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Interspecific Hybridization between Sympatric Coastal Cutthroat and Coastal Rainbow/Steelhead Trout on Vancouver Island, British Columbia: A Conservation and Evolutionary Examination

by

Corwyn Matthew Bettles

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ABSTRACT

Sympatric coastal cutthroat trout (*Oncorhynchus clarki clarki*) and coastal rainbow/steelhead trout (*O. mykiss irideus*) are thought to be reproductively isolated primarily by spatial and temporal separation. However, interspecific hybridization has been documented, thus raising the questions of how widespread hybridization is within their native range, and what are the nature and status of reproductive isolating mechanisms (i.e. prezygotic or postzygotic) in the hybridizing sympatric populations?

In a broad survey of 37 populations on Vancouver Island, hybridization between these trout species was found to be widespread (Chapter 2). The frequency of hybridization varied among locations (H_I = 3% - 88%; I_I = 2%-54%), with some populations displaying hybrid levels indicative of hybrid swarms and may be undergoing '*hybrid meltdown*'. Several environmental factors appear to influence hybridization (e.g. forest harvesting, stocking, habitat availability, watershed size), however, no single factor appears to have a dominant effect.

There is no consistent evidence for selection acting against first-generation (F1) hybrids, and in backcross hybrids inconsistent results implicate environment-dependent (i.e. extrinsic) selection (Chapter 3). Hybridization is reciprocal, but nuclear marker patterns show that the direction of hybridization is unidirectional in some populations (n = 5 out of 13 populations). Based on cytonuclear disequilibrium levels, a remarkable reproductive bias appears to exist (i.e. frequency of backcross hybrids with matched nuclear-mitochondrial marker composition exceeded mismatched genotypes). Selection against mismatch genotypes may be occurring, although a behavioural mating bias is more likely.

This study provides evidence that hybridization and introgression between coastal cutthroat and rainbow/steelhead trout occurs more frequently than first thought and that hybridization will contribute to the further decline of both trout species.

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CHAPTER 1

GENERAL INTRODUCTION – REPRODUCTIVE ISOLATION AND SPECIATION

1.0 GENERAL INTRODUCTION

1.1 Reproductive Isolation and Speciation

Speciation is the central concept to evolutionary theory, but is still not well understood. Understanding what species are and how they form is also central to efforts for preserving biodiversity. There are numerous species concepts that exist (Luckow 1995), which help define evolution and speciation; however, three concepts, in particular, are predominantly employed: (1) the *Phylogenetic Species Concept*; (2) the *Morphospecies Concept*; and (3) the *Biological Species Concept* (Freeman and Scott 2004). All three of these views agree that species are evolutionarily independent units that are isolated by a reduced or lack of gene flow; however, each utilizes different criteria for determining when groups are true species. The *Biological Species Concept* associates speciation to the evolution of reproductive isolating mechanisms that essentially prevent gene exchange among taxa (Turelli et al. 2001). Under this model, the standard for identifying species is the presence of prezygotic and postzygotic reproductive isolating mechanisms, which essentially prevent hybridization (i.e. among taxa gene flow). It is because of these attributes that I will use the *Biological Species Concept* exclusively. Prezygotic isolation results from factors that prevent interbreeding (i.e. hybridization) between genetically divergent populations, such as behavioral, ecological, temporal, and spatial isolation. Postzygotic isolation results from factors that occur after fertilization, which reduce or eliminate hybrid offspring viability (or fitness), and therefore reduce the incidence of hybrid organisms. Ultimately, postzygotic isolation is expected to lead to prezygotic isolation through selection against interspecific mating.

Speciation can work at three distinct spatial organizations: (1) allopatric speciation; (2) parapatric speciation; and (3) sympatric speciation. **Allopatric speciation** is the evolution of reproductive barriers between populations that are geographically isolated (vicariance). When physical barriers impede gene flow between populations, it allows natural selection (and genetic drift) to act on these populations to become genetically differentiated. If enough differentiation accumulates, it will alter populations, which would prevent gene flow if/when secondary contact took place. **Parapatric speciation** is a mode of gradual speciation in which new species arise from neighboring populations that maintain genetic contact in a zone of overlap (i.e. a hybrid zone). In this particular mode of speciation, progeny from the contact zone tend to show reduced fitness compared to the parental types (White 1968). Also, hybrid progeny do not move outside the zone of overlap because of strong environmental differences on either side of the contact zone. Over time, neighboring populations diverge and gradually become reproductively isolated. **Sympatric speciation** is the process whereby populations inhabiting (at least in part) the same geographic range become reproductively isolated. Previously, this model of speciation was thought to be driven primarily by ecological reproductive isolation between species (Turelli et al. 2001). More recently, sympatric speciation has been linked to "selection against intermediate phenotypes", where physically intermediate individuals (i.e. hybrids) are unable to adequately compete for resources or obtain mates (Higashi et al. 1999). The resulting selection ultimately drives the evolution of reproductive isolation for taxa even in sympatry.

Dobzhansky (1937) reasoned that if geographically isolated populations come into contact, then any hybrid progeny that are produced should have noticeably reduced fitness (i.e. the relative ability of an organism to survive and transmit genes to the next generation) relative to either parental species. In other words, if; 1) natural selection produced adaptations to local habitats, 2) sexual selection produced unique mating systems, or 3) genetic drift led to the fixation of alleles that were incompatible when heterozygous, then hybrid progeny should display low fitness. Consequently, there should be strong natural selection in favor of assortative mating – natural selection should favor individuals that choose mates only from the same population/species. Selection that reduces the frequency of hybridization is "*reinforcement*", which should ultimately finalize the speciation process; however evidence for reinforcement in nature is rare and controversial (Noor 1999). Nevertheless, selective arguments predict that when closely related species come into contact and hybridize to produce inferior offspring, some reproductive mechanism should evolve to prevent hybridization. However, hybrid offspring will remain rare even without reinforcement when the hybrid progeny are sterile or inviable (postzygotic reproductive isolation). It has been shown that prezygotic barriers do evolve much faster than postzygotic reproductive isolation due to the effects of reinforcement (Coyne & Orr 1989; 1997; Noor 1999), particularly in species that exhibit sympatry and where reciprocal hybridization events have previously occurred; however, the generality of this is still debated (Servedio 2000). Ultimately, the study of hybridization between divergent taxa facilitates understanding species, speciation, and the significance of reinforcement mechanisms.

This thesis focuses on the sympatric hybridization dynamics of coastal cutthroat (*Oncorhynchus clarki clarki*) and coastal rainbow/steelhead (*O. mykiss irideus*) trout on Vancouver Island, British Columbia (BC). Coastal cutthroat trout and coastal rainbow/steelhead trout are two species of salmonids native to the Pacific coast drainages of North America. The native range of steelhead trout extends from central California to the Alaska Peninsula. Coastal cutthroat's native range extends from northern California to southeastern Alaska. These two species are common in coastal BC waters; however, populations of both species have severely declined over the last two decades. In the United States, the *National Marine Fisheries Service* (NMFS) has combined 178 steelhead populations in Washington, Oregon, and California into 14 evolutionary significant units, which have been deemed as prime candidates for listing under the *U.S Endangered Species Act* (Di Silvestro 1997). Evidence of declining steelhead populations in British Columbia has been widely documented, specifically in the depleted stocks along the east coast of Vancouver Island. This has been attributed to two main factors: (i) steelhead stocks are typically small with low productivity and have migration patterns that coincide with other commercial salmonids, and hence many steelhead are lost as by-catch (Slaney et al. 1996); and more importantly (ii) relentless critical habitat modification and depletion, due to forestry activities and urbanization. Currently, over 50% of all steelhead stocks in BC have been identified as either a conservation concern or an extreme conservation concern (BC Ministry WLAP 2004).

In the United States, the *Endangered Species Committee of the American Fisheries Society* has identified all populations of coastal cutthroat trout in Washington, Oregon, and California as being at some level of risk of extinction (Wenburg et al. 1996). In BC, coastal cutthroat inhabit approximately 750 streams, however information is only available for approximately 120 populations, and more than half of those have been determined to be at some level of risk, while several populations within the lower Fraser River and Georgia Strait are considered extinct (Slaney et al. 1996). Slaney et al. (1996) further emphasized that coastal cutthroat trout in BC has the greatest percentage (12.5%) of extinct stocks as well as the highest proportion (80%) of stocks whose conservation status is unknown. In both Canada and the United States, decline in coastal cutthroat populations has been attributed primarily to loss of habitat due to land-use practices.

The thesis is divided into two main research sections. Chapter 2 addresses the incidence and distribution of hybridization between coastal cutthroat and coastal rainbow/steelhead trout on Vancouver Island, BC. This chapter also investigates particular anthropogenic (environmental) effects that may be associated with the breakdown of reproductive isolating mechanisms, and discusses conservation implications of hybridization and introgression for both species. Chapter 3 examines the evolutionary consequence(s) of hybridization (and introgression) between these two trout species by investigating the relative roles of prezygotic and postzygotic reproductive isolating mechanisms involved in maintaining distinct species. Finally, chapter 4 summarizes the key results of chapter 2 and 3 and offers recommendations for effective monitoring and management of sympatric populations of coastal cutthroat and coastal rainbow/steelhead trout.

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CHAPTER 2

CHARACTERIZATION OF ENVIRONMENTAL FACTORS ASSOCIATED WITH COASTAL CUTTHROAT AND COASTAL RAINBOW/STEELHEAD TROUT HYBRIDIZATION

2.1 ABSTRACT

Hybridization provides an exceptionally tough set of problems for biologists charged with conserving fish taxa. Coastal cutthroat (*Oncorhynchus clarki clarki*) and coastal rainbow/steelhead trout (*O. mykiss irideus*) are known to hybridize, and this complicates the conservation biology and genetics for systems where both species occur. Using a combination of mtDNA and co-dominant nuclear DNA markers plus a geographic information system (GIS), I investigated: (1) the broad-scale distribution and frequency of sympatric coastal cutthroat/coastal rainbow trout hybridization on Vancouver Island, BC; and (2) the environmental variables associated with increased hybridization levels among populations. I found 284 hybrids among 1004 genotyped fish (7% F1, 22% backcross), and hybrids were found in 29 of 30 sampled populations. Additionally, two populations showed the characteristics of hybrid swarms (i.e. a diverse array of recombinant genotypes) with evidence suggesting that these populations are temporally stable. Thus, I propose the new term '*hybrid meltdown*' to describe the process of loss of reproductive barriers, and consequently the irreversible loss of the pure species genotype in isolated sympatric populations. High variation in hybridization (and introgression) was observed among populations ($H_I = 3\%$ - 88%; $I_I = 2\%$-54%). No single environmental factor was found to dominate in the explanation of variation in hybridization (and introgression) levels; however, stocking of hatchery trout of either species, long-term effects of timber harvesting, and loss of available habitat all played a significant role in increased hybridization levels. The effects of all of these factors were magnified in small watersheds (i.e. less total stream length). Since watershed size by itself explained a significant proportion of the variation in hybridization levels, it is probable that other underlying mechanisms, undetected in this study, are influencing hybridization

levels (since stream length is not likely to directly affect reproductive isolation in trout). This study shows that hybridization and introgression is widespread between coastal cutthroat and coastal rainbow trout on Vancouver Island, and that environmental disturbance factors play a role in the process. Since similar environmental disturbance is common to most of the coastal trout habitat, such large-scale hybridization may be occurring elsewhere and may represent the most critical conservation issue for the Pacific trout species.

2.2 INTRODUCTION

Conservation biologists are becoming increasingly concerned with the rising incidence of interspecific hybridization and its impact on biodiversity and escalated extinction rates. Hybridization is usually deemed detrimental to native (i.e. parental) populations due to two processes: (1) the loss of reproductive opportunity (i.e. hybridization as a "wasted" reproductive effort); and (2) genetic introgression (i.e. the incorporation of genes of one species into the gene pool of another; Allendorf et al. 2001).

The ability for individuals from two different taxa to cross-breed (i.e. hybridize) does not inevitably result in genetic introgression; for example, gametic incompatibility can block the development of zygotes due to a lack of compatibility between sperm and egg (i.e. postzygotic incompatability; Zeh & Zeh 1997). Additionally, if zygotes do form and hybrids are produced, they may be sterile or inviable (i.e. also postzygotic reproductive isolation; e.g. Sasa et al. 1998; Price and Bouvier 2002). In these examples, the energy allocated to hybrid production is wasted, possibly resulting in the loss of population viability over time, even though gene pools are not mixed. Conversely, when hybrids are fertile and readily backcross with parental taxa, introgression can be widespread (Rhymer & Simberloff 1996). The incorporation of genes from one parental gene pool into another may ultimately result in the genetic extinction of parental genotypes (Rhymer & Simberloff 1996) by means of a hybrid swarm (i.e. a diverse array of recombinant genotypes). Although the major concerns over the loss of species has been the direct effects of habitat modification and loss, combined with species introductions, the increasing frequency of interspecific hybridization in general also appears to be influenced by modifications to habitat and species introductions (Allendorf et al. 2001).

For example, Simien jackal (*Canis simensis*) populations of Ethiopia have been so affected by depleted native habitat that they now inhabit areas where the domestic dog is common (Nowak 1991). Consequently, introgressive hybridization has occurred between male domestic dogs and female jackals (Gotelli et al. 1994) causing widespread reduction in genetically pure jackal populations. As another example, the introduction of non-native mallard ducks (*Anas platyrhynchos*) has been implicated in population declines of the New Zealand grey duck (*A. superciliosa superciliosa*) due to interspecific hybridization (Rhymer et al. 1994); the presence of the hybrid ducks further hinders efforts to conserve the remaining pure individuals.

Hybridization is known to occur among fish taxa (Hubbs 1955) more often than in any other vertebrate group (Allendorf & Waples 1996). Several factors have been hypothesized as contributing to higher incidence of hybridization in fish; including, (i) weak behavioral isolating mechanisms; (ii) external fertilization; (iii) unequal species abundance among parental taxa; (iv) competition for limited spawning habitat; and (v) loss of habitat complexity (Hubbs 1955; Campton 1987; Scribner et al. 2001). Hybridization is particularly common in the salmonids and has been observed in all genera (Taylor 2004); for example *Salmo* (Verspoor 1988), *Coregonus* (Lu & Bernatchez 1998), *Salvelinus* (Baxter et. al. 1998; Redenbach & Taylor 2004), and some species of *Oncorhynchus* (e.g. Dowling & Childs 1992; Rosenfield et al. 2000; Rubidge et al. 2001; Docker et al. 2003). In some cases, salmonid species have been shown to maintain their genetic integrity in the face of hybridization. For example, mating between naturally sympatric bull trout and Dolly Varden (genus *Salvelinus*) resulting in low levels of introgression has been documented, yet the two taxa have maintained species status despite several ancient hybridization events (Baxter et al. 1997). Similarly, hybridization has been reported between bull trout (*Salvelinus confluentus*) and introduced brook trout (*S. fontinalis*); however, reduced survival in hybrids and low fertility in surviving hybrids has limited levels of introgression (Kanda et al. 2002). The authors noted that wasted reproductive effort producing hybrids was a serious threat to native population stability of bull trout.

Cutthroat (*Oncorhynchus clarki* spp.) and rainbow trout (*Oncorhynchus mykiss* spp.) diverged from a common ancestor approximately 2 million years ago (Behnke 1992) allowing for considerable genetic (Leary et al. 1987), chromosomal (Gold 1977), and morphological (Behnke 1992) differences to accumulate. Western North American trout species of the genus *Oncorhynchus* have since evolved into several subspecies within the cutthroat and rainbow trout. Nearly all of these subspecies of trout evolved in allopatry (i.e. speciation by geographical isolation from related taxa; Young et al. 2001). As a consequence, stocking of nonnative rainbow trout (*O. mykiss* spp.) into areas of native allopatric cutthroat trout (*O. clarki* spp.) has resulted in extensive hybridization (and introgression) between trout species (e.g. Leary et al. 1984; Ferguson et al. 1988; Carmichael et al. 1993; Rubidge et al. 2001; Campbell et al. 2002). In some instances, hybrid swarms have been documented (Forbes and Allendorf 1991) and hybridization has been specifically recognized as the driving force for the extinction of one subspecies of cutthroat trout, the Alvord cutthroat trout (Gyllensten et al. 1985; Bartley & Gall 1991).

In contrast to the allopatric speciation of most western cutthroat and rainbow subspecies, the distribution of coastal cutthroat (*O. clarki clarki*) and coastal rainbow/steelhead trout (*O. mykiss irideus*) reveals a long evolutionary history of sympatry. Many reproductive barriers have been postulated to maintain species integrity. Without physical barriers to prevent hybridization, other reproductive isolating mechanisms (i.e. behavioral, ecological, and/or genetic) are expected to evolve to maintain species integrity. For example, species pairs that maintain sympatric relationships and have the potential to hybridize are believed to exhibit stronger prezygotic reproductive barriers, due to the effects of reinforcement (e.g. Coyne & Orr 1989, 1997; Noor 1999). However, the strength of the various reproductive isolating mechanisms in nature has been shown to vary widely among taxa; hence the relative significance of alternative reproductive isolating mechanisms, among recently diverged species, continues to be of interest to evolutionary and conservation biologists. In coastal cutthroat and coastal rainbow/steelhead trout, spatial and temporal differences in spawning behavior by adult spawners are most likely involved in minimizing interbreeding between species (Trotter 1989; Young et al. 2001).

