Molecular analysis of hybridization between native westslope cutthroat trout (*Oncorhynchus clarki lewisi*) and introduced rainbow trout (*O.mykiss*) in southeastern British Columbia

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ABSTRACT

Westslope cutthroat trout (*Oncorhynchus clarki lewisi*, WCT) and introduced rainbow trout (*O. mykiss*, RBT) readily hybridize and introgression has occurred in many drainages across the historic range of WCT. In British Columbia, the upper Kootenay River drainage is the heart of WCT distribution and is thought to be a refuge for genetically pure populations. In this study, I assess the extent and distribution of WCT x RBT hybridization in the upper Kootenay River drainage, examine the genotypic structure of hybridizing populations using population genetic analyses, and examine the potential for differential selection between pure WCT and hybrid individuals using cohort analysis. Caudal fin clips were collected from 981 fish at 23 sample sites in 12 different streams in the upper Kootenay River drainage. I used 4 diagnostic nuclear loci to determine the extent of hybridization at each sample site. Fourteen percent (142/981) of individuals were identified as hybrids, 3.4% (33/981) were identified as pure RBT, and the remaining individuals were identified as pure WCT. Mitochondrial DNA analysis indicated that hybrid matings occur between males and females of both species. Although pure RBT were absent from the majority of sites (20/23), I found evidence of hybridization at 78% (18/23) of samples sites and the percentage of heterospecific alleles (*% I*) ranged from 0.7-97.1%. Only 22% (5/23) of sample sites showed no evidence of hybridization. The majority of hybrid individuals were genotypically classified as WCT backcrosses (59%) and post-F_2 individuals (24%). The skewed ratio of pure WCT to RBT (27:1), and the rarity of F_1 individuals (4 of 142 hybrids), suggests that the spread of RBT alleles is facilitated by hybrids straying to neighbouring populations. Spatial analysis showed clustering among hybridized sites and decreasing hybridization with increasing distance from Koocanusa Reservoir, suggesting that the reservoir acts as a RBT source. I found little evidence of differential selection between pure WCT and hybrid individuals. My results suggest that hybridization is relatively recent in the upper Kootenay River drainage and that it is increasing in magnitude and distribution. In the absence of timely management intervention, the genetic integrity of WCT populations in the heart of their Canadian range may be lost.

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Chapter 1. General Introduction

Natural hybridization plays an important role in the speciation and adaptation of plants and animals (Reiseberg 1998), but it has also led to numerous extinctions of native flora and fauna (Rhymer and Simberloff 1996). Anthropogenic translocations of organisms and habitat modifications have increased rates of natural hybridization and introgression worldwide (Allendorf et al. 2001). Introgression, the accumulation of the alleles of one species into the genome of another, can occur to varying degrees within hybrid zones. There are many examples of introgression occurring in natural stable hybrid zones where the parental species remain distinct (reviewed in Barton and Hewitt 1985), and other examples where unnatural secondary contact (i.e. exotic species introduction) has resulted in a loss of biodiversity where two species merge into one hybrid species (i.e. hybrid swarm, reviewed in Rhymer and Simberloff 1996).

Hybridization in Freshwater Fish

Fish are known to hybridize more than any other vertebrate group (Hubbs 1955; Verspoor and Hammer 1991; Avise 1994). The common occurrence of hybridization in fish species is thought to be due to external fertilization and the often ephemeral nature of their habitat increasing the chance of secondary contact (Turner 2000). This is especially important in freshwater habitats where past glaciations, deglaciation and associated landmass movements provided routes of secondary contact for previously isolated populations (Hewitt 2000). The probability of secondary contact between closely related fish species has been increased by anthropogenic manipulation of fish habitats such as habitat degradation and introduction of non-native species (reviewed in Rhymer and Simberloff 1996).

Accidental and/or intentional introductions of non-native species for sport or commercial fishing or biological control are the greatest contributors to natural hybridization in fish (Leary et al. 1987a; Allendorf and Leary 1988). Salmonid fishes are among the most widely introduced group, primarily to establish populations for recreational angling (Fausch et al. 2001). Within this group, rainbow trout (*Oncorhynchus mykiss,* RBT) have been introduced into more areas than any other fish species (Welcomme 1992). They are native to western North America and northeastern Siberia but are now found across North America and on all continents but Antarctica.

Rainbow trout introductions have had a detrimental effect on the various subspecies of inland cutthroat trout (*Oncorhynchus clarki* spp) in western North America due to their tendency to hybridize with this closely related species (Busack and Gall 1981; Leary et al. 1984; Dowling and Childs 1992; Carmichael et al. 1993; Campbell et al. 2002; Hitt 2002). There have been drastic declines in inland cutthroat trout populations in the last century due to a number of factors including habitat loss and degradation, overexploitation, and competition with and predation by non-native species (Liknes and Graham 1988; Shepard et al. 1997) The single greatest threat to remaining westslope cutthroat trout populations, however, is introgressive hybridization with introduced rainbow trout (Allendorf and Leary 1988).

Study Taxa

The cutthroat trout, *Oncorhynchus clarki* (Richardson) is native to western North America. This species is arguably the most polytypic salmonid in the region, with up to 16 subspecies identified (Allendorf and Leary 1988; Behnke 1988). One of these subspecies, the westslope cutthroat trout (*O. clarki lewisi* Suckley), is native to tributaries of the upper Columbia, Snake, South Saskatchewan and Missouri rivers, in an area encompassing southeastern British Columbia, southwestern Alberta, and south of the international border in Idaho and Montana (Figure 1.1). There are also disjunct populations in Washington State and the South Thompson River (Fraser River drainage), the Columbia River, and the Kettle River, in British Columbia. Widespread stocking of non-native trout (mainly rainbow trout and Yellowstone cutthroat trout, *O. clarki bouveri*) has resulted in introgressive hybridization with westslope cutthroat trout (WCT) throughout its range in the United States and Alberta (Leary et al. 1984; Gyllensten et al. 1985; Leary et al. 1987a; Allendorf and Leary 1988; Carmichael et al. 1993; Mayhood 1999; Rubidge et al 2001; Hitt 2002). For example, Montana was once the heart of the distribution of *O. clarki lewisi*, but now it is left with pure populations in only an estimated 3% of its historic range (Liknes and Graham 1988). Because inland cutthroat trout subspecies evolved predominantly in the absence of RBT (Behnke 1992) there appears to be little impediment to introgression and hybrid swarms are often formed (e.g. Busack and Gall 1981; Leary et al. 1984; Carmichael et al. 1993; Leary et al. 1995; Hitt 2002).

Rainbow trout are native to the northwestern North America but have been extensively introduced outside their native range. Behnke (1992) suggested that no RBT are indigenous to sections of the Kootenay, Clark Fork, Spokane and Snake rivers above major barrier falls. The presence of WCT above these barrier falls is indicative of their early dispersal pattern (Liknes and Graham 1988). Westslope cutthroat trout were able to penetrate the upper Columbia River basin and the upper Kootenay River before barrier falls formed and were isolated in these areas from other species such as RBT. Behnke (1992) also suggested that WCT populations above and below falls on the Kootenay, Clark Fork and Spokane drainages were geographically isolated from RBT since at least the last discharge of glacial Lake Missoula 12,000-15,000 years ago, until RBT were introduced at the beginning of the twentieth century. There are, however, small areas where WCT and RBT are naturally sympatric (Figure 1.1). Coexistence between native WCT and native RBT has persisted in the John Day River drainage in Oregon and in the Salmon and Clearwater drainages of Idaho (Behnke 1992). Mechanisms that limit hybridization between these species in areas of natural sympatry are thought to be aggressive spawning behaviour and spatial and temporal separation of spawning sites (Liknes and Graham 1988).

Overview of Species Evolution and Life-history

Cutthroat trout and rainbow trout are sister taxa, and both exhibit high within species variation with many subspecies recognized (Behnke 1992). Cutthroat trout and rainbow trout diverged from a common ancestor at least one million years ago (Behnke 1992). Subsequently, the cutthroat trout lineage diverged into three main evolutionary branches about 900,000 years ago: the coastal (68 chromosomes), westslope (66) and Yellowstone (64) subspecies. The divergence of these three subspecies is speculated to have occurred in association with the formation of the Columbia River basin (Behnke 1992). From the Columbia basin, the coastal cutthroat spread south to California and north to Prince William Sound, the westslope cutthroat extended east across the continental divide to the South Saskatchewan and upper Missouri drainages, and the Yellowstone cutthroat trout extended southeast from the upper Snake River to the Yellowstone River drainage.

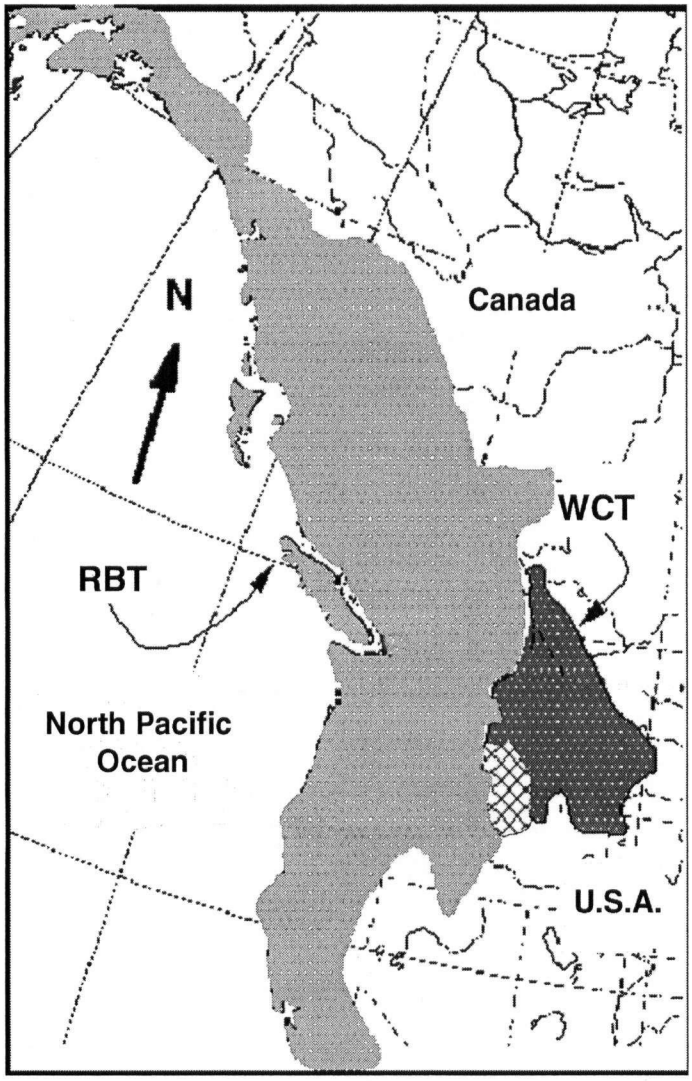


Figure 1.1 Native distribution of rainbow trout (RBT, light gray) and westslope cutthroat trout (WCT, dark gray) in North America. Area of possible natural sympatry is shown by crosshatching.

These three main lineages within the cutthroat trout complex are genetically distinct. Based on allozyme data, westslope cutthroat trout are more similar to rainbow trout (Nei's D=0.130) than they are to the coastal cutthroat trout (Nei's D= 0.164) and Yellowstone cutthroat trout (Nei's D =0.295) suggesting that cutthroat may be polyphyletic (Allendorf and Leary 1988). Morphological (Behnke 1992), karyotypic (Thorgaard 1983), and mitochondrial DNA (Gyllensten et al. 1985) analysis, however, all suggest that all cutthroat subspecies are more similar to each other than they are to rainbow trout. One explanation for this discrepancy between the allozyme, mitochondrial and morphological characters is that the evolutionary divergence of the protein loci and these other characters, have proceeded at different rates among subspecies (Allendorf and Leary 1988). Historical introgression between WCT and RBT has been suggested to account for the sharing of alleles between WCT and RBT (Leary et al. 1987b).

In contrast to other subspecies of the cutthroat trout, WCT do not appear to be highly predacious on other fish; their diet mainly consists of invertebrates (Liknes and Graham 1988). Behnke (1992) attributes the weak development of piscivory by WCT to the coevolution of this trout with two-fish eating species, the bull trout (*Salvelinus confluentus*) and the northern pikeminnow (*Ptychocheilus oregonensis*). WCT may have evolved as an insectivorous feeding specialist owing to trophic competition with these specialist piscivores. The introduction of RBT trout, also insectivorous in streams, has not only been detrimental to WCT due to hybridization, but has also introduced a strong competitor for food resources.

Westslope cutthroat trout exhibit three different life history forms: a lacustrine-adfluvial form, which migrates between lakes and streams, an adfluvial form, which migrates between small tributaries and rivers, and a fluvial form, which remains a resident of tributaries (Liknes and Graham 1988). Timing of spawning activity is dependent on water temperature. Adults generally migrate into tributaries during high stream flows and spawn between March and July (reviewed in Liknes and Graham 1988) when water temperatures are near 10°C (Scott and Crossman 1973). Spawning adults of naturally sympatric coastal cutthroat trout (CCT) and rainbow trout are generally separated spatially and temporally (Trotter 1987) preventing a high frequency of interspecific fertilizations. The spawning behaviour of WCT and introduced RBT has not been

intensively studied, but it appears that there is little pre-mating isolation that would limit hybridization.

Hybridization in British Columbia

The upper Kootenay River drainage in the southeast corner of British Columbia is thought to be one of the last drainages where genetically pure WCT populations predominate. Rainbow trout have been introduced into this drainage for at least the last 80 years (BC Ministry of Land, Water and Air Protection stocking records, unpublished data), but there has been very little study on the effects of these introductions on the native trout populations. The first genetic evidence of hybridization between native WCT and introduced RBT in British Columbia was presented in 1987 when Leary et al. (1987a) reported approximately 5% RBT introgression in one tributary of the upper Kootenay River using six allozyme markers; no evidence of hybridization was found at the other nine river systems in their study. Although RBT introductions have continued in the upper Kootenay River drainage since 1987, there has not been an extensive study on the hybridization issue. Currently, there is no information about the geographic extent of hybridization and the potential role of the environment in limiting hybridization in this drainage. This type of information is imperative to the future management and protection of this native subspecies.

The main objectives of my thesis are twofold. The first is to investigate the extent and distribution of hybridization in the upper Kootenay River drainage (Chapter 2). The focus of this objective is to identify where "pure" and "hybridized" populations exist and also to examine patterns in the distribution of these populations in relation to environmental parameters. The second main objective is to determine the structure of the hybrid zones in more detail (Chapter 3). The genotypic structure of the populations found to be of mixed descent in Chapter 2, are examined using population genetic analyses to determine if hybrid swarms (i.e. a unimodal distribution where hybrid individuals predominate and pure parental types are rare) have formed or are likely to form in the upper Kootenay River drainage. In Chapter 3, I also examine the potential for differential selection between hybrids and parental types within the St. Mary River, a large tributary of the upper Kootenay River. I conclude with Chapter 4 where I offer a general discussion on the findings of my thesis as well as future directions of research on this subject.

Chapter 2: The extent and distribution of hybridization

Introduction

Compared to the commonly cited warnings of imminent mass extinctions in terrestrial habitats, relatively little attention has been given to species loss in freshwater ecosystems (Ricciardi and Rasmussen 1999) despite several studies that demonstrate a growing number of freshwater extinctions (e.g., Miller et al. 1989; Williams et al. 1993). Freshwater fish are likely the most threatened group of vertebrates on the planet after amphibians (Bruton 1995). British Columbia (BC) is home to a diverse and unique freshwater fish fauna. The complex glacial history of this province created a number of novel evolutionary opportunities for ancestral fish immigrating from the ocean and the Mississippi, Yukon, and Columbia river systems (McPhail and Carveth 1992). Approximately 35% of the 83 recognized freshwater species in BC are either red (critically imperilled) or blue (vulnerable) listed provincially (BC Conservation Data Centre). One of these blue-listed species is the westslope cutthroat trout (*Oncorhynchus clarki lewisi*).

Westlope cutthroat trout (WCT) are one of two major subspecies of cutthroat trout native to British Columbia and the only native *Oncorhynchus* species in the extreme southeastern corner of the province. WCT are native both east and west of the Rocky Mountains in southeastern British Columbia, southwestern Alberta, throughout Montana, and northern Idaho (Figure 1.1). There are also disjunct populations in Washington State and the South Thompson River, Columbia River and Kettle River in British Columbia. Over the past decade, there have been significant declines in WCT populations throughout their historic distribution due to several factors, including: habitat loss and degradation, overexploitation, competition and predation by non-native salmonids, and introgressive hybridization with introduced rainbow trout (RBT, *O. mykiss*) and Yellowstone cutthroat trout (YCT, *O. clarki bouvieri* ; Allendorf and Leary 1988, Liknes and Graham 1988, Shepard et al. 1997).

In British Columbia, the upper Kootenay River drainage (Figure 2.1) is the heart of WCT distribution and is thought to be a refuge for genetically pure populations. Rainbow trout, native to many of BC's drainages, are non-native to the upper Kootenay

River drainage but have been introduced repeatedly over the last 80 years (BC Ministry of Water, Land and Air Protection (MWLAP), stocking records unpublished data). The recorded introductions of rainbow trout range in location from lower elevation tributaries of the upper Kootenay River and Koocanusa Reservoir (formed by the dam on the Kootenai River at Libby, Montana), to high elevation lakes including many mountain lakes (BC MWLAP stocking records, unpublished data) that lie upstream of Pleistocene-age barriers to fish colonization and that were naturally fishless (Bahls 1992). These stocking patterns leave two possible sources of RBT (and thus hybridization) in the upper Kootenay River: a main downstream source from the Koocanusa Reservoir, or upstream sources from multiple headwater lakes.

The first record of rainbow trout stocking in the upper Kootenay River drainage occurred in 1915 when 10,000 rainbow trout fry were introduced to Loon Lake (BC MWLAP stocking records, unpublished data). Since then, at least 3,000,000 RBT have been introduced into over 50 small lakes in this drainage (Figure 2.2). This is a conservative estimate because it represents only recorded stocking events while many introductions may go undocumented. One of the more recent focuses of RBT stocking programs is the Koocanusa Reservoir (Lake Koocanusa in U.S.A). Koocanusa Reservoir is a human-made lake, formed behind Libby Dam, that spans the international border between BC and Montana. Creating and maintaining a RBT sport fishery in this lake has been a goal of both the Montana and British Columbia governments since the mid-eighties. In British Columbia, from 1986-1998, 5,000 rainbow trout (Gerrard stock) were released each year into an inlet stream of Koocanusa Reservoir in the hopes of developing a RBT sport fishery in the reservoir (B. Westover, BC MWLAP, Cranbrook, BC, pers. comm. 2003). This stocking program has recently ended, in response to concern surrounding the hybridization issue (B. Westover, BC MWLAP pers. comm. 2003), but RBT stocking into Koocanusa Reservoir still continues south of the border (Montana Fish and Wildlife stocking information, unpublished data). In BC, RBT stocking continues in what are considered landlocked high elevation lakes throughout the upper Kootenay River drainage (B. Westover, BC MWLAP pers. comm. 2003).

There are many occurrences of introgressive hybridization between native cutthroat trout and introduced rainbow trout (e.g., Busack and Gall 1981, Leary et al. 1984, Carmichael et al. 1993, Campbell et al. 2002) therefore it appears that there are few

limitations to hybridization. In areas of natural sympatry, rainbow trout typically prefer larger, lower elevation, warmer streams and spawn earlier than cutthroat trout (Hartman and Gill 1968; Trotter 1987; Henderson et al. 2000; Paul and Post 2001). Partial and temporal segregation is thought to limit interspecific matings (Trotter 1987; Liknes and Graham 1988), but there has been little study of pre-mating isolation between native WCT and introduced RBT (for a more detailed discussion of pre- and post- mating isolation refer to Chapter 3). Certain habitat preferences, however, do exist which could potentially reduce hybridization.

There is evidence suggesting that rainbow trout do poorly in extreme headwater conditions. Paul and Post (2001) showed that although rainbow trout were stocked in many high elevation waters in Alberta, the highest densities of RBT were present in low elevation areas. Similarly, in the Flathead River system, naturalized populations of introduced rainbow trout occur in the lower reaches of the mainstem but have not been documented in higher elevation tributaries (Deleray et al 1999 cited in Hitt 2002). The Colorado River cutthroat (*O. c. pleuriticus*) also inhabits higher steeper and lower order streams than introduced RBT (Bozek and Rahel 1991). Therefore, lower elevation mainstem populations of WCT within the Kootenay River drainage may be at greater risk of hybridization.

There is some evidence of limited WCT x RBT hybridization in the upper Kootenay River (Leary et al. 1987a). Leary et al. (1987a) used six allozyme markers and determined that three samples sites within the White River system "unquestionably came from hybrid swarms". The source of RBT into the White River was not determined but it most likely came from the naturalized population of RBT in Whiteswan Lake. Whiteswan Lake is located above a waterfall on the White River system, which is a large tributary of the upper Kootenay River. It is likely that some of RBT introduced to the lake gained access to the river after falling over the waterfalls. The other nine rivers tested for hybridization by Leary et al. (1987a) showed no evidence of hybridization. Although RBT introductions have continued and expanded in the region (i.e. the stocking program 1986-1998 in Koocanusa Reservoir) there has been no follow-up study to determine if hybridization has increased or spread in the upper Kootenay River since 1986.

In order to ensure the future persistence of pure westslope cutthroat trout populations in British Columbia, it is important to understand the current distribution and extent of hybridization, and environmental factors that may influence hybridization. Locating pure populations and recognizing these as important sites for implementing protection is also an important facet for future management of WCT. Using molecular analysis I re-visit seven river systems sampled by Leary et al. (1987a) and extend analysis to16 previously unexamined sample sites and test for RBT hybridization. The main objectives of this chapter are to: 1) determine if RBT hybridization has increased or spread in the upper Kootenay River drainage since 1986, 2) locate genetically pure WCT populations and, 3) determine if the incidence of hybridization is related to certain site characteristics (i.e. elevation, stream order, stream magnitude, etc).

Materials & Methods

Study Site

The Kootenay River is one of two major tributaries of the Canadian portion of the Columbia River Basin, the third largest drainage basin in British Columbia. The headwaters of the Kootenay River are nestled in the Rocky Mountains in Kootenay National Park. It flows southwest through the Rocky Mountain Trench near Canal Flats, then continues south into the United States before re-entering BC to join the Columbia River at Castlegar, BC. This study takes place in the upper Kootenay River drainage, which extends from its source to the first border crossing (Figure 2.1).

