Genetic Analysis of two Rainbow Trout populations from the Kootenai River in northern Idaho

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Summary

Analysis of mitochondrial DNA variation among two rainbow trout populations from Fisher Creek and Long Canyon Creek, tributaries of the Kootenai River, provided evidence of complete introgression and replacement of native rainbow trout from introductions of coastal rainbow trout. These findings complement the allozyme analysis conducted by George Sage and Robb Leary of the University of Montana, who found the Fisher Creek and Long Canyon Creek samples to contain primarily alleles characteristic of coastal rainbow trout with a smaller contribution from westslope cutthroat. We found only coastal rainbow trout mitochondrial haplotypes in the two populations and no evidence of westslope cutthroat mitochondrial introgression. Westslope cutthroat mtDNA would have been easily detected, as it differs from coastal rainbow trout mtDNA by approximately 5% sequence divergence, nearly an order of magnitude greater than the differences we detected among the rainbow haplotypes observed in this study.

Mitochondrial DNA from the Long Canyon Creek sample was identical to that of rainbow trout from the Cowlitz River in southwestern Washington, and very similar (0.101% sequence divergence) to that of the Tacoma hatchery strain used by the Washington Department of Wildlife. Mitochondrial DNA from the Fisher Creek sample was very similar (0.099% sequence divergence) to that of the Tokul hatchery strain used by the Washington Department of Wildlife. Indeed, the mtDNA haplotypes from Fisher Creek and Long Canyon Creek were more similar to the Tacoma and Tokul hatchery strains, respectively, than they were to each other (0.503% sequence divergence). This is in marked contrast to most natural populations, where populations from adjacent tributaries usually exhibit mtDNA haplotypes that are identical or very similar to one another.

These data suggest that rainbow trout in the Fisher Creek and Long Canyon Creek were both derived from introductions of coastal rainbow trout, but that they originated from introductions of different strains of coastal rainbow trout. Because of their genetic status as revealed by both mtDNA and allozyme analysis, they do not represent a valuable indigenous genetic resource with respect to the goals of many agencies of identifying and preserving native populations and genetic diversity.

Introduction

The preservation and enhancement of native salmonid stocks has become an issue of much concern among federal, provincial, and state agencies. Yet the identification of genetically pure indigenous populations is difficult. This is partly due to the uncertainty of detecting differences between populations by relying on subtle changes in the range of counts from meristic and morphological data. Because man has transplanted salmonids virtually everywhere in North America, identification of intact native trout populations is a critical first step in many agencies' goals of preserving diversity among indigenous biota. Rainbow trout (*Oncorhynchus mykiss*) have been stocked more widely than any of the other North American members of the genus *Oncorhynchus* (Behnke 1992). This has led to extensive introgression with or replacement of native rainbow and cutthroat (*O. clarki*) stocks, further confounding identification of native populations (Allendorf and Leary 1988; Leary et al. 1984; 1987; 1993).

The development and application of various molecular methods has allowed a more direct assessment of population relatedness than morphological-based analyses because the genotype, rather than the phenotype, can be quantitatively assessed. With these techniques, the degree of relatedness of individuals within and between populations can be determined. When applied to a broad-based geographic survey it is possible to determine phylogenetic associations. By knowing phylogenies, it is possible to determine if certain genotypes would be expected to be native to a given region, or if their presence is more likely due to introductions by man. In the case of some DNA techniques, the presence of two or more genetically divergent groups often indicates introgression events, and that too can be quantified (Bermingham and Avise 1986; Billington and Hebert 1991). Populations can be given priority for conservation efforts where genetic analysis shows introgression is absent or slight. Populations that have been significantly introgressed due to man's introduction of hatchery reared or non-native stocks do not require the same degree of genetic conservation as do pure native stocks.

We used restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified segments of mitochondrial DNA (mtDNA) to determine the status of two rainbow trout populations from Fisher Creek and Long Canyon Creek, tributaries of the Kootenai River in northern Idaho. Native rainbow trout had a widespread distribution in Idaho occurring in all major tributaries of the upper Columbia and Snake River drainages. Substantial stocking of hatchery-reared rainbow trout and cutthroat trout has occurred. Previous genetic studies of indigenous rainbow trout populations in the desert west (Wishard et al. 1984; Leary et al. 1983; Williams et al. 1992) have often been unable to detect genetic evidence of introgression. However, Williams

et al. (*in press* a) observed introgression in several Idaho rainbow trout populations where long-term stocking had occurred.

Our research on mitochondrial genome diversity in rainbow trout and cutthroat trout indicates that hatchery populations frequently have multiple mtDNA haplotypes, often quite divergent from one another, with several haplotypes usually in a relatively high frequency within the population (i.e. 20% or greater). Conversely, our work has shown that native trout populations have low mitochondrial DNA diversity, frequently exhibiting only one mtDNA haplotype; however, when mtDNA diversity was apparent, it was due to minor changes in the DNA restriction sites and one haplotype dominated (80+% of the fish would have the dominant haplotype). Likewise, endemic populations within a given drainage basin generally shared identical or similar restriction patterns and differed from one another very little, if at all. Therefore, examination of mtDNA variation in natural populations should identify populations where introgression has occurred with hatchery rainbow trout because these populations should have mtDNA profiles more typical of hatchery trout populations than of native populations.

