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Dave;

We have analyzed the DNA extracted from fin clips from trout collected from the following locations:

Sample #	Water Name/Location/ Collection Date/ Collector	a N	b #Markers	c Taxa ID	d Power	e %	f # Fish
4618	**Rabbia Creek 45.53448 113.22359 7/9/2014 Jim Olsen**	**7(48)**	**R19W20Y20**	**WCT**	**R99Y99**		
4619	**Bender Creek 45.79534 113.71059 7/7/2014 Jim Olsen**	**12(13)**	**R19W20Y20**	**WCT**	**R99Y99**		
4621	**Jerry Creek 12t 355096 508731 6/30/2014 Jim Olsen**	**30**	**R19W20Y20**	**WCT X YCT**		**W99.2 X Y0.8**	
4622	**Whites Creek 26.619 111.474 6/11 & 16/14 Pat Clancey**	**59**	**R19W20Y20**	**WCT X YCT?**			

[a]Number of fish successfully analyzed. If combined with a previous sample, the number in parentheses indicates the combined sample size.
[b]Number of diagnostic loci analyzed for the taxon (R=rainbow trout *Oncorhynchus mykiss*, W=westslope cutthroat trout *O. clarkii lewisi*, Y=Yellowstone cutthroat trout *O. c. bouvieri*).
[c]Taxa: WCT = westslope cutthroat trout; RBT = rainbow trout; YCT = Yellowstone cutthroat trout . Only one taxon code is listed if the sample was considered to contain only individuals from it. However, we cannot definitely rule out the possibility that some or all

of the individuals are hybrids. We may not have detected any evidence of hybridization at the loci analyzed because of sampling error (see *d*). Taxa separated by "x" indicate hybridization between them was detected.
[d]Power: the number corresponds to the percent chance we have to detect 0.5% introgression in a hybrid swarm (a random mating population in which taxa markers are randomly distributed among individuals such that essentially all of them in the population are of hybrid origin) given the number of individuals and diagnostic markers analyzed. For example, with 12 individuals we have better than a 95 % chance to detect as little as a 0.5% rainbow (39 diagnostic loci) or Yellowstone cutthroat trout (40 diagnostic loci) genetic contribution to a hybrid swarm that once was a non-hybridized westslope cutthroat trout population Not reported when hybridization is detected. Taxa as in b.
[e]Indicates the genetic contribution of the hybridizing taxa (amount of admixture) denoted as in b. This number is usually reported only if the sample appears to have come from a hybrid swarm.
[f]Indicates the number of individuals with genetic characteristics corresponding to the taxa ID code column when the sample contains individuals from two or more genetically distinct groups.

Methods and Data Analysis

We developed a 'chip' specifically for analysis of westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) populations. This chip allows us to simultaneously genotype up to 95 single nucleotide polymorphic loci (SNPs) in 91 trout using a Fluidigm EP1 Genotyping System. Each SNP locus has only two states (alleles). Thus, considering hybridization among rainbow (*O. mykiss*), westslope cutthroat, and Yellowstone cutthroat trout (*O. c. bouvieri*) a single locus can only distinguish one of the taxa from the other two. In order to address hybridization issues among these fishes, therefore, each chip contained 19 loci that differentiate rainbow from westslope cutthroat and Yellowstone cutthroat trout (rainbow markers), 20 loci that distinguish westslope cutthroat from rainbow and Yellowstone cutthroat trout (westslope markers), and 20 loci that distinguish Yellowstone cutthroat from westslope cutthroat and rainbow trout (Yellowstone markers, Table 1). We verified the diagnostic property of each marker by analyzing them in reference samples that had previously been determined to be non-hybridized westslope cutthroat, Yellowstone cutthroat, or rainbow trout by analysis of allozymes, paired interspersed nuclear elements (PINEs), a combination of insertion/deletion (indel loci) events and microsatellite loci, or two or all of these techniques (Table 2).