Sample Collection

Caudal fin clips were collected from fish at 23 sample sites in 12 different river systems in the upper Kootenay River drainage (Figure 2.1). A total of 981 fish were included in this study; 356 collected between June and September 1999 and 625 collected between June and September 2000. Three sample sites were sampled in both years to assess temporal variation in the prevalence of hybridization. A combination of angling, electro-shocking and minnow-trapping was used to sample fish. To avoid any biases in sampling, fish were clipped as they were encountered until the desired sample size was reached without regard to presumed genotypic status. All tissue samples were stored in 95% ethanol and age class, fork length, and tentative species identification were

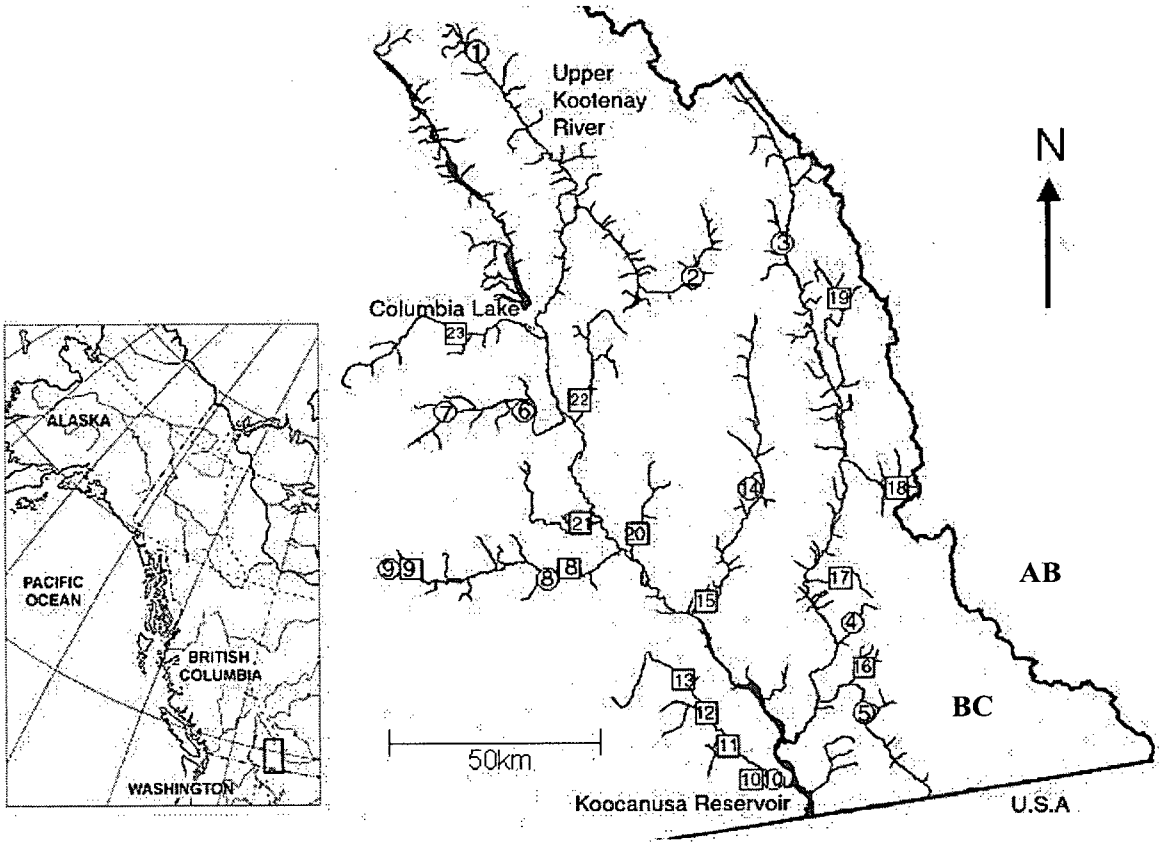


Figure 2.1. Sample sites examined for the presence of WCT, RBT and their hybrids in the upper Kootenay River drainage. 1, upper Kootenay River mainstem; 2, White River; 3, upper Elk River; 4, Morrissey Creek; 5, Wigwam River; 6, lower Skookumchuk Creek; 7, upper Skookumchuk Creek; 8, lower St. Mary River; 9, upper St. Mary River; 10, lower Gold Creek; 11, Bloom Creek at Gold Creek; 12, Teepee Creek at Gold Creek; 13, upper Gold Creek; 14, upper Bull River; 15, lower Bull River; 16, Lodgepole Creek; 17, Coal Creek; 18, Michel Creek; 19, Fording River; 20, Wild Horse River; 21, Mather Creek; 22, Lussier River; 23, Findlay Creek. Circles represent samples collected in 1999, squares represent samples collected in 2000. **Note**: Three systems were sampled both years; upper and lower St. Mary River and lower Gold Creek. Inset shows study area in western North America. BC-British Columbia, AB-Alberta.

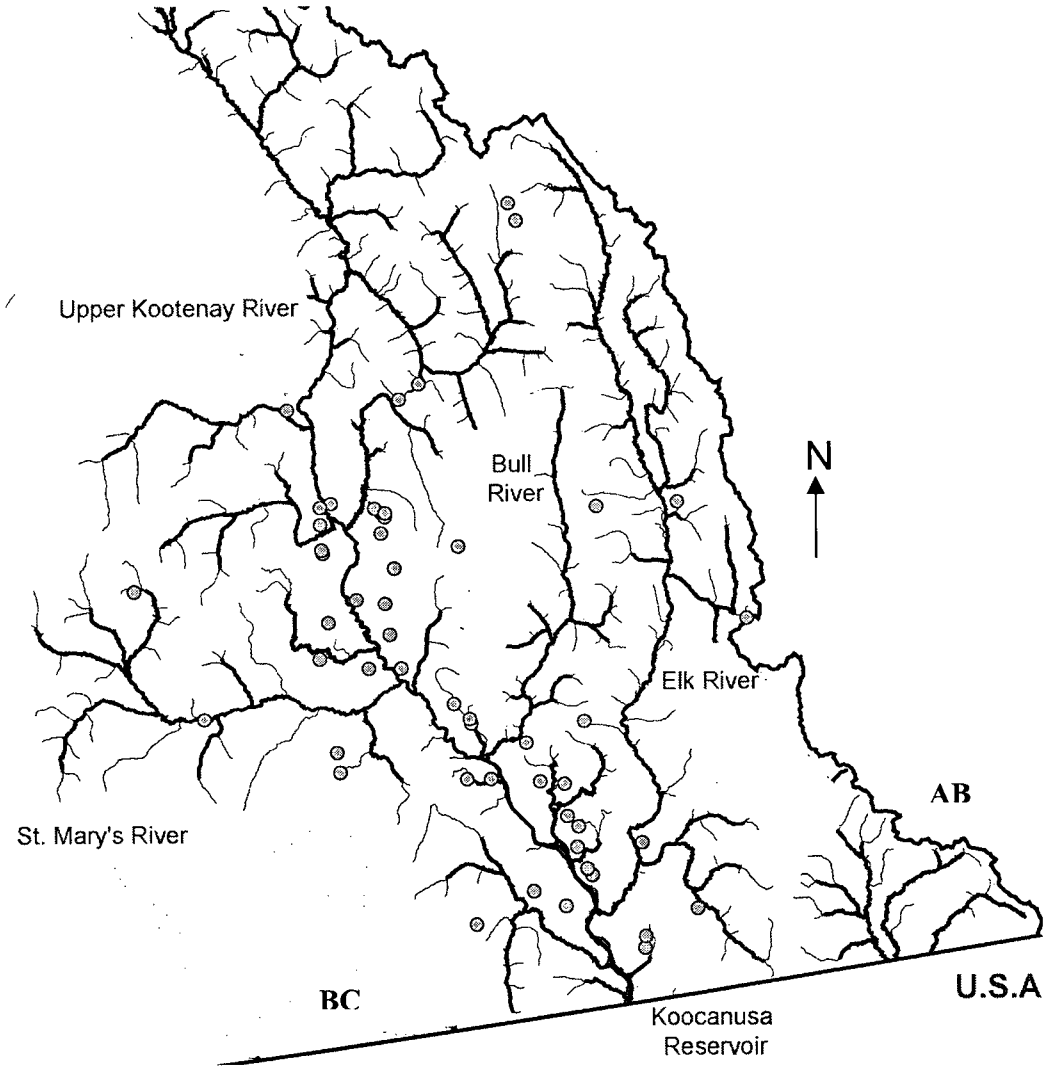


Figure 2.2. Rainbow trout stocking sites in the upper Kootenay River drainage, BC, between 1915-1998 (data from BC MWLAP stocking records). Each grey dot represents a site of RBT stocking; one site may have been stocked numerous times. BC- Britsh Columbia, AB-Alberta

determined for each fish. The species identification was based upon the following WCT characteristics: upper jaw extends past the posterior margin of the eye, bright red-orange slash under the base of the lower jaw, and reduced black spotting on the anterior body below the lateral line. Any individuals possessing intermediate or ambiguous phenotypes were tentatively classified as hybrids. Age classification was based on size and retention of juvenile characteristics such as parr marks (dark oval bands on the lateral surface of sub-adult fish). One of four age classes was assigned: 0+ (fry or young of the year, <55mm), 1+ (year old fish, approximately 60-130 mm), 2+ (fish larger than 130mm that retained parr marks), and 3+ (fish larger than 180 mm that have no retention of juvenile characteristics).

Genetic Analysis

DNA was extracted from each tissue sample (10-20mg) using the GENTRA Puregene DNA Extraction Kit following the manufacturer's protocol, diluted to 100ng/µl, and stored at –20°C.

In order to identify heterospecific alleles (in this case RBT alleles), fixed genetic differences between species must be identified. Markers were chosen from the literature based on the following criteria: species-specificity, repeatability, clarity (i.e. strength of banding patterns, ease of scoring), and availability. Preference was also given to codominant markers. I performed primer trials with 15 different potential markers (Table 2.1) and ranked them on the above criteria. A prospective power analysis on hybrid detection found that to reliably distinguish backcross individuals from first generation hybrids (F_1) relatively few markers are needed (Boecklen and Howard 1997). For example, the probability of confusing a backcross for an F_1 using four diagnostic markers is 0.0625. Therefore, I used four markers that best fit the above criteria. Once I found markers that met these criteria on a few test individuals, I assayed individuals from both species across their distribution to confirm fixation of alleles. I tested 30 WCT individuals from three populations that were believed to be pure (Findlay Creek, upper Bull River and Connor Lakes) and 20 RBT individuals from populations in California to Russia and several B.C. populations that are used for broodstock for hatcheries (i.e. Lardeau River and Pennask Lake populations). In addition to these tests, the authors that developed two of the chosen markers tested them on 118 RBT from six different populations and 57 WCT from two populations in Idaho (Ostberg and Rodriguez 2002).

Table 2.1. Molecular markers used in preliminary trials for species diagnostic markers including ranking for use in identifying species differences between WCT, RBT and their hybrids. Potential use was ranked from 1-3 where 1 = very poor (no amplification, no variability), 2 = good potential and 3 = fit all criteria and were chosen for study.

Fragment	Assay	Source	Rank
SINE (FOK)	PCR	Hamada et al; 1998	1
C-myc	PCR	J.Baker unpub. data U. Wash., Seattle	1
Epd	PCR	J.Baker unpub. data U. Wash., Seattle	2
GnRH Ex2/GnRHEx4	PCR	(Baker et al. 2002)	1
Growth Hormone 2 Intron C	PCR	McKay et al. 1996	2
P53	PCR	(Baker et al. 2002).	2
Metallothionine	PCR	(Baker et al. 2002).	1
OM15	PCR	(Ostberg and Rodriguez 2002).	2
OT 62	PCR	(Ostberg and Rodriguez 2002).	1
OM11	PCR	(Ostberg and Rodriguez 2002).	2
Occ19/Om27	PCR	(Ostberg and Rodriguez 2002).	2
Ikaros/Hinf I	PCR/RFLP	(Baker et al. 2002).	3
Hsc 71/Taq I	PCR/RFLP	(Baker et al. 2002).	3
Occ 16	PCR	(Ostberg and Rodriguez 2002).	3
Om 13	PCR	(Ostberg and Rodriguez 2002).	3

All four markers chosen are codominant markers with species diagnostic differences to identify hybrid individuals (Table 2.2). Ikaros and Heatshock cognate are coding genes, but the primers amplify introns of these genes. Species-specific variants of these introns enabled identification of individuals when cut with the appropriate restriction enzymes (RFLP's) (Baker et al. 2002). The other two markers used to identify WCT, RBT and their hybrids (Occ 16 and Om 13) are dual primer species diagnostic simple sequence repeats designed by Ostberg and Rodriguez (2002). Occ 16 and Om 13 are diagnostic based on fixed differences in allele frequencies of simple sequence repeats (SSR) (Ostberg and Rodriguez 2002), a type of microsatellite that is widespread throughout eukaryotic genomes.

DNA Amplification

PCR reactions were run with varying conditions for each marker (Table 2.2). A typical PCR reaction consisted of a total volume of 20µl with 100ng template DNA, 0.8 µM each primer, 0.2mM each dNTP, 1.5 mM $MgCl_2$, 1 x Invitrogen *Taq* DNA

Polymerase buffer (20mM Tris-HCl, pH 8.4, 50mM KCl), and 1 unit of *Taq* DNA polymerase. All PCR reactions were run on a PTC –100 thermal cycler (MJ Research). Restriction digests were performed as per manufacturer's instructions (New England Biolabs), overnight, using 6 µl of PCR product in a total volume of 15µl. The results of the PCR and the restriction fragment length polymorphisms were visualized using 2-3% agarose gels stained with ethidium bromide.

Table 2.2. Primer sequences, PCR conditions (annealing temperature/number of cycles), and species-specific diagnostic allele sizes for molecular markers used in DNA analyses of westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids.

Primer	Sequence 5'- 3'	Annealing Temp/No.of cycles	Enzyme	Diagnostic Allele sizes
Hsc 71F	ctg cgt atc atc aat gag cc	60,56/8,32	*Taq* I	WCT: 568, 367, 249*
Hsc 71R	gat cag gac ggt cat gac			RBT: 616, 352, 216
IK F	ctt cga gtg caa cct ctg	48/45	*Hinf* I	WCT: 500, 250
IK R	att ttc ttt gcc acc gag g			RBT: 750
Occ 16F	gac aga cac att aag agt agt	50/30	N/A	WCT: 380
Occ 16R	cag taa tac agg tac agt atg			RBT: 280
Om 13F	gct gtt agg cta tat ttg ata t	56/30	N/A	WCT: 190
Om 13R	gaa aga tga gta aaa cta ttc			RBT: 175

*Diagnostic band for all cutthroat trout subspecies, other two bands may vary within cutthroat subspecies, all fish in this study were fixed for all three bands
-N/A- non-applicable because no restriction enzymes were used

Data Analysis

Hybrid Identification

Individual fish were identified by their genotype at the four loci. If they were homozygous at all loci for the WCT alleles or the RBT alleles they were classified as pure WCT or pure RBT, respectively. If a heterozygote was observed at one or more of the four loci then that individual was classified as a hybrid. I use the term "hybrid" to include everything from a first generation hybrid (heterozygous at all loci) to a backcrossed individual (heterozygous at one or more loci and homozygous for one of the parental species at the remaining loci) to an n^{th} generation hybrid (homozygous for alternating parent species at two or more loci). The error associated with distinguishing between a parental genotype and a second or third generation backcross (BC-2 or BC-3)

individual is quite high. For example, with four markers, there is approximately a 25% chance that a BC-2 will be classified as a parental individual, with BC-3 and BC-4 there is a greater chance (~51% and ~72% respectively) of misclassification (Boecklen and Howard 1997). Therefore, my analyses underestimate the number of hybrid individuals and overestimates the number of parental individuals in each population. A more detailed analysis of hybrid zone structure is performed in Chapter 3.

RBT Introgression

I assessed the degree of hybridization at each sample site using Equation 1.

(1) $\%I = (\#\ RBT\ alleles/8) * 100$

The presence of RBT or heterospecific alleles (% heterospecific alleles, *I*) at each site was quantified by dividing the number of RBT alleles out of total possible alleles (8) for each individual then this value was multiplied by 100. The mean was calculated for each sample site. This analysis provided a comparative measure of the presence of RBT alleles in WCT populations across localities.

Statistical power to detect the presence of RBT alleles at each sample site was calculated using Equation 2 from Kanda et al. (2002):

(2) $\alpha = (1 - q)^{2nx}$

where q is the frequency of non-native alleles desired to detect, n is the number of fish sampled, x is the number of diagnostic markers and α is the desired probability of detection. For example, all sample sites (except one) have at least 30 individuals; therefore, I had a 91% chance of detecting as little as a 1% genetic contribution from RBT in each population. The main objective of this regional study was not to determine the precise genotype of each individual, but to detect rainbow trout introgression in each population.

Spread of hybridization

If hybridization is spreading from a downstream RBT source (i.e. Koocanusa Reservoir) to surrounding tributaries, then one would expect the highest percentage of heterospecific alleles (*% I*) to be in close proximity to Koocanusa Reservoir and that *% I*

would decrease at sample sites further upstream. If the opposite is true, and *% I* is higher further upstream, then an upstream RBT source is more likely. To test if the RBT source is Koocanusa Reservoir, I examined the relationship between *% I* and riverine distance to Koocanusa Reservoir. The values of *% I* across all 23 sites could not be normalized with the appropriate transformations; therefore, I used a nonparametric Spearman Rank Correlation (Zar 1999). I conducted the correlation twice, once with all sample sites (n=23) and again without sample sites that were located above upstream migration barriers (n=16) to determine if these barriers were protecting upstream populations from hybridization.

Temperature

In general, rainbow trout prefer warmer water temperatures whereas cutthroat trout are able to inhabit colder waters (Hartman and Gill 1968, Trotter 1987; McIntyre and Rieman 1995). If a temperature gradient is limiting the dispersal of RBT and hybrids into an area, then certain WCT populations may be naturally "protected" from hybridization. To determine if temperature was limiting the spread of RBT hybridization in the upper Kootenay River I collected temperature data from available sources. I was able to obtain temperature data for 10 of the 23 sample sites. I received temperature data from four creeks/rivers that were sampled from the Water Survey Board of Canada (Mather Creek, lower Skookumchuk, lower Bull, upper St. Mary rivers); four creeks/rivers (Morrissey Creek, upper Elk, lower St. Mary and Fording rivers) from Westslope Fisheries Ltd. (Cranbrook, BC) and lower Gold Creek from the Ministry of Water Land and Air Protection (Fisheries Branch). The Water Survey Board of Canada (WSBC) data sets were collected at least four times a year (in June, July, December, and March) over a varying number of years. For example, temperatures were collected from the lower Bull River from 1970-1998. The data received from Westslope Fisheries Ltd. was collected in 2001 from thermistors embedded in the streambed that record temperatures every 15 or 60 minutes for the year. The lower Gold Creek temperatures were recorded in the same way but in 1997. I plotted all data from the themistors against time and removed extraneous temperatures that were regarded as points when the thermistor was exposed to the air. I averaged all temperatures for each day then calculated the overall mean for the year.

Although water temperature is more stable than air temperature, to compare temperatures between sample sites across different years I have to assume that there is little variation between years. I tested this assumption using a One-way Analysis of Variance (ANOVA) on the 28 years of data from the lower Bull River. Also, to determine if the different sampling methods (four times a year over several years compared to mean annual) were comparable, I included the annual mean temperature for 2001 collected by Westslope Fisheries in a comparison across years at Michel Creek. The WSBC had available data from 1984-1994 for Michel Creek. I used a one-way ANOVA to compare these mean temperatures. To determine if the *% I* was correlated with temperature, I conducted a Spearman Rank Correlation. All statistics were calculated using SPSS Version 11.

Spatial Analysis

I used two methods to test for patterns in the distribution of hybridization across sample sites. To determine if sample sites containing hybrid individuals were found closer together than sites that did not contain hybrids (positive spatial autocorrelation) I used a Mantel Test (Mantel 1967). I constructed two distance matrices, one based on geographic distance between pairs of sites and the other based on the hybrid state of the pairs of sites. More specifically, the hybrid state matrix was a binary-coded matrix compiled of zeros and ones where 0 = both sites hybridized and 1 = any other combination. I compared the hybrid matrix with a straight-line distance (Euclidian distance) matrix and a fluvial distance matrix. Both fluvial and straight-line distances between all 23 pairs of sample sites were calculated from Arcview Spatial Analyst. I also tested the effects of upstream migration barriers by doing a Partial Mantel test. The Partial Mantel test compared the fluvial distance matrix and the hybrid matrix and controlled for a third matrix representing the presence of migration barriers between certain pairs of sites. All test were carried out using the statistical software R package (Legendre and Vaudor 1991) and 9999 permutations were conducted for each test.

The second analysis involved a Principal Components Analysis (PCA). I used the PCA to determine if a pattern existed between the physical site characteristics and the presence or absence of RBT hybridization. In addition, because most of the variables were intercorrelated, I used PCA to quantify the independent patterns of variation. If a pattern is revealed it may provide insight into certain site characteristics that promote or

hinder the rate of hybridization. I collected data on the physical characteristics of each sample stream to determine if any of these associated with the presence or absence of hybridization. The sample site characteristics used in the analysis were stream order, stream magnitude, elevation, stream gradient and distance to nearest hybridized population. Stream order and stream magnitude were obtained from the BC government FishWizard website (http://pisces.env.gov.bc.ca). Sample site elevations were recorded from topographical maps (Scale 1:50000) and estimated to the nearest 25 m. I calculated the average stream gradient from the change in elevation from the mouth of the stream to the sampling site, and divided by the distance between these two points. The last variable included was the distance to the nearest hybridized neighbouring site (NHN). This distance was measured using ArcView Spatial Analyst. All 23 sites were included in this analysis and the stream magnitude and NHN variables were both square root transformed to remove skewness.

Results

Hybrid Detection

Five hundred and sixty-three adults (age 3+), 304 juveniles (age 2+), 96 fingerling (age 1+) and 18 fry (age 0+) were sampled in total (Table 2.3). Most fish sampled were between 16 cm and 35 cm in length (Figure 2.3).

Table 2.3. Age classes of individual fish sampled each year of the regional assessment of hybridization between native westslope cutthroat trout and introduced rainbow in the upper Kootenay River.

Age Class	No. of individuals	
	1999	2000
3 + (Adults)	201	362
2 + (Juveniles)	100	204
1+ (Fingerling)	55	41
0+ (Fry)	0	18

One hundred and forty-two hybrids (14%) and 33 rainbow trout (3.4%) were identified from the 981 samples collected across sites in both years. The remaining 806 samples were identified as WCT. Field identification of hybrids significantly

underestimated the occurrence of hybrid individuals (χ^2=58.5, *df*=1, p<0.0001). Only 28 of the 142 hybrids identified genetically were correctly identified in the field. Fifteen fish genetically identified as cutthroat were misidentified in the field as hybrids. Another 14 were recorded as rainbow trout upon capture, and only six of these were confirmed genetically, the other eight were genetically identified as hybrids. These results suggest that the field-based assessment of hybrids used in this study was not very accurate.

The majority of the hybrid individuals were observed in the 2000 samples (114/625), where 18% of the individuals sampled were identified to be of mixed descent compared to only 8% in 1999 (28/356). The majority of the rainbow trout were also found in 2000 (31 in 2000 and only 2 in 1999).

RBT introgression in WCT populations

Eighteen of the 23 sample sites showed the presence of RBT alleles, leaving only five sites that showed no evidence of hybridization (Table 2.4, Figure 2.4 & 2.5). There was evidence of backcrossing at all 18 sample sites containing hybrid individuals, indicating that varying levels of introgression has occurred. There is a large range in the amount of RBT alleles present across sites with RBT hybridization from less than 1% in upper Skookumchuk Creek to an apparent naturalized population of RBT in lower Bull River (97.1%). A one-way analysis of variance revealed that *% I* differed significantly across sites ($F = 51.04$, $p<0.001$, Figure 2.4). The majority of sample sites containing hybrid individuals (13/18) had less than 10% heterospecific alleles.

Three sample sites that were sampled in both years (lower Gold Creek, lower St. Mary River and upper St. Mary River) showed no significant differences in *% I* between years ($p=0.53$, $p=0.71$, respectively, upper St. Mary was 0 % *I* in both years) and therefore, years were pooled when calculating *% I* (Table 2.4). I found evidence that a naturalized population of RBT exists in the lower Bull River. Twenty-five of the 30 individuals collected from lower Bull River were classified as RBT, and the remaining five were hybrids (97.1 % *I*). The next highest value of *% I* was found at Lodgepole Creek (37.5%), a tributary of the Wigwam River, then lower Gold Creek (20.6%), and then Michel Creek (13.1%) on the Elk River system (Figure 2.4 & 2.5). The only other site to show more than 10% heterospecific alleles was Bloom Creek (12.2%) a tributary of lower Gold Creek. In fact, on the Gold Creek system, I sampled four sites moving

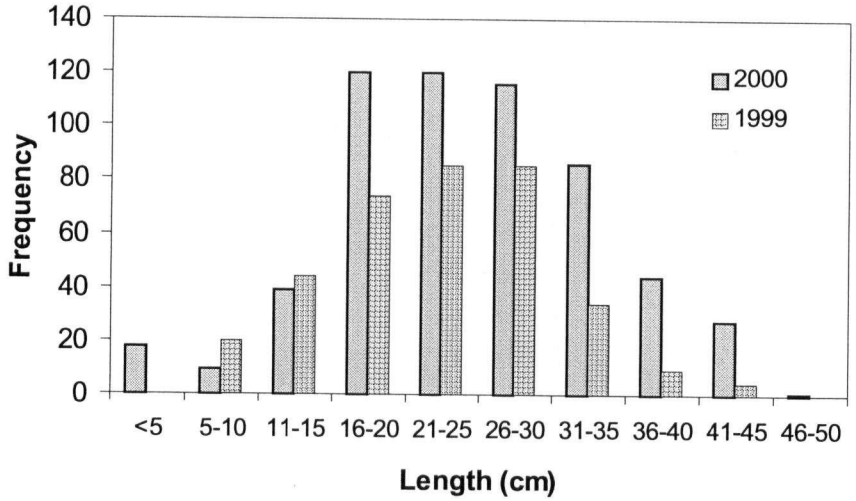


Figure 2.3. Lengths of *Oncorhynchus mykiss* (RBT), *O. clarki lewisi* (WCT), and their hybrids collected from all sites in the Upper Kootenay River drainage in the year 2000 (black) and 1999 (stipled).

