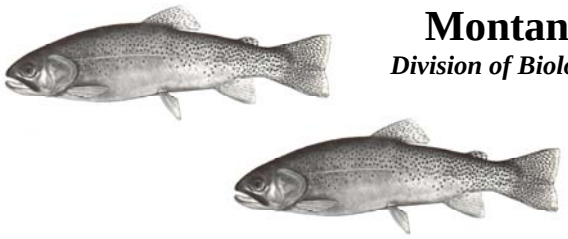

Montana Conservation Genetics Laboratory

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

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Brad Liermann
Montana Department of Fish, Wildlife, and Parks
601 North Columbia
Thompson Falls, Montana 59873

Brad:

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**
3128	**Jungle Creek** 7/17/2003 Laura Katzman	30	R6Y4	WCT	R97Y91	100	xx
3129	**McKay Creek** 26N31W31NE1/4NW1/4 9/5/2002 Doug Gropenhoff	30(55)	R6Y4	WCT	R99Y99	100	xx
3130	**East Fork Blue Creek** 27N34W03SW1/4SW1/4 9/9/2002 Doug Gropenhoff	21(36)	R6Y4	WCT	R98Y99	100	xx
3131	**South Branch Marten Creek** 25N33W32NW1/4SW1/4 8/20/2003 Doug Gropenhof	30(55)	R6Y4	WCT	R99Y99	100	xx

Sample #	Water Name/Location/Collection Date/ Collector	N [a]	# markers [b]	Species ID [c]	Power (%) [d]	% WCT [e]	Individuals [f]
3132	**North Branch Marten Creek** 25N33W30NW1/4SW1/4 8/7/2002 Doug Gropenhoff	30(55)	R6Y4	WCT	R99Y99	100	xx
3133	**South Fork Marten Creek** 24N33W14NW1/4NW1/4 8/15/2002 Doug Gropenhoff	29(56) 1	R6Y4	WCT WCTXRT	R99Y99	100 50X50	56 1
3134	**Johnson Creek** 8/14/2001 Laura Katzman	29	R6Y4	WCT	R95Y67	100	xx
3135	**Trestle Creek** 5/11/2001 Laura Katzman	29	R3Y0	WCT		-10	xx
3136	**Twin Creek** 10/31/2002 Laura Katzman	25	R3Y0	WCT		-10	xx
3137	**Deerhorn Creek** 23N27W32NW1/4SW1/4 9/3/2003 Laura Katzman	25	R3Y0	WCTXRT		89X11	xx

[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, 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 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 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

Jungle Creek 3128

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. With the sample size of 30, we have better than a 97% chance of detecting as little as a one percent rainbow trout but, only a 91% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a

hybrid swarm. Although we can not reasonably exclude the possibility that the Jungle Creek population may be slightly hybridized with Yellowstone cutthroat trout, at this time the conservative approach would be to consider it non-hybridized westslope cutthroat trout unless further data indicate otherwise.

McKay Creek 3129

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. A previous allozyme analysis of fish collected from McKay Creek (sample # 141, collected 8/9/85, N=25) also detected alleles characteristic of only westslope cutthroat trout. With the combined sample size of 55, we have better than a 99% chance of detecting as little as a one percent rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm. The McKay Creek population, therefore, is almost certainly non-hybridized westslope cutthroat trout.

East Fork Blue Creek 3130

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. A previous allozyme analysis of fish collected from East Fork Blue Creek (sample # 758, collected 7/7/93, N=15) also detected alleles characteristic of only westslope cutthroat trout. With the combined sample size of 36, we have better than a 98% chance of detecting as little as a one percent rainbow trout and better than a 99% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. The East Fork Blue Creek population, therefore, is almost certainly non-hybridized westslope cutthroat trout.