Purpose

Our purpose in conducting mtDNA analysis of the two Kootenai River population samples was:

a) to determine each population's taxonomic status through phylogenetic affinities, and

b) to determine to what extent, if any, each of the sample populations had undergone introgression with hatchery rainbow trout.

We would expect to identify the samples as native rainbow trout populations if they exhibit low levels of mtDNA diversity and divergence, and exhibit a mtDNA haplotype that is identical or similar to haplotypes observed in other rainbow trout populations from the upper Snake and Columbia Rivers.

Methods

Sample collection

Rainbow trout were collected from two tributaries of the Kootenai River (Table 1). Fin clips were collected and preserved in vials of ethyl alcohol before being shipped to Clear Creek Genetics for mtDNA analysis.

Table 1. Population sample, location, and sample size for rainbow trout collected from the Kootenai River system.

Sample	*Location*	*Sample Size*
1. Fisher Creek	T64N R1W Sec 22 SW 1/4	24
2. Long Canyon Creek	T64N R1W Sec 35 SE 1/4	24
	Total N =	48

Properties of mtDNA

Because mtDNA is distinct from other (nuclear) DNA and is universally distributed in the animal kingdom, homologous comparisons can be made both within and between species (Avise et al 1987). One of the most important qualities of mtDNA is that it is maternally inherited without recombination; this means that progeny of both sexes inherit mitochondria from their mothers, but only the daughters transmit mtDNA to future generations. Therefore, realistic models of mtDNA evolution can be made and phylogenies estimated. Although mtDNA does not participate in recombination, it does evolve rapidly; in fact, its nucleotide sequence changes 5-10 times faster than typical nuclear DNA (Vawter and Brown 1986). Much of the variation is due to base pair substitutions, but length differences due to additions or deletions also occur (Moritz, Dowling, and Brown 1987). Because mtDNA evolves quickly, mostly due to base-pair substitutions, it is relatively simple to detect differences between populations or species by using restriction fragment length polymorphism (RFLP) analysis.

Another important property of mtDNA is that it has a simple genetic structure, lacking repetitive DNA, introns, transposable elements, and pseudogenes. It is also remarkably uniform in gene content among all multicellular animals and even some protozoans (Wilson et al 1985). Therefore, direct comparisons can be made of the same genes between widely divergent groups. Finally, mtDNA is much easier to purify than any segment of nuclear DNA because of its unusual buoyant density, high copy number, and location in mitochondria, outside the nucleus (Wilson et al 1985).

Each typical vertebrate mitochondrion contains a circular genome, consisting of approximately 16,000 nucleotides. The most important function of the mitochondria is the synthesis of ATP by the process of oxidative phosphorylation (OXP). This process involves five multi-polypeptide enzyme complexes in the

mitochondrial inner membrane. Thirteen of the 69 separate polypeptides known to be required for OXP are coded by the mtDNA. All thirteen reading frames for these polypeptides have been mapped out and specific primers for each region have been synthesized. The regions of interest in this present study are: (1) NADH dehydrogenase subunit 1 gene (ND1), (2) NADH dehydrogenase subunits 5 and 6 genes (ND5/6), and (3) Cytochrome B gene (CytB).

Limitations of mtDNA analysis

Although the unique properties of mtDNA make it appear to be the perfect molecular system for phylogenetic analysis, it does have some potential limitations that should be recognized. One is heteroplasmy, a condition in which two or more genotypes coexist within an individual. Since each somatic cell (and mature oocyte) can contain several thousand mtDNA molecules and since new mutations arise relatively quickly in mtDNA, a heteroplasmic condition could exist. If heteroplasmy were extensive, mtDNA studies could be seriously compromised; however, empirical evidence shows that heteroplasmy is rare and therefore inconsequential (Avise et al 1987). In our studies of trout populations, we have recorded heteroplasmy from just one of over 150 populations examined.

A significant limitation of mtDNA analysis is the time scale in which it is useful for comparing phylogenetic relationships. Some regions of the mtDNA genome evolve faster than others; this is due mostly to relaxed selection constraints (Aquadro, Kaplan, and Risko 1984). Consequently, the initial rapid pace of mtDNA differentiation, which is about a 2% sequence divergence per million years in mammals (Brown, George, and Wilson 1979), is due mostly to changes in these sites, after which further mtDNA differences accumulate much more slowly (Avise et al 1987). The overall effect is that beyond about 8-10 million years, estimating the time of sequence divergence for a particular taxon becomes ambiguous and difficult to interpret. Therefore, unless only the slowly evolving mtDNA sequences are used, meaningful phylogenetic comparisons from mtDNA analysis are confined to related populations and species whose separations date to within the last few million years (Avise et al 1987). In the case of the rainbow trout, *Oncorhynchus mykiss*, the most significant divergence of the group is thought to have occurred in the late Pleistocene, and much of that since the end of the last glacial interval. This would place the most significant evolutionary events well within the last million years, and closer to the last 100,000 years.

