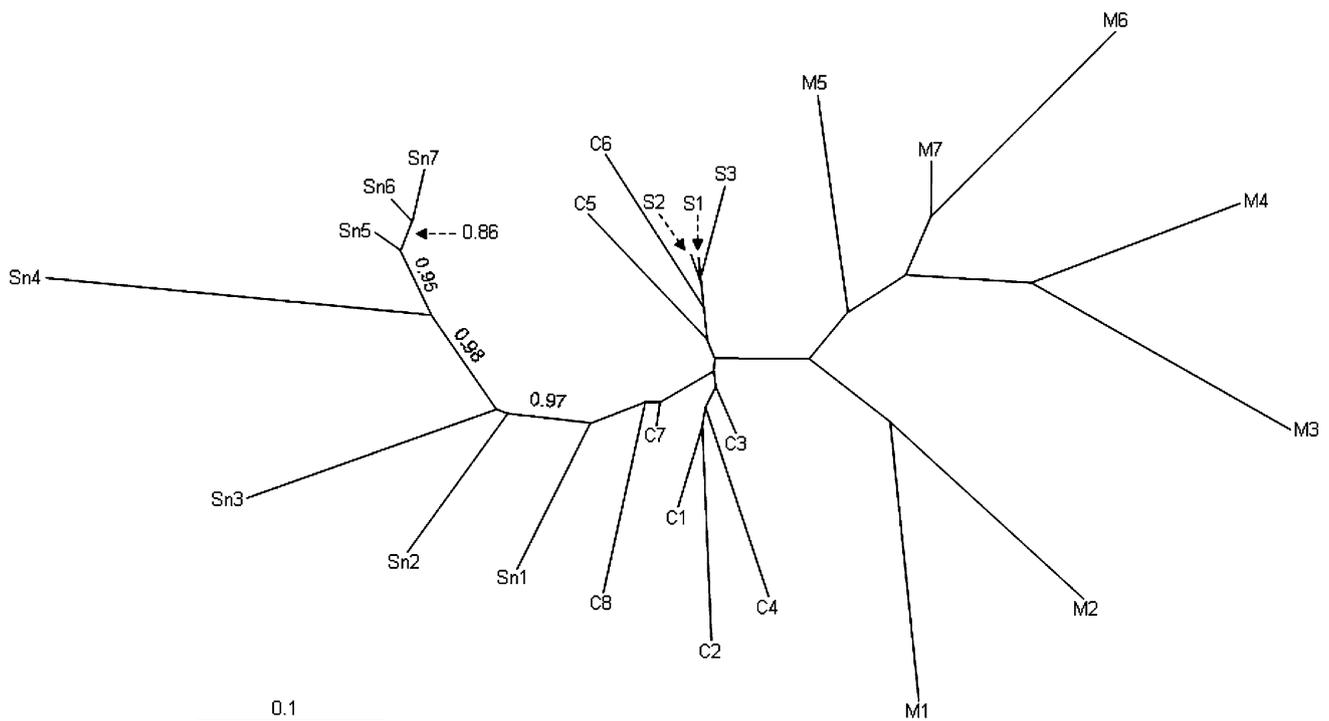
Fig. 3 A neighbor-joining tree of Missouri, Clark Fork, Saint Mary, and Snake river drainage populations. Distances based on the genetic distance θ (Weir and Cockerham 1984). *Numbers* indicate bootstrap support (results are shown only for *branches* with $\geq 80\%$ support)

The tree had a R^2 value of 0.935, which indicates that it is a reasonable summary of the matrix of pairwise genetic distances between populations. The tree based on pairwise θ also grouped populations by drainage. In contrast to the D_S based tree, the Missouri River populations formed the least cohesive group, while the Clark Fork River populations formed the tightest group. This is likely an artifact of the properties of θ (explored in the Discussion). The tree had a R^2 value of 0.97.

Bootstrap support for specific branches within the tree was generally modest. The only branches that had bootstrap support of greater than 0.95 were branches separating Snake River populations amongst themselves. Bootstrap support for the branch separating the Missouri and Clark Fork River populations was 0.85 in the D_S based tree and 0.78 in the θ based tree. Inspection of bootstrapped trees that did not separate the Missouri River populations from the Clark Fork populations showed that most of these

dissenting trees differed only slightly from the observed tree. In most of these dissenting trees, only the NF Little Belt Creek population did not cluster with the other Missouri River populations.