South Branch Marten Creek 3131

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. A previous allozyme analysis of fish collected from South Branch Marten Creek (sample # 621, collected 6/25/92, N=25) also detected alleles characteristic of only westslope cutthroat trout. With the combined sample size of 55, we have better than a 99% chance of detecting as little as a one percent rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm. The South Branch Marten Creek population, therefore, is almost certainly non-hybridized westslope cutthroat trout.

North Branch Marten Creek 3132

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. A previous allozyme analysis of fish collected from North Branch Marten Creek (sample # 63, collected 5/1/84, N=25) also detected alleles characteristic of only westslope cutthroat trout. With the combined sample size of 55, we have better than a 99% chance of detecting as little as a one percent rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm. The North Branch Marten Creek population, therefore, is almost certainly non-hybridized westslope cutthroat trout.

South Fork Marten Creek 3133

PINE fragments characteristic of only westslope cutthroat trout were detected in all the fish in the sample except one. The latter fish possessed rainbow trout fragments at all six diagnostic loci that usually distinguish rainbow from westslope cutthroat trout. It also possessed westslope cutthroat trout fragments at

all seven diagnostic loci that usually distinguish westslope cutthroat from rainbow trout. This fish, therefore, was almost undoubtedly a first generation hybrid between westslope cutthroat and rainbow trout.

A previous allozyme analysis of fish collected from South Fork Marten Creek (sample # 41, collected 8/16/83, N=27) detected alleles at all the loci analyzed characteristic of only westslope cutthroat trout. When these fish are combined with the 29 from the more recent sample showing no evidence of hybridization, we have better than a 99% chance of detecting as little as a one percent rainbow or Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Thus, when this reach of South Fork Marten Creek was most recently sampled it appears to have been a mixture of predominantly non-hybridized westslope cutthroat trout and a low proportion of first generation hybrids with rainbow trout. The presence of hybrids should be considered to represent a serious threat to the continued persistence of non-hybridized westslope cutthroat trout in South Fork Marten Creek.

Johnson Creek 3134

PINE fragments characteristic of only westslope cutthroat trout were detected in the sample. Because of DNA quality problems we were not able to obtain data from all the diagnostic loci between westslope cutthroat trout and rainbow or Yellowstone cutthroat trout from all the fin clips in the sample. Overall, data were available from 146 loci that usually distinguish rainbow from westslope cutthroat trout and 55 loci that usually distinguish Yellowstone from westslope cutthroat trout. From these data, we have about a 95% chance of detecting as little as a one percent rainbow trout but, only about a 67% chance of detecting as little as a one percent Yellowstone cutthroat trout genetic contribution to a hybrid swarm. Although we can not reasonably exclude the possibility that the Johnson Creek population may be hybridized with Yellowstone cutthroat trout, at this time the conservative approach would be to consider it non-hybridized westslope cutthroat trout unless further data indicate otherwise.

Trestle Creek 3135

The DNA extracted from the fin clips in the sample was of very poor quality. We were able to obtain information from only three diagnostic loci that usually distinguish rainbow from westslope cutthroat trout from 18 fin clips. No information was obtainable from the four diagnostic loci that usually distinguish Yellowstone from westslope cutthroat trout.

PINE fragments characteristic of rainbow trout were detected at two of the three diagnostic loci from which data were available that usually distinguish rainbow from westslope cutthroat trout. The markers characteristic of rainbow trout, however, were not randomly distributed ($P<0.001$) among the fish in the sample. In contrast, one fish possessed markers characteristic of rainbow trout at two of the three diagnostic loci and the remaining 17 fish had markers characteristic of only westslope cutthroat trout. Thus, at the time of sampling this portion of Trestle Creek appears to have contained a mixture of hybrids between westslope cutthroat and rainbow trout and non-hybridized westslope cutthroat trout. Because of the limited number of diagnostic loci between rainbow and westslope cutthroat trout and the number of fish from which data are available, at this time we can not reliably determine at the individual level how well hybrids and non-hybridized individuals in Trestle Creek can be distinguished. Because of this uncertainty, we suggest that from a management perspective that Trestle Creek presently be considered to contain a hybridized population between westslope cutthroat and rainbow trout with a predominant westslope cutthroat trout

genetic contribution. This suggestion of course is subject to change, especially if additional information indicates that hybrids and non-hybridized fish can reliably be distinguished at the individual level.

