DNA FINGERPRINTING OF ALBERTA BULL TROUT (*SALVELINUS CONFLUENTUS*) POPULATIONS

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Abstract

Bull trout (*Salvelinus confluentus*) populations from Alberta river drainage systems were compared using molecular techniques. Restriction fragment length polymorphisms (RFLP's) within the ND1 and ND5/6 regions of the mitochondrial genome were observed. In addition, randomly amplified polymorphic DNA profiles (RAPD's) from total genomic DNA extracts were compared.

Interdrainage comparisons using mtDNA revealed significant population heterogeneity among Alberta bull trout. Percent sequence divergence in mtDNA ranged from 0.14% to 0.92%. Most fish in each population were composed of a small number of common haplotypes, and the remaining fish displayed rare or locally unique haplotypes. RAPD profiles were used to calculate genetic distance values for Alberta, Canada and Montana, U.S.A. populations. Both Nei and Cavalli-Sforza distance values were used to generate neighbor-joining, FITCH and KITSCH distance trees. Two genetically distinct groups of bull trout were revealed by the RAPD analysis and the possibility that post-glacial bull trout populations are derived from two separate refugia is suggested.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABI	Applied Biosystems, Inc.
Alu I	*Arthrobacter luteus* I
ATP	adenosine triphosphate
ave	average
avedev	average deviation
bp	base pairs
BSA	bovine serum albumin
BTTF	Bull Trout Task Force
C	cytosine
Cfo I	*Clostridium formicoaceticum* I
COSEWIC	Committee on the status of endangered wildlife in Canada
CS	Cavalli-Sforza
CTP	cytidine triphosphate
Dde I	*Desulfovibrio desulfuricans* I
DNA	deoxyribonucleic acid
DW	Distance Wagner
FM	Fitch-Margoliash
G	guanine
GTP	guanosine triphosphate
H	heterozygosity
Hae III	*Haemophilus aegyptius* III
Hinf I	*Haemophilus influenzae* I
Hpa II	*Haemophilus parainfluenzae* II
ITS-1	internal transcribed spacer - 1
kb	kilobase pairs
MP	maximum parsimony
mtDNA	mitochondrial deoxyribonucleic acid
NADH-1	nicotinamide adenine dinucleotide - 1
ND1	nicotinamide adenine dinucleotide dehydrogenase - 1
ND2	nicotinamide adenine dinucleotide dehydrogenase - 2
ND3	nicotinamide adenine dinucleotide dehydrogenase - 3
ND5/6	nicotinamide adenine dinucleotide dehydrogenase - 5/6
NJ	neighbor-joining
OTU	operational taxonomic unit
P	polymorphism
PCR	polymerase chain reaction
PHYLIP	phylogenetic inference package
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal DNA
REAP	restriction enzyme analysis package
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
rRNA	ribosomal RNA
Rsa I	*Rhodopseudomonas sphaeroides* I
SD	standard deviation
TBE	tris-borate, EDTA
TEMED	N,N,N',N' -tetramethylethylenediamine
Tris-HCl	Tris-hydrochloric acid
tRNA	transfer RNA
TTP	thymidine triphosphate
UPGMA	unweighted pair group method using arithmetic averages
UTP	uridine triphosphate
VNTR	variable number tandem repeat

List of Fish Species

Anchovy	*Engraulis encrasicolus*
Arctic Char	*Salvelinus alpinus*
Arctic Grayling	*Thymallus arcticus*
Atlantic Salmon	*Salmo salar*
Brook Trout	*Salvelinus fontinalis*
Brown Trout	*Salmo trutta*
Bull Trout	*Salvelinus confluentus*
Chinook Salmon	*Oncorhynchus tshawytscha*
Chum Salmon	*Oncorhynchus keta*
Coho Salmon	*Oncorhynchus kisutch*
Cutthroat trout	*Oncorhynchus clarki*
Dolly Varden	*Salvelinus malma*
Japanese Ayu	*Plecoglossus altivelis*
Lake Trout	*Salvelinus namaycush*
Minnows	*Cyprinidae* spp.
Mountain Whitefish	*Prosopium williamsoni*
Pink Salmon	*Oncorhynchus gorbuscha*
Rainbow Trout	*Oncorhynchus mykiss*
Sculpins	*Cottus* spp.
Sockeye Salmon	*Oncorhynchus nerka*
Yellow Perch	*Perca flavescens*

1.0 The Problem

1.1 Rationale for a genetic study of bull trout

Bull trout (*Salvelinus confluentus*), also known as bull char, is one of two species of trout native to mountain streams of Alberta (Nelson and Paetz, 1992). Once thought to be conspecific with the coastal variety known as Dolly Varden (*Salvelinus malma*), they are now recognized as a distinct species (Cavender, 1978; Haas and McPhail, 1991). Bull trout often co-exist with brook trout (*Salvelinus fontinalis*) which were introduced from eastern North America (Nelson and Paetz, 1992). Both bull trout and brook trout are fall spawners and their spawning sites overlap spatially and temporally (Roberts, 1987).

Historically, populations of bull trout were abundantly distributed in the headwaters of all major drainage systems on the eastern slopes of the Rocky Mountains. Creel counts and anecdotal records dating back to the late 1800's show that the species flourished throughout the upper regions of the South Saskatchewan River drainage (Fitch, 1994) and north as far as the Peace River (Roberts, 1987). More recently, the number of bull trout has declined substantially. They were nearly eradicated from Kananaskis streams (Mayhood, 1995) and have been completely extirpated both from much of the Red Deer River drainage (Roberts, 1987) as well as substantial portions of the Alberta southwestern streams (Fitch, 1994). Roberts (1991) provided evidence that the normal range for bull trout extended well out onto the prairies. He cited records of bull trout angling in the Oldman River downstream as far as Lethbridge, in the vicinity of Carsland on the Bow River, in the badlands of the Red Deer River and in the Edmonton area of the North Saskatchewan River. None of these areas are

currently inhabited by bull trout and present estimates indicate that the species occupies only 30% of its historical range (Fitch, 1994).

A variety of reasons for this decline have been proposed including: (1) loss of habitat, (2) overfishing and (3) competition with introduced species. Fraley et al. (1989) described the effect of timber harvest and road building on the quality of stream beds used for spawning. They found that even moderate streambed silting greatly reduced fry production. Successful bull trout spawning depends on stringent requirements for gravel covered stream beds. The long incubation and development phase for bull trout embryos makes them particularly vulnerable to increases in fine sediments or changes in water quality (Fraley and Shepard, 1989).

Loss of habitat also occurs with the introduction of physical barriers in the streams such as dams and weirs. The Dickson and St Mary's River dams have introduced impassable barriers which effectively eliminate downstream portions of bull trout migratory routes (Roberts, 1991). The completion of the Oldman River Dam in 1991 blocked the last remaining access to prairie streams by bull trout (Nelson and Paetz, 1992). Fish that do manage to get downstream cannot return to their spawning areas and ultimately die without the ability to maintain viable downstream populations.

Overfishing is considered a major contributor to the decline of bull trout populations (Boag, 1987; Roberts, 1987). The fish are easy and exciting to catch because of their broad diets and aggressive feeding habits (Roberts, 1993). The situation is exacerbated by slow rates of maturation. Typically, it takes five to seven years for bull trout to reach spawning age (Roberts, 1987) by which time many are caught and removed from the population.

Bull trout compete with other fish on a variety of levels. Boag (1987) investigated food competition between bull trout and rainbow trout (*Oncorhynchus mykiss*). Though bull trout are more piscivorous than rainbow, they assume similar diets in the upper reaches of some streams. Of interest was the large number of fish eggs found in the stomachs of rainbow trout, raising the possibility that introduced species may contribute to the decline of bull trout through predation on eggs.

Donald and Alger (1993) reported that lake trout (*Salvelinus namaycush*) caused the displacement of bull trout from lakes located in regions where the two species have overlapping niches. In one lake, introduction of lake trout decimated an indigenous population of bull trout over a thirty year span.

Competition with brook trout also may contribute to declines in bull trout populations. Brook trout have been introduced into every major drainage of the Canadian Central Rockies ecosystems region (Mayhood, 1995). Hybridization between bull trout and brook trout was documented in several studies (Kitano et al., 1994; Markle, 1992). There is little evidence that such hybridization is solely responsible for the decline of bull trout populations but it may be a contributing factor in regions where bull trout numbers are already low. For example, Roberts (1993) suggested that hybridization most likely occurs if male bull trout are scarce and many male brook trout are in the vicinity of bull trout females. Thus, bull trout x brook trout hybridization may be more the result rather than the cause of a decline in bull trout (Roberts, 1987). In any case, such crosses perpetuate loss of bull trout because potential pure-line progeny are lost and the hybrids are usually sterile (Kitano et al., 1994).

The decline in Alberta bull trout populations parallels a similar situation in the eastern

slopes region of Montana. In 1989, bull trout were added to the American Fisheries Society updated list of fishes that are endangered, threatened, or of special concern (Williams et al., 1989). They were categorized as fish of "special concern" due to the threatened destruction of habitat and various factors affecting their existence including hybridization, introduction of exotic species, predation and competition. Application in the United States for protection of the species under the Endangered Species Act has been made (Leary et al., 1993). The status of bull trout in Canada has been under review by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) since 1988 (Campbell, 1988) and the species was recently described as "vulnerable" (Campbell, 1994), indicating official concern over declining populations. In Alberta, a comprehensive plan geared towards the management and recovery of bull trout populations was introduced in 1994 (Berry, 1994). The plan focused on conservation tactics and recommended zero catch limits in the recreational fishery. In addition, future studies were proposed including a suggestion that genetic studies be performed to investigate hybridization with brook trout. Concurrently, an alliance of organizations including sport-fishing groups, conservation groups, university academics and regional governments formed the Bull Trout Task Force (BTTF), through which priorities were set for future research related to Alberta bull trout (Bull Trout Task Force, 1995). The need for genetic studies of Alberta bull trout populations was recognized by this group although such studies were designated as low priority.

Currently, no genetic studies of Alberta bull trout have been completed. Consequently, management strategies were designed without knowledge of genetic structure or the extent of genetic variation among bull trout populations. In Montana, genetic studies

revealed little intrapopulation variation but significant interpopulation variation (Leary et al., 1993). The importance of these differences should not be underestimated. The presence of genetic differentiation among populations may indicate significant levels of local adaptation (Carvalho and Hauser, 1994). Consequently, management strategies should consider the local effects of such practices as habitat alteration, stocking and introduction of other species. In this context, understanding the extent of genetic variation within a species is important in the formulation of a rational management scheme (Ferguson, 1990). If the genetic integrity of locally adapted populations is to be preserved, they must be characterized relative to geographical references so that informed management decisions can be made.

Increasingly, management strategies are based on ecosystem preservation rather than species preservation (Stanford and Ward, 1992). The loss of local bull trout populations may affect the existence of many other species in the ecosystem (Mayhood, 1995). Preservation of entire communities requires long-term commitments to habitat protection. Monitoring these communities requires baseline status information for accurate assessment of future changes in fish populations (Williams et al., 1989). Genetic studies provide a sensitive measure of baseline status and they facilitate the detection of changes in local gene pools.

One American study suggested that a bull trout stocking program would provide a promising management option for the recreational fishery (Fraley et al., 1989). This proposal was met with severe criticism from several researchers. Leary et al. (1993) suggested that stocking may lead to loss of local adaptations and does not remove the original causes of population decline, such as habitat destruction. Though the essence of hatchery programs is to introduce fish for the rehabilitation of natural populations, the practice disregards the

concept that species are divided into genetically distinct localized units which are adapted to their particular environment (Ferguson, 1990). Such local adaptations may include the timing and extent of migration, temperature preferences, growth rates, foraging strategies and life history characteristics. Introduced fish have the potential to disrupt the genetics of these local populations, possibly resulting in local extirpation and a reduction in regional diversity. The presence of diverse locally adapted populations contributes to greater regional stability because multiple variant populations are less likely to go extinct simultaneously (Rieman and McIntyre, 1996). Conservation efforts should encourage the existence of local variant populations, but a stocking program likely would homogenize the gene pool promoting the opposite effect.

In summary, the decline in Alberta bull trout populations has been well documented and efforts to reverse this decline are ongoing. Current management practices cannot consider potential genetic differences among Alberta bull trout because no previous genetic studies exist. The implementation of genetic research on bull trout should greatly enhance future management efforts through the identification of specific localized gene pools and the establishment of baseline data for monitoring future changes in the genetics of these populations.

1.2 Theoretical framework

1.2.1 Measuring DNA polymorphism

Recent improvements in molecular techniques have provided new tools for population

genetic studies. Methods now exist which are rapid and highly sensitive for detection of genetic polymorphism. Variation can be measured in many types of DNA and the applications are widespread.

Prior to development of molecular techniques, population comparisons were dependent on analysis of differences in morphology, physiology and behavior (Avise, 1994). Limitations in such studies were inherent since many of these traits are heavily influenced by environmental conditions and developmental timetables. In addition, genetic factors regulating these traits are not always understood.

In the 1960's, molecular techniques for protein comparisons were developed (May, 1992). Variations were detected either with immunological methods which highlighted differential antigenic properties (Avise, 1994) or by allozyme electrophoresis which detected amino acid composition changes in enzymes (Carvalho and Hauser, 1994). Since the structure of proteins is regulated genetically, this method was considered revolutionary. It rapidly exceeded the sensitivity of conventional approaches and provided more direct measures of genetic difference among populations. Large numbers of proteins could be rapidly assayed at a relatively low cost. Data were used to study genetic differentiation, reproductive isolation and mating patterns (Carvalho and Hauser, 1994). Several limitations to protein studies have been described by Park and Moran (1994). Only coding regions of DNA are assessed in protein studies and these are estimated to comprise less than 5% of the total genome. Furthermore, resolution is limited by the redundant nature of the genetic code since identical proteins may be transcribed from different DNA sequences. As a consequence, conventional allozyme electrophoresis may underestimate variability and some alleles remain

undetected with the technique (Murphy et al., 1996). Other concerns include the frequent need to kill organisms for tissue (Park and Moran, 1994), the relative instability of protein molecules, especially when subjected to freeze and thaw storage and handling, and nongenetic sources of phenotypic variation in proteins such as posttranslational modifications which result in conformational isozymes (Murphy et al., 1996).

Direct analysis of DNA allows more precise detection of polymorphism in populations. Choices necessary prior to direct DNA analysis include selection of a DNA source, the region of the genome to be examined, and a method of analysis. The many choices currently available facilitate quantification of genetic relatedness among individual organisms at numerous levels, from close familial relationships to evolutionarily distant phylogenetic ones (Burke et al., 1991).

The source of DNA refers to its cellular origin: either nuclear or mitochondrial. Nuclear DNA comprises the bulk of cellular DNA and provides an enormous supply of genetic markers if the inherent variation can be accessed (Dowling et al., 1996). In fish, the nuclear genome is estimated to be about four billion base pairs (bp) in size (Park and Moran, 1994). Nuclear DNA studies in the salmonids are complicated by the polyploid nature of the genome. About 25-100 million years ago, ancestors to modern salmonid species experienced a genome duplication event resulting in tetraploidy and they are presently undergoing a rediploidization process (Allendorf and Thorgaard, 1984). About 50% of the additional loci created by genome doubling have become nonfunctional, presumably through mutational processes, and they no longer produce detectable protein products.

A variety of DNA types within the nuclear genome are described by Park and Moran

(1994). Some DNA specifically codes for proteins and may be present either in single copies (scnDNA) or as repetitive sequences such as those coding for histones. The bulk of the nuclear genome is noncoding and it too may appear either as single copies found in many regulatory regions and introns, or as repetitive sequences such as satellite DNA. Noncoding repeat sequences, called variable number tandem repeats (VNTR's), contain sufficient variability to distinguish between individual organisms within a single population; thus the term "DNA fingerprint" was coined (Jeffreys et al., 1985). VNTR's are composed of satellite DNA which is usually further classified based on the length of the repeat unit. Minisatellite regions display oligonucleotide repeats that are typically 9-25 base pairs (bp) in length and are usually G-C rich (Bentzen et al., 1991). Microsatellites are one to six base pair fragments usually found in a tandem sequence repeating up to 100 times (Goldstein et al., 1995). High levels of variation in satellite regions, particularly in fish, make them ideal for both intraspecific and interspecific DNA comparisons (Franck et al., 1991). VNTR's are noncoding and therefore are assumed to contain variation independent of natural selection (Carvalho and Hauser, 1994). The result is a large degree of variability between closely related groups of organisms rendering this class of DNA ideal for population studies. One disadvantage to using satellite DNA is the need to develop primers specific for each species of interest since transfer from one species to another often is not effective (Dowling et al., 1996).

The nuclear genome in salmonids is complex and highly variable. Its tetraploid nature results in twice the amount of DNA per cell than closely related fish. Chromosome number varies from 52 in pink salmon (*Oncorhynchus gorbuscha*) to 102 in European grayling

(*Thymallus thymallus*). Even within the genus *Salvelinus*, the chromosome number ranges from 78 (*S. alpinus*) to 84 (*S. fontinalis*) and intraspecies variation is not uncommon (Allendorf and Thorgaard, 1984).

Mitochondrial DNA is one of the most studied portions of the genome in animals (Park and Moran, 1994). Several characteristics of mtDNA make it ideal for population studies. It is inherited maternally in most organisms and exists in a haploid state which is not subject to recombination events (Park and Moran, 1994). Consequently, the effective population size for mtDNA is one-fourth of that for nuclear DNA, which makes it more likely to experience gene frequency changes caused by genetic drift between isolated gene pools (Park and Moran, 1994). Generally, there is strong conservation of gene order in closely related animals but a rapid rate of sequence divergence may occur in some gene regions (White and Densmore III, 1992). Since different regions evolve at different rates, the type of study should determine the region targeted. For example genus and family level studies may focus on gene order or base sequences coding for tRNA and rRNA (Dowling et al., 1996). The D-loop, which contains control regions for mtDNA replication and transcription, is highly variable in some mammals making it useful for population studies and the cytochrome *b* and ND genes also are widely used at this level (Park and Moran, 1994). The relative speed at which mtDNA evolves at the sequence level is attributed, at least in part, to a lack of known repair mechanisms for mutations that arise during mitochondrial replication and most sequence differences result from point mutations in which transitions predominate over transversions (Avise, 1994).

Mitochondrial DNA provides an additional advantage when doing restriction fragment

length polymorphism (RFLP) analysis. Methylation sensitive restriction endonucleases may provide a biased perspective on the relatedness of organisms if the degree of methylation varies in the DNA fragments of interest. Variable methylation states could mimic the appearance and disappearance of restriction sites resulting in the overestimation of genetic variability between organisms. This methylation problem does not exist when using mtDNA (Dowling et al., 1996). In fact, use of PCR products from any DNA source entirely eliminates the problem since no methylation occurs during the amplification process.

The main disadvantages of using mtDNA stem from its lack of genetic recombination. Even though it is composed of over thirty genes it is treated as a single locus (Park and Moran, 1994). Consequently, estimates of genetic diversity obtained from mtDNA are expected to exhibit greater variance than when such estimates are made from a similar number of nuclear genes (Dowling et al., 1996). Another disadvantage arises from the potential for "within" individual copy number variation in some tandem repeat sections which may reflect *de novo* replication errors rather than heritable changes (White and Densmore III, 1992). Avise (1994) suggested that the resultant change in the size of the repeat regions could be distinguished from restriction site changes because they alter concordantly the sizes of particular restriction fragments in all digestion profiles. A third problem with mtDNA is the possibility for biparental inheritance in some organisms, which may complicate analysis (White and Densmore III, 1992). Though a more common occurrence with chloroplast DNA of plants (Dowling et al., 1996), biparental inheritance of mtDNA has been documented in animals such as mice (Gyllensten et al., 1991) and even in fish (Margoulas and Zourous, 1993), although they were anchovy (*Engraulis encrasicolus*) rather than salmonids.

Several methods have been developed that facilitate the direct comparison of DNA among groups of organisms. DNA hybridization is a common technique used to assess the extent of relatedness among organisms (Werman et al., 1996). It utilizes the complementary nature of double stranded DNA and involves the annealing of two nonidentical complementary strands under different salt and temperature conditions (Park and Moran, 1994). The melting temperature of double stranded DNA is determined by the number of hydrogen bonds between complementary base pairs. Base mismatches within the double strand lower the melting temperature due to a reduced number of hydrogen bonds. Heteroduplex hybrid DNA (from groups of different organisms) have more base mismatches and a lower melting temperature than homoduplex hybrid DNA (from groups of the same organisms). The difference in melting temperature between homoduplex and heteroduplex hybrids can be used to estimate genetic distance between the two groups (Avise, 1994).

Another technique which has become essential in current DNA studies is the use of restriction endonucleases. These enzymes cleave double stranded DNA at specific recognition sites characterized by a particular DNA base sequence. DNA containing these sites is enzymatically cut into smaller fragments which can be separated and visualized using electrophoretic techniques. Fragment patterns depend on the presence or absence of restriction sites. Variations in fragment patterns are called restriction fragment length polymorphisms (RFLPs) (Aquadro et al., 1992) and form the basis of interspecies and intraspecies comparisons. RFLP analysis is particularly useful for population level studies when used with mtDNA (Avise, 1994). Differences in fragment patterns may result both from base substitutions which cause the gain or loss of restriction sites, or by insertion and

deletion mutations (Park and Moran, 1994). Comparisons may be based on fragment sizes or on a map of restriction sites (Dowling et al., 1996).

Development of the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) has dramatically increased the potential for DNA comparisons since it facilitates the amplification of specifically targeted DNA sequences and allows use of very small amounts of source tissue. DNA polymorphism may be detected as variable sized PCR products amplified from primers complementary to highly conserved base sequences flanking a variable region of DNA. For example, coding regions in ribosomal DNA (rDNA) are highly conserved but the intervening spacer regions potentially contain high levels of polymorphism (Zhuo et al., 1994). Also, primers have been developed which target a wide variety of DNA types including satellite regions (Morris et al., 1996), specific genes such as the tumor suppressor gene *p53* (Kusser et al., 1994), spacer regions of rDNA (Pleyte et al., 1992), as well as highly conserved mitochondrial DNA regions (Simon et al., 1994).

A recent technique which facilitates rapid assessment of polymorphism with relative ease is the use of randomly amplified polymorphic DNA (RAPD). Single primers with arbitrary nucleotide sequences are used in PCR amplification resulting in unique DNA fragment patterns with dominant and recessive characteristics (Avise, 1994). Primers are usually 10-12 bp long (Hoelzel and Green, 1992) and should contain a GC content of at least 40% (Williams et al., 1990). RAPD's have been used to discriminate among plant cultivars, to describe patterns of relationships among animal and plant populations and for the estimation of parentage (Dowling et al., 1996). Unlike other PCR based techniques, no prior knowledge of flanking regions is required for primer development but replication of PCR

products is highly dependent on exact duplication of the PCR reaction conditions (Williams et al., 1990).

A wide variety of methods are available for genetic comparisons at the population level. Protein electrophoresis and RFLP analysis of mtDNA have been widely used and are highly recommended for this purpose (Avise, 1994; Park and Moran, 1994). More recently, the value of microsatellites and RAPDs has been recognized (Caetano-Anollés et al., 1991; Park and Moran, 1994). Ultimately, the design of a population study must weigh the pros and cons of each method as well as consider practical aspects such as the cost of analysis, tissue requirements, and availability of probes.

1.2.2 Genetic distance

Genetic distance is an estimate of genetic divergence (Avise, 1994). Distance measurements are used to objectively quantify genetic differences among groups of organisms. Several algorithms have been developed for calculating distance values from quantitative data sets, which are usually composed of amino acid sequences, DNA sequences or allele frequencies. Typically a distance tree or dendrogram is developed to graphically represent distance relationships.

The use of quantitative characters to estimate evolutionary relationships has been progressively examined for decades. Felsenstein (1981, 1984) describes a chronology beginning in the mid-1960's with work published by Cavalli-Sforza. The Cavalli-Sforza (CS) chord measure calculates distances based on gene frequencies (Cavalli-Sforza and Edwards, 1967). At the same time Fitch and Margoliash (FM) produced their own algorithm to

quantify variation in amino acid sequences by calculating the percentage of sites differing between two species (Fitch and Margoliash, 1967). According to Felsenstein (1984), both the CS and FM approaches grew out of earlier clustering techniques.

Additional approaches were introduced over the next twenty years. Nei (1972) defined "genetic distance" as an accumulated number of allele differences per locus. He stated that the "ideal" measure of genetic difference would be the number of nucleotide differences per unit length of DNA. At the time DNA sequencing was considered too expensive and time consuming, therefore he relied on gene frequency data obtained from protein studies. In the same year the Distance Wagner (DW) method was introduced (Farris, 1972), followed closely by a refined clustering technique called UPGMA or unweighted pair-group arithmetic average clustering method (Sneath and Sokal, 1973). Although many debates and refinements followed the introduction of these techniques (Felsenstein, 1985a; Felsenstein, 1985b; Reynolds et al., 1983), the next novel approach for analyzing distance data did not appear until 1987 when the neighbor-joining (NJ) method was introduced (Saitou and Nei, 1987).

As new directions in molecular research evolved, there have been corresponding changes in the methods used to calculate genetic distance values. These include a coefficient of gene diversity calculated from single-probe multilocus DNA fingerprints (Jin and Chakraborty, 1994), an algorithm based on a step-wise mutation model for microsatellite data (Goldstein et al., 1995), and a coefficient of similarity designed for RAPD data (Lamboy, 1994a; Lamboy, 1994b).

When phylogenetic studies began to favor maximum likelihood and parsimony analyses, attempts were made to model these approaches using continuous character data for

genetic distance calculations. Felsenstein (1981, 1992) developed a maximum likelihood approach for use with gene frequencies and quantitative characters. Swofford and Berlocher (1987) described the use of gene frequency data to infer an evolutionary tree using principles of maximum parsimony. Their method looks for the ancestor which requires the least amount of allele frequency change necessary to result in the contemporary frequency distributions.