The potential for coastal cutthroat and coastal rainbow/steelhead trout to hybridize has complicated matters in terms of conservation biology and genetics for both species (Baker et al. 2002). These trout have maintained their species integrity in sympatry for at least 10,000 years (i.e. since the last glaciation; Behnke 1992), yet have only recently begun to hybridize and produce reproductively viable hybrid offspring. Campton & Utter (1985) first reported genetic evidence of hybridization between coastal cutthroat and coastal rainbow trout from two streams in Washington State, USA. The authors speculated that hybridization between these trout occurs where spawning habitat overlaps for both species, but this was not formally tested. Young et al. (2001) observed limited hybridization and introgression in an additional five sympatric populations from Washington State, USA (3% F1 hybrids, 3% backcross hybrids from 252 trout sampled over all five streams). They hypothesized that variation in hybridization levels among populations may be due to localized environmental factors that influence interspecific mating. Their conclusion, however, was based on the various levels of hybridization in their five sample populations and not on direct evidence of specific environmental factors. Docker et al. (2003) investigated hybridization in 10 streams located in British Columbia, Canada, testing for effects of supplementation (i.e. stocking) of hatchery trout, on naturally sympatric coastal cutthroat and rainbow/steelhead trout populations. The authors observed a significantly higher incidence of hybrids where hatchery rainbow trout were introduced into naturally sympatric trout populations compared to sympatric populations with no supplementation. However, one population with no history of stocking also had a high level of hybridization, suggesting that other environmental factors (e.g. forest harvesting) may affect reproductive isolation (Docker et al. 2003).

Hybridization between coastal cutthroat and rainbow/steelhead trout appears to be relatively widespread; however, neither the magnitude of nor the environmental factors contributing to the hybridization is well known. Thus, there were two principal goals of this study. The first was to investigate the distribution and frequency of hybridization and introgression between sympatric populations of coastal cutthroat and coastal rainbow trout on Vancouver Island, British Columbia. A spatial assessment of hybridization and introgression between these trout species has never been performed. A broad range of hybridization is expected (Docker et al. 2003), both in incidence and geographic extent, across Vancouver Island. The second objective was to quantitatively investigate anthropogenic (environmental) effects on hybridization levels testing several continuous and categorical environmental variables. More than one environmental factor, either independently or in combination, is expected to contribute to hybridization and introgression between these trout species. The results of these analysis provides fisheries managers and conservation biologists with quantitative data on the magnitude of the hybridization problem and possible mitigation approaches based on the identification of environmental factors associated with elevated hybridization among populations.

2.3 METHODS AND MATERIALS

2.3.1 Study Location – Vancouver Island

Vancouver Island is located on the Pacific Coast of Canada, separated from the British Columbia (BC) mainland by the Georgia Strait. Watersheds on the west coast of BC, particularly Vancouver Island, hold high resource values for forestry, fisheries, wildlife, tourism, and cultural heritage (Hartman et al. 1996). Streams on Vancouver Island generally flow out from interior lakes and snowpacks to the ocean. Stream flow commonly peaks during winter months, with low flows during the summer and fall. Forest cover on Vancouver Island is approximately 91% of the total land base. Half of this cover is reported as old growth forest, found primarily in higher elevation and more remote western and northern locations, while the remainder is managed second growth forest. Resident freshwater and anadromous fish populations in Vancouver Island streams are extensive, and are particularly dependent on the forest ecosystems for survival at all life history stages (Porter et al. 2000). Past and present human activities have resulted in destruction of spawning and rearing habitats, and the decline of several native fish populations has been attributed to these anthropogenic effects (Slaney et al. 1996; Porter et al. 2000).

2.3.2 General Life History – Coastal Cutthroat and Rainbow/Steelhead Trout

Coastal rainbow and coastal cutthroat trout are both native to the Pacific coast drainages of North America. The native range of coastal rainbow trout covers an area as far south as central California to as far north as the Alaska Peninsula. Coastal cutthroat's native range occurs from northern California to southeastern Alaska. Both species have anadromous and resident freshwater life histories; anadromous coastal rainbow trout are specifically referred to as steelhead while anadromous cutthroat trout are referred to as simply sea-run cutthroat trout.

Steelhead trout generally spawn in late winter to early spring (February – April) (Pearcy et al. 1990) using primarily deep, fast water of larger rivers. Resident freshwater coastal rainbow trout generally spawn during a similar timeframe as steelhead (February – May) and they occupy various ecosystems; however they typically spawn in small to moderately large (but shallow) streams and rivers. Sea-run coastal cutthroat trout return to freshwater in late fall to early winter (i.e. October – December), feed over the winter, and spawn mid/late winter to early spring (January – May) (Trotter 1989) depending on locale. Mature resident freshwater cutthroat trout spawn during the same time period as their anadromous counterpart, and both life history types prefer to utilize smaller headwater streams for spawning (Trotter 1989). Hartman & Gill (1968) reported that where cutthroat and coastal rainbow/steelhead were sympatric, juvenile cutthroat were predominant in headwater tributaries and rainbow/steelhead juveniles in larger river reaches. It has been postulated, however, that habitat preferences for cutthroat and coastal rainbow/steelhead trout may overlap considerably (Campton & Utter 1985).

2.3.3 Sample Collection

Samples were collected from 37 sympatric populations of coastal cutthroat and rainbow/steelhead trout on Vancouver Island (Fig. 2.1). All fish were collected during early/mid summer 2002 (22 June - 30 July) and 2003 (20 June - 7 July) using a 2-pass backpack electroshocking technique (Smith-Root, Model LR-24, Vancouver, WA). Captured fish were anaesthetized using a mixture of clove oil and stream water (10-15 ppm), fin clips were collected and stored in 95% ethanol (28-38 individuals per locality), and fish were released back to sites from which they were collected once fully recovered from anaesthetic. To avoid any potential bias in sampling, fish were fin clipped as they were encountered until a desired sample size was reached without regard to morphological species identification. Chase River was sampled in both 2002 and 2003, to determine temporal stability in this highly introgressed population. All sample locations were recorded in the field using a global positioning system (GPS) (Garmin eTrex, Kansas City, KS) to accurately locate sample sites within specific Vancouver Island watersheds for eventual use in a geographic information system (GIS).

2.3.4 Genetic Analysis

Extraction of DNA from fin clips was conducted using the Wizard DNA Purification Kit (Promega Corp. Madison, WI) following manufacturer's instructions. Seven PCR-based nuclear co-dominant markers and one mitochondrial DNA (mtDNA) marker, diagnostic for coastal cutthroat and coastal rainbow trout, were used in this study to assess the hybridization status of each fish. Five markers (one size polymorphism and four Restriction Fragment Length Polymorphisms – RFLP hereafter) were developed

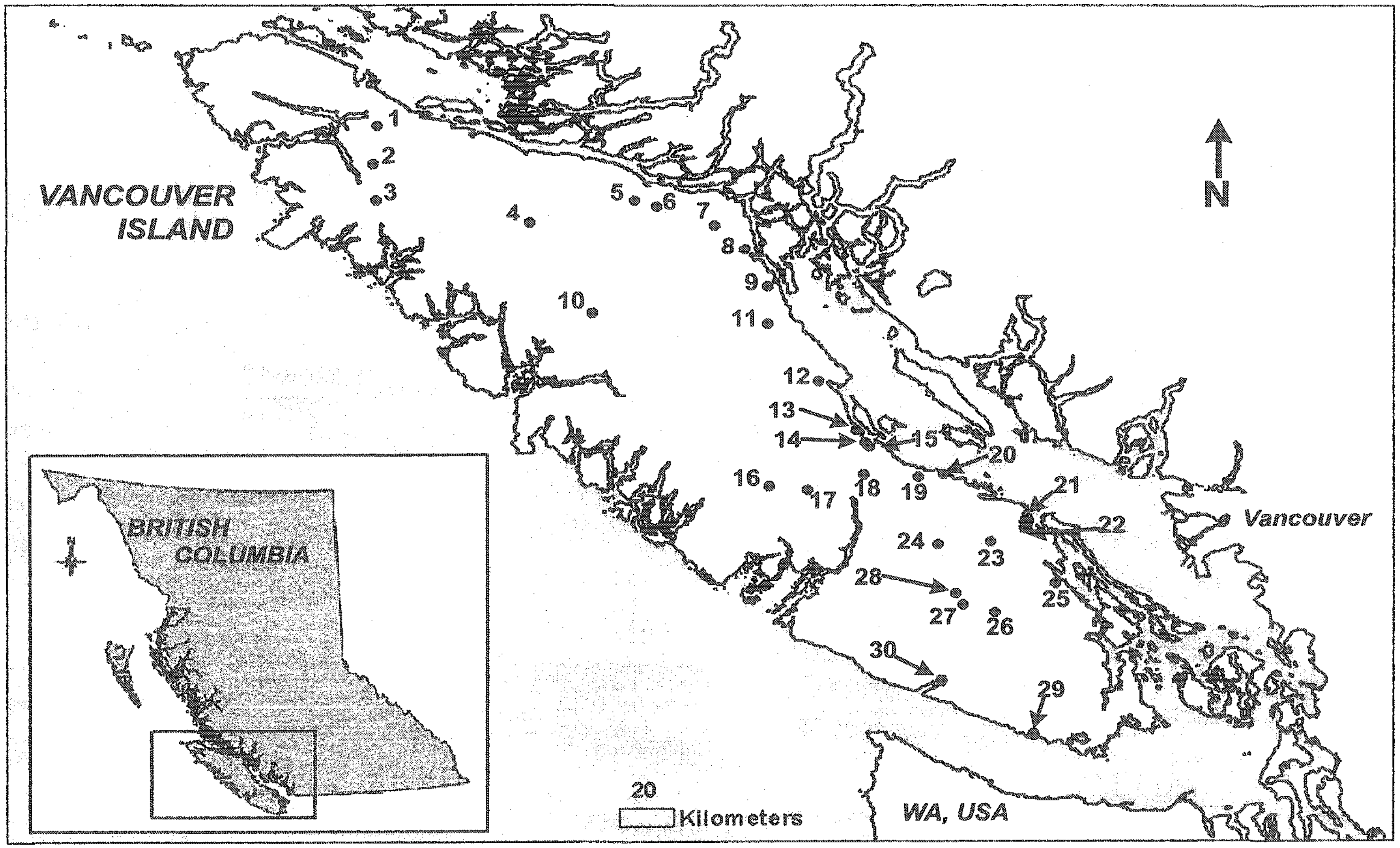

Figure 2.1 Inset map shows primary geographical study location in British Columbia, Canada. Map of Vancouver Island, British Columbia showing surveyed locations. All stream identification numbers correspond to Map ID in Tables 2.3 and 2.4

by Baker et al. (2002), who validated them using coastal cutthroat and rainbow/steelhead trout populations from Oregon and Washington State, USA, a steelhead out-group from Russia, and two inland subspecies of cutthroat trout (Westslope and Yellowstone cutthroat trout; *O. clarki lewisi; O. clarki bouvieri* respectively). The two remaining RFLPs (GH1D and TFex 3-5) were developed in the current study. Additionally, a mtDNA marker (ND3) was used to detect the directionality of hybridization (Docker et al. 2003). A complete listing of genetic markers, with corresponding restriction enzymes, can be found in *Appendix I.* I further validated all species-specific RFLPs and size polymorphisms (including the two novel markers and the mtDNA marker) as diagnostic using an additional 30 allopatric coastal rainbow and 30 allopatric coastal cutthroat trout taken from five populations located throughout coastal British Columbia.

Polymerase chain reactions (PCR) were performed using a standard 25 µL reaction that contained: 10 mM Tris-HCl (pH-8.4) 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.05 µg of each primer, 0.5 units of DNA Taq polymerase, and approximately 100 ηg of genomic DNA template. The optimized thermocycler (MJ Research model PTC-0225) profile consisted of a 'hot-start' and a 2-minute initial denaturation (94°C), followed by 35-40 cycles of 1-minute denaturation cycle (94°C), a 1-minute annealing (49°C – 63°C; refer to *Appendix I* for specific annealing temperatures for each marker), a 1.5-minute extension (72°C), and ending with a final 5-minute extension cycle (72°C). Five microliters of individual PCR product were then digested (excluding size polymorphism, GH2D) for 6 hours in a 10-µL reaction mix containing ddH_2O (3.5 µL), enzyme optimizing buffer (1 µL), restriction enzyme (0.25 µL), and BSE (0.25 µL).

PCR products, size polymorphisms, and RFLPs were separated by agarose gel electrophoresis at 80-90 volts through a 1.8% agarose gel. All fragments were visualized using ethidium bromide staining and UV transillumination. All hybrid genotypes that could be interpreted as 'partial' restriction digests were re-amplified and re-digested to confirm genotype.

2.3.5 Hybrid Calculation

All fish were genotyped as homozygous rainbow trout, homozygous cutthroat trout, or heterozygous at each of the seven nuclear loci. Fish that were identified as homozygous at all seven loci, for one species, were considered pure-type for that species. First-generation (F1) hybrid fish were those individuals identified as heterozygous at all seven loci, while backcross hybrids were those that were identified as having a mix of homozygous and heterozygous marker loci. Individual fish that were homozygous at all seven co-dominant loci (of either species), but carried the mitochondrial haplotype of the other species, were identified as an "ancient" backcross hybrid. Mitochondrial DNA haplotypes were assigned as cutthroat or rainbow trout for all fish. I quantified hybridization in each sample population using two statistics (Fig 2.2). I first calculated a "*Hybridization Index*" (H_I) where there was no discrimination of hybrids based on hybrid type or level of introgression. I calculated this index by dividing the number of observed hybrids within a population, by the total number of fish collected in the population. This was computed to give a general indication the extent of hybridization in each population. Second, I assessed the degree of introgression for each population using an *'Introgression Index'* (I_I), calculated as;

$$\textit{Introgression Index}\ (\%\,I_I) \quad = \quad \frac{(\#\text{ of }A_R)\times 2}{A_T} \times 100\%, \qquad (1)$$

where A_R is the number of rare species alleles observed within individual hybrids (i.e. < 7 alleles) and A_T is the total number of alleles within individual hybrids (A_T is constant for our system; 14 alleles). Pure-type individuals within populations were assigned a value of $I_I = 0\%$. In the case where F1 hybrids were encountered, they were assigned a value of $I_I = 100\%$. Mean introgression was calculated for each population (Chase River was calculated for sample years 2002 and 2003 separately). This particular index provides a relative measure of genome introgression in both coastal cutthroat and rainbow trout populations. The use of this index differs from other indices used in hybridization studies of inland native cutthroat and introduced rainbow trout (e.g. Hitt et al. 2003). In those cases, emphasis was placed on identifying introgression levels of nonnative alleles (e.g. introduced rainbow trout) into native populations (e.g. inland cutthroat spp.). Since both coastal cutthroat and rainbow/steelhead trout are native to our sample locations the more common measures of introgression were not applicable. Our '*Introgression Index*' takes into account introgression into both species and reciprocal introgression.

2.3.6 Environmental Effect Estimation

Collection of environmental data was organized based on the watershed where sample streams were located. The term "watershed" describes an area of land that drains downslope through a common outflow. Water moves by means of a network of drainage pathways (e.g. stream network) most notably above ground via streams and rivers. Because water moves downstream, any activity that affects the water quality, quantity, or rate of movement at one location can change the characteristics of the watershed at locations downstream (Chamberlain et al. 1991). Consequently, watershed level assessments have been shown to have effective predictive capability for evaluating relative environmental (e.g. anthropogenic) effects on freshwater fish populations (Hunsaker & Levine 1995; Roth et al. 1996; Wang et al. 1997; Regetz 2003; Feist et al. 2003).

Watershed data for British Columbia are in a province-wide GIS database, which holds extensive baseline information, particularly for variables pertaining to the effects of forest harvesting (BC Watershed Statistics data dictionary, http://srmwww.gov.bc.ca/risc). Vancouver Island watersheds were extracted from a provincial database in ArcMap (ArcGIS Version 8.1, ESRI, Redlands, CA), using the '*join by attribute*' command, and the '*select by graphics*' command. Once Vancouver Island was isolated within ArcMap, watershed attributes were attached to each spatial unit using the '*join*' command. Global Positioning System (GPS) coordinates obtained for all sample locations in the field (in decimal degrees) and were added to the database using the '*add XY data*' command. This was performed to allow precise identification of sampled stream locations within their respective watersheds.

Most environmental variables were chosen based on current understanding of habitat factors deemed most important to western North American trout and the habitat factors believed to be most vulnerable to disturbance. A total of 8 variables were selected for inclusion in the analyses (see Table 2.1). The first five variables included: (i) % young forest ("%YF"), (ii) % recently logged forest ("%RL"), (iii) length (km) of stream within a watershed ("StLg"), (iv) road density (km/km^2) ("RdDs"), and

Table 2.1 List of environmental variables chosen for inclusion in analyses. All variables are accompanied with corresponding description.