Twin Creek 3136

The DNA extracted from the fin clips in this sample was of poor quality. Thus, information was not available from all six diagnostic loci that usually distinguish rainbow from westslope cutthroat trout from all of the fish sampled. Information from three diagnostic loci, however, was available from 19 individuals in the sample. Considering the number of diagnostic loci and individuals these data represent the most powerful data set available to determine whether or not the population contains fish of hybrid origin and if it does contain hybrids whether or not it appears to be a hybrid swarm. We used these data, therefore, to assess the status of the population.

PINE fragments characteristic of rainbow trout were detected at all three of the diagnostic loci between rainbow and westslope cutthroat trout that were analyzed in the sample. The PINE fragments characteristic of rainbow trout, however, were not randomly distributed ($P<0.001$) among the fish from which information was available. Rather, there were significantly more fish completely lacking rainbow trout fragments at all diagnostic loci analyzed than expected by chance (Figure 1). There was also significantly more fish possessing rainbow trout fragments at all three of the diagnostic loci analyzed than expected by chance (Figure 1). Finally, significantly fewer individuals than expected by chance possessed rainbow trout markers at only one of the three diagnostic loci analyzed (Figure 1). These results suggest that when this reach of Twin Creek was sampled it contained a mixture of hybrids between westslope cutthroat trout and rainbow trout and non-hybridized westslope cutthroat trout.

The hybrid index scores (Figure 1) indicate that individuals definitely of hybrid origin generally contain a substantial rainbow trout genetic contribution. Thus, if good quality DNA is available one may reasonably be able to distinguish hybrid from non-hybridized individuals using six diagnostic loci. If this is the case, then the population could be managed as a mixture of hybrids and non-hybridized fish. Until this is known with better certainty, however, we feel at this time the conservative approach would be to consider the Twin Creek population to be hybridized.

Deerhorn Creek 3137

The DNA extracted from fin clips in this sample was of poor quality. Thus, PINE information was obtainable from only three diagnostic loci that usually distinguish rainbow from westslope cutthroat trout. Furthermore, information from all three of these loci was obtainable from only 12 fish.

PINE fragments characteristic of rainbow trout were detected at all three of the diagnostic loci analyzed from the 12 fish from which all the information was available. The PINE fragments characteristic of rainbow trout were not randomly distributed ($P<0.001$) among the fish in the sample. In contrast, significantly more fish than expected by chance possessed PINE fragments at all three of the diagnostic loci analyzed (Figure 2). This in conjunction with the distribution of hybrid index scores (Figure 2) suggests that this portion of Deerhorn Creek when it was sampled contained individuals from at least two genetically different populations. One of the populations appears to be a hybrid swarm between rainbow and westslope cutthroat trout with a predominant westslope cutthroat trout genetic contribution (hybrid index scores of 0-2). The two individuals with hybrid index scores of three are definitely of hybrid origin as they possess PINE fragments

at other loci usually characteristic of westslope cutthroat trout. Based on the available information these two fish could be first generation hybrids or individuals from a hybridized population with a predominant rainbow trout genetic contribution. Regardless of whether or not these two fish are first generation hybrids, they appear to be relatively recent migrants into the population. Considering all the available information, essentially all of the fish in the sample appear to be of hybrid origin. The Deerhorn Creek population, therefore, should simply be considered to be hybridized.