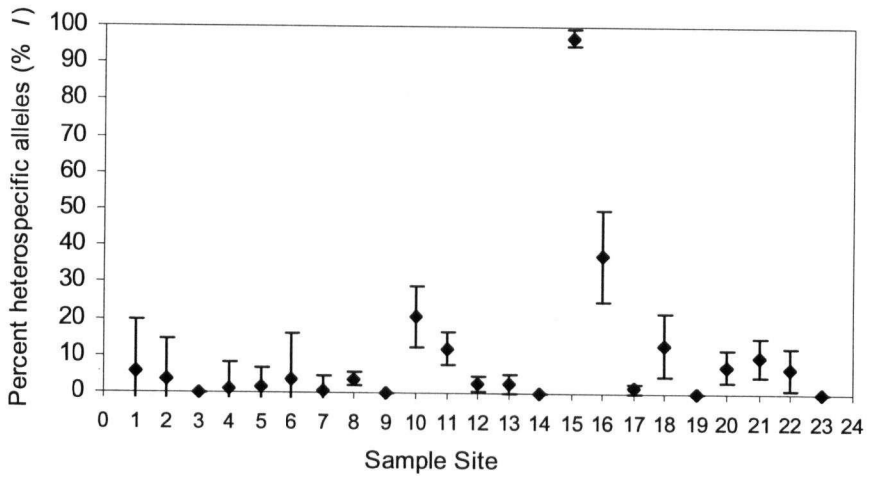


Figure 2.4. Mean percent value of heterospecific alleles present *(% I)* and 95% confidence intervals in WCT populations in the upper Kootenay River Drainage in British Columbia; 1, upper Kootenay River (mainstem); 2, White River; 3, upper Elk River; 4, Morrissey Creek; 5, Wigwam River; 6, lower Skookumchuk Creek; 7, upper Skookumchuk Creek; 8, lower St. Mary River; 9, upper St. Mary River; 10, lower Gold Creek; 11, Bloom Creek; 12, Teepee Creek; 13, Upper Gold Creek; 14, Upper Bull River; 15, Lower Bull River; 16, Lodgepole Creek; 17, Coal Creek; 18, Michel Creek; 19, Fording River; 20, Wildhorse River; 21, Mather Creek; 22, Lussier River; 23, Findlay Creek.

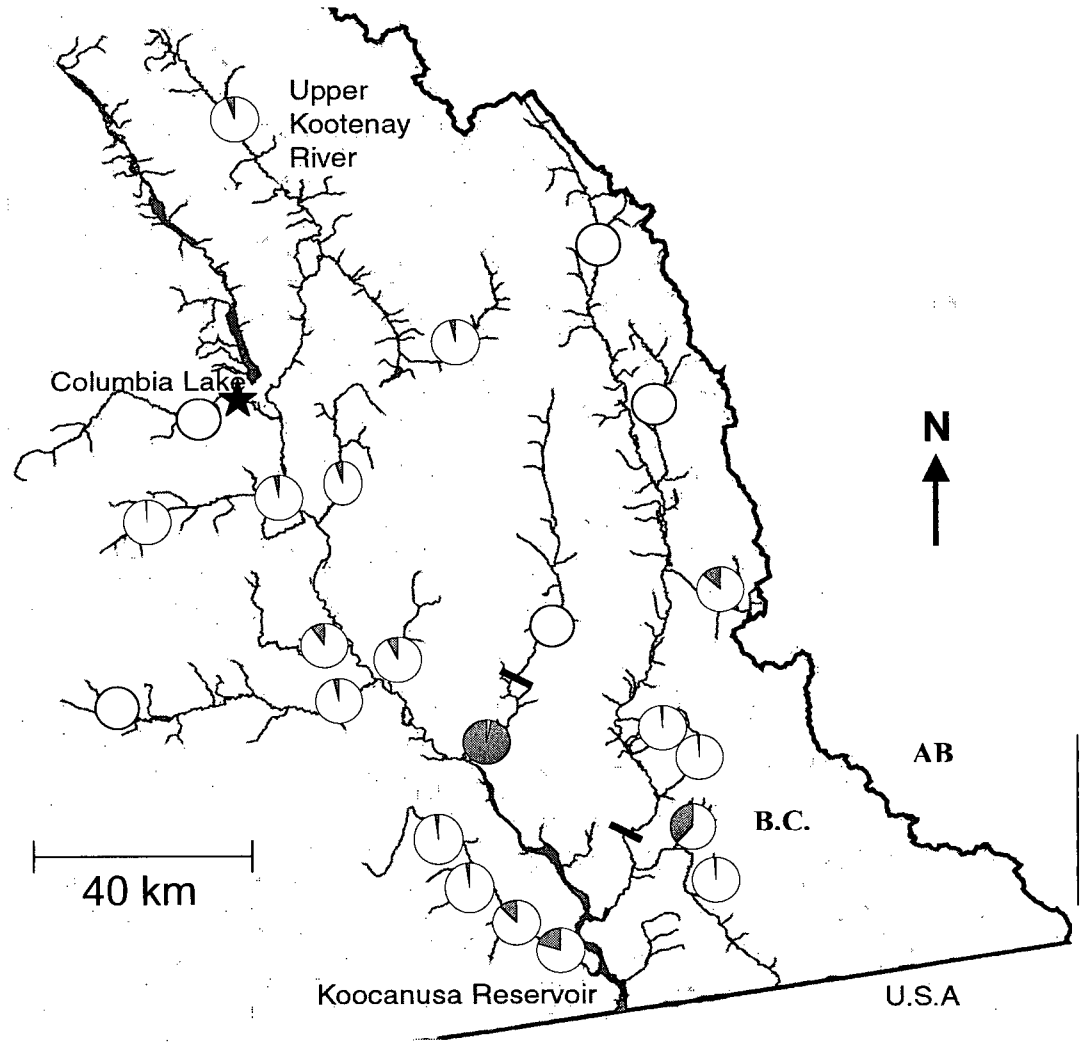


Figure 2.5. Percentage of heterospecific alleles *(% I)* into westslope cutthroat trout populations in the upper Kootenay River drainage. Pie charts represent the proportion of species alleles at each site; shaded area indicates % RBT alleles, white area indicates % WCT alleles. Black bars represent hydro dams and the star represents a canyon, both barriers to upstream fish migration.

upstream from Koocanusa Reservoir and there was a striking decrease in hybridization with increasing distance from the reservoir (20.6% in the lower reaches of Gold Creek near the mouth to 2.5% at the site furthest away from the reservoir $r = -0.957$, $p=0.043$).

No evidence of hybridization was found at 5/23 sample sites (Findlay Creek; upper St. Mary River, Fording River, upper Elk River, and the upper Bull River; Figure 2.5). The upper St. Mary River has been sampled extensively (131 fish total) and not one hybrid has been detected. A power analysis revealed virtually 100% confidence in detecting as little as 1 % introgression at this site (Table 2.4). The lower St. Mary River (below St. Mary lake) however, has experienced significantly more RBT hybridization ($t=3.814$, $df=134$, $p<0.0001$).

Spread of Hybridization

There was a significant negative correlation between distance to Koocanusa Reservoir and the presence of RBT alleles ($r_s = -0.486$, $p = 0.019$). This relationship became stronger when sites upstream from migration barriers were removed ($r_s = -0.568$, $p = 0.023$, Figure 2.6).

There was no significant difference in the annual mean temperature between years for the 28 years of data for the lower Bull River ($F=0.407$, $p=0.995$, Figure 2.7). This supports my assumption that water temperature does not vary significantly annually and therefore, comparing different years across sites is adequate in examining temperature difference between sites. Comparing 10 years of temperature data from Michel Creek obtained from the WSBC and the 2001 thermistor readings from Michel Creek indicated that there was no significant difference between the means of the two data sets (Kruskall-Wallis ANOVA, $p = 0.261$).

Table 2.4. Mean percent heterospecific alleles (*% I*) in westslope cutthroat trout populations throughout the upper Kootenay River drainage

Sample Site (*see Fig. 2.1 for location*)	Year (n)	Mean % RBT alleles (% I)	Power to detect 1% introgression (1-α)
1. upper Kootenay mainstem	1999 (15)	5.8	0.70
2. White River	1999 (33)	3.8	0.93
3. upper Elk River	1999 (38)	0.0	0.95
4. Morrissey Creek	1999 (30)	1.3	0.91
5. Wigwam River	1999 (34)	1.5	0.94
6. lower Skookumchuk Creek	1999 (33)	3.4	0.93
7. upper Skookumchuk Creek	1999 (40)	0.7	0.96
8. *lower St. Mary River	1999 (31)	4.4, 3.8 (pooled mean)	0.92
*lower St. Mary River	2000 (104)	3.6	1.0
9. *upper St. Mary River	1999 (31)	0.0	0.92
*upper St. Mary River	2000(100)	0.0	1.0
10. *lower Gold Creek	1999 (36)	18.4, 20.6 (pooled mean)	0.94
*lower Gold Creek	2000 (30)	23.3	0.91
11. Bloom Creek	2000 (30)	12.2	0.91
12. Teepee Creek	2000 (30)	2.5	0.91
13. upper Gold Creek	2000 (30)	2.5	0.91
14. upper Bull River	1999 (36)	0.0	0.94
15. lower Bull River	2000 (30)	97.1	0.91
16. Lodgepole Creek	2000 (30)	37.5	0.91
17. Coal Creek	2000 (40)	1.4	0.96
18. Michel Creek	2000 (30)	13.2	0.91
19. Fording River	2000 (30)	0.0	0.91
20. Wild Horse River	2000 (45)	7.5	0.97
21. Mather Creek	2000 (30)	9.7	0.91
22. Lussier River	2000 (30)	6.7	0.91
23. Findlay Creek	2000 (32)	0.0	0.92

*Sites sampled in both years that showed no significant differences in % I between years were pooled by site for calculating % I and graphed with 95% C.I. in Figure 2.4.

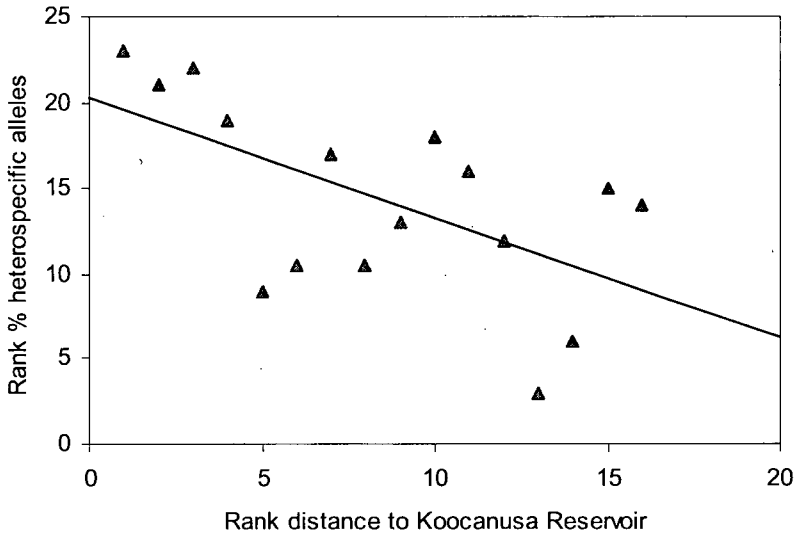


Figure 2.6. Spearman's Rank Correlation of distance to Koocanusa Reservoir and percent heterospecific alleles (*% I*) (Spearman's Rho =-0.568, p=0.023). Only below migration barrier sites are included (n=16).

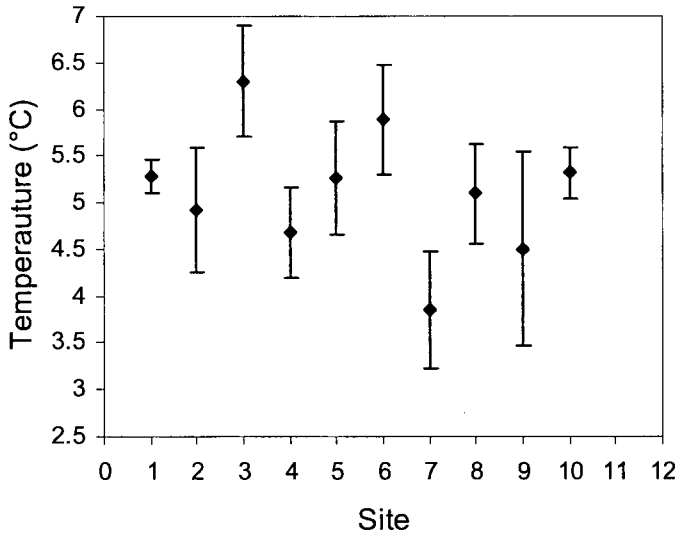


Figure 2.7. Mean temperatures for 10 streams in the upper Kootenay River drainage in British Columbia. The year(s) measured and the number of data points are in parentheses. 1) lower Gold Creek (1997, n=366); 2) Mather Creek (1977-1995, n=169); 3) Morrissey Creek (2001, n=355); 4) Fording River (2001, n=335); 5) lower Bull River (1970-1998, n= 173); 6) lower St. Mary River (2001, n=343); 7) upper St. Mary River (1977-1989, n=130; 8) Michel Creek (2001, n=364); 9) lower Skookumchuk Creek (1977-1985, n=75); 10) Elkford (near upper Elk River site, 2001, n=338).

The above results allowed me to compare the temperature data between sites, even though they were not measured in exactly the same manner. The mean temperatures for ten sites ranged from 3.8 in the upper St. Mary River to 6.3 at Morrissey Creek (Figure 2.7) and there were significant differences between the mean temperatures among sites ($F = 5.6$, $df = 9$, $p = 0.000$). Multiple tests of mean differences (Bonferonni corrected alpha level $0.05/10 = 0.005$) revealed that upper St. Mary River is significantly colder than lower Gold Creek ($p = 0.001$), Morrissey Creek ($p = 0.000$), and lower St. Mary River ($p < 0.0001$). There was a positive but non-significant relationship between the presence of RBT alleles and mean temperature ($r_s = 0.322$, $p = 0.204$). I removed the genetically pure sample sites from the analysis, however, and observed a non-significant negative relationship between % *I*, and temperature ($r_s = -0.333$, $p = 0.293$) suggesting that mean annual temperature is not a limiting factor in RBT hybridization at these sample sites.

Spatial Analysis

The presence of RBT alleles was significantly correlated with the distance between sample sites. There was positive spatial autocorrelation between the binary hybrid matrix and straight-line distance ($r = 0.192$, $p = 0.05$) suggesting that sample sites with RBT alleles present are clustered geographically. Stream networks connecting sample sites appear to be important in influencing hybridization because a higher correlation coefficient was observed when comparing fluvial distance and presence of RBT alleles ($r = 0.230$, $p = 0.032$) than when using straight-line distance. The Partial Mantel test that controlled for the presence of upstream migration barriers revealed a slightly stronger positive correlation between fluvial distance and the presence of RBT alleles ($r = 0.259$, $p = 0.023$).

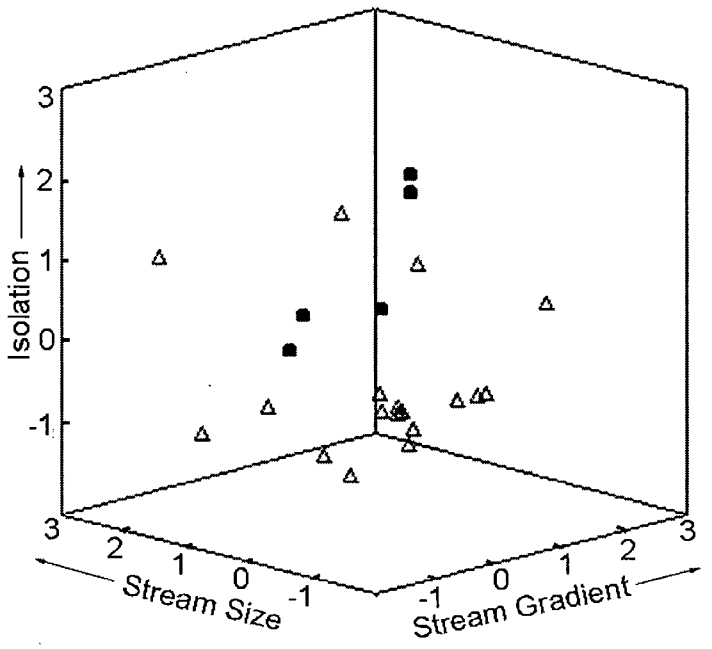
Three principal components that together explained 88.2% of the variation in the environmental data were extracted from the correlation matrix of the five variables (Table 2.5). Stream order and magnitude loaded heavily on principal component 1 (PC 1) making this a stream size component. Principal component 2 (PC 2) represents the isolation component of the sample sites because both elevation and nearest hybridized neighbour (NHN) loaded heavily on PC 2. Average stream gradient loaded heavily on the third component (PC 3). Both genetically pure westslope cutthroat trout sample sites and hybridized sample sites appear to be present in the range of stream gradients and sizes

(Figure 2.8a), but there is a clear separation between localities along PC 2 (Figure 2.8b). PC 1 and PC 2 explained 65.1% of the variation in the data. Higher values of PC 2 indicate sites that are located at higher elevations and at greater distances from hybridized sites (i.e. more isolated). Four of the five pure sample sites are located on the positive side of PC 2 axis indicating that more pure populations were sampled at high elevations and further from sample sites containing hybrid individuals. The one pure site with a negative PC 2 score is the upper Bull River, which is located above a migration barrier. Fourteen of the hybridized sites are found in lower elevation areas and closer to neighbouring hybridized sites. There was a significant difference between the mean value of PC 2 scores for pure sites and the mean value of PC 2 scores for hybridized sites ($t = -3.2$, $df = 21$, $p = 0.004$). These results suggest that RBT hybridization is more common at, but not exclusive to, lower elevation streams and rivers.

Table 2.5. Principal components analysis results of environmental variables for 23 sample sites showing the loadings of these variables for the first three components after Varimax rotation. Variables related to stream size loaded on PC 1, elevation and distance to nearest hybridised neighbour site (NHN) loaded heavily on PC 2, and stream gradient loaded heavily on PC 3.

	Loading on		
Variable (units)	***PC 1***	***PC 2***	***PC 3***
Stream Order	0.903	-0.017	0.235
Elevation (m)	-0.215	0.843	0.342
Stream Gradient (m/km)	0.065	0.034	0.967
NHN (km)	0.293	0.853	-0.243
Stream Magnitude	0.906	0.080	0.155
% cumulative variance	36.6	65.1	88.2

a)



b)

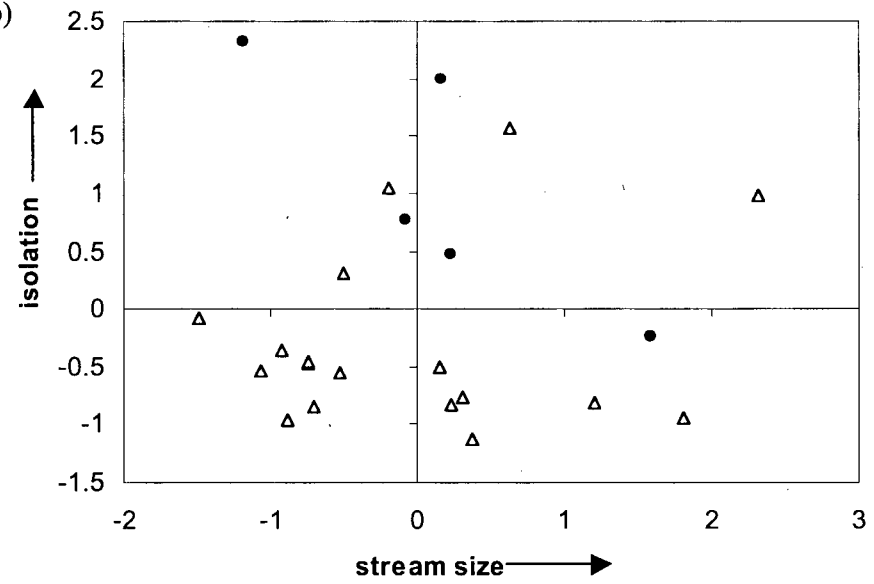


Figure 2.8. a) Plot of all 23 sample sites against values of the principal components extracted from correlation matrix of the site data. Black circles represent locations with no RBT hybridization; open triangles represent locations with RBT hybridization. PC 1 increases with increasing stream size; PC 2 increases with increasing elevation and NHN; PC 3 increases with increasing stream gradient b) Plot of all 23 locations against values of PC 1 (stream size) and PC 2 (isolation).

Discussion

I analyzed 16 sample sites in the upper Kootenay River drainage that were previously untested for hybridization between cutthroat and rainbow trout; 14 of these showed evidence of hybridization and two were identified as pure westslope cutthroat populations. Of the seven river systems previously sampled by Leary et al. (1987a), I found new evidence of hybridization at three, confirmed hybridization at one, and found continued absence of hybridization at three sample sites. It is important to note that my power to detect 1% heterospecific alleles (Table 2.4) was similar or greater to that of Leary et al. (1987a), which ranged from 62% in the upper St. Mary River to 99.99% at Skookumchuk Creek. Leary et al. (1987a) used 2 more diagnostic markers than I did in this study (6 vs. 4); however, my sample sizes were larger in most cases. A comparison of all available genetic data on WCT x RBT hybridization indicates an increase in the number of hybridized populations in the upper Kootenay River drainage from 1986 to 1999 (Table 2.6).

Table 2.6. Summary table for all existing data on percent heterospecific alleles between native westslope cutthroat trout and introduced rainbow trout in seven river systems in the upper Kootenay River drainage in British Columbia

River system	1986 (Leary *et al.* 1987a)	1999	2000
Skookumchuk	0%	3.4%	Not sampled
White	5.3%	3.8%	Not sampled
Wigwam	0%	1.5% (mainstem)	37.5%(tributary)
upper St. Mary	0%	0%	0%
lower St. Mary	Not sampled	4.4%	3.6%
upper. Elk	0%	0%	Not sampled
lower Elk	Not sampled	1.2%	1.3%
upper Bull	0%	0%	Not sampled
lower Gold	0%	18.4%	23.3%

More hybrid fish were detected in the year 2000 than in 1999. This observed pattern does not necessarily mean that the presence of rainbow trout and hybridization has increased over one year, but rather that the expansion of the sampling regime included more hybridized populations. In rivers that were sampled in both years there was no

difference in the percent of heterospecific alleles detected suggesting that the rate of hybridization had not increased over one year.

Field identification of hybrids greatly underestimated the number of hybrids in this study. A more rigorous analysis of morphology may have resulted in a higher accuracy of identifying hybrids. For example, Weigel et al. (2002) showed that the most reliable characters in identifying WCT x RBT hybrids are slash intensity, basibranchial teeth, spot shape and ratio of head length to total length (HL:TL). Because genetic studies are expensive and labour intensive, an accurate protocol for field identification such as the one described by Weigel et al. (2002) is very useful and should be employed in future studies.

Spread of hybridization

I found a negative correlation between the degree of hybridization and geographic distance from Koocanusa Reservoir, implying that hybridization is spreading upstream. It thus appears that RBT alleles could spread throughout the drainage unless restricted by physical barriers, or removed via natural selection. Exogenous selection against hybrids at upstream sample sites (i.e., a reduction in RBT alleles at greater distances from Koocanusa Reservoir, perhaps due to an environmental gradient) would also be consistent with the observed correlation. If selection were causing the pattern, however, I would expect the frequency of RBT alleles to decrease over time in locations upstream of the reservoir, whereas my results show that the frequency of RBT alleles has in fact increased in three upstream samples sites since the previous study. Therefore, it appears that the RBT introductions into Koocanusa Reservoir from 1986-1998 have provided a source population of rainbow trout and subsequent hybrids that are spreading from the reservoir to surrounding areas. My results also suggest that migration barriers (hydro dams on the Bull River and the Elk River and an impassable canyon on Findlay Creek) are effective in preventing rainbow trout or hybrids from moving upstream. Evidence of hybridization in tributaries above the hydro dam on the Elk River; however, is indicative of other RBT sources in the upper Kootenay River system.

Many of the remaining pure populations throughout the westslope cutthroat trout's range are restricted to isolated headwaters (Brown and Mackay 1995, Mayhood 1999, Hilderbrand and Kershner 2000), and pure populations in the upper Kootenay River

drainage seem to be no exception. All five sample sites determined to be pure are located on tributaries further upstream from the mainstem Kootenay River than their hybridized counterparts. If hybridization continues to spread without any physical or environmental impediment, RBT alleles will likely permeate into these upstream areas via pure rainbow trout dispersal or hybrid trout straying. The upper Bull River and Findlay Creek cutthroat, however, are located above impassable physical barriers that prevent access from downstream fish, and are thus protected from hybridization as long as rainbow introductions do not occur above the barriers. The other pure sample sites (upper St. Mary River, Fording River and upper Elk River) are not separated by physical barriers and may be vulnerable to hybridization.

I found no evidence of environmental limitation of hybridization based on the following site characteristics: stream order, stream magnitude, stream gradient and mean annual water temperature. The only factor that appears to be limiting hybridization in this system is the degree of isolation from other hybridized populations or Koocanusa Reservoir. Results from a recent study on WCT x RBT hybridization in the Flathead River system in Montana, U.S.A., are consistent with my results in that they also provide evidence of a downstream RBT source (Flathead Lake) and suggest that the thermal regime of the river and presence of hybridization are not correlated (Hitt 2002).