The use of mitochondrial DNA for detection of hybridization has several caveats. Because mtDNA does not undergo recombination and is maternally transmitted, hybridization cannot be detected on the basis of individual specimens. Rather, hybridization is assessed by looking at the mtDNA haplotype profile of the population as a whole. Indicators of hybridization (i.e., gene flow with non-

native fish) are (a) high haplotype diversity within the sample population, (b) multiple haplotypes that diverge from one another more than might be expected from *in situ* mutations of the mtDNA genome, and (c) the presence of more than one predominant haplotype. As a general rule among rainbow and cutthroat trout populations, we have observed mtDNA haplotype divergence within populations to fall into two classes: (a) less than 0.6% sequence divergence, and (b) greater than 0.9% and up to 1.5% sequence divergence. We have interpreted the former class as *in situ* mutational derivatives from the native mtDNA haplotype in the population. This is nearly always supported by the frequency of the haplotypes within the population, where one haplotype is predominant, with any others representing minor variants. The second class of within-population divergence (0.9% - 1.5% sequence divergence) occurs when a non-native (and therefore divergent) mtDNA haplotype is introduced into the sample population, usually through hatchery stocking. The frequency of haplotypes in these populations can be quite variable, depending upon the degree of introgression, however, often more than one haplotype occurs at frequencies greater than 20%.

The maternal mode of inheritance for mtDNA also can pose problems for the detection of hybridization, if non-native males and females are not contributing equally to the hybridization event. For example, if a population was introgressed through non-native males selectively mating within the native population, the introgression event would not be detected in the mtDNA survey. In our studies of cutthroat trout, where we used allozymes as nuclear markers, we have not found asymmetric introgression to be a problem. However this is a possibility, since male and female fish do show different segregation times over redds. It is likely that undetectable male crossovers will be similar in frequency to the probability of detecting hybridization with mtDNA due to very low levels of introgression of both males and females. If only a very small portion of the genetic makeup is due to non-native genes, a very large sample size may be necessary to detect it.

Finally, the study of whole mtDNA RFLPs gives excellent phylogenetic results, usually with hexa- and penta-nucleotide restriction enzymes. We used this technique via Southern (1975) blot analysis in our previous examination of genetic variation among rainbow trout from the upper Owyhee River system in Nevada (Williams et al. 1992a) and for our analysis of Humboldt and Lahontan cutthroat trout populations in Nevada (Williams et al. 1992b). Unfortunately, total mitochondrial DNA isolation using cesium chloride gradient separation requires significant amounts of fresh tissue and the organism being studied usually must be sacrificed. Recent advances in molecular techniques allow us to collect field samples non-lethally (Shiozawa et al 1992), but require that the laboratory analysis examine a subunit of the mtDNA genome, rather than the

entire genome. We rely on amplification of the ND1, ND5/6, and cytB regions of the mtDNA genome. These subunits comprise nearly 35% of the rainbow trout mitochondrial genome (Williams et al. 1994). It is possible that the subunits will not provide the same or as robust information about genetic relatedness; however, our work to date with mtDNA subunits (Shiozawa and Evans 1994; Williams et al. *in press* b) has provided excellent discrimination of haplotype differences from total DNA extracts from small, non-lethal tissue samples.

Isolation of DNA

<u>The Phenol-Chloroform-Isoamyl alcohol (P-C-I) extraction procedure.</u>

1. 500 mg of fin tissue was ground with a teflon homogenizer in a 30 ml Corex centrifuge tube using 5 ml of digestion buffer until no clumps larger than 4 mm in diameter remained. The digestion buffer contained 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% proteinase K, and 0.5% sodium dodecyl sulfate at pH 8. Proteinase K efficiently degrades proteins; sodium dodecyl sulfate disrupts cell membranes and also helps in the degradation of proteins. The samples were incubated with shaking for 24 hours in tightly capped tubes.

2. The DNA was purified by extracting with an equal volume of P-C-I. Each sample was vortexed and then centrifuged for 10 minutes at 10,000 rpm in a SS34 rotor at room temperature. The P-C-I was extracted and this step was repeated to ensure complete purification. This is the most commonly used method for deproteinizing a solution of DNA. Phenol denatures and dissolves proteins and chloroform stabilizes the rather unstable boundary between an aqueous phase and a pure phenol layer. Isoamyl alcohol is added to prevent foaming of the mixture upon vortexing and to aid in the separation of the organic and aqueous phases. The denatured protein forms a layer at the interface between the aqueous and organic phases and is thus isolated from the majority of the DNA in the aqueous layer.

3. The aqueous (top) layer of each sample was transferred to a new tube containing 1/10 volume of 3M potassium acetate and 2 volumes of 100% ethanol. The DNA did not immediately precipitate so the solutions were cooled overnight at -20°C. The DNA was then concentrated by centrifugation at 12,000 rpm for 5 minutes. The supernatant was removed, and the pellet was washed with 70% ethanol. After the 70% ethanol was decanted the pellet was dried in a Savant Speed Vac Concentrator for 10 minutes. Ethanol precipitation is used for concentrating DNA solutions and for removing residual phenol and chloroform from the deproteinized aqueous solution. In the presence of relatively high (0.1 to 0.5 M) concentrations of monovalent cations such as sodium acetate, ethanol induces a structural transition in nucleic acid molecules which causes them to aggregate and precipitate from solution. Also, since most salts and small organic molecules are soluble in 70% ethanol, ethanol precipitation and washing of the pellet will effectively desalt DNA.