The selection of an appropriate measure of genetic distance is complicated by the large number of distance algorithms which exist and requires an understanding of the assumptions upon which each method is based. Most distance methods begin with an OTU x OTU data matrix (Avise, 1994). An OTU (operational taxonomic unit) is the group from which molecular data were collected for comparison. These data consist of DNA base sequences, amino acid sequences, or gene frequencies. The matrix body contains distance estimates determined by comparing each OTU to every other OTU in the data set. Excluding self comparisons, for n OTU's there are n(n-1)/2 pairwise distances (Avise, 1994). Thus a data set with 10 populations has 45 pairwise comparisons in its data matrix. In order that data may be logically organized for analysis, a dendrogram or distance tree is usually developed. Such trees resemble phylogenetic trees in appearance but are not intended to represent putative evolutionary pathways. Some distance algorithms are specifically designed for calculating distance values (CS, Nei) while others do the tree building (NJ, DW, FM, UPGMA).

The exact method of calculating distance values varies with each algorithm since each approach is based on unique assumptions which may not be applicable to every situation. Common to all distance methods are the assumptions that different groups or taxa evolve independently and that all genetic distances result, at least in part, from genetic drift

(Felsenstein, 1981). Several differences among distance methods are highlighted with the following examples. The UPGMA method is a clustering procedure in which clusters or groups are created based on the smallest mean distance between the taxa involved (Ferguson, 1980). The method is called unweighted because each OTU contributes equally to the means. UPGMA assumes an equal rate of evolution along all branches (Avise, 1994). This imposes a strong limitation on its use since there is substantial empirical evidence which suggests that equal rates of evolution do not occur in many instances (Forey et al., 1992). Even with this apparent weakness, UPGMA may provide some advantage because distance estimates are subject to large stochastic errors and the "distance averaging" aspect of UPGMA tends to reduce the effect of such error (Nei, 1987).

Fitch and Margoliash (1967) also make pairwise comparisons of taxa. They attempt to build the shortest possible tree by seeking one which minimizes deviation between observed and tree-derived (patristic) distances. Each possible tree arrangement is considered and the one with the lowest percent standard deviation from the observed data is accepted (Ferguson, 1980). Genetic distances are reflected by branch lengths on the tree and they are additive. An FM tree does not assume a constant rate of evolution; therefore branches to two or more descendants from a common ancestral node may vary in length.

Neighbour-joining is related to cluster analysis but like FM, it allows for unequal rates of evolutionary change along adjacent tree branches (Avise, 1994). The method begins with a "starlike" tree in which no hierarchical structure is present (Saitou and Nei, 1987). Subsequently, pairs of OTU's are grouped so as to minimize branch distances. The algorithm is heuristic in nature so that it arrives directly at a final topology without attempting to build

and examine every possible tree. Consequently large data sets can be analyzed with greater expedience than with most other procedures. Heuristic methods are not ideal however, since the order in which taxa are added to the tree constrains the subsequent topologies. The resulting tree is optimal based on the order the taxa were added but is not necessarily the optimal tree if all possible topologies were evaluated (Wiley et al., 1991). Even with this shortcoming NJ may still be the algorithm of choice if a large data set makes it unrealistic to examine all possible topologies.

The Distance Wagner (DW) method (Farris, 1972) assumes that observed distances represent the lower boundary of the true values (Avise, 1994). Therefore Wagner trees always have branch lengths which equal or exceed observed values. As in neighbour-joining, the algorithm searches for the tree with the shortest total length.

Choosing the most appropriate distance measure for a particular study may be difficult. The choice should be based on advantages and disadvantages inherent in each method rather than availability or historical inertia (Hillis et al., 1993). Ideally the underlying assumptions for each algorithm are matched to the biology of the organisms in question. For example, Nei's genetic distance assumes a constant population size whereas CS allows for fluctuation. With Nei's algorithm mutations are thought to contribute to shifts in allele frequencies with the same influence as genetic drift, but CS assumes that genetic drift is the sole cause of genetic change. FM and NJ allow for unequal rates of mutation whereas UPGMA assumes a constant rate of change. It is apparent that genetic distance methods should not be chosen randomly but instead with a clear realization of the limitations inherent in each.

Choosing the best tree drawing algorithm requires additional considerations. For example, NJ allow negative branch lengths where others are constrained. Branch lengths reflect the genetic distances between taxa. Generally there is an expectation that branch lengths are additive and that the rule of triangle inequality is satisfied (Forey et al., 1992). Distance values for adjacent branches may be added together but for any three taxa (A, B and C), triangle inequality requires that:

$$d(A,B) \leq d(A,C) + d(B,C)$$

Allowing negative branch lengths ensures triangle inequality, but at the expense of maintaining additivity. To circumvent this difficulty, it has been argued that additivity is only an 'expectation' and that negative branch lengths represent sampling error (Felsenstein, 1984). Alternatively, it was proposed that branch lengths do not always have to be additive if homoplasy is present (Forey et al., 1992). Homoplasy is the component of overall similarity between taxa caused by convergent evolution or by a reversal of character states back to an ancestral form (Wiley et al., 1991). Like genetic divergence, the frequency of homoplasy increases with time and may bias distance calculations so that they appear smaller than the true values. In such cases additivity should not be expected.

Another issue in selecting a tree drawing algorithm relates to tree rooting. For example, UPGMA produces rooted trees and NJ produce unrooted trees. In cladograms, tree rooting is based on outgroup comparisons which establish the direction of character change (Forey et al., 1992). Thus determination of monophyletic groups (a common ancestor and all of its descendants) is facilitated and the presence of homoplasy may be identified. In distance trees a root may 'imply' a direction of change but such an interpretation is open to

greater criticism. Certainly it is assumed that genetic distances contain information related to the phylogeny of taxa (Felsenstein, 1984; Xu et al., 1994), however rooting the distance tree may be based on invalid assumptions. For example, distance methods do not require the identification of outgroups and in their absence, midpoint rooting becomes the basis for identifying the root location (Forey et al., 1992). In this procedure the root is placed at the midpoint of the two most distant groups on the tree. This is reasonable if the taxa in question are assumed to evolve at the same rate but such an assumption may not always be well supported. Therefore rooted distance trees should be interpreted with caution since the evolutionary directions implied may be based on invalid assumptions.

A number of studies have compared the relative efficiencies of various distance methods. Tateno et al. (1982) used computer modelling to simulate random substitutions in a hypothetical ancestral gene. DW and modified DW produced the best trees if the rates of substitution varied and UPGMA performed best if substitution rates were constant. In a similar study it was determined that FM and DW outperformed UPGMA if the number of nucleotide substitutions was large (Sourdis and Krimbas, 1987). When NJ was compared to UPGMA and DW it was as good as DW if nucleotide substitution rates were constant and better if nucleotide substitution rates varied (Saitou and Nei, 1987). UPGMA scored poorly in these simulations, especially when variable mutation rates were included. The poor performance of UPGMA also was reported in a more recent study in which FM, CS and NJ were similar in their performance as long as the assumptions on which they were based were not violated, but UPGMA provided inconsistent results (Huelsenbeck and Hillis, 1993). NJ was compared to maximum parsimony (MP) methods and had a higher probability of

identifying the correct tree if the rate of nucleotide substitutions varied between branches (Jin and Nei, 1991). When substitution rates were constant, NJ and MP performed equally well. In an empirical study FM, CS and NJ performed equally well, whereas parsimony methods were significantly better and UPGMA was significantly worse (Hillis et al., 1992).

Genetic distances provide a method for determining the degree of similarity among groups of organisms. The selection of an appropriate distance method requires an understanding of their underlying assumptions which should be matched to the biology of the study organisms. In particular, it must be decided if the organisms are evolving due to genetic drift alone or if other factors are affecting the gene pool. Also, consideration of the rate of evolution along adjacent branches is necessary since some algorithms assume a constant rate. Tree building is available in many formats and options to consider include whether or not the tree should be rooted and whether or not to allow negative branch lengths. Figure 1 provides a summary of several choices required when selecting genetic distance algorithms.

Evaluation of some common algorithms (FM, CS, UPGMA, DW, and NJ) indicated that UPGMA is a poor estimator of the 'true' tree under most conditions and that FM, DW and NJ were similar in their ability to estimate phylogenies although NJ proved slightly better in some studies.

1.2.3 Other statistical tools

In addition to the use of genetic distance values, a variety of other statistical methods exist for objectively comparing DNA variation. On the simplest level, variation may be

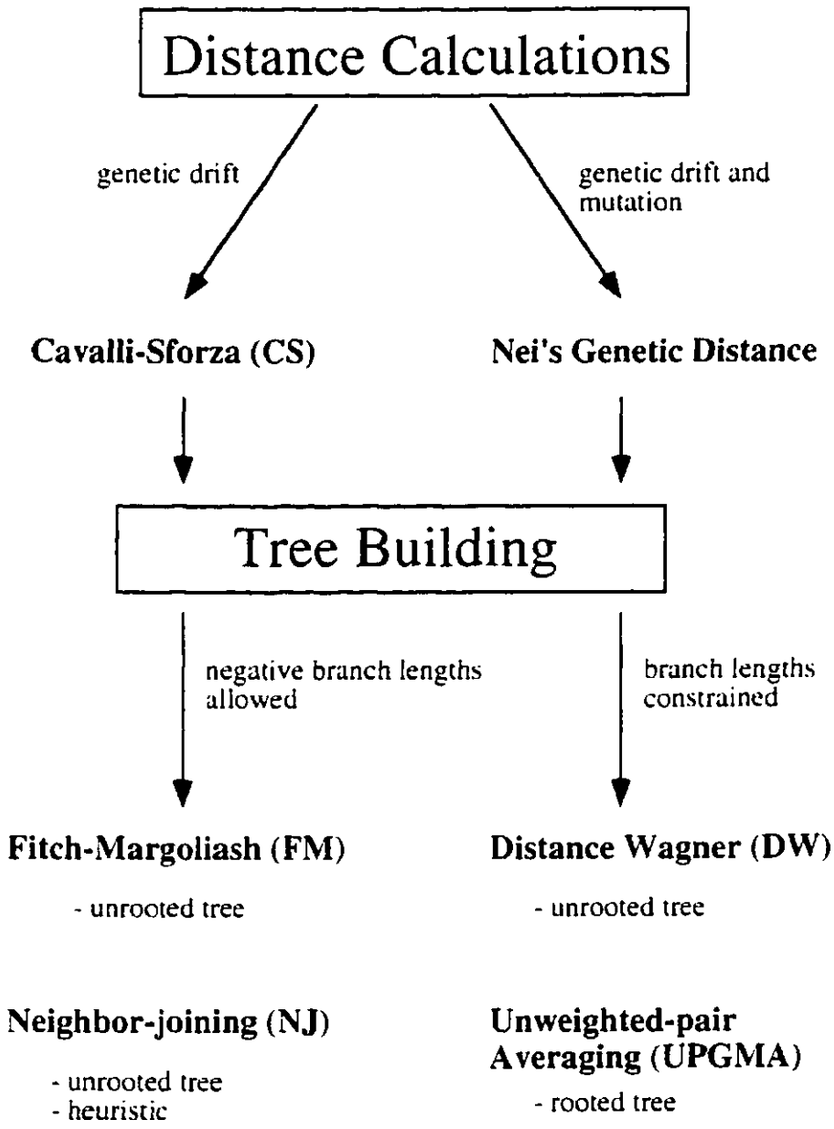


Figure 1. Summary flow chart indicating some of the choices available for calculating genetic distance values and drawing distance trees.

expressed in descriptive terms such as the number and types of mtDNA haplotypes in target populations. On the next level, simple measures of variation are attained by counting genotypes or determining allele frequencies. The calculation of frequencies allows the introduction of more complex functions such as determination of gene diversity values for a population. Gene diversity can be used as a measure of within population variation (Weir, 1996).

Several other measures of variation have been described including measures of polymorphism (P), based on the proportion of polymorphic loci, and heterozygosity (H) described as the proportion of heterozygous individuals in a population (Hoelzel and Bancroft, 1992). Both are commonly applied to allozyme data. With restriction fragment data, a value similar to heterozygosity may be calculated based on the frequency of different haplotypes, and further, the proportion of shared restriction sites (or fragments) can be used to determine nucleotide diversity among populations (Hoelzel and Bancroft, 1992).

The most widely employed statistical description of population structure is the "F-statistic" (Avise, 1994). Generally, F-statistics are derived during analyses of variance and are used to determine the significance of differences in population means (Howell, 1992). In population genetic studies, these values reflect the variance of allele frequencies among populations and are commonly used as a measure of population subdivision and for estimating interpopulational gene flow (Avise, 1994).

Several "goodness of fit" statistics are widely used for genetic comparisons. The two most common are the log likelihood ratio test (G statistic) and Chi square (χ^2). The former is more commonly applied in phylogenetic studies and does not require the expected

probability of all distinct nucleotide patterns to be calculated (Swofford et al., 1996). The latter is frequently used to test for Hardy-Weinberg equilibrium and is believed to have greater power than the G-statistic (Weir, 1996). In other words, a χ^2 test is more likely to detect real departures from a Hardy-Weinberg equilibrium than the log likelihood ratio test.

Chi square forms the basis for detecting significant heterogeneity among populations. Roff and Bentzen (1989) have developed an algorithm which assesses the extent of heterogeneity among populations. Repeated randomizations of the original matrix are used to determine a mean χ^2 value based on chance alone. The probability of encountering a χ^2 value as large as that calculated for the original can be determined. The algorithm is designed to facilitate analysis of populations based on relatively small sample sizes, and where haplotype frequency data matrices are likely to have relatively large numbers of empty cells. A computerized version of the algorithm is available (McElroy et al., 1991).

Another method to assess genetic differences among populations is to determine percent sequence divergence values. These are determined directly from sequencing data or inferred from restriction fragment analysis (Nei, 1987; Nei and Tajima, 1981). Sequence divergence reflects the accumulated effects of point mutation, deletions and insertions such that organisms which are more closely related should have lower levels of DNA base sequence divergence than those more distantly related.

1.3 Description of the problem and hypotheses

This study was designed to investigate the extent of genetic variation among Alberta bull trout populations. Four goals were established at the outset. The first was to explore the

level of genetic divergence among drainages in which bull trout are located. Interdrainage variation was expected to be measurable and possibly considerable. Trout living in separate drainage systems have been geographically isolated for up to 15 000 years (Ferguson, 1994; Pielou, 1991). The subsequent lack of interdrainage gene flow, coupled with potentially unique local selection pressures may have resulted in divergent genetic profiles. Previous studies revealed this type of genetic variation in other regions. Leary et al. (1993) reported significant interdrainage variation in bull trout from the Columbia and Klamath River systems. In Alberta, the genetics of bull trout have not been studied with the exception of one allozyme study on Pinto Lake bull trout (Carl et al., 1989), but interdrainage genetic differences in rainbow trout were inferred from protein studies (Carl et al., 1994).

The second goal of the study was to measure the extent of intradrainage genetic variation. Fish within the same drainage system might be expected to comprise a single interbreeding population. Previous studies in the United States reported few genetic differences from intradrainage comparisons (Leary et al., 1993; Williams et al., 1995). However, conditions in Alberta streams may differ substantially. If a wider variety of environmental conditions are present within a single drainage, within stream population substructuring may exist. Both geographical and reproductive isolation may occur within a single drainage region. Geographical isolation may be caused by man-made structures such as dams or weirs (Roberts, 1991), but natural barriers such as beaver dams also may isolate fish populations (Rhude and Rhem, 1995). Dams prevent downstream populations from returning to upstream spawning sites, eliminating them from the gene pool. Fish trapped upstream may form unique demes due to reduced intradrainage gene flow. Even if it is

unlikely that man-made barriers have been in place long enough to cause significant changes in gene pools, recently isolated demes subjected to rapid population declines may result in a significant founder effect which could give rise to divergent genetic profiles.

Intradrainage population substructuring may occur without geographical isolation. Multiple life history patterns represent an important source of diversity within a species if they result in reproductive isolation (Rieman and McIntyre, 1993). In bull trout, two distinct morphs have been identified. Resident populations spend their entire lives in upstream headwaters. Migratory populations spend 1-3 years in tributary streams and subsequently migrate downstream to larger rivers (fluvial forms) or lakes (adfluvial forms) where they spend several more years maturing before returning to upstream spawning sites (Pratt, 1992; Rieman and McIntyre, 1993). Resident and migratory forms may live together but it is not known if they represent single or separate populations (Rieman and McIntyre, 1993).

A third goal of the study was to identify potential introgression or hybridization with brook trout. This goal was a natural extension of attempts to measure intradrainage variation since hybridization with brook trout could cause the appearance of new bull trout haplotypes. Hybridization was previously documented in regions similar to Alberta (Dambacher et al., 1992; Kitano et al., 1994; Leary et al., 1993; Markle, 1992; Ziller, 1992) and it has been suggested that the same occurs within Alberta streams (Fitch, 1994; Mayhood, 1995; Roberts, 1987), however no genetic evidence currently exists to verify that suggestion.

The fourth goal of the study was to establish baseline genetic data for Alberta bull trout populations. Baseline data are necessary for detection of future changes in population gene pools. Temporal shifts in genetic structure may prove to be diagnostic if trends in either

population recovery or decline can be correlated to such changes.

1.4 The study design - limitations and delimitations

Several factors influenced the study design. The decline of Alberta bull trout populations demanded that no fish be sacrificed. Thus protein comparisons were eliminated as an acceptable mode of analysis. It was clear that tissue collection should be as noninvasive as possible which led to the development of a rapid and efficient DNA extraction protocol from fin tissue.

Mitochondrial DNA was a logical choice to incorporate in this study design because: (1) it was highly recommended for population studies (Avise, 1994; Dowling et al., 1996; Ferguson et al., 1995); (2) it was widely reported in previous studies of other fish species (Avise, 1987; Billington and Hebert, 1991; Carvalho and Hauser, 1994; Park and Moran, 1994); and (3) it was previously used in at least one other bull trout study (Williams et al., 1995). Restriction endonucleases were selected from those screened by Williams et al. (1995) which reportedly revealed the greatest level of polymorphism among bull trout populations.

Since the mitochondrial genome is considered as a single locus, it remained highly desirable to include a nuclear based method of DNA analysis. The use of microsatellites for this purpose was rejected since no microsatellite primers have been developed for bull trout. The screening of probes which were initially developed for other species may have required an extensive effort and was not guaranteed to be fruitful. Project timelines prohibited the development of new bull trout specific microsatellite primers so they were eliminated as a practical method of comparison in this study.

Alternatively, the use of RAPDs fulfilled this role without the problems previously recognized for microsatellite protocols. The use of arbitrary primer sequences eliminated the need to identify specific DNA base sequences which flanked bull trout microsatellite regions. The only screening necessary was to establish which RAPD primers would reveal polymorphism at a population level in bull trout.

The locations from which fish were sampled for this study were based on a previously developed agenda of field work established by Alberta Fish and Wildlife researchers. To stay within budget constraints, all samples were collected during routine field work, which was already scheduled by Fish and Wildlife field technicians. Several advantages stemmed from this arrangement. Each region was sampled by technicians who were familiar with the local river drainage systems and who were trained in the proper handling of the fish. More importantly, species identification was performed by trained and experienced field personnel so that identification errors were negligible. The disadvantage to the collection method was that some drainages were not scheduled for field work and thus, were not included in the study.

2.0 Review of the Literature

2.1 The taxonomic placement of bull trout

The Salmonidae is one of 15 families of the order Salmoniformes and consists of a monophyletic assemblage of 10 genera and approximately 68 species of freshwater and anadromous fishes (Shedlock et al., 1992). Three major tetraploid lines were thought to arise from a polyploidization event about 50-100 million years, forming the subfamilies Coregoninae (whitefish), Thymallinae (grayling), and Salmoninae (salmon and trout) (Allendorf and Thorgaard, 1984). The subfamily Salmoninae consists of six genera: *Brachymystax*, *Hucho*, *Oncorhynchus*, *Salmo*, *Salmothymus* and *Salvelinus* (Grewe et al., 1990). The genus *Salvelinus* is composed of five morphologically distinguishable species in North America including *S. alpinus* (Arctic char), *S. malma* (Dolly Varden), *S. naymaycush* (lake trout), *S. fontinalis* (brook trout), and *S. confluentus* (bull trout) (Pleyte et al., 1992). Bull trout and Dolly Varden were once thought to be conspecific until Cavender (1978) suggested that they form two distinct species, an idea further substantiated by Haas and McPhail (1991).

The species of *Salvelinus* have been grouped into three subgenera: *Cristovomer*, including lake trout, *Baione*, including brook trout, and *Salvelinus*, including Arctic char, Dolly Varden and bull trout (Pleyte et al., 1992). Phylogenetic analyses support these groupings using allozymes (Crane et al., 1994), mtDNA (Grewe et al., 1990) and DNA sequences from the first internal transcribed spacer (ITS-1) of rDNA (Phillips et al., 1994; Pleyte et al., 1992).

2.2 Bull trout habitat requirements and life history

The survival of bull trout is dependent upon specific habitat requirements. Distribution and abundance of bull trout populations is influenced by channel and hydrologic stability, stream substrate, available cover in the stream, water temperature, and the presence of migration corridors (Rieman and McIntyre, 1993). The survival of young bull trout is reduced with flooding and scouring during the early spring, and by low or variable stream flow rates. Increased sedimentation, reduced pool depth and alteration of substrate composition also negatively affect bull trout survival. In general, preferred habitat includes cold, fast flowing mountain streams with deep pools and complex forms of cover (Rieman and McIntyre, 1993).

Bull trout spawn from August to November and eggs incubate throughout the winter, hatching in late winter or early spring (Pratt, 1992). Spawning occurs in the coldest headwater tributaries of mountain streams (less than 10°C. for spawning) fed by snowmelt and underground aquifers (Ratliff and Howell, 1992). Two distinct forms, resident and migratory, exist throughout the range of bull trout. Once spawning is completed, migratory forms travel downstream to a larger river (fluvial populations) or to a lake (adfluvial populations) where they spend the remainder of the year. Resident fish reside in the upstream tributaries year round.

Smaller bull trout (less than 110 mm) are known to feed on aquatic insects while larger fish are piscivorous. In addition, resident populations remain largely insectivorous due to the relative lack of prey species in the upper reaches of the tributary streams (Boag, 1987). In contrast, migratory fish tend to acquire piscivorous habits, feeding on whitefish

(*Prosopium williamsoni*), yellow perch (*Perca flavescens*), rainbow trout, and other bull trout (Pratt, 1992).

Bull trout coexist with a variety of other fish species including cutthroat trout (*O. clarki*), rainbow trout, Dolly Varden, chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), mountain whitefish and several species of sculpins (*Cottus* spp.) and minnows (*Cyprinidae* spp.) (Rieman and McIntyre, 1993). Hybridization with brook trout has been previously reported (Kitano et al., 1994; Markle, 1992; Ziller, 1992) and is considered to be an important threat to the persistence of many bull trout populations (Rieman and McIntyre, 1993).

2.3 Salmonid genetic studies

2.3.1 Overview

The development of biochemical and molecular techniques has facilitated the direct detection of genetic variability within and among salmonid populations. Although early studies predominantly relied upon allozyme analysis (May, 1992), contemporary approaches include a wide array of methods which focus on mtDNA, microsatellites, minisatellites and RAPDs. One of the most widely studied salmonids is the rainbow trout which often appears to lead the way in the application of techniques to salmonid genetic analysis. For example, complete sequences of major gene regions are reported for rainbow trout (Buhler et al., 1994; Digby et al., 1992; Glamann, 1995; Hansen et al., 1994; Kusser et al., 1994; Zardoya et al., 1995) where little, if any, sequencing has been reported for bull trout. A large number of

microsatellite primers have been developed for rainbow trout (Morris et al., 1996; Sakamoto et al., 1994a; Sakamoto et al., 1994b; Sakamoto et al., 1994c; Sakamoto et al., 1994d; Sakamoto et al., 1994e) and many of these are proposed for use with other salmonid species but their utility must be empirically determined. Development of VNTR probes is increasing for other species including microsatellite primers for brown trout (Estoup et al., 1993) and minisatellite probes both for brown trout (Prodöhl et al., 1994) and Arctic char (Hartley et al., 1995). To date there are no parallel studies for the development of such probes for bull trout.

Considerable research has been completed on the mtDNA genomes of salmonids. Animal mitochondria carry genes which code for 13 proteins, 22 tRNAs and 2 rRNA molecules, in addition to a noncoding control region which contains the origin of heavy strand replication (D-loop) in vertebrates (Beckenbach, 1991). Gene order is highly conserved in all vertebrates including fish. Fish mitochondrial genomes vary little in size, with a range of 15.2 to 19.8 kilobase pairs (kb) in length (Billington and Hebert, 1991). Salmonid mtDNA exists within a smaller size range from 16.0 to 18.0 kb and members of the genus *Salvelinus* reportedly contain 16.8 kb mitochondrial genomes (Grewe et al., 1990). Though genome size is relatively conserved, variation exists within the mtDNA base sequences, mostly resulting from point mutations (Avise, 1994). The average rate of base substitutions in animal mtDNA is higher than in their single-copy nuclear DNA (Dowling et al., 1996), and different regions of the mitochondrial genome are known to evolve at different rates (Park and Moran, 1994). NADH dehydrogenase (ND) and cytochrome *b* regions exhibit variation at the population level (Park and Moran, 1994), but other regions, such as the D-loop, show little variability

in many salmonids (Ferguson, 1994). Shedlock et al. (1992) sequenced the entire D-loop region of all eight species of anadromous Pacific salmon and reported minimal sequence variability. Cytochrome *b* and 16S rRNA sequence analysis in brown trout revealed low levels of genetic variation (Patarnello et al., 1994). Analysis of both ND and cytochrome *b* regions of the mitochondrial genome of cutthroat trout indicated that only ND-1 regions contained sufficient polymorphism to differentiate among populations (Williams et al., 1994). ND-2 and ND-3 regions provided weak resolving power and the cytochrome *b* region was strongly conserved. In bull trout, similar results were obtained with the greatest variation measured when the ND1 region was targeted (Williams et al., 1995).

There is increasing evidence that important genetic variation exists within and among many salmonid populations (Ferguson et al., 1995). Appraisal of such variation may be accomplished through the use of a variety of molecular markers including allozymes, mtDNA or VNTRs. Direct measurement of genetic variation contributes to an improved understanding of salmonid behavior, ecology and systematics. From a wildlife management perspective, an understanding of genetic variation is essential in dealing with the current trends towards rapid erosion of genetic diversity by population reduction, local extinction, and both deliberate and inadvertent mixing of natural populations with cultured stocks (Ferguson et al., 1995).

