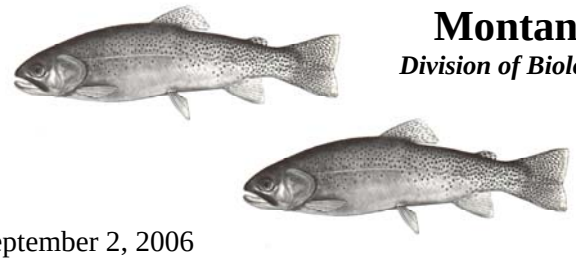

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Jim:

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

Summary of results.

Sample #	Water Name/Location/Collection Date/ Collector	N [a]	# markers [b]	Taxa ID [c]	Power (%) [d]	% YCT [e]	Individuals [f]
3308	**Brushy Fork of Willow Creek** 45.227 109.290 8/6/2005 Jim Olsen	22	R8W4	YCT	R97W82	100	
3309	**Lower Deer Creek** 45.675 109.882 3/30/2005 Jim Olsen	21	R8W4	YCT YCTXRBT			13 8

[a]Number of fish successfully analyzed. If combined with a previous sample, the number in parentheses indicates the combined sample size.
[b]Number of markers analyzed that are diagnostic for the non-native species (R=rainbow trout *Oncorhynchus mykiss*, W=westslope cutthroat trout *O. clarki lewisi*, Y=Yellowstone cutthroat trout *O. c. bouvieri*).
[c]Codes: WCT = westslope cutthroat trout; RBT = rainbow trout; YCT = Yellowstone cutthroat trout. Only one taxon code is listed when the entire sample possessed alleles from that taxon 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 %). Taxa codes separated by "x" indicate hybridization between those taxa.
[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 yield a 98% chance of detecting as little as 1% hybridization with rainbow trout but only an 87% chance of detecting as little as 1% hybridization with westslope cutthroat trout into what once was a Yellowstone 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 non-hybridized population or a hybrid swarm. The latter is a random mating population in which taxa markers are randomly distributed among individuals.
[f]Indicates number of individuals with genetic characteristics corresponding to the taxa ID 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 and hybrids and non-hybrids can be reliably distinguished.

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 using four different pairs of primers, twelve fragments are usually characteristic of Yellowstone cutthroat trout and eight fragments are usually characteristic of rainbow trout (Table 1). Likewise, when DNA from Yellowstone and westslope cutthroat trout, *O. c. lewisi*, is compared using the same procedure four fragments are usually characteristic of westslope cutthroat trout and seven fragments are usually 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 (F_1) 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 (post F_1), the amount and particular regions of DNA acquired from the parental taxa will vary among individuals. Thus, DNA from post F_1 hybrids 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 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 computed a hybrid index for each individual in the sample. Each diagnostic locus at which an individual possessed a PINE fragment characteristic of the non-native taxon was given a value of one. Each diagnostic locus at which an individual did not possess a PINE fragment characteristic of the non-native taxon was given a value of zero. Each diagnostic locus at which an individual possessed a PINE fragment characteristic of the native taxon was given a value of zero. Each diagnostic locus at which an individual lacked a PINE fragment characteristic of the native taxon was given a value of one. These values summed over all diagnostic loci represent an individual's hybrid index. Considering rainbow and Yellowstone cutthroat trout, therefore, Yellowstone cutthroat trout would be characterized by a hybrid index of zero, rainbow trout by a hybrid index of 20, F_1 hybrids by a hybrid index of eight, and post F_1 hybrids could have hybrid indices ranging from zero to 20. The observed distribution of hybrid index scores was then statistically compared to the expected random binomial distribution based on the estimated native and non-native genetic contributions to the sample. If the allele frequencies were statistically homogeneous among the diagnostic loci and the observed distribution of hybrid indices statistically conformed to the expected random binomial distribution, then the sample was considered to have come 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. Thus, samples with these characteristics were also considered to have come from hybrid swarms.

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 PINE 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.

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.

Results and Discussion

Brushy Fork of Willow 3308

Only PINE fragments characteristic of Yellowstone cutthroat trout were detected in the sample form the Brushy Fork of Willow Creek. With a sample size of 22 fish, we have a 97.1% chance of detecting as little as a one percent rainbow trout genetic contribution to a hybrid swarm, but a 95% chance of detecting only as little as a ten percent westslope cutthroat trout genetic contribution to a hybrid swarm. The Brushy Fork of Willow Creek population, therefore, is most likely not hybridized with rainbow trout but, there is a chance that the population may be slightly hybridized with westslope cutthroat trout but this was not detected because of sampling error. Although there is some

uncertainty about the status of the Brushy Fork Willow Creek population, at this time the conservative approach would be to consider the population to be non-hybridized Yellowstone cutthroat trout unless future data suggest otherwise.

