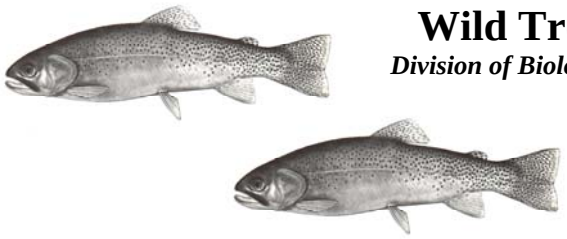

Wild Trout and Salmon Genetics Laboratory

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Clint:

The paired interspersed nuclear DNA elements (PINE) technique has been used to analyze DNA from the following trout samples:

Summary of results.

		a	b	c	d	e	f
Sample #	**Water Name/Location/Collection Date/ Collector**	**N**	**# markers**	**Species ID**	**Power (%)**	**% WCT**	**Individuals**
2960	**North Fork Flathead River** Clint Muhlfeld	14	R5Y4	WCT	76R68Y	100	xx
2961	**Parker Creek** Clint Muhlfeld	20	R5Y4	WCT	87R80Y	100	xx
2962	**Ketchikan Creek** Clint Muhlfeld	15	R5Y4	WCT	78R70Y	100	xx
2963	**Trail Creek** Clint Muhlfeld	10	R5Y4	WCT	63R55Y	100	xx
2964	**Colts Creek** Clint Muhlfeld	25	R5Y4	WCT	92R87Y	100	xx
2965	**Burnham Creek** Clint Muhlfeld	25	R5Y4	WCT	92R87Y	100	xx
2966	**Commerce Creek** Clint Muhlfeld	25	R5Y4	WCT	92R87Y	100	xx
2967	**Middle Pass Creek** Clint Muhlfeld	24	R5Y4	WCT	91R85Y	100	xx
2968	**Steamer Creek** Clint Muhlfeld	9	R5Y4	WCT X RBT		55x45	xx
2969	**Rabe Creek** Clint Muhlfeld	25	R5Y4	WCT X RBT		59x41	xx

2970	**Red Meadow Creek upper**	24	R5Y4	WCT X RBT	97X3	22
				WCTXYCT	69X31	2
	Clint Muhlfeld					
2971	**Red Meadow Creek lower**	23	R5Y4	WCT,WCTXRBT		
	Clint Muhlfeld					
2972	**Third Creek**	24	R5Y4	WCT X RB	50X50	23
				WCT	100	1
	Clint Muhlfeld					

[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 92% 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 hydridization 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 significant 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.

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 loci per individual at which non-native 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 will still be randomly distributed among individuals.

There are two likely reasons why a non-random distribution of non-native fragments 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 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

North Fork Flathead River (2960), Parker Creek (2961), Ketchikan Creek (2962), Trail Creek (2963), Colts Creek (2964), Burnham Creek (2965), Commerce Creek (2966), and Middle Pass Creek (2967)

With the exception of *Hpa15'/Hpa13'* 66*, PINE fragments characteristic of only westslope cutthroat trout were detected in these samples. The presence of *Hpa15'/Hpa13'* 66* in the samples could indicate a small amount of hybridization with rainbow trout or it could simply be westslope cutthroat trout genetic variation that is electrophoretically indistinguishable from that characteristic of rainbow trout. In these situations, the latter interpretation is much more likely. In many of these samples, the frequency of the *Hpa15'/Hpa13'* 66* is estimated to be 0.10 or greater (Table 2) and in these situations if its presence were indicative of hybridization it is highly unlikely ($P<0.001$) that even with a sample size of 10 fish evidence of the hybridization would not be detected at other diagnostic loci. Furthermore, the presence of this allele in numerous populations in conjunction with the absence of other rainbow trout markers is much more compatible with it representing westslope cutthroat trout genetic variation than hybridization since among slightly hybridized populations the particular rainbow trout fragments detected are expected to vary among loci rather than be consistent. Thus we conclude, the presence of H*pa15'/Hpa13'* 66* in these populations represents westslope cutthroat trout genetic variation and there is no evidence of hybridization in any of them. Because the *Hpa15'/Hpa13'* 66* allele apparently exists in numerous populations in this drainage it was not used in the subsequent analyses of hybridization.

Considering sample sizes, with the exception of the North Fork Flathead River, Ketchikan Creek, and Trail Creek samples we have about a 90% chance of detecting as little as one percent hybridization with rainbow or Yellowstone cutthroat trout in the samples. Thus, we are reasonably certain that the Parker Creek, Burnham Creek, Commerce Creek, Middle Pass Creek, and Colts Creek samples came from non-hybridized westslope cutthroat trout populations. Because of the smaller sample sizes, we have less than an 80% chance of detecting as little as one percent hybridization with rainbow or Yellowstone cutthroat trout in the North Fork Flathead River, Ketchikan Creek, and Trail Creek populations. Thus, we cannot reasonably exclude the possibility that some or all three of these populations may be slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both species. Although the status of the North Fork Flathead River, Ketchikan Creek, and Trail Creek populations is somewhat uncertain at this time the conservative approach would be to consider these populations to be non-hybridized westslope cutthroat trout unless future data indicate otherwise.

Steamer Creek (2968) and Rabe Creek (2969)

Alleles characteristic of both westslope cutthroat and rainbow trout were detected at all the diagnostic loci between these fishes analyzed in the Rabe Creek sample and at four of the five diagnostic loci analyzed in the Steamer Creek sample (Table 3). In both samples, the allele frequencies are statistically homogeneous ($P>0.05$) among the diagnostic loci and the rainbow trout fragments appear to be randomly distributed ($P>0.05$) among individuals. These populations, therefore, appear to be westslope cutthroat-rainbow trout hybrid swarms in which essentially all fish are of hybrid origin.