Environmental Variables		Variable Description
Watershed Stream Length (km)	"StLg"	Total length of all streams within a given watershed
Young Forest (%)	"%YF"	Percent of watershed that has been logged approximately 40-140 years ago but has partially recovered
Recently Logged (%)	"%RL"	Immediate clear-cut logging effects within a watershed primarily within the last 10-20 years (includes logging to streambank)
Road Density (km/km^2)	"RdDs"	Density of all roads within the watershed (includes urban and non-urban areas)
Stream Crossings (#/km^2)	"CrDs"	Total number of stream crossings per square km of watershed
Stream Availability (%)	"%SAV"	The amount of stream available below an impassable barrier divided by the total length of sampled stream
Anadromous Life History Influence		Streams that are influenced by the presence of sea-run cutthroat trout and/or steelhead trout life history types
Trout Stocking		Streams that have been stocked with coastal cutthroat and/or coastal rainbow trout (including any life history type)

(v) stream crossing density (#/km^2) ("CrDs"). It should be noted that "StLg" was chosen to represent the size of a watershed, since "StLg" and watershed area were highly positively correlated ($r^2 = 0.98$). The first three variables chosen include potential effects of forest harvesting activities to Vancouver Island watersheds. Road density and number of stream crossings per watershed, which also pertain to forest harvesting activities, also reflect possible urban, agricultural, and rangeland impacts. Choice of these variables were justified based on literature (e.g. Hartman et al. 1996) that identifies forest harvesting impacts on streams based on recent logging (i.e. 3-20 years) and long-term logging (i.e. 20-140 years) effects. Recent logging relates to immediate effects on streams after logging, for example increased fine sediment due to erosion sources (soil leaching, exposed slope soils, road surfaces and ditches etc; Bescheta 1978; Porter et al. 2000) and increased stream temperature due to loss of canopy cover (increased direct sunlight; Holtby 1988). These effects are known to continue for 3-20 years until forest recovery is established and vegetation has begun stabilizing disturbed areas of stream (Hartman et al. 1996). Long-term logging effects are those that are not immediate and they reflect the occurrence of flooding events and/or the deterioration of stump root strength, years after timber removal (Swanston 1991). These effects can accumulate over 20 years and persist for several decades (Hartman et al. 1996). Flood events and unstable soils due to root deterioration have been known to cause severe transport of sediment to streams, and hence change the composition of spawning gravel when sediments are deposited from upstream channels (Slaney et al. 1977). Data were extracted for individual from GIS watershed data for Vancouver Island using the '*identify*' tool in ArcMap.

The remaining three environmental variables, which include trout stocking, life-history type presence (i.e. anadromous and/or resident freshwater), and stream availability (%SAV) were obtained (and generated) from the BC government *FishWizard* website (http://pisces.env.gov.bc.ca). These environmental factors were selected for their known effects on the incidence of hybridization between other species of salmonids, as well as factors believed to be biologically essential to the reproductive success for both trout species (i.e. %SAV). The presence/absence of impassable barriers (obtained from *FishWizard*) was used to calculate percent stream availability (%SAV). All populations were sampled below impassable barriers when barriers were present. The geographic coordinates for all barriers were incorporated into ArcMap. Using the '*measure*' tool, the distance of stream below impassable barriers was measured (in km) to its first confluence (i.e. a major river, a lake, or the ocean). The measured distance was then divided by the total stream length (in km) to give the proportion of available stream habitat. The derived percent stream availability ("%SAV") thus represents the proportion of stream that is available to fish populations below impassable barriers. For streams identified with no impassable barrier %SAV = 100%.

2.3.7 Statistical Analysis

Two types of analyses were used to test for associations between environmental effects (factors) and levels of hybridization: (1) a continuous model (regression) and (2) a categorical model (ANOVA). Continuous models included both simple and multiple regression models. Before models were constructed, particular environmental variables (% YF, % RL, StLg, RdDs, and CrDs) were tested for correlation. Significant correlated relationships were observed between %RL and %YF ($r = -0.46$; $p < 0.05$) as well as %RL

Table 2.2 Correlation matrix (r-values) for continuous environmental variables. Bold-type with asterisk (*) represents a significant correlation between variables ($p < 0.05$). Significantly correlated variables were not combined for multiple regression models.

Variables	W/S Stream Length	Young Forest	Recently Logged	Road Density	Stream Crossing Density
W/S Stream Length	1.00				
Young Forest	-0.20	1.00			
Recently Logged	0.21	**-0.46***	1.00		
Road Density	-0.18	0.27	-0.07	1.00	
Stream Crossing Density	0.25	-0.20	**0.57***	0.21	1.00

and CrDs ($r = 0.57$; $p < 0.05$; see Table 2.2). Hence, these parameters were not combined in multiple regression models. Percent variables were *arcsine square root* transformed and density variables (roads and stream crossings) were *log* transformed to meet assumptions of normality and equal variance (Berry 1987). Simple linear regression analyses were performed using each hybridization statistic (H_I and I_I) as the dependent variable and all continuous variables as independent variables (SYSTAT® Version 7.01 SPSS, IL, USA). Multiple regression analyses were performed using the introgression statistic (I_I) as the dependent variable, but with specific combinations of continuous variables grouped into two anthropogenic effect categories: (1) timber harvesting, which tests for associations directly related to the practice(s) of timber removal; and (2) infrastructure, which tests for associations related to road development. Infrastructure combines road development from urban, agriculture, rangeland, and forested areas. Stream availability (%SAV) was explored independently using a simple regression model. A 'Habitat Availability' model was assessed by combining %SAV and StLg in a multiple regression model using the introgression statistic (I_I) as the dependent variable. The hybridization statistic (H_I) was not used for multiple regression models because the introgression statistic (I_I) is a more sensitive indicator of environmental disturbance effects. Since H_I and I_I are highly correlated ($r^2 = 0.88$; see Fig. 2.2), the use of I_I as the exclusive dependent variable for multiple regression models is justified. The General Linear Model routine (SYSTAT® Version 7.01 SPSS, IL, USA) was used for analysis of all single and multiple regression models.

Effects from categorical environmental factors were investigated using an analysis of variance (ANOVA). An ANOVA was used to test for combined effects of anadromous life-history presence, stocking, and %SAV. The introgression statistic (I_I) was used as the dependent variable and anadromous life-history presence, stocking, and %SAV were used as independent variables for categorical (ANOVA) models. Interactions between variables were also tested for significance. The General Linear Model routine (SYSTAT® Version 7.01) was used for all models.

2.4 RESULTS

2.4.1 Hybrid Identification

Seven of 37 populations consisted of 100% pure genotypes of only one trout species (i.e. either cutthroat or rainbow/steelhead with no presence of hybrids).

Consequently, these populations were excluded from further analyses because field and genetic sampling did not identify a sympatric relationship between trout species nor any evidence of hybridization.

Two hundred and eighty-four hybrids out of 1004 fish genotyped (29%) were identified across all sample locations. First generation (F1) hybrids were least abundant making up 7% (*n* = 62) of the fish genotyped during this study. Backcross hybrids made up 22% (*n* = 222) of the total number of genotyped fish and pure coastal cutthroat and rainbow/steelhead consisted of 36% (*n* = 365) and 35% (*n* = 355) of the sample respectively.

Only one stream (Misery Creek) had no evidence of hybrids despite the presence of both trout species (Table 2.3). Five populations (Menzies Creek, Morrison Creek, Cowie Cougar-Smith Creek, Chase River, and Meade Creek) demonstrated hybridization levels of 50% or higher, with Cowie Cougar-Smith Creek and Chase River '02 displaying the highest levels at 88% and 86% respectively (Table 2.3). Only 7 populations (Waukwaas Creek, Marble River tributary, Elk Creek, Roberts Creek, Rosewall Creek, Wardroper Creek, and Fairy Creek) demonstrated hybridization less than 10% (Table 2.3).

Introgression index (I_I) values indicate widespread gene flow between the two trout species throughout Vancouver Island (Fig. 2.2; Table 2.3). Eight populations exhibited very high levels of introgression. Chase River showed high introgression levels in both 2002 and 2003 sample years (54% and 41% respectively). Cowie Cougar-Smith Creek also displayed a high level of introgression (48%). The high incidence of introgression in these two populations, and the low incidence of pure trout of both

Table 2.3 Sample size (*n*) and observed ratios of fish species and hybrid type based on genetic identification for Vancouver Island streams. Map identification (ID) numbers correspond to those provided in **Figure 2.1**. Pure CTT – pure cutthroat trout; Pure RBT – pure rainbow/steelhead trout; H_I – Hybridization Index; I_I – Introgression Index.

Map ID	Population	*n*	Pure CTT (%)	Pure RBT (%)	H_I	I_I
1	Waukwaas Cr	37	0.00	0.97	0.03	0.03
2	Howlal Cr	29	0.48	0.04	0.48	0.23
3	Marble R trib.	28	0.86	0.07	0.07	0.03
4	Lukwa Cr	31	0.48	0.13	0.39	0.31
5	Elk Cr	33	0.94	0.00	0.06	0.03
6	Stowe Cr	30	0.10	0.67	0.23	0.09
7	Roberts Cr	34	0.91	0.00	0.09	0.04
8	Menzies Cr	30	0.43	0.00	0.57	0.30
9	Cold Cr	30	0.27	0.37	0.36	0.37
10	Nameless Cr	32	0.25	0.63	0.12	0.08
11	Woodhus Cr	30	0.00	0.90	0.10	0.04
12	Morrison Cr	33	0.42	0.03	0.55	0.25
13	Cowie CS Cr	32	0.03	0.09	0.88	0.48
14	Rosewall Cr	27	0.00	0.96	0.04	0.02
15	Cook Cr	32	0.13	0.47	0.40	0.26
16	Taylor R trib.	30	0.87	0.00	0.13	0.05
17	Friesen Cr	33	0.30	0.21	0.49	0.34
18	Esary Cr	37	0.92	0.00	0.08	0.03
19	Whisky Cr	36	0.81	0.08	0.11	0.11
20	French Cr	28	0.00	0.89	0.11	0.06
21	Millstone R	35	0.37	0.26	0.37	0.35
22	Chase R '02	35	0.00	0.14	0.86	0.54
22	Chase R '03	37	0.00	0.19	0.81	0.41
23	N Nanaimo R	38	0.05	0.74	0.21	0.16
24	Rockyrun Cr	37	0.00	0.70	0.30	0.13
25	Stocking Cr	32	0.00	0.81	0.19	0.04
26	Meade Cr	30	0.47	0.03	0.50	0.30
27	Misery Cr	32	0.97	0.03	0.00	0.00
28	Wardroper Cr	34	0.97	0.00	0.03	0.02
29	Kirby Cr	31	0.16	0.74	0.10	0.06
30	Fairy Cr	31	0.06	0.87	0.07	0.05

species, indicates they are likely hybrid swarms. Menzies Creek (I_I = 30%), Morrison Creek (25%), Friesen Creek (34%), Millstone River (35%), and Meade Creek (30%) all displayed relatively high levels of introgression, indicating the genetic integrity of pure trout in these systems is deteriorating and may result in hybrid swarms as in Chase River and Cowie Cougar-Smith Creek.

2.4.2 Environmental Factor Analysis

Hybridization Index (H_I)

The hybridization index (H_I) provides a general indication of the extent of hybridization in each population. Percent YF and StLg (values in Table 2.4) were associated with increased hybridization (Table 2.5) in simple linear regression analysis ($p = 0.02$ and $p = 0.008$ respectively). Percent YF had a positive relationship with H_I indicating that hybridization increases with increased %YF among watersheds. Total stream length in a watershed (StLg) had a negative slope, indicating elevated hybridization is associated with smaller stream networks. ANOVA results for anadromous life-history influence (i.e. presence/absence in either species; Table 2.4) revealed no significant effect on levels of hybridization (H_I). Fish stocking (i.e. with either trout species) also did not significantly affect H_I in the ANOVA (Table 2.5).

Introgression Index (I_I)

The introgression index (I_I) provides a relative measure of genome introgression in both coastal cutthroat and rainbow trout populations and is thus a more sensitive indicator of reproductive isolation breakdown. Percent stream availability (%SAV), %RL,

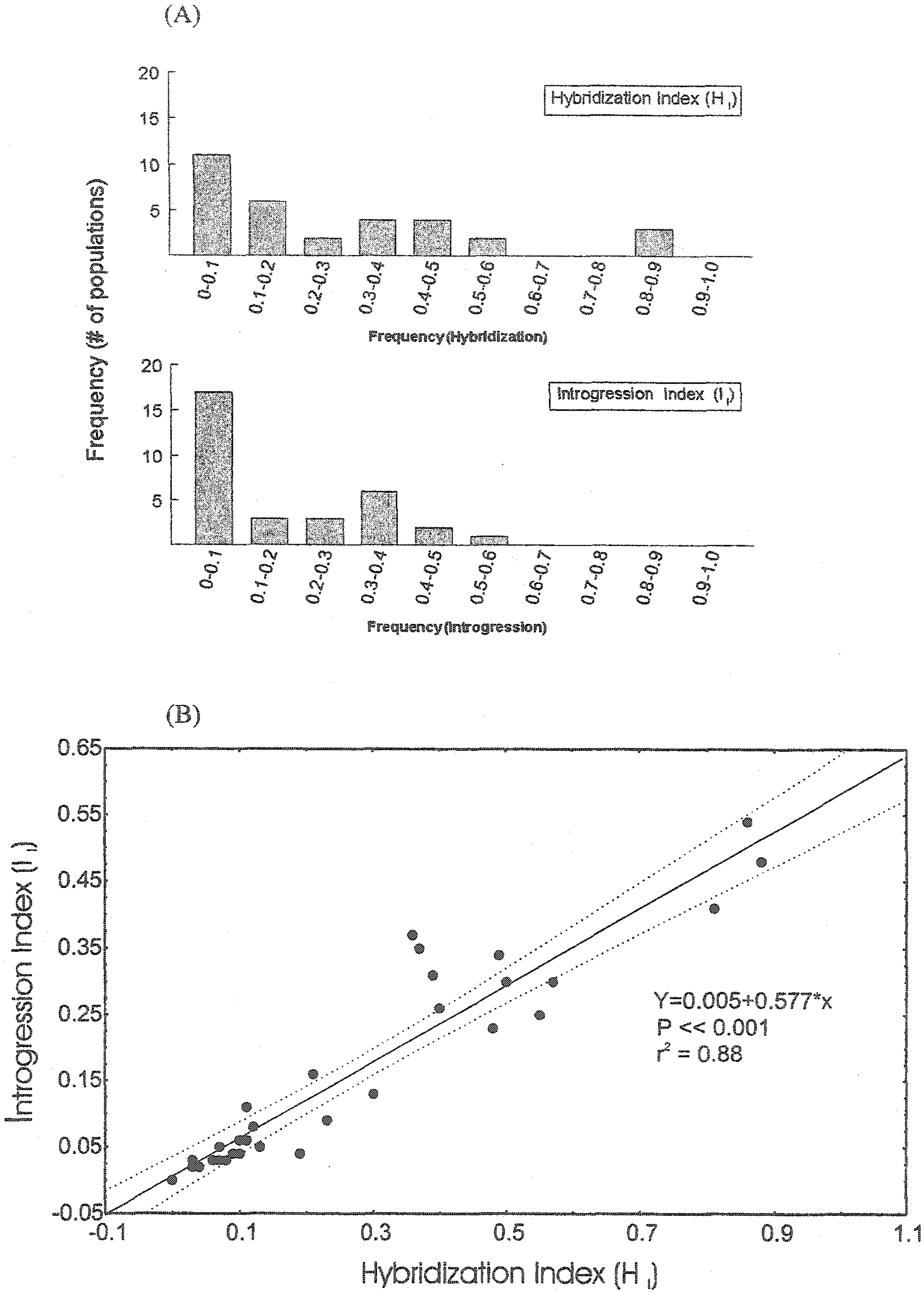

Figure 2.2 (A) Frequency distribution for Hybridization Index (H_I) and Introgression Index (I_I) for 30 populations sampled from Vancouver Island, BC. (B) Linear regression of H_I versus I_I to display relationship between indices. Dotted lines represent the 95% confidence interval.

Table 2.4 Environmental variable values for all sampled streams. Continuous variables include: StLg (km) – stream length in watershed; %YF – % young forest; %RL - % recent logging; RdDs (km/km^2) – road density; StCr (#/KM^2) - stream crossing density; %SAV – % stream availability. Categorical values include anadromous life-history presence and fish stocking, which are based on presence (Y) or absence (N) of the variable in individual streams.

