Reference # 701028
Repository # 82626

University of Montana Conservation Genetics Laboratory
Division of Biological Sciences, University of Montana, Missoula, Montana 59812
Phone (406) 243-6749 or 6725; Fax (406) 243-4184

July 17, 2017

Anne Tews
Montana Fish, Wildlife & Parks
Butte Area Resource Office
Butte, Montana 59701

Anne

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

		a	b	c	d	e	f
Sample	**Water Name/Location/**	**N**	**#Markers**	**Taxa ID**	**Power**	**%**	**# Fish**
#	**Collection Date/**						
	Collector						
4863	**Big Coulee Creek**	**32**	**R19W20Y20**	**WCT**	R99Y99		
	47.42920--42304						
	110.57418-55746						
	6/27/2016						
	Mike Schilz						

[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 are of hybrid origin) given the number of individuals and diagnostic markers analyzed. For example, with 13 individuals we have better than a 99 % chance to detect as little as a 0.5% rainbow (38 diagnostic loci) or westslope cutthroat trout (36 diagnostic loci) genetic contribution to a hybrid swarm that once was a non-hybridized Yellowstone 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 Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) 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 (*O. c. lewisi*), and Yellowstone cutthroat trout 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), 17 loci that distinguish westslope cutthroat from rainbow and Yellowstone cutthroat trout (westslope markers), and 19 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 Yellowstone cutthroat trout at all Yellowstone markers and had no alleles characteristic of rainbow trout at the rainbow markers or westslope cutthroat trout at the westslope markers, then it was considered to have come from a non-hybridized Yellowstone cutthroat trout population. Evidence for potential hybridization between rainbow and Yellowstone 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 Yellowstone markers also had to be genetically variable (polymorphic). Finally, no westslope cutthroat trout alleles were detected at the westslope 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 Yellowstone cutthroat trout and the alleles shared between rainbow and westslope cutthroat trout at the Yellowstone markers can confidently be assigned to having originated from rainbow trout. Thus, in terms of hybridization between Yellowstone cutthroat and rainbow trout the data set contains information from 38 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 Yellowstone markers polymorphic, and westslope cutthroat trout alleles present at, at least, some westslope markers) the data set contains information from 36 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 westslope markers (17) provide information about westslope cutthroat trout hybridization.

An important aspect of SNPs is that they demonstrate a codominant mode of inheritance. That is, all genotypes are 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 westslope cutthroat trout admixture using only the 17 westslope markers. The amount of Yellowstone 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. 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 Yellowstone cutthroat and rainbow trout, therefore, non-hybridized Yellowstone cutthroat trout would have a hybrid index of zero, non-hybridized rainbow trout a hybrid index of 76, F_1 (first generation) hybrids a hybrid index of 38, and post F_1 hybrids could have values ranging from zero to 76. 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 numerically small hybrid swarms, 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, the distribution of genotypes at marker loci and the observed distribution of hybrid indices can provide insight into which of these two factors appears mainly responsible for the non-random 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 non-random distribution of alleles among individuals. The latter situation is expected to be fairly common as the two factors usually responsible for the non-random 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 40 loci that are generally polymorphic within Yellowstone cutthroat trout populations. Information from these loci can be used to address issues concerning the relative amount of genetic variation within and divergence among Yellowstone cutthroat trout populations.

When two or more samples were collected from the same area in different years or different reaches of a stream, we used the log likelihood G test of Goudet et al. (1996) in GENEPOP version 4.0 (Rousset 2008) to test for genetic differences among the samples. In instances where multiple loci were compared among samples and some demonstrated significant differences, significance was determined using Rice's (1989) method for correcting for multiple comparisons (modified level of significance). When no differences were detected at the modified level, any observed differences were considered to most likely represent chance departures from homogeneity and the samples were combined for further analysis. When evidence of genetic differences was detected between samples they were kept separate for analysis and the relative amount of divergence between them was estimated as F_{ST} using the method of Weir and Cockerham (1984) available in GENEPOP version 4.0.