Lower Deer Creek 3309

At the 10 diagnostic loci at which the *p* allele is usually characteristic of Yellowstone cutthroat trout from which we were able to obtain data, all individuals in the sample possessed the Yellowstone cutthroat trout fragment. At seven of the eight diagnostic loci that usually distinguish rainbow from Yellowstone cutthroat trout, PINE fragments characteristic of rainbow trout were detected. Thus, there is conclusive evidence of hybridization in the Lower Deer Creek sample. The sample, however, does not appear to have come from a hybrid swarm. The allele frequencies are statistically heterogeneous ($P<0.05$, $X^2_7=15.33$) among the diagnostic loci and the rainbow trout fragments do not appear to be randomly distributed ($P<0.001$, $X^2_3=26.529$) among individuals in the sample. In contrast to the expected random distribution of hybrid indices, the individuals in the sample fall into two distinct groups. One group (N=13) is composed of individuals with a hybrid index value of zero and the other group (N=8) is composed of individuals with hybrid indices of two through four (Figure 1). The hybrid index scores of the latter individuals are highly characteristic of first generation hybrid backcrosses to Yellowstone cutthroat trout. Thus, the Lower Deer Creek sample appears to have contained a mixture of non-hybridized Yellowstone cutthroat trout and first generation backcrosses to Yellowstone cutthroat trout.

Robb Leary

John Powell

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	
110.5			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	
Fok1 5'/Hpa1 3'			
323	x		
242		x	
173	x		
170	x		
161.5		x	
143.5			x

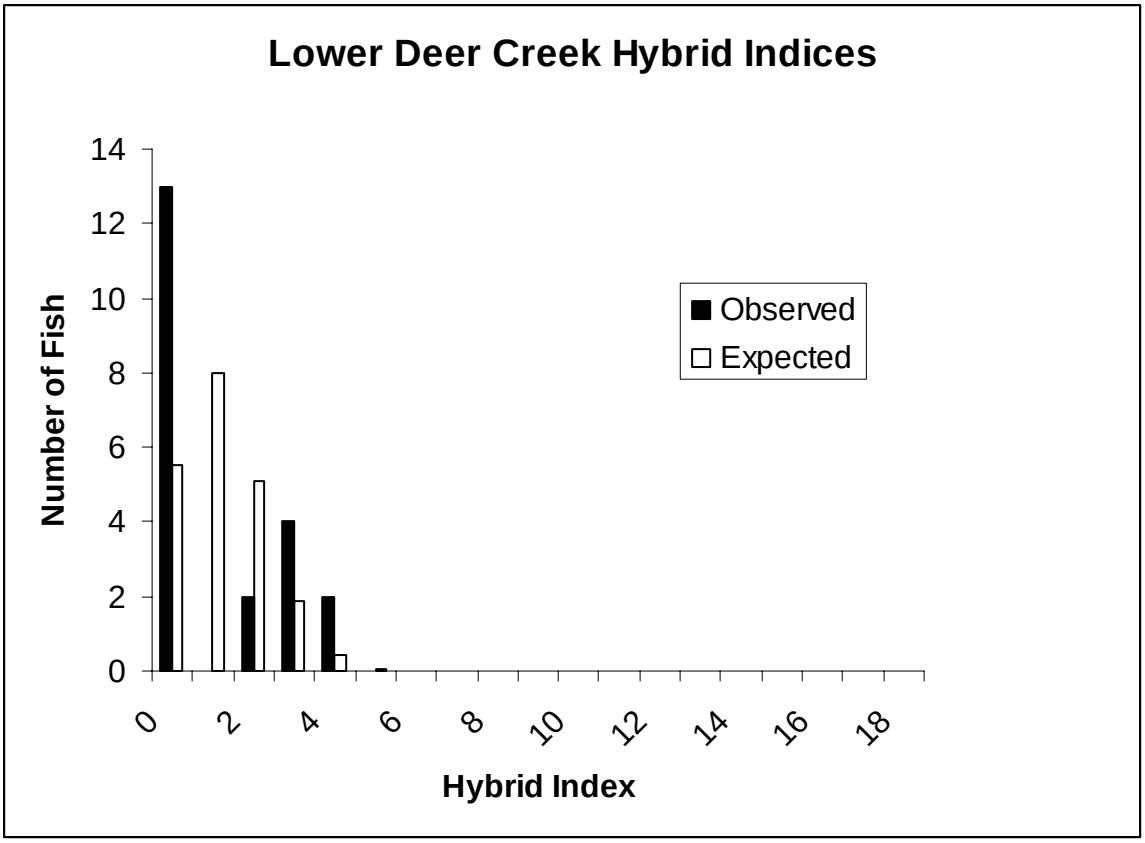

Figure 1. Observed and expected random distribution of hybrid indices among the fish in a sample from Lower Deer Creek.