		Variables							
Map ID	Population	StLg (km)	YF (%)	RL (%)	RdDs (km/km^2)	StCr (#/km^2)	SAV (%)	Anadromous Life History Presence (Y/N)	Fish Stocking (Y/N)
1	Waukwaas Cr	80.2	55.1	21.2	1.3	2.1	100.0	Y	Y
2	Howlal Cr	28.8	81.0	3.5	1.9	2.7	100.0	Y	N
3	Marble R trib.	343.2	50.0	16.1	1.7	2.2	100.0	N	N
4	Lukwa Cr	80.5	29.0	21.7	1.2	1.7	100.0	Y	N
5	Elk Cr	81.8	26.5	22.3	1.6	1.0	100.0	N	N
6	Stowe Cr	581.1	35.4	20.1	1.3	0.8	24.5	Y	N
7	Roberts Cr	58.0	64.5	23.8	1.6	1.1	100.0	N	Y
8	Menzies Cr	33.1	83.5	5.7	1.3	0.8	100.0	Y	N
9	Cold Cr	8.0	90.4	0.0	2.4	0.2	100.0	Y	N
10	Nameless Cr	151.4	24.1	23.3	1.5	1.9	100.0	N	N
11	Woodhus Cr	41.6	71.7	9.7	1.7	0.7	100.0	Y	Y
12	Morrison Cr	16.9	38.5	21.0	3.2	2.0	100.0	Y	Y
13	Cowie CS Cr	27.4	84.8	13.3	1.1	0.9	43.7	Y	N
14	Rosewall Cr	86.8	54.8	14.0	1.2	1.2	100.0	Y	Y
15	Cook Cr	55.6	79.5	10.2	1.3	1.2	27.0	Y	N
16	Taylor R trib.	262.6	18.4	14.5	0.9	1.7	100.0	Y	N
17	Friesen Cr	311.2	51.3	13.5	1.9	1.3	13.6	Y	N
18	Esary Cr	228.5	62.5	7.1	1.6	1.0	100.0	N	N
19	Whisky Cr	122.7	48.7	15.9	2.4	1.0	66.5	Y	Y
20	French Cr	97.2	46.7	19.8	2.4	1.5	51.0	Y	Y
21	Millstone R	102.0	65.2	0.4	3.5	1.0	18.1	Y	Y
22	Chase R	25.3	70.7	0.7	3.9	0.8	30.5	Y	Y
23	N Nanaimo R	101.2	77.3	13.8	2.3	1.7	100.0	Y	Y
24	Rockyrun Cr	24.8	19.7	29.0	2.2	4.3	100.0	N	N
25	Stocking Cr	32.2	46.0	9.6	4.1	1.2	100.0	Y	Y
26	Meade Cr	113.2	87.8	12.2	2.3	2.8	27.2	Y	Y
27	Misery Cr	618.6	70.2	11.7	2.2	1.4	100.0	Y	N
28	Wardroper Cr	618.6	70.2	11.7	2.2	1.4	38.7	Y	N
29	Kirby Cr	36.0	45.9	51.6	2.5	1.4	59.0	Y	N
30	Fairy Cr	45.0	20.2	1.3	0.6	0.3	41.4	N	N

%YF, and StLg significantly influence introgression in simple linear regression analyses (Table 2.5), although neither %RL or %SAV were found to have a significant association with the hybridization index (H_I). Percent recently logged (%RL) area produced a significantly negative slope, indicating that increased levels of recent logging are associated with decreased introgression. Percent stream availability (%SAV) displayed a negative slope, indicating that as stream availability increased, introgression decreased. This may reflect that as more habitat is available, opportunity for hybridization is reduced. The combination of StLg (slope = -0.12) and %YF (slope = 0.24) showed a significant association (Table 2.6; $p < 0.01$; $r^2 = 0.30$), indicating that when %YF is increased in watersheds with simple stream networks, introgression is extensive. The combination of %RL (slope = -0.35) and StLg (slope = -0.12) also revealed a significant association, indicating that when %RL increases in watersheds with smaller stream networks, introgression is lower (Table 2.6). Multiple regression models revealed the greatest proportion of variation in introgression was explained by the habitat availability model (see Table 2.7; $p = 0.0001$; $r^2 = 0.42$). When watersheds are comprised of simple stream networks in combination with limited stream availability, introgression is substantial. Additionally, a significant interaction was observed between %SAV and StLg (see Table 2.7) indicating that these habitat variables strongly affect introgression when working together rather than as independent effects. Results of one-way analysis of variance (ANOVA) for anadromous life-history influence (i.e. presence/absence of either species) revealed no significant difference in introgression (I_I) between the two life histories (see Table 2.5). Fish stocking was significantly associated with elevated levels of introgression (I_I) (Table 2.5; ANOVA $p < 0.05$). A significant effect on introgression

Table 2.5 Results of (A) simple linear regression analyses; and (B) one-way ANOVA for Hybridization Index (H_I) and Introgression Index (I_I). *P*-values and coefficient of determination values (r^2) are also given, with significant values identified in bold-type.

	H_I			I_I		
(A)	r^2	*p*	Slope	r^2	*p*	Slope
Stream Length in W/S (km)	**0.19**	**0.008**	**-0.24**	**0.17**	**0.01**	**-0.14**
Young Forest (%)	**0.13**	**0.02**	**0.41**	**0.18**	**0.01**	**0.29**
Recently Logged (%)	0.08	0.07	-0.49	**0.17**	**0.01**	**-0.42**
Stream Availability (%)	0.08	0.07	-0.24	**0.13**	**0.03**	**-0.19**
Road Density (km/km^2)	0.01	0.28	0.26	0.02	0.22	0.19
Stream Crossing Density (no./km^2)	0.00	0.88	-0.03	0.00	0.38	-0.09
(B)						
Anadromous Life-History Presence	0.03	0.45	—	0.02	0.48	—
Fish Stocking	0.07	0.17	—	**0.09**	**0.04**	—

was observed with the combined variables of stocking and %SAV (Table 2.7; ANOVA $p < 0.05$; $r^2 = 0.27$). Results indicate that introgression is higher when stocking of trout occurs in locations of reduced stream availability.

2.5 DISCUSSION

2.5.1 Spatial Distribution of Hybridization

Though little is known of the extent of hybridization (and introgression) between sympatric coastal cutthroat and coastal rainbow/steelhead throughout their entire native range, my work shows that hybridization is widespread within their native range on Vancouver Island. Though the broad geographic incidence of hybridization is startling, it is not completely uncommon. For example, a study conducted by Spruell et al. (1998) on the Lower Columbia River indicated that in no case did coastal cutthroat and rainbow trout co-exist without evidence of hybridization. In the current study, 29 of 30 sympatric trout populations sampled over a broad spatial scale on Vancouver Island (see Fig. 2.1) showed evidence of hybridization, a pattern similar to that observed by Spruell et al. (1998). Interestingly, the frequency of hybridization among populations in this study is highly variable.

In populations with low levels of hybridization, only one or two hybrids were identified, with the remaining trout samples comprised of one pure-type (i.e. parental type) species (i.e. Waukwaas Creek, Roberts Creek, Rosewall Creek; see Table 2.2). One possible explanation why these populations exhibited low frequencies of hybrids and such a high frequency for one pure-type species, may be hybrids straying into locations

Table 2.6 Results of multiple linear regression analysis for Introgression Index (I_I) with selected timber harvesting and infrastructure environmental factors. Two models were constructed for timber harvesting and three models were constructed for infrastructure. Probabilities (black squares[a]) and regression coefficients (i.e. slopes) are provided for each variable. Total *P*-values and coefficient of determination (r^2) for each model are also provided. Dashes indicate variables were not included in models. Independent variable abbreviations correspond to: StLg (km) – stream length in watershed; %YF – % young forest; %RL - % recent logging; RdDs (km/km^2) – road density; StCr (#/km^2) - stream crossing density.

Timber Harvesting

		A	B	C				
Dependent Variable:		YF (%)	RL (%)	StLg (km)	Interaction A + B	Interaction A + C	Interaction B + C	**TOTAL MODEL**
Introgression	Model1	■ (0.24)	—	■ (-0.12)	—	ns	—	**$p = 0.002$ $r^2 = 0.30$**
Index (I_I)	Model2	—	■ (-0.35)	■ (-0.12)	—	—	ns	**$p = 0.005$ $r^2 = 0.28$**

Infrastructure

		A	B	C				
Dependent Variable:		RdDs (km)	StCr (#/km)	StLg (km)	Interaction A + B	Interaction A + C	Interaction B + C	**TOTAL MODEL**
Introgression	Model1	ns	ns	■ (-0.12)	ns	ns	ns	NS
Index (I_I)	Model2	ns	—	■ (-0.13)	—	ns	—	NS
	Model3	—	ns	■ (-0.14)	—	—	ns	**$p = 0.04$ $r^2 = 0.15$**

[a] ■ $p < 0.05$; ■■ $p < 0.01$; ■■■; $p < 0.001$; NS/ns - not significant

Table 2.7 Results of ANOVA for Introgression Index (I_I) for selected categorical environmental factors and multiple linear regression analysis for Introgression Index (I_I) for the habitat availability model. Three ANOVA models were constructed for categorical effects and one model was constructed for habitat availability. Probabilities (black squares[a]) and regression coefficients (i.e. slopes) are provided for each selected variable (regression coefficients not available for ANOVA results). Total model *P*-values (excluding ANOVA analyses) and coefficient of determination (r^2) for each model are also provided. Dashes indicate variables were not included in models. Independent variable abbreviations correspond to: StLg (km) – stream length in watershed; % SAV – % stream availability; Anadromy – anadromous life history presence; Stocking – trout stocking.

Categorical

		A	B	C				
Dependent Variable:		Anadromy	Stocking	SAV (%)	Interaction A + B	Interaction A + C	Interaction B + C	**TOTAL MODEL**
Introgression Index (I_I)	Model1	ns	■	■	ns	ns	ns	**$r^2 = 0.17$**
	Model2	ns	—	■	—	ns	—	NS
	Model3	—	■	■	—	—	ns	**$r^2 = 0.27$**

Habitat Availability

		A	B	C				
Dependent Variable:		StLg(km)	SAV (%)	—	Interaction A + B	Interaction A + C	Interaction B + C	**TOTAL MODEL**
Introgression Index (I_I)	Model1	**■■■ (-0.18)**	**■■ (-0.24)**	—	**■■■ (-0.13)**	—	—	**p = 0.0001 $r^2 = 0.42$**

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[a] ■ $p < 0.05$; ■■ $p < 0.01$; ■■■; $p < 0.001$; NS/ns - not significant

exclusively inhabited by one pure-type. In other words, the location where samples were collected in the field was not in proximity to where hybridization took place. The reason straying of hybrids may pose a problem is because hybrid straying has known to be a factor in the spread of hybridization in other trout hybrid systems. Studies of hybridization between westslope cutthroat (*O. clarki lewisi*) and rainbow trout (e.g. Hitt et al. 2003) as well as yellowstone cutthroat (*O. clarki bouvieri*) and rainbow trout (Campbell et al. 2002) have implicated hybrids straying into previously pure cutthroat populations, as a major factor in the spread of hybridization within streams. Additionally, Weigel et al. (2003) found that the spread of hybridization between westslope cutthroat and rainbow trout was inversely related to stream elevation, suggesting that the spread of hybridization is limited to lower elevated streams. The bulk of streams that I sampled on Vancouver Island were at lower elevations (data not shown), thus hybridization in low elevation streams may enhance the spread of hybridization throughout whole watersheds. It should be made clear that sampling for this study was conducted specifically to: (1) determine if hybridization was present and (2) if present, provide a general indication as to the incidence of hybridization over a broad spatial scale. Sampling was not intended to investigate the spatial incidence of hybridization at a local population scale because the extent of hybridization in streams sampled (except Chase River and Stocking Creek) was previously unknown. Thus, investigating the spatial distribution of hybridization between coastal cutthroat and coastal rainbow/steelhead trout at a stream-reach approach will allow for better understanding of the significance of hybrid straying as a means of spreading hybridization.

2.5.2 Temporal Stability of Hybrids

Hybrid swarms have been previously reported between various subspecies of cutthroat trout and rainbow trout (e.g. Forbes and Allendorf 1991; Carmichael et al. 1993); however evidence of hybrid swarms in coastal cutthroat and coastal rainbow/steelhead populations has rarely been reported (e.g. Campton and Utter 1985; Young et al. 2001, but see Docker et al. 2003). In the current study, Chase River ('02 and '03) and Cowie Cougar-Smith Creek exhibited extremely high levels of introgression (I_I = 48% and 54% respectively) with a diverse array of recombinant genotypes, along with relatively few pure-types – all indicative of hybrid swarms. My data clearly indicate that hybrid swarms can, and do, form between these sympatric trout species (see Chapter 3). Campton and Utter (1985) and Young et al. (2001) did not detect hybrid swarms in sympatric populations of coastal cutthroat and coastal rainbow/steelhead trout in Washington State, USA, and the authors suggested that the lack of hybrid swarms might have been due to factors that inhibit or prevent complete introgression in these species (i.e. postzygotic reproductive barriers; Young et al. 2001). My data for Chase River ('02 and '03), combined with data from Docker et al. (2003) (Chase River sampled in 2000—92% total hybrids), not only demonstrate that hybrid swarms in coastal cutthroat and coastal rainbow/steelhead trout populations do exist, but in fact they display considerable temporal persistence. The apparent temporal persistence of these hybrid swarms is alarming; apparently, as the frequency of hybridization reaches some threshold level, all mechanisms of reproductive isolation appear to dissolve. Thus, I propose the concept of '*hybrid meltdown*' – that is – the total breakdown and irreversible loss of interspecific reproductive isolating mechanisms between recently diverged species. This term differs significantly from the term 'hybrid swarm', because 'hybrid swarm' only describes the level and extent of hybridization and not the actual consequence(s) of hybridization and introgression to a species or population. Furthermore, the *hybrid meltdown* process is analogous to the '*Invasional Meltdown*' model (Simberloff & Von Holle 1999; Ricciardi 2001): As the number of hybrids and environmental change increases cumulatively, reproductive isolating mechanisms break down to where they are irrecoverable. Remaining pure-types in a population will then reproduce with hybrids because hybrids significantly outnumber pure-types, thus the chances of mating with another pure-type of the same species is rare. Several populations, which displayed relatively high levels of introgression, appeared to not constitute a hybrid swarm (i.e. Friesen Creek, Meade Creek, Morrison Creek). However, data for Chase River indicates that the persistence of introgression (and hybridization) in these other hybrid populations (i.e. Friesen Creek, Meade Creek, Morrison Creek) is likely to drive them toward hybrid swarms. As a result, these populations may, too, be driven towards *hybrid meltdown* and ultimately to non-recoverable status for both trout species.

2.5.3 Environmental Effects on Introgression

Despite obvious associations between habitat perturbations and threatened or endangered species, conservation biologists have been hard pressed to link population health with environmental variables (Feist et al. 2003). Several studies of stream habitat variables and salmonid life history (i.e. spawning and rearing) have focused on fine-scale or local impacts (Hillman et al. 1987; Shirvell 1994; Geist and Dauble 1998). However, identifying relationships between habitat conditions and salmonid demography has proven extremely difficult (Regetz 2003). In this study, several environmental variables were used in an analysis of factors associated with introgression between sympatric coastal cutthroat and coastal rainbow/steelhead trout. It is evident that no single environmental factor controls introgression or hybridization between coastal cutthroat and coastal rainbow/steelhead trout on Vancouver Island. However, results of simple/multiple regression analyses and ANOVA (Tables 2.5, 2.6. 2.7) demonstrate that several environmental factors affect introgression, and each factor accounts for only a percentage of the variance when tested independently.

Trout stocking influences introgression between trout species, and these effects are magnified in locations with limited stream availability (i.e. below impassable barriers). Trout stocking on naturally sympatric trout populations and its effect on the increased incidence of hybridization is not unexpected. Docker et al. (2003) found that the frequency of hybridization and introgression was significantly higher in systems where hatchery rainbow trout were introduced. Furthermore, it has been shown that the introduction of nonnative rainbow trout into allopatric populations of native cutthroat (e.g. Carmichael et al. 1993; Rubidge et al. 2001) also results in extensive introgression. The present study did show that trout stocking was strongly associated with elevated hybridization levels in locations with minimal stream availability. Since sympatric trout species are often reproductively isolated by spatial separation they are less likely to be spatially separated in areas where stream availability is limited. When hatchery trout from exogenous populations are introduced, particularly in streams with reduced stream availability, ecological reproductive isolation appears to break down between trout species, ultimately resulting in elevated levels of hybridization.

Timber harvesting practices clearly have an effect on the incidence of introgression in coastal cutthroat and coastal rainbow/steelhead trout on Vancouver Island. The results of multiple regression models for timber harvesting (Table 2.6) revealed an interesting trend. An association between young forest (%YF), watershed stream length (StLg), and introgression indicated that where there is high percentage of young forest, associated with low total stream length (i.e. small watershed), the incidence of introgression is highly elevated. This result is perhaps not surprising given the fact that forestry activities have previously been correlated with declining populations of other Pacific salmonids (e.g. Slaney et al. 1996; Porter et al. 2000). Interestingly, my data indicate that the persistent, long-term effects of logging (i.e. as opposed to recent logging effects) in smaller watersheds significantly influence introgression. The persistent long-term effects of erosion and transport of sediment over several decades is a likely problem, as increased sediment load into streams has been shown to reduce critical spawning habitat for salmonids (Hogan 1986). In general, spawning and rearing habitat in smaller watersheds is most often less abundant than that found in larger watersheds. As a consequence, the effects of forest harvesting (i.e. sediment transport) in small watersheds are magnified, thus reducing available habitat for spawning coastal cutthroat and coastal rainbow trout even further. Surprisingly, recent logging (%RL) had no observable effect on increasing levels of introgression; however, recent logging was significantly associated with decreased introgression. Two possibilities come to mind as to why there is an observed reduction in introgression: (1) the immediate effects of recent logging, which is magnified in smaller watersheds, may be so severe that hybrid fish do not survive; or (2) populations of both trout species have declined dramatically (due to similar forestry effects; Hartman et al. 1996) and that the opportunity to hybridize is reduced. If hybrids were present before logging, severe environmental effects, as a result of recent timber harvesting, could play a role in hybrid mortality, for example, increase in stream temperatures (Holtby 1988) and changes in ion/nutrient concentrations (Hartman et al. 1996). It has been widely documented that salmonid fry often preferentially inhabit lower-velocity back channels and smaller streams (Chamberlin et al. 1991) to minimize predation and competition with other salmonid species (Rosenfeld et al. 2000). Additionally, hybrids have been found to be intermediate morphologically and in swimming performance when compared to both pure coastal cutthroat and rainbow/steelhead trout species (e.g. Hawkins and Quinn 1996; Hawkins and Foote 1998). Hence, dramatic changes to instream conditions, as a result of very recent timber harvesting, may result in substantial mortality of hybrids (see Chapter 3).

Reduced habitat availability has the strongest association with increased levels of introgression. The effects of limited habitat availability are quite often the result of impassable barriers (e.g. culverts, waterfalls, log jams). Hence, impediments to upstream migration poses serious conservation problems, not only for coastal cutthroat trout and coastal rainbow/steelhead trout, but for all salmonids that utilize forested watersheds for spawning; it is well documented that loss of spawning habitat has resulted in the decline of several populations of salmonids (e.g. Slaney et al. 1996) due to their inability to reproduce. Most spawning by salmonids, in particular coastal cutthroat and coastal rainbow/steelhead trout, takes place in second- to fourth-order streams (Chamberlin et al. 1991), which are found primarily further upstream in watersheds. Since second- to fourth-order streams account for the majority of total aggregate stream length available in most watersheds (Chamberlin et al. 1991), the constraints on migration to spawning sites triggers a broad overlap of spawning habitat, thus creating greater opportunity for interbreeding.