Analysis of molecular variance test showed that most variation occurred within populations (53%) followed by variation among populations (24%) and variation among basins (22%). Variation from all three sources was significant (P-value for all three $= 0.01$).

Our analysis of genetic differences within and among drainages showed that, on average, the populations that we sampled were more similar to populations in the same basin than to populations in the other basins (Table 4). For example, the average D_S among Missouri River populations was 0.23. The average D_S among Clark Fork River populations was 0.42. The average D_S between Missouri and Clark Fork river populations is 0.52, which is 2.26 times as great as the amount of genetic divergence within

Table 4 Average genetic distances when comparing populations both within and among drainages

	Average pairwise θ between drainages			Average pairwise D_S between drainages		
	Missouri R.	Clark Fork R.	Snake R.	Missouri R.	Clark Fork R.	Snake R.
Missouri R.	0.36	0.37	0.52	0.23	0.52	1.88
Clark Fork R.	0.37	0.19	0.32	0.52	0.41	1.44
Snake R.	0.52	0.32	0.25	1.88	1.44	1.09

For all drainages, genetic distances are least when comparing within the same river basin

the Missouri River and 1.28 times as great as the amount of genetic divergence in the Clark Fork River.

Mantel tests showed limited support for genetic isolation by distance. Across the entire range, Euclidean distance was correlated with genetic distance ($P = 0.05$). However, there was no support for the model when isolation by stream distances was examined within each basin (All comparisons had $P > 0.10$).

In all respects, the three populations from the Saint Mary River (Saskatchewan River Basin) were similar to Clark Fork River populations. The amount of genetic diversity within the Saint Mary River drainage populations was similar to the Clark Fork ($H_{exp} = 0.58$, $A = 4.63$). All three Saint Mary River drainage populations nested within the Clark Fork River populations in the neighbor-joining trees (Figs. 2 and 3).

Discussion

The data clearly show that westslope cutthroat trout populations are very different from each other—especially populations in different river basins. Some populations shared almost no alleles. For example, Ray Creek in the Missouri River Basin and Roaring Creek in the Snake River Basin shared only two alleles at 20 loci, and had a pairwise θ' of 0.99 (out of a maximum value of 1.0). This is an extreme amount of genetic differentiation, and many other populations were almost as different from each other. For example, the average value of θ' between Missouri River populations and Snake River populations was 0.92, and 17% of all comparisons have θ' values greater than 0.90.

These extreme levels of genetic differentiation may explain the high level of PCR failure that we observed, and the apparent high frequency of null alleles. Both of these artifacts can be caused by mutations in the primer sites of the microsatellite, and are more likely when populations have been isolated for a long time (Amos 2006; Chapuis and Estoup 2007).

We do not believe that the patterns of higher genetic differentiation observed among populations in the Snake River were the result of undetected non-native rainbow trout introgression. Many of the populations in our study have been screened previously for rainbow trout hybridization and introgression and an effort was made to avoid populations in which introgression had been observed. In addition, all samples were screened with genetic markers diagnostic between rainbow trout and cutthroat trout, with no rainbow trout alleles observed. Intuitively, it seems more likely that introgression from non-native rainbow trout might erode natural levels of genetic differentiation, a pattern observed in studies examining intraspecific

hybridization and introgression in *O. mykiss* (Kozfkay et al. 2011; Nielsen et al. 2009).