4. The DNA was then resuspended in 1 ml of 10 mM Tris and 1 mM EDTA at pH 7.2.

Amplification of mtDNA

Polymerase chain reaction was used to amplify four regions of the mitochondrial genome from each individual. These regions were: NADH Dehydrogenase subunit 1 gene (ND1), NADH Dehydrogenase subunit 5/6 gene (ND5/6), and the cytochrome B gene (CytB). The ND1 primers 381 and 563b generate a 2.0 kbp fragment, the ND5/6 primers 763 and 764 generate a 2.4 kbp fragment, and the CytB primers, 765 and 766 generate a 1.3 kbp fragment. Primers are obtained from LGL Ecological Genetics (Bryan, TX).

Amplification was performed in a 40 µl reaction containing:

- 1-6 µl of isolated DNA
- 0.8 µl of each of the two primers (16 pmol)
- 4.0 µl of 10 mM dNTP mix
- 4.0 µl of 10 X reaction buffer
- 0.1 µl of *Thermus aquaticus* (Taq) DNA polymerase
- 29.3 - 24.3 µl of sterile distilled water

Each cycle of the PCR consisted of denaturation for 45 seconds at 92-95°C, annealing for 40-60 seconds at 40-50°C, and elongation for 2.30-3.00 minutes at 72°C. This cycle was repeated 38 times, then an extra 2.30 minutes at 72°C was allowed for complete elongation. To insure that successful amplification had taken place, 3 µl of the amplified mixture was electrophoresed in a 2% agarose gel (NuSieve, FMC) in 40 mM Tris-acetate-EDTA (TAE) buffer (pH 8) and the DNA was stained with ethidium bromide.

RFLP (Restriction Fragment Length Polymorphism) Analysis

The three amplified regions of each individual's mtDNA was then cut separately by 8 different restriction endonucleases (RE's): *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, and *Rsa*I. Each RE is a four base cutter, except *Hinf* I, which is a five base cutter. Each restriction cut was performed in a 10 µl well on a microtiter plate. Each well contained:

- 3 µl of amplified mtDNA
- 0.2-0.3 µl of the RE
- 1.0 µl of a 10X reaction buffer
- 5.7 - 5.8 µl of sterile distilled water

The samples were allowed to incubate for at least 2-4 hours at 37°C. Electrophoresis of the digestion mixture was done in a 3% agarose gel. A pUC molecular weight fragment ladder was coelectrophoresed for fragment size reference and to facilitate sample location. The restriction fragment banding patterns resulting from electrophoresis were fluoresced under uv-light and photographed.

Data Analysis

Alphabetic designations were assigned to each unique band pattern generated by restriction endonuclease digests of each amplified mtDNA region for each specimen. The haplotype designations for each fish was based on the combined letter designation for the eight restriction enzymes used in each of the three PCR amplified regions (Table 3). These composite haplotypes (hereafter haplotypes) were compared to the database of rainbow haplotypes for genetic identification (see Table 2).

Table 2. Populations included in data analysis: Kootenai River, Cowlitz River, and hatchery rainbow trout populations by geographic area and sample size

Geographic area	*Population label*	*N=*
A. Kootenai (naturally spawning)		
1. Fisher Creek	FC	24
2. Long Canyon Creek	LC	24
B. Cowlitz (naturally spawning)	CW*	76
C. Cowlitz (hatchery)		
1. Tokul	TK	16
2. Tacoma	TC	20
		Total N = 160

* Representatives of the predominant haplotype observed native coastal rainbow trout populations from in this river system were compared to haplotypes in the Kootenai populations.

Fragment patterns were converted to restriction site data (presence or absence of a restriction site) after log transformation of digitized measurements of fragment bands and construction of inferred restriction site maps. Estimates of sequence divergence, based on the unique composite mtDNA haplotypes (Table 3) and the presence or absence of restriction sites (Table 4), were quantified with the formulae of Nei and Li (1979) and Nei (1987) as implemented by McElroy et al. in their software package REAP (Restriction Enzyme Analysis Package). REAP was used to produce a diagonal matrix of Nei's (Nei 1987) genetic distances (Table 6), which in turn was input into PHYLIP (Phylogenetic Inference Package; Felsenstein 1991) where distance methods (KITSCH) were used to estimate relationships among mtDNA haplotypes. REAP was also used to create a binary data matrix (presence or absence of specific restriction sites) for all restriction enzymes and PCR amplified mitochondrial regions. This data matrix was used in PAUP (Phylogenetic Analysis Using Parsimony; Swofford 1993) to generate a set of the most parsimonious relationships among mitochondrial DNA haplotypes.

Table 3. Input file for REAP of grouped composite mtDNA haplotypes for rainbow trout samples. Actual data start after the pound sign (#) in the table. Data are presented for the five unique composite haplotypes observed in the sample populations. Each haplotype is also listed in the comments (prefaced by "{") and shows the populations in which it was observed. Haplotype frequencies within each population are listed in Table 5.

```
{ MtDNA RFLP data for 93-94 Kootenai Rainbow collections
{ Williams and Jaworski                    9-21-95
{
{ enzyme file = cowsites.dat
{DUPLICATE HAPLOTYPES REMOVED BY GROUP
{
{Haplotype 1:  Fisher Creek (N=24)
{Haplotype 2:  Long Canyon Creek (N=23), Cowlitz River (N=76)
{Haplotype 3:  Long Canyon Creek (N=1)
{Haplotype 4:  Tacoma Hatchery Strain (N=20), Washington
{Haplotype 5:  Tokul Hatchery Strain (N=16), Washington
{
5 24 S
#
{ haplotype     cytB        ND1         ND5/6
Haplotype 1    AAAAAAAA    ABAABAAA    AAABBAAE
Haplotype 2    AAAAAAAA    AAAAAAAA    AAAAAAAC
Haplotype 3    AAAAAAAA    AAAAAAAA    AAAAAAAD
Haplotype 4    AAAAAAAA    AAABAAAA    AAAAAAAC
Haplotype 5    AAAABAAA    ABAABAAA    AAABBAAE
```