One of the most consistent and intriguing trends observed in this study was that total stream length (StLg) showed a significant negative association with elevated levels of introgression throughout the analyses. The frequency of introgression between coastal cutthroat and coastal rainbow/steelhead trout was higher in smaller watersheds irrespective of the effects of the other environmental factors included in the models (see Tables 2.5, 2.6, 2.7). Since stream length, by itself, is unlikely to affect hybridization and introgression (since stream length has not changed much in the last few hundred years), it must reflect some other, not measured, property of the environment that is affecting hybridization. The question, then, is what is happening in these smaller watersheds that influences hybridization and introgression? It does not appear that a location bias exists for small watersheds on Vancouver Island, since the small watersheds examined in this study were interspersed uniformly throughout the sampled area. It may be that smaller watersheds, in general, experience greater cumulative environmental impacts, due to their lack of "buffering capacity" when disturbed. Furthermore, Rosenfeld et al. (2002) pointed out that smaller watersheds have previously been viewed by planners and resource managers as having poor fisheries values, and have thus been excluded from specific protection during resource extraction. Finally, it could simply be that small watersheds have smaller trout populations; therefore, a single hybridization event would ultimately produce higher hybridization levels reflecting absolute population size. Although this study was unable to identify the causal mechanisms associated with smaller watersheds, it remains clear, and vitally important, that smaller watersheds be prioritized in conservation management strategies for sympatric coastal cutthroat and coastal rainbow/steelhead trout populations.

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CHAPTER 3

HYBRIDIZATION BETWEEN SYMPATRIC SPECIES OF TROUT: SELECTION, HYBRID MELTDOWN, AND BACKCROSS MATING BIAS

3.1 ABSTRACT

Sympatric species are expected to exhibit stronger reproductive barriers than allopatric species of similar genetic divergence due to reinforcement resulting from hybridization events. Using a combination of mtDNA and co-dominant nuclear DNA markers, I investigated: the role of selection against hybrids, the reproductive directionality (i.e. uni-directional vs. reciprocal) of hybridization, and potential backcross mating biases between sympatric coastal cutthroat and rainbow/steelhead trout in 13 populations in British Columbia, Canada. There was no evidence for selection (either extrinsic or intrinsic) acting against F1 hybrids based on the frequency of hybrid genotypes at different sizes. Although selection against backcross hybrids (i.e. outbreeding depression) was present, it was not consistent across populations. Furthermore, two populations were hybrid swarms, thus I propose that these populations are undergoing "*hybrid meltdown*" and that other populations could also experience such consequences. My analysis of the direction of hybridization shows that, overall, interbreeding is reciprocal, although some populations showed unidirectional hybridization. Analysis of nuclear-mitochondrial marker associations (including cytonuclear disequilibria, $D^{ii}{}_{j}$), showed a remarkable reproductive bias (the frequency of backcross hybrids with matched nuclear and mitochondrial marker composition greatly exceeded mismatched genotypes). Although selection against marker mismatch genotypes is possible, a behavioral mating bias is more plausible. To my knowledge, no other study has shown such a pattern, and the mechanism by which it could arise is not clear. Cutthroat-rainbow/steelhead trout hybrid zones clearly represent a valuable tool for

investigating the genetic and evolutionary implications of interspecific hybridization dynamics.

3.2 INTRODUCTION

As recently as the 1960s, hybridization among taxa was not considered an important evolutionary or ecological process because it was presumed that hybrid fauna were rare (Mayr 1963). However, there have been many examples of animal hybridization reported in nature over the last three decades (e.g. Howard 1986; Heath et al. 1995; Wilhelm & Hilbish 1998—(invertebrates); Grant & Grant 1992—(birds); Hatfield & Schluter 1999, Avise & Saunders 1984—(fish)). The occurrence of natural hybridization has since raised important questions regarding the role of reproductive isolating mechanisms in maintaining species, such as: (1) why have reproductive mating barriers failed in many interspecific crosses; and (2) what are the fitness consequences of those failures? Biologists have widely recognized the importance of both prezygotic and postzygotic reproductive isolation for maintaining species and both forms are believed to intensify with divergence time between taxa (Coyne & Orr 1997). Furthermore, it has been shown that prezygotic barriers evolve much faster than postzygotic reproductive isolation due to the effects of reinforcement in species pairs that maintain a sympatric relationship and where reciprocal hybridization events have historically occurred (e.g. Coyne & Orr 1989, 1997; Noor 1999; Servedio 2000). However, the strength of these reproductive isolating mechanisms (prezygotic and postzygotic) in nature has been shown to vary widely among taxa, hence the relative significance of the two types of reproductive isolating mechanisms continues to be of interest.

Two models widely applied to explain hybrid zone stability are the "*tension zone*" and "*mosaic*" models (Burke et al. 1998). The *tension zone* model (Barton & Hewitt 1985, 1989) postulates that the stability and size of hybrid zones are maintained by a balance between intrinsic selection (i.e. environmentally-independent selection) against hybrids and the dispersal of parental genotypes, where the intensity of selection against hybrids determines the width of the hybrid zone. The *mosaic* model (Howard 1986) assumes that hybrids are also comparatively inferior; however, it differs from the *tension zone* model in that the distribution of parental genotypes is governed by extrinsic selection (i.e. environment-dependent selection). Distribution of hybrids in the *mosaic* model reflects the adaptation of the parental genotypes to habitat heterogeneity (Moore & Price 1993; Burke et al. 1998) resulting in the hybrid genotypes inhabiting "transition zones". The value of these models lies in predicting the distribution and size of hybrid zones; however they cannot determine the nature of the selection acting on hybrids (i.e. intrinsic or extrinsic; Moore & Price 1993). Reviews of hybrid zone stability (e.g. Barton & Hewitt 1981; 1985) have concluded that intrinsic selection is likely the principal factor contributing to observed hybrid zone stability. However, extrinsic selection has also been demonstrated in some hybrid zones (Harrison 1990; Arnold 1997) and is increasingly being recognized as an important factor in speciation (e.g. Hatfield & Schluter 1999; Rundle 2002). The fitness consequences of hybridization are often extremely difficult to predict *a priori* (Edmands 1999), since hybrids may show an increased fitness (i.e. hybrid vigor or heterosis), credited to overdominance, or a reduced fitness (i.e. hybrid inferiority) relative to their parents. Reduced fitness in first-generation (F1) hybrids has been widely reported (e.g. Dowling & Moore 1985; Leary et al. 1993; Lamnissou et al. 1996), where the decline in fitness is attributed to disruption of local adaptations (i.e. gene x environment interactions; extrinsic selection; Edmands 1999, 2002). Outbreeding depression, resulting from a cross between genetically divergent groups (Edmands 2002), is expected to have maximum impact on fitness in the second generation backcrosses (i.e.

F2 and backcrossed hybrids; Dobzhansky 1940). Outbreeding depression is hypothesized to arise due to the recombination of the parental genes, resulting in disrupted epistasis and the creation of deleterious gene interactions (i.e. intrinsic selection; Edmands 1999, 2002).

Scribner et al. (2001) showed that hybridization is more common among fish species than in any other vertebrate group (see also Campton 1987; Allendorf & Waples 1996). Several factors have been proposed as contributing to the high incidence of hybridization in fish, including; competition for spawning habitat, external fertilization, weak behavioral isolating mechanisms, and unequal abundance of species (Hubbs 1955; Campton 1987). Scribner et al. (2001) identified the existence of weak prezygotic barriers among numerous species pairs of fish. Additionally, Scribner et al. (2001) identified relatively minor postzygotic reproductive barriers among several species pairs; however they acknowledged that hybrid inferiority was often cited as the primary postzygotic isolating mechanism. Very few of the studies reviewed by Scribner et al. (2001) directly examined the extent of hybrid inferiority in fish or the relative roles of intrinsic or extrinsic selection against the hybrids. A study conducted by Dowling & Moore (1985) did test for selection effects in hybrids produced by two species of *Cyprinidae.* They discovered that reinforcement mechanisms were weak and that the hybrids produced were selected against post-reproductively; however, they did not discriminate individuals based on hybrid type (i.e. F1, F2, or backcross) nor did they determine whether the selection against hybrids was intrinsic or extrinsic. Hatfield and Schluter (1999) established that the fitness reduction observed in F1 stickleback hybrids was based primarily on extrinsic mechanisms (hybrids inability to adapt to either parental habitat) and not the result of intrinsic selection or genetic incompatibility. Their findings, however, only included the

F1 generation; hence no evidence for either intrinsic or extrinsic selection effects in backcrossed hybrids was presented.

Here, I focus on the sympatric coastal cutthroat trout and steelhead/rainbow trout to investigate the role of selection in hybridization dynamics. What makes these trout species ideal for exploring selection is the maintenance of their species status in sympatry for over 10,000 years (Behnke 1992). Cutthroat trout (*Oncorhynchus clarki* ssp.) and rainbow trout (*Oncorhynchus mykiss* spp.) are believed to have diverged from a common ancestor approximately 2 million years ago (Behnke 1992). Nearly all trout subspecies from the *Oncorhynchus* genus evolved in allopatry, hence the evolution of reproductive isolating mechanisms (pre/ and postzygotic via intrinsic/extrinsic selection) has been assumed to be negligible (Behnke 1992; Young et al. 2001). As a result, secondary contact between introduced and native forms of trout (Behnke 1992) has resulted in the decline or direct loss of species due to extensive introgressive hybridization (Busack & Gall 1981; Leary et al. 1984; Gyllensten et al. 1985). However, coastal cutthroat trout (*O. clarki clarki*) and coastal rainbow (and/or steelhead) trout (*O. mykiss irideus*) have a relatively long evolutionary history of sympatry. The lack of geographical barriers separating the two species is believed to have driven the evolution of genetic (i.e. intrinsic and/or extrinsic), ecological, and/or behavioral reproductive isolating mechanisms to maintain species distinction (Young et al. 2001). Temporal and spatial differences in spawning behavior are thought to be the primary mechanisms that minimize interspecific mating (Trotter 1989).

Here I investigate possible selective effects in thirteen hybridizing populations of sympatric coastal cutthroat and coastal rainbow/steelhead trout using seven species-specific co-dominant markers and one mitochondrial DNA (mtDNA) marker. The combination of co-dominant nuclear markers with a mtDNA marker provides unique power to evaluate hybridization dynamics by genotype and haplotype analysis. To address intrinsic and extrinsic selective consequences for hybridized (and backcrossed) trout, I compared body size of pure-type and hybrid-type fish to indirectly test for differences in survival in the 13 populations. To test for reproductive directionality (i.e. unidirectional vs. reciprocal) among hybrids, I determined the mtDNA haplotype of hybrid fish in the thirteen populations. Furthermore, I examined the association of mtDNA with nuclear genotype to test whether mate preference exists beyond the F1 generation. This analysis provides insight into the relative roles of extrinsic and intrinsic selection against F1 and backcrossed hybrid trout in these natural populations. I also investigate reciprocal hybridization patterns as well as non-random mating bias (i.e. mate preference) in post-F1 backcross hybrids. I document two examples of a complete breakdown of reproductive barriers leading to a "*hybrid meltdown*" of local trout populations.

3.3 MATERIALS AND METHODS

3.3.1 Sample Collection

Thirteen sympatric populations of coastal cutthroat and rainbow (and/or steelhead) trout were sampled on Vancouver Island, British Columbia (Fig. 3.1). The populations were chosen for known hybridization, based on preliminary genetic analyses (refer to Chapter 2). We sampled Chase River in both 2002 and 2003, and thus include data from both sample years to address questions of temporal stability. Each fish was measured for fork length (± 1 mm) and fin clips were collected and stored in 95% ethanol. I extracted

DNA using the Wizard Genomic DNA Purification Kit (Promega Corp. Madison, WI) following manufacturer's instructions.

3.3.2 Species Markers

Seven PCR-based nuclear and one mitochondrial DNA (mtDNA) markers diagnostic for coastal cutthroat and rainbow trout were used in this study. Five of these nuclear loci (one size polymorphism and four restriction fragment length polymorphisms – RFLPs hereafter) were developed by Baker et al. (2002) (GH2D; GTH-β; IGF-2; Ikaros; RAG).The mtDNA marker (ND3) was developed by Docker et al. (2003). Two RFLPs, Growth hormone 1 intron D (GH1D [enzyme - *Mbo I*]; primers 5'-CAGCCTAATGGTCAGAAACG-3' and 5'-CTTATGCATGTCCTTCTTGAA-3'; Docker and Heath (2003) and McKay et al. (1996), respectively) and Transferrin, Exons 3-5 (Tfex3-5 [enzyme - *Nci I*]; primers 5'- GCCTCCACAACTACAACCTGCA-3' and 5'-TGGAAGGCCCCGGAATAGTCAT-3'; Ford et al. 1999) were developed for the current study. The two DNA fragments (GH1D—1375 bp; TFex3-5—1634 bp) were amplified by PCR in five coastal cutthroat and five rainbow trout from allopatric populations, and sequenced using the DCTS QuickStart cycling sequencing kit and the CEQ 8000 Automated DNA Sequencer (Beckman Coulter, Inc.). DNA sequence data were aligned using OMIGA 1.1 software (Oxford Molecular, Rainbow Tech. USA) and analyzed for species-specific RFLPs that would be easily discernable on an agarose gel GH1D; cutthroat—1375 bp, rainbow—985 bp & 390 bp; TFex3-5; cutthroat—717 bp, 487 bp, 430 bp, rainbow—917 bp, 717 bp).

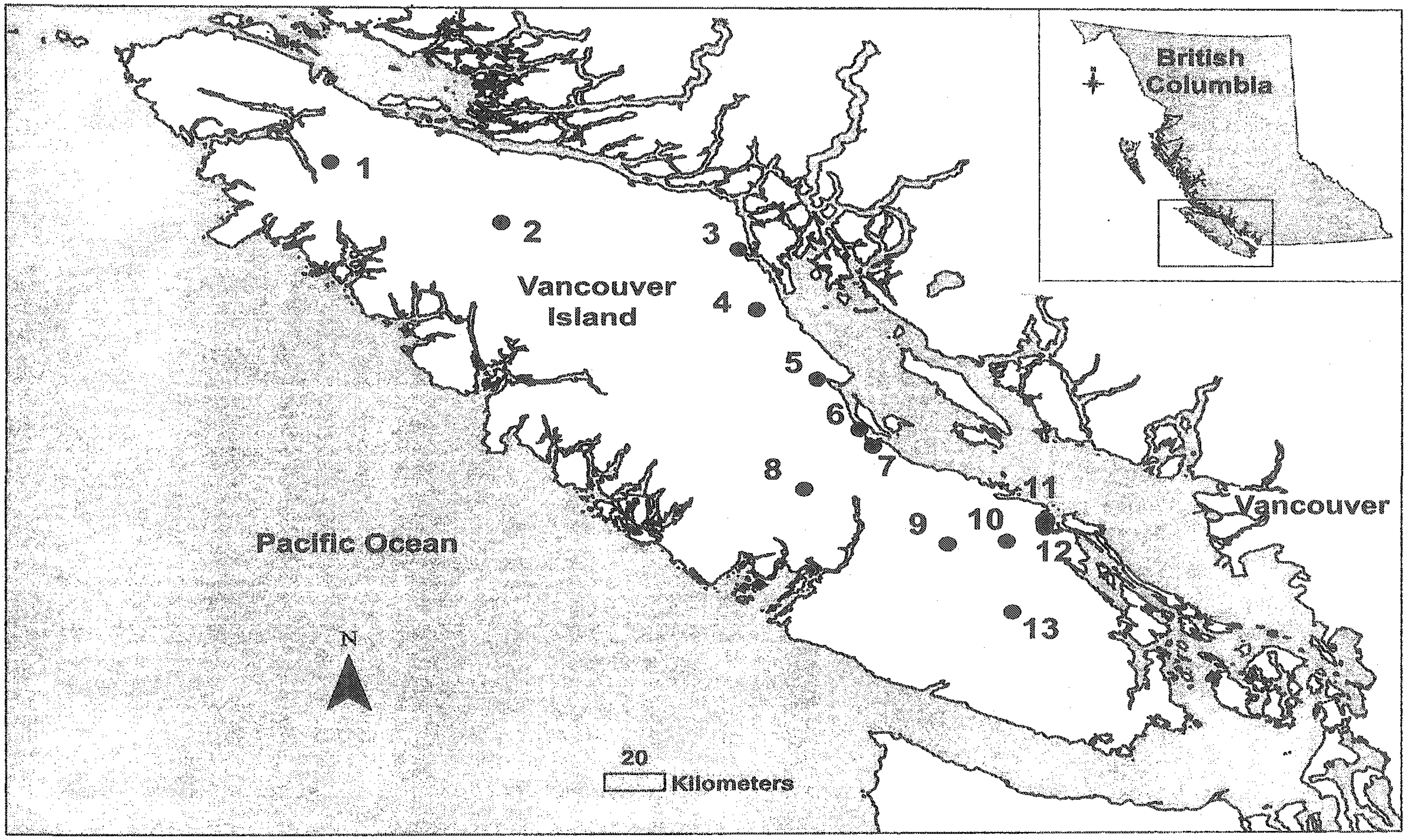

Figure 3.1 Map of Vancouver Island, British Columbia showing stream locations where cutthroat and rainbow trout sampling occurred. (1) Howlal Creek; (2) Lukwa Creek; (3) Menzies Creek; (4) Cold Creek; (5) Morrison Creek; (6) Cowie Cougar-Smith Creek; (7) Cook Creek; (8) Friesen Creek; (9) Rockyrun Creek; (10) North Nanaimo River; (11) Millstone River; (12) Chase River; (13) Meade Creek. Inset map shows primary geographical study location in British Columbia, Canada.