2.3.2 Allozyme studies

In the early 1970's, allozymes formed the initial basis for quantitative assessment of population structure among salmonids but their usefulness sometimes was constrained by the

lack of sufficient gene frequency divergence at polymorphic loci to discriminate between populations (Billington and Hebert, 1991). Notwithstanding, the successful analysis of intraspecies genetic divergence was accomplished for several salmonid species. Numerous studies have explored allozyme variation in rainbow trout (Reisenbichler et al., 1992; Snowden and Adam, 1992; Williams et al., 1996). Alberta rainbow trout populations were different at several allozyme loci when compared to both coastal and Columbia River rainbow trout (Carl et al., 1994). In a similar study, significant genetic differences were found among rainbow trout populations ranging from Minnesota tributaries to Lake Superior (Krueger et al., 1994). Two main population clusters of rainbow trout were identified, leading to the recommendation that hatchery strains should not be introduced to these streams if the maintenance of genetic differences among wild populations was desirable. In contrast, Ferguson et al. (1991) determined that hatchery stocks of rainbow trout and brown trout did not display reduced heterozygosity when compared to wild populations, and they concluded that the Ontario Ministry of Natural Resources hatchery program was successful in maintaining allelic variation during broodstock propagation.

A total of 36 protein encoding loci in Atlantic salmon (*Salmo salar*) were described by Davidson et al. (1989), of which only five showed significant polymorphism. Sufficient sensitivity was obtained to differentiate only among three large population clusters. On the other hand, O'Connell et al. (1995) obtained sufficient resolution with allozymes to detect significant interpopulation and intrapopulation variation among Atlantic salmon populations from three catchments in Wales. They reported that allozyme data were as informative as mtDNA data, which was in contrast to previous studies.

Another salmonid species extensively investigated using allozyme techniques is the brown trout (*Salmo trutta*) (Apostolidis et al., 1996; Jorde and Ryman, 1996; Karakousis and Triantaphyllidis, 1990; Riffel et al., 1995). Of particular interest was a study designed to detect genetic variation among brown trout with different life history patterns. Landlocked, resident and anadromous fish were compared. Resident and anadromous fish, spawning in the same locality, displayed no genetic differentiation. However fish spawning in geographically separated localities revealed significant variation (Hindar et al., 1991).

Other *Salvelinus* species analyzed using allozyme techniques include lake trout (Grewe et al., 1994; Kincaid et al., 1993; Marsden et al., 1993) and brook trout (McCracken et al., 1993; Perkins et al., 1993).

Attempts to characterize bull trout populations using allozyme techniques have resulted in limited success. Intradrainage comparisons of bull trout populations from within the Columbia and Klamath River drainages of Montana revealed little genetic variation at 51 loci (Leary et al.1993) . In fact, six of the nineteen populations were identical. In contrast, substantial allele frequency differences were detected when Klamath River populations were compared to Columbia River populations, leading to the conclusion that little intrapopulation but significant interpopulation variation existed. Kanda et al. (1994) analyzed 45 loci encoding enzymes present in bull trout muscle, liver or eye tissue. Only two loci were highly polymorphic and, though these provided useful information, the authors recommended direct examination of mitochondrial and nuclear DNA to potentially reveal greater levels of polymorphism.

The relative success of allozyme studies may be traced to quick processing times and

low cost but applying the techniques usually requires sacrificing organisms, an option not acceptable for species which are rare or endangered (Park and Moran, 1994). Generally, allozyme studies have proven valuable in estimating population divergence and identifying discrete fish stocks and they have focused attention on the underlying evolutionary forces that promote differentiation among the salmonid species (Carvalho and Hauser, 1994).

2.3.3 Mitochondrial DNA studies

Mitochondrial DNA studies have provided substantial insight into salmonid population structures. Though some species show little or no mtDNA differentiation, mtDNA genotypes often provide sufficient resolving power to distinguish geographically separated conspecific populations (Avise, 1987). Using two informative restriction enzymes, Birmingham et al. (1991) were able to correctly identify the origin for 67 of 68 physically tagged Atlantic salmon caught in the West Greenland fishery. The study clearly indicated the usefulness of mtDNA analysis for discriminating between European and North American strains. Giuffra et al. (1994) applied similar techniques to brown trout but confined their study to eighteen populations from one drainage system in Northern Italy. They confirmed the phylogenetic differentiation of two regional variants, and perhaps of greater interest, discovered that morphologically identical *Salmo trutta fario* populations displayed extensive genetic heterogeneity. This latter point indicates the value of DNA based analyses over purely morphometric approaches. This concept is further supported by the study of Arctic char from Sawtooth Lake, Idaho (Kircheis et al., 1995) where fish similar in appearance were shown to be sufficiently distinct in their genetic profiles that a proposed transplanting program was

not recommended. McVeigh et al. (1995) reported that mtDNA markers were more discriminating than morphological, allozyme or multilocus probes for discriminating among populations of brown trout in Ireland. Danzmann et al. (1991), following mtDNA RFLP analysis, reported a high degree of genetic discrimination between hatchery and native brook trout (*Salvelinus fontinalis*) in southern Ontario. Data were used to ascertain the reproductive and competitive success of hatchery and wild fish in that region, a common application of mtDNA techniques (Danzmann et al., 1993; Evans and Willox, 1991; Nielsen et al., 1994). Mixed stock analysis was the goal of a mtDNA study of Ontario lake trout, where differences in mtDNA haplotype frequencies permitted greater discrimination of strains than allozyme data. Based on these data, it was suggested that such information could facilitate evaluation of reproductive success of hatchery strains introduced into Lake Ontario (Grewe et al., 1993).

Some mtDNA analyses reveal little or no variation among fish populations. Low intraspecific mtDNA sequence divergence was inferred from RFLP analysis of both chinook salmon and chum salmon (*O. keta*), even though the typically highly variable ND-1 region was targeted (Cronin et al., 1993). Park et al. (1993) also examined chum salmon mitochondria and found low levels of variation when they sequenced the entire D-loop region. Though mtDNA analysis provides a powerful tool for discriminating among salmonid populations, it is apparent that the resolving power of mtDNA regions varies with the species and that successful analyses require preliminary identification of regions with sufficient polymorphism. Initial surveys should include ND-1 if maximum variation is desired or cytochrome *b* and the D-loop if more conserved regions are appropriate.

2.4 Sequence variation and genetic distance among salmonids

Sequence variation among salmonids may be directly measured by DNA sequencing or inferred from analysis of restriction sites or fragments. Interspecific sequence divergence is generally an order of magnitude greater than intraspecific variation (Beckenbach, 1991). Mean nucleotide sequence divergence between conspecific individuals commonly ranges from 0.3 to 0.4%, however numerous studies indicate these values can be much higher (Avise, 1987). Direct sequencing of 16S rRNA in brown trout revealed an interpopulation divergence of 1.1% (Patarnello et al., 1994). The fish showed surprisingly little DNA sequence variation for the amount of phenotypic difference present since populations were composed of five landlocked morphs which differed in color, body size, meristics and behavior.

The same populations were studied by Giuffra et al. (1994) using both direct sequencing and RFLP analysis. They found a high degree of congruence between the sequence divergence values measured by direct sequencing and those inferred by RFLP analysis. Mean intraspecific divergence values were 0.93% based on sequencing data as compared to 1.2 % from RFLPs. Interspecies comparisons with brown trout and Atlantic salmon revealed similar congruency between the two methodologies with divergence values of 6.2-7.5% from sequencing and 6.2-6.5% from RFLP analysis.

Intraspecific sequence divergence has been estimated for several brook trout populations including hatchery strains (Danzmann et al., 1991). The greatest divergence was 0.41% with some populations displaying only a 0.26% difference.

Arctic char populations were compared to determine the extent of sequence

divergence for populations both within North America and between North America and Europe (Kircheis et al., 1995). Within the North American group divergence values up to 0.39% were reported. Trans-Atlantic populations differed by 0.42 to 0.78%.

Beckenbach (1991) compared rainbow trout to several other species revealing sequence divergence values of 3.04% with coho salmon (*O. kisutch*), 3.18% with chinook salmon, 5.08% with sockeye salmon, and 7.51% with pink salmon (*O. gorbuscha*). Values were based on restriction analysis of mtDNA. A parallel study was completed by McKay et al. (1996) but included direct sequencing of both the ND3 mtDNA region and the type-2 growth hormone region of nuclear DNA. Mitochondrial DNA revealed sequence divergence values which averaged threefold higher than nuclear sequence divergence.

Genetic variation in populations of bull trout was studied using RFLP analysis of mtDNA (Williams et al., 1995). Intraspecies sequence divergence values were reported for populations within the same stream and ranged from 0.22 to 0.66%. Populations from separate drainages differed by 1.0 - 1.5%. Comparison to brook trout produced divergence values of approximately 5%.

2.5 Gaps in the literature

The use of genetic analyses in the fisheries has become increasingly widespread. Allozyme, mtDNA and nuclear data have contributed to a deeper understanding of the nature of intraspecific gene pools, have provided significant evidence for determining phylogenetic relationships and have led to some key recommendations in the management of salmonid species. In spite of these advances, significant gaps exist in the growing base of knowledge

which stems from such genetic analyses. For example, a tremendous amount of attention has recently surfaced on the need to maintain intraspecies genetic variation in localized gene pools (Hanski, 1991; Dizon et al., 1992; Ryman, 1991), but data establishing a relationship between DNA diversity and population fitness are generally lacking (Moritz, 1994). Ideally, conservation efforts should be based on scientific principles which are empirically supported thus pointing to a need for further research efforts in this area.

Another gap in the literature is a lack of temporal studies. Numerous research efforts have documented spatial relationships through comparison of genetic profiles but few studies have followed populations over time. Demonstrating that patterns of differentiation are temporally stable is necessary to ensure that heterogeneity truly represents population differentiation (Ferguson, 1994). For example, Hansen and Loeschcke (1996) demonstrated temporal variation in mtDNA haplotype frequencies from brown trout populations in which nuclear allele frequencies remained unchanged. They argued that the common assumption that haplotype frequencies should display temporal stability may not be valid for small populations, even if nuclear stability has been observed. Mitochondrial DNA analysis may yield data which differ from nuclear and therefore, should not be applied uncritically to problems where previous studies were allozyme based. Also, a combination of mtDNA and nuclear analysis may provide insight to the time of divergence. It takes much longer for divergence to appear in the nuclear genome compared to the mtDNA genome. Genetic differences in the mtDNA but not the nuclear DNA may suggest more recent divergence whereas differentiation in both types of DNA indicates a longer period of separation.

The characterization of genetic differences among populations is dependent upon the

selection of appropriate methodologies. It is clear that several studies were unable to reveal significant polymorphism among their target groups. Variation may exist but the methods selected had insufficient resolving power to recognize existing differences. For example, allozyme methods may yield few differences where microsatellites do. The ND region of mtDNA may display more polymorphism than the cytochrome *b* region. It seems that population structuring may be identified with confidence but lack of population structuring is difficult to verify without using several methods simultaneously.

Liskauskas and Ferguson (1991) found significant differences in allele frequencies and in the number of heterozygous loci per individual in five age classes of brook trout. Their study suggests another gap in the literature concerning age class variation which naturally exists in a population. Many studies use random sampling techniques and little attention is paid to the age class of the fish used as population representatives. Gender differences also may exist (Danzmann et al., 1994; Liskauskas and Ferguson, 1991) but few studies account for this potential.

Finally, further research is required to clarify the taxon levels at which conservation efforts are to be directed towards. An updated taxonomy based on molecular studies should facilitate proper recognition of the extent of biotic diversity among salmonids (Avise, 1989). The introduction of metapopulation theory to wildlife management has resulted in renewed focus on the importance of maintaining small, localized populations (Rieman and McIntyre, 1995; Stacey and Taper, 1992) and led to rethinking the traditional concept of fish stocks (Dizon et al., 1992).

Bull trout have received little attention in the literature. Studies on distribution and

life history patterns exist (Dambacher et al., 1992; Donald and Alger, 1993; Fitch, 1994; Fox et al., 1996; Haas and McPhail, 1991; Ratliff and Howell, 1992; Rhude and Rhem, 1995; Rieman and McIntyre, 1993; Rieman and McIntyre, 1996; Pratt, 1992; Ziller, 1992) and several phylogenetic analyses of the genus *Salvelinus* have included bull trout (Crane et al., 1994; Phillips et al., 1994; Pleyte et al., 1992). Few studies exist on the genetics of bull trout (Kanda et al., 1994; Leary et al., 1992; Williams et al., 1995) and no studies have been published which investigate the population genetic structure of bull trout in the eastern slopes region of the Alberta Rocky Mountains.

2.6 Contributions of this study to the literature

This study is the first to investigate genetic variation in Alberta bull trout. Using both mtDNA and nuclear DNA, the extent of interpopulation and intrapopulation genetic variation was explored, thus facilitating identification of potentially unique gene pools. Results from this study provide baseline data for comparison of fish with future data sets and allow a temporal component to be added to existing findings. This study also contributes to the body of knowledge necessary for development of proper management and conservation plans.

In addition to these primary goals, several other contributions to the literature stem from this study. The development of new DNA extraction protocols facilitated more rapid processing of small fin clippings as a DNA source. The relatively non-invasive tissue sampling technique facilitated extensive bull trout sampling without sacrificing fish, a necessary condition when working with declining populations. Also, a new DNA visualization technique was developed for use with mtDNA RFLP's. The method was much

more sensitive than traditional ethidium bromide techniques, revealing a wider range of polymorphism with greater accuracy. Finally, this study supports the concept that considerably greater levels of variation exist within the mtDNA genome than was first thought.

3.0 Methods

3.1 Overview

Bull trout populations from five Alberta river drainage systems were sampled. Total genomic DNA, extracted from dried fin tissue, was subjected to PCR amplification and subsequently used to produce DNA fingerprint profiles. Populations were compared using mtDNA restriction fragment patterns and with RAPD profiles. Restriction fragment data were used to calculate population heterogeneity and percent sequence divergence while RAPD data were used to calculate genetic distance values. Analysis of RAPD data included samples collected from the St. Mary River drainage in Glacier National Park, Montana, U.S.A.

3.2 Research Sample

Bull trout fin tissue was collected by Alberta Fish and Wildlife field technicians and by U.S. National Park Service, Resource Management personnel. Fins were clipped with scissors and placed in scale envelopes where they were allowed to dry. Upon shipment to the University of Lethbridge, samples were stored at -80°C.

In Alberta, sample sites were located along the eastern slopes of the Rocky Mountains and included twelve locations in five major river drainage systems: Peace River, Athabasca River, North Saskatchewan River, Bow River and the Oldman River. The U.S.A. fish were collected from the St. Mary River drainage. Figure 2 indicates the locations of these river drainage systems within the Alberta and Montana boundaries. Table 1 identifies the

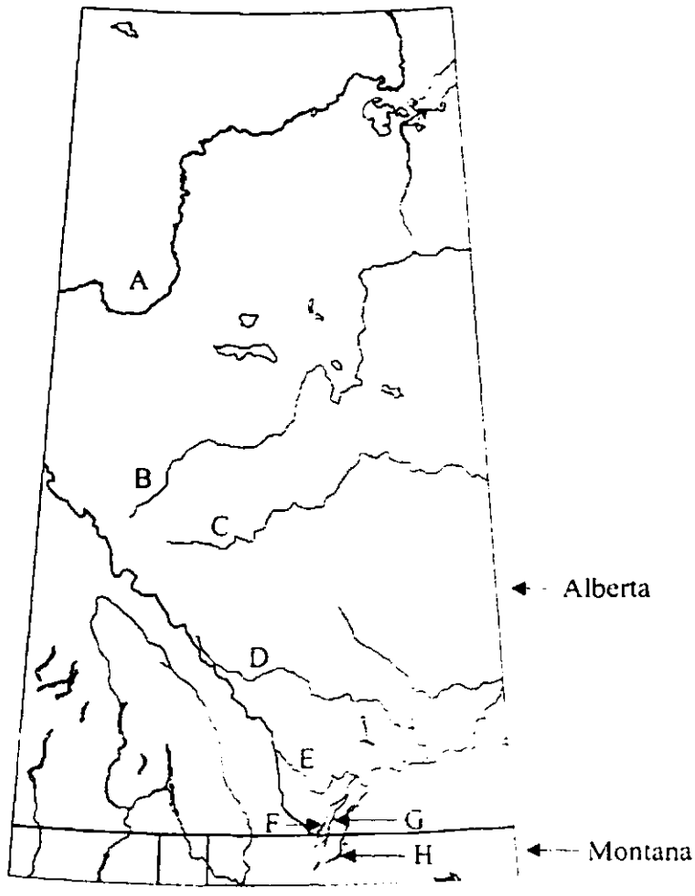


Figure 2. Locations where bull trout (*Salvelinus confluentus*) were sampled. Drainages include (A) Peace River, (B) Athabasca River, (C) North Saskatchewan River, (D) Bow River, (E) Oldman River, (F) Waterton River, (G) Belly River, and (H) St. Mary River.

dates and locations where tissue samples were collected. Sample sizes ranged from eight to twenty fish for most regions although two regions with fewer fish were included in the mtDNA analysis for qualitative comparison only.

The Athabasca drainage was sampled in the Berland River and tissue from the North Saskatchewan drainage was collected from the Cardinal River. Two regions were sampled in the Peace River drainage including the Kakwa River and the Simonette River. The South Saskatchewan drainage was sampled in several locations. Sites upstream of the Bow River included Smith Dorian Creek, which flows into Lower Kananaskis Lake, Prairie Creek, which drains into the Elbow River, and the Sheep River which drains directly into the Bow River. Tissue samples also were collected in the Oldman River drainage system. Some were taken directly from the Oldman River (upstream of the dam) and the rest were collected from the Carbondale River, the Belly River, and upstream tributaries of the Waterton River including Blakiston Creek and Yarrow Creek. The U.S.A. samples were collected from Slide Lake, Cracker Lake and Red Eagle Lake, all of which are located in Glacier National Park in Montana. These lakes drain into the St. Mary River on the eastern slopes of the Rocky Mountains.

Tissue collection occurred between July 15 and October 15 in 1995, except for samples from the Cardinal River (April, 1993) and Glacier National Park (June-August, 1996). Fish in the Belly River were captured in a live trap during upstream migration but all others were sampled by electroshocking. Most samples were composed of adipose fin tissue, for which the entire fin was removed with scissors and placed in a standard scale envelope. If the adipose fin was unavailable, a 1.0 cm diameter cutting was removed from the pelvic fin.

Table 1. Bull trout (*Salvelinus confluentus*) sampling information indicating the major drainage to which the collection river belongs, the sample collection site and the downstream river into which the collection river drains. Samples collected from Quirk Creek were brook trout (*Salvelinus fontinalis*).

Major Drainage System	Sample Site	Downstream Drainage	Sampling Date (1995)
Oldman River	Belly River	Oldman River	Sep-Oct
Oldman River	Glacier Park Lakes (USA)	St. Mary River	Jun-Aug (1996)
Oldman River	Carbondale River	Oldman River	Aug
Oldman River	Oldman River	Oldman River	Jul
Oldman River	Blakiston and Yarrow Creeks	Waterton River	Aug-Sep
Bow River	Sheep River	Bow River	Sep-Oct
Bow River	Quirk Creek	Bow River	Aug
Bow River	Prairie Creek	Elbow River	Aug
Bow River	Smith-Dorian Creek	Kananaskis River	Sep
Athabasca River	Berland River	Athabasca River	Jul-Aug
N. Saskatchewan River	Cardinal River	Brazeau River	Apr (1993)
Peace River	Kakwa River	Smoky River	Jul-Aug
Peace River	Simonette River	Smoky River	Sep

In addition to bull trout samples, tissue was collected from a resident population of brook trout located in Quirk Creek, upstream of the Bow River. Samples were cut and stored using identical methods to those used for bull trout. The Quirk Creek samples were used for outgroup comparison and to provide baseline data for evidence of bull trout and brook trout hybridization.

3.3 Specific procedures

3.3.1 DNA extraction

DNA was extracted using TRIzol™ reagent (Gibco/BRL, Life Technologies, Burlington, Ontario, Canada). The manufacturer's recommended protocol was followed with two modifications: (1) smaller initial tissue samples and (2) redissolving DNA in water.

Using ethanol rinsed dissection scissors, dried fin samples ranging in size from 1.0 to 10.0 mg were cut into pieces smaller than 0.5 cm in diameter and placed in 15 mL polyethylene centrifuge tubes. Upon addition of 1.0 mL of TRIzol™ reagent, samples were homogenized using a Kinematica CH-6010 polytron fitted with a 0.5 cm blade at a power setting of three. After 30 seconds, the samples were observed and homogenization was repeated if large pieces of fin tissue were still present. Between samples, the polytron blade was rinsed with distilled water followed by methanol. Samples were transferred to 1.5 mL screw-cap microcentrifuge tubes and stored overnight at -20°C.

Homogenized samples were thawed and 200 μL of chloroform were added followed by 15 sec of vigorous shaking. After a 2 min incubation at room temperature, the samples

were centrifuged at 12 000 x g for 15 min at 4°C. The upper aqueous phase was removed by careful pipetting and 300 μL of ice cold 99% ethanol were mixed into the sample by gently inverting the tubes several times. After a 2 min incubation at room temperature, the samples were centrifuged at 2 000 x g for 5 min at 4°C.

The supernatant was discarded and the DNA pellet was washed by addition of 1.0 mL of 0.1 M sodium citrate in 10% ethanol. Samples were incubated in the washing solution for 30 min with periodic mixing by inversion. Subsequently, the sample tubes were centrifuged at 2 000 x g for 5 min at 4°C and the supernatant was discarded. The washing process was repeated three times.

Washed DNA pellets were rinsed by addition of 1.5 mL of 75% ethanol. The tubes were frequently inverted during a 20 min incubation period at room temperature and centrifuged at 2 000 x g for 5 min at 4°C. The supernatant was removed and the DNA pellet was dried under vacuum conditions.

Samples were redissolved in 100 μL of HPLC grade bottled water (Optima Water, Fisher Scientific, Fair Lawn, New Jersey). DNA yields and purity were determined using a Beckman DU-65 spectrophotometer with a nucleic acid software pac. Dilutions were prepared for each sample by addition of 10 μL of DNA extract to 90 μL of water. The 100 μL volumes were loaded in microcuvettes and absorbance was measured at wavelengths of 320 nm, 280 nm and 260 nm. DNA quantities were calculated from A_{260} values assuming that an absorbance value of 1.0 indicated 50 μg/mL of double stranded DNA (Sambrook et al., 1989). DNA purity was estimated from $A_{260/280}$ ratios where a value of 1.8 indicates pure DNA (Sambrook et al., 1989). The TRIzol™ extraction protocol was expected to yield DNA

with $A_{260/280}$ ratios between 1.7 and 1.9 (Life Technologies, 1994).

3.3.2 PCR Amplification of mtDNA

Two primer sets, designed by Cronin et al. (1993), targeted highly conserved regions of the mtDNA NADH dehydrogenase gene. NADH dehydrogenase-1 (ND1) and NADH dehydrogenase-5/6 (ND5/6) were separately used to amplify 2.0 kb and 2.5 kb segments of mtDNA respectively. Primer sequences are listed below:

NADH dehydrogenase - 1 (ND1):

5' -ACCCCGCCTGTTTACCAAAAACAT-3'

5' -GGTATGAGCCCGATAGCTTA-3'

NADH dehydrogenase - 5/6 (ND5/6):

5' -ATTAGTTTATCC(A/G)TTGGTCTTAGG-3'

5' -TTACAACGATGGTTTTTCAT(G/A)TCA-3'

ND1 fragments were amplified in 100 μL reaction volumes using a Techne Progene thermocycler. In thin-walled 500 μL PCR tubes, a reaction mixture was prepared containing 200 ng of DNA template, 5 μL each of 20 μM forward and reverse primer, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.3 μM (F)dUTP (Perkin Elmer Corporation, Applied Biosystems Division, Foster City, California), 3.5 mM $MgCl_2$, 6.0 μL of 1.0 μg/μL bovine serum albumin (BSA), 0.5 units of Ultra Therm™ Thermophilic DNA polymerase (Bio/Can Scientific Inc., Mississauga, Ontario, Canada) and

deionized sterile water to make up the remaining volume. The amplification cycle consisted of initial denaturation at 95°C for 5 min, followed by 36 cycles of 95°C for 45 s, 58°C for 60 s, and 72°C for 90 s. The amplification was completed with a final elongation at 72°C for 10 min.

For samples difficult to amplify with ND1 primers, a modified step-up PCR protocol was used. Step-up PCR increases the probability of obtaining amplified products with poorly matched primers (Palumbi, 1996). Often, samples which would not initially amplify did yield a useable quantity of PCR product with the step-up method. The preparation of the reaction mixture was the same except that 1.0 μL of formamide was added. Thermocycler conditions were altered so that initial denaturation occurred at 95°C for 5 min, followed by 8 cycles of 95°C for 40 s, 45°C for 60 s, and 68°C for 90 s. In turn, this was followed by 35 cycles of 95°C for 40 s, 58°C for 60 s, and 72°C for 90 s. The amplification was completed with a final elongation at 72°C for 10 min.

ND5/6 fragments were amplified in 50 μL reaction volumes with an Ericomp single block thermocycler using 500 μL PCR tubes. The reaction mixture was prepared containing 100 ng of DNA template, 5 μL each of 20 μM forward and reverse primer, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.6 μM (F)dUTP (Perkin Elmer Corporation, Applied Biosystems Division, Foster City, California), 2.0 mM $MgCl_2$, 3.0 μL of 1.0 μg/μL bovine serum albumin (BSA), 1.5 units of *Taq* DNA polymerase (Gibco/BRL, Life Technologies, Burlington, Ontario, Canada) and deionized sterile water to make up the remaining volume. Reaction volumes were covered with 80 μL of light mineral oil. The amplification cycle consisted of an initial denaturation at 95°C for 5 min, followed

by 38 cycles of 95°C for 45 s, 52°C for 60 s, and 72°C for 90 s. The amplification was completed with a final elongation at 72°C for 10 min.