If a sample possessed alleles characteristic of only westslope cutthroat trout at all westslope markers and had no alleles characteristic of rainbow trout at the rainbow markers or Yellowstone cutthroat trout at the Yellowstone markers, then it was considered to contain non-hybridized westslope cutthroat trout. Evidence for potential hybridization between rainbow and westslope cutthroat trout was generally considered to be present when three criteria were met. First, the sample had to contain alleles characteristic of rainbow trout at, at least, some of the rainbow markers. Next, at least some of the westslope markers also had to be genetically variable (polymorphic). Finally, no Yellowstone cutthroat trout alleles were detected at the Yellowstone markers. In this situation, the alleles at the rainbow markers shared between westslope cutthroat and Yellowstone cutthroat trout can confidently be assigned to having originated from westslope cutthroat trout and the alleles shared between rainbow and Yellowstone cutthroat trout at the westslope markers can confidently be assigned to having originated from rainbow trout. Thus, in terms of hybridization between westslope cutthroat and rainbow trout the data set contains information from 39 diagnostic loci. Likewise, when evidence of hybridization was detected only between westslope and Yellowstone cutthroat trout (no rainbow alleles at rainbow markers, at least some westslope markers polymorphic, and Yellowstone cutthroat trout alleles present at, at least, some Yellowstone markers) the data set contains information from 40 diagnostic loci. When all three sets of markers were polymorphic, this generally indicates hybridization among all three taxa. In this situation, the rainbow markers (19) provide information about rainbow trout hybridization and the Yellowstone markers (20) provide information about Yellowstone cutthroat trout hybridization.

An important aspect of SNPs is that they demonstrate a codominant mode of inheritance. That is, all genotypes are readily distinguishable from each other. Thus, at marker loci the genotype of individuals in a sample can directly be determined. From these data, the proportion of alleles from different taxa in the population sampled can be directly estimated at each marker locus analyzed. These values averaged over all

marker loci yields an estimate of the proportion of alleles in the population that can be attributed to one or more taxa (proportion of admixture). In samples showing evidence of hybridization among all three taxa, we estimated the amount of rainbow trout admixture using only the 19 rainbow markers and the amount of Yellowstone cutthroat trout admixture using only the 20 Yellowstone markers. The amount of westslope cutthroat trout admixture was then estimated by subtracting the sum of the former two values from one. We used this procedure so the estimates would sum to one. Because of sampling error, it is unlikely that all three estimates from the marker loci would sum to one.

When evidence of hybridization is detected, the next issue to address is whether or not the sample appears to have come from a hybrid swarm. That is, a random mating population in which the alleles of the hybridizing taxa are randomly distributed among individuals such that essentially all of them are of hybrid origin.

A common, but not absolute, attribute of hybrid swarms is that allele frequencies at marker loci are similar among them because their presence can all be traced to a common origin or origins. Thus, one criterion we used for the assessment of whether or not a sample appeared to have come from a hybrid swarm was whether or not the allele frequencies among diagnostic loci reasonably conformed to homogeneity using contingency table chi-square analysis.

In order to determine whether or not alleles at the marker loci were randomly distributed among the fish in a sample showing evidence of hybridization, we calculated a hybrid index for each fish in the sample. The hybrid index for an individual was calculated as follows. At each marker locus, an allele characteristic of the native taxon was given a value of zero and an allele characteristic of the non-native taxon a value of one. Thus, at a single diagnostic locus the hybrid index for an individual could have a value of zero (only native alleles present, homozygous), one (both native and non-native alleles present, heterozygous), or two (only non-native alleles present, homozygous). These values summed over all diagnostic loci analyzed yields an individual's hybrid index. Considering westslope cutthroat and rainbow trout, therefore, non-hybridized westslope cutthroat trout would have a hybrid index of zero, non-hybridized rainbow trout a hybrid index of 78, F_1 (first generation) hybrids a hybrid index of 39, and post F_1 hybrids could have values ranging from zero to 78. The distribution of hybrid indices among the fish in a sample was statistically compared to the expected random binomial distribution based on the proportion of admixture estimated from the allele frequencies at the diagnostic loci. If the allele frequencies appeared to be statistically homogeneous among the marker loci and the observed distribution of hybrid indices reasonably conformed to the expected random distribution, then the sample was considered to have come from a hybrid swarm.