My study was somewhat limited in its analysis of temperature effect. The mean annual temperature may be less important to RBT dispersal than the variation around that mean (e.g., the percentage of daily averages above 15°C or below 10°C). Hitt (2002) was able to obtain more detailed temperature data at each sample site in his Montana hybridization study, and still did not detect a relationship between hybridization and temperature. Therefore, the available evidence suggests that temperature does not limit the spread of hybridization. Hitt (2002) also tested habitat degradation, geomorphology and neighbour statistics. He only found a significant association in the neighbours data, indicating that the spread of RBT hybridization is facilitated via hybrids straying to neighbouring populations in the Flathead River system.

Although to date there is little evidence of environmental factors limiting the spread of hybridization, other environmental parameters such as habitat availability and stream flow may be important. Recent research has revealed that flow regime is a factor in the invasion success of rainbow trout; in particular, a match between timing of fry

emergence and months of low flow appears to be associated with successful invasion (Fausch et al. 2001). There is also evidence that availability of winter habitat limits rainbow trout recruitment in the Snake River (Idaho), and that age-0 trout survived only where complex bank habitat was present (Mitro and Zale 2002). An analysis of flow regimes and habitat types within the upper Kootenay River drainage may aid in predicting which tributaries are at greatest risk of RBT invasion.

There is some evidence that pure rainbow trout are unlikely to successfully colonize and exploit colder, high elevation habitats (Bozek and Rahel 1991, Paul and Post 2001). My results are consistent with these findings given the evidence of a naturalized population of rainbow trout in the lower Bull River and the absence of pure RBT at higher elevations. Although there appears to be some spatial segregation between the pure parental species in my study system, possibly due to differing environmental preferences, WCT x RBT hybrids may not be limited in the same way. For example, rainbow trout may be deterred from dispersing into colder headwater habitat, but phenotypically intermediate hybrids may not be. A study on hybridization between rainbow trout and Yellowstone cutthroat trout (YCT, *O. clarki bouveri*) in Idaho found that the spawning behaviour of hybrids was more similar to that of pure rainbow (Henderson et al. 2000). Hybrids spawned earlier and in lower stream reaches alongside rainbow trout, but the study's authors also determined that hybrid fish were more introgressed with RBT alleles than with YCT alleles. The majority of hybrids in my study were more genetically similar to westslope cutthroat trout than to rainbow trout (see Chapter 3) and therefore, applying the same logic, may be behaving more like cutthroat trout and thus able to exploit habitat that is typically considered cutthroat habitat. The apparent lack of pure rainbow trout in the system and the presence of RBT alleles at 78% of sample sites may be explained by hybrid individuals straying upstream from downstream areas where both parental species exist. The positive results of the Mantel test, indicating that hybridized sites are found in closer proximity to each other than they are to pure sites, also supports the idea of hybrid straying. The correlation was strengthened using fluvial distance and controlling for the presence of upstream migration barriers. This result confirms the importance of connectivity between sample sites and suggests that hybridization may be facilitated via hybrid straying in the upper Kootenay River drainage. As mentioned, Hitt (2002) found a similar result in the Flathead River system.

Upstream Source

There is an exception to the downstream RBT source model for the spread of hybridization. Although the majority of hybridized sites were observed in lower elevation areas, the Michel Creek site, where I found 13.1% heterospecific alleles, is at a higher elevation. Only two other sample sites on the Elk River system above the hydro dam (out of 5 total) showed evidence of RBT hybridization (Morrissey Creek and Coal Creek), and levels at these sites were much lower (1.5% and 1.2 % respectively). Both of these sample sites are found downstream from the Michel Creek, suggesting an upstream RBT source in the Elk River system.

Recently, out of concern for hybridization, the British Columbia government ceased the rainbow trout stocking program in Koocanusa Reservoir, but stocking continues in many "landlocked" high elevation lakes throughout the region (B. Westover, BC MWLAP pers. comm.2003). These high elevation lakes are often isolated and naturally fishless and are considered to have a low risk of introduced fish dispersing from them to other areas. Adams et al. (2001) have shown, however, that headwater lake stocking has the potential to allow non-native fish to access more stream area within a watershed than does mainstem or low elevation stocking. The most obvious factor preventing upstream migration of non-native fish is impassable barriers such as waterfalls or high gradient streams, but stocking above these barriers does not prevent downstream movement of non-native fish into previously inaccessible habitat. For instance, exotic brook trout (*Salvelinus fontinalis*) have been shown to disperse through much steeper streams in a downstream direction than an upstream direction, and this downstream movement may increase the rate and extent of invasion by forcing the colonization of new habitat (Adams et al. 2001). Once the fish have invaded downstream, they can no longer return to their upstream origin because the barrier or steep gradient prevent it.

British Columbia rainbow trout stocking records show that the closest stocking site to Michel Creek is Summit Lake, a small lake 5km upstream from the site sampled on Michel Creek. The last recorded stocking event into Summit Lake was in 1995, when 3,000 RBT were released. Between 1961-1995 nearly 50,000 RBT were released into this lake (BC MWLAP stocking records, unpub. data). Although no pure rainbow trout were found at this site, the presence of a hybrid individual classified as a RBT backcross (see Chapter 3) suggests that rainbow trout are present. It is impossible from this study to

determine if the hybrids found at Michel Creek are a result of matings between rainbow trout stocked in Summit Lake and the natural westslope population, but it is likely that some rainbow have spilled out of the lake at some point (possibly during snow melts when flows are high) and have been swept downstream into Michel Creek. This evidence of hybridization above the hydro dam on the Elk River indicates that ceasing lower elevation RBT introductions will not stop the spread of hybridization in this river system. Adams et al. (2001) suggest that the stocking even a small number of high elevation lakes could allow non-native fishes to access nearly an entire stream network.

Conservation Implications

Hybridization between introduced rainbow trout and westslope cutthroat trout appears to have increased and spread since its original documentation in British Columbia. This apparent increase is most likely a result of the continued and expanded introductions of rainbow trout into the Koocanusa Reservoir and adjacent tributaries. Given the high levels of introgression documented in other drainages (Leary et al. 1984; Mayhood 1999, Hitt 2002), and the increase of hybridization documented here, the most obvious step to minimize impacts on native *O. clarki lewisi* populations would be to cease all rainbow trout introductions into the geographic range of westslope cutthroat trout. Although ceasing exotic rainbow introductions may reduce hybridization, it would not necessarily solve the hybridization issue. In the absence of selection against hybrid genotypes, introgressed westslope cutthroat trout populations will persist indefinitely (see Chapter 3). Consequently, locating and protecting pure populations should be the highest priority for fisheries managers.

Evidence from this study suggests that the environment does not play a significant role in limiting the spread of hybridization, indicating that populations unaffected by hybridization have most likely avoided it simply because they are more isolated from rainbow trout stocking sites and hybridized populations. Therefore, if rainbow trout introductions continue, all westslope populations are likely vulnerable to hybridization unless they are protected by upstream migration barriers (and this is only effective if there is no upstream rainbow trout source). There are many management strategies for protecting remaining pure populations in the United States. For example, in Montana, where only from 2.5% - 13.1% of westslope populations remain pure (Liknes and Graham 1988; Shepard et al. 2002) and the majority of the populations have formed hybrid

swarms, restoration strategies include moving pure westslope to isolated headwater reaches to expand habitat, chemical treatment to remove introduced species, and constructing barriers to prevent invasion from downstream non-native trout (Tews et al. 2000). Each of these strategies has its limitations and problems that will not be discussed here (see Leary et al. 1995; Tews et al. 2000) but in British Columbia, because hybridization is relatively recent, and introgression levels remain relatively low (13/18 were less than 10% heterospecific alleles), we may be able to prevent hybridization from progressing to the extent it has south of the border.

Hybrid swarms are populations in which genes from the parental taxa are randomly distributed among the individuals in the population (Lassuy 1995). Pure parental taxa are rare in a hybrid swarm unless they are recent immigrants. From an evolutionary genetics perspective, hybrid swarms represent extinctions; although the genes of the native fish still exist in the population, they no longer exist in the novel combinations distinct to the native fishes' evolution (Leary et al. 1995). These novel combinations are lost once a randomly mating hybrid swarm has formed. If a complete hybrid swarm has yet to form, and pure parental types remain in the population, then removing hybrid individuals and exotic parental types may be an effective management tool in preventing further hybridization. Hybrid swarms have been shown to form in cutthroat trout and rainbow trout in as little as five generations (Hitt 2002), therefore hybridization has to be recent for removal programs to be useful. The distribution of hybrid genotypes and population genetic parameters (measures of heterozygote deficiency and linkage disequilibrium) can determine whether a hybrid swarm has formed. Harrison and Bogdananowicz (1997) have devised a useful classification of hybrid zones into unimodal (hybrid swarm), flat (even mixture of genotypes) and bimodal (parental types predominate with few hybrid genotypes). In the next chapter, I determine the structure of the hybrid zones in the 18 streams found to contain both WCT and RBT alleles. This analysis determines if hybrid swarms have formed in the upper Kootenay River drainage and therefore determines if local extinctions of this native trout have occurred in the area.

Chapter 3: Hybrid zone structure & the potential for differential selection

Introduction

Reproductive Isolation and Hybrid Zone Structure

A hybrid zone occurs when genetically distinct groups of individuals meet and mate, producing offspring of mixed ancestry (i.e. hybrids, Barton and Hewitt 1989). These zones can vary from a few metres to several kilometres wide (Hewitt 1989) and can persist through evolutionary time (e.g., Campton and Utter 1985; Baxter et al. 1997) or rapidly result in the collapse of one or both parental species (e.g., Whitmore 1983; Echelle and Connor 1989). Natural hybridization has been reported to occur in a variety of taxa (Arnold 1997) and is often associated with areas where previously isolated populations have come into secondary contact (Hewitt 1989).

Hybrid-zone populations are classified by the structure of their genotypic distribution using a defined hybrid index (where the minimum and maximum values represent the pure parental genotypes). A "hybrid swarm", defined by Harrison (1993) as a "diverse array of recombinant types", refers to a unimodal hybrid zone distribution where hybrid genotypes predominate. A bimodal hybrid zone consists largely of genotypes that are similar to the parental forms, with few intermediate hybrid genotypes present (i.e., F_1's or early generation post F_1's or backcrosses; e.g., Forbes and Allendorf 1991a; Redenbach and Taylor 2003). Jiggins and Mallet (2000) described a continuum of hybrid zones from a unimodal distribution to a bimodal distribution, which may represent different stages in speciation. This continuum has also been shown to happen in reverse when two previously isolated and closely related species come into contact (e.g., Rhymer et al. 1994) and merge into a single species.

The relative fitness of first generation hybrids (F_1) determines whether occasional hybridization strengthens isolating mechanisms or leads to introgression of the gene pools (Avise 1994). These isolating processes or barriers to hybridization fall into two major categories: prezygotic (pre-mating) and postzygotic (post-mating) (Arnold 1997). Prezygotic barriers normally involve mating behaviour and/or gamete recognition. The mating behaviour of the two parental species can prevent, or limit hybridization by via assortative mating or temporal or spatial separation of mating. Gamete recognition has also been shown to play an important role, especially in closely related species (e.g.

Howard et al. 1998). Jiggins and Mallet (2000) reviewed the hybrid zone literature and suggested that bimodal zones are strongly associated with assortative mating, whereas parental species in unimodal zones show little assortative mating. The importance of pre-mating isolation factors in limiting hybridization events in salmonids is exemplified by the relatively high incidence of hybridization in systems where such isolating factors are absent or less developed (reviewed in Taylor 2003).

Endogenous and/or exogenous selection against hybrids falls into the postzygotic barrier category (Arnold 1997). The relative importance of these two types of selection in the maintenance of hybrid zones over time has been a topic of much research in the hybrid zone literature (Howard et al. 1993; Bert and Arnold 1995; Harrison and Bogdananowicz 1997; Good et al. 2000). The roles that endogenous and exogenous selection play in shaping hybridized populations are examined in many hybrid zone models. For example, the tension zone model posits that hybrid zones are maintained by a balance between dispersal of parental individuals into the zone and selection against hybrids (Barton and Hewitt 1985). This model assumes that hybrid individuals are less fit than the parental species. The ecotone model (or the bounded hybrid superiority model, Moore 1977) posits that selection against hybrids is environmentally dependent and that hybrids can be more fit than parental genotypes in intermediate habitats. In this case, it is the scarcity of intermediate habitat that then limits the width of the hybrid zone.

Recently, both exogenous and endogenous selection, have been recognized as important factors in postzygotic barriers to hybridization (Arnold 1997). Bert and Arnold (1995) found that both types of selection maintain a clam hybrid zone using cohort analysis (i.e. the measurement of proportions of hybrids in a cohort as it ages). Arnold and Hodges (1995) provided many examples from both plant and animal taxa; they concluded that hybrids are not uniformly less fit than parental species and that this fitness can vary over an environmental gradient.

Westslope Cutthroat Trout and Rainbow Trout Hybridization

Westslope cutthroat trout (WCT) and introduced rainbow trout (RBT) readily hybridize and introgression has occurred in many drainages across the native range of WCT (Shepard et al. 1997). WCT and RBT evolved predominantly in allopatry throughout their historic ranges (Behnke 1992), and in the few naturally sympatric

populations in the Clearwater and Salmon rivers' drainages (Idaho), spatial and temporal segregation appears to limit interspecific mating (Liknes and Graham 1988). The coastal subspecies of cutthroat trout (CCT, *Oncoryhchus clarki clarki*) evolved in sympatry with native rainbow trout populations. Although natural hybridization does occur between CCT and RBT (Campton and Utter 1985), the factors preventing widespread hybridization are not well understood. In general, the ability of these two closely related species to coexist in sympatry, yet maintain species integrities is attributed to the spatial and temporal separation of spawning adults and not to postzygotic isolation (Behnke 1972; Behnke 1992). Spawning adults of coastal cutthroat and rainbow are generally separated both temporally and spatially within the spawning area (Trotter 1987), decreasing the chance of interspecific matings. Also, there is potential for size assortative mating between steelhead trout (anadromous rainbow trout) and coastal cutthroat trout because steelhead are often much larger. Therefore, pre-mating isolation plays an important role in limiting hybridization where cutthroat trout and rainbow trout are naturally sympatric.

By contrast, the evidence for post-mating isolation limiting the spread of cutthroat trout and rainbow trout hybridization is less convincing. Cutthroat trout and rainbow have different numbers of chromosomes, which has been suggested to decrease viability in hybrid progeny (Hawkins and Foote 1998), but the available evidence suggests few intrinsic barriers to gene flow. A study on the development of WCT and RBT hybrids found that pure RBT crosses were at a significantly less advanced stage of yolk sac absorption after the same time post-fertilization than hybrids and pure cutthroat crosses (Ferguson et al. 1985). These authors concluded that the relatively high developmental success of hybrids between cutthroat trout and rainbow trout suggests an absence of post-mating isolation between these taxa (Ferguson et al. 1985). Forbes and Allendorf (1991a) found no evidence from allele frequencies or gametic disequilibria that diagnostic allozyme alleles affect fitness in trout hybrid swarms between subspecies of cutthroat trout and concluded that western trout taxa have minimal genomic incompatibility in hybrid matings. A follow-up study showed that alternate mitochondrial DNA haplotypes had no detectable effect on development as measured by meristic counts in WCT and YCT (Yellowstone cutthroat trout, *O. clarki bouveri*) hybrid populations (Forbes and Allendorf 1991b). One study reported reduced developmental stability in laboratory raised F_1 WCT x RBT hybrids, but suggested that because of the numerous examples of

genetic exchange between RBT and WCT in nature, F_2 and backcross individuals must not be severely affected by this reduction (Leary et al. 1985). Another study showed that experimentally produced WCT x RBT hybrids had equal or higher survival to hatching but experienced slower growth and survival to 112 days post-fertilization under laboratory conditions (Leary et al. 1995). The few studies that have been done on exogenous selection (environment dependent) between cutthroat trout and rainbow trout have been done on coastal cutthroat trout and rainbow trout hybrids. Hawkins and Quinn (1996) found that artificially produced hybrids were intermediate to the parental species in both swimming performance and morphology, and they suggested that there is potential for a competitive advantage of hybrids in intermediate habitats.

Due to the lack of strong post-mating isolation and the presence of pre-mating isolation limiting hybridization in naturally sympatric populations, it is assumed that westslope cutthroat trout and introduced rainbow trout readily hybridize because they lack pre-mating barriers to gene flow, including the lack of great differences in size-at-maturity (Taylor 2003). If two species lack both pre- and post-mating isolation, there is little to stop a hybrid swarm from forming. Many of the studies on artificial natural hybridization (natural hybridization as a result of unnatural secondary contact, such as exotic species introductions) are of management or conservation relevance, where the main of objective is to determine the status of the native population (i.e. pure or hybridized, e.g., Chapter 2 of this thesis). By examining the genotypic distribution of the individuals in the population and using population genetic analyses to determine if the population is mating randomly, one can determine if the population is completely introgressed such that parental types no longer exist, or if hybridization is relatively recent. If hybridization is recent and a hybrid swarm has yet to form, then certain management actions (i.e. exotic species removal) can potentially preserve the native fish.

Allendorf et al. (2001) explained this with a useful example: Consider a sample of 100 individuals from a two hybrid populations (Population 1, Population 2) examined at 10 loci. Ten F_1 hybrids and 90 parental individuals are detected in Population 1, and in Population 2, every individual carries a single foreign allele at one of the 10 loci. The proportion of admixture is 5% in both populations but the genotypic distribution of hybridization is very different in these two populations (bimodal in Population1, but unimodal in Population 2). In Population1 hybridization is recent or rare, and

introgression has not yet occurred; management action in this population may include the removal of the foreign parental species and hybrid genotypes. In Population 2 a hybrid swarm has formed, and although the level of introgression is low, there is no feasible management action that can effectively remove the foreign alleles from this population. This example illustrates the crucial need to understand the genetic structure of hybrid zones before management actions can be implemented.

The main objective of this chapter is to determine if unimodal hybrid swarms have formed between introduced rainbow trout and native westslope cutthroat trout in the upper Kootenay River drainage. In Chapter 2 of this thesis, 18 sample sites in 9 river systems were found to have some level of RBT introgression. The "percent heterospecific alleles" value calculated in Chapter 2 is useful in conservation biology, as a comparative measure of population purity, but it does not reveal any information about the genotypic distribution within each hybridized population (see example above). In this chapter, I examine the distribution of the hybrid individuals within these introgressed populations and use population genetic analysis to determine the genotypic structure. This examination will allow me to determine if hybrid swarms have formed in this system and to make inferences about the associations between the hybrid zone structure and the prevelance of pre- or post-mating processes.

A second objective of this chapter examines hybridization in one river system in greater detail, the St. Mary River. Specifically, I sought to: 1) determine if hybridization was increasing in the St. Mary River system over time, 2) determine if it had spread to the upper river, and 3) determine if there was differential survival occurring between hybrids and pure parental fish. Although studies have concluded that there is limited endogenous selection against WCT x RBT hybrids (Ferguson et al. 1985, Forbes and Allendorf 1991a), there have been few studies examining the role of exogenous selection (environmental dependent selection) in WCT x RBT hybridization. Rainbow trout are not native to the upper Kootenay River drainage. Therefore, it seems possible that WCT x RBT hybrids would not do as well in the cutthroat environment as pure WCT.

To test the hypothesis that pure WCT are more fit than WCT x RBT hybrids, I used a cohort analysis. Cohort (individuals of a given age class) analysis examines the relationship between a particular variable (in this case proportion of hybrids) and the age of the individuals. For example, if selection is eliminating hybrid individuals, then as age

increases, the proportion of hybrids should decrease and genetic associations among parental alleles (i.e. heterozygote deficiency and linkage disequilibrium) should increase. If the opposite is true and selection favours hybrid genotypes (i.e. heterozygotes) then the proportion of hybrids would increase as the cohort aged and genetic associations among the parental alleles would disappear.

Methods

Study Site

The Kootenay River is one of two major tributaries of the Canadian portion of the Columbia River Basin, the third largest drainage basin in British Columbia (BC). The headwaters of the Kootenay River are nestled in the Rocky Mountains in Kootenay National Park. It flows southwest through the Rocky Mountain Trench near Canal Flats, then continues south into the United States before re-entering BC to join the Columbia River at Castlegar, BC. This study takes place in the upper Kootenay River drainage, which reaches from its source to the first border crossing (Figure 2.1).

Sample Collection

Tissue samples from 981 fish were collected from 23 sample sites in 12 different River systems (Figure 2.1). A detailed description of collection is given in Chapter 2 (page 12), but generally a combination of angling, electro-shocking and minnow-trapping was used to sample fish. To avoid any biases in sampling, fish were clipped as they were encountered until the desired sample size was reached without regard to presumed genotypic status. All tissue samples were stored in 95% ethanol and age class, fork length, and tentative species identification was determined for each fish (see methods in Chapter 2). In Chapter 2, 5/23 sample sites were considered pure WCT populations. Only the 18 samples sites where both species alleles were present are analysed in this chapter.

Cohort Collection & Study Site

The headwaters of the St. Mary River are in the Purcell Mountains. The river flows east to drain into the upper Kootenay River about 2 km north of Fort Steele near Cranbrook, BC. The St. Mary River displays a typical interior snowmelt-driven flow

regime with peak flows occurring in June and low flows occurring in February (Prince 2001). The total river length is 116.42 km. The upper and lower portions of the river are separated by St. Mary Lake (Figure 3.1).

In the St. Mary River, approximately 100 tissue samples were collected from each of the four age classes in 2000 (0+, 1+, 2+, >3+) above and below St. Mary Lake. Age classification was based on size, location of sampling and retention of juvenile characteristics such as parr marks (dark oval bands on the lateral surface of sub-adult fish). The general criteria used to define age classes were: 0+ (fry or young of the year, <55mm in length), 1+ (year old fish, approximately 60-130 mm), 2+ (fish larger than 130 mm that retained parr marks), and >3+ (adults, fish larger than 180 mm that have no retention of juvenile characteristics). Age classes were recorded by individual samplers and, therefore, there is some potential for observer bias. The next summer (2001), each age class was re-sampled to determine if the proportion of hybrids decreased, increased or remained the same over one year. Tissue samples for this analysis were assayed with four diagnostic nuclear markers in the same way as described in Chapter 2.

DNA Extraction and Hybrid Identification

DNA was extracted from each tissue sample (10-20mg) using the GENTRA Puregene DNA Extraction Kit following the manufacturer's protocol, diluted to 100ng/µl, and stored at –20°C. Parental species and hybrids were identified using four diagnostic nuclear markers: two restriction fragment length polymorphisms (RFLPs) and two species-specific simple-sequence repeats (SSR) dual primer PCR (dpPCR) (see Chapter 2 for details of marker selection). To determine if markers were diagnostic for species identification, I tested 30 WCT individuals from three populations that were believed to be pure and 20 RBT individuals from throughout their range, including individuals from known hatchery stocks. In addition to these tests, the authors that developed the diagnostic SSR dpPCR markers tested them on 118 RBT from six different populations and 57 WCT from two populations in Idaho (Ostberg and Rodriguez 2002) and the authors that developed the diagnostic RFLP markers tested them on 12 WCT individuals presumably from U.S. populations (Baker et al. 2002). In all cases, there appeared to be fixed differences between species. The RFLP markers are based on sequence variation in the Ikaros (IK) intron and the Heat Shock protein (Hsc 71) (Baker et al 2002).