We validated all species-specific RFLPs and size polymorphisms (including the two novel markers) as diagnostic using 30 allopatric rainbow and 30 allopatric coastal cutthroat trout from coastal British Columbia. See Appendix I for a list of fragment sizes for all loci used in this study.

3.3.3 Molecular Protocols

Polymerase chain reactions (PCR) were performed using standard 25-μL reactions that contained: 10 mM Tris-HCl (pH-8.4) 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.05 μg of each primer, 0.5 units of DNA Taq polymerase, and approximately 100 ηg of genomic DNA template. The optimized thermocycler (MJ Research model PTC-0225) profile consisted of a 'hot-start' and 2-minute initial denaturation (94°C), followed by 35-40 cycles of 1-minute denaturation cycle (94°C), a 1-minute annealing (49°C Ikaros; 53°C ND3; 55°C GH2D; 55°C GTH-β; 57°C RAG; 58°C GH1D; 62°C IGF-2; 63°C TFex3-5), a 1.5-minute extension (72°C), and ending with a final 5-minute extension cycle (72°C).

PCR products, size polymorphisms, and RFLPs were separated by agarose gel electrophoresis at 80-90 V through a 1.8% agarose gel. All fragments were visualized using ethidium bromide staining and UV transillumination.

3.3.4 Data Analysis

All fish were genotyped as homozygous rainbow trout, homozygous cutthroat trout, or heterozygous at each of the seven nuclear loci. Fish that were identified as homozygous at all seven loci for one species were considered pure-type for that species.

First-generation (F1) hybrid fish were the individuals that were heterozygous at all seven loci, while backcross hybrid fish were those individuals having a mix of homozygous and heterozygous marker loci (Fig. 3.2). All genotypes that could be interpreted as a partial restriction digest on the agarose gel were re-amplified and digested to confirm genotype. Mitochondrial DNA haplotypes were assigned as cutthroat or rainbow trout for all fish. Individual fish that were scored as homozygous for cutthroat or rainbow trout at all seven nuclear loci, but had the opposite species mtDNA were identified as "ancient" backcross hybrids.

Wright's *Fixation Index* ($F_{IS} = H_E\text{-}H_O/H_E$) was calculated at each locus using observed and expected heterozygosity levels generated from *Tools for Populations Genetic Analyses* (TFPGA) software, version 1.3 (Miller 1997). Conventional Monte Carlo exact test (10 batches, 2000 permutations per batch) for Hardy-Weinberg equilibrium (HWE) were utilized at each locus (TFPGA). A Bonferroni correction, to account for multiple simultaneous tests, (7 loci x 14 populations = 98 comparisons) was performed to test for significant departure from HWE (Rice 1989). Many locus-by-population calculations were in HWE before, and all were in HWE after Bonferroni adjustments, which was unexpected given that interspecific hybridization occurring among two distinct species should violate the HWE assumptions of random mating and no selection. To further examine the HWE status of our hybridizing populations we tested for trends in the sign of F_{IS} among the seven nuclear marker loci within each population using *sign tests* (SYSTAT Version. 7.1). This was done to determine if more heterozygote deficits or excesses were present than expected by chance.

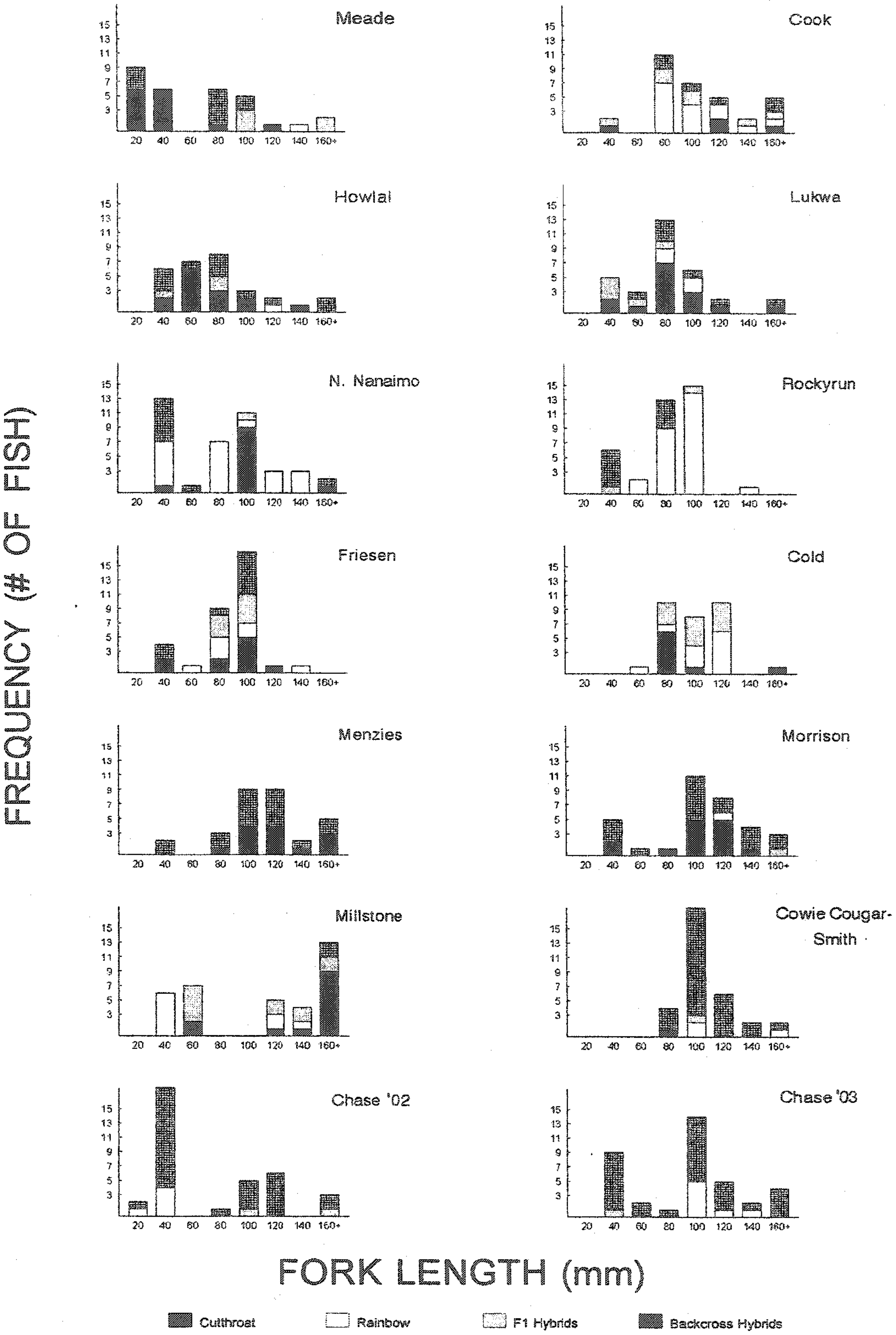

Figure 3.2 Length-Frequency distribution for the four genotypes (i.e. pure cutthroat trout, pure rainbow trout, F1, backcross) of trout taken from 13 populations on Vancouver Island, BC. Frequency is based on a fork length bin size of 20 mm.

Associations between nuclear genotypes and mtDNA haplotypes within each hybrid population were estimated using measures of cytonuclear disequilibria (Asmussen et al. 1987; Asmussen & Basten 1994). Genotypic disequilibria ($D^{CC}{}_{C}$, $D^{RR}{}_{C}$) were calculated (which reflects departures from the expectation of random association) (Harrison & Bogdanowicz 1997).

$$D^{CC}{}_{C} = \mathrm{freq}(CC/c) - \mathrm{freq}(CC)\mathrm{freq}(c) \qquad (1)$$

and

$$D^{RR}{}_{C} = \mathrm{freq}(RR/c) - \mathrm{freq}(RR)\mathrm{freq}(c), \qquad (2)$$

where C and R are coastal cutthroat (*O. clarki clarki*) and the rainbow trout (*O. mykiss*) nuclear alleles, respectively, and c and r are the mtDNA haplotypes of each species. When $D^{CC}{}_{C}$ is positive and $D^{RR}{}_{C}$ is negative, the cutthroat (CC) genotypes carry the cutthroat (c) mtDNA haplotype more often than would be expected by chance, indicative of assortative mating or selection against disassortative mtDNA and nuclear hybrid genotypes.

Fish were assigned to two size categories based on a size-age distribution for coastal cutthroat (adapted from Rosenfeld et al. 2000), where fish less than 55 mm correspond to young-of-the-year (YOY; i.e. age 0+) and fish greater than 55 mm correspond primarily to older year-classes (i.e. fish that have over-wintered at least once). Though Rosenfeld et al. (2000) developed this relationship for cutthroat trout; rainbow/steelhead trout applications are justified since juvenile fish of both species demonstrated no differences in fork length-at-age measurements early in life (Pearcy et al. 1990). Once fish were assigned to size/age categories, they were further categorized as either pure or hybrid trout. A two-way Pearson chi-square was used to test for evidence of intrinsic selection against hybrids. Intrinsic selection effects should be manifest across all populations, thus populations were pooled for this analysis.

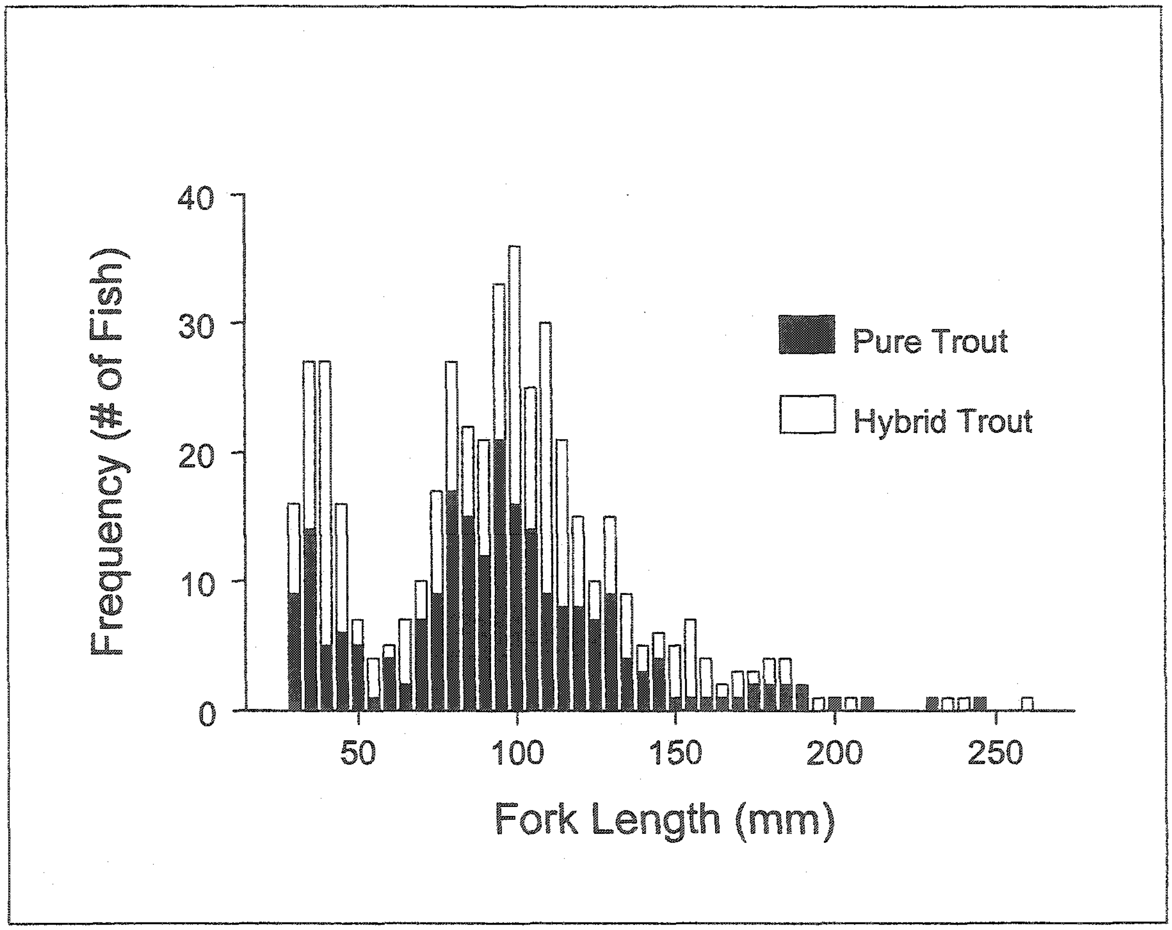

Figure 3.3 Frequency distribution of pure and hybrid fish pooled from all 13 populations. Fish less than 55 mm correspond to young-of-the-year (i.e. fish that have not over-wintered) and fish greater than 55 mm correspond primarily to older year-classes (i.e. fish that have survived at least one winter).

Additionally, to test for extrinsic selection acting against hybrid genotypes, we used ANOVA to test for differences in fork length among genotypes (i.e. pure cutthroat, pure rainbow, F1, and backcross) within individual populations. Significance levels were adjusted for multiple simultaneous comparisons using a Bonferroni correction (Rice 1989). A length-frequency histogram for all genotypes in each population was generated using a fork length bin size of 20 mm.

To establish whether: (a) hybridization was recent and ongoing; (b) hybridization was occurring in a unidirectional or reciprocal pattern; and (c) hybrid swarms existed among any of the thirteen populations, the level of introgression was examined using a 'hybrid index' identifying the total number of possible cutthroat alleles (i.e. seven co-dominant markers = 14 alleles) within each population. Furthermore, to verify whether interspecific reproduction exhibited a bias towards one species beyond the F1 generation, we utilized mtDNA data to assign backcross hybrids (excluding F1 and pure-types) to their maternal lineage (i.e. mother was cutthroat or rainbow). Furthermore, we pooled the total number of cutthroat and rainbow genomic alleles (based on the seven co-dominant markers) in each population for each maternal haplotype to identify any association between maternal haplotype and nuclear genotype.

3.4 RESULTS

All eight markers (seven nuclear and one mtDNA) were 100% diagnostic among the thirty cutthroat and thirty rainbow trout. A total of 236 (52%) hybrids (including both F1 and backcross) were identified among the 13 populations. Eleven of ninety-eight locus-by-population calculations exhibited significant departures from HWE, before Bonferroni correction (Table 3.1). Additionally, *sign test* results showed significant

Table 3.1 F_{IS} values with Monte Carlo exact test probabilities (in brackets) for Hardy-Weinberg (HWE) departures. Significant departures from HWE before Bonferroni adjustments are denoted by *. No populations were found to be significantly out of HWE after Bonferroni correction. Sign tests showed that 8 populations had significant bias for positive F_{IS} (i.e. heterozygote deficiency) across all loci (denoted by †).

Population	Locus						
	GH2D	GH1D	RAG	GTH-β	TFex3-5	IKAROS	IGF-2
Meade Cr	0.07 (p = 0.63)	0.04 (p = 1.00)	0.04 (p = 1.00)	0.04 (p = 1.00)	-0.002 (p =1.00)	-0.08 (p = 1.00)	-0.002 (p = 1.00)
Cook Cr†	0.33 (p = 0.08)	0.32 (p = 0.10)	0.21 (p = 0.32)	0.43 * (p = 0.02)	0.43 * (p = 0.02)	0.26 (p = 0.21)	0.21 (p = 0.32)
Howlal Cr	0.03 (p = 1.00)	0.13 (p = 0.43)	0.34 (p = 0.17)	-0.05 (p = 1.00)	-0.05 (p = 1.00)	0.08 (p = 0.52)	0.26 (p = 0.25)
Lukwa Cr†	0.21 (p = 0.38)	0.21 (p = 0.38)	0.26 (p = 0.17)	0.43 * (p = 0.03)	0.43 * (p = 0.03)	0.37 (p = 0.07)	0.26 (p = 0.18)
N. Nanaimo R†	0.26 (p = 0.15)	0.53 * (p = 0.02)	0.37 (p = 0.06)	0.21 (p = 0.20)	0.21 (p = 0.21)	0.47 * (p = 0.02)	0.26 (p = 0.15)
Rockyrun Cr	0.27 (p = 0.20)	0.36 (p = 0.14)	0.36 (p = 0.14)	0.21 (p = 0.27)	-0.09 (p = 1.00)	-0.10 (p = 1.00)	0.16 (p = 0.35)
Friesen Cr†	0.13 (p = 0.49)	0.51 * (p = 0.004)	0.35 (p = 0.06)	0.19 (p = 0.30)	0.24 (p = 0.17)	0.35 (p = 0.06)	0.37 (p = 0.06)
Cold Cr†	0.26 (p = 0.16)	0.26 (p = 0.16)	0.26 (p = 0.16)	0.26 (p = 0.16)	0.26 (p = 0.16)	0.26 (p = 0.16)	0.26 (p = 0.16)
Menzies Cr	0.26 (p = 0.24)	-0.20 (p = 0.56)	-0.002 (p = 1.00)	-0.22 (p = 0.55)	-0.20 (p = 0.56)	0.04 (p = 1.00)	-0.04 (p = 1.00)
Morrison Cr†	0.02 (p = 1.00)	0.10 (p = 0.47)	0.20 (p = 0.30)	0.14 (p = 0.57)	0.24 (p = 0.19)	0.02 (p = 1.00)	0.10 (p = 0.46)
Millstone R†	0.35* (p = 0.04)	0.35* (p = 0.04)	0.30 (p = 0.09)	0.25 (p = 0.17)	0.31 (p = 0.09)	0.25 (p = 0.17)	0.35* (p = 0.04)
CC-Smith Cr	0.12 (p = 0.61)	0.50* (p = 0.004)	-0.02 (p = 1.00)	-0.11 (p = 0.71)	-0.20 (p = 0.45)	0.08 (p = 0.69)	-0.12 (p = 0.68)
Chase R '02	0.12 (p = 0.32)	-0.12 (p = 0.69)	-0.19 (p = 0.43)	-0.33 (p = 0.11)	-0.12 (p = 0.69)	-0.47 (p = 0.17)	-0.23 (p = 0.44)
Chase R '03†	0.50* (p = 0.005)	0.04 (p = 1.00)	0.05 (p = 0.73)	0.54* (p = 0.001)	0.31 (p = 0.07)	0.09 (p = 0.71)	0.08 (p = 0.73)

trends of heterozygote deficiency (i.e. $F_{IS} > 0$) in Cook Creek, Lukwa Creek, Friesen Creek, Cold Creek, Morrison Creek, Chase River '03, and the North Nanaimo River. The remaining populations (Meade, Howlal, Rockyrun, Menzies, Cowie Cougar-Smith Creeks, and Chase River '02) did not show any trends in heterozygote (i.e. $F_{IS} < 0$) or homozygote (i.e. $F_{IS} > 0$) deficiency.