In old or hybrid swarms with small effective population size, allele frequencies at marker loci can randomly diverge from homogeneity over time because of genetic drift. In this case, however, the observed distribution of hybrid indices is still expected to reasonably conform to the expected random distribution. Thus, if the allele frequencies were statistically heterogeneous among the marker loci in a sample but, the observed distribution of hybrid indices reasonably conformed to the expected random distribution the sample was also considered to have come from a hybrid swarm.

The strongest evidence that a sample showing evidence of hybridization did not come from a hybrid swarm is failure of the observed distribution of hybrid indices to reasonably conform to the expected random distribution. The most likely reasons for this are that the population has only recently become hybridized or the sample contains individuals from two or more populations with different amounts of admixture. At times, previous samples and the distribution of genotypes at marker loci and the observed distribution of hybrid indices can provide insight into which of the latter two factors appears mainly responsible for the nonrandom distribution of the alleles from the hybridizing taxa among individuals in the sample. At other times, the distribution of genotypes at marker loci and the observed distribution of hybrid indices may provide little or no insight into the cause of the nonrandom distribution of alleles among individuals. The latter situation is expected to be fairly common as the two factors usually responsible for the nonrandom

distribution of alleles are not necessarily mutually exclusive. Regardless of the cause, when alleles at the marker loci do not appear to be randomly distributed among individuals in a sample, estimating the amount of admixture has little if any biological meaning and, therefore, is generally not reported.

Failure to detect evidence of hybridization in a sample does not necessarily mean the population is non-hybridized because there is always the possibility that we would not detect evidence of hybridization because of sampling error. When no evidence of hybridization was detected in a sample, we assessed the likelihood the population is non-hybridized by determining the chances of not detecting as little as a 0.5 percent genetic contribution of a non-native taxon to a hybrid swarm. This is simply 0.995^{2NX} where N is the number of fish in the sample and X is the number of marker loci analyzed.

The chip also contained 34 loci that are generally polymorphic within westslope cutthroat trout populations. Information from these loci can be used to address issues concerning the relative amount of genetic variation within and divergence among westslope cutthroat trout populations.

Finally, the chip contained two mitochondrial DNA (mtDNA) loci that differentiate cutthroat and rainbow trout. Data from these loci were used only if an individual appeared to be an F_1 hybrid. Because mtDNA is inherited only from females (maternal inheritance), in this situation we can determine the taxon of the female, and by default the taxon of the male, that produced the hybrid.

Results and Discussion

Rabbia Creek (Spawners) 4618

In the sample from Rabbia Creek, no alleles characteristic of rainbow trout were detected at the rainbow markers, none of the westslope markers were polymorphic, and no alleles characteristic of Yellowstone cutthroat trout were detected at the Yellowstone markers analyzed. Previous allozyme (#1137, col. 8/24/95, T4(?)S R13W S4, N=7) and PINE (#2170, col. 8/2/01, T3S R13W S33, N=7; #2173, col. 8/1/01, T3S R13W S33 NW1/4 NW1/4, N=27) analyses also detected no evidence of hybridization in samples from Rabbia Creek. With the total of 1038 rainbow trout diagnostic alleles and 972 Yellowstone cutthroat trout diagnostic alleles analyzed, we had better than a 99 percent chance of detecting as little as a 0.5 percent rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm that once was non-hybridized westslope cutthroat trout. Rabbia Creek, therefore, very likely contains non-hybridized westslope cutthroat trout.

Bender Creek (Spawners) 4619

No alleles characteristic of rainbow trout were detected at the rainbow markers, none of the westslope markers were polymorphic, and no alleles characteristic of Yellowstone cutthroat trout were detected at the Yellowstone markers analyzed in the sample from Bender Creek. A previous allozyme analysis of a single fish (#1090, col. 7/26/95, T1N R17W S33) from Bender Creek also detected no evidence of hybridization. Because of the large number of SNP diagnostic loci analyzed, we had better than a 99 percent chance of detecting as little as a 0.5 percent rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm that once was non-hybridized westslope cutthroat trout. Thus, with the available data Bender Creek appears to contain non-hybridized westslope cutthroat trout.