The extreme levels of genetic differentiation among populations of westslope cutthroat trout make it difficult to quantify genetic similarities and differences among populations. F_{ST} estimated by θ (Weir and Cockerham 1984) is the most commonly used genetic distance to describe population structure, but it has a maximum value equal to the average homozygosity within populations (Kalinowski 2002a). This probably explains why θ and D_S (Nei 1978) gave contrasting descriptions of the relative amount of genetic divergence within river basins (Figs. 2, 3; Table 3). The likely explanation for this contrast is because Missouri River populations have less genetic diversity within populations, they can have a higher value of θ between populations. Standardized θ, or θ' (Hedrick 2005) is not affected by the amount of diversity within populations, but it still has a maximum value (1.0) which highly diverged populations will approach asymptotically (S. Kalinowski unpublished). This will cause θ' to underestimate the amount of divergence among highly diverged populations. Nei's standard distance avoids these problems, but it has a large sampling variance at high values. All of these issues are symptoms of the fundamental problem that microsatellite data are not well suited for describing genetic differences among populations that have been separated for a long time. Once populations have evolved to the point that they share no alleles, they are maximally differentiated, and further inference is impossible. Populations of westslope cutthroat trout, especially in the Snake River basin, seem to be reaching this point. If this is true, the relative genetic differences between the most isolated populations may be underestimated, or, perhaps, estimated with great uncertainty. Analysis of DNA sequence data should be useful for further investigating these relationships.

One of the most striking features of the neighbor-joining tree of populations based on D_S (Fig. 2) is the high level of genetic differentiation among Snake River populations. In comparison, populations in the Missouri River Basin are much more similar to each other. Populations in the Missouri River Basin are also more similar to Clark Fork populations than Snake River populations are to each other. These relative differences should not be interpreted as evidence that Missouri River populations are genetically similar (in an absolute sense) to each other or to Clark Fork River populations. Estimated θ for the Missouri River populations was 0.36. This is a substantial amount of genetic differentiation for populations of the same species. And as discussed above, Clark Fork River populations are more different from Missouri River populations than Missouri River populations are from each other.

This study of population structure in westslope cutthroat trout is the most extensive to date, both in number of loci

and range of populations. It agrees well with previous investigations (Leary et al. 1988; Taylor et al. 2003). Previous investigations of population structure in westslope cutthroat trout have found high levels of genetic differentiation among populations. For example, Taylor et al. (2003) estimated θ for British Columbia populations to be 0.32. Leary et al. (1988) estimated G_{ST} (which is nearly equivalent to θ) for populations in the Missouri, Clark Fork, and Saskatchewan river basins to be 0.33. The most substantive difference between our results and those of previous investigators is how we interpreted genetic differences associated with the continental divide that separates the Missouri from the Clark Fork river basins. Leary et al. (1988) concluded that there was little or no more genetic divergence between populations in the Clark Fork and Missouri river basins than there is within the Clark Fork River basin. We interpret our data as indicating that the continental divide in Montana is associated with a substantial amount of genetic differentiation. This is evident in the topology of the neighbor-joining trees (Figs. 2, 3) and our analysis of average genetic differences within and between river basins (Table 4). We propose there are three explanations for the contrasting view of the genetic significance of the continental divide in Montana. First, Leary et al. (1988) analysis was based on limited data. The accuracy of estimates of genetic distance are proportional to the number of alleles at the loci genotyped (Kalinowski 2002b) and the allozyme loci of Leary et al. appear to have two alleles per locus. In contrast, the microsatellite loci presented here have an average of over 12 alleles per locus in the Clark Fork and Missouri river basins. Numerous other studies have found more polymorphic loci using microsatellite compared to allozyme data (Hughes and Queller 1993; Sánchez et al. 1996). Second, Leary et al. sampled only one Missouri River population and had limited data for comparing populations in the river basins. Third, the genetic significance of the continental divide may depend on from where the divide is viewed. As we reported above, Clark Fork Populations are 2.36 times more different from Missouri River populations than Missouri River populations are from each other (based on D_S). In some contrast, Missouri River populations are 1.28 times more different from Clark Fork populations than Clark Fork populations are from each other (based on D_S). This asymmetry is a consequence of the higher amount of differentiation among Clark Fork populations than in the Missouri River populations.