In samples containing 10 or more individuals appearing to have come from non-hybridized Yellowstone cutthroat trout populations, we compared the observed to the expected random mating genotypic proportions (Hardy-Weinberg proportions) at the polymorphic loci using the Markov Chain method of Guo and Thompson (1992) available in GENEPOP version 4.2. A deficit of observed heterozygotes can arise in a sample if it contains individuals from two or more genetically divergent populations or is experiencing a fair to high amount of inbreeding. Conversely, a population produced from a very small number of parents may show an excess of heterozygotes compared to expected random mating proportions (Pudovkin et al. 1996, 2010; Luikart and Cornuet 1999). Since multiple comparisons were performed in most cases, significance was again determined at the modified level. In cases showing significant departures from expected Hardy- Weinberg genotypic proportions because of a tendency for there to be either a deficit or excess of heterozygotes, we used the program ML-RELATE of Kalinowski et al. (2006) to estimate the degree of relationship among the fish in the sample as this could possibly provide some insight into the cause for the deviations.

Results and Discussion

Big Coulee Creek 4863

In the sample from Big Coulee Creek, no alleles characteristic of rainbow trout were detected at all the rainbow markers, only alleles characteristic of westslope cutthroat trout were detected at all the westslope markers, and no alleles characteristic of Yellowstone cutthroat trout were detected at all the Yellowstone markers analyzed. Big Coulee Creek was previously sampled and PINE analysis (#2149, col, 8/17/02,T20N R8E S9, N=40) also detected no evidence of hybridization in the fish. With the combined sample and the two techniques used there were the 2,976 diagnostic rainbow and 2,880 diagnostic Yellowstone cutthroat trout alleles analyzed, we had better than a 99.99% chance of detecting as little as a 0.5% rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm that once was non-hybridized westslope cutthroat trout. The samples from Big Coulee Creek, therefore, almost certainly contained only non-hybridized westslope cutthroat trout.

Robb Leary

Andrew Whiteley

Sally Painter

Angela Lodmell

.

Literature Cited

Amish, S. J., P. A. Hohenlohe, S. Painter, R. F. Leary, C. Muhlfeld, F. W. Allendorf, and G. Luikart. 2012. RAD sequencing yields a high success rate for westslope cutthroat and rainbow trout species-diagnostic SNP assays. Molecular Ecology Resources 12:653-660.

Campbell, N. R., S. J. Amish, V. L. Pritchard, K. S. McKelvey, M. K. Young, M. K. Schwartz, J. C. Garza, G. Luikart, and S. R. Narum. 2012. Development and evaluation of 200 novel SNP assays for population genetic studies of westslope cutthroat trout and genetic identification of related taxa. Molecular Ecology Resources 12:942-949.

Finger, J. A., M. R. Stephens, N. W. Clipperton, and B. May. 2009. Six diagnostic single nucleotide polymorphism markers for detecting introgression between cutthroat and rainbow trouts. Molecular Ecology Resources 9:759-763.

Goudet, J., M. Raymond, T. deMeeus, and F. Rousset. 1996. Testing differentiation in diploid populations.
Genetics 144:1933-1940.

Guo, S. W. and E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics 48:361-372.

Harwood, A. S., and R. B. Phillips. 2011. A suite of twelve single nucleotide polymorphism markers for detecting introgression between cutthroat and rainbow trout. Molecular Ecology Resources 11:382-385.

Kalinowski, S. T., B. J. Novak, D. P. Drinan, R. deM Jennings, and N. V. Vu. 2011. Diagnostic single nucleotide polymorphisms identifying westslope cutthroat trout (*Oncorhynchus clarkii lewisi*), Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) and rainbow trout (*Oncorhynchus mykiss*).
Molecular Ecology Resources 11:389-393.

Kalinowski, S. T., A. P. Wagner, and M. L. Taper. 2006. ML-RELATE: a computer program for maximum likelihood estimation of relatedness and relationship. Molecular Ecology Notes 6:576-579.

Luikart, G. and J.-M. Cornuet. 1999. Estimating the effective number of breeders from heterozygote excess in progeny. Genetics 151:1211-1216.

Pudovkin, A. I., O. L. Zhdanova, and D. Hedgecock. 1996. On the potential for estimating the effective number of breeders from heterozygote excess in progeny. Genetics 144:383-387.

Pudovkin, A. I., O. L. Zhdanova, and D. Hedgecock. 2010. Sampling properties of the heterozygote-excess estimator of the effective number of breeders. Conservation Genetics 11:759-771.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223-225.

Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Window and Linux. Molecular Ecology Resources 8:103-106.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 28:1358-1370.

Table1

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 trout from westslope cutthroat and rainbow trout (Yellowstone markers).