Jerry Creek 4621

In the sample from Jerry Creek, two of the Yellowstone markers analyzed possessed alleles characteristic of Yellowstone cutthroat trout and six of the westslope markers were polymorphic. No alleles characteristic of rainbow trout were detected at the rainbow markers analyzed. Although the Yellowstone cutthroat trout allele frequencies were statistically heterogeneous (X^2_{39}=96.000, P<0.001) among the diagnostic loci, the

Yellowstone cutthroat trout alleles appeared to be randomly distributed (X^2_3=7.530, P>0.05) among the fish in the sample. This sample, therefore, appears to have come from a hybrid swarm between westslope and Yellowstone cutthroat trout with a predominant (0.992) westslope cutthroat trout genetic contribution.

Jerry Creek has now been sampled six times with highly variable results. Initial allozyme analyses (#874, col. 10/5/93, T2N R10W S21 SE1/4 SE1/4, N=8; #1190, col. 10/24/96, T1N R10W S8, N=10) suggested the fish were non-hybridized westslope cutthroat trout. The next allozyme analysis (#2856, col. 7/18/99, N=5) gave some indication that the fish might be slightly hybridized with Yellowstone cutthroat trout. Subsequently fish collected below the confluence of Flume Creek (#4149, col. 7/7/10, 45.91358 112.85498, Indel analysis, N=25) mainly appeared to have come from a hybrid swarm between westslope cutthroat and rainbow trout with a predominant (0.994) westslope cutthroat trout genetic component. Another fish in the sample was also of hybrid origin between westslope cutthroat and rainbow trout with a higher amount of admixture. Indel analysis of fish (#4150. col. 7/7/10, 45.922757 112.875898, N=35) collected above a culvert above the confluence with Flume Creek indicated the fish were non-hybridized westslope cutthroat trout. Thus, it appears that the genetic characteristics of the trout in Jerry Creek vary both temporally and spatially. Because of these attributes, they probably do not represent a good source of fish or gametes for westslope cutthroat trout conservation or restoration purposes.

Whites Creek 4622

Fish were collected on two days during June 2014 from Whites Creek. We used the log likelihood G test of Goudet et al. (1996) in GENEPOP version 4.0 (Rousset 2008) to determine if there was evidence of allele frequency differences between the two collections. Since multiple comparisons were made between the two samples, we accounted for the possibility that a significant difference may simply represent a chance departure from homogeneity using Rice's (1989) correction for multiple comparisons (modified level of significance). There were no significant allele frequency differences at the nine polymorphic loci between the samples. Since there was no evidence of genetic differences between the samples, they were combined for subsequent analysis.

No alleles characteristic of rainbow trout were detected at the rainbow markers and none of the westslope markers were polymorphic in the sample from Whites Creek. In contrast, alleles characteristic of Yellowstone cutthroat trout were detected at two of the Yellowstone markers analyzed. *OclYGD100974_Garza* possessed two Yellowstone alleles each in a different individual. The other Yellowstone allele was detected at *OclYSD107607_Garza* and was present in a third individual. The presence of these alleles could indicate a very small amount (0.001) of hybridization with Yellowstone cutthroat trout or they could simply represent westslope cutthroat trout genetic variation. Unfortunately, in this situation we cannot distinguish between these possibilities.

We have not detected the Yellowstone alleles at *OclYGD100974_Garza* and *OclYSD107607_Garza* in other populations that otherwise appear to be non-hybridized westslope cutthroat trout. This observation lends some support to the hybridization hypothesis.