Robb Leary

Dawson Dunning

TABLE 1

Diagnostic PINE markers between Yellowstone and westslope cutthroat trout, Yellowstone cutthroat and rainbow trout, and westslope cutthroat and rainbow trout. X= fragment usually characteristic of the 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	

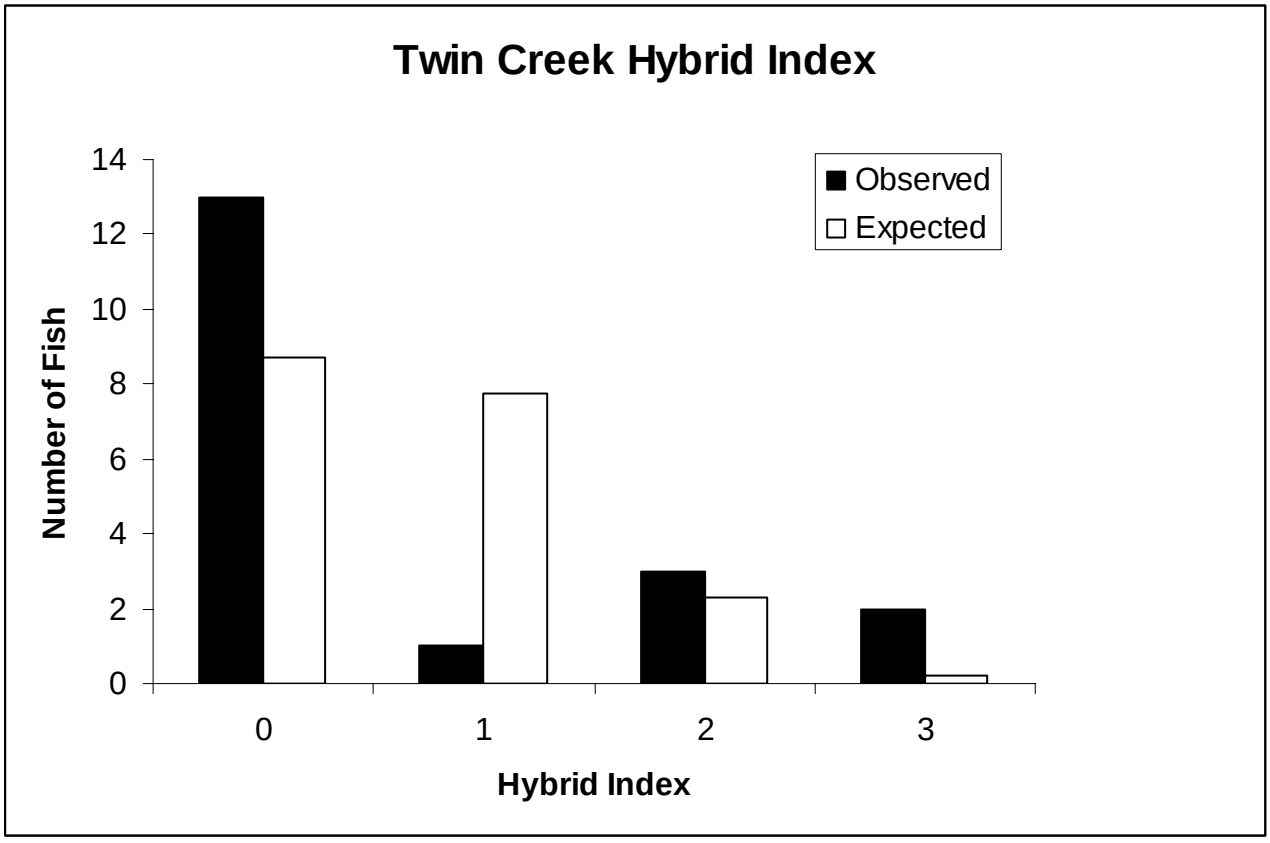

Figure 1). Distribution of hybrid index scores among 19 individuals sampled from Twin Creek in the Lower Clark Fork River drainage. The observed distribution significantly differs ($P<0.001$) from the expected random distribution suggesting this sample contains a mixture of hybrids between rainbow and westslope cutthroat trout and non-hybridized westslope cutthroat trout.