Red Meadow Creek, upper (2970)

Alleles characteristic of both westslope cutthroat trout and rainbow trout were detected at four of the five diagnostic loci between these fishes that were analyzed in the upper Red Meadow Creek sample. The allele frequencies are statistically homogeneous ($P>0.05$) among the diagnostic loci and the rainbow trout fragments appear to be randomly distributed ($P>0.05$) among the individuals in the sample.

Alleles characteristic of both westslope and Yellowstone cutthroat trout were detected at three of the four diagnostic loci between these fishes that were analyzed in the sample. Although the allele frequencies are statistically homogeneous ($P>0.05$) among the diagnostic loci, the Yellowstone cutthroat trout fragments are not randomly distributed ($P<0.05$) among the fish in the sample. In contrast, they were detected in only two fish one of which possessed a Yellowstone cutthroat trout marker at one locus and the other at three loci. Both of these fish showed no evidence of hybridization with rainbow trout. This in conjunction with the non-random distribution of Yellowstone cutthroat trout markers among individuals suggests these two fish are probably recent migrants from a hybridized population of westslope and Yellowstone cutthroat trout into the upper Red Meadow Creek population.

Considering all the data it appears that the upper Red Meadow Creek population is mainly composed of hybridized individuals between westslope cutthroat and rainbow trout and a small proportion of hybridized individuals between westslope and Yellowstone cutthroat trout.

Red Meadow Creek, lower (2971)

The lower Red Meadow Creek sample also showed evidence of hybridization with rainbow trout. PINE fragments characteristic of rain bow trout were detected at four of the five diagnostic loci between westslope cutthroat trout and rainbow trout analyzed in the sample. Although the allele frequencies were statistically homogeneous ($P>0.10$) among the diagnostic loci, the rainbow trout markers were not randomly distributed ($P<0.01$) among the fish in the sample. Rather they were confined to just three fish, one of which possessed a rainbow marker at one locus, another at two loci, and the last at three loci. Thus, this sample appears to have contained a mixture of non-hybridized westslope cutthroat trout and fish of hybrid origin. Hitt et al. (2003) obtained similar results from samples collected from lower Red Meadow Creek in 1998 and 2000. Their results and ours suggest that hybridization has only relatively recently begun in lower Red Meadow Creek and that migrants from a hybridized population or populations continue to enter the system. The presence of

hybridized fish in the population and the continued migration of hybridized fish into it must be viewed as a serious threat the continued genetic integrity of the non-hybridized westslope cutthroat trout.

Third Creek (2972)

The Third Creek sample appears to have come from a largely hybridized population of westslope cutthroat and rainbow trout. PINE fragments characteristic of rainbow trout were detected at all five diagnostic loci between these fishes that were analyzed in the sample. Although the allele frequencies were statistically homogeneous ($P>0.05$) among the diagnostic loci, the rainbow trout markers were not randomly distributed ($P<0.001$) among the fish in the sample. In contrast, significantly more fish ($N=1$) lacked rainbow trout markers at all diagnostic loci than expected by chance. These results suggest the population may have only recently become hybridized and it still contains a small proportion of non-hybridized westslope cutthroat trout or it is a hybridized population that contains a small proportion of migrants from a non-hybridized westslope cutthroat trout population. Regardless of the explanation, fish of hybrid origin are by far the most common in the population and, therefore, from a management perspective the population should simply be considered to be hybridized with a substantial rainbow trout genetic contribution.

Sincerely,

Robb Leary

Literature Cited

Hitt, N. P., C. A. Frissell, C. C. Muhlfeld, and F. W. Allendorf. 2003. Spread of hybridization between native westslope cutthroat trout, *Oncorhynchus clarki lewisi,* and nonnative rainbow trout, *O. mykiss.* Canadian Journal of Fisheries and Aquatic Sciences 60:1440-1451.

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

Estimated frequencies of *Hpa15'/Hpa13'*66* in samples from what appear to be non-hybridized westslope cutthroat trout populations from the North Fork Flathead River drainage.

Sample	Allele frequencies
North Fork Flathead River	0.087
Parker Creek	0.142
Ketchikan Creek	0.118
Trail Creek	0.106
Burnham Creek	0.205
Commerce Creek	0.174
Middle Pass Creek	0.023
Colts Creek	0.025

TABLE 3

Allele frequencies at the diagnostic PINE loci between westslope cutthroat and rainbow trout analyzed in samples from hybridized populations of these fishes in Rabe Creek and Steamer Creek in the North Fork Flathead River drainage. At each locus, the *a* allele is characteristic of westslope cutthroat trout and the *p* allele is characteristic of rainbow trout. NA= locus not scoreable in the sample.

		Sample and allele frequencies	
Locus	Alleles	Rabe	Steamer
*Hpa15'/Hpa13'*70*	*a*	0.477	--
	p	0.523	1.000
*Fok15'/Tc1*369*	*a*	NA	0.707
	p		0.293
230	*a*	NA	0.655
	p		0.345
*Hpa15'/33.6+2*395*	*a*	0.748	1.000
	p	0.252	--
266	*a*	0.552	0.408
	p	0.448	0.592
Average westslope		0.592	0.554
Average rainbow		0.408	0.446