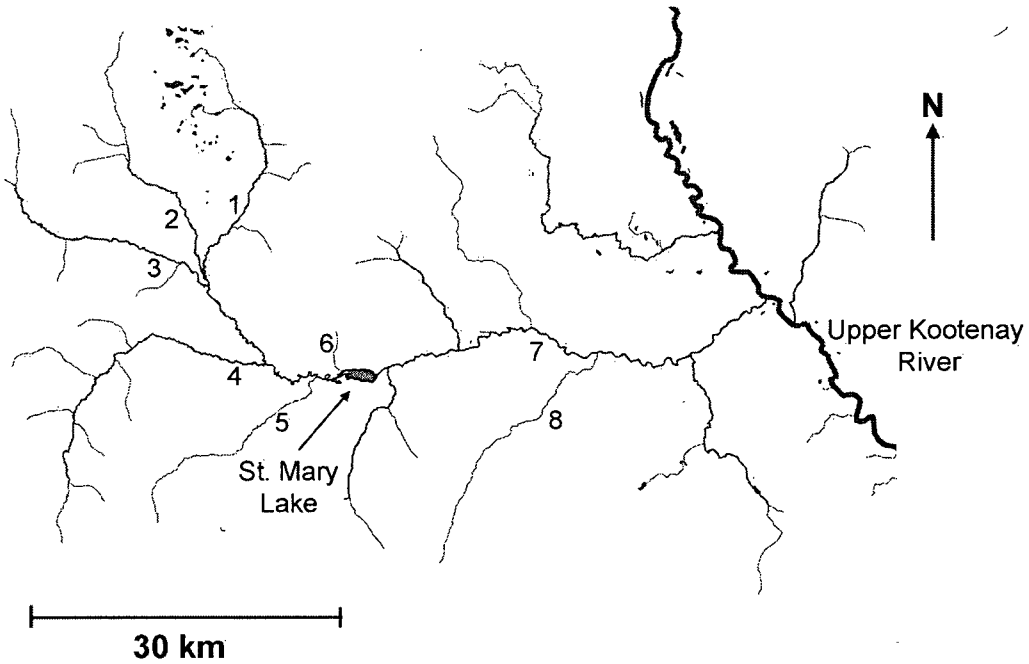


Figure 3.1. St. Mary River. Upper and lower St. Mary River are separated by St. Mary Lake. Tissue samples for cohort analysis were collected from the numbered tributaries 1)White Creek; 2)Dewar Creek; 3)Upper St. Mary River mainstem; 4)Redding Creek; 5)Meachen Creek; 6)Alki Creek; 7) Lower St. Mary River mainstem; 8) Perry Creek. Tributaries 1-6 are from the upper St. Mary River, and 7 & 8 are from of the lower St. Mary River. Please refer to Figure 2.2, page 11 for location of St. Mary River within upper Kootenay River drainage.

These intron regions were amplified using the polymerase chain reaction (PCR) and incubated with restriction enzymes (*Hinf I* for IK and *Taq I* for Hsc 71) following the enzyme supplier protocol (New England Biolabs) with 6 µl of PCR product in a 15µl reaction volume. The two dpPCR markers (OCC16 and OM13) were amplified following conditions indicated in Table 2.2. For more details regarding this procedure please refer to Chapter 2 page 13. PCR products and restriction fragments were visualised on 1.5%-2.5% agarose gels stained with ethidium bromide.

Individuals were classified as 'pure' westslope cutthroat trout (WCT) or rainbow trout (RBT) only if they contained the respective diagnostic alleles at all loci. Individuals were considered to be hybrids if they contained any combination of alleles from the two parental species.

Mitochondrial DNA Analysis

A mitochondrial DNA marker was used to identify the mitochondrial cytotype of a subset of hybrid individuals. The primer combination GluDG/12Sar (Palumbi 1996) amplifies an approximately 3.0 kilobase pair fragment of the mitochondrial DNA spanning the cytochrome b gene, the control region, and a portion of the 12S rRNA gene. Species diagnostic haplotypes are revealed when this fragment is cut with the restriction enzyme, *Ava II* (Table 3.1, E.B. Taylor, unpubl. data). I assayed 35 individuals (24 hybrids, 6 RBT and 5 WCT, as identified with nuclear markers) with mitochondrial DNA to determine if hybridization is symmetrical in these species. Because mitochondrial DNA is maternally inherited, this analysis allowed me to determine which species was the mother in the interspecific matings. Unidirectional mating (i.e. only females of species A mate with males of species B) can often be explained by the behaviour of the fish in the interspecific mating. For example, sneaking is a common parasitic mating behaviour observed in salmonids. Sneaking occurs when a smaller male rushes into the nest of a larger mating pair and attempts to fertilize the eggs (Taborsky 1998). If species B is generally smaller at maturity than species A, the small males may adopt this mating strategy in interspecific matings (e.g. Redenbach and Taylor 2003). In that case, all hybrid individuals would have the mitochondrial haplotype diagnostic of species A.

Table 3.1. Primer sequences, PCR conditions (annealing temperature/number of cycles), and species-specific diagnostic allele sizes for molecular markers used in mitochodrial DNA analyses of westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids.

Primer	Sequence 5'- 3'	Annealing Temp/No.of cycles	Enzyme	Diagnostic Allele sizes
GluDG 12Sar	tga ctt gaa gaa cca ccg ttg ata gtg ggg tat cta act cca gtt	56,54/5,30	*Ava* II	WCT: 1400, 800 RBT: 1600, 600

Data Analysis

Hybrid Classes

At each locus, individuals were scored as WCT if they were homozygous for the WCT alleles, RBT if they were homozygous for the RBT alleles and a hybrid if they were heterozygous at least one of the four loci. Hybrid individuals were subdivided into different hybrid classes; F_1, F_n or backcross genotype classes. The goal of this classification is to crudely classify the hybrids into those that could have recently formed (all heterozygous at all loci, labelled F_1 genotypes) and more advanced hybrids (at least one homozygous locus). These more advanced hybrids were then further classified according to whether they carried homozygous loci from both parental types (labelled F_n genotypes) or only from one parental type (labelled BC (backcross) genotypes). The homozygous loci are diagnostic for WCT in a westslope cutthroat trout backcross genotype (BC_{WCT}) or RBT in a rainbow trout backcross genotype (BC_{RBT}). The intent of these hybrid classes is to describe the genotype of the individuals, and they do not necessarily describe the parentage of an individual because it is nearly impossible to be sure of a hybrids' ancestry in most cases. There are errors associated with these classifications, for example, using four markers, there is a 6.25% chance that an individual classified as an F_1 is actually, the result of a mating between a true F_1 and a parental species. There is also a 6.25% chance of classifying a true later generation hybrid (F_n) a "pure" WCT or RBT. Again, an individual classified as a backcross genotype could be anything from a first generation backcross to an n^{th} generation backcross (complete introgression) and could even be an F_n hybrid. The error associated with distinguishing between a parental genotype and a second or third generation backcross (BC-2 or BC-3) individual is quite high. For example, with four markers, there is approximately a 25% chance that a BC-2 will be classified as a parental individual,

with BC-3 and BC-4 there is a greater chance (~51% and ~72%, respectively) of misclassification (Boecklen and Howard 1997). Consequently, using only four nuclear markers for this type of classification results in an overestimate of pure parental and F_1 individuals and an underestimate of backcross individuals. My analysis, therefore, is a conservative estimate of the number of hybrid individuals present.

Hybrid Zone Structure

To characterize the continuum from a unimodal hybrid zone structure to a bimodal structure, Jiggins and Mallet (2000) recommended that future studies provide individual data or some means of visualizing the distribution of genotypes. This is difficult to do with hybrid classes so I have calculated the hybrid index used by (Szymura and Barton 1991). It is simply a frequency distribution of the number of individuals in the population with 0, 1, 2, . . ., 8 RBT alleles. Because I have used four markers, the maximum hybrid index value possible is 8 (all 8 possible alleles are RBT alleles). An individual with no RBT alleles present at any loci would have a hybrid index of zero (or be considered a "pure" WCT). A problem with this index is that it does not distinguish heterozygotes from homozygotes with alternate alleles. For example, an F_1 individual is heterozygous at all four markers giving a hybrid index value of four; but there are other genotypes that also give a hybrid index value of four, for example an F_n that is homozygous for WCT at two loci and homozygous for RBT at the other two loci. The possible number of F_1 hybrids present, however, will be estimated from the results of the hybrid class analysis.

Population Genetic Analysis

If all individuals collected at a sample site were produced by random mating then allele frequencies should conform to Hardy-Weinberg (H-W) proportions. If, however, immigration of RBT alleles (via pure RBT or hybrids) is recent, or individuals were mating assortatively, or if selection was acting against/for hybrids (heterozygotes in this case) then one would expect deviations from H-W proportions. I calculated Weir & Cockerham's (1984) estimate of the inbreeding coefficient as a measure of heterozygote deficit (Jiggins and Mallet 2000) in populations that deviated significantly from H-W equilibrium using GENEPOP Version 3.1d (Raymond and Rousset 1995). The inbreeding coefficient (F_{IS}) is equal to $1-(P_{Aa}/2p_Ap_a)$ where P_{Aa} is the observed frequency and $2p_Ap_a$ the expected frequency of heterozygotes. F_{IS} values range from -1 to $+1$. Positive values

indicate a heterozygote deficit, negative values a heterozygote excess, and 0 indicates what is expected under random union of gametes.

A state of random gametic association between alleles of different genes is called linkage equilibrium (Hartl and Clark 1997). Linkage disequilibrium (i.e. non-random gametic association between alleles of different genes) between pairs of loci is helpful to describe the distribution of hybrid genotypes and to estimate the "age" of the hybridized population (Forbes and Allendorf 1991a). Strongly bimodal populations will have high linkage disequilibrium and near maximum heterozygote deficiencies, whereas hybrid swarms or unimodal populations will not reveal any genetic associations among parental alleles if they are a randomly mating hybrid swarm (Jiggins and Mallet 2000; Allendorf et al 2001). When previously isolated and genetically distinct populations (i.e. WCT and RBT) come into secondary contact, gametic disequilibria will be high initially. Eventually it will erode over time if: there is some level of mixing between gene pools, there is no selection, and there is no dispersal of parental types continually into the zone of contact. Recent immigration of parental types, positive assortative mating, or selection against hybrids, however, will slow or prevent the disappearance of disequilibria. Given that hybrid swarms have been documented between WCT and RBT in other drainages (i.e. randomly mating populations; e.g., Leary et al. 1984), the presence of linkage disequilibrium in hybridized populations of these species suggests that random mating has not yet been established and hybridization is recent. The absence of linkage disequilibrium in hybridized populations, however, does not exclude the possibility of recent hybridization. In populations where there is recent hybridization of post-F_2 or backcrossed individuals linkage disequilibrium may not be present.

I calculated linkage disequilibrium between all six possible pairs of loci using GENETIX (Belkir et al 1999). GENETIX uses the recompiled version of the FORTRAN program LINKDOS (Black and Krafsur 1985) described in Garnier-Gere and Dillman (1992). The correlation coefficient (R_{ij}), described by Weir (1979), is determined from the composite linkage disequilibrium coefficient, $\Delta_{ij,}$ which takes into account associations between alleles whether they are in coupling or repulsion (Black and Krafsur 1985). Cockerham and Weir (1977) recommended using the composite linkage disequilibrium coefficient, Δ_{ij} , (as opposed to D, the standard linkage disequilibrium coefficient) when working with genotypic data from natural populations because it is not

biased by departures from random mating and gamete frequencies are seldom known from field data. A sequential Bonferroni correction for multiple tests was used to prevent artificial inflation of alpha values in both the H-W equilibrium test and the LD tests. To compare the mean F_{IS} values and the mean R_{ij} values between years (1999 and 2000) in sample sites that were sampled in both years (lower Gold Creek and lower St. Mary River) I used an Independent-Samples T-Test. These statistics were conducted using the statistical software SPSS 11.0.

Detecting Selection using Cohort Analysis

To conclude that selection is acting against hybrids in a hybrid zone, one must demonstrate that a heterozygote deficit exists and that the deficit is the product of selection against hybrids (Bert and Arnold 1995). By comparing the proportion of hybrid individuals among cohorts of defined ages and by examining the relationship between age class and heterozygote deficiencies, I can assess whether there is evidence for selection against hybrids. For example, if selection is eliminating hybrids from the population, as age increases the proportion of hybrid individuals should decrease, and measures of heterozygote deficiency and linkage disequilibrium should increase.

I used both a static and a dynamic cohort analysis to determine if differential survival between hybrids and parental individuals was occurring. A static cohort analysis involves sampling each age class in the same year. The fish were collected in August 2000 and assigned to an age cohort based on size and retention of juvenile characteristics (see collection methods for more details). The limitations associated with static cohort analysis are presented in the discussion. In August 2001, I collected tissue samples from each of the age classes again, re-sampling each cohort from the previous year (a dynamic cohort analysis). The dynamic analysis allowed me to follow each age class over one year, and determine if the proportion of hybrids changed during the course of that year.

I calculated the following parameters at each age class to determine if a relationship existed between these parameters and the age of the cohort: (1) proportion of hybrid individuals at each age class, (2) Weir and Cockerham's (1984) estimate of the inbreeding coefficient as a measure of heterozygote deficit (F_{IS}), and (3) the linkage disequilibrium correlation coefficient R_{ij} (Weir 1979). I calculated the mean value for F_{IS} and R_{ij} across all loci for each age class. I compared the means between age classes using

a one-way analysis of variance (ANOVA) if all assumptions were met, and a non-parametric Kruskal-Wallis test if ANOVA assumptions (i.e. homogeneity of variances) were violated. The proportion of hybrid individuals was compared across age classes using χ^2 contingency tests and Fisher's exact tests. Both SPSS 11.0 and JMPIN 4.0 statistical software were used. For a finer scale analysis I compared genotypic frequencies of homozygotes (WCT), heterozygotes (hybrids), and homozygotes (RBT), across cohorts locus by locus using Monte Carlo-statistics on R x C matrices (http://itb.biologie.hu-berlin.de/~nils/stat/). One thousand random matrices were generated with the same row and column totals as in the empirical matrix. Methods of the randomization and generation of the test statistics are described in more detail in Blüthgen et al. (2000).

Results

Hybrid Classes

Westslope cutthroat trout backcrosses (BC_{WCT}) were the most common hybrid genotype. In 1999, 50% and in 2000, 60% of the hybrid fish were classified as BC_{WCT} (Table 3.2). The next most common class of hybrids was later generation hybrids (F_n) at 29% in 1999 and 23% in 2000. Rainbow trout backcrosses (BC_{RBT}) were more common in 1999 at 21% than in 2000 at 12%. In both years, first generation hybrids (F_1) were absent or very rare in this system. Only four potential F_1 hybrids were observed across all 142 hybrids examined. All F_1 individuals were collected in 2000 from three sites; Lodgepole Creek on the Wigwam River system, Bloom Creek (a tributary of Gold Creek) and lower Gold Creek (Figure 3.2, See appendix 1 for breakdown of hybrid classes by sample site).

Table 3.2. Classification of hybrid individuals collected from the upper Kootenay River drainage in 1999 and 2000.

Hybrid Class	*1999(n=358, hyb=28)*	*2000 (n=625, hyb=114)*
Backcross WCT (BC_{WCT})	14/28	70/114
Backcross RBT (BC_{RBT})	6/28	14/114
$\geq 2^{nd}$ gen. (F_n)	8/28	26/114
1^{st} gen. hybrid (F_1)	0/28	4/114

Fish classified as WCT were found at every site sampled except for lower Bull River where all but five fish were identified as RBT, the remaining five were of hybrid descent. Also, the majority of fish at all sites (with the exception of two: lower Bull River and Lodgepole Creek) were classified as WCT (Table 3.3). Of the 30 fish sampled at Lodgepole Creek, only a third were classified as WCT. Lodegpole Creek and lower Gold Creek were the only sample sites that contained every hybrid class and both WCT and RBT parental genotypes (Figure 3.2). Four sites (upper Kootenay River, upper Skookumchuk Creek, Coal Creek, Wild Horse River) have WCT and BC_{WCT} only, and seven sites have WCT, BC_{WCT} and F_n. Although, pure RBT were only found at three locations, BC_{RBT} were found at an additional three locations (lower Skookumchuk Creek, lower St. Mary River and Michel Creek) providing evidence that RBT are in these systems or at least in nearby streams.

Hybrid Zone Structure and Population Genetic Analysis

The hybrid zone structure for the majority of the populations was unimodal but left-skewed towards "pure" WCT (e.g. Wild Horse River Figure 3.3a.) with no RBT parental types present. These populations did not significantly deviate from H-W proportions and did not show significant linkage disequilibrium indicating that they most likely are randomly mating populations. The upper Kootenay River, the White River, Morrissey Creek, the Wigwam River, the upper Skookumchuk River, Teepee Creek, upper Gold Creek, Coal Creek, the lower St. Mary River (2000) and Mather Creek all have very similar genotypic distributions to the Wild Horse River (See Appendix 2 for these and the other sample site distributions). Lodgepole Creek (Figure 3.3b) has a flat distribution. Lower Gold Creek (Figure 3.3c) has a bimodal distribution. The lower Bull River genotypic distribution (Figure 3.3d) is right skewed towards RBT, with no WCT parental types present.

Table 3.3. Number of individuals classified as westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids based on four diagnostic nuclear markers. The percentage of hybrid individuals is further broken down into hybrid classes in Figure 3.2 where the letter shown here corresponds to the sample site in the figure.

Site (*n*)	**WCT (%)**	**Hybrids (%)**	**RBT (%)**
A. upper Kootenay River (*15*)	12 (80.0)	3 (20.0)	0
B. White River (*33*)	29 (87.9)	4 (12.1)	0
C. Lussier River (30)	25 (83.3)	5 (16.7)	0
D. upper Skookumchuk Creek (*40*)	39 (97.5)	1 (2.5%)	0
E. lower Skookumchuk Creek (*33*)	30 (90.9)	3 (9.1)	0
F. Mather Creek (30)	18 (60.0)	12 (40.0)	0
G. Wild Horse River (45)	28 (62.2)	17 (37.8)	0
H. lower St. Mary River 1999 (*31*)	27 (87.1)	4 (12.9)	0
lower St. Mary River 2000 (*104*)	87 (83.7)	17 (16.3)	0
I. Michel Creek (30)	21 (70.0)	9 (30.0)	0
J. lower Bull River (30)	0	5 (16.7)	25 (83.3)
K. Coal Creek (36)	32 (88.9)	4 (11.1)	0
L. Morrissey Creek (*30*)	29 (96.7)	1 (3.3)	0
M. Lodgepole Creek(30)	10 (33.3)	17 (56.7)	3 (10.0)
N. Wigwam River (*34*)	31(91.2)	3 (8.8)	0
O. lower Gold Creek 1999 (36)	25 (69.4)	9 (25.0)	2 (5.6)
O. lower Gold Creek 2000 (30)	20 (66.7)	7 (23.3)	3 (10.0)
P. Bloom Creek (30)	19 (63.3)	11 (36.7)	0
Q. Teepee Creek (30)	25 (83.3)	5 (16.7)	0
R. upper Gold Creek (30)	26 (86.7)	4 (13.3)	0

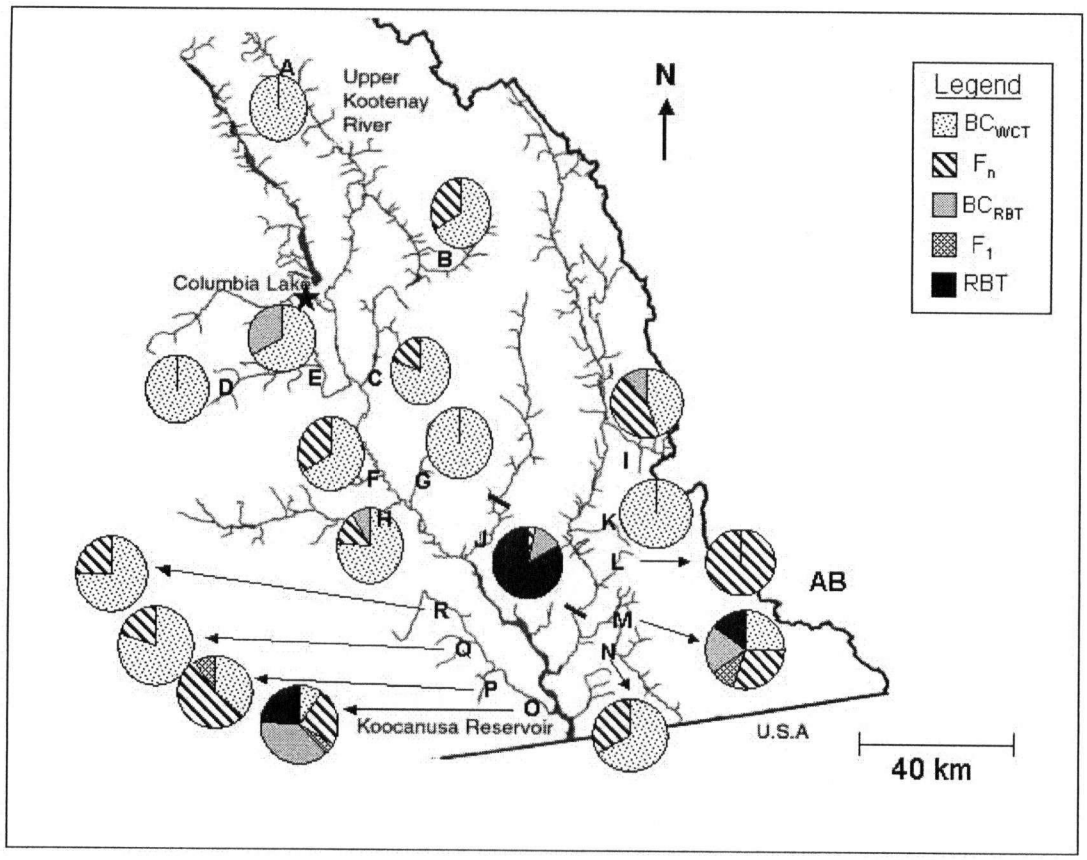
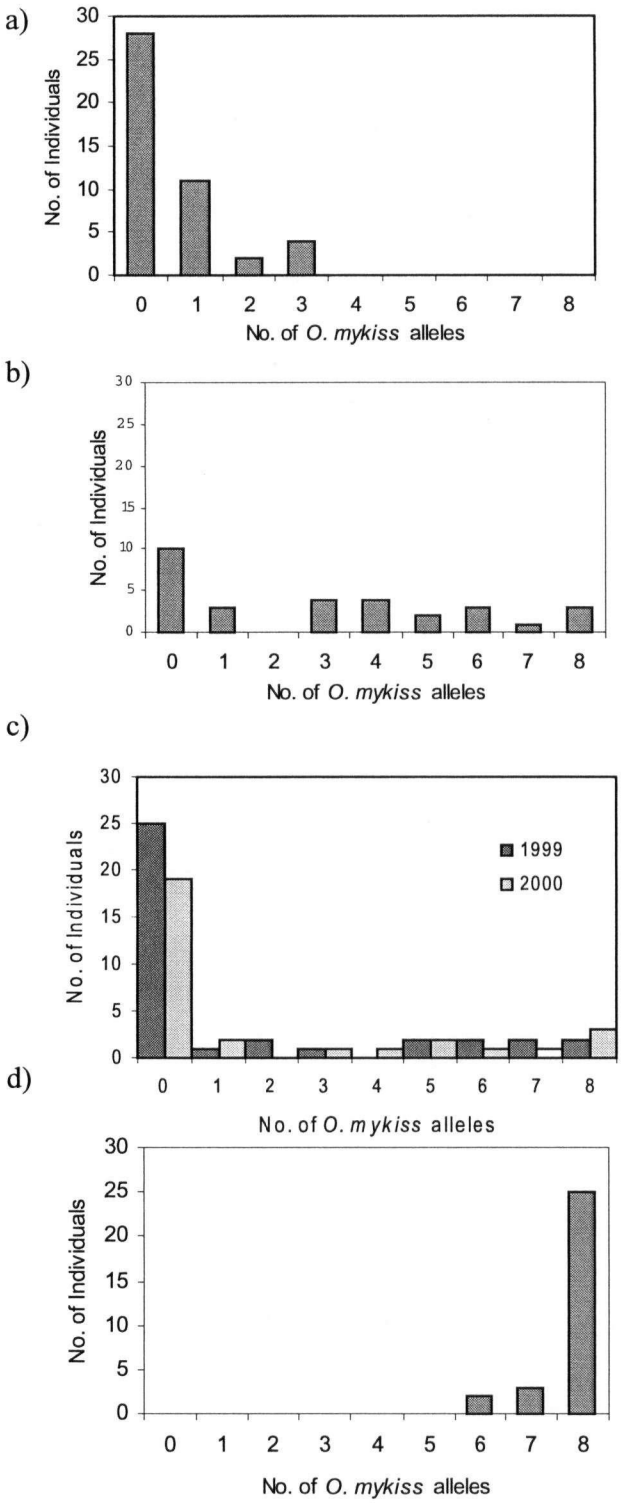


Figure 3.2. Classification of genotypes collected in the upper Kootenay River drainage, British Columbia, that were *not* identified as pure westslope cutthroat trout. Pie charts represent the proportion of hybrid classes and rainbow trout identified. The proportion of individuals identified as hybrids differs between sample sites; this information is shown in Table 3.3. The letter indicates the sampling location and the pie chart closest to the letter represents the hybrid classes at that sample location (unless otherwise indicated, i.e. arrows). Refer to Table 3.3 for site labelling. Black bars represent impassable hydro dams and the star represents an impassable canyon.