Although inferring the evolutionary histories of populations from the geographic distribution of genetic variation is difficult (because many evolutionary histories can produce similar patterns of population structure), patterns of genetic structure can provide some insight into the evolutionary history of these populations. We found greater genetic diversity among populations in the Snake than the Clark Fork and Missouri river drainages and greater genetic diversity among populations in the Clark Fork than the Missouri river drainage. One possible explanation for this pattern is the hypothesis that westslope cutthroat trout have a longer evolutionary history in the Snake River, which could have served as a refugium during the latest Pleistocene glaciations ($\sim$ 10,000–14,000 ybp). Populations in areas that were less influenced by glaciation have a longer evolutionary history and likely contain a relatively greater amount of genetic variation and geographic structuring of genetic diversity (Bernatchez and Wilson 1998). The peak of glaciation in western North America occurred between 15,000 and 18,000 years ago at the end of the Pleistocene era when the Laurentide and Cordilleran ice sheets covered much of Canada, southeastern Alaska and the northern continental United States (McPhail and Lindsey 1986), and in the Rocky Mountain region of the United States, lobes of the continental ice sheet extended into present day Montana (including the Clark Fork basin; McPhail and Lindsey 1986). The advancing glacial front as well as the accompanying climatic changes dramatically altered the distribution of taxa, forcing many species south or into unglaciated refugia (Pielou 1991). As the ice sheets retreated for the final time around 10,000 years before present, vast amounts of habitat were exposed, permitting re-colonization by terrestrial and aquatic organisms, such as westslope cutthroat trout (McPhail and Lindsey 1986).

The genetic data are consistent with a history of sequential founding events through which cutthroat trout experienced a genetic "bottleneck" as a limited number of source populations colonized new habitat after glaciers receded (Bernatchez and Wilson 1998; Hewitt 2000). This hypothesis is supported by the distribution of private alleles (Table 3), with the Snake River populations having the most private alleles and the Missouri River populations the fewest. A similar pattern could be produced by hybridization, but based on the screening that occurred; it is unlikely that these results were influence by hybridization with congeners (Supplementary data). In addition, this pattern has been seen in other westslope cutthroat trout genetic analyses (Dunning et al. unpublished).

Finally, the amount of genetic differentiation within major basins suggests that after westslope cutthroat trout colonize a river basin, there is limited movement among populations within that basin. If this is true, it explains why Snake River populations are the most different from each other, and why Missouri River populations are the most similar to each other (based on D_S). In addition, it explains why an isolation by distance model was not appropriate for explaining genetic distances within each drainage.

As stated earlier, populations from the Saint Mary River basin (Saskatchewan River basin) were similar to Clark

Fork River populations. This is likely due to recent stocking of MO12 westslope cutthroat trout in the Saint Mary River basin (Montana Fisheries Information System (MFISH) 2011). The MO12 stock is a hatchery population created from broodstock from the Clark Fork River drainage.

Conservation and management implications

Our objectives were to describe the variation in genetic diversity within westslope cutthroat trout by estimating the amount of genetic diversity within and among populations in the Snake, Clark Fork, and Missouri river drainages. Furthermore, we were interested in providing this information so that managers can better assess the spatial scale for which conservation units may be designated across most of this subspecies' range. Our results highlight many important considerations for the conservation and management of this sensitive species. First, our genetic data corroborate previous studies that have shown a large degree of genetic differentiation among populations. Second, we detected substantial genetic differentiation among drainages, suggesting that it may be necessary to manage these units separately to maintain the evolutionary legacy of westslope cutthroat trout. For example, translocation of drainage specific stocks to recover declining stocks in other drainages could be detrimental to the unique genetic and geographic separation of westslope cutthroat trout populations that we have described herein.

The United States Endangered Species Act (ESA) protects designated species from activities that increase the probability of extinction. The ESA gives the same degree of protection to "distinct population segments" (DPS) within species. To date, there has been little discussion regarding whether westslope cutthroat trout comprise a single species (for the purpose of the ESA), or whether the currently recognized sub-species is composed of multiple distinct population segments. Given the current threats to the persistence of westslope cutthroat trout, and the substantial genetic differentiation between populations, this question deserves attention. As a starting point for such a discussion, we will explore aspects of these data that support and counter the qualification of westslope cutthroat trout as distinct population segments based on drainage.

The USFWS has specific criteria for designating distinct population segments. To qualify as a DPS, a population segment must be discrete from other population segments, and must be biologically or ecologically significant. There is no question that cutthroat trout in the Missouri, Clark Fork, and Snake river drainages are discrete; the relevant question is whether these populations are significantly different from each other. USFWS policy (USFWS (US Fish, Wildlife Service), NOAA (National Oceanic, Atmospheric

Administration) 1996) lists several possible criteria for establishing biological significance. The criterion relevant to this investigation is that a discrete population segment is considered significant if it "differs markedly from other populations of the species in its genetic characteristics."

