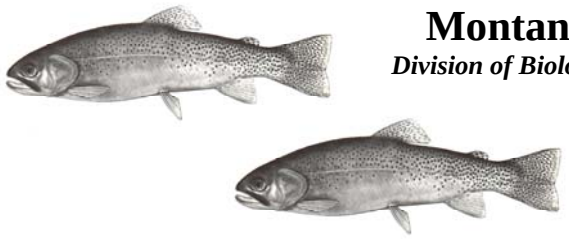

Montana Conservation Genetics Laboratory

*Division of Biological Sciences * University of Montana * Missoula, MT 59812*
(406)243-5503/6749 Fax (406)243-4184

January 2, 2006

Mark Deleray
Montana Department of Fish, Wildlife, and Parks
409 North Meridian
Kalispell, Montana 59901

Mark:

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**
3116	**South Fork Gin Gulch**	22	R6Y4	WCT	R93Y83	100	xx
	7/24/2002 Laura Katzman						
3117	**Henry Creek**	27	R6Y4	WCT	R96Y89	100	xx
	7/26/2002 Laura Katzman						
3118	**Rock Creek**	25	R5Y4	WCT?	R92Y87	100	xx
	8/19/2002 Laura Katzman						
3119	**Dog Creek**	20	R6Y4	WCT	R91Y80	100	xx
	21N16W06 7/2/2003 Scott Rumsey						
3120	**Herrick Run Creek**	20	R6Y4	WCT	R91Y80	100	xx
	19N17W28 7/31/2003 Scott Rumsey						
3121	**South Fork Lost Creek**	20	R5Y4	WCT	R87Y80	100	xx
	8/8/2003 Scott Rumsey						

Sample #	Water Name/Location/Collection Date/ Collector	N [a]	# markers [b]	Species ID [c]	Power (%) [d]	% WCT [e]	Individuals [f]
3122	**Smith Creek**	30	R6Y4	WCT	R97Y91	100	xx
	21N16W29 7/9/2003 Scott Rumsey						
3123	**Cooney Creek**	17	R6Y4	WCT	R87Y74	100	xx
	21N16W33 7/10/2003 Scott Rumsey						
3124	**North Fork Lost Creek**	25	R5Y4	WCT	R92Y87	100	xx
	25N17W25SW1/4SW1/4 7/31/2001 Scott Rumsey						
3125	**Deep Creek**	24	R6Y4	WCT	R94Y85	100	xx
	33N19W34 7/6/2004 Mark Deleray						

[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 95% chance to detect as little as 1% hybridization with rainbow or an 87% chance to detect as little as 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 usually 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 two fragments are usually characteristic of westslope cutthroat trout and four 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 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.

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 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. These values summed over all diagnostic loci represent an individual's hybrid index. 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.

Results and Discussion

South Fork Gin Gulch 3116

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 22, we have about a 93% chance of detecting as little as a one percent rainbow trout and about an 83% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the South Fork Gin Gulch population may be slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

Henry Creek 3117

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 27, we have about a 96% chance of detecting as little as a one percent rainbow trout and about an 89% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the Henry Creek population may be slightly hybridized with Yellowstone cutthroat trout. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

Rock Creek 3118

Fish were collected from three reaches of the stream: just above the confluence with the West Fork Rock Creek (#17-29), about one mile further upstream (#8-16), and about another mile further upstream (#1-7). Considering all three samples, a PINE fragment usually characteristic of rainbow trout was detected at one of the six diagnostic loci analyzed that usually distinguish rainbow from westslope cutthroat trout. The fragment was detected in one fish collected about one mile above the confluence with the West Fork Rock Creek. Its presence, therefore, could indicate a small amount of hybridization with rainbow trout or it could simply be westslope cutthroat trout PINE genetic variation that is electrophoretically indistinguishable from that usually characteristic of rainbow trout.

