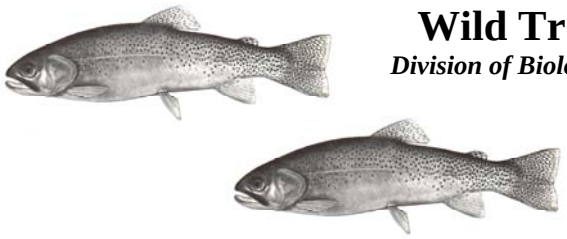

Wild Trout and Salmon Genetics Laboratory

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April 8, 2005

Scott Rumsey
Genetics Contact, Region 1
Mt. Dept. of Fish, Wildlife, and Parks
490 N. Meridian
Kalispell, MT 59901

Dear Scott:

Protein electrophoretic analysis of 15 fish and the paired interspersed nuclear DNA elements (PINE) technique using DNA extracted from these individuals and an additional 25 fin clips has been used to analyze the following trout samples from four streams in the South Fork Flathead River drainage:

Summary of results.

		a	b	c	d	e	f
Sample #	**Water Name/Location/Collection Date/ Collector**	**N**	**# markers**	**Species ID**	**Power (%)**	**% WCT**	**Individuals**
2980	**Addition Creek** 8/10/2004 Scott Rumsey	40	R6Y4	WCT	99	100	xx
2981	**Addition Creek** 8/10/2004 Scott Rumsey	15	R10Y8	WC	99	100	xx
2982	**Goldie Creek** 8/9/2004 Scott Rumsey	40	R6Y4	WCT	99	100	xx
2983	**Goldie Creek** 8/9/2004 Scott Rumsey	15	R10Y8	WCT	99	100	xx
2984	**South Creek** 8/10/2005 Scott Rumsey	40	R6Y4	WCT	99	100	xx
2985	**South Creek** 8/10/2005 Scott Rumsey	15	R10Y8	WCT	99	100	xx
2986	**Upper Twin Creek** 8/10/2004 Scott Rumsey	40	R6Y4	WCT	99	100	xx
2987	**Upper Twin Creek** 8/10/2004 Scott Rumsey	15	R10Y8	WCT	99	100	xx

[a]Number of fish successfully analyzed. If combined with a previous sample (Indicated in "Location" column), the number indicates the combined sample size. If present, the number in () is the average number of individuals successfully analyzed per locus (some individuals do not amplify for all marker loci).
[b]Number of markers analyzed that are diagnostic for the non-native species (R=rainbow trout, W=westslope cutthroat trout, Y=Yellowstone cutthroat trout).
[c]Codes: WCT = westslope cutthroat trout (*Oncorhynchus clarki lewisi*); RBT = rainbow trout (*O. mykiss*); YCT = Yellowstone cutthroat trout (*O. clarki bouvieri*). Only one species code is listed when the entire sample possessed alleles from that species only. However, it must be noted that we cannot definitively rule out the possibility that some or all of the individuals are hybrids. We may not have detected any non-native alleles at the loci examined because of sampling error (see Power %). Species codes separated by "x" indicate hybridization between those species.
[d]Number corresponds to the percent chance we have to detect 1% hybridization given the number of individuals successfully analyzed and the number of diagnostic markers used. For example, 25 individuals are required to yield a 97% chance to detect 1% hybridization with rainbow or an 87% chance to detect 1% hybridization with Yellowstone cutthroat trout into what once was a westslope cutthroat trout population. Not reported when hybridization is detected.
[e]Indicates the genetic contribution of the hybridizing taxa in the order listed under c to the sample assuming Hardy-Weinburg proportions. This number is reported if the sample appears to have come from a hybrid swarm. That is, a random mating population in which species markers are randomly distributed among individuals.
[f]Indicates number of individuals with genetic characteristics corresponding to the species code column when the sample can be analyzed on the individual level. This occurs when marker alleles are not randomly distributed among individuals and hybridization appears to be recent and/or if the sample appears to consist of a mixture of populations.