Westslope cutthroat trout allele frequencies (averaged over 4 loci) varied with sample location from 0.03 in the lower Bull River to 0.63 - 0.99 across the other 17 sample sites containing both species alleles (See Appendix 3 for break down of allele frequencies for each sample site). Four admixed sites significantly deviated from Hardy-Weinberg (H-W) proportions, and seven sites showed significant linkage disequilibrium. Lower Gold Creek (both 1999 & 2000 samples), lower St. Mary River (1999 only) Lodgepole Creek, and Michel Creek all significantly deviated from H-W proportions and had significant linkage disequilibrium (Table 3.4 & 3.5). Three sample sites, Lussier River, Bloom Creek, and lower Skookumchuk Creek conformed to H-W equilibrium (average F_{IS} = 0.139, 0.119, and –0.009 respectively), but they exhibited significant linkage disequilbrium between pairs of loci. It is important to note that H-W equilibrium can be restored after one generation of random mating, whereas linkage relationships decay more slowly, depending on the rate of recombination and the mating regime. The samples collected from lower St. Mary River in 1999 deviated from H-W equilibrium (average F_{IS} = 0.794), but the 2000 samples from the same population did not (average F_{IS} = 0.142) suggesting that RBT hybridization is relatively recent at this site.

All markers in the four populations that deviated from H-W equilibrium had significant positive F_{IS} values indicating a heterozygote deficiency. Only lower Gold Creek 2000 had a significant heterozygote deficiency at all four markers (F_{IS} ranged from 0.521-0.637). In Michel Creek, Occ16 was fixed for the WCT allele, therefore, F_{IS} was not calculated in this case. In all four populations, it is a heterozygote deficiency that is driving the deviation from H-W equilibrium indicating that hybrid genotypes are not as abundant as expected (under H-W) based on the observed alleles frequencies.

Gametic disequilibria were calculated for all six nuclear locus pairs for all 18 localities that showed evidence of hybridization (Table 3.5). Contingency tests revealed significant genetic associations (H_o = genotypes at one locus are independent from genotypes at another locus) between at least two of the possible six locus pairs in 7/18 admixed sites. There is a wide range of calculated R_{ij} values from 0.046-0.999. The marker Occ16 was fixed for the WCT allele at the Lussier River site and therefore R_{ij} cannot be calculated in Occ16 pairs at this site. All calculated R_{ij} values were positive indicating an association between alleles from the same parental species.



Figures 3.3*a-d*. The hybrid zone structure of westslope cutthroat trout (*O. clarki lewisi*) and rainbow trout (*O. mykiss*) in four populations in the upper Kootenay River drainage. Values on the x-axis represent the number of RBT alleles present ranging from zero ("pure" WCT) to eight ("pure" RBT). a) Wild Horse River; b) Lodgepole Creek; c) lower Gold Creek; d) lower Bull River.

Four out of the six pairs of loci in the lower Gold Creek 1999 samples showed significant linkage disequilibrium; the 2000 samples from lower Gold Creek showed all six pairs in significant linkage disquilibrium. The average R_{ij} value in 1999 was 0.474 whereas the average R_{ij} value in 2000 was significantly higher at 0.933 (t=-4.2, df=10, p=0.002). In contrast, in the two years that the lower St. Mary's river was sampled, the opposite was true. The average R_{ij} for 1999 was 0.659, and for 2000 it was significantly lower at 0.309 (t=3.683, df=10, p=0.004) indicating that linkage disequilibrium had decayed over the year.

Mitochondrial DNA Analysis

Fish from two sample sites that contained individuals from all hybrid classes, lower Gold Creek and Lodepole Creek, were assayed to determine their mitochondrial DNA haplotypes. Mitochondrial DNA from 21 hybrids from these sites were successfully amplified (15 from Lodgepole Creek, 6 from lower Gold Creek). Two hybrids (an F_n and BC_{WCT}) from Michel Creek was assayed and one F_1 hybrid fingerling from the lower St. Mary River were also examined. There appears to be no directionality to hybridization between these two species because both species' haplotypes were observed in the hybrid individuals tested (Table 3.6). A Fisher's exact test revealed that there was no difference in the ratio of WCT haplotypes to RBT haplotypes in hybrids between the two sites ($p = 0.36$) (Figure 3.4). Five pure WCT (nuclear DNA identified) from Findlay Creek and six pure RBT from lower Bull River were also assayed to determine if there were any discrepancies between the nuclear and mitochodrial identifications.

The mtDNA analysis on the four F_1 hybrids (only three successfully amplified) showed that male westslope cutthroat trout mate with female rainbow trout and vice versa. All nuclear identified WCT had WCT mtDNA, but one of the six nuclear identified RBT had the diagnostic WCT haplotype. This individual was most likely a misclassified BC_{RBT}. From the 23 hybrids (classified into any one of the four hybrid classes) assayed with mtDNA, 13 had the RBT haplotype and 10 had the WCT haplotype (Figure 3.5).

Table 3.4. Inbreeding coefficient (F_{IS}; Weir and Cockerham 1984), a measure of heterozygote deficit, for four nuclear loci in three populations that significantly deviated from Hardy-Weinberg proportions (in all cases $p \leq 0.0002$). F_{IS} values range from −1 to +1, with positive values indicating heterozygote deficit, negative values heterozygote excess, and 0 equal to expected under random mating. Stars (*) indicate F_{IS} values statistically significant from zero at an alpha level of 0.05, corrected for multiple tests with a sequential Bonferroni (0.05/80=0.0006)

Location	F_{IS}			
	Ikaros	Hsc 71	Occ 16	Om13
L.St. Mary's River 1999	-0.034	1*	1*	1*
L.Gold Creek 1999	-0.029	0.862*	0.316	0.645*
L.Gold Creek 2000	0.521	0.601*	0.637*	0.594*
Lodgepole Creek	0.367	0.657*	0.356	0.440
Michel Creek	0.354	0.766*	-	0.529*

Table 3.5. Linkage disequilibrium correlation coefficient R_{ij} for all pairs of nuclear loci (Weir 1979), in all populations that indicated significant linkage between pairs of loci calculated by GENETIX. R_{ij} values range from −1 to+1. When $R_{ij} = 0$ then the loci are in linkage equilibrium. Stars (*) indicate pairs that are statistically significant from zero at an alpha level of 0.05, corrected for multiple tests with a sequential Bonferroni (0.05/120=0.0004)

Location	R_{ij}					
	Ikaros/Hsc	Ikaros/Occ16	Ikaros/Om13	Hsc/Occ16	Hsc/Om13	Occ16/Om13
L. Skookumchuk Creek	0.480	0.046	0.482	*0.719	*0.999	*0.718
L.Gold Creek 1999	*0.515	0.216	0.103	*0.793	*0.570	*0.648
L.St. Mary River 1999	0.370	*0.576	*0.576	*0.718	*0.718	*0.999
L.Gold Creek 2000	*0.937	*0.999	*0.934	*0.960	*0.856	*0.914
Lussier River	*0.850	Fixed	*0.959	Fixed	*0.973	Fixed
Bloom Creek	0.472	0.268	*0.541	0.329	*0.598	0.541
Lodgepole Creek	*0.739	*0.643	*0.737	*0.445	*0.631	*0.684
Michel Creek	*0.754	0.557	*0.948	0.457	*0.821	0.492

Table 3.6. Mitochondrial DNA analysis on westslope cutthroat trout, rainbow trout and their hybrids. LC = Lodgepole Creek; MC = Michel Creek; Fin = Findlay Creek; GCLW = lower Gold Creek; STLW = lower St. Mary River

Individual	Nuclear DNA genotypic class	mtDNA haplotype
LC 01	BC_{RBT}	RBT
LC 02	F_1	WCT
LC 03	F_n	WCT
LC 06	RBT	RBT
LC 09	RBT	RBT
LC 11	BC_{RBT}	WCT
LC 12	F_n	RBT
LC 13	RBT	WCT
LC 15	BC_{WCT}	WCT
LC 16	BC_{WCT}	WCT
LC 17	BC_{RBT}	WCT
LC 18	BC_{WCT}	WCT
LC 19	F_n	RBT
LC 20	WCT	WCT
LC 21	BC_{RBT}	RBT
LC 22	F_n	WCT
LC 23	F_n	WCT
LC 26	BC_{WCT}	RBT
LC 29	F_n	RBT
MC 08	BC_{WCT}	RBT
MC 17	F_n	RBT
Fin 04	WCT	WCT
Fin 09	WCT	WCT
Fin 25	WCT	WCT
Fin 33	WCT	WCT
GCLW 02	BC_{RBT}	WCT
GCLW 06	F_1	RBT
GCLW 07	BC_{WCT}	WCT
GCLW 04	BC_{WCT}	RBT
GCLW 37	BC_{RBT}	RBT
GCLW 40	RBT	RBT
GCLW 41	BC_{RBT}	RBT
GCLW 42	RBT	RBT
GCLW 05	RBT	RBT
*STLW 226	F_1	RBT

*Individual was not including in regional study. This particular individual is an age 1+ individual from the cohort analysis in the St. Mary River system (see next section).

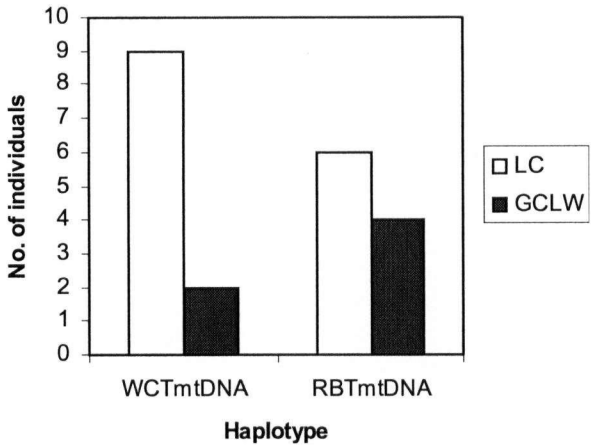


Figure 3.4. Mitochondrial analysis of *O. clarki lewisi* (WCT) and *O. mykiss* (RBT) hybrid individuals at two sites, Lodgepole Creek (LC-white) and lower Gold Creek (GCLW-Black)

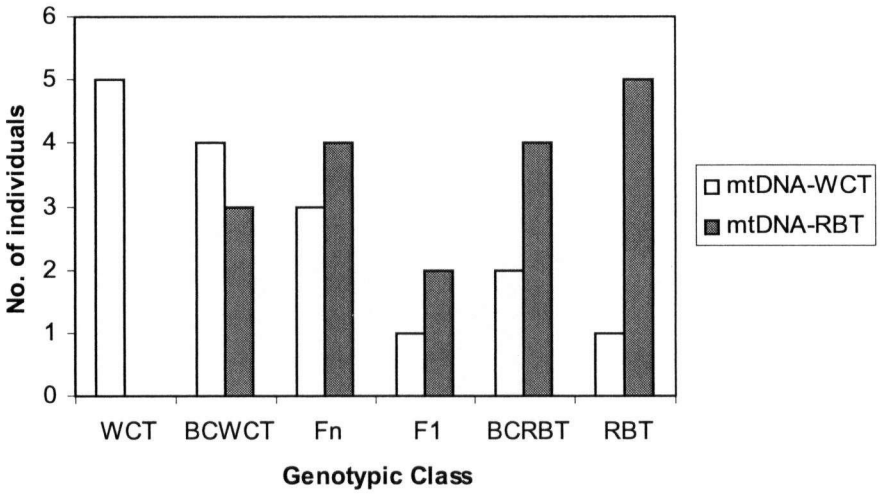


Figure 3.5. Classification of *O. clarki lewisi* and *O. mykiss* hybrid individuals with mitochondrial DNA haplotypes of *O. clarki lewisi* (WCT, white) or *O. mykiss* (RBT, gray)

Cohort Analysis in St. Mary River

Between August 7 and August 14, 2000, 409 fish were sampled from the lower river and 409 fish were sampled from the upper St. Mary River (Table 3.7). In the lower river, 19.8 % were identified as hybrid individuals. No hybrids were detected in the upper river above St. Mary Lake. Pure RBT were not identified anywhere in this system in 2000. I was unable to collect samples from every age classes in the summer of 2001, and the difficulty of catching older age classes in 2001 was attributed to warmer summer water temperatures and lower water levels than the year before. There was below normal precipitation in the winter of 2001 and above normal temperatures in the summer of 2001 (Environment Canada). Most laboratory experiments indicate that trout reduce and eventually cease feeding as water temperatures rise above 22°C (Dickson and Kramer 1971). At the time of sampling the WCT had most likely moved from feeding areas into deeper cooler pools, making them difficult to sample via angling. There were no >3+ and only five 2+ trout that were successfully sampled and assayed in 2001.

A total of 100 fish were sampled between August 16-August 22, 2001 from the lower river, 29% of these fish were identified as hybrid individuals and one pure RBT juvenile was identified (Table 3.8). I did not resample the upper St. Mary River in 2001 because no hybrids were detected in 2000. My results suggest that hybrids and rainbow trout are extremely rare or absent in the upper river considering that 409 fish were sampled and not one fish was identified as such. The majority of the hybrids identified in all age classes in both years were classified as BC_{WCT} (Figure 3.5 a & b). One F_1 hybrid was found in the 1+ cohort in 2000. This individual had the mitochondrial DNA haplotype diagnostic of RBT (Table 3.8).

The percentage of hybrids in the static analysis ranged from 16.3% in the >3+ cohort to 24.7% in the 2+ cohort (Table 3.7). There was no significant difference in the proportion of hybrid individuals identified between each age class in 2000 ($\chi^2 = 2.41$, $p = 0.49$) suggesting that the rate of hybridization when the adults were produced (approximately 3 years ago) is similar to rate of hybridization in 2000. The stability in the proportion of hybrids over time also suggests that selection does not play a significant role in eliminating hybrids from the population. Although sample sizes were smaller in 2001, a significant difference between cohorts sampled in 2001 was detected ($\chi^2 = 11.68$,

p = 0.003). Thirteen percent of the 2001 0+ cohort were hybrids whereas 43% and 40% of the 1+ and 2+ samples were identified as hybrids (Table 3.8).

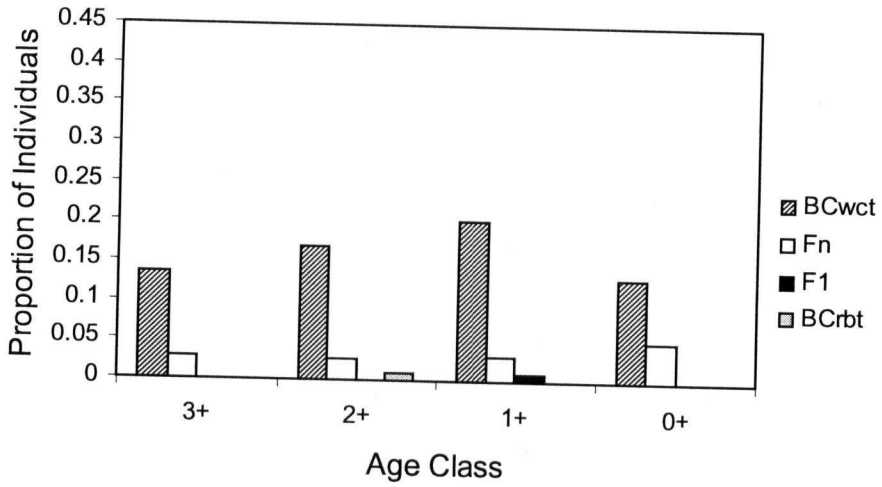
Table 3.7. Mean length (cm) of each cohort +/- the standard deviation (SD) and the genetic identification of westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids collected from defined cohorts in the St. Mary River in 2000.

Age Class *(n)*	Mean Length (cm) +/-SD	No. of WCT (%)	No. of hybrids (%)	No. of RBT (%)
Lower River				
>3+ *(104)*	33.3 +/- 6.10	87 (83.7 %)	17 (16.3 %)	0
2+ *(93)*	18.4 +/- 4.52	70 (75.3 %)	23 (24.7%)	0
1+ *(112)*	11.2 +/- 2.3	89 (79.5%)	23 (20.5%)	0
0+ *(100)*	Not recorded	82 (82%)	18 (18%)	0
Total *(409)*		328 (80.25)	81 (19.8%)	0
Upper River				
>3+ *(101)*	31.1 +/- 5.29	101	0	0
2+ *(129)*	20.2 +/- 3.12	129	0	0
1+ *(104)*	12.8 +/- 1.70	104	0	0
0+ *(75)*	Not recorded	75	0	0
Total *(409)*		409 (100%)	0	0

Table 3.8. The genetic identification of westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids collected from four defined cohorts the St. Mary River in 2001

Age Class (*n*)	No. of WCT (%)	No. of hybrids (%)	No. of pure RBT (%)
>3+ *(0)*	N/A	N/A	N/A
2+ *(5)*	2 (40%)	2 (40%)	1 (20%)
1+ *(49)*	28 (57%)	21 (43%)	0
0+ *(46)*	40 (87%)	6 (13%)	0
	70 (70%)	29 (29%)	1 (1%)

a)



b)

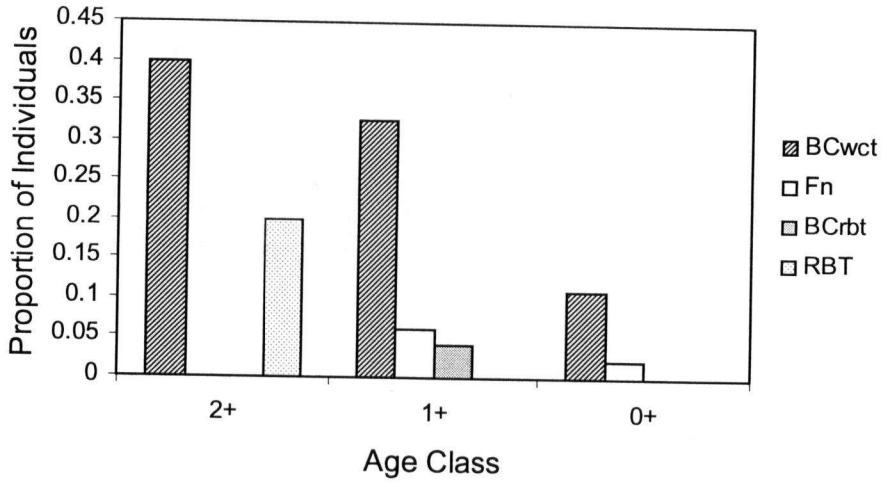


Figure 3.6. Genotypic classification of *O. clarki lewisi* (WCT) and *O. mykiss* (RBT) hybrids across age classes for (a) individuals collected in 2000, and (b) individuals collected in 2001.

There was no detectable difference in the frequencies of homozygous WCT genotypes, heterozygous hybrid genotypes, and homozygous RBT genotypes in the 2000 samples at any of the four loci across age classes ($p = 0.112 - 0.472$; Table 3.9 a-d). There were, however, significant differences in genotypic frequencies between loci ($p<0.0001$, Table 3.10). The Hsc 71 marker identified more homozygous RBT genotypes than the other 3 markers. Upon removal of the Hsc 71 frequencies from the contingency test, significant differences were still detected ($p=0.006$) suggesting that the Occ16 marker identified fewer heterozygous hybrid genotypes than the other two markers. Upon removal of both Hsc 71 and Occ16, the result of the contingency test was non-significant ($p = 0.48$). These results indicate that there is a possibility that parts of the genome are introgressing at different rates.

Of the 2000 samples, the 2+ and the 0+ cohorts deviated significantly from H-W proportions (Table 3.7). In both cases the Hsc 71 marker showed a significant heterozygote deficiency. Both the Ikaros and the Occ16 markers appear to have F_{IS} values approaching zero in every cohort, but the other two markers (Hsc 71 and Om13) have positive F_{IS} values in every cohort. Furthermore, in nearly every cohort, the Hsc 71 marker shows a higher F_{IS} value than any of the other markers. The mean F_{IS} value, however, did not differ significantly between cohorts ($F=0.357$, $df = 3$, $p = 0.79$) indicating there was no detectable decrease or increase in the heterozygote deficit with age.

All four cohorts showed significant positive linkage disequilibrium. The R_{ij} values ranged from 0.026-0.627 (Table 3.8). A Kruskal-Wallis test comparing the mean R_{ij} value between cohorts indicated that was no significant difference in this value between cohorts. My results, therefore, provide little evidence that hybrids (i.e. heterozygotes) are being removed via selection over time.

Table 3.9a-d. Locus by locus genotypic frequencies of homozygous westslope cutthroat trout genotypes (ww), hybrid genotypes (wr) and homozygous rainbow trout genotypes (rr) by age class. Adults (3+), juveniles (2+), fingerling (1+) and fry (0+) from lower St. Mary River were collected in August 2000. Monte-Carlo randomizations (1000 runs) were used on R x C contingency tables to determine if the genotypic frequency differed with age class. The observed test statistic (T_{obs}) and associated p-value is shown.

a) Locus Ikaros

		Genotypic Frequency			T_{obs}, p-value
Age	*n*	ww	wr	rr	
3+	104	95	9	0	
2+	93	75	17	1	
1+	112	100	12	0	
0+	99	91	8	0	1741.83, p = 0.122
All Ages	408	361	46	1	

b) Locus hsc 71

		Genotypic Frequency			T_{obs}, p-value
Age	*n*	ww	wr	rr	
3+	100	90	8	2	
2+	88	77	7	4	
1+	112	96	13	3	
0+	98	89	4	5	1664.54, p = 0.472
All Ages	398	352	32	14	

c) Locus Occ16

		Genotypic Frequency			T_{obs}, p-value
Age	n	ww	wr	rr	
3+	103	101	2	0	
2+	90	85	4	1	
1+	112	105	7	0	
0+	100	95	5	0	1741.83, p = 0.122
All Ages	405	386	18	1	

d) Locus Om 13

		Genotypic Frequency			T_{obs}, p-value
Age	n	ww	wr	rr	
3+	104	99	4	0	
2+	89	82	6	2	
1+	111	100	11	0	
0+	99	93	6	0	1741.83, p = 0.122
All Ages	403	374	27	2	

Table 3.10. Pooled genotypic frequencies of: homozygous westslope cutthroat trout genotypes (ww), hybrid genotypes (wr) and homozygous rainbow trout genotypes (rr) from lower St. Mary River by loci. Monte-Carlo randomizations (1000 runs) were used on R x C contingency tables to determine if the genotypic frequency differed among loci. The observed test statistic (T_{obs}) and associated p-value is shown

		Genotypic Frequency			T_{obs}, p-value
Locus	*n*	ww	wr	rr	
Ikaros	408	361	46	1	
Hsc 71	398	352	32	14	
Occ 16	405	386	18	1	
Om 13	403	374	27	2	9155.20, p = <0.0001

Table 3.11. Inbreeding coefficient (F_{IS}; Wier & Cockerham 1984), a measure of heterozygote deficit, for four nuclear loci in the four cohorts sampled from lower St. Mary River in 2000. F_{IS} values range from –1 to +1, with positive values indicating heterozygote deficit, negative values heterozygote excess, and 0 equal to expected under random mating. Stars (*) indicate F_{IS} values statistically different from 0 using a sequential Bonferroni corrected alpha level of 0.003 (0.05/16). The Mean F_{IS} value and the standard deviation are also shown for each cohort.

Cohort	Ikaros	Hsc 71	Occ16	Om13	Mean (SD)
>3+	-0.04	0.295	-0.005	0.318	**0.144 (0.189)**
2+	0.009	0.494*	-0.017	0.369	**0.032 (0.257)**
1+	-0.05	0.257	-0.028	-0.048	**0.213 (0.150)**
0+	-0.037	0.695*	-0.021	-0.026	**0.136 (0.362)**

Table 3.12. Linkage disequilibrium R_{ij} (Weir 1979) for all pairs of nuclear loci in all four cohorts collected from lower St. Mary River 2000 calculated by GENETIX (Belkir *et al.* 1999). R_{ij} values range from –1 to+1. When $R_{ij} = 0$, the loci are in linkage equilibrium. Stars (*) indicate pairs that are statistically significant from zero at an alpha level of 0.05, corrected for multiple tests with a sequential Bonferroni (0.05/24=0.002). Mean R_{ij} and standard deviation (SD) are also included.

Cohort	R_{ij}						
	Ik/Hsc	Ik/Occ16	Ik/Om13	Hsc/Occ16	Hsc/om13	Occ16/Om13	Mean R_{ij} (SD)
> 3+	0.363*	0.208	0.443*	0.391*	0.218	0.230	**0.309(0.163)**
2+	0.627*	0.077	0.026	0.486*	0.066	0.595*	**0.313(0.285)**
1+	0.592*	0.289*	0.271*	0.569*	0.322*	0.489*	**0.422(0.145)**
0+	0.348*	0.331*	0.069	0.101	0.127	0.466*	**0.240(0.163)**

Due to sampling difficulties in the older age classes in 2001, the dynamic cohort analysis follows only one age class for one year (0+(fry 2000) – 1+ (fingerling 2001)). The proportion of hybrids more than doubled over the year, from the fry to fingerling stage (Table 3.13) suggesting that hybrid individuals survived better than pure WCT over their first year ($\chi^2 = 10.1$, $p = 0.0015$). As mentioned, the 0+ cohort deviated from H-W proportions indicating a heterozygote deficiency at the Hsc marker, but after one year as observed in the 1+ stage, this deficiency is no longer significantly different from zero (Table 3.14). The 1+ samples conformed to H-W proportions suggesting that there are more heterozygotes (i.e. the cohort is no longer heterozygote or hybrid deficient) relative to homozygotes (parentals) after one year of life; thus more hybrids than parentals survived the first year of life. Both age classes showed significant linkage disequilibrium (Table 3.15) and there is no significant difference in the mean R_{ij} value between ages ($F= 4.7$, $df = 1$, $p= 0.06$).