There were no significant differences in hybrid incidence between young-of-the-year and older fish, indicating intrinsic selection acting in the first year of life is absent or very weak (Fig. 3.3; $p = 0.528$). However, there were significant differences in fork length among genotypes in Meade Creek, North Nanaimo River, Rockyrun Creek, and the Millstone River (see Fig. 3.4). Mean fork length of backcross hybrids in the North Nanaimo River and Rockyrun Creek were significantly smaller than pure rainbow trout ($p < 0.01$ and $p < 0.05$ respectively) but not significantly different than pure cutthroat or F1 hybrids ($p > 0.05$). Meade Creek and the Millstone River displayed significantly different size patterns among genotypes suggesting selection effects against hybrids may be environment-dependent. The remaining ten populations showed no significant differences in mean fork length among all four genotype categories, signifying that selection effects against hybrids are likely weak and system dependent.

Cytonuclear (genotypic) disequilibria (i.e. $D^{CC}{}_{C}$ and $D^{RR}{}_{C}$) revealed significantly positive associations (range $p < 0.05$ to $p < 0.001$) between genotype and cytotype (i.e. cutthroat genotype with cutthroat haplotype & rainbow genotype with rainbow haplotype) in eight of thirteen populations (Table 3.2). One population, Howlal Creek, revealed a significantly negative association ($p < 0.05$) between genotype and cytotype (i.e. cutthroat genotype with rainbow haplotype). The remaining populations displayed

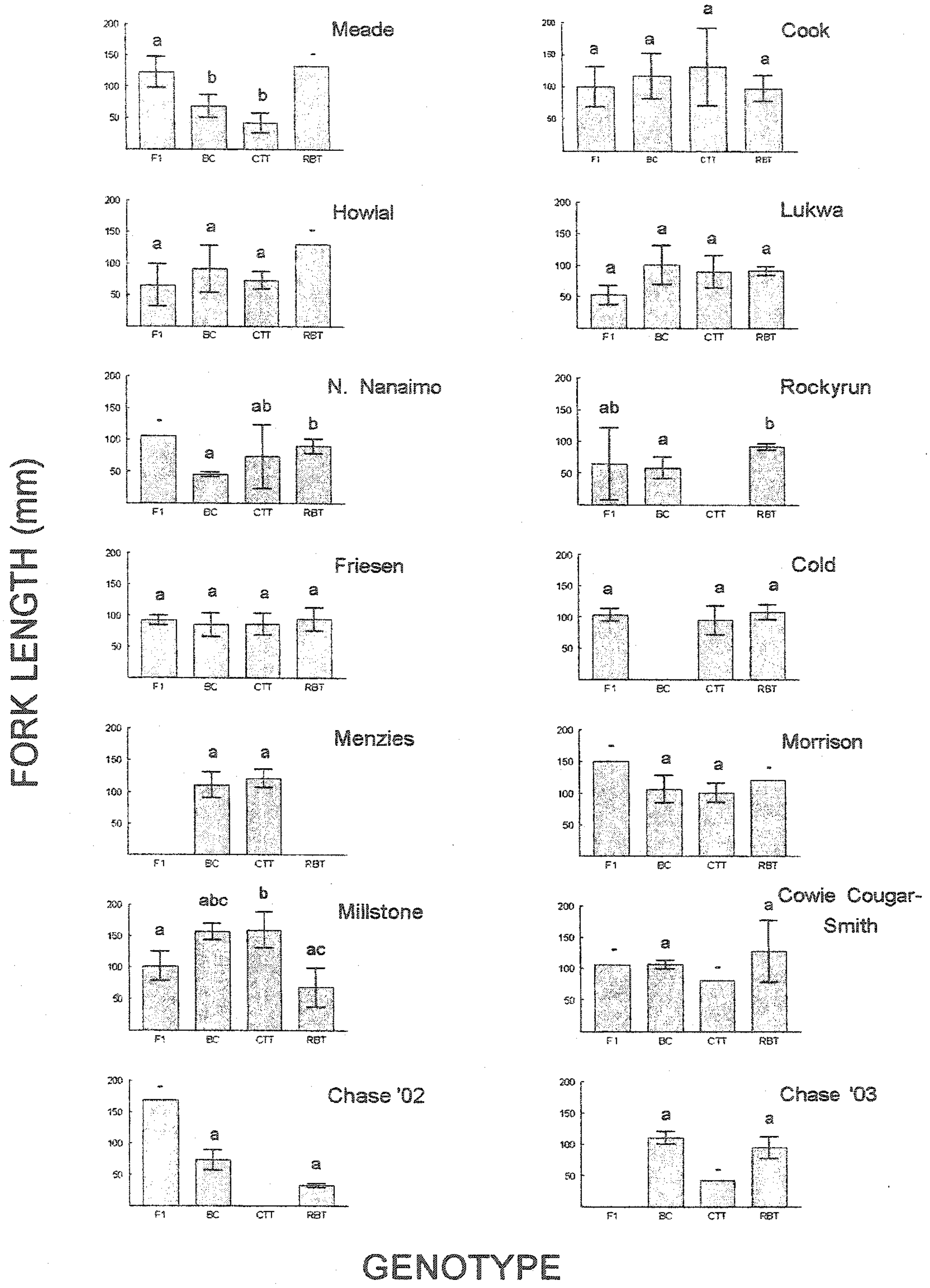

Figure 3.4 Mean fork length (mm, ± 2 SE) in the four genotypes (i.e. CTT—pure cutthroat, RBT—pure rainbow, F1—first-generation hybrid, BC—backcross hybrid) from 13 populations of sympatric cutthroat and rainbow/steelhead trout on Vancouver Island, BC. Significant differences between genotypes, based on Bonferroni correction, are denoted by letters (different letters = significant differences, $p < 0.05$) A dash ("-") indicates only one individual with that particular genotype.

Table 3.2 Cytonuclear (i.e. genotypic) disequilibria (D^{ii}_{j}) for all thirteen hybridizing trout populations on Vancouver Island. Values for cytonuclear disequilibria have been averaged over all 7 loci within each population. Significant disequilibria values are bold-types, with *P*-values (fisher exact test) in brackets.

Population	D^{CC}_{C}	D^{RR}_{C}
Meade Cr	0.04	-0.03
	NS	NS
Cook Cr	**0.12**	**-0.16**
	($p < 0.001$)	($p < 0.001$)
Howlal Cr	0.02	0.03
	NS	($p < 0.05$)
Lukwa Cr	**0.10**	**-0.12**
	($p < 0.01$)	($p < 0.001$)
N. Nanaimo R	**0.04**	**-0.14**
	($p < 0.05$)	($p < 0.001$)
Rockyrun Cr	0.01	-0.02
	NS	NS
Friesen Cr	**0.11**	**-0.16**
	($p < 0.01$)	($p < 0.001$)
Cold Cr	**0.15**	**-0.16**
	($p < 0.001$)	($p < 0.001$)
Menzies Cr	0.05	-0.02
	NS	NS
Morrison Cr	0.02	**-0.03**
	NS	($p < 0.05$)
Millstone R	**0.10**	**-0.19**
	($p < 0.01$)	($p < 0.001$)
CC-Smith Cr	**0.07**	**-0.10**
	($p < 0.05$)	($p < 0.05$)
Chase R '02	0.04	**-0.12**
	NS	($p < 0.05$)
Chase R '03	**0.10**	**-0.17**
	($p < 0.001$)	($p < 0.001$)

a non-significant positive association between genotype and haplotype, consistent with the eight previous significant populations.

Twelve of thirteen populations contained individuals that were heterozygote at all seven nuclear markers (i.e. 50% cutthroat alleles), signifying first-generation (F1) hybrids (Table 3.3; Fig. 3.5). Several populations displayed high frequencies of F1 hybrids (Table 3.3). The presence of F1 hybrids in the majority of our populations provides evidence of current, ongoing hybridization. Menzies Creek contained no F1 hybrids, suggesting pure-type (parental) fish have not interbred recently. The presence of a variety of backcross genotypes in Menzies Creek suggests that introgression among hybrids and pure cutthroat is ongoing. Chase River '02 and '03, as well as Cowie Cougar-Smith Creek exhibited a diverse array of recombinant genotypes and very few F1 or pure-type, suggesting that these two systems are hybrid swarms (see Fig. 3.5).

The North Nanaimo River displayed a strong bias for hybrids (F1 and backcross) to mate with pure rainbow trout (Fig. 3.5). Hybrids in Meade Creek, Howlal Creek, Friesen Creek, Menzies Creek, and Morrison Creek, exhibited a mating bias with pure cutthroat trout (Fig. 3.5). Chase River '02 and '03, Lukwa Creek, Rockyrun Creek, and Cowie Cougar-Smith Creek displayed a reciprocal (i.e. bi-directional) bias between hybrids and either rainbow or cutthroat trout pure individuals.

We observed a highly significant association between mtDNA haplotype and nuclear DNA genotype in several populations (Fig. 3.6). In Meade Creek Howlal Creek, Friesen Creek, Morrison Creek, and the Millstone River ($p < 0.001$ respectively), we observed a strong significant association between the cutthroat haplotype and a higher

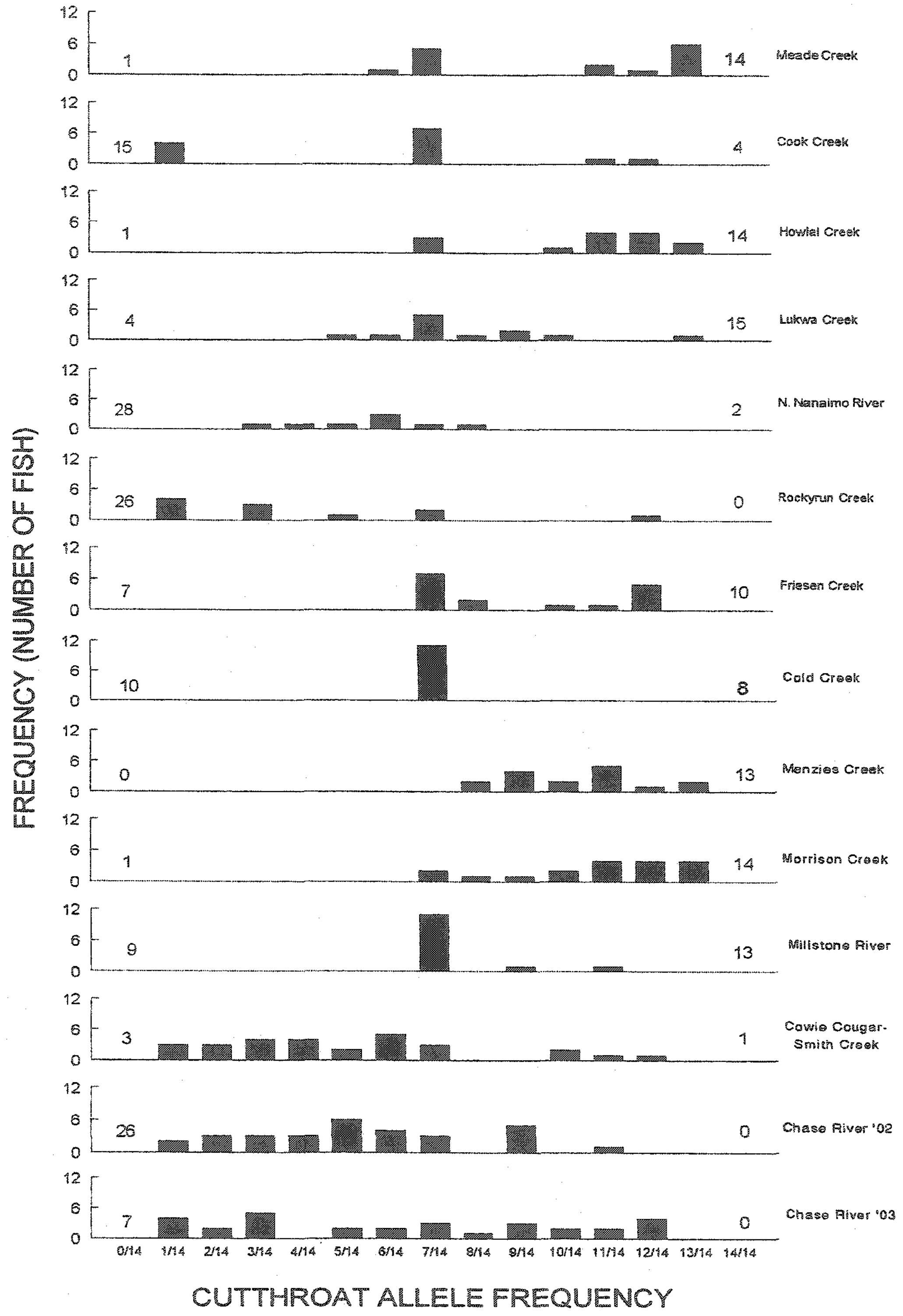

Figure 3.5 Frequency distribution of genotypes ('hybrid index') for the thirteen populations of sympatric cutthroat and rainbow/steelhead trout on Vancouver Island, BC. The number of observed pure-type (i.e. parental genotypes) has also been included (i.e. 0/14 denotes pure rainbow trout; 14/14 denotes pure cutthroat trout).

Table 3.3 Sample size (*n*), proportion of total hybrids identified from sample size (actual number in parentheses), proportion of all hybrids identified as F_1 hybrids (actual number in parentheses), frequency of F_1 hybrids based on haplotype, and influence of life history type (i.e. anadromous vs. resident life-history types) for each population (+ denotes life history type is present in that population; - denotes life history is absent from that population).