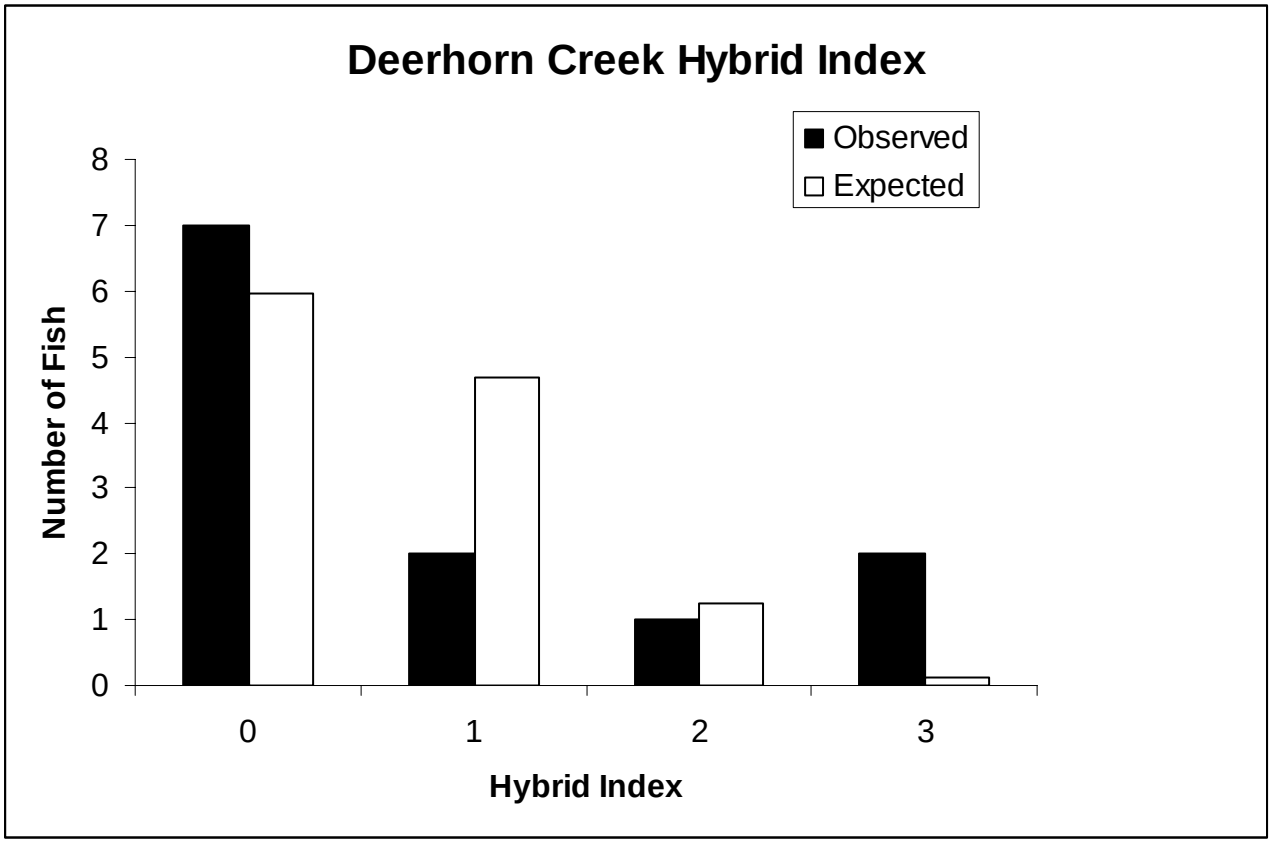

Figure 2). Distribution of hybrid index scores among 12 individuals sampled from Deerhorn Creek in the Lower Clark Fork River drainage. The observed distribution significantly differs ($P<0.001$) from the expected random distribution. In this situation, the sample appears to contain a mixture of individuals from at least two hybridized populations between rainbow and westslope cutthroat trout with different westslope cutthroat trout genetic contributions.