Table 3.13. Genetic identification of westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids from the same cohort sampled over one year (2000-2001).

Age of cohort	Sample Number	No. of hybrids (%)	No. of pure RBT (%)
1+	49	21 (43%)	0
0+	100	18 (18%)	0

Table 3.14. Inbreeding coefficient (F_{IS}; Wier & Cockerham 1984), a measure of heterozygote deficit, for four nuclear loci in aged 0+ cohort from lower St. Mary River 2000 (as shown in Table 3.7). The same cohort was measured in 2001, at age 1+ to compare values of F_{IS}. F_{IS} values range from –1 to +1, with positive values indicating heterozygote deficit, negative values heterozygote excess, and 0 equal to expected under random mating. Stars (*) indicate F_{IS} values statistically different from 0 at a Bonferroni corrected alpha level of 0.0125 (0.05/4). The Mean F_{IS} value and the standard deviation are also shown for each cohort.

Age of cohort	IK	Hsc 71	Occ16	Om13	Mean (SD)
1+	-0.011	0.186	-0.033	0.196	**0.064 (0.099)**
0+	-0.037	0.695*	-0.021	-0.026	**0.136 (0.362)**

Table 3.15 Linkage disequilibrium, R_{ij}, (Weir 1979) for all six pairs of nuclear loci, in aged 0+ cohort collected from lower St. Mary River 2000 calculated by GENETIX (Belkir *et al.* 1999). The same cohort was measured in 2001 at age 1+ to compare values of R_{ij}. R_{ij} values range from –1 to+1. When $R_{ij} = 0$ then the loci are in linkage equilibrium. Stars (*) indicate pairs that are statistically significant from zero at an alpha level of 0.05, corrected for multiple tests with a sequential Bonferroni (0.05/6=0.008). Mean R_{ij} and standard deviation (SD) are also included.

Age of Cohort	R_{ij}						
	Ik/Hsc71	Ik/Occ16	Ik/Om13	Hsc71/Occ16	Hsc71/om13	Occ16/Om13	Mean R_{ij} (SD)
1+	0.501*	0.367*	0.637*	0.189*	0.412*	0.524*	**0.438(0.154)**
0+	0.348*	0.069	0.101	0.127	0.466*	0.331*	**0.240(0.163)**

Discussion

Estimates of hybrid prevalence

Hybridization is widespread in the upper Kootenay River drainage, but the number of hybrids identified in this study is most likely a conservative estimate of the actual number of hybrids present. The use of four diagnostic nuclear markers to distinguish between a first generation hybrid and a backcrossed individual is fairly accurate (6.25% chance of error), but distinguishing between a later generation backcross and a pure parental species is much more difficult. The chance of misidentifying a later generation backcross (BC_{WCT}-3 and higher), as a pure westslope cutthroat trout is over 50% (Boecklen and Howard 1997). Therefore, my estimates of the number "pure" WCT present in the upper Kootenay River system are almost certainly overestimated. Even if the number of diagnostic markers were doubled (8 markers) there is still a 34% chance of misidentifying a third generation BC_{WCT} as a genetically pure WCT (Boecklen and Howard 1997).

Cohort analysis & role of selection

The static cohort analysis where cohorts are compared across years assumes that each cohort not only has the same initial hybridization rate (same number of hybrids offspring produced each year), but also that the environmental selective conditions are constant over the four years of my study. Although this environmental constancy is

unlikely, the non-significant results between the proportion of hybrids across age classes suggests that even in the face of natural environmental fluctuation, there does not appear to be strong selection against hybrid genotypes in the lower St. Mary River. A post-hoc power analysis using standard recursion on the two-sample model (classic one gene with two alleles viability model, Hartl and Clark 1997) revealed that selection would have to be very strong ($s = 0.5$) to have an 80% chance of detecting a difference when sampling 100 individuals over the four years of study (Figure 3.6). Therefore, it is likely that even if selection is occurring I am unable to detect it due to sample size and length of study. The dynamic cohort analysis may have been able to corroborate the results of the static analysis if all age classes could have be re-sampled the next year, but unfortunately in 2001 older age class fish were very difficult to catch.

Another limitation of both the cohort analyses is the error associated with the defined age classes. Because the classification was based on size and retention of juvenile characteristics, and fish were classified by a number of different samplers, there is potential for observer bias. As the fish ages, it is more and more difficult to determine its age based on size alone. The growth and size at maturity can vary between streams depending on the productivity and temperature of the river (Behnke 1992). Also, there is potential for differential growth rates between hybrids and pure parental individuals. Hybrids may have reduced growth rates and, therefore, could be misclassified as younger fish, which could result in an underestimation of hybrids in the older age classes. By contrast, stream resident RBT and WCT are similar in size (Figure 3.6); therefore, even if the growth rates of the hybrids were intermediate between the two parental types, the error in age classification would be minimal. In 2001, I attempted to determine the error associated with the defined age classes by taking scale samples. Scale samples can be used to age fish, and then based on these known ages, an age-length distribution can be generated to age the other samples collected. Unfortunately no older fish were caught in 2001 therefore scale samples were not collected. In retrospect, scale samples should have been taken from a subset of adult and juvenile fish upon sampling to avoid this problem.

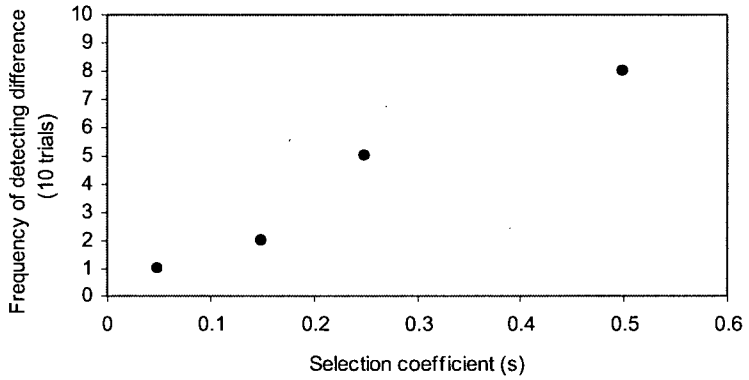


Figure 3.7. Power analysis on detecting a difference in the ratio of WCT:hybrids as age increases. The y-axis displays the number times out of ten trials that a significant difference was detected as age increased, at varying selection levels (x-axis). Standard recursion equations were used on the two-sample model, inputting various *s*-values (0.05, 0.15, 0.25, and 0.5) to calculate frequencies of hybrid genotypes in older age classes. The observed 0+ age class frequencies were used in the original calculations, and the trials were carried out using JMPIN 4.0 binomial random number generator.

Despite the mentioned limitations of the cohort analyses, my results provide little evidence of selection acting against WCT x RBT hybrids in the lower St. Mary River. There was no significant difference in the number of hybrids identified, the degree of heterozygote deficiency, or linkage disequilibrium with increasing age in the static cohort analysis. The dynamic analysis where the same cohort was sampled after one year suggests that the possibility of a selective advantage exists. The proportion of hybrids increased from 18% in the fry stage (0+) to 43% in the fingerling stage (1+) suggesting that the hybrid genotypes survived better in their first year of life. The heterozygote deficiency disappeared over the year providing evidence that selection may act in favour of hybrids, but the value of linkage disequilibrium did not differ significantly. This could be because hybridization is relatively recent in the lower St. Mary River.

Hybrids tend to predominate in the younger age classes in a Dolly Varden (*Salvelinus malma*)/ bull trout (*S. confluentus*) hybrid zone whereas adult hybrids are relatively rare (Redenbach and Taylor 2003). By contrast, my results suggest that the proportion of WCT x RBT hybrids either does not vary, or possibly increases with age. Dolly Varden and bull trout, however, may show marked differences in life history (in the study system of Redenbach and Taylor (2003) one is adfluvial, the other is not). The complexities associated with lakeward migration that occurs later in life are likely an important source of selection against hybrids (Redenbach and Taylor 2003). Similar arguments have been advanced to explain the greater prevalence of hybrids at younger ages classes when one of the species is anadromous (Hawkins and Quinn 1996; Utter 2000). Non-migratory or less complex migratory life histories, like the ones exhibited by westslope cutthroat trout and introduced rainbow trout, may provide limited opportunities for ecological selection against hybrids (Utter 2000). My results support this hypothesis in that the prevalence of adult hybrids is comparable to that in the younger age classes.

Studies have shown that endogenous selection against hybrids between cutthroat trout and rainbow trout or between subspecies of cutthroat trout is relatively weak (Ferguson et al. 1985, Forbes and Allendorf 1991a). The results of my study, coupled with evidence of hybrid swarms in other areas of the WCT distribution, suggest that there is little exogenous selection removing hybrid genotypes. A study on the spawning ecology of rainbow trout, cutthroat trout and their hybrids in Idaho found that hybrids spawned earlier and alongside the non-native rainbow trout (Henderson et al. 2000).

They also determined that the hybrids were more genetically similar to RBT (RBT backcrosses) than to cutthroat trout. Most of the hybrids identified in the upper Kootenay River were classified as backcrosses to WCT and, therefore, they are more similar to WCT than they are to RBT. This genetic similarity may allow the hybrids to have comparable fitness to pure WCT. Also, if hybrids are ecologically more similar to WCT, and tend to show similar spawning preferences, this may further facilitate the introgression of RBT alleles into the genome of WCT through extensive backcrossing.

Asymmetrical Introgression

Although the static cohort analysis did not reveal any selection against hybrids, there did appear to be selection acting at the locus level, due to differential rates of introgression between loci. Homozygous RBT genotypes were approximately 17 times more likely at the Heatshock (Hsc 71) locus than at the other three loci. This implies that selection is acting against heterospecific alleles, but it acting less strongly on the Hsc locus. Also, heterozygote genotypes were approximately 2.5 times less likely at the Occ 16 locus than at the other three loci, which implies that selection was stronger against heterospecific alleles at this locus. Differential introgression between nuclear loci has been detected in other salmonids. Redenbach and Taylor (2003) reported that bull trout growth hormone 2 introgressed 2.7 times more into Dolly Varden than the other three loci used to detect hybrids in the char hybrid zone. Similar observations of interlocus variation in introgression have been suggested to be the result of differential selection in other taxa (e.g., Shoemaker et al. 1996; Poteaux et al. 1998; Martinsen et al. 2001). The precise mechanism is unknown and although my results are suggestive that differential introgression is occurring in the lower St. Mary River population, this result should be taken with caution until confirmed through replication.

Bidirectional hybridization

Hybridization events that arise by the mating of females of species A with males of species B and vice versa, is called reciprocal or bidirectional hybridization (Wirtz 1999). Unidirectional hybridization (hybrids arise by matings of females of one species and males of the other species) has been reported within family Salmonidae in *Salvelinus* (Hammer et al 1989, Wilson and Hebert 1993, Redenbach and Taylor 2002, 2003), *Salmo* (McGowan and Davidson 1992), and in other species of *Oncorhynchus* (Dowling and

Childs 1992). Several hypotheses, including both prezygotic and postzygotic processes, have been proposed to explain different degrees of directionality in hybridizing species (reviewed in Wirtz 1999). One hypothesis involves hybridization via sneak fertilizations by the males of the smaller sympatric species. A simple prediction based on this hypothesis is that in hybridizing species that do not show differences in size-at maturity, one would expect bi-directional hybridization. In hybridizing species that do show differentiation in size-at-maturity, one would expect unidirectional hybridization. For example, bull trout and Dolly Varden naturally hybridize when found in sympatry (Baxter et al. 1997). Males and females of Dolly Varden in these sympatric populations mature at much smaller sizes than bull trout (Figure 6a, Hagen and Taylor 2001, Taylor 2003) and hybridization, as shown by mtDNA analysis, is in the direction of female bull trout and male Dolly Varden (Redenbach and Taylor 2002, 2003). An explanation for this unidirectionality is small male Dolly Varden sneaking into nests of larger bull trout females (Baxter et al 1997, Redenbach and Taylor 2003). Westslope cutthroat trout and rainbow trout show a greater overlap in size-at maturity (Figure 7b, Taylor 2003) and consistent with this hypothesis, the mtDNA analysis reveals that hybridization is bi-directional. The overlap in size at maturity and the bi-directional hybridization implies that the potential for size-assortative mating occurring between species is low.

Hybrid Zone Structure

The majority of the 142 fish identified as hybrids were classified as backcrosses to westslope cutthroat trout (59%). This suggests that not only are hybrids viable and fertile, but that rainbow alleles are being spread through extensive backcrossing with pure WCT (introgressive hybridization). Less than 3% of hybrids were classified as first generation hybrids (F_1). This lack of F_1's could be a result of the skewed ratio of WCT to RBT (27:1) found across sites. The ratio is typically more extreme than this because 76% of the pure RBT were found at a single site, the lower Bull River. Although rainbow trout are not currently distributed evenly among sites and appear to be absent from most hybridized sites (15 out of 18), their alleles have successfully spread through the upper Kootenay River system. Possible explanations of how RBT alleles have spread can be inferred from the structure of the genotypic distribution of the populations at each site.

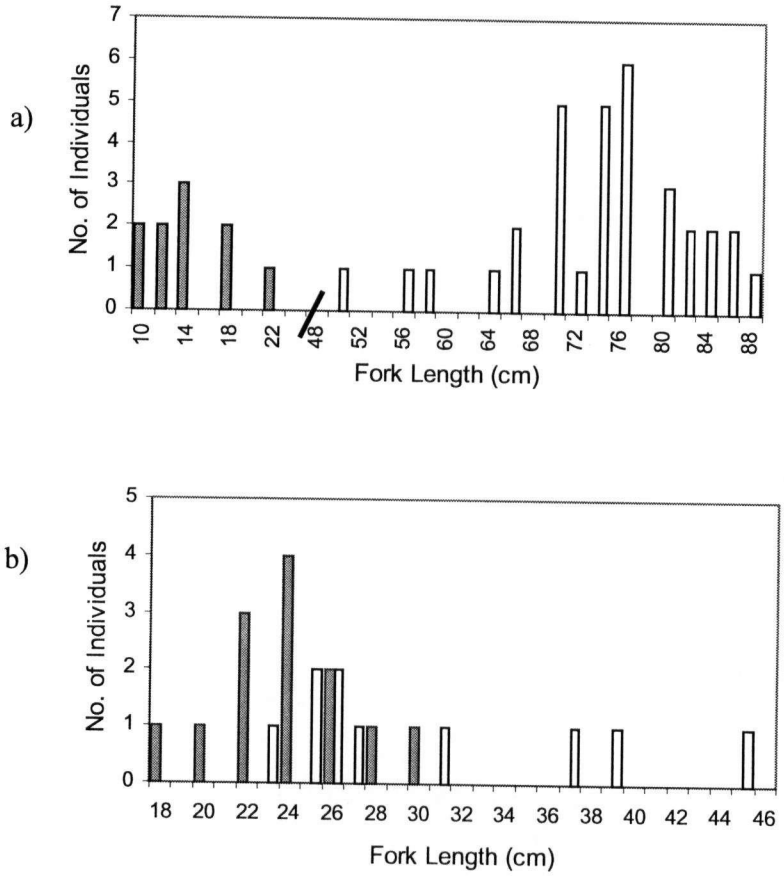


Figure 3.7. Size-frequency distribution of adult fish a) Dolly Varden (*Salvelinus malma*, grey bars) and bull trout (*S. confluentus*, white bars) and b) rainbow trout (*Oncorhynchus mykiss*, grey bars) and westslope cutthroat trout (*O. clarki lewisi*). Data for Dolly Varden and bull trout are from Hagen and Taylor (2001). Data for westslope cutthroat trout and rainbow trout are from Rubidge et al. 2001

Ten populations that exhibit a genotypic distribution skewed towards WCT (Figure 3a, see also Appendix) conform to Hardy-Weinberg proportions and show no significant linkage disequilibrium. These populations show levels of rainbow introgression under 10%. There are two possible explanations for this genotypic distribution. One is that there was an influx of rainbow trout into the system several generations ago and the rainbow alleles now present are a signal of this past hybridization. The second scenario is recent immigration and subsequent hybridization of post-F_2 individuals from neighbouring hybridized sites. The presence of linkage disequilibrium can be a result of recent hybridization, but the absence of it does not exclude the possibility of recent hybridization. Linkage disequilibrium may not be present in a situation where there is recent introgression of post-F_2 individuals.

Rainbow trout introductions began as early as 1915 in the upper Kootenay River drainage, in fact for about 40 years (1920's-1964) the Cranbrook Hatchery sporadically introduced "cranbrook trout". In Cranbrook, BC, hatchery operators purposely crossed rainbow and westslope cutthroat trout in the hatchery, to introduce "cranbrook trout" or hybrid individuals. These hybrid fish were introduced into many streams and lakes in both BC and Alberta (BC stocking records, unpublished data, Ward 1974). Therefore it is possible that the genetic structure observed in many populations (the right-skew distribution) is a result of this past stocking. If, however, this were true one would expect that the last genetic study on hybridization in this drainage would have detected it. The last genetic study of WCT x RBT hybridization in the upper Kootenay River was in 1986 (Leary et al. 1987a). Of the seven sample sites that our studies had in common, Leary et al. (1987a) found evidence of RBT introgression at one sample site (the White River) and I found RBT introgression at this site as well as three others (see Chapter 2 for more details). Although different genetic markers were used, both studies had similar power to detect introgression (Chapter 2), consequently, if the genotypic distribution observed in the present study was a result of an influx of RBT alleles several generations ago, then one would expect that Leary et al. (1987a) would have detected introgression at more than just the one site. The absence of RBT alleles in 1986 and the presence of them in 1999 suggests that hybridization (about 3-4 generations later) is recent in the three streams where new hybridization was detected. In addition, the presence of post-F_2 (F_n) and backcrossed individuals in the populations displaying the left-skewed unimodal genotypic

structure coupled with the rarity of observed F_1 hybrids, suggests that these populations are not closed to immigration.

Straying has been defined as the migration of mature individuals to spawn in a stream other than the one where it originated (Quinn 1993). Straying is important for salmonids because it can lead to the colonization of new habitats (e.g. Milner and Bailey 1989), but the high degree of genetic differentiation among populations within salmonid species (reviewed in Taylor 1991) suggests that straying (and subsequent gene flow between populations) is relatively rare. Homing has a genetic component and hybridization has been shown to greatly decrease homing accuracy (Bams 1976). Therefore, hybridization appears to increase the rate of straying by disrupting the locally adapted population. My results from Chapter 2 found that hybridized populations are spatially clustered, and only the most isolated samples sites contained no RBT alleles suggesting that hybrid straying may be responsible for the spread of RBT introgression. Hitt (2002) conducted a similar study on westslope cutthroat and rainbow trout hybridization in the Flathead River in Montana, and his results also suggested that the spread of rainbow introgression was facilitated through hybrid individuals straying among populations. Further, Hitt (2002) suggested that RBT introgression could introduce a genetic predisposition to stray and, therefore, further facilitate RBT introgression throughout the drainage.

The fish sampled from Bloom Creek, lower St. Mary River, lower Skookumchuk Creek and Lussier River all had a left-skewed genotypic distribution where WCT are the most common genotype, and conformed to H-W equilibrium, but had significant linkage disequilbrium suggesting that hybridization is recent at these sample sites. The Michel Creek samples also had a left-skewed genotypic distribution but deviated from Hardy-Weinberg equilibrium and had significant linkage disequilibrium indicating that hybridization or immigration of RBT is very recent. These populations are particularly interesting because although no pure rainbow trout were found at these sites, backcrosses to rainbow trout (BC_{RBT}) were found at lower St. Mary River, lower Skookumchuk Creek and at Michel Creek, and an F_1 was found at Bloom Creek. So either rainbow trout are present at these populations in very low numbers, or these hybrid individuals have come from a near-by population.

Lower Gold Creek had a bimodal distribution of genotypes. Bimodality is achieved in populations with extremely strong heterozygote deficits and near maximum linkage disequilibrium (Jiggins and Mallet 2000; Redenbach and Taylor 2003). Jiggins and Mallet (2000) suggested that bimodality is indicative of strong assortative mating, but there is little evidence of assortative mating between WCT and RBT because hybrid swarms have formed in other drainages (reviewed in Shepard et al. 2002). The bimodal distribution in lower Gold Creek is most likely a result of recent and continuous immigration of RBT into the area and subsequent hybridization. Gold Creek flows straight into Koocanusa Reservoir. The site sampled on lower Gold Creek is only about 5 km up from the mouth of the creek. It is possible that rainbow trout from Koocanusa Reservoir are migrating up Gold Creek to spawn. Leary et al. (1987a) detected no evidence for hybridization or rainbow trout 13 years ago with similar power to detect hybridization and RBT, therefore, appear to be relatively new in this system. Consequently, in the absence of selection against hybrids (as suggested by the cohort analysis), this population is at risk of forming a hybrid swarm (i.e. unimodal genotypic distribution).

The last pattern of hybrid zone structure observed in the upper Kootenay River WCT populations was a bimodal to flat distribution observed at Lodgepole Creek. A flat genotypic distribution consists of a more even mixture of parental and hybrid genotypes (Jiggins and Mallet 2000). The deviations from Hardy-Weinberg proportions and linkage disequilibrium values were not that of a strong bimodal distribution (i.e. extreme heterozygote deficiencies, and maxiumum linkage disquilibrium) and only one third of the population consisted of fish classified as pure westslope cutthroat trout. This population has a higher presence of RBT alleles (37.5%) than lower Gold Creek (20.3 %) and is possibly closer to becoming a hybrid swarm. Lodgepole Creek is a tributary of the Wigwam River. Approximately six kilometres upstream from the Lodgepole /Wigwam confluence, fish were sampled from the mainstem Wigwam River and only 1.5% introgression was detected. If hybrid individuals or rainbow trout are straying between sites, then this Wigwam River site is at great risk of further hybridization.

Although rainbow trout have been introduced in various lakes in the upper Kootenay River drainage for at least 80 years (BC stocking records, unpublished data) it appears that the recent focus of rainbow trout stocking in Koocanusa Reservoir from

1986-1998 has greatly enhanced WCT x RBT hybridization in this drainage. Hybridization appears to be spreading from Koocanusa Reservoir (Chapter 2), and populations in close proximity to the reservoir are at greatest risk of forming hybrid swarms. The rainbow trout stocking program into Koocanusa Reservoir was cancelled due to hybridization issue (B. Westover MWLAP, pers. com 2003), but the threat of continued hybridization and introgression continues. For instance, my results strongly suggest that introduced rainbow trout have established a feral population in the lower Bull River, which can serve as a source of pure rainbow trout into surrounding tributaries. In addition, the spread of RBT alleles through hybrid straying complicates conservation efforts to protect pure WCT.

There is little that can be done to remove the RBT alleles from the populations in this study that have experienced less than 10% introgression and do not deviate from the measured population genetic parameters. By contrast, in situations where a hybrid swarm has not formed and pure parental types are still present, a species removal program may be effective in removing RBT alleles before they can introgress into the WCT genome. For example, in lower Gold Creek where the genotypic structure is bimodal, and the heterozygote deficiency and linkage disequilibria are near maximal, removing pure RBT and hybrid individuals may be successful in slowing or preventing further hybridization. It will also aid in preventing hybrids produced at this site from straying to neighbouring sites (e.g., upstream to Bloom Creek).

In conclusion, my results suggest that in the absence of management intervention hybrid swarm formation and local extinctions of pure westslope cutthroat trout populations are likely in at least two tributaries of the upper Kootenay River (lower Gold Creek and Lodgepole Creek) and possibly more (Bloom Creek and Michel Creek). The remaining populations with less than 10% RBT introgression are at threat of accumulating more RBT alleles via hybrids straying from nearby hybridized populations and from continued RBT stocking in high elevation "landlocked" lakes in this drainage.

Chapter 4: General Discussion and Conclusions

Hybridization with non-native rainbow trout (RBT) has been listed as the greatest threat to remaining westslope cutthroat trout (WCT) populations in United States (Leary and Allendorf 1998). The upper Kootenay River drainage in British Columbia was one of the last areas in the WCT range where populations were thought to be free from RBT hybridization, but unfortunately my results clearly indicate that this is not the case. Hybridization and introgression have increased and spread to at least nine tributaries of the upper Kootenay River drainage over recent years. This spread appears to have occurred since 1986 (approximately three generations) and is most likely the result of the Gerrard RBT stocking program in Koocanusa Reservoir from 1986-1998. There is also evidence of an upstream rainbow trout source in the Elk River system from previous stocking in Summit Lake. Although pure rainbow trout were absent from many of the sample sites, WCT populations appear to be very susceptible to introgression by RBT in the study area.