Table 4. Input file for REAP which lists the presence (1) or absence (0) of a restriction site for each restriction enzyme used in each amplified region. Amplified mitochondrial regions are cytochromeB (CB), ND1 (ND1), and ND5/6 (ND5).

```
#
CB-AluI     1 4 4
   A        1111
CB-CfoI     1 3 4
   A        111
CB-DdeI     3 6 4
   A        111111
   B        111011
   C        110011
CB-Hae3     5 5 4
   A        11111
   C        11110
   D        11011
   E        11001
   F        11000
CB-HinfI    3 4 4
   A        1110
   B        1100
   D        1111
CB-MboI     4 5 4
   A        11111
   B        11101
   C        10001
   D        10111
CB-MspI     1 4 4
   A        1111
CB-RsaI     2 5 4
   A        11101
   B        11111
ND1-AluI    3 8 4
   A        10111111
   B        11111111
   C        10101111
ND1-CfoI    3 6 4
   A        101011
   B        101111
   C        111011
ND1-DdeI    4 9 4
   A        111010111
   B        111110111
   C        111011111
   D        111010101
```

Table 4. (continued)

ND1-Hae3	6 9 4
A	111010111
B	111011111
C	100001111
D	111100111
E	101001111
G	110001111
ND1-Hinf	4 5 4
A	11011
B	11111
C	11000
D	11010
ND1-MboI	4 11 4
A	11111010101
B	11111110101
C	11111011110
D	11111010110
ND1-MspI	2 9 4
A	111111111
B	111111010
ND1-RsaI	4 12 4
A	110100011011
B	111111111011
C	110111111010
D	110100001111
ND5-AluI	1 7 4
A	1111111
ND5-CfoI	1 3 4
A	111
ND5-DdeI	1 6 4
A	111111
ND5-Hae3	2 4 4
A	1111
B	1011
ND5-Hinf	2 8 4
A	11111110
B	11111111
ND5-MboI	1 4 4
A	1111
ND5-MspI	1 2 4
A	11
ND5-RsaI	3 6 4
C	101011
D	111011
E	101111

We also used REAP to produce a diagonal matrix of average genetic distances among populations (Table 7) based on an average of the weighted haplotypes from each population in Table 2. Again, we used PHYLIP (Phylogenetic Inference Package; Felsenstein 1991; distance method, KITSCH) to estimate relationships among populations.

Results and Discussion

We examined mitochondrial DNA variation among 48 rainbow trout specimens from Fisher and Long Canyon Creeks, tributaries of the Kootenai River in northern Idaho (Table 1). Specimens from Fisher Creek all exhibited a single unique haplotype (hap-1). Specimens from Long Canyon exhibited two mtDNA haplotypes, with 23 of 24 specimens having haplotype-2 in common with native coastal rainbow trout from the Cowlitz River (CW) in western Washington. The remaining Long Canyon specimen exhibited a unique haplotype (hap-3; Table 5). The two hatchery samples, Tacoma (TC) and Tokul (TK), exhibited haplotypes 4 and 5, respectively (Table 5)

Table 5. Frequencies of the mtDNA haplotypes observed in the Kootenai River, Cowlitz River, and two hatchery populations sampled. Population codes are listed in Table 2.

	POPULATIONS					
HAPLOTYPE	FC	LC	CW	TC	TK	TOTAL
1	24	0	0	0	0	24
2	0	23	76	0	0	99
3	0	1	0	0	0	1
4	0	0	0	20	0	20
5	0	0	0	0	16	16
TOTAL	24	24	76	20	16	160

Haplotype diversity within the Fisher and Long Canyon samples were low, typical of many natural populations (Table 5). Earlier in the report, we noted that mtDNA haplotype frequencies were useful in assessing hybridization between native and non-native fishes. Hatchery populations often have a) multiple mtDNA haplotypes, that b) are often quite divergent from one another, and c) each haplotype usually occurs in a relatively high frequency within the population (i.e. 20% or greater). Neither of the Kootenai samples exhibited any of the three criteria. Nevertheless, neither appear to have mitochondrial genomes typical of interior rainbow trout. Rather, the mtDNA haplotypes observed in Fisher and Long Canyon Creeks appear to be haplotypes typical of coastal rainbow trout. Both populations appear to be derived from coastal rainbow trout (reasons discussed below), in spite of apparent downstream migration barriers and low levels of recorded stocking for both creeks (records provided by IDFG and USFWS).

