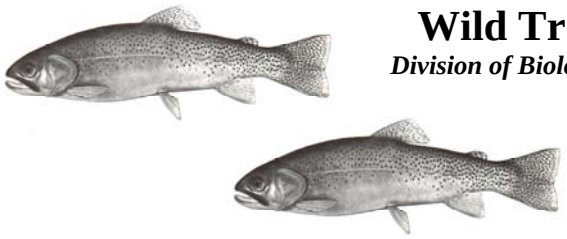

Wild Trout and Salmon Genetics Laboratory

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Pat:

The paired interspersed nuclear DNA elements (PINE) technique has been used to analyze DNA from the following trout samples collected from the Yellowstone 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**
2974	**Mulherin Creek** 08S07E24 6/16/2004 Pat Byorth	47	R7Y12W4	YCT X WCT X RBT			xx
2973	**Mulherin Creek** 08S07E24 5/20/2004 Pat Byorth	5	R6C5Y4W2	RBT X YCT X WCT			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 (C=westslope or Yellowstone cutthroat trout, 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 in 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 Yellowstone cutthroat trout, *Oncorhynchus clarki bouvieri* and rainbow trout, *O. mykiss,* is compared with PINE analysis and four different pairs of primers seven fragments are characteristic of rainbow trout and 12 fragments are usually characteristic of Yellowstone cutthroat trout (Table 1). Likewise, when DNA from Yellowstone and westslope cutthroat trout, *O. c. lewisi*, is compared using the same procedure four fragments are characteristic of westslope cutthroat trout and six 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 analyzed 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

Mulherin Creek 2973 and 2974

These represent a temporal sequence of four samples of fish migrating into Mulherin Creek, presumably to spawn, collected from a trap set near the mouth of the creek: end of first run (col. 5/20/04; N=5) and early (6/16-18/04; 12), middle (6/20-21/04; 16), and late second run (6/24-27/04; 19). The primary issues of interest were: 1) as suspected does the first run predominantly contain rainbow trout or hybrids with a predominant rainbow trout genetic contribution, 2) is the second run mainly composed of non-hybridized Yellowstone cutthroat trout or does it contain individuals of hybrid origin, 3) if it does contain individuals of hybrid origin do they appear to be more prevalent early in the run than later and does the rainbow trout genetic contribution to the hybrids temporally decrease, and 4) how well does field identification in terms of separating hybridized and non-hybridized fish compare to PINE analysis.

PINE fragments characteristic of rainbow trout were detected at all six diagnostic loci for this species analyzed among

four of the five fish in the sample from the end of the first run. The remaining fish (#4) had PINE fragments characteristic of only Yellowstone cutthroat trout. This fish, therefore, could be a non-hybridized Yellowstone cutthroat trout. Based on the data that will subsequently be presented, however, we suspect it probably is not non-hybridized.

Of the four fish in this sample containing rainbow trout fragments, three were definitely of hybrid origin. One fish (#5) possessed rainbow trout fragments at five of the six diagnostic loci analyzed for this species. At one locus it also possessed a fragment characteristic of westslope cutthroat trout and at another it possessed a fragment characteristic of Yellowstone cutthroat trout. This fish, therefore, is almost undoubtedly a rainbow-westslope cutthroat-Yellowstone cutthroat trout hybrid.

The other two fish definitely of hybrid origin in the sample possessed fragments characteristic of rainbow trout at all six diagnostic loci analyzed for this species. One (#2), however, also possessed a fragment characteristic of westslope cutthroat trout at one locus and the other (#3) also possessed this fragment and one characteristic of both westslope and Yellowstone cutthroat trout.

The remaining fish (#1) in this sample possessed PINE fragments characteristic of only rainbow trout. This fish, therefore, may be non-hybridized, but this cannot be stated with much conviction. Based on the average genetic contribution of cutthroat trout (0.08) to the fish definitely of hybrid origin in the sample and the number of diagnostic loci analyzed, 16% of hybrids are expected to provide no evidence of hybridization and appear to be non-hybridized rainbow trout.

Overall, this sample appears to have contained individuals from two genetically different populations. One of the populations appears to be of hybrid origin with a predominant rainbow trout genetic contribution. In contrast, the other population appears to be predominantly Yellowstone cutthroat.

In the early sample from the second run, PINE markers characteristic of both Yellowstone cutthroat trout and rainbow trout were detected at six of the seven diagnostic loci between these species analyzed. PINE markers characteristic of both Yellowstone and westslope cutthroat trout were also detected at two of the four diagnostic loci between these subspecies that were analyzed. Although the frequency of rainbow trout markers were statistically homogeneous ($P>0.05$) among the diagnostic loci, as were the frequency of westslope cutthroat trout markers, the markers for both species were not randomly distributed ($P<0.001$) among the fish in the sample. In contrast, one fish (#13) possessed rainbow trout markers at six diagnostic loci and it also possessed westslope cutthroat trout markers at two diagnostic loci. This fish, therefore was undoubtedly a hybrid individual with a Yellowstone cutthroat, westslope cutthroat, and rainbow trout genetic contribution. Another fish in the sample (#7), was undoubtedly of hybrid origin between Yellowstone cutthroat and rainbow trout. It possessed rainbow trout markers at two diagnostic loci. The remaining fish in the sample contained fragments characteristic of only Yellowstone cutthroat trout.