For samples difficult to amplify with ND5/6, a modified step-up PCR protocol was used. Preparation of the reaction mixture was the same except that 0.5 μL of formamide was added. Thermocycler conditions were altered so that initial denaturation occurred at 95°C for 5 min, followed by 8 cycles of 95°C for 40 s, 42°C for 60 s, and 68°C for 90 s. In turn, this was followed by 35 cycles of 95°C for 40 s, 52°C for 60 s, and 72°C for 90 s. The amplification was completed with a final elongation at 72°C for 10 min.

Prior to restriction enzyme digestion, successful amplification was verified using agarose gel electrophoresis. A 10 μL volume of PCR product, combined with 10 μL of 2X loading dye, was loaded onto a 1.2 % high melting point agarose gel made with 1X TBE and containing 0.4 μg/mL ethidium bromide. The DNA was electrophoresed for 1.0 h at 100 V and visualized with a Fisher FBTIV-614 transilluminator emitting UV light at 312 nm. Agarose gels were photographed with a Polaroid MP-4 Land camera. DNA fragments sizes were estimated by comparison of migration distances to molecular size markers (1.0 kb DNA ladder, Gibco/BRL, Life Technologies, Burlington, Ontario, Canada) in an adjacent lane. Fragment migration was plotted against fragment size with Gel-Frag Sizer software (Gilbert, 1989) and the resulting standard curve facilitated interpolation of PCR product sizes.

3.3.3 Restriction enzyme digestion

ND1 products were digested with five enzymes including Cfo I, Dde I, Hae III, Hpa II, and Rsa I. ND5/6 products were digested with Alu I, Hinf I, and Rsa I. Table 2 provides

full names, buffer requirements, and a description of the restriction site for each enzyme.

Prior to digestion, PCR products were dragged across parafilm to remove light mineral oil. Digestions were completed in 1.5 mL microcentrifuge tubes and each reaction contained 8.5 μL of PCR product, 1.0 μL of the appropriate buffer and 0.5 μL of enzyme. Samples were incubated overnight at 37°C.

3.3.4 PCR Amplification of RAPD fragments

For RAPD analysis, a 10mer oligonucleotide primer (DAF2) from Caetano-Anollés et al. (1991) was used . The primer sequence was:

5' -AGTCAGCCAC- 3'

RAPD fragments were amplified in 25 μL reaction volumes using a Techne Progene thermocycler. In thin-walled 500 μL PCR tubes, a reaction mixture was prepared containing 100 ng of DNA template, 1.5 μL each of 20 μM primer, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.3 μM (F)dUTP (Perkin Elmer Corporation, Applied Biosystems Division, Foster City, California), 6 mM $MgCl_2$, 1.5 μL of 1.0 μg/μL bovine serum albumin (BSA), 0.5 units of Ultra Therm™ Thermophilic DNA polymerase (Bio/Can Scientific Inc., Mississauga, Ontario, Canada) and deionized sterile water to make up the remaining volume. Reaction volumes were covered with 80 μL of light mineral oil. The amplification cycle consisted of initial denaturation at 95°C for 5 min, followed by 28 cycles of 95°C for 40 s, 52°C for 30 s, and 72°C for 60 s. The amplification was completed with a final elongation at 72°C for 10 min.

Table 2. Restriction enzymes used for digestion of ND1 and ND5/6 PCR products. Recognition sites and buffer requirements are listed. All enzymes from Gibco/BRL (Life Technologies, Burlington, Ontario, Canada).

PCR products	Enzyme	Recognition Site	Buffer
ND1	Cfo I	5' -GCG‖C- 3' 3' -C‖GCG- 5'	React 1
	Dde I	5' -C‖TNAG- 3' 3' -GANT‖C- 5'	React 3
	Hae III	5' -GG‖CC- 3' 3' -CC‖GG- 5'	React 2
	Hpa II	5' -C‖CGG- 3' 3' -GGC‖C- 5'	React 8
	Rsa I	5' -GT‖AC- 3' 3' -CA‖TG- 5'	React 1
ND5/6	Alu I	5' -AG‖CT- 3' 3' -TC‖GA- 5'	React 1
	Hinf I	5' -G‖ANTC- 3' 3' -CTNA‖G- 5'	React 2
	Rsa I	5' -GT‖AC- 3' 3' -CA‖TG- 5'	React 1

3.3.5 Visualization of DNA fragments

DNA fragments were separated by polyacrylamide gel electrophoresis on an Applied Biosystems 373A DNA sequencer and visualized with GENESCAN 672 software (Perkin Elmer Corporation, Applied Biosystems Division, Foster City, California).

A 5% polyacrylamide native gel was prepared in 12 cm well to read plates by combining 8.0 mL of GeneAmp™ Detection Gel (Perkin Elmer Corporation, Applied Biosystems Division, Foster City, California), 2 mL 10X TBE, and 30 mL deionized sterile water with 400 μL of fresh 10% ammonium persulfate and 20 μL of TEMED (N,N,N',N' - tetramethylethylenediamine). The swirled mixture was loaded using a 60 mL syringe and the plates were clamped for 1.0 h to allow complete polymerization.

For most samples, 2.0 μL of PCR product were added to the gel loading mixture, however modifications were made when necessary. For example, the success in amplifying mtDNA varied and the quantity of PCR product prior to enzyme digestion was assessed by the brightness of the uncut band on an agarose gel. Although 2.0 μL was normally the quantity used, 1.0 μL was added if the uncut bands were very intense, and 3.0 μL were added if the bands were faint. When RAPD PCR products were loaded, 1.0 μL volumes were added to the loading mixture. The total volume of the loading mixture was always 5.0 μL, including 0.5 μL ROX 2500 (Perkin Elmer Corporation, Applied Biosystems Division, Foster City, California), PCR product as previously described, and the remaining volume made up with loading dye. After brief vortexing and pulse spinning in a microcentrifuge, samples were placed on ice and quickly loaded.

Genescan runs were performed with the DNA sequencer parameters listed in Table

3. Lane tracking and correct sizing of internal standards were checked for each sample prior to analysis.

3.4 Treatment of data

Mitochondrial data were used to calculate population heterogeneity and percent sequence divergence. Analysis of restriction fragment patterns was performed using Restriction Enzyme Analysis Package (REAP) 4.0 software (McElroy et al., 1991).

RAPD fragments were assumed to represent independent biallelic loci with dominant inheritance patterns (Dowling et al., 1996). RAPD data were used in calculating genetic distance values using the Phylogenetic Inference Package (PHYLIP) developed by Felsenstein (1993). Distance trees were drawn with the aid of DRAWTREE and DRAWGRAM, also from the PHYLIP package.

Table 3. Applied Biosystems 373A DNA Sequencer parameters used for mtDNA RFLP and RAPD fragment analysis.

Electrical Settings		**Other Parameters**	
Laser setting	40 mW	Plate size	30 cm
PMT Voltage	825 V	Well to read	12 cm
Electrophoresis voltage	800 V	Buffer	1X TBE
Electrophoresis current	40 mA	Run Time	3.0 h
Electrophoresis power	30 Watts	Scan Range	0 - 3000

4.0 Fin tissue as a DNA source

4.1 Overview

DNA fingerprinting techniques are dependent on the extraction and purification of adequate quantities of DNA. In fish studies, many protocols utilize nucleated red blood cells as a DNA source (Cummings and Thorgaard, 1994; Medrano et al., 1990). Others make use of various internal tissues such as the liver (Zhuo et al., 1994), muscle (Goodier and Davidson, 1994) and heart (Cronin et al., 1993).

Though widely used, these protocols may be limited in their application. For example, the use of internal tissue requires sacrificing fish, a practice particularly undesirable with species which are endangered or in population decline (Park and Moran, 1994). Blood collection may not kill fish but requires technical skill, methods of tissue preservation such as anticoagulants, and exposes fish to considerable handling stress if they are removed from the water or anesthetized.

In an effort to reduce the harmful effects of tissue collection and to simplify the field work involved in such collection, the use of fin tissue as an alternative DNA source was explored. The quantity and purity of DNA extracted from fin tissue was compared to internal tissues and the suitability of DNA from fin tissue for PCR was investigated.

4.2 Methods and materials

DNA was extracted from rainbow trout and bull trout. Several tissue types were collected from frozen specimens including heart, liver and muscle tissues as well as adipose,

pelvic and caudal fins. In addition, adipose and pelvic fins were clipped from live fish and subsequently dried. Dried samples were stored at -80°C or left at room temperature. DNA was extracted and quantified using the protocols previously described in section 3.3.1

The suitability of extracted samples for PCR was determined by amplifying DNA with an Ericomp single block thermocycler using 60R primers described by Estoup et al. (1993). Primer sequences were as follows:

5' -CGGTGTGCTTGTCAGGTTTC- 3'

5' -GTCAAGTCAGCAAGCCTCAC- 3'

A 25 μL reaction volume was prepared with 50 ng of template DNA, 25 pmol of each oligonucleotide primer, 2 mM $MgCl_2$, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.25 units of *Taq* DNA polymerase (Gibco/BRL, Life Technologies, Burlington, Ontario, Canada), 2% formamide and deionized sterile water to make up the remaining volume. Samples were covered with 80 μL of light mineral oil. The amplification cycle consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 120 s. Amplification was completed with a final elongation at 72°C for 15 min. PCR products were visualized using agarose gel electrophoresis as described in section 3.3.2.

4.3 Results and discussion

DNA yields averaged 3.3 μg/mg dried fin tissue (N = 64, SD = 3.1). Mean tissue size was 5.12 mg (SD = 3.0), but samples as small 1.0 mg provided adequate DNA to perform PCR. Such small tissue requirements facilitates selectively removing part of a fin when whole

when whole fin removal is undesirable. Freezing the dried tissue at -80°C prior to extraction resulted in no significant differences in either DNA yield or purity ($p > 0.05$, two-tailed t-test). Yields obtained using the TRIzol™ protocol were greater than those previously reported by Shiozawa et al. (1992). Their method, using proteinase K digestion followed by phenol-chloroform extraction, was lengthy (three days) and the mean yield was 1.1 μg DNA/mg dried fin tissue. The TRIzol™ method can be completed in three to four hours and yields three times the DNA quantity.

The average $A_{260/280}$ ratio was 2.0 (SD = 0.13), which was just higher than the preferred range of 1.7-1.9 (Life Technologies, 1994). Incomplete removal of the aqueous phase during extraction likely resulted in small levels of RNA remaining in the sample, thus accounting for slightly elevated $A_{260/280}$ values. To ensure maximum DNA recovery for the purpose of determining extraction efficiency, a thin film of the aqueous phase was occasionally left on the organic phase so that none of the organic layer was inadvertently removed.

Caudal, adipose and pelvic fins each provided adequate amounts of DNA for PCR amplification. Table 4 provides average yield data for each tissue type. Adipose fins are preferred since their removal has no effect on fish locomotion (Wydoski and Emery, 1983). For fins other than the adipose, only partial clipping is recommended due to their role in maneuvering. Partial removal of the dorsal, caudal, pectoral, or pelvic fins does not adversely affect swimming ability (Radcliffe, 1950), and as long as the fin is not removed to the point of attachment, some regeneration will occur (Wydoski and Emery, 1983). Survival rates from fin clipping have been extensively studied and the techniques remain a common fisheries tool

Table 4. Mean DNA yields from three types of dried fin tissue. Tissue mass and $A_{260/280}$ ratios are included. Standard deviations are shown in parentheses following each mean value. Adipose and pectoral fins all came from bull trout (*Salvelinus confluentus*). Caudal fins were clipped from 8 bull trout, 8 brook trout (*S. fontinalis*) and 3 cutthroat trout (*Oncorhynchus clarki*).

Fin Type	**DNA Yield** (μg/mg dried tissue)	**Tissue mass** (mg)	**Purity** ($A_{260/280}$ ratio)
Adipose (N = 16)	3.7 (3.3)	1.9 (0.8)	2.1 (0.1)
Caudal (N = 19)	3.7 (3.0)	8.4 (2.4)	2.0 (0.2)
Pectoral (N = 11)	0.9 (1.2)	2.9 (0.4)	1.9 (0.3)

for marking and aging fish (Bergstedt, 1985).

Using frozen specimens, fin extractions were compared to muscle, liver and heart extractions. Yield data were measured as μg DNA/mg wet tissue. Since the tissue was wet the yields appeared to be significantly lower than with dry samples because tissue mass was elevated by the presence of water. Therefore, these data are not comparable to the dry tissue data discussed earlier, but the results are useful for analyzing the relative difference in yields among various tissue types. Liver and heart tissue yield significantly higher DNA quantities ($p < 0.05$, two-tailed t-test) than fin tissue. Figure 3 indicates the relative DNA yields of softer internal tissues compared to muscle and several fins types. The large quantities of DNA extractable from the heart and liver explains their common use as a DNA source. However, PCR amplification makes such enormous quantities unnecessary. PCR products derived from fin tissue were the same as those from internal tissues, as seen in Figure 4.

TRIzol™ was used to extract DNA from scales, otoliths and fin samples that had been stored in dry conditions for several years. Local Fish and Wildlife offices have large numbers of archived tissue samples in permanent storage and carefully catalogued. Most were collected for aging fish and some are decades old. The possibility of using archived tissue samples for PCR was explored. Archived fin tissue (collected in 1993) yielded high quantities of DNA with a mean of 2.6 μg/mg dried tissue (SD = 0.38), a value not significantly different from fresh dried tissue ($p > 0.05$, two-tailed t-test). Yields from scales and otoliths were 0.7 and 0.1 μg DNA/mg dried tissue respectively, as shown in Table 5.

The successful extraction of DNA from archived samples creates a potential for investigating temporal changes in population allele frequencies and heterozygosity spanning

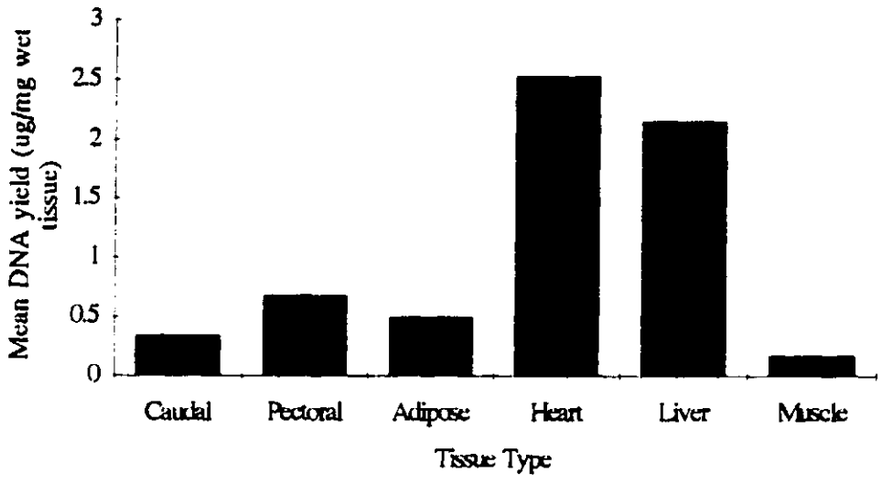


Figure 3. Comparison of DNA yields from fins and internal tissues. Samples were frozen but not dried prior to extraction. Internal tissues were from bull trout (*Salvelinus confluentus*). Fin tissue was from both bull trout and rainbow trout (*Oncorhynchus mykiss*).

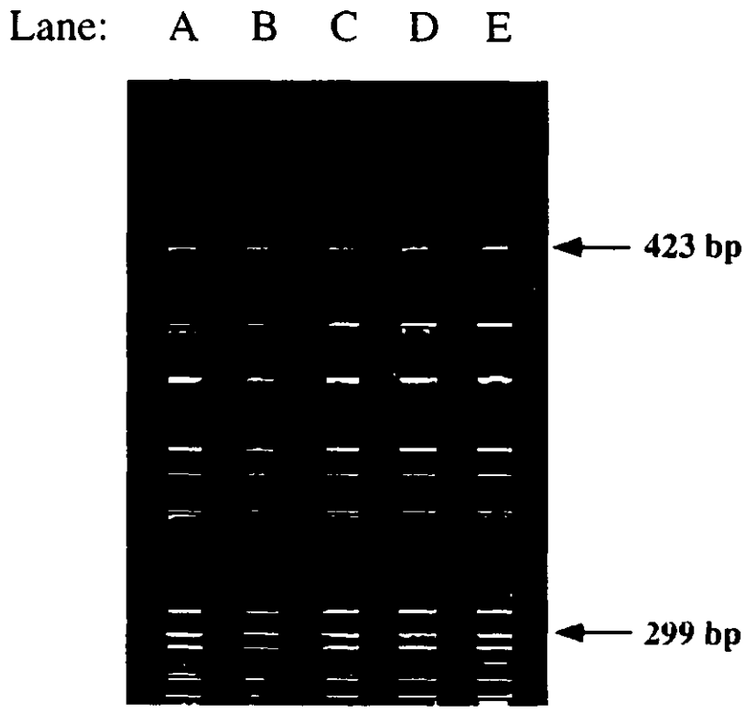


Figure 4. Polyacrylamide gel image created with ABI DNA sequencer Genescan software showing bull trout (*Salvelinus confluentus*) DNA fragments from five tissue types including (A) pelvic fins, (B) adipose fins, (C) muscle, (D) heart, and (E) liver. PCR was performed as described in section 4.2. PCR products were visualized as described in section 3.3.5.

Table 5. Mean DNA yields for three types of archived fish tissue. Samples were collected and dried in 1993. Standard deviations are shown in parentheses following each mean value. All fin tissue and scale samples were from bull trout (*Salvelinus confluentus*). Otoliths were from one rainbow trout (*Oncorhynchus mykiss*) and two cutthroat trout (*Oncorhynchus clarki*).

Fin Type	DNA Yield (μg/mg dried tissue)	Tissue mass (mg)	Purity ($A_{260/280}$ ratio)
Fin tissue (N = 24)	2.6 (1.9)	2.9 (0.8)	2.0 (0.1)
Scales (N = 5)	0.7 (0.7)	2.4 (1.7)	1.8 (0.5)
Otoliths (N = 3)	0.1 (0.01)	4.9 (2.0)	1.9 (0.5)

several decades. Such studies may provide additional insight to the interpretation of genetic divergence measured among fish populations using only contemporary samples (Ferguson, 1994).

4.4 Summary

Fin tissue as a DNA source provided a sufficient quantity and quality of DNA for PCR-based genetic studies of fish. Fins may be clipped from live fish at the water's surface and the organisms immediately released at the point of capture, thus making this the least invasive method for obtaining tissue samples. Field collection and storage of fin tissue is simplified by drying the tissue after clipping.

5.0 Results

5.1 Review of the study plan

The purpose of this study was to compare the genetic profiles of Alberta bull trout populations. Fish DNA from 12 sampling sites was analyzed to determine the extent of both intrapopulation and interpopulation variation. The characterization of regional genetic differences and the detection of significant population heterogeneity were key goals of the project.

Mitochondrial and nuclear DNA were examined to detect the level of polymorphism present among populations of Alberta bull trout. Both ND1 and ND5/6 regions of mtDNA were amplified and separately digested with five and three restriction endonucleases respectively. Resulting DNA fragments were visualized and composite haplotypes for each fish were determined. Pair-wise nucleotide sequence divergence values were calculated and population heterogeneity was determined using a Monte Carlo simulation technique (Roff and Bentzen, 1989). RAPD data were used to calculate genetic distance values and distance trees were drawn.

5.2 PCR products

Total genomic extracts from each fish were amplified separately with both ND1 and ND5/6 primers. Mitochondria were not isolated prior to PCR since it was previously shown that these primers yield the same PCR products with either mtDNA purified in a cesium chloride gradient or with total cellular extracts of DNA (Williams et al., 1994). In this study,

a single amplification product was obtained both for ND1 and ND5/6 regions, with respective sizes of 2.0 kb and 2.5 kb, which were consistent with previously reported ND1 and ND5/6 fragments (Cronin et al., 1993; Williams et al., 1995). Uncut PCR products were visualized with ethidium bromide on an agarose gel to ensure the presence of single bands following PCR amplification. Consequently, successful amplification was verified prior to restriction enzyme digestion and if bands were absent, or extra bands present, the sample was not used. Also, the brightness of the uncut band determined the volume of PCR product to load on polyacrylamide gels following enzyme digestion. The laser detection system, used with incorporated fluorescent tags, was so sensitive that the PCR was considered successful even when uncut bands were barely visible. If ethidium bromide in agarose had been the method of visualization, uncut bands must be very bright because subsequent enzyme digestion subdivides the DNA quantity into multiple restriction fragments and band brightness reduces proportionately. The sensitivity of the ABI Genescan system was a tremendous advantage since many of the samples were difficult to amplify and often produced lower quantities of PCR product than would normally be useful for restriction enzyme digestion. Even when the bands were barely visible with ethidium bromide in agarose, they produced restriction fragments detectable with the ABI DNA sequencer.

The selection of ND1 and ND5/6 as target regions for PCR was based on previous studies utilizing these areas. Williams et al. (1994) reported that ND1 revealed more detailed population structuring in cutthroat trout than ND2 or ND5/6. However, using the cytochrome *b* region, they were completely unable to differentiate populations. In another study (Williams et al., 1995), bull trout mtDNA was amplified with primers for ND1, ND2,

ND5/6 and cytochrome *b* regions. ND1 was reported to show the greatest level of variation while ND5/6 and cytochrome *b* showed the least.

Primers selected for this study (ND1 and ND5/6) revealed approximately 27% of the mitochondrial genome by amplifying sequences which were 2.0 kb and 2.5 kb respectively. The entire mitochondrial genome in bull trout was estimated to be 16.8 kb (Grewe et al., 1990).

5.3 Mitochondrial DNA haplotypes

ND1 PCR products were digested with five restriction enzymes including Cfo I, Dde I, Hae III, Hpa II and Rsa I. ND5/6 PCR products were digested with Alu I, Hinf I and Rsa I. Selection of restriction enzymes was based on previous work done by Williams et al. (1995) in which bull trout mtDNA profiles were analyzed using four primer sets and ten restriction endonucleases. In their study, no variation was detected with 60% of the enzyme primer combinations. Those enzymes which revealed the greatest level of polymorphism with the ND1 and ND5/6 primers were chosen for this study.

Table 6 displays the sample sizes for each region used in the mtDNA analysis. Sample sizes ranged from 7 to 16 fish per region with an average sample size of 10.7 (SD = 2.4), not including two regions with only a few fish for each enzyme data set. The Yarrow Creek sample size was very small, including only three fish in the ND1 enzyme group and four in the ND5/6 group. Similarly, one Manteau River fish was included in ND1 runs and two in ND5/6 runs. Both Yarrow Creek and Manteau River sample sizes were too small to include in

Table 6. Sample sizes for each region used in the mtDNA analysis. The three regions listed at the bottom of the table were included for qualitative comparison of mtDNA and not for statistical analyses due to small sample sizes. The Quirk Creek samples were brook trout (*Salvelinus fontinalis*), all others were bull trout (*Salvelinus confluentus*).

	ND1					ND5/6			
	Cfo I	Dde I	Hae III	Hpa II	Rsa I	Hinf I	Rsa I	Alu I	Ave.
Berland R.	10	10	10	10	6	13	13	11	10.4
Belly R.	16	16	16	15	16	15	15	15	15.5
Carbondale R.	8	8	8	8	8	8	8	8	8.0
Kakwa R.	10	11	11	11	10	11	11	11	10.8
Oldman R.	7	7	6	7	7	7	7	7	6.9
Prairie Cr.	10	10	10	10	10	10	11	11	10.3
Quirk Cr.	12	12	12	11	11	11	7	12	11.0
Sheep R.	12	12	12	11	11	11	12	13	11.8
Simonette R.	13	12	12	11	13	9	11	13	11.8
Total	98	98	97	94	92	95	95	101	
Ave.	10.9	10.9	10.8	10.4	10.2	10.6	10.6	11.2	10.7
SD	2.7	2.6	2.8	2.2	3.1	2.5	2.7	2.5	2.4
Smith-Dorian Cr.	14	14	14	14	14	1	1	1	9.1
Manteau R.	1	1	1	1	1	2	2	2	1.4
Yarrow Cr.	3	3	3	3	3	4	4	4	3.4

statistical analyses for sequence divergence and heterogeneity, however it was felt that even one or two fish would make an interesting qualitative comparison.

Samples from several regions did not amplify with the ND1 and ND5/6 primers, including those from the Waterton River, Cardinal River and the Glacier National Park region. Consequently, these regions were excluded from the mtDNA analysis. Also, most Smith-Dorian Creek samples did not amplify with ND5/6 primers so that DNA from only one fish was digested with these enzymes.

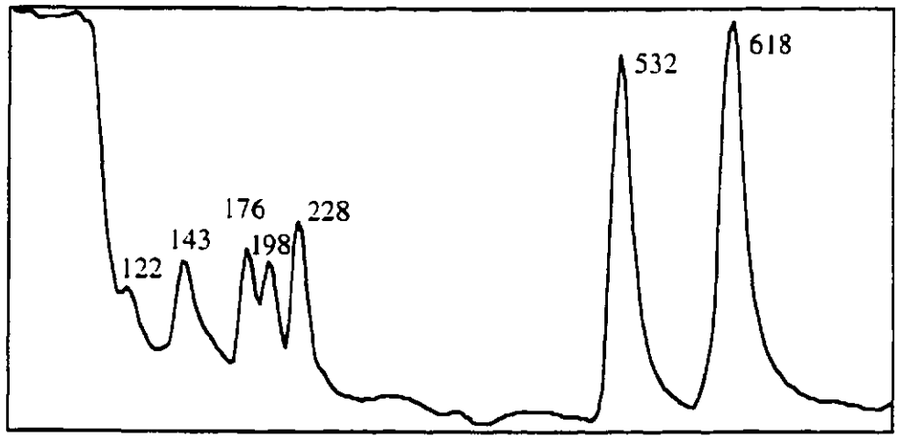
For each restriction enzyme, fish profiles were compared using a spread sheet format and similar fragment sizes were aligned vertically to facilitate the calculation of average fragment sizes for each size range. Subsequently, the average size values were used to define each haplotype. The grouping of DNA fragments into common size categories was performed so that potential errors had the most conservative effect on the results. If one sample had a 123 bp fragment and another had a 127 bp fragment these were considered to represent the same fragment rather than as evidence of polymorphism. In fact, any bands within 10 bp were considered to represent the same fragment and the small variations were ignored. In this way, all fish profiles were initially assumed to be the same and any differences which still existed following this grouping technique were more likely to be real rather than artifactual. Consequently, these data most likely underestimate differences among bull trout populations since bands similar in size often were grouped into the same size category.