Rainbow Markers

	Taxa and characteristic alleles		Reference
	Rainbow	Westslope/Yellowstone	
OmyRD_RAD_29252_Hoh	1	2	Amish et al. 2012
OmyRD_RAD_77157_Hoh	1	2	
OmyRD_RAD_30378_Hoh	1	2	Amish et al. 2012
OclRD_P53T7R1_Har	1	2	Amish et al. 2012
OmyRD_RAD_30423_Hoh	1	2	Harwood and Phillips 2011
OmyRD_RAD_59515_Hoh	1	2	Amish et al. 2012
OclRD_Thymo_320Kal	1	2	Amish et al. 2012
OmyRD_RAD_48301_Hoh	1	2	Kalinowski et al. 2011
OmyRD_RAD_49759_Hoh	1	2	Amish et al. 2012
OclRD_P53T7R2_Har	1	2	Amish et al. 2012
OmyRD_URO_302May	1	2	Harwood and Phillips 2011
OmyRD_RAD_20663_Hoh	1	2	Finger et al. 2009
OmyRD_RAD_51740_Hoh	2	1	Amish et al. 2012
OmyRD_RAD_22111_Hoh	1	2	Amish et al. 2012
OmyRD_RAD_55820_Hoh	2	1	Amish et al. 2012
OmyRD_RAD_5666_Hoh	2	1	Amish et al. 2012
OmyRD_F5_136May	1	2	Amish et al. 2012
OmyRD_RAD_42014_Hoh	2	1	Finger et al. 2009
OmyRD_RAD_54584_Hoh	2	1	Amish et al. 2012

Table 1-continued

Westslope Markers

	Taxa and characteristic alleles		Reference
	Westslope	Rainbow/Yellowstone	
OclWD_CLK3W5_Har	2	1	Harwood and Phillips 2011
OclWD_CLK3W1_Har	2	1	Harwood and Phillips 2011
OclWD101119_Garza	2	1	Campbell et al. 2012
OmyWD_RAD_76689_Hoh	2	1	Amish et al. 2012
OclWD_114315L _Garza	2	1	Campbell et al. 2012
OclWD_Tnsf_387Kal	2	1	Kalinowski et al. 2011
OmyWD_RAD_55391_Hoh	2	1	Amish et al. 2012
OclWD_P53_307Kal	2	1	Kalinowski et al. 2011
OclWD111312_Garza	2	1	Campbell et al. 2012
OclWD_107031L _Garza	2	1	Campbell et al. 2012
OclWD_PrLcW1_Har	2	1	Harwood and Phillips 2011
OmyWD_RAD_54516_Hoh	2	1	Amish et al. 2012
OclWD_105075L_Garza	2	1	Campbell et al. 2012
OmyWD_RAD_52968_Hoh	2	1	Amish et al. 2012
OclWD_114336_Garza	1	2	Campbell et al. 2012
OclWD103713_Garza	2	1	Campbell et al. 2012
OclWD107074_Garza	2	1	Campbell et al. 2012
OclWD109651_Garza	2	1	Campbell et al. 2012
OclWD_129170L _Garza	1	2	Campbell et al. 2012
OclWD_ppie_32NC	1	2	Campbell et al. 2012

Table 1-continued

Yellowstone Markers

	Taxa and characteristic alleles		Reference
	Yellowstone	Westslope/Rainbow	
OclYD_CLK3Y1_Har	2	1	Harwood and Phillips 2011
OclYGD100974_Garza	2	1	Campbell et al. 2012
OclYGD110571_Garza	2	1	Campbell et al. 2012
OclYSD117432_Garza	2	1	Campbell et al. 2012
OclYGD127236_Garza	2	1	Campbell et al. 2012
OclYGD112820_Garza	2	1	Campbell et al. 2012
OclYGD104216_Garza	2	1	Campbell et al. 2012
OclYGD113600_Garza	2	1	Campbell et al. 2012
OclYSD129870_Garza	2	1	Campbell et al. 2012
OclYGD104569_Garza	2	1	Campbell et al. 2012
OclYGD117286_Garza	2	1	Campbell et al. 2012
OclYGD117370_Garza	2	1	Campbell et al. 2012
OclYSD107607_Garza	2	1	Campbell et al. 2012
OclYGD106457_Garza	2	1	Campbell et al. 2012
OclYSD106367_Garza	1	2	Campbell et al. 2012
OclYGD107031_Garza	1	2	Campbell et al. 2012
OclYGD106419_Garza	1	2	Campbell et al. 2012
OclYSD123205_Garza	1	2	Campbell et al. 2012
OclYGD109525_Garza	1	2	Campbell et al. 2012
OclYSD113109_Garza	1	2	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 rainbow 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