Previous allozyme analyses of fish collected from Rock Lake (samples #1680 and 1681) in the headwaters of the drainage indicated the lake population to be a hybrid swarm between westslope (about 80%) and Yellowstone cutthroat trout (about 20%). A previous allozyme analysis of fish collected from Rock Creek Meadows (sample # 108) in the upper reaches of the stream indicated the fish in this area to be a hybrid swarm among westslope cutthroat (93%), Yellowstone cutthroat (5%), and rainbow trout (2%). In contrast, fish collected about one mile above the mouth of Rock Creek (sample # 121) appeared to be non-hybridized westslope cutthroat trout. Thus, if the middle reaches of Rock Creek contain hybridized trout the most likely source of hybridization appears to be from upstream and not downstream.

We suspect the middle reaches of Rock Creek contain non-hybridized westslope cutthroat trout because the potential upstream sources of hybridization appear to contain a higher Yellowstone cutthroat trout genetic contribution than a rainbow trout genetic contribution. Gene flow from these potential sources of hybridization, therefore, should have introduced Yellowstone cutthroat trout genes into the middle reaches as well as rainbow trout genes, but this does not appear to be the case. Unfortunately, however, the available data do not allow us to conclusively determine whether or not the middle reaches of Rock Creek contain non-hybridized westslope cutthroat trout. In this situation of uncertainty, the conservative approach would be to consider the middle reaches of Rock Creek to contain non-hybridized westslope cutthroat trout.

Dog Creek 3119

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 20, we have about a 91% chance of detecting as little as a one percent rainbow trout and about an 80% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the Dog Creek population may be

slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

Herrick Run Creek 3120

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 20, we have about a 91% chance of detecting as little as a one percent rainbow trout and about an 80% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the Herrick Run Creek population may be slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

South Fork Lost Creek 3121

A PINE fragment usually characteristic of rainbow trout was detected at one of the six diagnostic loci analyzed in the sample that usually distinguish rainbow from westslope cutthroat trout . The *Hpa1 5'/Hpa1 3'*70* fragment was detected in only two fish. Thus, its presence could indicate a small amount of hybridization with rainbow trout or it could simply be westslope cutthroat trout PINE genetic variation that is electrophoretically indistinguishable from that usually characteristic of rainbow trout. In this situation, we strongly favor the latter interpretation because this fragment exists at appreciable frequency (0.15) in the westslope cutthroat trout population in North Fork Lost Creek. With the sample size of 20, we have about an 87% chance of detecting as little as a one percent rainbow trout and about an 80% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the South Fork Lost Creek population may be slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

Smith Creek 3122

Fish were sampled from Smith Creek above (N=10) and below (N=20) the road 9762 camp. In both samples, PINE fragments characteristic of only westslope cutthroat trout were. With the combined sample size of 30, we have about a 97% chance of detecting as little as a one percent rainbow trout and about a 91% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the Smith Creek population may be slightly hybridized with Yellowstone cutthroat trout. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

Cooney Creek 3123

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 17, we have about an 87% chance of detecting as little as a one percent rainbow trout and about a 74% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to

a hybrid swarm. Thus, we can not reasonably exclude the possibility that the Cooney Creek population may be slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

North Fork Lost Creek (upper) 3124

A PINE fragment usually characteristic of rainbow trout was detected at one of the six diagnostic loci analyzed in the sample that usually distinguish rainbow from westslope cutthroat trout . The *Hpa1 5'/Hpa1 3'*70* fragment was detected in seven fish. Thus, its presence could indicate hybridization with rainbow trout or it could simply be westslope cutthroat trout PINE genetic variation that is electrophoretically indistinguishable from that usually characteristic of rainbow trout. In this situation we strongly favor the latter interpretation because if the presence of this fragment was due to hybridization given its frequency (0.15) it is highly unlikely (contingency table chi-square; $P<0.001$) that we would not detect fragments characteristic of rainbow trout at the other diagnostic loci analyzed. With the sample size of 25, we have about a 92% chance of detecting as little as a one percent rainbow trout and about an 87% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the North Fork Lost Creek population may be slightly hybridized with rainbow trout, Yellowstone cutthroat trout, or both. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

Deep Creek 3125

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 24, we have about a 94% chance of detecting as little as a one percent rainbow trout and about an 85% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, we can not reasonably exclude the possibility that the Deep Creek population may be slightly hybridized with Yellowstone cutthroat trout. Despite this uncertainty, unless future data indicate otherwise the conservative approach would be to consider the population non-hybridized westslope cutthroat trout.

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	