The middle sample from the second run was genetically very similar to the early one. Of the 16 fish in the sample, 13 possessed markers characteristic of only Yellowstone cutthroat trout. One fish (#20) possessed fragments characteristic of rainbow trout at all seven diagnostic loci between Yellowstone cutthroat and rainbow trout analyzed. It also, however, lacked fragments characteristic of Yellowstone cutthroat trout at seven of the 12 diagnostic loci for this subspecies that were analyzed. Another fish (#21), possessed fragments characteristic of rainbow trout at two of the seven diagnostic loci for this species that were analyzed. These two fish, therefore, were undoubtedly of hybrid origin between Yellowstone cutthroat and rainbow trout. Since the rainbow trout markers were grouped into only two

fish, they were not randomly distributed ($P<0.001$) among the fish in the sample.

There was also evidence of hybridization between westslope and Yellowstone cutthroat trout in the sample. One fish (#24) possessed PINE fragments characteristic of westslope cutthroat trout at one of the four diagnostic loci between these fishes that were analyzed. Generally we would not consider this strong evidence of hybridization, but in this situation we favor this interpretation because conclusive evidence of hybridization between these fishes was detected in the first run and early second run samples.

No evidence of hybridization between Yellowstone and westslope cutthroat trout was detected in the late sample from the second run. There was, however, good evidence of hybridization between Yellowstone cutthroat and rainbow trout. One fish (#44) possessed rainbow trout fragments at two and another (#70) at one of the seven diagnostic loci between these fishes analyzed. Like in the early and middle second run samples, the rainbow trout fragments do not appear to be randomly distributed ($P<0.05$) among the individuals in the late sample.

We used two approaches to investigate the temporal pattern of hybridization among the second run samples. Contingency table chi-square analysis was used to compare the proportion of fish definitely of hybrid origin and the frequency of westslope cutthroat and rainbow trout fragments among the samples.

The proportion of fish definitely of hybrid origin does not significantly differ ($P>0.50$) among the samples suggesting this attribute was temporally stable. Likewise the frequency of westslope cutthroat trout fragments does not significantly differ ($P>0.10$) among the early (0.021), middle (0.008), and late samples (0.000). Because of the low frequency of westslope cutthroat trout fragments in all the samples this comparison is statistically weak. The apparent temporal stability of the frequency of westslope cutthroat trout fragments among the samples, therefore, requires cautious interpretation.

In contrast to the above results, the frequency of rainbow trout fragments is statistically heterogeneous ($P<0.05$) among the early (0.051), middle (0.042), and late (0.012) samples. This significant difference is basically due to the two individuals definitely of hybrid origin detected that had a substantial rainbow trout genetic contribution. Thus, it is mainly a contrast of the late to the other two samples and the results suggest that hybrid individuals with a substantial rainbow trout genetic contribution tend to appear somewhat earlier in the second run than later.

From a genetics perspective, the Mulherin Creek spawning run represents somewhat of a complex situation. We feel, however the data suggest the following scenario is likely. The spawning run as a whole is composed of two genetically very different groups of fish. One group contains mainly hybridized individuals with a substantial rainbow trout genetic contribution (77%) and a relatively small Yellowstone (11%) and westslope cutthroat trout (9%) genetic contribution. The other group contains mainly hybridized individuals with a substantial Yellowstone cutthroat trout genetic contribution (98.4%) and a small rainbow (1.3%) and westslope cutthroat trout (0.3%) genetic contribution. The former group appears to generally enter the creek earlier than the latter. There is, however, overlap between the two groups. One fish in the first run sample genetically appeared to be a member of the latter group while two fish in the second run samples appeared to be members of the former group. Because of this overlap, there is probably some gene flow between the groups. The non-random distribution of species markers among individuals in the samples, therefore, reflects the mixture of individuals from the two groups and continued gene flow that prevents the two groups from coming to genetic equilibrium. Overall we suspect it is unlikely any non-hybridized Yellowstone cutthroat or rainbow trout exist in the run, and thus both groups should simply be considered hybridized.

Because fish in both groups are probably mainly of hybrid origin, the pertinent issue to address is how well can individuals be assigned to group of origin based on field identification. Individual #4 in the first run sample genetically appears to have originated from the predominant Yellowstone cutthroat trout hybrid group. It was classified as a rainbow-Yellowstone cutthroat trout hybrid in the field suggesting it was erroneously assigned to the predominant rainbow trout hybrid group. Individuals #13 and #20 in the second run samples appear to have originated from the predominant rainbow trout hybrid group. Individual #20 was field identified as a rainbow-Yellowstone cutthroat trout hybrid and, therefore, was correctly identified. Individual #13 was incorrectly identified as being a Yellowstone cutthroat trout. The minimal data, therefore, suggest identifying individuals to group of origin in the field is fairly inaccurate. Thus, potentially culling individuals from the second run that are believed to have originated from the predominant rainbow trout hybrid group will only retard, but not prevent, the continued infusion of rainbow trout genes into the second predominantly Yellowstone cutthroat trout hybrid group.

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	