Whites Creek has been sampled seven previous times (#3245, col. 9/8/05, 46.619 111.477, N=50, PINE analysis; #3295, col. 6/12/06, 46.619 111.474, N=31, PINE analysis; #3445, col. 6/12/07, 46.619 111.474, N=24, Indel analysis; #3709, col. 6/11/08, 46.619 111.474, N=54, Indel analysis; #3916, col. 6/10/09, 46.61903 111.47739. N=57, Indel analysis; #4241, col. 6/22/11, 46.61906 111.47946, N=12, Indel analysis; #4501, col. 6/6/13, 46.6202 111.4738, N=24, SNP analysis). All of these analyses indicated the fish were non-hybridized westslope cutthroat trout. With the sample size of only 24 in the only other sample from Whites Creek from which we have SNP data, there was a good chance the "Yellowstone" alleles at *OclYGD100974_Garza* (X^2_1=0.827, P>0.10) and *OclYSD107607_Garza* (X^2_1=0.411, P>0.50) detected in

the present sample would not have been included in the prior sample because of sampling error. These observations, therefore, lend some support to the westslope cutthroat trout genetic variation interpretation.

Thus, we are uncertain about the present status of the trout in Whites Creek although we tend to favor the westslope cutthroat trout genetic variation interpretation. With this uncertainty, conservatively we would not introduce the fish produced from those spawned from Whites Creek into Cherry Lake.

Robb Leary

Sally Painter

Angela Lodmell
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Table 1

SNP loci that differentiate rainbow from westslope and Yellowstone cutthroat trout (rainbow markers), westslope cutthroat from rainbow and Yellowstone cutthroat trout (westslope markers), and Yellowstone cutthroat from westslope cutthroat and rainbow trout (Yellowstone markers).

Rainbow Markers			Reference
	Taxa and characteristic alleles		
Locus	Rainbow	Westslope/Yellowstone	
OmyRD_RAD_29252_Hoh	11	22	Amish et al. 2012
OmyRD_RAD_77157_Hoh	11	22	Amish et al. 2012
OmyRD_RAD_30378_Hoh	11	22	Amish et al. 2012
OclRD_P53T7R1_Har	11	22	Harwood and Phillips 2011
OmyRD_RAD_30423_Hoh	11	22	Amish et al. 2012
OmyRD_RAD_59515_Hoh	11	22	Amish et al. 2012
OclRD_Thymo_320Kal	11	22	Kalinowski et al. 2011
OmyRD_RAD_48301_Hoh	11	22	Amish et al. 2012
OmyRD_RAD_49759_Hoh	11	22	Amish et al. 2012
OclRD_P53T7R2_Har	11	22	Harwood and Phillips 2011
OmyRD_URO_302May	11	22	Finger et al. 2009
OmyRD_RAD_20663_Hoh	22	11	Amish et al. 2012
OmyRD_RAD_51740_Hoh	11	22	Amish et al. 2012
OmyRD_RAD_22111_Hoh	22	11	Amish et al. 2012
OmyRD_RAD_55820_Hoh	22	11	Amish et al. 2012
OmyRD_RAD_5666_Hoh	11	22	Amish et al. 2012
OmyRD_F5_136May	22	11	Finger et al. 2009
OmyRD_RAD_42014_Hoh	22	11	Amish et al. 2012
OmyRD_RAD_54584_Hoh	22	11	Amish et al. 2012
Westslope Markers			
	Taxa and characteristic alleles		
Locus	Westslope	Rainbow/Yellowstone	
OclRD_CLK3W5_Har	22	11	Harwood and Phillips 2011
OclWD_CLK3W1_Har	22	11	Harwood and Phillips 2011
OclWD101119_Garza	22	11	Campbell et al. 2012
OmyWD_RAD_76689_Hoh	22	11	Amish et al. 2012
OclWD_114315L _Garza	22	11	Campbell et al. 2012
OclWD_Tnsf_387Kal	22	11	Kalinowski et al. 2011
OmyWD_RAD_55391_Hoh	22	11	Amish et al. 2012
OclWD_P53_307Kal	22	11	Kalinowski et al. 2011
OclWD111312_Garza	22	11	Campbell et al. 2012
OclWD_107031L _Garza	22	11	Campbell et al. 2012
OclWD_PrLcW1_Har	22	11	Harwood and Phillips 2011
OmyWD_RAD_54516_Hoh	22	11	Amish et al. 2012
OclWD_105075L_Garza	22	11	Campbell et al. 2012
OmyWD_RAD_52968_Hoh	22	11	Amish et al. 2012
OclWD114336_Garza	11	22	Campbell et al. 2012
OclWD103713_Garza	22	11	Campbell et al. 2012
OclWD107074_Garza	22	11	Campbell et al. 2012
OclWD109651_Garza	22	11	Campbell et al. 2012
OclWD_129170L _Garza	11	22	Campbell et al. 2012
OclWD_ppie_32NC	11	22	Campbell et al. 2012