Methods and Data Analysis

The PINE technique uses short synthetically made segments of DNA called primers, in pairs, to search for relatively small segments of organismal DNA flanked by particular, often viral, DNA inserts. During the polymerase chain reaction (PCR), the primers bind to the ends of the inserts and many copies of the organismal DNA between the primers are made. While the DNA from some organisms may have two appropriately spaced inserts to which the primers can attach, the DNA from other organisms may have only one or none of the appropriately spaced inserts in particular regions. During PCR we will fail to copy DNA in the latter two cases. Thus, the PINE technique coupled with PCR is used to search for evidence of genetic variation based on the presence or absence of particular DNA fragments. The fragments are labeled by the primers used to produce them and their length in terms of the number of nucleotides in the fragment.

The fragments are made using dye labeled nucleotides and after PCR are separated from each other via electrophoresis in polyacrylamide gels. Smaller fragments move through the gels at a faster rate than larger fragments. The use of dye labeled nucleotides allows one to visualize the position of the fragments in the gels after electrophoresis using a spectrophotometer and the size of the fragments is determined by comparison to the position of synthetic fragments of known size that were also migrated into the gel.

When DNA from westslope cutthroat trout, *Oncorhynchus clarki lewisi,* and rainbow trout, *O. mykiss,* is compared with PINE analysis and three different pairs of primers seven fragments are characteristic of westslope cutthroat trout and six fragments are usually characteristic of rainbow trout (Table 1). Likewise, when DNA from westslope and Yellowstone cutthroat trout, *O. c. bouvieri*, is compared using the same procedure one fragment is characteristic of westslope cutthroat trout and four fragments are characteristic of Yellowstone cutthroat trout (Table 1).

Fragments produced from the DNA of one taxon and not another are commonly termed diagnostic or marker loci because they can be used to help determine whether a sample came from a non-hybridized population of one of the taxa or a population in which hybridization between them has or is occurring. Individuals from a non-hybridized population will possess fragments characteristic of only that taxon. In contrast, since half the DNA of first generation hybrids comes from each of the parental taxa the DNA from such individuals will yield all the fragments characteristic of the two parental taxa. In later generation hybrids, the amount and particular regions of DNA acquired from the parental taxa will vary among individuals. Thus, DNA from later generation hybrid

individuals will yield only a subset of the parental fragments and the particular subset will vary among individuals. In a sample from a random mating hybrid swarm, that is a population in which the genetic material (i.e. fragments) of the parental taxa is randomly distributed among individuals such that essentially all of them are of hybrid origin, the frequency of the fragment producing allele from the non-native taxon is expected to be nearly equal among the diagnostic loci since their presence can all be traced to a common origin or origins. Thus, if a sample contains substantial variation at only a single marker locus where the presence of the fragment is usually characteristic of a non-native taxon and lacks such fragments at all other markers this is probably not indicative of hybridization. Rather, it much more likely represents the existence of genetic variation for the presence or absence of the fragment within this particular population of the native taxon.

An important aspect of PINE marker loci is that individuals homozygous for the presence allele (*pp*) or heterozygous (*pa*) will both yield the fragment. That is, *p* is dominant to *a*. Thus, in order to estimate the genetic contribution of the native taxon to a hybrid swarm we concentrate on the marker loci at which the *p* allele is characteristic of the non-native taxon. Furthermore, we must assume that genotypic distributions in the population reasonably conform to expected random mating proportions. Under this assumption the frequency of the native *a* allele is approximately the square root of the frequency of individuals in the population lacking the fragment (*aa).* The frequency of the non-native allele then is one minus this value. We focus on the *p* alleles characteristic of the non-native taxon because with low levels of hybridization it is the presence of these alleles that are likely to provide evidence of hybridization. With low levels of hybridization, it is likely all individuals in the sample will genotypically be *pp* or *pa* where the *p* allele is characteristic of the native taxon. Thus, like in non-hybridized populations all individuals in the sample will yield the fragment providing no evidence of hybridization.