Increased fitness is not generally expected when genetically divergent genomes hybridize and introgress; rather, the general expectation is intermediate or reduced fitness (i.e., outbreeding depression). The breakdown of local adaptation and the breakdown of coadapted gene complexes are causes of outbreeding depression. A review of the literature, however, indicated that hybrids vary in degree of fitness across animal and plant taxa, and in many cases hybrids are more fit than the parents (Arnold and Hodges 1995). My results found no immediate evidence for differential selection between pure and hybrid genotypes. Laboratory crosses have demonstrated intermediate traits in WCT x RBT hybrids and found little evidence of genetic incompatibilities between these species (Leary et al. 1985; Ferguson 1985). The effects of RBT introgression on the long-term viability of these populations, however, are unknown. Even if hybridization does not result in an immediate and detectable reduction in fitness, it can have deleterious evolutionary consequences. Genetic changes caused by hybridization or introgression can disrupt local adaptation such as thermal tolerances or homing behaviour in the native population (Leary and Allendorf 1995).

In the upper Kootenay River drainage hybridization is spreading upstream from Koocanusa Reservoir and evidence suggests that the spread of RBT alleles is facilitated by hybrids straying to neighbouring tributaries. Hybrid straying has also been found to

spread RBT alleles into WCT populations in the Flathead River drainage in Montana (Hitt 2002). The effects of RBT introgression on straying rates and the potential for certain populations to act as sources of rainbow trout or hybrids (i.e. Koocanusa Reservoir, lower Bull River, Gold Creek and Lodgepole Creek) should be investigated to further our understanding of the spread of RBT alleles through the upper Kootenay River system. Subjecting potential sources to more rigorous genetic analysis may assist in this investigation. The use of high-resolution microsatellite DNA assays combined with statistical analysis (specifically, individual assignment procedures) has the potential to identify exotic genotypes (Hansen et al. 2000; Taylor et al. 2003). For example, if hybrid individuals from lower Gold Creek were genetically assigned to the Gerrard RBT stock in Koocanusa Reservoir, and hybrids from Bloom Creek were genetically assigned to lower Gold Creek it would be implied that RBT and hybrid individuals moved from the reservoir to lower Gold Creek and then up to Bloom Creek.

Introduced species and subsequent hybridization with native species threaten many native populations worldwide (reviewed in Rhymer and Simperloff 1996). Within salmonid species, there are many management actions that can be employed in an attempt to stop the spread of the exotic alleles, or genetically restore the native genome (reviewed in Leary and Allendorf 1995). For example the lower Gold Creek and Lodgepole populations (and possibly the Michel Creek population) are likely sources of hybrids that stray to nearby areas. This is particularly obvious for the lower Gold Creek population where hybridization was shown to be spreading upstream to the Gold Creek headwaters. As the results suggest, however, a complete hybrid swarm has not formed in this population. A possible management action to control further hybridization in this creek would be to remove pure RBT and hybrid individuals, although this may not be feasible. Constructing and monitoring a fish fence is expensive, and my results suggest that identifying hybrids in the field can be inaccurate. Another possible management approach is genetic restoration, where a pure WCT stock is introduced to the river in the hopes of flooding the gene pool with native genes, but this can also be problematic. Unless the introduced stock was generated from individuals from the creek population (which is unlikely in most cases) then stocking even pure WCT can dilute the locally adapted population. For instance, Taylor et al. (2003) demonstrated strong population differentiation among WCT populations in the upper Kootenay River system using microsatellite DNA. Consequently, the benefits of introducing pure WCT from other, even nearby, populations must be balanced with the potential costs of introducing

genetically distinct WCT. Obviously attempts to restore the native population are complicated, expensive and may not be ultimately effective in the end. The best approach in protecting remaining native populations is prevention: cease all further introductions of RBT. There is, however, strong political pressure to continue these stocking programs. Although the stocking program into Koocanusa Reservoir (the likely source of increased RBT hybridization in recent years) was cancelled in BC, headwater lakes are stocked annually.

My study revealed the importance of genetic surveys in determining the status of the remaining WCT populations in Canada and I recommend further sampling throughout their distribution to identify other pure populations. In Alberta, WCT populations have greatly declined in the last 100 years and populations have been reduced almost exclusively to highly isolated and fragmented headwater populations (reviewed in Mayhood 1999). It is assumed that hybridization is widespread, but there has not been an extensive genetic study on remaining populations throughout the historic range of WCT in Alberta. I suggest that all future genetic monitoring programs, particularly in the upper Kootenay River drainage, repeat the genetic identification protocol used in this study. This will allow direct temporal comparison of the percentage of heterospecific alleles and will avoid any potential inconsistencies in using different genetic markers. Currently, WCT are under review for a Committee on the Status of Endangered Wildlife in Canada (COSEWIC) listing, and understanding the extent of hybridization and locating pure populations is imperative to accurately assess the threats to and status of this subspecies. Also, once pure populations are located, clearly they should be given conservation priority and efforts should be made to preserve their habitat and protect them from RBT hybridization.

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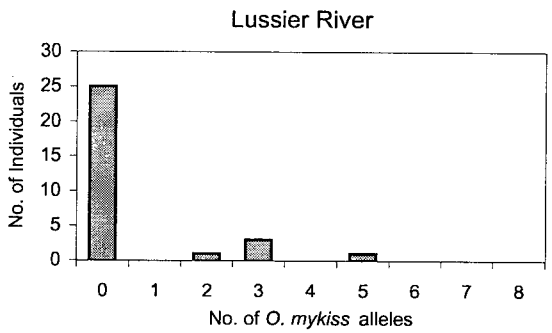
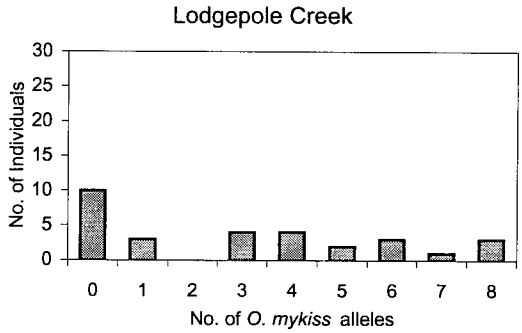
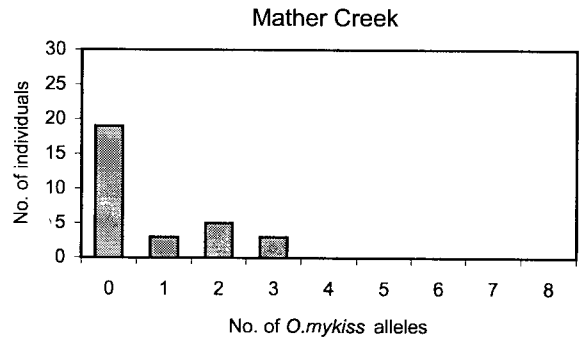
Appendix 1. Genotypic classification of westslope cutthroat trout (WCT), rainbow trout (RBT) and their hybrids (BC_{WCT}, F_1, F_n , & BC_{RBT}) from hybridized sites sampled in Upper Kootenay River drainage. Based on four diagnostic nuclear markers.

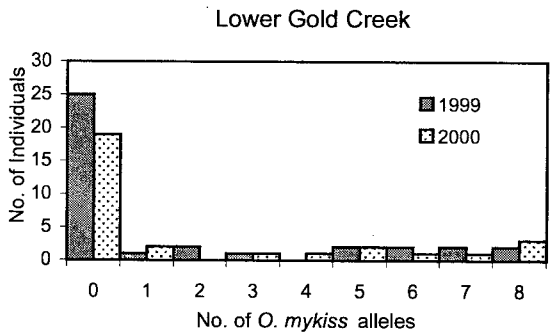
Site (*n*)	WCT	BC_{WCT}	F_n	F1	BC_{RBT}	RBT
upper Kootenay River (*15*)	12	3	0	0	0	0
White River (*33*)	29	2	2	0	0	0
Morrissey Creek (*30*)	29	0	1	0	0	0
Wigwam River (*34*)	31	2	1	0	0	0
lower Skookumchuk Creek (*33*)	30	2	0	0	1	0
upper Skookumchuk Creek (*40*)	39	1	0	0	0	0
lower St. Mary River 1999 (*31*)	27	2	1	0	1	0
lower St. Mary River 2000 (*104*)	47	14	2	0	1	0
lower Gold Creek 1999	25	2	3	0	4	2
lower Gold Creek 2000	19	0	2	1	4	3
Bloom Creek	19	4	6	1	0	0
Teepee Creek	25	4	1	0	0	0
upper Gold Creek	26	3	1	0	0	0
lower Bull River	0	0	1	0	4	25
Lodgepole Creek	10	5	6	2	4	3
Coal Creek	32	4	0	0	0	0
Michel Creek	21	4	4	0	1	0
Wild Horse River	28	17	0	0	0	0
Mather Creek	18	8	4	0	0	0
Lussier River	25	4	1	0	0	0

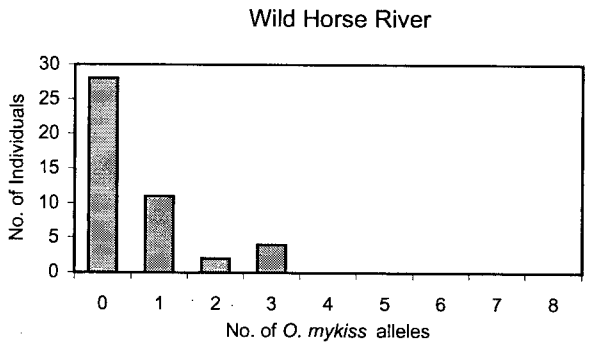
Appendix 2. The frequency distribution (hybrid zone structure) of individuals collected from each sample site

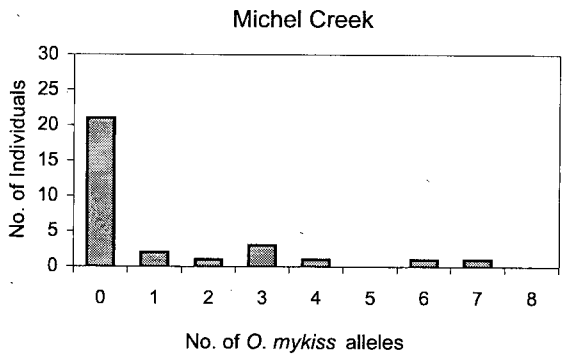


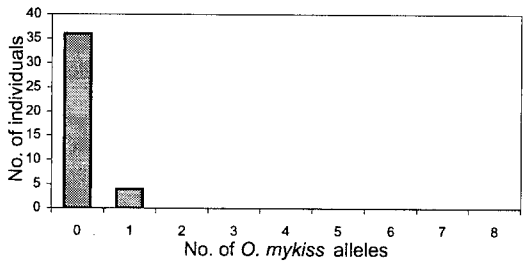


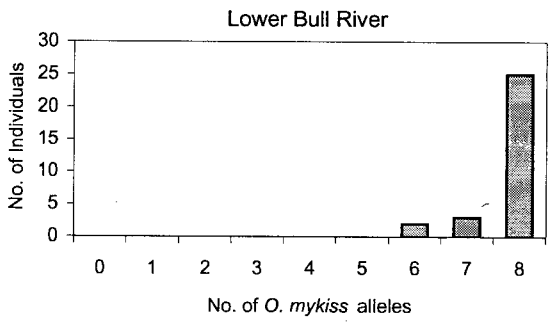




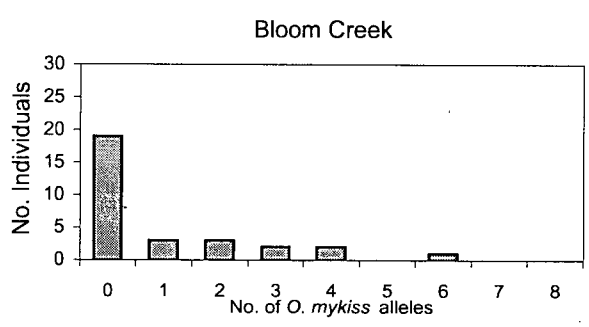


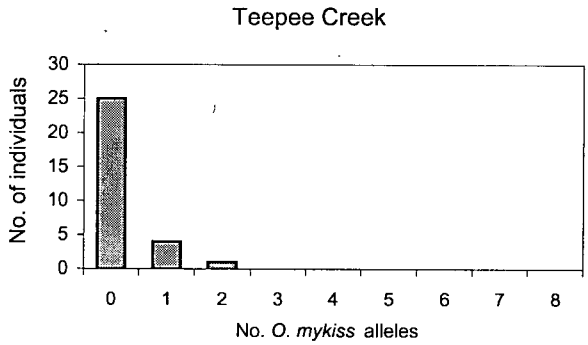


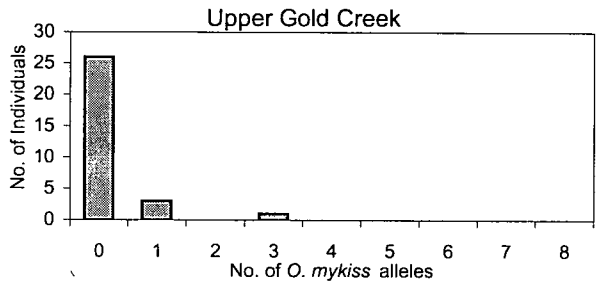


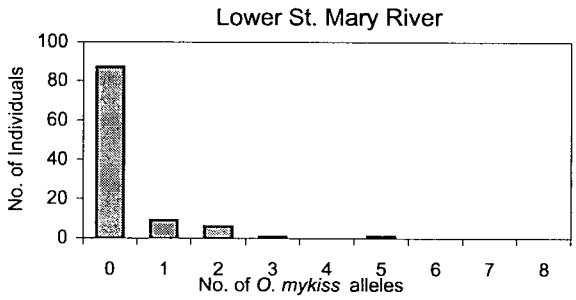


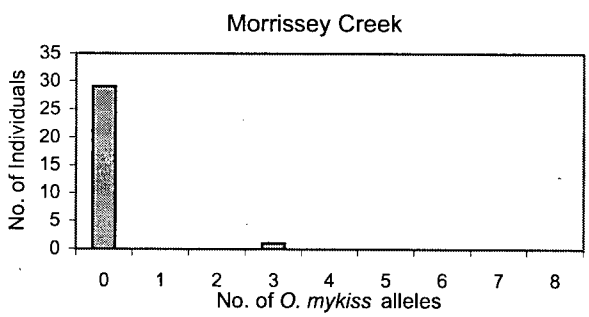
Appendix 2. (cont'd)

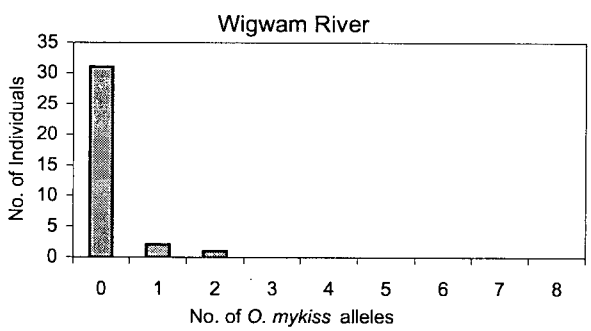


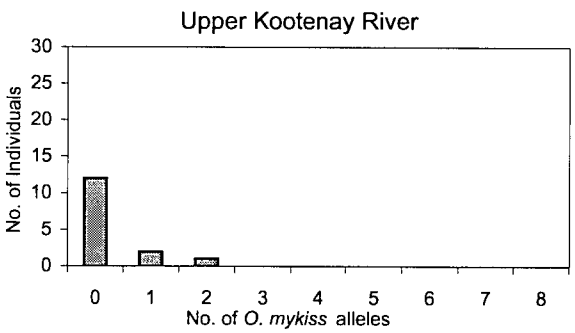


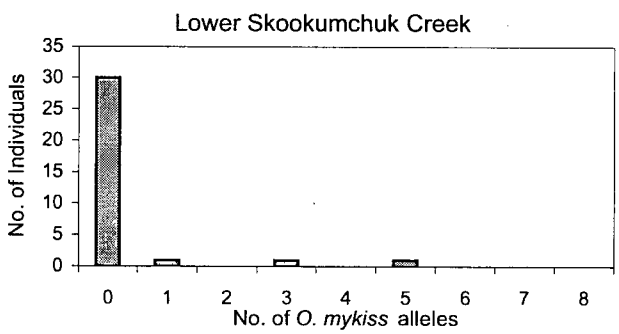




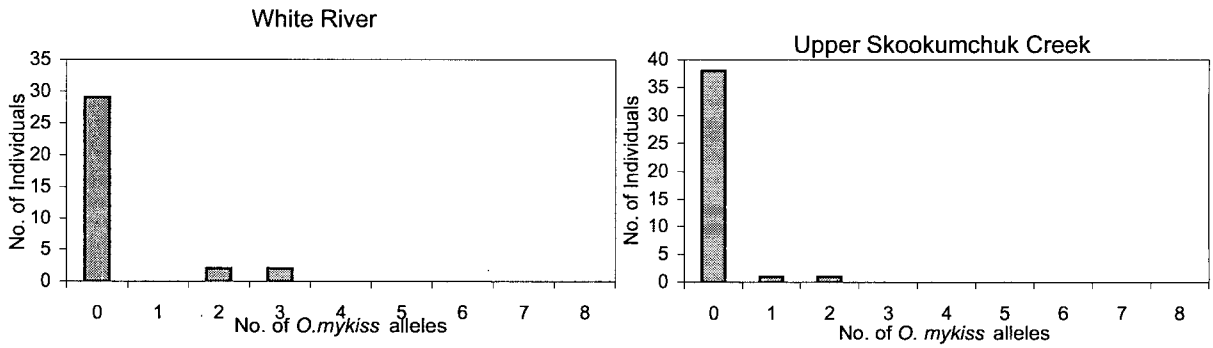








Appendix 2. (cont'd)



Appendix 3. Alleles sizes and frequencies at the four species diagnostic loci for each sampling site and over all sites

1999 samples

Locus Ikaros

Site	*n*	Allele Size 500 (WCT)	750 (RBT)
Upper Elk	38	1	0
Morrissey	30	0.983	0.017
Wigwam	34	1	0
U.Kootenay	15	0.9	0.1
U. St Mary	31	1	0
L.Skookumchuk	33	0.97	0.03
U.Bull	36	1	0
Gold	36	0.958	0.042
White	33	0.955	0.045
L.S tMary	31	0.952	0.048
U.Skookumchuk	40	0.975	0.025
All Sites	357	0.976	0.024

Locus Occ16

Site	*n*	Allele Size 380 (WCT)	280 (RBT)
Upper Elk	38	1	0
Morrisey	30	1	0
Wigwam	34	0.985	0.015
U.Kootenay	12	1	0
U. St Mary	30	1	0
L.Skookumchuk	33	0.985	0.015
U.Bull	36	1	0
Gold	36	0.861	0.139
White	33	1	0
L.St. Mary	31	0.968	0.032
U.Skookumchuk	40	1	0
All Sites	353	0.98	0.02

Locus hsc 71

Site	*n*	Allele Size 249 (WCT)	216 (RBT)
Upper Elk	33	1	0
Morrisey	25	0.96	0.04
Wigwam	34	0.956	0.044
U.Kootenay	14	0.964	0.036
U. St Mary	28	1	0
L.Skook	30	0.967	0.033
U.Bull	36	1	0
Gold	34	0.706	0.294
White	26	0.904	0.096
L.St. Mary	31	0.935	0.065
U.Skookumchuk	39	0.987	0.013
All Sites	330	0.942	0.058

Locus Om 13

Site	*n*	Allele Size 190 (WCT)	175 (RBT)
Upper Elk	38	1	0
Morrisey	30	1	0
Wigwam	34	1	0
U.Kootenay	12	1	0
U. St Mary	31	1	0
L.Skookumchuk	33	0.97	0.03
U.Bull	36	1	0
Gold	36	0.917	0.083
White	33	0.97	0.03
L.St. Mary	31	0.968	0.032
U.Skookumchuk	40	1	0
All sites	354	0.983	0.017

Appendix 3. (cont'd)

2000 samples

Locus Ikaros

Site	*n*	Allele Size 500 (WCT)	750(RBT)
Lussier	30	0.917	0.083
Mather	30	0.817	0.183
Wild Horse	45	0.933	0.067
Lodgepole	29	0.603	0.397
Michel	28	0.839	0.161
Coal	40	1	0
Findlay	32	1	0
Fording	33	1	0
Lower Bull	29	0.086	0.914
Bloom	30	0.95	0.05
Teepee	30	1	0
Upper Gold	30	0.967	0.033
Lower Gold	30	0.783	0.217
All Sites	416	0.85	0.15

Locus Occ16

Site	*n*	Allele Size 380 (WCT)	280 (RBT)
Lussier	30	1	0
Mather	27	0.963	0.037
Wild Horse	45	0.967	0.033
Lodgepole	30	0.65	0.35
Michel	30	0.983	0.017
Coal	37	0.986	0.014
Findlay	32	1	0
Fording	34	1	0
Lower Bull	29	0.017	0.983
Bloom	30	0.95	0.05
Teepee	30	1	0
Upper Gold	28	1	0
Lower Gold	30	0.767	0.233
All Sites	412	0.876	0.124

Locus hsc 71

Site	*n*	Allele Size 249 (WCT)	216 (RBT)
Lussier	29	0.914	0.086
Mather	27	0.87	0.13
Wild Horse	45	0.922	0.078
Lodgepole	30	0.617	0.383
Michel	29	0.828	0.172
Coal	40	0.962	0.038
Findlay	32	1	0
Fording	26	1	0
Lower Bull	28	0	1
Bloom	30	0.717	0.283
Teepee	30	0.9	0.1
Upper Gold	30	0.967	0.033
Lower Gold	30	0.717	0.283
All Sites	416	0.812	0.188

Locus Om 13

Site	*n*	Allele Size 190 (WCT)	175 (RBT)
Lussier	29	0.897	0.103
Mather	24	0.896	0.104
Wild Horse	45	0.878	0.122
Lodgepole	30	0.633	0.367
Michel	29	0.828	0.172
Coal	39	1	0
Findlay	32	1	0
Fording	34	1	0
Lower Bull	30	0.017	0.983
Bloom	30	0.933	0.067
Teepee	30	1	0
Upper Gold	30	0.967	0.033
Lower Gold	30	0.8	0.2
All Sites	412	0.841	0.159

Appendix 3. (cont'd)

Cohort Analysis: Lower St. Mary River 2000; Adults (3+), juveniles (2+), fingerling (1+) and fry (0+)

Locus Ikaros

Age	*n*	Allele Size 500 (WCT)	750(RBT)
3+	104	0.957	0.043
2+	93	0.898	0.102
1+	112	0.946	0.054
0+	99	0.96	0.04
All Ages	408	0.941	0.059

Locus Occ16

Age	n	Allele Size 380 (WCT)	280 (RBT)
3+	103	0.99	0.01
2+	90	0.978	0.022
1+	112	0.969	0.031
0+	100	0.975	0.025
All Ages	405	0.978	0.022

Locus hsc 71

Age	*n*	Allele Size 249 (WCT)	216 (RBT)
3+	100	0.94	0.06
2+	88	0.915	0.085
1+	112	0.915	0.085
0+	98	0.929	0.071
All Ages	398	0.925	0.075

Locus Om 13

Age	n	Allele Size 190 (WCT)	175 (RBT)
3+	104	0.971	0.029
2+	89	0.944	0.056
1+	111	0.95	0.05
0+	99	0.97	0.03
All Ages	403	0.959	0.041

Cohort Analysis: Lower St. Mary River 2001; juveniles (2+), fingerling (1+) and fry (0+)

Locus Ikaros

Age	*n*	Allele Size 500 (WCT)	750(RBT)
2+	5	0.7	0.3
1+	46	0.826	0.174
0+	46	0.978	0.022
All Ages	97	0.892	0.108

Locus Occ16

Age	*n*	Allele Size 380 (WCT)	280 (RBT)
2+	5	0.8	0.2
1+	48	0.958	0.042
0+	45	0.989	0.011
All Ages	**98**	**0.964**	**0.036**

Locus hsc 71

Age	*n*	Allele Size 249 (WCT)	216 (RBT)
2+	5	0.7	0.3
1+	43	0.849	0.151
0+	42	0.976	0.024
All Ages	**90**	**0.9**	**0.1**

Locus Om 13

Age	*n*	Allele Size 190 (WCT)	175 (RBT)
2+	5	0.8	0.2
1+	47	0.809	0.191
0+	42	0.976	0.024
All Ages	**94**	**0.883**	**0.117**