An argument can be made that westslope cutthroat trout in the Missouri, Clark Fork, and Snake River basins each qualify as a distinct population segment under the ESA. Populations cluster genetically (Figs. 2 and 3) by basin, and there are substantial genetic differences among populations in each basin.

Two counter arguments can be made against designating Missouri, Clark Fork, and Snake river populations of westslope cutthroat trout as distinct populations segments. First, genetic differences within the Snake River basin are greater than differences between the Clark Fork and Missouri river basins. If distinct population segments were defined based solely on genetic differences between populations, we might create multiple distinct populations segments within the Snake River Basin and cluster all Clark Fork and Missouri River populations together. The second counter argument against the Missouri/Clark Fork/Snake river partition is that there are relatively few unique alleles in the Missouri River basin: 0.56 unique alleles per locus were present in the Missouri River basin compared to 3.51 and 2.16 for the Snake River and Clark Fork populations. These results highlight why defining distinct population segments based solely on genetic differences among populations would be difficult and reinforce the use of a variety of types of data (ecological, distribution, etc.) to designate DPS units.

With such ambiguity, further research is necessary. The use of sequence data could improve estimates of genetic distances and provide temporal estimates for important dendrogram branches. In addition, by investigating genes under selection, details of potential adaptive differences among groups could be discovered, which would be valuable information for any conservation plan. Finally, the inclusion of additional samples from both interior and periphery populations could provide a more detailed understanding of both the genetic structure of westslope cutthroat trout and the natural processes that helped shape their evolutionary history.

References

Allendorf FW, Leary RF (1988) Conservation and distribution of genetic variation in a polytypic species, the cutthroat trout. Conserv Biol 2:170–184

Amos W (2006) The hidden value of missing genotypes. Mol Biol Evol 23:1995–1996

Behnke RJ (1992) Native trout of western North America. American Fisheries Society Monograph 6, Bethesda, Maryland

Behnke RJ (2002) Trout and salmon of North America. The Free Press, New York

Bernatchez L, Wilson CC (1998) Comparative phylogeography of Nearctic and Palearctic fishes. Mol Ecol 7:431–452

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. Mol Biol Evol 24:621–631

COSEWIC (2006) COSEWIC assessment and update status report on the westslope cuttthroat trout *Oncorhynchus clarkii lewisi* (British Columbia population and Alberta population) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

Fausch KD, Rieman BE, Dunham JB, Young MK, Peterson DP (2009) Invasion versus isolation: trade-offs in managing native Salmonids with barriers to upstream movement. Cons Biol 23:859–870

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hedrick PW (2005) A standardized genetic differentiation measure. Evolution 59:1633–1638

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Hughes CF, Queller DC (1993) Detection of highly polymorphic microsatellite loci in a species with little allozyme polymorphism. Mol Ecol 2:131–137

Kalinowski ST (2002a) Evolutionary and statistical properties of genetic distances. Mol Ecol 11:1263–1273

Kalinowski ST (2002b) How many alleles per locus should be used to estimate genetic distances? Heredity 88:62–65

Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical sampling designs. Conserv Genet 5:539–543

Kalinowski ST (2005a) Do polymorphic loci require larger sample sizes to estimate genetic distances? Heredity 94:33–36

Kalinowski ST (2005b) HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. Mol Ecol Notes 5:187–189

Kalinowski ST (2009) How well do evolutionary trees describe genetic relationships between populations? Heredity 102:506–513

Kalinowski ST, Taper ML (2006) Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. Conserv Genet 7:991–995

Kalinowski ST, Novak BJ, Drinan DP, Jennings R, Vu NV (2010) Diagnostic single nucleotide polymorphisms for identifying westslope cutthroat trout (*Oncorhynchus clarki lewisi*), Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) and rainbow trout (*Oncorhynchus mykiss*). Mol Ecol Resour 11:389–393