In addition horizontal starch gel electrophoresis was used to determine each fishes genetic characteristics (genotype) at 44 loci (genes) coding for proteins present in eye, liver, or muscle tissue (Table 2). At some of these loci the westslope cutthroat trout and rainbow trout rarely share alleles (form of a gene) in common (Table 3). This situation also pertains to a comparison of westslope and Yellowstone cutthroat trout (Table 3). Loci at which such fixed genetic differences exist between taxa are commonly termed diagnostic loci because the alleles detected at them can be used to help determine whether a sample came from a non-hybridized population of one of these fishes or a population in which hybridization between two or all three of them has or is occurring. A non-hybridized population will possess alleles at all loci characteristic of only that taxon. Hybridized populations on the other hand will possess alleles characteristic of the hybridizing taxa at two or more diagnostic loci.

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. In order to assess the likelihood the population is non-hybridized, we determine the chances of not detecting as little as a one percent genetic contribution of a non-native taxon to a hybrid swarm. This is simply 0.99^{2NX} where N is the number of fish in the sample and X is the number of marker loci where the *p* allele is characteristic of the non-native taxon.

In samples showing evidence of hybridization, that is; fragments characteristic of a non-native taxon were detected at two or more marker loci, we used two approaches to determine if the population appeared to be a hybrid swarm. First, contingency table chi-square analysis was used to test for heterogeneity of allele frequencies among the marker loci. Next, we compared the observed distribution of the number of non-native protein alleles per individual and the number of loci per individual at which non-native PINE fragments were detected to the expected random binomial distribution based on the estimated native and non-native genetic contributions to the population. If both analyses were non-significant we concluded the population came from a hybrid swarm.

Heterogeneity of allele frequencies among marker loci can arise in very old hybrid swarms as the frequencies over time diverge from each other due to genetic drift. In this case, however, the non-native fragments and protein alleles will still be randomly distributed among individuals.

There are two likely reasons why a non-random distribution of non-native fragments and protein alleles may be observed among individuals in a sample. It may contain individuals from genetically divergent populations with different amounts of hybridization or hybridization may have only recently occurred in the population. Based on genetic data alone, these two situations will generally be difficult to distinguish from each other. Regardless of the explanation, when the non-native fragments and protein alleles are not randomly distributed among individuals in a sample estimating a mean level of hybridization has little, if any, biological meaning and, therefore, is often not estimated.

Results and Discussion

Addition Creek (2980 and 2981), Goldie Creek (2982 and 2983), South Creek (2984 and 2985), and Upper Twin Creek (2986 and 2987)

PINE fragments and protein alleles (Table 4) characteristic of only westslope cutthroat trout were detected in all the samples. Considering both data sets, we have better than a 99% chance of detecting as little as a one percent rainbow or Yellowstone cutthroat trout genetic contribution to each population. These populations, therefore, are almost undoubtedly non-hybridized westslope cutthroat trout and would be suitable sources for crossing with the Montana Department of Fish, Wildlife, and Parks captive westslope cutthroat trout broodstock (MO12).

Sincerely,

Ben Wright
Robb Leary

TABLE 1

Diagnostic PINE markers for westslope cutthroat, Yellowstone cutthroat, and rainbow trout. **X** indicates the fragment is present in the particular taxon.

Markers	Yellowstone	Westslope	Rainbow
Hpa1 5'/Hpa1 3'			
232	x		
153		x	
72	x	x	
70			x
69	x	x	
66			x
Fok1 5'/Tc1			
369			x
366	x	x	
230			x
159	x		
138	x		
110		x	
Hpa1 5'/33.6+2			
395			x
388	x	x	
266			x
248	x		
148	x	x	

Table 2

Enzymes and loci examined. Tissues: E=eye, L=liver, M=muscle.