At times it was clear that two fragments within 10 base pairs should be considered distinct based on two types of evidence. First, two DNA fragments of similar size may have

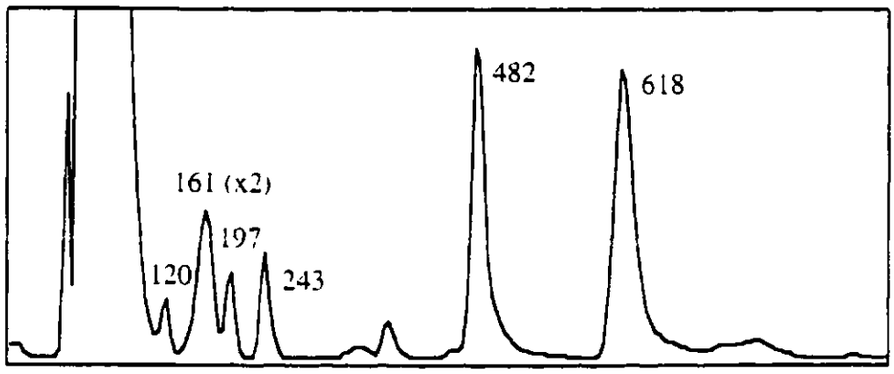
been detected in the same fish sample. For example, several of the Cfo I digestions contained fragments of both 139 bp and 136 bp sizes. If two bands were detected separately in the same fish sample, they must be different bands. Second, comigrating fragments were differentiated using peak height data from computer generated electropherograms. Fluorescent signal "brightness" was relative to the quantity of fluorescent tag present. Two comigrating bands produce a fluorescent signal approximately twice as strong as a single band. The ABI Genescan system converts signal intensity to peak height. Assuming equal rates of tag incorporation, smaller fragments produce correspondingly smaller peak heights, and within this context, comigrating bands produce peak heights twice as high as expected. Figure 5 provides an example of two restriction fragment profiles. The first has the expected decline in peak heights as smaller fragments are detected. The second indicates a profile created from comigrating fragments. Fragment sizes were independently checked against the original uncut DNA. The sum of the restriction fragments must equal the total size of the uncut DNA.

Fragments smaller than 100 bp were difficult to assess due to high intensity fluorescence caused by unincorporated primers. With some samples, this "primer peak" effectively masked any small DNA fragments so to maintain consistency, all fragments under 100 bp were ignored. In the future, further optimization of primer and (f)dUTP concentrations may facilitate the detection of smaller DNA fragments.

Haplotypes were labelled with an alphabetic code. The order in which letters were assigned corresponded to the order in which haplotypes were identified with no other significance attached. Table 7 provides an overview of the number of haplotypes revealed by enzyme digestions. The maximum number of haplotypes detected with any one enzyme was



YC3-Hae Expected total uncut fragment size = 2000 bp



K25-Dde Expected total uncut fragment size = 2000 bp

Figure 5. RFLP profiles of ND1 gene PCR products from samples YC3 and K25. The top profile was digested with Hae III and contains no comigrating DNA fragments. The bottom profile was digested with Dde I and contains comigrating fragments as suggested by the unexpectedly tall peak at 161 bp.

Table 7. Overview of the number of haplotypes per region revealed for each restriction enzyme. Sample sizes varied for each category as previously indicated in Table 5. The Quirk Creek samples were brook trout (*Salvelinus fontinalis*), all others were bull trout (*Salvelinus confluentus*).

	Cfo I	Dde I	Hae III	Hpa II	Rsa I	Hinf I	Rsa I	Alu I	Total	Ave	SD
Berland R.	4	4	4	6	3	2	3	3	29	3.6	1.2
Belly R.	4	5	4	4	3	3	2	4	29	3.6	0.9
Carbondale R.	2	1	1	3	2	2	5	3	19	2.4	1.3
Kakwa R.	2	4	6	4	3	3	3	4	29	3.6	1.2
Smith-Dorian Cr.	3	4	2	2	3	1	1	1	17	2.1	1.1
Manteau R.	1	1	1	1	1	1	2	1	9	1.1	0.4
Oldman R.	2	4	3	3	3	3	3	3	24	3.0	0.5
Prairie Cr.	4	6	3	4	3	1	2	3	26	3.3	1.5
Quirk Cr.	2	2	5	4	4	4	1	7	29	3.6	1.9
Sheep R.	4	5	4	5	2	4	4	7	35	4.4	1.4
Simonette R.	6	4	5	5	3	1	2	5	31	3.9	1.7
Yarrow Cr.	2	1	1	1	2	1	1	2	11	1.4	0.5
Ave	3.0	3.4	3.3	3.5	2.7	2.2	2.4	3.6	24.0		
SD	1.4	1.7	1.7	1.6	0.8	1.2	1.2	2.0	8.2		

14 and the minimum was 6, with an average of 11.0 haplotypes (SD = 3.2) per enzyme. No region contained every haplotype revealed by a particular enzyme. The average number of haplotypes per region ranged from 2.4 (Carbondale River) to 4.4 (Sheep River), excluding the three areas with small sample sizes (Manteau R., Smith-Dorian Cr., Yarrow Cr.). Each enzyme revealed an average of two unique haplotypes, which are those appearing in only one region.

5.3.1 Cfo I (ND1)

Cfo I was used to digest 116 mtDNA samples representing 12 regions. Table 8 summarizes the Cfo I haplotypes. Fragment sizes ranged from 105 bp to 852 bp in length and the average sum of fragments from all samples was 2025 bp (avedev = 28.1 bp). Most DNA profiles included bands at 833, 382, 296, 223, and 139 bp. Brook trout displayed a distinctive set of bands at 532 and 515 bp which never appeared in bull trout profiles.

Table 9 displays the distribution of Cfo I haplotypes for all regions analyzed. Thirteen bull trout haplotypes and two brook trout haplotypes were detected. Almost half of the fish (47%) contained haplotype M and two other haplotypes, E and J, were relatively common at 12.5% and 18.3% respectively. Thus, a total of 77.8% of the fish belonged to only three categories and every region contained at least one of these common haplotypes.

The number of Cfo I haplotypes varied for each region. The largest number was six, found in the Simonette River. The Berland River, Belly River, Prairie Creek, and Sheep River contained four haplotypes, whereas three were found in Smith-Dorian Creek. The Carbondale River, Kakwa River, Oldman River, and Yarrow Creek each contained two

Table 8. Summary of haplotypes produced by Cfo I digestion of PCR products amplified from the ND1 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes. Fragment sizes in bp are written vertically above each column.

Haplotype	105	113	123	136	139	158	180	197	223	296	368	382	403	420	447	515	532	833	852
C				x	x		x			x		x						x	
D		x			x				x	x		x						x	
E				x	x				x	x		x						x	
F			x			x			x	x		x						x	
G						x		x				x			x			x	
H					x	x	x			x			x						x
I		x					x		x	x		x						x	
J					x	x			x	x		x						x	
K					x		x		x	x	x							x	
L		x					x		x	x			x					x	
M					x		x		x	x		x						x	
N			x							x		x		x				x	
O			x					x	x	x		x						x	
A*	x	x		x	x				x	x		x					x		
B*									x	x		x				x	x		

Table 9. Distribution of haplotypes produced by Cfo I digestion of PCR products amplified from the ND1 region of mtDNA. Values represent the number of fish from each region which show a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes															
Cfo I	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	Hap Total*	Fish No.
Berland R.				1	4					3			2			4	10
Belly R.					3		1			2			10			4	16
Carbondale R.										5			3			2	8
Kakwa R.										7			3			2	10
Smith-Dorian Cr.					1				2				11			3	14
Manteau R.													1			1	1
Oldman R.				6									1			2	7
Prairie Cr.					5				2	1			2			4	10
Quirk Cr.	3	9														2	12
Sheep R.			2					2					7	1		4	12
Simonette R.				1		1				1	1		7		2	6	13
Yarrow Cr.												1	2			2	3
Total	3	9	2	8	13	1	1	2	4	19	1	1	49	1	2		116

*This value represents the total number of haplotypes resolved for each population.

haplotypes.

Several regions contained unique DNA fragment patterns. These included the Belly River, Sheep River, Simonette River, and Yarrow Creek. Of these, the Belly River and Yarrow Creek had one unique haplotype whereas the others each had three.

5.3.2 Dde I (ND1)

Dde I was used to digest 116 mtDNA samples from 12 regions and the haplotypes produced are summarized in Table 10. Fragment sizes ranged from 104 bp to 661 bp in length. The average sum of fragments from all samples was 1986 bp (avedev = 31.2 bp). Most DNA profiles included bands at 616, 482, 242, 196, and 177 bp and brook trout displayed a distinctive band at 543 bp.

Table 11 displays the haplotype distribution revealed by Dde I. Fourteen bull trout haplotypes and two brook trout haplotypes were detected. About 52% of the fish were either haplotype F (21.2%) or H (30.8%) and two other haplotypes, D and J, were relatively common at 11.5% and 9.6% respectively. Thus, a total of 73.1% of the samples belonged to four categories and every region contained at least one of these common haplotypes.

The largest number of Dde I haplotypes was six, found in Prairie Creek. The Belly River and Sheep River each contained five haplotypes, whereas four haplotypes were identified in the Berland River, Kakwa River, Smith-Dorian Creek, Oldman River and Simonette River. The Carbondale River and Yarrow Creek each revealed only one fragment pattern.

Unique haplotypes were found in the Berland River, Belly River, Prairie Creek, Sheep

Table 10. Summary of haplotypes produced by Dde I digestion of PCR products amplified from the ND1 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	104	116	123	141	164	177	196	234	242	294	320	365	397	482	543	616
C	x		x			x	x		x					x		x
D		x	x			x	x		x					x		x
E		x		x		x	x		x					x		x
F			x	x		x	x		x					x		x
G	x				x	x	x		x					x		x
H			x		x	x	x		x					x		x
I						x	x		x	x				x		x
J				x	x	x	x		x					x		x
K	x	x	x		x		x		x					x		x
L			x		x	x	x		x	x		x		x		
M				x			x		x				x	x		x
N							x	x	x		x			x		x
O	x	x			x	x	x		x					x		x
P						x	x		x				x	x		x
A*			x	x		x	x		x					x	x	
B*			x		x	x	x		x					x	x	

Table 11. Distribution of haplotypes produced by Dde I digestion of PCR products amplified from the ND1 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes																	
Dde I	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Hap Total*	Fish No.
Berland R.				6	2					1			1				4	10
Belly R.						1		6		4	4		1				5	16
Carbondale R.								8									1	8
Kakwa R.				3			4	3		1							4	11
Smith-Dorian Cr.			1	2		9		2									4	14
Manteau R.								1									1	1
Oldman R.						1	1	3		2							4	7
Prairie Cr.			4	1		2			1	1						1	6	10
Quirk Cr.	7	5															2	12
Sheep R.						2	2	6		1		1					5	12
Simonette R.						4	1	3							4		4	12
Yarrow Cr.						3											1	3
Total	7	5	5	12	2	22	8	32	1	10	4	1	1	1	4	1		116

*This value represents the total number of haplotypes resolved for each population.

River, and Simonette River. Each revealed only one unique haplotype except Prairie Creek which had two.

5.3.3 Hae III (ND1)

Hae III was used to digest 115 mtDNA samples representing 12 regions and the resulting haplotypes are summarized in Table 12. Fragment sizes ranged from 119 bp to 617 bp in length and the average sum of fragments from all samples was 1984 bp (avedev = 31.2 bp). Most DNA profiles included bands at 617, 528, 229, 199, and 176 bp. Brook trout displayed a distinctive band at 289 bp however, a band this size did appear in two bull trout profiles.

Table 13 displays the distribution of haplotypes produced with Hae III. Twelve bull trout haplotypes and five brook trout haplotypes were detected. About 62% of the fish belonged to either haplotype I (30%) or J (32%) and two other haplotypes, K and L, made up 8.7% and 7.8% of the samples respectively. Thus, a total of 78% of the samples belonged to four categories and every region, except the Manteau River fish, contained at least one of these common haplotypes.

The largest number of Hae III haplotypes was six, found in the Kakwa River. The Simonette River had five haplotypes whereas the Berland, Belly and Sheep Rivers each contained four. There were three haplotypes in the Oldman River and Prairie Creek, and two in Smith-Dorian Creek. The Carbondale River and Yarrow Creek each revealed one. Rivers with unique haplotypes included the Belly, Carbondale, Kakwa, Sheep, and Simonette, however none revealed more than one.

Table 12. Summary of haplotypes produced by Hae III digestion of PCR products amplified from the ND1 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	100	119	120	142	143	174	176	199	229	252	289	398	528	617
A			x				x	x	x				x	x
B					x		x	x	x				x	x
D	x		x				x	x	x				x	x
E				x	x		x	x			x	x		x
G	x				x		x	x	x				x	x
H			x					x	x		x		x	x
I		x	x				x	x	x				x	x
J			x		x		x	x	x				x	x
K				x	x		x	x	x				x	x
L			x			x	x	x	x				x	x
N			x				x		x			x	x	x
P							x			x	x		x	x
C*			x				x	x			x		x	x
F*				x	x					x	x		x	x
M*		x	x				x	x			x		x	x
O*			x		x			x			x		x	x
Q*				x	x		x	x			x		x	x

Table 13. Distribution of haplotypes produced by Hae III digestion of PCR products amplified from the ND1 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes																	
Hae III	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	Hap Total*	Fish No.
Berland R.		1							5	2	2							4	10
Belly R.							6		6	2	2							4	16
Carbondale R.												8						1	8
Kakwa R.	1			1				1	2	5	1							6	11
Smith-Dorian Cr.									13		1							2	14
Manteau R.		1																1	1
Oldman R.	1									4	1							3	6
Prairie Cr.		3							1	6								3	10
Quirk Cr.			1			1							3		4		3	5	12
Sheep R.					1				2	7				2				4	12
Simonette R.				3					2	4	2					1		5	12
Yarrow Cr.										3								1	3
Total	2	5	1	4	1	1	6	1	31	33	9	8	3	2	4	1	3		115

*This value represents the total number of haplotypes resolved for each population.

5.3.4 Hpa II (ND1)

Hpa II was used to digest 112 mtDNA samples representing 12 regions and the resulting haplotypes are summarized in Table 14. Fragment sizes ranged from 113 bp to 1043 bp in length. The average sum of fragments from all samples was 2000 bp (avedev. = 48.1 bp). Most DNA profiles included bands at 1043, 445, and 202 bp but brook trout displayed a distinctive band at 800 bp which never appeared in bull trout profiles.

Table 15 displays the distribution of haplotypes revealed by Hpa II digestion. Thirteen bull trout haplotypes and four brook trout haplotypes were detected. About 63% of the samples displayed haplotype G (12.9%), H (26.7%) or O (23.4%), while two other haplotypes, A and J, made up 7.9% and 8.9% of the samples respectively. Thus, 79.8% of the fish belonged to five categories and every region contained at least one of these common haplotypes.

The largest number of Hpa II haplotypes was six, found in the Berland River. The Sheep and Simonette Rivers each had five haplotypes whereas the Belly River, Kakwa River, and Prairie Creek had four. There were three haplotypes each in the Carbondale and Oldman Rivers. Smith-Dorian Creek had two patterns and only one was detected in Yarrow Creek.

Unique haplotypes were found in the Belly, Carbondale, Sheep, and Simonette Rivers. Each revealed one unique DNA fragment pattern except the Simonette River which revealed two.

Table 14. Summary of haplotypes produced by Hpa II digestion of PCR products amplified from the ND1 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	113	125	144	171	202	218	248	282	347	445	473	525	800	1043
A	x			x	x					x				x
B			x	x	x		x	x	x			x		
C	x	x			x					x				x
D					x		x			x				x
E	x	x			x						x			x
G	x			x	x					x				x
H		x		x	x					x				x
J			x	x	x					x				x
M	x				x	x				x				x
N		x			x	x				x				x
O					x				x	x				x
P		x			x		x			x				x
Q	x	x		x	x					x				x
F*		x	x		x		x			x			x	
I*		x		x	x		x			x			x	
K*			x	x	x		x			x			x	
L*				x			x		x				x	

Table 15. Distribution of haplotypes produced by Hpa II digestion of PCR products amplified from the ND1 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes																	
Hpa II	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	Hap Total*	Fish No.
Berland R.			3		2		1	1		1							1	6	10
Belly R.	4			1				6		4								4	15
Carbondale R.	1	1						6										3	8
Kakwa R.	2							2		3							4	4	11
Smith-Dorian Cr.					1												13	2	14
Manteau R.																	1	1	1
Oldman R.							4	2									1	3	7
Prairie Cr.			2		3								2				3	4	10
Quirk Cr.						2			5		3	1						4	11
Sheep R.	1						1	6					1	2				5	11
Simonette R.							7	1		1					1	1		5	11
Yarrow Cr.								3										1	3
Total	8	1	5	1	6	2	13	27	5	9	3	1	3	2	1	1	24		112

*This value represents the total number of haplotypes resolved for each population.

5.3.5 Rsa I (ND1)

Rsa I was used to digest 110 mtDNA samples representing 12 regions and the resulting haplotypes are summarized in Table 16. Fragment sizes ranged from 110 bp to 499 bp in length. The average sum of fragments from all samples was 2028 bp (avedev = 18.9 bp). Most DNA profiles included single bands at 449 and 323, and double bands at 270 and 223 bp and brook trout displayed a distinctive double band at 294 bp.

Table 17 displays the distribution of haplotypes revealed by Rsa I. Six bull trout haplotypes and three brook trout haplotypes were detected. About 83.8% of the samples displayed haplotype C (35.4%) or G (48.4%), while another 11% were haplotype A. Thus, 94.8% of the fish belonged to three categories and every region contained at least one of these common haplotypes.

All regions revealed three haplotypes except for the Carbondale River, Sheep River and Yarrow Creek which each had two. Only the Simonette River contained unique DNA haplotypes of which there were two.

5.3.6 Hinf I (ND5/6)

Hinf I was used to digest 102 mtDNA samples representing 12 regions and the resulting haplotypes are summarized in Table 18. Fragment sizes ranged from 129 bp to 1130 bp in length. The average sum of fragments from all samples was 2577 bp (avedev = 14.0 bp). Most DNA profiles included bands at 968, 475, 389, 307, and 226 bp and brook trout displayed distinctive bands at 613, 285, and 262 bp.

Table 19 displays the distribution of haplotypes revealed by Hinf I. Seven bull trout

Table 16. Summary of haplotypes produced by Rsa I digestion of PCR products amplified from the ND1 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	110	115	128	129	142	144	222	223	270	270	294	294	323	449	499
A	x	x					x	x	x	x			x	x	
C			x	x			x	x	x	x			x	x	
D		x				x	x	x	x	x			x	x	
F	x	x							x	x			x	x	x
G					x	x	x	x	x	x			x	x	
B*	x	x	x	x			x	x				x	x	x	
E*	x	x					x	x			x	x	x	x	
H*			x	x			x	x			x	x	x	x	
I*					x	x	x	x			x	x	x	x	

Table 17. Distribution of haplotypes produced by Rsa I digestion of PCR products amplified from the ND1 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes										
Rsa I	A	B	C	D	E	F	G	H	I	Hap Total*	Fish No.
Berland R.			3		2		1			3	6
Belly R.			7		1		8			3	16
Carbondale R.	7						1			2	8
Kakwa R.	1		1				8			3	10
Smith-Dorian Cr.	1		11				2			3	14
Manteau R.			1							1	1
Oldman R.	1		3				3			3	7
Prairie Cr.	1		3				6			3	10
Quirk Cr.		4						5	2	3	11
Sheep R.			4				7			2	11
Simonette R.				1		1	11			3	13
Yarrow Cr.			2				1			2	3
Total	7	4	34	1	3	1	48	5	2		110

*This value represents the total number of haplotypes resolved for each population.

Table 18. Summary of haplotypes produced by Hinf I digestion of PCR products amplified from the ND5/6 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	129	141	154	170	204	226	262	285	307	356	389	428	475	503	534	613	968	1130
A					x	x		x		x		x					x	
C	x					x			x		x		x				x	
E		x				x			x		x		x				x	
F	x	x				x					x			x				x
G			x			x			x		x		x				x	
I				x		x			x		x		x				x	
J					x	x		x		x				x			x	
B*	x	x	x		x		x	x	x		x					x		
D*				x			x	x	x		x		x			x		
II*				x	x		x	x	x		x	x	x					
K*							x		x					x	x		x	

Table 19. Distribution of haplotypes produced by Hinf I digestion of PCR products amplified from the ND5/6 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes												
Hinf I	A	B	C	D	E	F	G	H	I	J	K	Hap Total*	Fish No.
Berland R.					3				10			2	13
Belly R.			1				3		11			3	15
Carbondale R.			2						6			2	8
Kakwa R.			2		8		1					3	11
Smith-Dorian Cr.			1									1	1
Manteau R.									2			1	2
Oldman R.					1		1		5			3	7
Prairie Cr.									10			1	10
Quirk Cr.		3		4				3			1	4	11
Sheep R.	1					1			8	1		4	11
Simonette R.									9			1	9
Yarrow Cr.									4			1	4
Total	1	3	6	4	12	1	5	3	65	1	1		102

*This value represents the total number of haplotypes resolved for each population.

haplotypes and four brook trout haplotypes were detected. About 71% of the fish revealed haplotype I and another 13% were haplotype E. Thus, 84% of the fish belonged to two categories and every region contained at least one of these common haplotypes.

The Sheep River had the largest number of Hinf I haplotypes with four. The Belly, Kakwa and Oldman Rivers each had three whereas two haplotypes were detected in the Berland and Carbondale Rivers. The remaining regions each had one. Three unique profiles were observed with this enzyme, all from the Sheep River region.

5.3.7 Rsa I (ND5/6)

Rsa I was used to digest 102 mtDNA samples from 12 regions and the resulting RFLP profiles are shown in Table 20. Fragment sizes ranged from 127 bp to 952 bp. The average sum of the fragments for all samples was 2525 bp (avedev = 71.5 bp). Most DNA profiles included single bands at 952, 936, 389, and 139 bp. Brook trout displayed distinctive bands at 613 and 345 bp.

Table 21 displays the distribution of haplotypes revealed by Rsa I. Nine bull trout haplotypes and one brook trout haplotype were detected. About 67.4% of the fish were either haplotype A (28.4%) or G (39.0%), while two other haplotypes, E and H, made up 9.5% and 8.4% of the total respectively. Thus, 85.3% of the fish belonged to four categories and every region contained at least one of these common DNA patterns.

The Carbondale River had the largest number of Rsa I haplotypes with five, followed by four in the Sheep River. Three fragment patterns were resolved in the Berland, Kakwa and Oldman Rivers whereas two were detected in the Belly River, Manteau River, Prairie Creek

Table 20. Summary of haplotypes produced by Rsa I digestion of PCR products amplified from the ND5/6 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	127	139	157	187	217	292	345	398	468	543	613	936	952
A		x						x				x	x
C			x					x				x	x
D	x	x				x						x	x
E		x			x	x		x		x		x	
F		x								x		x	x
G		x	x					x				x	x
H			x							x		x	x
I		x		x				x				x	x
J		x	x						x			x	x
B*	x	x	x	x			x	x	x		x		

Table 21. Distribution of haplotypes produced by Rsa I digestion of PCR products amplified from the ND5/6 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes											
Rsa I	A	B	C	D	E	F	G	H	I	J	Hap Total*	Fish No.
Berland R.	8		3		2						3	13
Belly R.	6						9				2	15
Carbondale R.	3			1	1		1		2		5	8
Kakwa R.	3					4	4				3	11
Smith-Dorian Cr.	1										1	1
Manteau R.					1		1				2	2
Oldman R.	5				1		1				3	7
Prairie Cr.					3		8				2	11
Quirk Cr.		7									1	7
Sheep R.	1				1		9		1		4	12
Simonette R.								8		3	2	11
Yarrow Cr.							4				1	4
Total	27	7	3	1	9	4	37	8	3	3		102

*This value represents the total number of haplotypes resolved for each population.

and Simonette Rivers. The remaining regions each had one.

Four regions displayed unique DNA fragment patterns including the Berland, Kakwa and Simonette Rivers, each with one, and the Carbondale River with two.

5.3.8 Alu I (ND5/6)

Alu I was used to digest 108 mtDNA samples representing 12 regions and the resulting DNA fragment profiles are shown in Table 22. Fragment sizes ranged from 109 bp to 736 bp in length. The average sum of the fragments from each fish was 2490 bp (avedev = 35.5 bp). Most profiles included single bands at 656, 517, 486, 380, 191 and 139 bp, and brook trout displayed a distinctive band at 1190 bp.

Table 23 displays the distribution of haplotypes from Alu I digestion. Fourteen bull trout and seven brook trout haplotypes were detected. About 57.3% of the samples were either haplotype M (32.3%) or P (25.0%), while another 19.8% were haplotype K. Thus, 77.1% of the fish belonged to three categories and every region had at least one of these common haplotypes.

The Sheep River had the largest number of Alu I haplotypes with seven, followed by five in the Simonette River. Four fragment patterns were revealed in the Belly and Kakwa Rivers whereas three were detected in the Belly, Berland, and Kakwa Rivers, as well as in Prairie Creek. Yarrow Creek had two haplotypes.

Five regions displayed unique fragment patterns. The Belly River, Kakwa River and Prairie Creek each had one unique profile whereas the Simonette River had three and the Sheep River had four.

Table 22. Summary of haplotypes produced by Alu I digestion of PCR products amplified from the ND5/6 region of mtDNA. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those marked with * which represent brook trout (*Salvelinus fontinalis*) haplotypes.