Most hatchery populations of rainbow trout have been derived from coastal forms of rainbow trout (rainbow trout occurring west of the Cascades) and carry mtDNA haplotypes that differ from those of most interior rainbow trout (those occurring east of the Cascades), such as those found in Idaho, Montana, and Nevada, by 1.0 -1.5% sequence divergence (Williams et al *in press* a). Sequence divergence among all haplotypes observed in the Kootenai, Cowlitz, and hatchery rainbow trout samples did not exceed 0.71% and averaged 0.42% (Table 6).

Table 6. Percent sequence divergence among the 5 mtDNA haplotypes observed in the Kootenai River, Cowlitz River, and hatchery populations. Population codes are listed in Table 2.

Hap	*1(FC)*	*2(LC/CW)*	*3(LC)*	*4(TC)*
2(LC/CW)	0.5030			
3(LC)	0.6024	0.1006		
4(TC)	0.6024	0.1006	0.2008	
5(TK)	0.0990	0.6073	0.7071	0.7071

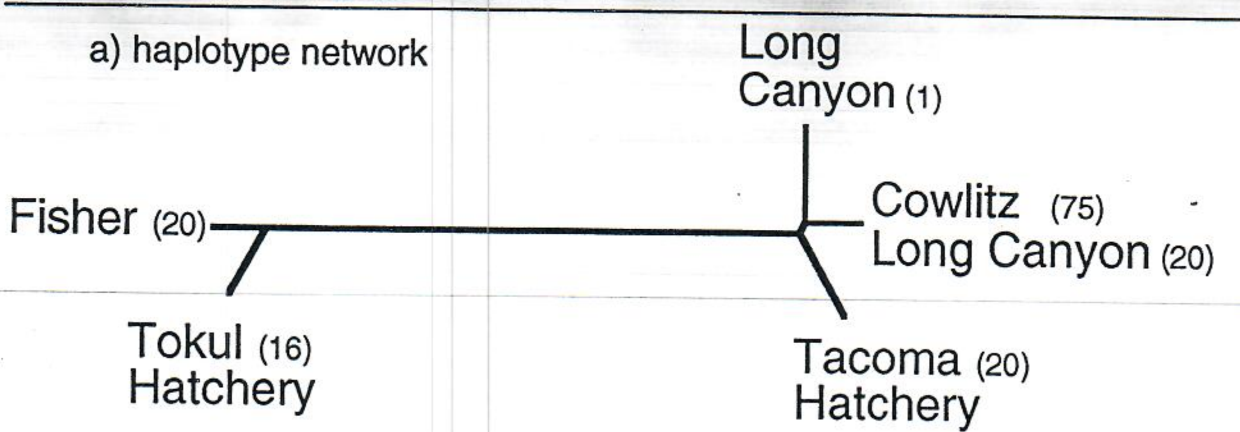
The Cowlitz, Tacoma, and Tokul mtDNA haplotypes (3, 4, and 5, respectively) are derived from coastal rainbow trout. Mitochondrial haplotypes of native interior rainbow trout, such as occurred historically in the Kootenai River, should differ from coastal rainbow trout haplotypes by approximately 1.25% $\pm$ 0.25% sequence divergence. Instead, haplotype-1 observed in the Fisher Creek sample differed from the Tokul Hatchery strain mtDNA (hap-5) by 0.099% sequence divergence and from the Tacoma strain (hap-4) by 0.602% sequence divergence. Similarly, haplotype-2, the predominant haplotype observed in the Long Canyon sample, was also the predominant haplotype observed in coastal rainbow trout from the Cowlitz River. It differed from the Tacoma Hatchery strain mtDNA (hap-4) by 0.101% sequence divergence and from the Tokul strain (hap-5) by 0.607% sequence divergence. Thus, sequence divergence between the Kootenai River samples and the three coastal rainbow trout samples show that the mtDNA haplotypes observed in Fisher and Long Canyon Creeks are coastal rainbow trout mtDNA haplotypes, rather than interior rainbow trout as would be expected based on their geographic location.

Relationships among haplotypes were examined by distance analysis of percent sequence divergence. Haplotypes were clustered using the Fitch-Margoliash algorithm from the Kitsch program in PHYLIP (Phylogenetic Inference Package; Felsenstein 1990) and depicted as both a dendrogram and network (Figure 1). The dendrogram and network are simply different depictions of the same set of relationships.

The relationship of the various mtDNA haplotypes depicted in the network (Figure 1a) and dendrogram (Figure 1b) reflect the similarities discussed above. The mtDNA haplotype observed in Fisher Creek rainbow trout is closely related to the predominant haplotype observed in rainbow trout from the Tokul hatchery strain; whereas, the haplotypes observed in rainbow trout from Long Canyon Creek are similar or identical to those from the Cowlitz River in western Washington and the Tacoma hatchery strain. These relationships are seen in both the network and the dendrogram, although the network is scaled so that branch or arm lengths reflect genetic distance, unlike the dendrogram. Consequently, the network provides a more accurate depiction of genetic similarities among haplotypes than the dendrogram.

We also examined relationships among the populations from the Kootenai River, the Cowlitz River, and the two hatchery samples, based on the average percent sequence divergence among the haplotypes within each population (weighted by the frequency of their occurrence). Again, clustering relied on the Fitch-Margoliash algorithm from PHYLIP using a diagonal matrix of the average percent sequence divergence among populations (Table 7). Those results are displayed in dendrogram and network (Figure 2) formats. The population level analysis revealed the same pattern of relationships as the haplotype analysis (Table 6, Figure 1). This was not surprising, as little variation was observed within any of the sample populations that would have changed the averaged estimates for the population from those provided by the haplotypes. The minor exception was for Long Canyon, which had one individual with a second haplotype.