Table 1-continued

Locus	Yellowstone Markers Taxa and characteristic alleles		Reference
	Yellowstone	Westslope/Rainbow	
OclYD_CLK3Y1_Har	22	11	Harwood and Phillips 2011
OclYGD100974_Garza	22	11	Campbell et al. 2012
OclYGD110571_Garza	22	11	Campbell et al. 2012
OclYSD117432_Garza	22	11	Campbell et al. 2012
OclYGD1127236_Garza	22	11	Campbell et al. 2012
OclYGD112820_Garza	22	11	Campbell et al. 2012
OclYGD104216_Garza	22	11	Campbell et al. 2012
OclYGD113600_Garza	22	11	Campbell et al. 2012
OclYSD129870_Garza	22	11	Campbell et al. 2012
OclYGD104569_Garza	22	11	Campbell et al. 2012
OclYGD117286_Garza	22	11	Campbell et al. 2012
OclYGD117370_Garza	22	11	Campbell et al. 2012
OclYSD107607_Garza	22	11	Campbell et al. 2012
OclYGD106457_Garza	22	11	Campbell et al. 2012
OclYSD106367_Garza	11	22	Campbell et al. 2012
OclYGD107031_Garza	11	22	Campbell et al. 2012
OclYGD106419_Garza	11	22	Campbell et al. 2012
OclYSD123205_Garza	11	22	Campbell et al. 2012
OclYGD109525_Garza	11	22	Campbell et al. 2012
OclYSD113109_Garza	11	22	Campbell et al. 2012

Table 2

Reference samples used for the identification of marker SNPs among westslope cutthroat, rainbow, and Yellowstone cutthroat trout. Taxa: WCT=westslope cutthroat trout, YCT=Yellowstone cutthroat trout, IRT=redband trout, CRT=coastal rainbow trout. N=sample size.

Sample	Taxa	N	Location
Washoe Park State Trout Hatchery	WCT	12	Anaconda, Montana
Big Foot Creek	WCT	2	Upper Kootenai River, Montana
Runt Creek	WCT	3	Yaak River, Montana
Hawk Creek	WCT	2	North Fork Flathead River, Montana
Werner Creek	WCT	3	North Fork Flathead River, Montana
Morrison Creek	WCT	3	Middle Fork Flathead River, Montana
Sixmile Creek	WCT	3	Swan River, Montana
South Fork Jocko River	WCT	3	Lower Flathead River, Montana
Cottonwood Creek	WCT	3	Upper Clark Fork River, Montana
Copper Creek	WCT	2	Flint-Rock Creek, Montana
Gillispie Creek	WCT	3	Flint-Rock Creek, Montana
Davis Creek	WCT	4	Bitterroot River, Montana
Humbug Creek	WCT	2	Blackfoot River, Montana
Ringeye Creek	WCT	2	Blackfoot River, Montana
Flat Creek	WCT	3	Middle Clark Fork River, Montana
McGinnis Creek	WCT	3	Lower Clark Fork River, Montana
Bear Creek	WCT	1	Red Rock River, Montana
McVey Creek	WCT	1	Big Hole River, Montana
McClellan Creek	WCT	1	Upper Missouri River, Montana
Yellowstone River State Trout Hatchery-Goose Lake	YCT	6	Big Timber, Montana
Slough Creek	YCT	4	Yellowstone River, Montana
Lake Koocanusa	IRT	4	Upper Kootenai River, Montana
North Fork Yahk River	IRT	5	Yahk River, British Columbia
Jocko River State Trout Hatchery Arlee Rainbow	CRT	7	Arlee, Montana