Haplotype	109	127	139	157	191	230	295	330	380	406	457	486	517	656	736	1190
A	x		x			x			x		x	x	x			
D		x	x	x			x					x				x
E			x	x							x	x	x	x		
F	x	x	x						x			x	x	x		
G			x	x					x			x	x		x	
H	x		x	x					x			x	x	x		
I		x	x	x	x				x		x	x	x			
J		x	x	x					x			x	x	x		
K	x		x		x				x			x	x	x		
L					x	x				x		x	x	x		
M		x	x		x				x			x	x	x		
P			x	x	x				x			x	x	x		
S	x	x	x	x					x			x	x	x		
T			x		x	x			x			x	x	x		
U				x	x	x			x			x	x	x		
B*			x	x	x		x			x						x
C*	x		x		x		x			x			x		x	
N*		x	x	x	x		x			x						x
O*			x	x			x	x		x						x
Q*	x		x	x	x		x			x			x		x	
R*			x		x		x	x		x						x

Table 23. Distribution of haplotypes produced by Alu I digestion of PCR products amplified from the ND5/6 region of mtDNA. Values represent the number of fish from each region which displayed a particular haplotype. All haplotypes were detected in bull trout (*Salvelinus confluentus*) except those from Quirk Creek which represent brook trout (*Salvelinus fontinalis*) haplotypes.

	Haplotypes																					
Alu I	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	Hap Total*	Fish No.
Berland R.											4		6			1						3	11
Belly R.						1					5		8			1						4	15
Carbondale R.											4		2			2						3	8
Kakwa R.								2			2		6			1						4	11
Smith-Dorian Cr.													1									1	1
Manteau R.													2									1	2
Oldman R.											1		2			4						3	7
Prairie Cr.									2							7					2	3	11
Quirk Cr.	1	5	2											1	1		1	1				7	12
Sheep R.				1							1	2				5			1	1	2	7	13
Simonette R.					3		1			4			2			3						5	13
Yarrow Cr.											2		2									2	4
Total	1	5	2	1	3	1	1	2	2	4	19	2	31	1	1	24	1	1	1	1	4		108

*This value represents the total number of haplotypes resolved for each population.

5.3.9 Intrapopulation haplotype variation

Generally, bull trout populations showed a pattern of mtDNA diversity in which most individuals (70 - 90 %) had one of a few common haplotypes, and a small proportion of the population displayed rare or unique haplotypes. This diversity pattern was consistent with those previousiy reported (Williams et al., 1995). The larger number of rare haplotypes are thought to be mutational derivatives of the common haplotypes in the population (Billington and Hebert, 1991).

The total number of haplotypes per region varied substantially. Values ranged from 19 (Carbondale River) to 35 (Sheep River), including all eight enzymes. Clearly, the Sheep River and Simonette River showed the greatest quantity of intrapopulation variation with 35 and 31 haplotypes respectively. The Berland, Belly and Kakwa Rivers were close behind with 29 haplotypes and twenty six haplotypes were detected in Prairie Creek. The least variability was observed in the Oldman River (24 haplotypes) and the Carbondale River (19 haplotypes).

Each enzyme revealed the presence of unique haplotypes although not in every region. Table 24 shows the number of unique haplotypes detected in each region for each of the eight enzymes. Again, the Sheep and Simonette rivers displayed the greatest variability with 13 and 14 unique haplotypes respectively. If the number of unique haplotypes was calculated as a percent of the haplotype total, the Simonette River had the greatest number of unique DNA patterns relative to the other regions.

Two regions revealed no unique patterns. The Oldman River was represented by smaller sample sizes, usually 6 or 7 fish samples for each digestion and still revealed 24 haplotypes in total, yet none of these were unique to the region. Smith-Dorian Creek was

Table 24. Number of unique bull trout (*Salvelinus confluentus*) mtDNA haplotypes identified in each study region. The number of unique haplotypes is shown as a percent of the total number of haplotypes detected. Brook trout (*Salvelinus fontinalis*) data are not included.

	ND1					ND5/6				
	Cfo I	Dde I	Hae III	Hpa II	Rsa I	Hinf I	Rsa I	Alu I	Unique * Haplotypes	% of total haplotypes
Berland R.		2					1		3 (29)	10.3
Belly R.	1	2	1	1				1	6 (29)	20.7
Carbondale R.			1	1			1		3 (19)	15.8
Kakwa R.			1				1	1	3 (29)	10.3
Smith-Dorian Cr.									0 (17) **	0
Oldman R.									0 (24)	0
Prairie Cr.		2						1	3 (26)	11.5
Sheep R.	3	1	1	1		3		4	13 (35)	37.1
Simonette R.	4	1	1	2	2		1	3	14 (31)	45.2

* Total number of haplotypes is shown in parentheses.

** Smith-Dorian Creek sample sizes included only one fish for the ND5/6 data.

represented by relatively large sample sizes (N=14) for ND1 digestions but only one sample amplified for ND5/6 analysis. Seventeen haplotypes were detected in the Smith-Dorian Creek region, 14 from ND1 analysis, but no unique fragment profiles were observed. In comparison, Yarrow Creek (not included in Table 24) had no more than four fish in each enzyme data set, yet 11 haplotypes were revealed and one was unique to the region. The absence of unique fragment patterns in the Oldman River and Smith-Dorian Creek may be due to slightly smaller sample sizes than the other regions but in each case a large enough number of common haplotypes was detected that at least some rare patterns were expected. It seems more likely that these regions revealed proportionately fewer unique patterns because fewer exist.

Another region with a relatively low number of haplotypes was Prairie Creek. These fish putatively comprise a resident population (Terry Clayton, personal communication, 26 October 1996) which may explain their low diversity levels since their gene pool was not likely affected by immigrant fish displaying natal stream infidelity. Even without the presence of physical barriers, resident populations remain somewhat reproductively isolated from their downstream counterparts through spatial and temporal differences in spawning behavior (Northcote, 1992). Thus, the effective population size of the Prairie Creek sample may be smaller than other populations sampled for this study.

Differences in intradrainage variability appear rather substantial and may be caused by a number of factors. The smallest level of intradrainage variation was detected in the Oldman and Smith-Dorian Creek. These streams may have experienced more severe population declines than others in the province, resulting in loss of genetic variants. With

fewer individuals in the population, variants may be lost through genetic drift (Dowling and Childs, 1992) or stochastic lineage extinction (Avise, 1987). An extreme population decline may create a bottleneck effect resulting in reduced genetic diversity.

The Sheep and Simonette Rivers display the greatest diversity in mtDNA haplotypes, perhaps indicating that these streams have experienced less drastic population reductions. Their drainage metapopulation structures may contain a larger number of distinct spawning populations, and hence more genetic variability. Factors which could explain this scenario include more suitable spawning sites (in quantity and quality), fewer physical barriers in migratory corridors, and lower angling pressure.

5.3.10 Mitochondrial DNA composite haplotypes

Composite haplotypes were determined for individual fish based on the combined DNA fragment patterns of all eight restriction enzymes. Percent sequence divergence and heterogeneity values were calculated using composite profiles rather than individual restriction enzyme profiles. Fish DNA that did not amplify with both ND1 and ND5/6 primers, or which wasn't digested with all eight restriction enzymes, could not be included in these calculations. Therefore, sample sizes for these analyses were smaller than those reported for individual enzymes.

Composite haplotypes are shown in Table 25. Sample sizes ranged from 4 fish (Berland River) to 14 fish (Belly River). The average composite haplotype sample size was 7.9 (SD = 3.2) over nine regions. A high level of variability within each enzyme category led to a wide array of composite haplotypes so that 63 individual patterns were compared.

Table 25. Composite haplotypes used for calculation of population heterogeneity and percent sequence divergence. Haplotypes are listed in the following enzyme order: Cfo I, Dde I, Hae III, Hpa II, Rsa I, Hinf I, Rsa I, Alu I. The first five enzymes were used on PCR fragments amplified with ND1 primers, the others with ND 5/6. All samples are bull trout (*Salvelinus confluentus*) except for the Quirk Creek group which are brook trout (*Salvelinus fontinalis*).

Berland R. (N=4)
MDIEEEAM
MDIQEIAM
JEJJCIAM
JMBCCICP

Belly R. (N=14)
MHGJGIGK
MKIHCIGP
EKIHCIGK
EKIHGIAK
MJIHGIAM
MHKJCIGM
JJJACIAM
MJGACCAM
EHJAGIAM
MHIAGIGM
MHKDCGGM
JJGJGGAM
MFGJCIGK
MKGHEGGF

Carbondale R. (N=8)
JHLHAIIK
MHLBAIIK
JHLHACAM
MHLHAIGK
JHLAAIEK
JHLHAIAP
JHLHACDM
MHLHGIAP

Kakwa R. (N=9)
JHIQGCGH
JGJHGCAM
MHJJGEGH
JDIQCEAP
MGJJGEAM
MDAHGEGK
JGJAGEAM
JHJJGEAM
JJHQGGGK

Oldman R. (N=7)
MFAQAIAP
DHJGCGAM
DJJHCIAP
DGJHCIGK
DHKGGEAM
DHJGGIEP
DJBGGIAP

Prairie Cr. (N=8)
IJJMAIEP
ECJECIEP
ECJQGIEP
ECJEGIGP
EPBCCIGP
EIBCCIGU
MFJQGIGU
MFJQGIGI

Quirk Cr. (N=4)
BBOFEBBB
BBOKBBBB
BBOKHBBN
BBQIIDBB

Sheep R. (N=9)
MGJHGJIT
MHJHGIGK
CHIHCIAP
MHJMGIGP
HJNNCIGU
MFNNGIGU
CHJHGIGP
MFJGGIGL
MHJACIGP

Simonette R. (N=7)
FHDJGIHM
OHDGGIHP
OHPPFIHP
MGJGGIHJ
MFJHGIHP
MFJOGIHJ
MOIGGIJJ

5.3.11 Percent sequence divergence

Percent sequence divergence was determined using composite haplotypes. Calculations were performed with REAP software version 4.0 (McElroy et al., 1991), with which total nucleotide divergence between two populations was estimated and the component of this diversity not explained by within-population polymorphism was extracted. A diagonal matrix of these pairwise comparisons is shown in Table 26. All bull trout populations differed from brook trout by more than 5.0%. The average sequence divergence at this interspecies level was 5.46% (SD = 0.29). The minimum interspecies divergence level was between brook trout and the Oldman River samples (5.17%) and the maximum was with Simonette River samples (5.91%).

Comparison of bull trout populations indicated wide variations in sequence divergence ranging from 0.14% between the Berland and Belly River samples to 0.92% between Carbondale and Simonette Rivers. Other pair-wise comparisons which showed the higher levels of diversity included Carbondale and Prairie Creek (0.88%), Carbondale and Berland Rivers (0.70%), and the Berland and Simonette Rivers (0.68%). The lower range of sequence divergence was represented by the Belly and Oldman River (0.16%), Belly and Kakwa Rivers (0.23%) and the Berland and Oldman Rivers (0.23%). Several regions differed by 0.5% ± 0.1 including the Belly and Simonette Rivers, Belly and Carbondale Rivers, Kakwa and Carbondale Rivers, Oldman and Carbondale Rivers, Kakwa and Prairie Creek, Kakwa and Simonette Rivers, Oldman and Simonette Rivers, and Prairie Creek and Simonette River.

There does not appear to be any strong geographical structuring revealed by interpopulation composite haplotype comparisons. Populations must be separated for long

Table 26. Pairwise comparison of percent sequence divergence based on composite haplotypes. All samples were bull trout (*Salvelinus confluentus*) except for those from Quirk Creek which were brook trout (*Salvelinus fontinalis*).

Berland R. (Ber)	Ber	Bel	Car	Kak	Old	PC	Shp	Sim
Belly R. (Bel)	0.14							
Carbondale R. (Car)	0.70	0.57						
Kakwa R. (Kak)	0.35	0.23	0.57					
Oldman R. (Old)	0.23	0.16	0.55	0.23				
Prairie Cr. (PC)	0.25	0.45	0.88	0.58	0.21			
Sheep R. (Shp)	0.36	0.21	0.66	0.33	0.26	0.22		
Simonette R. (Sim)	0.68	0.53	0.92	0.56	0.58	0.58	0.49	
Quirk Cr. (QC)	5.83	5.24	5.61	5.29	5.17	5.39	5.25	5.91

periods of time to show strong geographical structuring in their genetic divergence patterns. Clear evidence for this exists with previous studies comparing northern freshwater species to southern species. The latter inhabited unglaciated regions and have experienced geographical separation for relatively long periods of time compared to the northern species whose ranges were largely glaciated up to 15 000 years ago (Pielou, 1991). Mitochondrial analysis reveals much larger sequence divergence in the southern species and clear geographical structuring exists (Billington and Hebert, 1991). Since Alberta bull trout populations presumably separated into distinct drainages following the last ice age, they have been isolated from one another long enough for distinct haplotypes to become fixed in separate drainages. The common haplotypes shared by large numbers of fish in each drainage are a consequence of recent geographical isolation. The differences apparent among the drainages likely result from random events such as the mutational variations of common haplotypes, stochastic extinction of mitochondrial lines and bottleneck effects for those populations which have experienced the greatest population declines.

5.3.12 Population heterogeneity

Population heterogeneity was calculated using a Monte Carlo simulation from REAP software version 4.0 (McElroy et al., 1991). The algorithm assesses heterogeneity through Chi square analysis by comparing the original data matrix to that estimated from repeated randomizations of the original matrix. All pairwise comparisons were completed using 1000 randomizations. Table 27 provides a summary of the analysis for significant population heterogeneity. Clearly, significant heterogeneity ($p < 0.05$) exists between most of the

Table 27. Pairwise comparisons showing significant population heterogeneity. Analysis was based on haplotype frequencies and calculated using REAP software (McElroy et al., 1991). Monte Carlo simulations were completed with 1000 randomizations.

Berland R. (Ber)	Ber	Bel	Car	Kak	Old	PC	Shp
Belly R. (Bel)	=						
Carbondale R. (Car)	**	=					
Kakwa R. (Kak)	**	*	*				
Oldman R. (Old)	*	*	=	=			
Prairie Cr. (PC)	**	**	*	**	*		
Sheep R. (Shp)	*	=	=	*	=	=	
Simonette R. (Sim)	**	**	**	**	**	**	=

The level of significance is indicated as follows:
= not significantly different ($p > 0.05$)
* significantly different ($p < 0.05$)
** significantly different ($p < 0.01$)

regions sampled. For example, both Prairie Creek and the Simonette River differed significantly from all regions except the Sheep River. The Simonette and Sheep were similar in that they both had large number of haplotypes. This increases the chance of similar haplotype frequencies appearing through the Monte Carlo simulation. The Prairie Creek fish had lower numbers of haplotypes, but those which were common to other streams also were common in the Sheep River. Since both rivers belong to the Bow River drainage this result is not surprising.

The Oldman River showed significant heterogeneity when compared to the Berland, Belly and Simonette Rivers, and to Prairie Creek, but not to the Carbondale, Kakwa or Sheep Rivers. Since the Oldman sample revealed no unique haplotypes, the differences compared to other streams was a function of which common haplotypes were present in each drainage. Common haplotypes were defined as those found in more than one drainage but they are not necessarily present in every drainage and certainly not with the same frequency. The level of heterogeneity described for the Oldman River reflects its place in the distribution of the common haplotypes. It contains enough of the same haplotypes as the Kakwa, Sheep and Carbondale Rivers that Monte Carlo Simulations cannot differentiate between them. The fact that the Oldman River was significantly different than the Belly River, is of interest since they belong to the same South Saskatchewan drainage.

Other notable results include the potential uniqueness of populations in Prairie Creek and the Simonette River. It was suggested previously that the Prairie Creek fish may form a resident population. Their intrapopulation diversity levels appeared lower than other populations, which in turn affects the analysis of heterogeneity in such a way as to increase

the likelihood of obtaining a significant result through the Monte Carlo randomizations. The situation with the Simonette River is similar except that the result is caused by an above average level of intrapopulation diversity rather than limited intrapopulation diversity.

Unlike sequence divergence, heterogeneity among populations is affected by the frequency of haplotypes appearing in a population. Even though geographical structuring was not indicated by sequence divergence data, the analysis for heterogeneity indicates that these populations have significant differences in their gene pools. Many common haplotypes were present in each region but the frequency with which they appear and the proportion of unique haplotypes both have an influence on the level of significance in the analysis.

5.4 RAPD data

RAPD profiles were generated by performing PCR on total genomic DNA extracts with a 10mer oligonucleotide primer. Artifactual bands were identified through replicate runs so that only bands consistently present in the same fish samples were selected for RAPD analysis. Consequently, nine DNA fragments were selected and their respective sizes were 189, 212, 229, 315, 406, 526, 544, 607, and 990 bp. DNA fragments within 10 bp of these were recorded whereas all other bands were ignored.

A summary of regions included in the RAPD analysis is shown in Table 28. Sample sizes and the frequencies of each DNA fragment size are indicated. The average sample size was 12 fish (SD = 3.6) with most regions represented by 10 to 12 fish. The Waterton sample contained fish from two streams, Blakiston Creek and Yarrow Creek, which were combined to increase the number of individuals from that region. In addition to those regions

Table 28. Regions included in the RAPD analysis using total genomic DNA extracts. The top row indicates targeted DNA fragment sizes in bp and the values below show the proportion of each population that displayed the respective band sizes. Samples sizes are shown in the first column. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek which were brook trout (*Salvelinus fontinalis*). Cracker Lake, Red Eagle Lake and Slide Lake samples were from Glacier National Park, Montana, U.S.A.

	N	189	212	229	315	406	526	544	607	990
Cracker L.	20	0.000	0.850	0.100	0.800	0.900	0.000	0.450	0.850	0.900
Red Eagle L.	7	0.000	0.857	0.000	0.857	1.000	0.000	0.143	0.571	1.000
Slide L.	18	0.000	0.944	0.000	0.889	0.889	0.000	0.278	0.556	0.944
Belly R.	15	0.133	1.000	0.000	0.933	1.000	0.000	0.733	0.667	1.000
Berland R.	12	0.083	1.000	0.000	1.000	1.000	0.083	0.917	0.083	0.333
Carbondale R.	10	0.100	0.900	0.500	1.000	1.000	0.000	0.900	0.500	0.400
Cardinal R.	11	0.000	0.909	0.000	1.000	1.000	0.000	1.000	0.546	0.000
Kakwa R.	12	0.083	1.000	0.083	0.917	0.917	0.000	1.000	0.333	0.083
Oldman R.	9	0.000	1.000	0.444	1.000	1.000	0.000	1.000	0.889	0.556
Prairie Cr.	12	0.167	0.917	0.000	0.917	0.917	0.333	1.000	0.750	0.833
Sheep R.	12	0.083	1.000	0.000	0.917	1.000	0.000	0.917	0.250	0.083
Simonette R.	12	0.364	1.000	0.273	1.000	1.000	0.000	1.000	0.455	0.000
Smith-Dorian	11	0.000	1.000	0.182	0.909	1.000	0.000	1.000	0.182	0.091
Waterton R.	8	0.000	1.000	0.000	1.000	1.000	0.000	1.000	0.625	0.000
Quirk Cr.	12	0.000	1.000	0.000	0.917	1.000	0.917	0.083	0.917	0.250

sampled for the mtDNA analysis, the RAPD data set also included samples from (1) Smith-Dorian Creek, which was only partially represented in the mtDNA data set, (2) Yarrow and Blakiston Creeks in the Waterton River region, (3) the St. Mary River in Glacier National Park, Montana, U.S.A., and (4) the Cardinal River which drains into the North Saskatchewan River system. The Glacier Park and Cardinal River fin clippings were the only samples not collected in 1995. The former were obtained in the fall of 1996 and the latter were from archived fin tissue collected in 1993. The population from Quirk Creek consisted of brook trout and was used as an outgroup during genetic distance analyses.

RAPD profiles revealed fewer "haplotypes" than mtDNA. However RAPD data were similar in that each region displayed both common profiles (found in more than one region) and unique profiles (found only in one region). DNA fragment patterns were assigned alphabetic codes based on the order they were first observed. Table 29 shows all DNA patterns and indicates the number of regions in which each was found. A summary of the RAPD profiles found in each region is provided in Table 30. In total, thirty-four profiles were recognized in the fifteen regions sampled. Of these, twenty six were unique to a particular location and 8 were common to two or more locations. Table 31 indicates the number of common and unique profiles identified for each region. The total number of patterns found in each region ranged from two (Quirk Creek and Waterton) to nine (Cracker Lake). In most cases, 1-3 unique profiles contributed to the total number of patterns present so that all regions averaged about three common profiles and, in all but four locations, one to three unique profiles were observed.

Table 29. RAPD profiles generated with the DAF2 primer developed by Caetano-Anolles et al. (1991). The number of regions in which these profiles were found is shown. Fragment sizes are shown in bp in the top row. All profiles are from bull trout (*Salvelinus confluentus*) except for codes "I" and "L" which were generated from brook trout (*Salvelinus fontinalis*).

Profile Code	Regions	189	212	229	315	406	526	544	607	990
A	1				X	X		X		
B	1			X	X	X		X	X	
C	1		X					X		
D	1		X			X		X	X	
E	6		X		X	X		X		
F	5		X		X	X		X		X
G	9		X		X	X		X	X	
H	9		X		X	X		X	X	X
I	1		X		X	X	X		X	
J	1		X		X	X	X	X		
K	1		X		X	X	X	X		X
L	1		X		X	X	X	X	X	
M	1		X		X	X	X	X	X	X
N	1		X	X	X	X			X	
O	2		X	X	X	X		X		
P	1		X	X	X	X		X		X
Q	3		X	X	X	X		X	X	
R	1		X	X	X	X		X	X	X
S	1	X	X			X		X		X
T	1	X	X		X	X			X	
U	1	X	X		X	X		X		X
V	1	X	X		X	X		X		
W	1	X	X		X	X	X	X	X	
X	3		X		X	X			X	X
Y	2		X		X	X				X
Z	1									X
AA	1				X	X		X		
BB	1		X			X		X	X	X
CC	1							X		
DD	1			X				X	X	
EE	1		X	X	X	X			X	X
FF	1		X			X				X
GG	1				X	X		X	X	X
HH	1		X							

Table 30. Summary of RAPD profiles found in each region. All profiles were obtained from bull trout (*Salvelinus confluentus*) except those from Quirk Creek which were from brook trout (*Salvelinus fontinalis*).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	BB	CC	DD	EE	FF	GG	HH
Belly R.						x		x											x		x			x										
Berland R.					x	x				x										x														
Carbondale R.		x				x		x						x	x	x																		
Cardinal R.	x				x		x																											
Cracker L.						x		x																x				x	x	x	x	x	x	
Red Eagle L.								x																	x		x							
Slide L.						x		x																x	x	x								x
Kakwa R.			x		x		x								x							x											
Oldman R.								x									x	x															
Prairie Cr.							x	x			x		x										x										
Sheep R.					x		x	x																									
Simonette R.					x		x										x																
Smith-Dorian Cr.				x			x	x									x																
Waterton R.					x		x																										
Quirk Cr.									x			x																						

Table 31. Number of common and unique RAPD profiles present in each of 12 sample sites. Common profiles appeared in more than one region whereas unique profiles did not. Values in parentheses indicate sample sizes. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek which were brook trout (*Salvelinus fontinalis*).

	Common Profiles	Unique Profiles
Belly R. (15)	3	2
Berland R. (12)	2	2
Carbondale R. (10)	3	3
Cardinal R. (11)	2	1
Cracker L. (20)	3	6
Red Eagle L. (7)	2	1
Slide L. (18)	4	2
Kakwa R. (12)	3	2
Oldman R. (9)	2	1
Prairie Cr. (12)	2	3
Sheep R. (12)	3	0
Simonette R. (12)	3	0
Smith-Dorian Cr. (11)	3	1
Waterton R. (8)	2	0
Quirk Cr. (12)	0	2

The level of diversity represented by RAPD profiles appears to be lower than that indicated by mtDNA analysis. Such a result is not surprising if RAPD profiles reflect differences in nuclear DNA which is known to evolve at a much slower rate than mitochondrial DNA (Park and Moran, 1994). Since mtDNA is maternally inherited and lacks recombination, the effective population size for mtDNA is one fourth of that for nuclear genes. The result is more rapid accumulation of frequency changes in mtDNA profiles due to genetic drift. Additionally, mtDNA seems to acquire mutations more rapidly than single-copy nuclear DNA (Park and Moran, 1994). All of these factors explain why fewer distinct genetic profiles were revealed by RAPD analysis.

The variability that exists in the RAPD profiles, particularly the unique profiles specific to one region, may be caused by a number of factors. Since total genomic extracts were amplified, it is possible that some products were mitochondrial in origin, resulting in more variants. Another possibility arises from the presence of highly variable repeat sequences in the nuclear genome. Several types of repeating sequences normally exist in nuclear DNA (Estoup et al., 1993), and the extent to which they affected the RAPD profiles in this study was not investigated.

5.4.1 Genetic distances from RAPD profiles

A prerequisite to the calculation of genetic distance values was the selection of an appropriate algorithm based on biologically sound assumptions. Two models are commonly utilized for this purpose including an infinite alleles model (Nei, 1987) and a pure random drift model (Felsenstein, 1985a; Xu et al., 1994). The former states that alleles in a population are

kept in balance by a combination of losses through genetic drift and gains through a mutation process. Nei's algorithm (Nei, 1972), based on this model, also assumes equal effective population sizes among lineages. The latter model imparts no restrictions on population sizes and assumes that genetic drift is the primary force behind genetic divergence of taxa.

In this study, genetic distance values were calculated with both methods. Table 32 displays the distance values based on a drift and mutation model using Nei's algorithm. Table 33 shows Cavalli-Sforza distance values which assume a pure drift model. Both calculations were performed with PHYLIP ver 3.5. Previous arguments (Crane et al., 1994) suggest that genetic drift is the predominant force in the evolution of *Salvelinus* species, especially given the typically small effective population sizes within this group. Thus the Cavalli-Sforza algorithm may incorporate more realistic assumptions about the nature of evolutionary change in *Salvelinus*. Unlike Nei's distances, it is not heavily influenced by within-taxon heterozygosity, so that if no alleles are shared between two taxa, the distance takes its limiting value of one regardless of variability within each population (Swofford et al., 1996).

Several graphical representations of the distance relationships were generated with PHYLIP software using distance matrices both from Nei and Cavalli-Sforza. Neighbor-joining (NJ), FITCH and KITSCH trees were developed for each. The NJ trees are shown in Figure 6 (Nei's distances) and Figure 7 (Cavalli-Sforza distances). These trees are unrooted and branch lengths are additive so that they reflect genetic distances between regions. The order of data entry was randomized prior to running the algorithm since NJ trees are heuristic and their topologies are heavily influenced by OTU input order.