Figure 1. Network and distance dendrograms of the five composite mtDNA haplotypes observed in rainbow trout populations from the Kootenai and Cowlitz Rivers and from two hatchery strains.



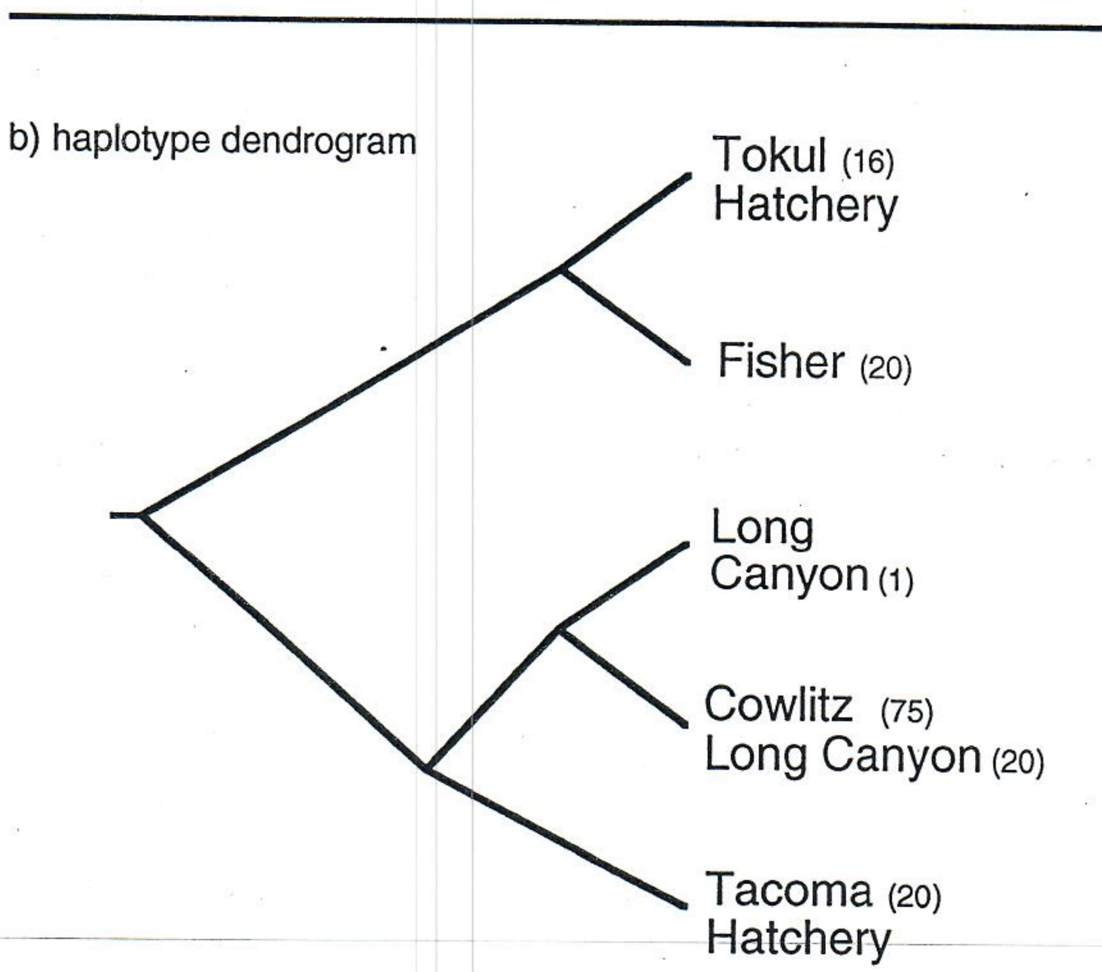


Table 7. Average percent sequence divergence among Kootenai River, Cowlitz River, and hatchery rainbow trout populations. Population codes are listed in Table 2.

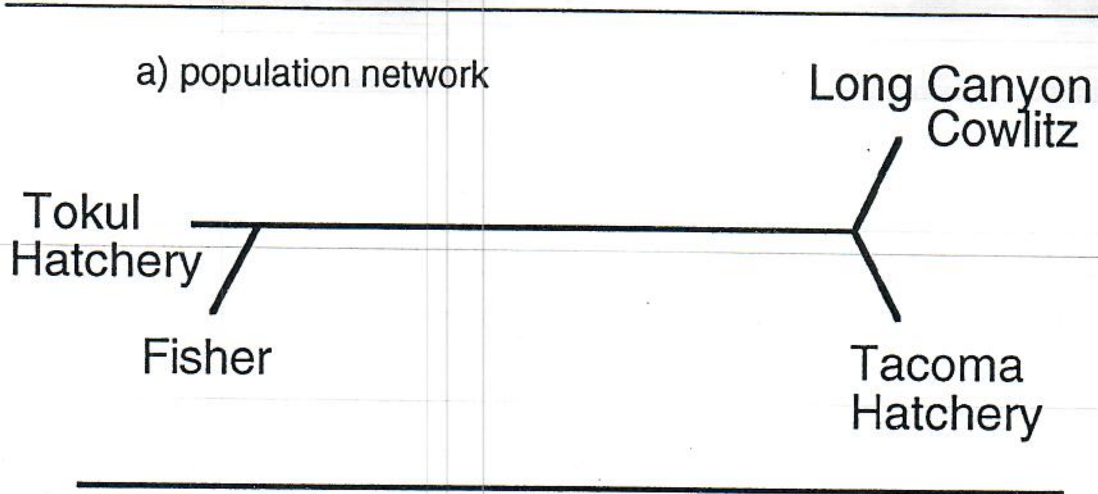
Pop	FC	LC	CW	TC
LC	0.5029			
CW	0.5030	0.0000		
TC	0.6024	0.1006	0.1006	
TK	0.0990	0.6073	0.6073	0.7071