Enzyme	Loci	Tissue
Adenylate Kinase	*AK-1*, AK-2**	M
Alcohol Dehydrogenase	*ADH**	L
Aspartate Aminotransferase	*sAAT-1*, sAAT-2**	L
	*sAAT-3,4**	M
Creatine Kinase	*CK-A1*, CK-A2**	M
	CK-B, CK-C1*, CK-C2**	E
Dipeptidase	*PEPA-1*, PEPA-2**	E
N-acetyl-beta-Glucosaminidase	*bGLUA**	L
Glucose-6-phosphate Isomerase	*GPI-A**	E
	GPI-B1, GPI-B2**	M
Glyceraldehyde-3-phosphate Dehydrogenase	*GAPDH-3*, GAPDH-4**	E
Glycerol-3-phosphate Dehydrogenase	*G3PDH-1*, G3PDH-2**	L
Iditol Dehydrogenase	*IDDH**	L
Isocitrate Dehydrogenase	*mIDHP-1*, mIDHP-2**	M
	sIDHP-1, sIDHP-2**	L
Lactate Dehydrogenase	*LDH-A1*, LDH-A2**	M
	LDH-B1, LDH-B2*, LDH-C**	E
Malate Dehydrogenase	*sMDH-A1,2**	L
	*sMDH-B1,2**	M
Malic Enzyme	*sMEP-1**	M
	*sMEP-2**	L
Phosphoglucomutase	*PGM-1*, PGM-2**	M
		L

Table 2-continued

Enzyme	Loci	Tissue
Phosphogluconate Dehydrogenase	*PGDH**	M
Superoxide Dismutase	*sSOD-1**	L
Tripeptide Aminopeptidase	*PEPB**	E
Xanthine Dehydrogenase-*like*	*XDHl**	L

Table 3

Alleles at the diagnostic loci that differentiate westslope cutthroat trout and rainbow trout, westslope and Yellowstone cutthroat trout, and rainbow and Yellowstone cutthroat trout. When more than one allele exists at a locus within a taxon, the most common allele is listed first.

Locus	Taxa and characteristic alleles	
	Westslope	Rainbow
*sAAT-1**	*200,250*	*100*
*CK-A2**	*84*	*100*
*GPI-A**	*92,100*	*100*
*IDDH**	*40,100*	*100,200,40*
*sIDHP-1**	*86,71*	*100,114,71,40*
	Westslope	Yellowstone
*sAAT-1**	*200,250*	*165*
*CK-C1**	*100,38*	*38*
*GPI-A**	*92,100*	*100*
*IDDH**	*40,100*	*100*
*mIDHP-1**	*100*	*-75*
*sIDHP-1**	*86,71*	*71*
*sMEP-1**	*100*	*90*
*sMEP-2**	*100*	*110*
*PEPA-1**	*100*	*101*
*PEPB**	*100*	*135*
*PGM-1**	*100,null*	*null*
	Rainbow	Yellowstone
*sAAT-1**	*100*	*165*
*CK-A2**	*100*	*84*
*CK-C1**	*100,38,150*	*38*
*mIDHP-1**	*100*	*-75*
*sIDHP-1**	*100,114,71,40*	*71*
*sMEP-1**	*100*	*90*
*sMEP-2**	*100,75*	*110*
*PEPA-1**	*100,115*	*101*
*PEPB**	*100,120*	*135*
*PGM-1**	*100,null*	*null*

Table 4

Allele frequencies at the loci showing evidence of genetic variation in samples from non-hybridized westslope cutthroat trout populations in Addition Creek, Goldie Creek, South Creek, and upper Twin Creek.

		Sample and allele frequencies			
Locus	Alleles	Addition	Goldie	South	upper Twin
*bGLUA**	*100*	0.929	1.000	0.679	0.933
	90	0.071	--	0.321	0.067
*sIDHP-2**	*100*	0.821	1.000	0.700	0.500
	40	0.179	--	0.300	0.500