Kozfkay CC, Campbell MR, Yundt SP, Peterson MP, Powell MS (2007) Incidence of hybridization between naturally sympatric westslope cutthroat trout and rainbow trout in the Middle Fork Salmon River drainage, Idaho. T Am Fish Soc 136:624–638

Kozfkay CC, Campbell MR, Meyer KA, Schill DJ (2011) Influences of habitat and hybridization on the genetic structure of Redband trout in the upper Snake River basin, Idaho. T Am Fish Soc 140:282–295

Leary RF, Allendorf FW, Phelps SR, Knudsen KL (1988) Population genetic structure of westslope cutthroat trout: genetic variation within and among populations. Proc Mont Acad Sci 48:57–70

Liknes GA, Graham PJ (1988) Westslope cutthroat trout in Montana: life history, status and management. Status and management of interior stocks of cutthroat trout. Am Fish Soc Symp 4:53–60

May BE (2009) Westslope cutthroat trout status update summary. Wild Trout Enterprises LLC, Bozeman

McPhail JD, Lindsey CC (1986) Zoogeography of the freshwater fishes of Cascadia (the Columbia system and rivers north to the Stikine). In: Hocutt CH, Wiley EO (eds) The zoogeography of North American freshwater fishes. J Wiley and Sons, New York, pp 615–637

Montana Fisheries Information System (MFISH) (2011) Montana Department of Fish, Wildlife and Parks. http://fwp.mt.gov/fishing/mFish/ Accessed Jan 2011

Muhlfeld CC, Kalinowski ST, McMahon TE, Painter S, Leary RF, Taper ML, Allendorf FW (2009) Hybridization reduces fitness of cutthroat trout in the wild. Biol Lett 5:328–331

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Nielsen JL, Byrne A, Graziano SL, Kozfkay CC (2009) Steelhead genetic diversity at multiple spatial scales in a managed basin: Snake River, Idaho. T Am Fish Soc 29:680–701

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mole Ecol Notes 6:288–295

Pielou EC (1991) After the ice age. University of Chicago Press, Chicago

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249

R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

Rieman BE, Apperson KA (1989) Status and analysis of salmonid fisheries: westslope cutthroat trout synopsis and analysis of fishery information. Idaho Department of Fish and Game, Federal Aid in Sport Fish Restoration, Project F-73-R-11, Boise, Idaho

Rousset F (2008) GENEPOP'007: a complete reimplementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour 8:103–106

Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. Genetics 140:1413–1419

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sánchez JA, Clabby C, Ramos D, Blanco G, Flavin F, Vázquez E, Powell R (1996) Protein and microsatellite single locus variability in *Salmo salar* L. (Atlantic salmon). Heredity 77:423–432

Shepard BB, Sanborn B, Ulmer L, Lee DC (1997) Status and risk of extinction for westslope cutthroat trout in the upper Missouri River basin, Montana. N Am J Fish Manag 17:1158–1172

Shepard BB, May BE, Urie W (2005) Status and conservation of westslope cutthroat trout within the western United States. N Am J Fish Manag 25:1426–1440

Taylor EB, Stamford MD, Baxter JS (2003) Population subdivision in westslope cutthroat trout (*Oncorhynchus clarki lewisi*) at the northern periphery of its range: evolutionary inferences and conservation implications. Mol Ecol 12:2609–2622

USFWS (US Fish, Wildlife Service), NOAA (National Oceanic, Atmospheric Administration) (1996) Policy regarding the recognition of distinct vertebrate population segments under the Endangered Species Act. Fed Regist 61:4721–4725

Van Eimeren P (1996) Westslope cutthroat trout *Oncorhynchus clarki lewsi*. In: Duff DA (ed) Conservation assessment for inland cutthroat trout: distribution, status and habitat management implications. US Department of Agriculture, Forest Service, Intermountain Region, Ogden, Utah, pp 1–10

Vu NV, Kalinowski ST (2009) Twelve tetranucleotide microsatellite loci for westslope cutthroat trout *Oncorhynchus clarki lewisi* (Salmonidae). Conserv Genet Resour 1:249–251

Weir BS, Cockerham CC (1984) Estimating F-Statistics for the analysis of population structure. Evolution 38:1358–1370