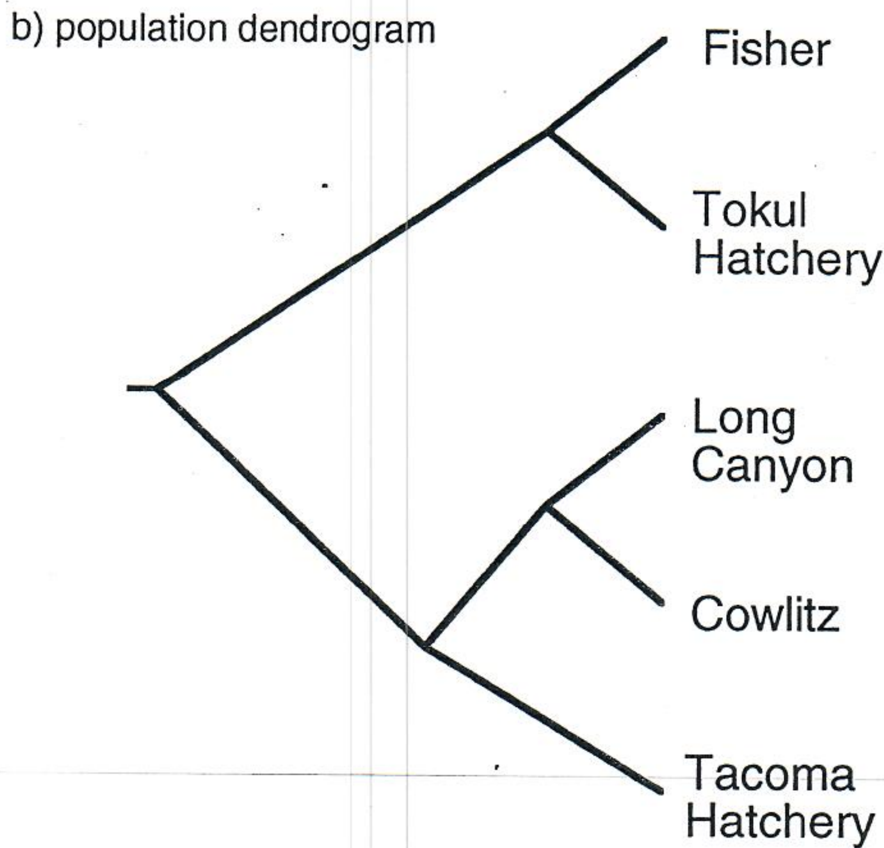
Conclusions and Management Implications

Analysis of mitochondrial DNA polymorphisms among two rainbow trout populations from the Kootenai River system in northern Idaho revealed little variation within each population and showed complete mitochondrial replacement due to introgression from hatchery rainbow trout. These findings complement the allozyme analysis conducted by George Sage and Robb Leary of the University of Montana, who found the Fisher Creek and Long Canyon Creek samples to contain primarily alleles characteristic of coastal rainbow trout with a smaller contribution from westslope cutthroat. We found only coastal rainbow trout mitochondrial haplotypes in the two populations and no evidence of westslope cutthroat mitochondrial introgression. Westslope cutthroat mtDNA would have been easily detected, as it differs from coastal rainbow trout mtDNA by approximately 5% sequence divergence, nearly an order of magnitude greater than the differences we detected among the rainbow haplotypes observed in this study.

Mitochondrial DNA from the Long Canyon Creek sample was identical to that of rainbow trout from the Cowlitz River in southwestern Washington, and very similar to that of the Tacoma hatchery strain used by the Washington Department of Wildlife (0.101% sequence divergence). Mitochondrial DNA from the Fisher Creek sample was very similar to that of the Tokul hatchery strain used by the Washington Department of Wildlife (0.099% sequence divergence). Indeed, the mtDNA haplotypes from Fisher Creek and Long Canyon Creek were more similar to the Tacoma and Tokul hatchery strains respectively, than they were to each other. This is in contrast to most natural populations, where populations from adjacent tributaries are usually identical or very similar to one another.

Figure 2. Distance network and dendrogram of the average percent sequence divergence among mtDNA haplotypes observed in five rainbow trout populations from the Kootenai and Cowlitz Rivers and from two hatchery strains..





These data suggest that rainbow trout in the Fisher Creek and Long Canyon Creek were both derived from introductions of coastal rainbow trout, but that they originated from different strains of coastal rainbow trout. Because of their genetic status as revealed by both mtDNA and allozyme analysis, they do not represent a valuable indigenous genetic resource.

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