Table 32. Genetic distance values generated with Nei's distance algorithm using PHYLIP ver. 3.5. All populations were bull trou (*Salvelinus confluentus*) except Quirk Creek which were brook trout (*Salvelinus fontinalis*).

Quirk Cr. (QC)	QC	SM	Car	OR	PC	LKSC	Ber	Shp	NS	Kak	Wat	BRFT	GSL	GCL
Simonette R. (SM)	0.3243													
Carbondale R. (Car)	0.3043	0.0413												
Oldman R. (OR)	0.2877	0.0891	0.0270											
Prairie Cr. (PC)	0.2336	0.1487	0.0948	0.0615										
Smith-Dorian Cr. (LKSC)	0.2296	0.0553	0.0593	0.0461	0.1090									
Berland R. (Ber)	0.3061	0.0546	0.0595	0.1236	0.1094	0.1136								
Sheep R. (Shp)	0.2894	0.0276	0.0567	0.1135	0.1324	0.0656	0.0131							
Cardinal R. (NS)	0.2636	0.0274	0.0541	0.0810	0.1189	0.0263	0.0441	0.0150						
Kakwa R. (Kak)	0.3035	0.0196	0.0450	0.0916	0.1225	0.0497	0.0205	0.0034	0.0104					
Waterton R. (Wat)	0.2503	0.0281	0.0555	0.0721	0.1137	0.0196	0.0524	0.0201	0.0017	0.0138				
Belly R. (BRFT)	0.2755	0.1761	0.0951	0.0713	0.0306	0.1391	0.1116	0.1424	0.1483	0.1430	0.1429			
Slide L. (GSL)	0.2155	0.2471	0.1437	0.1411	0.1020	0.1898	0.1452	0.1777	0.1992	0.1911	0.1969	0.0341		
Cracker L. (GCL)	0.2121	0.2269	0.1199	0.0846	0.0701	0.1288	0.1756	0.1879	0.1761	0.1872	0.1692	0.0264	0.0197	
Red Eagle L. (GBL)	0.2184	0.2995	0.1794	0.1780	0.1348	0.2315	0.1863	0.2221	0.2456	0.2426	0.2450	0.0529	0.0054	0.027

Table 33. Genetic distance values generated with Cavalli-Sforza's distance algorithm using PHYLIP ver. 3.5. All populations were bull trout (*Salvelinus confluentus*) except Quirk Creek which were brook trout (*Salvelinus fontinalis*).

Quirk Cr. (QC)	QC	SM	Car	OR	PC	LKSC	Ber	Shp	NS	Kak	Wat	BRFT	GSL	GCL
Simonette R. (SM)	0.9289													
Carbondale R. (Car)	0.7576	0.1819												
Oldman R. (OR)	0.7871	0.2978	0.1179											
Prairie Cr. (PC)	0.5830	0.4989	0.3372	0.3188										
Smith-Dorian Cr. (LKSC)	0.6069	0.2708	0.1966	0.1822	0.3469									
Berland R. (Ber)	0.6360	0.2561	0.2262	0.3628	0.2849	0.3748								
Sheep R. (Shp)	0.6662	0.1602	0.2211	0.3417	0.3551	0.2197	0.0726							
Cardinal R. (NS)	0.7751	0.1778	0.2765	0.3171	0.4315	0.1779	0.2218	0.1172						
Kakwa R. (Kak)	0.7949	0.1024	0.1766	0.2452	0.3174	0.1580	0.1405	0.0586	0.1256					
Waterton R. (Wat)	0.7402	0.1619	0.3023	0.2838	0.4441	0.1826	0.2208	0.1087	0.0221	0.1140				
Belly R. (BRFT)	0.7071	0.6156	0.3492	0.3527	0.2246	0.4658	0.3311	0.3724	0.5782	0.4449	0.5545			
Slide L. (GSL)	0.5493	0.7708	0.3990	0.4728	0.3489	0.4137	0.3870	0.4102	0.6018	0.4923	0.6144	0.1342		
Cracker L. (GCL)	0.5601	0.6970	0.3000	0.3244	0.3042	0.2597	0.4765	0.4482	0.5704	0.4622	0.5925	0.1723	0.0650	
Red Eagle L. (GBL)	0.6141	0.9452	0.5064	0.5994	0.4664	0.5796	0.5253	0.5593	0.7555	0.6972	0.7875	0.1575	0.0497	0.1183

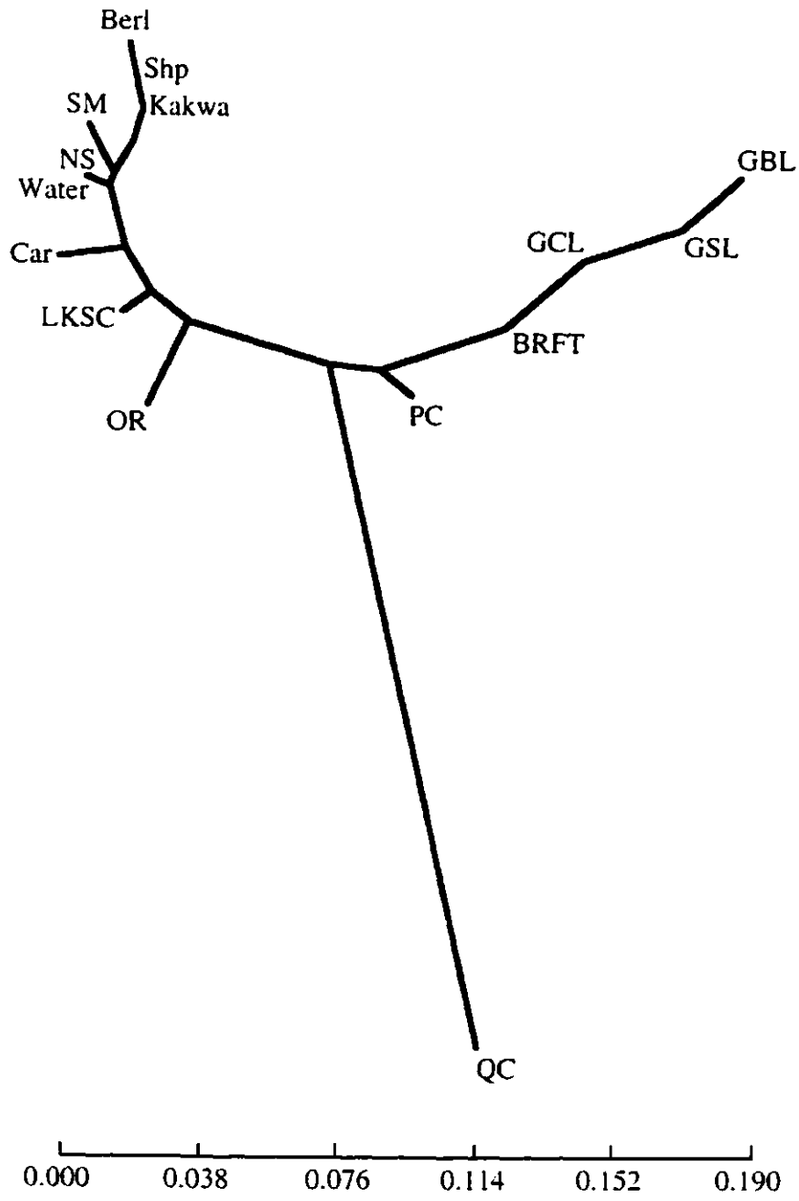


Figure 6. Neighbor-joining tree based on Nei's genetic distance values. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek (QC) which were brook trout (*Salvelinus fontinalis*). Populations were sampled in Alberta (Berl, Shp, Kakwa, SM, NS, Water, Car, LKSC, OR, PC, QC, BRFT) and Montana (GCL, GSL, GBL).

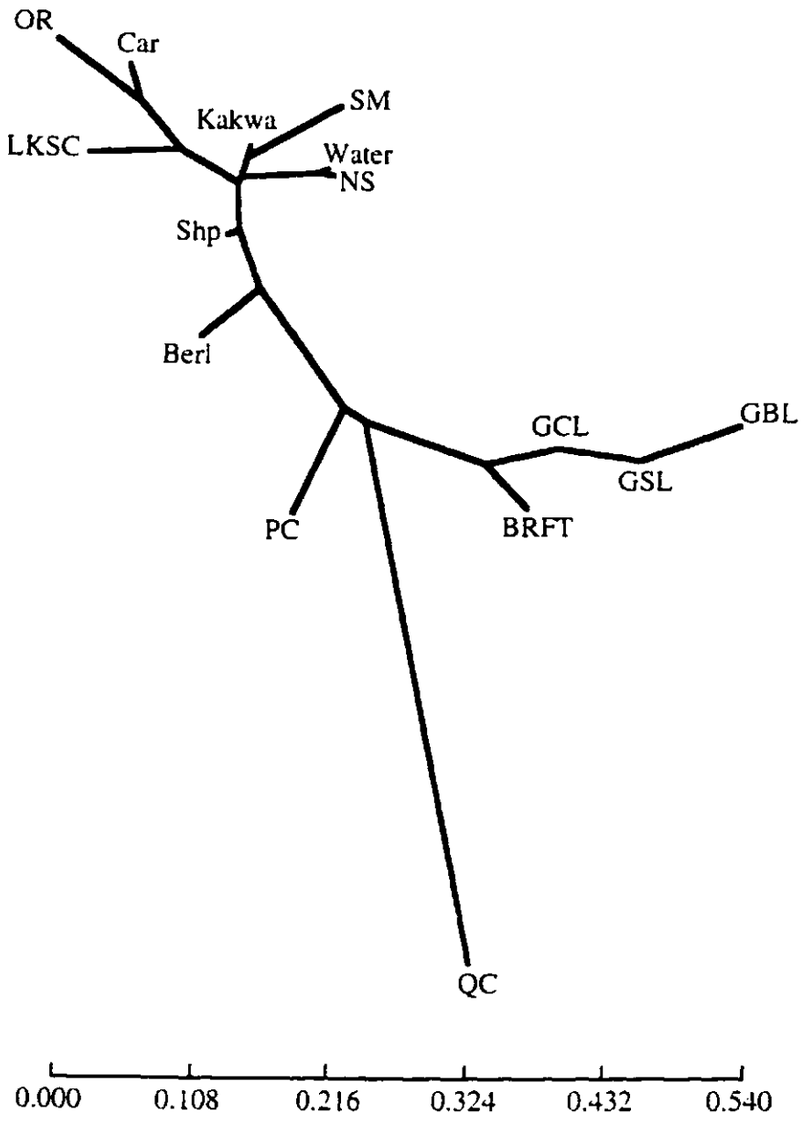


Figure 7. Neighbor-joining tree based on Cavalli-Sforza's genetic distance values. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek (QC) which were brook trout (*Salvelinus fontinalis*). Populations were sampled in Alberta (Berl, Shp, Kakwa, SM, NS, Water, Car, LKSC, OR, PC, QC, BRFT) and Montana (GCL, GSL, GBL).

In both NJ trees, many of the regions appear grouped in a manner which resembles their geographical distribution. For example, the Carbondale River and Oldman River regions are in close proximity, as are the Kakwa and Simonette regions. The three lakes from the Glacier National Park region in Montana cluster as well. However some groupings on the distance trees do not follow geographical boundaries, such as the tight group formed by the Waterton and North Saskatchewan River samples. Also, the Belly River fish cluster more closely to the U.S.A. samples than to the Southern Alberta samples. Prairie Creek fish seem to stand alone from other regions. Both the Nei and Cavalli-Sforza distance values tend to result in similar tree topologies with some exceptions. Most notable is a shift in the placement of the Berland River branch and some smaller shifts in the placement of Smith-Dorian Creek (LKSC) and Sheep River fish.

FITCH trees are shown in Figure 8 (Nei's distances) and Figure 9 (Cavalli-Sforza distances). The FITCH algorithm is based on the Fitch-Margoliash (FM) method of tree building. Like NJ trees, these are unrooted and their branch lengths reflect genetic distances between populations. Branch lengths are additive and the trees were constrained so that negative branch lengths were not permitted. Unlike NJ trees, the FITCH algorithm is not heuristic. By using a global optimization option, and with ten trials in which the taxon input order was randomized, the algorithm examined 7705 trees (with Nei's distances) and 8069 trees (with Cavalli-Sforza distances) before determining the best tree topologies. The extensive survey of possible tree topologies performed by FITCH is more time consuming than the NJ algorithm, but more likely results in the best tree (Felsenstein, 1993).

A comparison of the FITCH trees indicates similar topologies to those of the NJ trees.

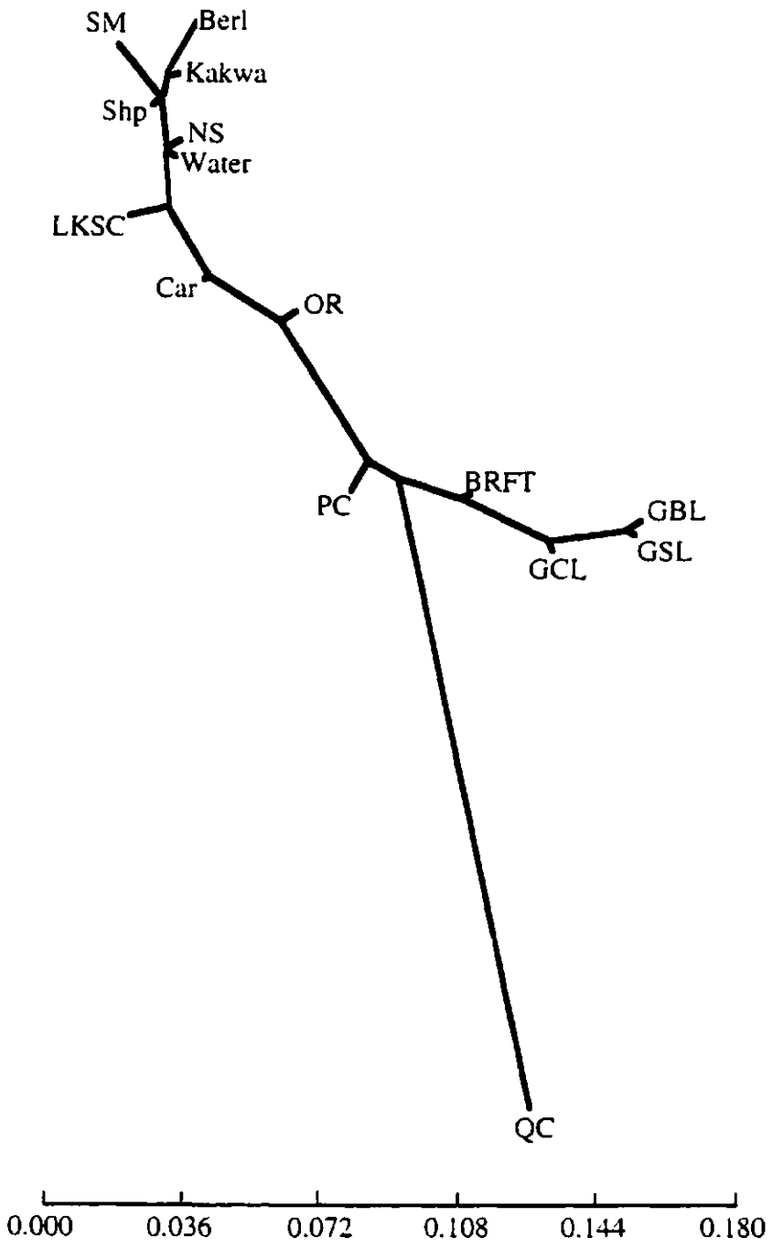


Figure 8. FITCH tree based on Nei's genetic distance values. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek (QC) which were brook trout (*Salvelinus fontinalis*). Populations were sampled in Alberta (Berl, Shp, Kakwa, SM, NS, Water, Car, LKSC, OR, PC, QC, BRFT) and Montana (GCL, GSL, GBL).

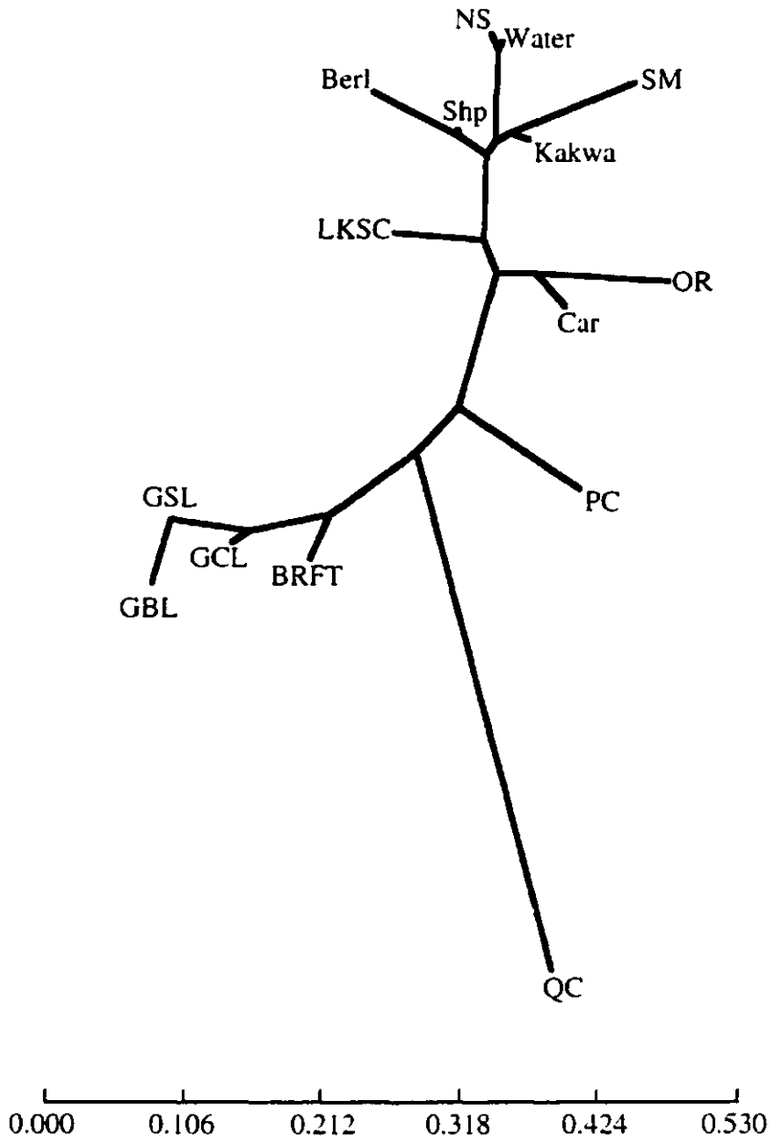


Figure 9. FITCH tree based on Cavalli-Sforza's genetic distance values. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek (QC) which were brook trout (*Salvelinus fontinalis*). Populations were sampled in Alberta (Berl, Shp, Kakwa, SM, NS, Water, Car, LKSC, OR, PC, QC, BRFT) and Montana (GCL, GSL, GBL).

With the exception of the Waterton region, the populations are placed on the trees in a manner which parallels their geographical distribution from north to south. This is especially apparent in Figure 9. Again, the Belly River and Glacier National Park regions form a relatively tight grouping. The Oldman and Carbondale groups remain in close proximity, as do the Kakwa and Simonette Rivers. Once more, the Waterton and North Saskatchewan regions are clustered. The position of the Prairie Creek fish changes little with either NJ or FITCH.

A final comparison was performed with the KITSCH tree building algorithm. This is a modified form of the Fitch-Margoliash algorithm and assumes that an evolutionary clock is in effect. The rate of evolutionary change along each branch of the tree is assumed to be constant and consequently, the branch lengths do not reflect actual genetic distance values. Instead, the branch lengths are constrained so that the total length from the root of the tree to each population is the same. This algorithm functions in a similar manner to Unweighted Pair Group Arithmetic Group Averaging (UPGMA), except that it is able to assess a large variety of alternative tree topologies and therefore may yield different and better results than simple sequential clustering (Felsenstein, 1993). The trees produced by KITSCH are most useful for representing phenetic clusters of the taxa at the tips of the branches.

KITSCH trees are shown in Figure 10 (Nei's distances) and Figure 11 (Cavalli-Sforza distances). There were 10814 and 10859 possible trees examined respectively before these "best" were selected. Close inspection of the branching patterns reveals that the two trees are identical, except for a slight change in the branch placement for the Berland River population. The same relationships shown in the NJ and FITCH trees are shown in the KITSCH trees as

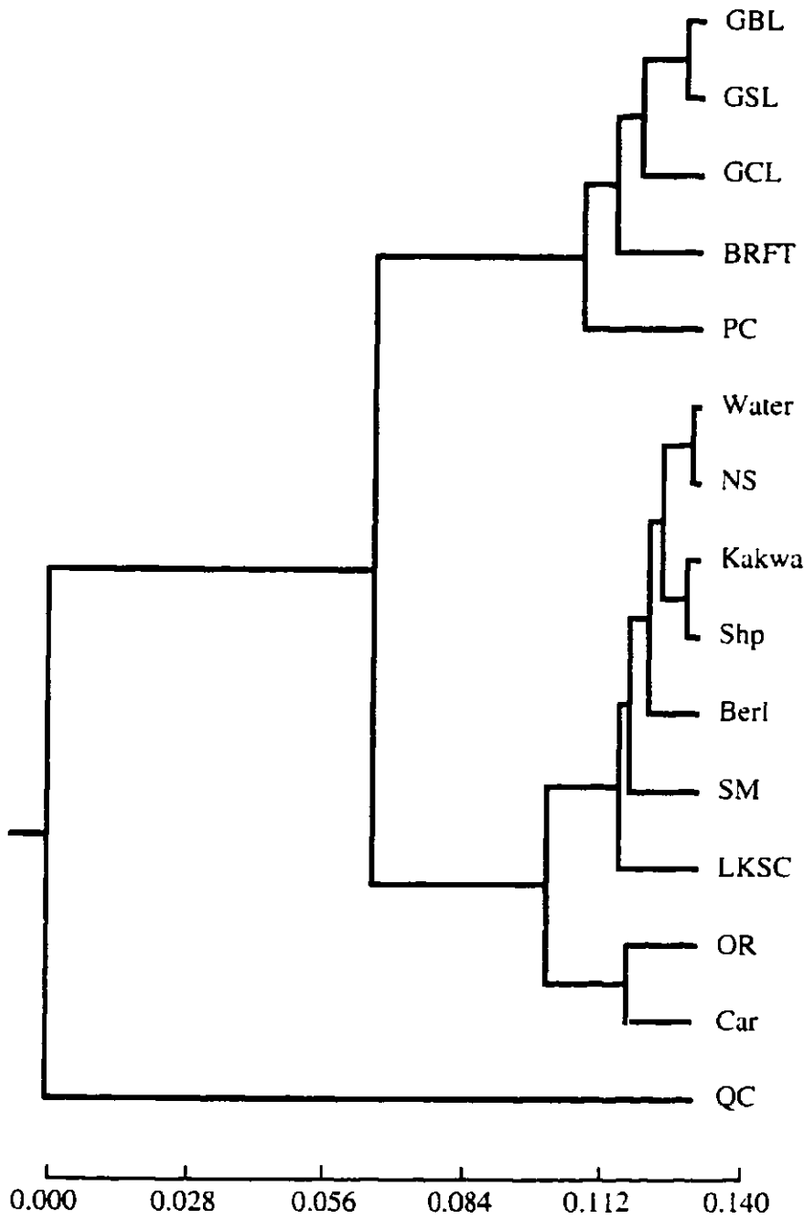


Figure 10. KITSCH tree based on Nei's genetic distance values. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek (QC) which were brook trout (*Salvelinus fontinalis*). Populations were sampled in Alberta (Berl, Shp, Kakwa, SM, NS, Water, Car, LKSC, OR, PC, QC, BRFT) and Montana (GCL, GSL, GBL).

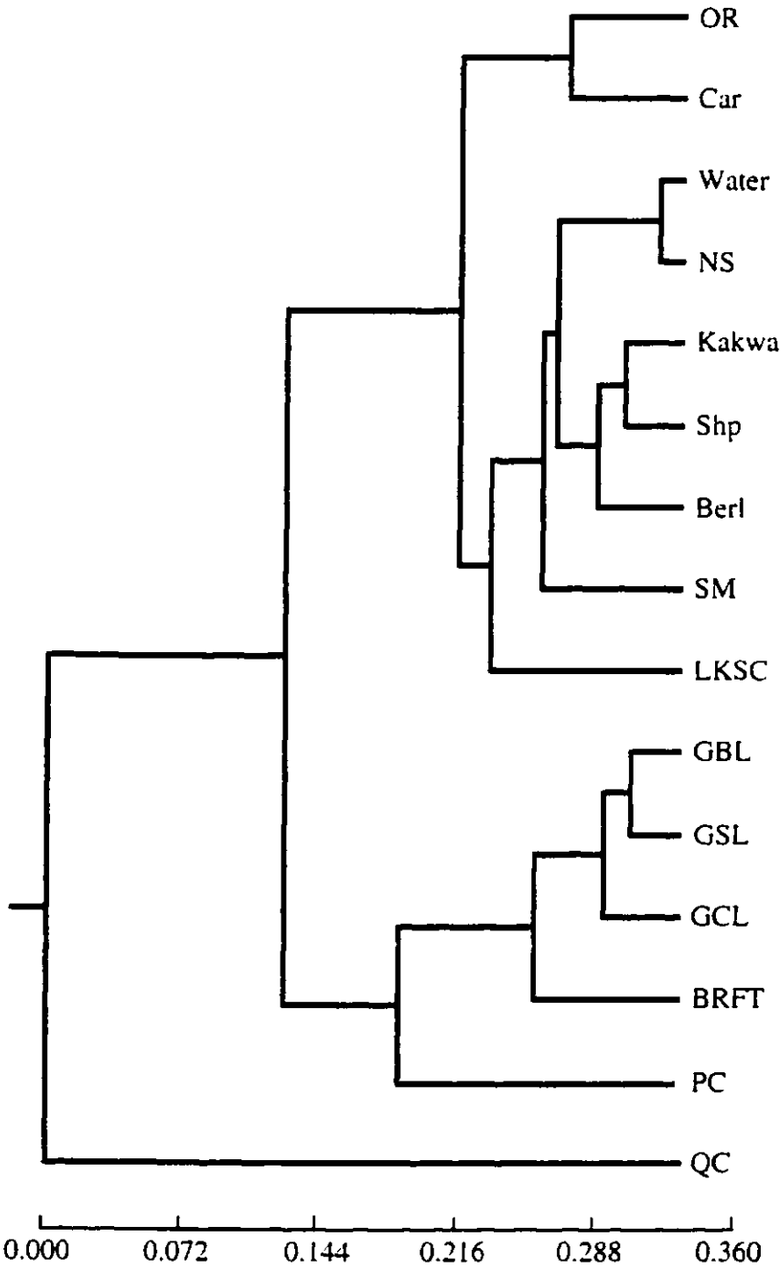


Figure 11. KITSCH tree based on Cavalli-Sforza's genetic distance values. All samples were bull trout (*Salvelinus confluentus*) except those from Quirk Creek (QC) which were brook trout (*Salvelinus fontinalis*). Populations were sampled in Alberta (Berl, Shp, Kakwa, SM, NS, Water, Car, LKSC, OR, PC, QC, BRFT) and Montana (GCL, GSL, GBL).

well. The Belly River and Glacier National Park regions form a cluster which includes the Prairie Creek region. The remaining Alberta regions form a separate grouping, within which the Oldman River and Carbondale region are paired. Again, the Waterton region is closely associated with the North Saskatchewan region.

5.5 Unanticipated results

5.5.1 The number of haplotypes

The use of fluorescent labelling and laser detection of DNA fragments resulted in an extremely sensitive detection method. The accuracy of assessing fragment sizes was further enhanced by the inclusion of internal lane standards. Previous studies have utilized ethidium bromide staining, transfer hybridization and autoradiography for visualization of DNA fragments but these methods are limited in their ability to detect small DNA fragments. Ethidium bromide staining intensity is proportional to DNA concentration and requires the presence of about 2 ng of DNA to be detectable (Dowling et al., 1996). Consequently, very small DNA fragments will not be observed unless large volumes of PCR product are loaded onto electrophoretic gels. Cronin et al. (1993) used this method to analyze restriction fragment polymorphism in mtDNA of chinook and chum salmon, however, they reported that DNA fragments smaller than 300 bp were not detected. Grewe et al. (1990) used autoradiography to study mtDNA divergence in the genus *Salvelinus* but they were unable to estimate the total mtDNA genome size with more accuracy than ± 200 bp. Hybridization had similar disadvantages in that detection of DNA smaller the 250 bp was considered

difficult (Dowling et al., 1996).

In addition to the problem of observing smaller DNA fragments, additional error may be introduced during the estimation of DNA fragment sizes. Usually, fragment migration distances are compared to size standards located in adjacent lanes. The accuracy of this method is affected both by the placement of a measuring device and the assumption that gel conditions are consistent in every lane. Error in measuring DNA fragments in this way may be approximately 5% (Davidson et al., 1989).

The use of fluorescent labelling and laser detection vastly increased both the accuracy of determining DNA fragment sizes and the ability to consistently detect small fragments. Consequently, greater levels of variation were detected than was previously possible. For example, small changes in fragment sizes such as 20 or 30 bp would be difficult to observe using traditional protocols yet such differences were easily recognized using the ABI Genescan system. In this study it was suspected that some mtDNA haplotype variation was due to the presence of restriction sites close to the ends of PCR amplified fragments, resulting in very small DNA fragments separating during digestions. Thus, two fish haplotypes may differ by only one fragment which appears slightly shorter in one fish than in the other. One may have a 168 bp fragment, and the other a 143 bp fragment. Even with high resolution agarose or polyacrylamide gels, such small differences are not measurable with any level of certainty. Thus, in previous studies, these fish would likely be considered as having the same haplotypes.

Consequently, use of the ABI Genescan system resulted in detection of far greater mtDNA variability than was initially expected. The selection of mtDNA primers was based

on previous studies indicating their utility in differentiating bull trout and other salmonids at the population level. However, their application with the ABI Genescan system resulted in the appearance of greater numbers of haplotypes than previously reported. Williams et al. (1995) recognized 21 composite haplotypes in 133 bull trout from 15 regions using 4 primer sets and 10 restriction enzymes. Most restriction enzymes they screened showed little or no polymorphism among fish populations. In comparison, this study analyzed 116 bull trout from 12 regions using 2 primer sets and 8 restriction enzymes. The results revealed the presence of 63 composite haplotypes, indicating far greater diversity both within and between populations than expected. Similar findings were reported by Vitic and Strobeck (1996) during analysis of mtDNA from lake trout (*S. naymaycush*). Initial RFLP analysis of the ATPase-6-COIII region resolved four mtDNA haplotypes but subsequent sequencing of the same PCR products revealed that seventeen haplotypes actually existed where only four were previously recognized. It seems plausible that many other mtDNA studies potentially underestimate the level of polymorphism present in their target populations due to insensitive detection protocols.

Most likely, the amount of polymorphism identified in Alberta bull trout populations is not greater than other populations, but the sensitivity of the detection methods revealed more information than previously attainable. The data indicate significant differences among the groups surveyed. It is clear that Alberta bull trout populations do not comprise a single common gene pool, although their common ancestry is apparent in the large number of similar haplotypes present in most regions.

5.5.2 Unamplified regions

Several regions were not included in the mtDNA analysis because the DNA would not amplify with ND1 or ND5/6 primers. The Belly River samples amplified most of the time, although a few did not and were omitted from the data set. The Sheep River samples amplified only when modified step-up PCR protocols were used. Some regions did not amplify under any conditions such as samples from the Highwood River, the Cardinal River, Smith-Dorian Creek, most of the Waterton fish, and most fish from the Glacier National Park region. Since these regions did amplify with the RAPD primers, the quality and purity of the DNA wasn't considered a problem. No evidence of contaminants was observed as positive and negative PCR controls provided expected outcomes. It seems likely that the ND1 and ND5/6 primer sites were more variable than first thought, and that the inability to amplify fish from some regions resulted from poor primer recognition sites. Williams et al. (1995) reached the same conclusion when one entire bull trout population in Montana failed to amplify. Cronin et al. (1993) also reported that ND1 regions in some chum salmon failed to amplify, but they did not speculate as to the reasons. These results suggest that more polymorphism exists among Alberta bull trout than was measured in this study. In the future, if these additional drainages are to be included in the mtDNA analysis, more highly conserved regions must be amplified, such as cytochrome *b* or ATPase regions, thus reducing the likelihood of DNA-primer mismatches.

5.5.3 Unexpected relationships among populations

Analysis of mtDNA and RAPD's indicated several surprising relationships. Most

notable was the Belly River population which was expected to be similar to the Oldman River group. Mitochondrial DNA analysis revealed significant population heterogeneity between the Belly River bull trout and the Oldman River fish. RAPD analysis confirmed this divergence and showed that the Belly River populations cluster with bull trout from the St. Mary River headwaters in Glacier National Park, Montana.

Another unexpected result was the placement of Waterton fish in relation to other populations. The close geographical proximity among the Waterton River, Belly River and Oldman River might suggest that these populations would be most similar to each other. However, RAPD data analysis revealed that Waterton bull trout did not group with populations from either geographical neighbor, but instead paired most closely with the North Saskatchewan population. An independent assessment of the status of Waterton bull trout using mtDNA was not possible because the mtDNA from the Waterton fish did not amplify with the ND1 and ND5/6 primers. Interestingly, the North Saskatchewan samples would not amplify with these primers either.

The Prairie Creek population appears distinct from other Alberta populations. Of interest is the suggestion that these fish form a resident population of bull trout, whereas all other samples are from fluvial populations. Mitochondrial DNA revealed that the Prairie Creek population showed significant heterogeneity in haplotypes when compared to all others included in the mtDNA analysis. The only exception was when the Prairie Creek fish were compared with the Sheep River fish, and no significant differences were found. RAPD analysis consistently placed the Prairie Creek population in the middle region of the distance trees, not closely grouped with any other populations. KITSCH trees suggest that these fish

have RAPD profiles more similar to the Belly River and U.S.A. fish than to other Alberta populations.

6.0 Conclusions and implications

6.1 Conclusions

Four main goals for this study were described at the outset including: (1) measurement of genetic divergence across major drainage systems in Alberta, (2) investigation into the extent of intradrainage genetic variation among Alberta bull trout, (3) detection of hybridization between Alberta bull trout and brook trout, and (4) establishment of baseline data for comparison to future genetic studies.

6.1.1 Goal one: Interdrainage comparisons

The first goal was to explore the level of genetic divergence across major drainage systems in Alberta. This goal was met with considerable success. Interdrainage comparisons of mtDNA revealed significant population heterogeneity among Alberta bull trout. Most regions contained one or more common haplotypes as well as several locally unique haplotypes, which is consistent with other fish species. The larger number of rare haplotypes were thought to be mutational derivatives of the common haplotypes (Billington and Hebert, 1991; Williams et al., 1996).

Further progress towards this goal requires analysis of samples from several regions not represented in this study. No fish tissue was collected from the Red Deer River drainage and samples from the Highwood River, Cardinal River, Waterton region and Smith-Dorian Creek would not amplify with the mtDNA primers. The samples sizes for the Waterton region, Oldman River and Red Eagle Lake were smaller than desired for the RAPD analysis.

All regions may have benefitted from larger sample sizes especially in view of the higher than expected levels of diversity.

6.1.2 Goal two: Intradrainage comparisons

The second goal of the study was to measure the extent of intradrainage variation. This goal was met with limited success. Time and funding constraints prevented extensive analysis of intradrainage population structure. Data were generated for several populations from the Oldman drainage including the Belly River, Carbondale River, and fish sampled directly from the Oldman River. The Belly River fish showed significant differences in heterogeneity from the Carbondale group ($p<0.05$) and the Oldman River group ($p = 0.05$). Several reasons may exist for the apparent divergence of the Belly River fish. Perhaps they have been reproductively isolated from other fish in this drainage for a long enough period that significant genetic differences have accumulated. Another possibility is that they experienced a bottleneck effect caused by a population decline severe enough to alter the nature of the gene pool.

Results from the RAPD analysis suggest another explanation for the genetic divergence displayed by bull trout in the Belly River. Belly River fish clustered with the St. Mary River bull trout which were sampled in Glacier National Park, Montana. It is possible that bull trout in the Belly and St. Mary Rivers are derived from a different ancestral population than those in other Alberta rivers. Nelson and Paetz (1992) review post-glacial dispersal routes of freshwater fishes in Alberta and describe two major refugia from which bull trout may have returned to Alberta streams following the last ice age. Both the Missouri

refugium in the east, and the Columbia refugium in the west were suggested as potential sources of contemporary bull trout populations. It was speculated that fish from the Missouri refugium could have gained access to Alberta streams via the Milk River and St. Mary River systems. Alternatively, fish from the Columbia refugium may have crossed into the east slope streams of Alberta through a Kootenay-Bow or Kootenay-Oldman access, or through headwater capture via a Flathead-St. Mary crossing. RAPD analysis suggests that two distinct groups of Alberta bull trout may exist, including the Belly River-St. Mary River group which may stem from the Missouri refugium, and the remaining Alberta populations which may have come from the Columbia refugium. Such genetic divergence has been previously reported in pike where stocks from two distinct refugia are believed to have invaded the same post-glacial regions however they still maintain considerable genetic differences (Pielou, 1991). A more complete comparison of Alberta bull trout populations to those in British Columbia populations may provide additional evidence in this regard.

The Waterton population seems to contradict the possibility that streams with Montana headwaters contain bull trout from the Missouri refugium. The headwaters of the Waterton River are located in Glacier National Park and are very close to the Belly River headwaters. In spite of the close geographical relationship, the Waterton sample did not cluster with Belly River and St. Mary River fish. One explanation may be that populations downstream from Waterton Lakes are different than upstream populations. The Waterton populations in this study were collected from Blakiston and Yarrow Creeks, both of which are downstream from Waterton Lakes, and both of which flow in an easterly direction. These streams may have been stocked from the Columbia refugium along with other sections of the

Oldman drainage, whereas bull trout in the headwater regions of the Waterton River may have been stocked from the Missouri refugium. An analysis of genetic profiles of bull trout from the Waterton River in Glacier National Park, Montana, is required to shed further light on this discrepancy.

Another explanation for the presence of two distinct groups of bull trout in Alberta may relate to specific adaptations to local habitat. The Glacier National Park region of Montana is characterized geologically by the presence of mudstone. In contrast, most of the Alberta streams are located in regions where the predominant lithological features are carbonic in nature (Dr. R. Barendregt, personal communication, April 3, 1997). Consequently, streams in these two regions may differ chemically in both the composition of minerals and solutes, and pH. Fish populations may be specifically adapted to the conditions of their local habitat. As a result, two divergent groups may have evolved in response to the selection pressures created by the two distinct lithologies.

The Oldman and Carbondale River fish did not show significant population heterogeneity but did display 0.55% mtDNA sequence divergence and the Carbondale group contained some unique haplotypes. Intrapopulation structuring may explain these results since the Carbondale fish display some level of uniqueness which partially separates them from the Oldman River fish. Fish sampled in the Oldman River probably represent a mixture of separate breeding populations which have not yet returned to their spawning headwaters. Resident populations of bull trout in the Carbondale River have not been previously reported, but the absence of unique Carbondale haplotypes in the Oldman group may suggest that they exist. Further sampling of additional headwater regions is required to better establish the

possibility of such intrapopulation structuring in the Oldman River drainage.

The Kakwa and Simonette Rivers belong to the same drainage and significant population heterogeneity was detected between them. Mitochondrial DNA sequence divergence was measured at 0.56% and several unique haplotypes were identified, especially in the Simonette River. Thus, strong evidence exists for the presence of intradrainage population structuring in this region and further study is recommended.

It was not possible to gather evidence for intradrainage population structuring in several drainages due to a lack of samples. Fish tissue collected from the Athabasca drainage all came from the Berland River. These showed significant differences with most regions outside of the drainage, but no other samples from within the Athabasca system were available for intradrainage comparisons. A similar situation existed for the North Saskatchewan drainage where only one sample site was used. Further, these samples failed to amplify with the mtDNA primers and therefore were omitted from interdrainage comparisons as well.

The Sheep River was considered a logical alternative for representing fish populations in the upper reaches of the Bow River drainage once it became apparent that the Highwood River samples would not amplify. Sheep samples did not display significant heterogeneity from most regions, probably due to the large number of haplotypes identified in this region. Many of the haplotypes were common to other areas which explains the reduced heterogeneity levels. Though the Sheep River displayed one of the highest numbers of unique haplotypes, little evidence of intradrainage population structuring was apparent for the upper Bow region. The Sheep samples could only be compared with the Prairie Creek samples and

significant population heterogeneity did not exist ($p > 0.05$) when tested with Monte Carlo simulations using REAP software. Sequence divergence between these two groups was low at 0.22%.

6.1.3 Goal three: Detection of hybridization

A third goal of the study was to identify hybridization between bull and brook trout. It was thought that mtDNA profiles found in brook trout populations would appear in some of the bull trout or visa versa. There were no such occurrences in this study, initially suggesting that hybridization between the two species does not occur with any significant frequency. Initial planning for achieving this goal was based on an expectation that lower levels of variability would be detected. Consequently, brook trout samples were collected only from one region. In retrospect, it seems that bull trout and brook trout must be sampled from the same location to identify hybridization with this approach. Only hybrids with very common haplotypes had any reasonable chance of being detected with the sampling method used in this study. Like bull trout, there is empirical evidence that brook trout also contain high levels of interdrainage diversity (Perkins et al., 1993). Consequently, some of the unique bull trout haplotypes identified in this study may be partial brook trout profiles, but no brook trout from those regions were collected to verify such an occurrence.

6.1.4 Goal four: Baseline data

The fourth goal of the study was to establish base line data for Alberta bull trout populations. A large amount of data were generated although deficiencies exist. The

fluorescent detection system increased sensitivity resulting in a greater resolution of haplotypes. Several common haplotypes and many more unique haplotypes were identified. Larger sample sizes would facilitate a more detailed characterization of each population by increasing the certainty that all haplotypes were detected.

Sufficient baseline data were generated for the establishment of future temporal studies. RAPD data have provided regional allele frequency values which may be reassessed over time. Future studies should focus on the stability of these frequencies and explore correlations with population recovery or decline.

In summary, this study has provided a first glimpse at Alberta bull trout population genetics. Although additional investigation is required, it is now clear that significant interdrainage population heterogeneity exists among Alberta bull trout. Also evident is the presence of two genetically distinct groups of bull trout which may be the result of post-glacial recolonization from different refugia. There is some indication that intradrainage population structuring exists within some drainages. The study provides a substantial amount of baseline data upon which several future investigations should be designed.

6.2 Implications

The results presented here should be useful in the development and refinement of future bull trout management strategies. The conservation of bull trout has become an important goal for wildlife officials, conservation organizations, and private individuals. Within the context of wildlife management and conservation it is necessary to consider genetic conservation. The goal of fisheries management should be to maintain genetic resources

without avoidable and irreversible losses of genetic diversity (Ryman, 1991). Several issues arise from this research which should be considered for future fish management strategies. First, there is considerable interdrainage diversity among Alberta bull trout populations. Most fish in the eastern slope streams were likely isolated from common ancestors 10000-15000 years ago, as glaciers of the last ice age receded (Pielou, 1991). Species with formerly continuous spatial distribution may become metapopulations through such habitat fragmentation (Hanski and Gilpin, 1991). The isolation of ancestral bull trout to distinct drainages has resulted in their geographical separation for thousands of years. Historical patterns of drainage isolation are known to result in geographically concordant genetic differences (Avise, 1989). Such differences stem from the effects of population bottlenecks, stochastic events, and selective processes resulting in locally adapted demes. Alberta bull trout share a number of common haplotypes which suggests recent separation from a common ancestor (Billington and Hebert, 1991). However, most regions contain locally unique haplotypes not found in other drainages. If the genetic diversity of locally native populations is to be preserved, then future management strategies must recognize the interdrainage divergence present among Alberta bull trout populations.

A second issue relevant to future management strategies is the potential for intradrainage population structuring. Metapopulations are described as a population of populations occurring in a range of environments, such that the loss of all populations at any one time is less likely (Hanski and Gilpin, 1991). Dispersal of members from local populations provides a mechanism for supporting weaker populations, or for refounding those which do become locally extinct. The distribution and hypothetical structuring of most bull

trout populations are consistent with the metapopulation concept (Rieman and McIntyre, 1993; Rieman and McIntyre, 1995). Bull trout within a single drainage may belong to separate breeding populations which either remain as upstream residents or return seasonally to spawn in the same headwater regions. The importance of this possibility is two-fold. The likelihood of local extinctions is reduced by the maintenance of maximum levels of diversity within the drainage, and even if the best habitats are preserved, the migratory corridors which connect upstream populations must be maintained to facilitate the rescue effects which occur with local extinctions. Locally reduced populations can be replenished (Hanski and Gilpin, 1991) or continually supplied (Stacey and Taper, 1992) with enough migrants to reestablish or maintain the local deme. In this context, conservation management should favor the maintenance of strong local populations in close proximity to one another to facilitate dispersal and demographic support (Rieman and McIntyre, 1996). It was suggested that if all populations in a metapopulation were to decline or expand simultaneously, the entire metapopulation may go extinct (Stacey and Taper, 1992). Ideally, management strategies will recognize the concept of locally adapted demes within a single drainage system and facilitate the maintenance of strong local populations as well the maintenance of migratory corridors. This should include the recognition that isolated headwater residential populations may represent important sources of genetic diversity for other members of the metapopulation network (Northcote, 1992). In Alberta, further genetic data are required to better characterize the extent of intradrainage population structuring within each major drainage system. Until such time, defining the boundaries of management units will be difficult.

The development of conservation strategies for declining populations inevitably raises

the topic of supplementing wild populations with hatchery stock. Stocking programs may have deleterious effects on the diversity of local gene pools, particularly where distinct population structuring exists. Natural populations possess a propensity for localized, and often subtle, adaptive differentiation in response to fine-scale environmental heterogeneity. There is a danger of destroying local gene pool integrity through the introduction of cultured fish which may be genetically homogeneous (Carvalho and Hauser, 1994). Such an occurrence has been previously documented in lake trout (Evans and Willox, 1991) and Japanese ayu, *Plecoglossus altivelis* (Pastene et al., 1991). Problems arise when genetic differences among hatchery and wild populations are not explored prior to introductions. Hatchery populations have been shown to possess considerably less mtDNA haplotype diversity than natural populations (Danzmann et al., 1993), although this is not always the case (Nielsen et al., 1994). Important differences in behavioral traits may exist between hatchery and native populations, such as the onset of spawning behavior (Danzmann et al., 1994). Clearly, such differences would be subtle and difficult to detect without extensive genetic and behavioral analysis of both native and hatchery populations, thus making it nearly impossible to predict the extent of introgression which may occur between the native and introduced fish. In some cases introgression has been documented (McCracken et al., 1993; Williams et al., 1996) whereas other studies have shown low levels of introgression (Danzmann et al., 1991). In view of these difficulties, the prospect of supplementing with hatchery fish has been highly criticized (Ferguson, 1990), and several genetic studies have led to the recommendation that stocking programs be discontinued or avoided (Dowling and Child, 1992; Kircheis et al., 1995; Krueger et al., 1994). Even so, successful hatchery

programs have been implemented (Ferguson et al., 1991; Grewe et al., 1994). Bull trout hatchery programs were previously recommended as a promising management option in the upper Flathead drainage (Fraley et al., 1989), however the idea was subsequently discouraged in favor of habitat improvement and more restrictive regulations (Leary et al., 1993). Hatcheries may play an essential role in recovery programs but only if appropriate genetic and behavioral information is attained prior to their implementation. In view of the interdrainage diversity now recognized among Alberta bull trout populations, extreme caution is recommended in any supplementation efforts. Not only should further genetic studies on native populations be completed but genetically appropriate hatchery strains must be established for each region where supplementation would occur. For example, brood stock for hatchery strains may be obtained from wild-caught fry taken from the same stream which will be supplemented at a later date (Marsden et al., 1993). Alternatively, eggs may be collected and reared in the same manner.

6.3 Recommendations

A number of recommendations arise from this research, some of which relate to methodologies and others which point to future research directions.

Methodologies should be altered in consideration of the large number of haplotypes resolved for each of the regions. Sample sizes should be increased to ensure that all common haplotypes and most rare haplotypes are identified. In addition, repeat sampling of at least some localities would permit direct assessment of sampling variance in allele frequencies or to detect artifacts which may arise from nonrandom sampling of the gene pool (Baverstock

and Moritz, 1996).

Also, the use of more highly conserved primer regions is suggested because the amount of variability detected with ND1 and ND5/6 primers was much higher than expected and many regions did not amplify with these primers. The use of cytochrome *b* or the ATPase III region of the mitochondrial genome may facilitate wider success in the PCR amplification of mtDNA regions. Thus, areas such as the Highwood River and Smith-Dorian Creek could be included in the mtDNA analysis. The alternative to using different primer sets would be to sequence the DNA regions in question and perhaps determine a bull trout specific primer sequence. Unfortunately, this would be time consuming and expensive, and it does not guarantee that all populations contain fish that could be amplified with the newly developed primer.

Future attempts to explore the extent of hybridization between bull trout and brook trout should include sampling of both species in the same region. There was considerable interdrainage diversity in mtDNA haplotypes of bull trout and brook trout profiles would likely be as divergent. On this basis, the chance of detecting hybrids would be greatly enhanced if bull trout and brook trout profiles from the same drainage were analyzed.

Several future studies logically spring from the data accumulated in this investigation. Interdrainage genetic differences should be explored further, especially including additional drainages such as the Red Deer River region. Further insight into differences between the Belly River group and the rest of the Alberta populations could be gained by more extensive sampling of the southern Alberta streams. It is recommended that specific comparisons be made between bull trout upstream and downstream of the Waterton Lakes.

More extensive analysis of spawning populations in upstream regions of each drainage would provide a clearer picture of the breeding relationships among bull trout within the same drainage. As a consequence, it may be possible to identify precise management units within the drainage. Management units are populations that exchange so few migrants as to be genetically distinct (Moritz, 1994). They are best defined by significant divergence in allele frequencies and mtDNA are especially useful for detecting the boundaries between them. In depth intradrainage analyses could determine if distinct management units exist within Alberta drainage systems, thus allowing the development of management strategies designed to enhance their preservation. The study design should include tissue sampling from headwater regions during spawning and from the downstream habitats before or after the spawning run. Genetic profiles of the individual spawning populations could be compared with the larger downstream population. The presence of resident populations may be revealed in this way, since their unique haplotypes would never appear in the downstream sections of the river.

Another direction for future research involves the addition of a temporal component. Resampling from the same fish populations over several years will provide a measure of the genetic stability of each population. Shifts in allele frequencies may be correlated with drastic increases or declines in population levels. Also, the relative stability of regional gene pools could be assessed. For example, it is not known if bull trout populations in southern streams such as the Belly River are genetically stable or if regions such as the Oldman River drainage are experiencing more rapid genetic changes than the Peace River drainage. Temporal studies are necessary to establish data on regional genetic stability, to correlate rates of genetic change with population declines, and to allow monitoring of populations as an indicator of

the effects of various management strategies.

6.4 Summary

The bull trout populations in Alberta displayed considerable interdrainage variation. Most regions revealed a few common haplotypes and a larger number of rare haplotypes. Significant population heterogeneity exists across the province. Pairwise comparisons of percent sequence divergence in mtDNA and genetic distance values from RAPD profiles both characterize the extent to which differences exist in the populations sampled. Evidence of intradrainage population structuring was sparse, mainly due to a lack of multiple sample sites within a single drainage. The exception was the Peace River drainage for which samples were analyzed from the Kakwa and Simonette Rivers. Significant differences in the genetic profiles of these populations suggest population structuring and more extensive intradrainage sampling is recommended. No evidence of bull trout-brook trout hybridization was observed but sampling methods were inadequate for comprehensive testing in this area. Sufficient data now exist for expansion of the study to include a temporal component allowing assessment of regional gene pool stability.

It is hoped that genetic data be considered in the development of future management and conservation strategies. The preservation of regional diversity is vital if local extinction of native populations is to be avoided. Further studies of population structure, and assessment of regional gene pool stability will provide a clearer understanding of the nature of bull trout genetics in Alberta streams.

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