						Life-History Influence			
Population	*n*	Frequency (All Hybrids)	Frequency (F_1 Hybrids)	Frequency F_1 Genotype		Resident		Anadromous	
				CTT Haplotype	RBT Haplotype	CTT	RBT	CTT	STHD
Meade Creek	30	0.50 (15)	0.33 (5)	4	1	+	+	+	+
Cook Creek	32	0.41 (13)	0.54 (7)	4	3	+	+	+	+
Howlal Creek	29	0.48 (14)	0.21 (3)	3	0	+	+	-	+
Lukwa Creek	31	0.39 (12)	0.42 (5)	5	0	+	+	+	+
N. Nanaimo River	38	0.21 (8)	0.12 (1)	1	0	+	+	-	+
Rockyrun Creek	37	0.30 (11)	0.18 (2)	0	2	+	+	-	-
Friesen Creek	33	0.49 (16)	0.44 (7)	6	1	+	+	+	-
Cold Creek	30	0.37 (11)	1.00 (11)	5	6	+	+	+	+
Menzies Creek	30	0.57 (17)	0	-	-	+	-	+	+
Morrison Creek	33	0.55 (18)	0.06 (1)	1	0	+	+	+	+
Millstone River	35	0.37 (13)	0.85 (11)	11	0	+	-	+	+
CC-Smith Creek	32	0.88 (28)	0.04 (1)	1	0	+	+	-	+
Chase River '02	35	0.86 (30)	0.03 (1)	1	0	+	+	+	+
Chase River '03	37	0.81 (30)	0.03 (1)	1	0	+	+	+	+

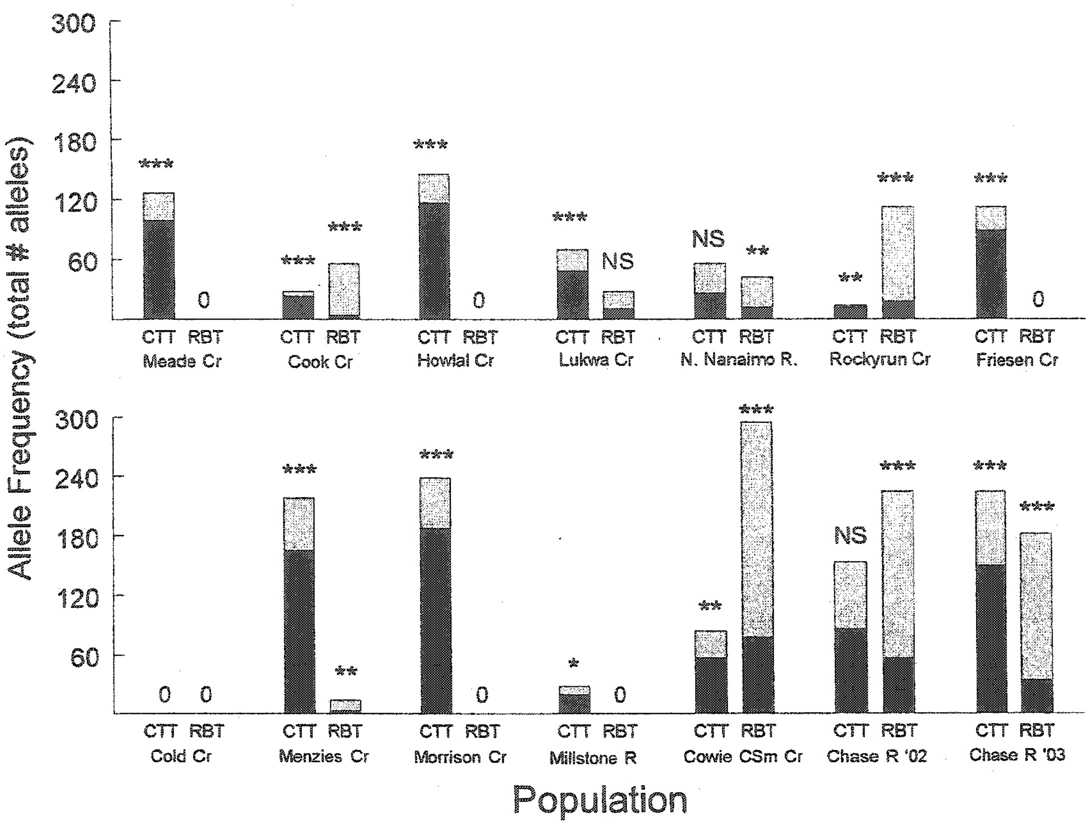

Figure 3.6 Nuclear allele frequency for each mtDNA haplotype for the 13 populations of sympatric cutthroat (Black bars) and rainbow/steelhead (Grey bars) trout. CTT – cutthroat trout mtDNA haplotype; RBT – rainbow trout mtDNA haplotype (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS – not significant).

frequency of cutthroat nuclear alleles among backcrossed hybrids. A similar pattern was observed in Cook Creek ($p < 0.001$ both haplotypes), Rockyrun Creek ($p < 0.01$ cutthroat haplotype, $p < 0.001$ rainbow haplotype), Menzies Creek ($p < 0.001$ cutthroat haplotype, $p < 0.01$ rainbow haplotype), Cowie Cougar-Smith Creek ($p < 0.01$ cutthroat haplotype, $p < 0.001$ rainbow haplotype), and Chase River '03 ($p < 0.001$ both haplotypes); however these populations displayed a reciprocal association within each population, where backcrossed hybrids with the rainbow trout haplotype were significantly associated with a higher frequency of rainbow trout nuclear alleles while those with the cutthroat trout haplotype were significantly associated with a higher frequency of cutthroat trout nuclear alleles. Lukwa Creek demonstrated a significant association ($p < 0.001$) between backcrossed hybrids with the cutthroat haplotype and a higher frequency of cutthroat nuclear alleles, but no significant association was observed in backcrossed hybrids with the rainbow trout haplotype. The North Nanaimo River and Chase River '02 exhibited a similar pattern as in Lukwa Creek ($p < 0.01$ respectively); however, the strong significant association was observed in backcrossed hybrids with the rainbow trout haplotype and a higher frequency of rainbow trout nuclear alleles.

3.5 DISCUSSION

3.5.1 Breakdown of Reproductive Barriers

Sympatric species pairs are believed to exhibit stronger species reproductive barriers, for example mate discrimination, than allopatric species pairs of the same genetic divergence (e.g. Coyne & Orr 1989; Butlin 1995). This has been attributed to natural selection, which drives reinforcement mechanisms in response to hybridization events (Noor 1999; Servedio 2000). Our data shows compelling, but indirect, evidence that there is no strong selection (intrinsic or extrinsic) currently acting against F1 hybrids, suggesting that reinforcement of the reproductive isolation between sympatric coastal cutthroat and rainbow/steelhead trout is not likely to occur, despite a long history of sympatry between these two species. Two lines of evidence support our theory for weak selection against F1 hybrids: (1) several observed populations were in HWE and others displayed relatively weak departures from HWE indicating that strong selection, which is expected to drive populations out of HWE, was absent; (2) fish size data (i.e. length-frequency histogram and size-category data) displayed no indication of decline in F1 frequency as the fish age. Young et al. (2001) and Docker et al. (2003) have previously documented sympatric populations of coastal cutthroat and rainbow trout showing relatively high incidence of juvenile F1 hybrids, further suggesting that substantial prezygotic barriers have not evolved. Additionally, Hawkins and Foote (1998) established that there was no evidence of reduced hatchability or viability of F1 hybrids despite maternal and paternal species effects on size and development. However, Campton and Utter (1985) stated that F1 hybrids face a selective disadvantage later in life during anadromous migrations due to intermediate life history characteristics, while Hawkins and Quinn (1996) found that F1 hybrids were intermediate to the pure-type species in both swimming performance and morphology, thus generating the potential for a competitive disadvantage in the hybrids. It appears that the F1 hybrids on Vancouver Island have not been strongly selected against by extrinsic or intrinsic effects, despite the expectation for such selective effects in hybrids of sympatric species pairs (Young et al. 2001; Edmands 2002).

Although I found no evidence for F1 hybrid inferiority, fitness declines attributed to intrinsic selection may not occur until the second or backcross generations (Edmands 2002). The observed differences in mean fork length in the North Nanaimo River and Rockyrun Creek backcross hybrids compared to pure-types, suggest that backcross hybrids experience reduced survival or growth, consistent with outbreeding depression. However, this inferred reduction in survival was not consistent across all populations; in fact the majority of sampled populations showed no such effects. Extrinsic selection effects are thus most likely causing the observed reduction in survival (or growth) of backcross hybrids in the few populations where differences in fork length were observed. It is generally difficult to determine whether fitness in backcross hybrids is affected by intrinsic selection, extrinsic selection, or both. Allendorf et al. (2001) hypothesized that outbreeding depression stems purely from extrinsic selection effects. Additionally, Edmands and Timmerman (2003) suggested that disruption of local adaptation (extrinsic selection) was more severe than disruption of co-adapted gene complexes (intrinsic selection). Further evidence to support extrinsic selection as the most likely cause of our backcross reduced fitness is found in the two populations characterized by "hybrid meltdown" (i.e. the actual consequence/outcome of a persistent hybrid swarm; refer to Chapter 2). In Chase River and Cowie Cougar-Smith Creek, I observed a diverse array of backcross genotypes indicating that outbreeding depression was either undetectable or absent, resulting in no fitness cost to hybridization and hence the formation of a hybrid swarm. The strength of fitness gradients among pure-type and hybrid genotypes can greatly influence the development of hybrid swarms, and it has been postulated that even the narrowest margin of increased fitness in later generation hybrids can lead to the establishment of a hybrid swarm (Epifano and Philipp 2001). The hybrid meltdown in Chase River and Cowie Cougar-Smith Creek indicates fitness among the hybrids in these populations is at least equal to pure-types. However, the relative fitness of hybrid fish likely depends on local environmental conditions and hence reflects extrinsic selection. Furthermore, the abundance of backcross hybrids, relative to the low frequency of F1 and pure-types in these systems demonstrate that the hybrid meltdown is not a transient phenomenon. Our data for Chase River ('02 and '03), combined with data from Docker et al. (2003) (Chase River sampled in 2000—92% total hybrids; 38% F1 hybrids), demonstrate considerable temporal persistence of the hybrid swarm over time, thus the hybrid meltdown apparently drives a permanent loss of species reproductive barriers. Most populations in our study did not exhibit the characteristics of hybrid meltdown or swarms; however, the majority of them displayed no detectable hybrid inferiority (with the exception of Rockyrun Creek and the North Nanaimo River populations). Thus these populations have no discernable barriers to future hybrid meltdown.

3.5.2 Direction of Hybridization and Mate Bias

Size differences between mature adults of sympatric species pairs have been hypothesized to influence the direction of hybridization (i.e. unidirectional or reciprocal; Wirtz 1999). Our results show that the initial hybridization events (i.e. the production of F1 hybrids) occur reciprocally (see Table 3.3). There does, however, appear to be a weak tendency for hybridization to occur between a female cutthroat trout mating with male rainbow/steelhead trout. This observation may be attributed to body size differences between adult anadromous and nonanadromous female cutthroat and male steelhead trout. Steelhead commonly spend 2-3 years in the ocean and attain a much larger body size than anadromous (and nonanadromous) cutthroat trout upon return to freshwater (Pearcy et al. 1990). Grant and Grant (1997b) reasoned that the female of smaller species might accept males of larger species, but not *vice-versa* because the smaller males transmit subnormal reproductive stimuli. An excellent example of female mate preference for larger heterospecific males was described by Ryan and Wagner (1987), where female *Xiphophorus pygmaeus* preferred to mate with the larger male *X. nigrensis*, even in the presence of smaller conspecific males. Though the two swordtail species are not naturally sympatric, the authors hypothesized that if they were to become sympatric, preference of female *X. pygmaeus* for *X. nigrensis* males could result in extensive introgression, and possible convergence of these species. In our case, when steelhead trout return to freshwater to spawn, female cutthroat may prefer the larger steelhead males over the smaller cutthroat males based on the same principles (Ryan and Wagner 1987).

Alternatively, initial hybridization events could simply be due to a greater abundance of one species, with female mate choice or male mating behavior playing no significant role. Several of our populations displayed a much higher abundance of one species relative to the other (Fig. 3.5). When we compared the abundance of parental species to the mitochondrial haplotype of F1 hybrids, it was evident that when cutthroat trout were the abundant species, hybridization occurred most frequently between a male rainbow/steelhead and a female cutthroat (see Fig. 3.5; Table 3.3). The North Nanaimo River displayed a similar pattern of unequal abundance, however rainbow trout were more abundant rather than cutthroat trout and the single F1 hybrid in this system was also a product of a male rainbow trout and a female cutthroat trout, despite the reversal of species abundance. Avise and Saunders (1984) identified fourteen hybrid sunfish (*Lepomis spp.*) produced by matings between a common and rare species of *Lepomis*; and there was a tendency for the rare species to be the female. Additionally, Avise et al. (1997) analyzed a hybridizing population of bass (*Micropterus punctulatus* and *M. dolomieui*) and found that six of seven probable F1 hybrids carried the mtDNA of the *M. dolomieu*, the rarer species. Our results do not agree with these studies, which suggest two possibilities: mating patterns between abundant and rare species may be species-specific and dependent on reproductive life history strategies; or perhaps a more plausible explanation may be that interbreeding between abundant and rare species may be dependent upon the sex of the available spawners of the rare species. Dowling et al. (1989) found that all F1 hybrids of *Luxilus cornutus* and *L. chrysocephalus* (Family *Cyprinidae*) from Raisin River had the *L. chrysocephalus* mtDNA, while approximately 90% of the F1 hybrids from the Kalamazoo River had the *L. cornutus* mtDNA. These data are consistent with my data, where hybridization may appear uni-directional in one or a few populations, but when multiple populations are examined, hybridization is clearly reciprocal. Furthermore, when the frequency of pure-type individuals is equal, hybridization may end up being reciprocal within a single population (e.g. Friesen Creek, Cook Creek, and Cold Creek). Thus, it is important to screen multiple populations to correctly define hybridization dynamics, given the possibility of extrinsic effects.

My analyses of post-F1 backcross hybrids demonstrate a reproductive association, where backcross hybrids tend to have disproportionately more nuclear alleles that match their mtDNA species haplotype (i.e. cytonuclear disequilibrium; see Fig. 3.6). What could be driving this apparent mitochondrial-nuclear marker association? Two possible explanations present themselves: (1) random mating, but strong selection against hybrids with a mismatched mitochondrial-nuclear marker pattern; or (2) a behavioral mating preference, which is tied to the mtDNA haplotype or, more likely, to the maternal lineage. My demonstration of little or no selection against hybrids appears to discount the first possibility of selection against mismatched mitochondrial-nuclear marker patterns. Furthermore, my calculation of cytonuclear disequilibria, which displayed significant nonrandom association of intraspecific nuclear alleles with corresponding haplotype (Table 3.2), further discounts the possibility of random mating with selection against mismatched hybrids. However, a behavioral mating preference may exist. Although all F1 males could mate randomly with either pure-type and generate backcross progeny with mtDNA and nuclear genotypes that resemble our observed patterns (Fig. 3.6), F1 females must mate assortatively with the species that match their mtDNA haplotype to produce offspring that are consistent with my data. It is possible that all F1 hybrids are exclusively of one sex or the other. Forbes and Allendorf (1991) suggested that sexual differentiation is one process that may be particularly susceptible to disruption in hybrids. Turner and Liu (1977) and Cockendolpher (1980) observed a consistent excess of females in F1 progeny among species of killifish (genus *Cyprinodon*) indicating that some form of intrinsic prezygotic barrier may explain this novel and curious mating bias.

3.5.3 Conclusion

This study provides compelling, but indirect, insight into the relative roles of extrinsic and intrinsic selection in interspecific hybridization between sympatric coastal cutthroat and rainbow/steelhead trout. The presence of two populations in complete hybrid meltdown, coupled with weak or no evidence for selection against hybrids, indicates that prezygotic and postzygotic reproductive barriers are very weak or non-existent in these species, despite a long history of sympatry. The evidence for backcross selection effects may represent weak outbreeding depression. Furthermore, my data suggest there is no evidence for reinforcement mechanisms existing to prevent hybridization. My results further suggest that hybridization between coastal cutthroat and rainbow/steelhead trout is common and has the potential to displace the native trout populations, as has been seen in other subspecies of cutthroat trout (Leary et al. 1984; Carmichael et al. 1993). I cannot, as yet, provide any conclusive explanation for the apparent mating bias in the hybrid populations. To my knowledge, no other study has shown such effects, and the mechanism by which it could arise is not obvious. The study of coastal cutthroat-rainbow/steelhead trout hybridization clearly represents a valuable area for evolutionary as well as conservation research.

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4.0 GENERAL CONCLUSION AND RECOMMENDATIONS

Hybridization, with or without introgression, occurs frequently in numerous species of fish. The high incidence of hybridization in fish taxa has been attributed to various anthropogenic activities and apparently weak reproductive isolating mechanisms (compared to other vertebrate taxa). These factors have contributed to the conservation crisis of several western native trout species. Hence, this thesis investigated the frequency, potential consequences, and the conservation implication(s) of hybridization between naturally sympatric populations of coastal cutthroat and coastal rainbow/steelhead trout.

My survey of hybridization between sympatric coastal cutthroat and coastal rainbow/steelhead trout on Vancouver Island, BC had two primary goals: (1) to investigate the broad-scale distribution and frequency of hybridization and explore the environmental factors associated with elevated hybridization levels; and (2) investigate possible selective effects in hybridized populations. Hybridization between these trout species is evidently widespread throughout Vancouver Island as a result of various environmental effects (Chapter 2), indicating that hybridization between these species may be extensive throughout their entire native range. Coupled with an apparent lack of selection (intrinsic or extrinsic) against F1 hybrids, evidence of weak extrinsic selection against backcross hybrids, and an indication of temporally stable hybrid swarms (Chapter 3), it is clear that other sympatric populations may face the same fate. The ability for these naturally sympatric species to hybridize successfully, and develop hybrid swarms, poses great conservation concern for *both* species. This is because introgression occurs in both species, resulting in the simultaneous genetic extinction of two native fish taxa.

Consequently, conservation and management strategies developed for these species must include the prevention of hybridization, a complex addition to difficult management issues.

The urgency of this conservation situation, coupled by the unique and exciting opportunities available for studying the ongoing breakdown of reproductive barriers between sympatric coastal cutthroat and coastal rainbow/steelhead trout, will generate further understanding of the consequences of extensive hybridization and introgression. Based on my results, I propose the following actions to facilitate the ongoing and future conservation and management of sympatric coastal cutthroat and coastal rainbow/steelhead trout populations:

1) In order to effectively develop conservation management strategies for both species, it is vital to know how many sympatric populations remain pure; the smaller the number of pure populations, the greater the conservation risks. Consequently, it is important to structure a genetic monitoring program to extensively assess the status of more populations. It is impossible to reliably identify hybrids based on phenotypic characteristics, however genotyping a sample of fish would be cost-effective and is critical for future conservation efforts.

2) Small watersheds should be of priority when testing new populations for evidence of hybridization. Though results from this thesis could not pinpoint all the environmental effects contributing to hybridization in small watersheds, several environmental factors were identified (Chapter 2). Long-term effects of logging, trout stocking, and

lack of habitat availability all influence hybridization, and their effects are magnified in smaller watersheds. It is critical to continue efforts of identifying the genetic status of sympatric populations. Furthermore, examining additional fine-scale environmental processes within small watersheds may shed light on other environmental/biotic/geological effects associated with high levels of hybridization.

3) It is imperative that the stocking of hatchery trout be more carefully administered. Though the stocking of triploid trout does help to reduce the effects of stocking on increased hybridization, one area of stocking programs that should be given further attention is the genetic background of broodstock. The possibility that existing broodstock (i.e. Taylor River) may be of hybrid origin is quite likely. As a result, stocking of hatchery trout from hybrid broodstock could drastically spread the incidence of hybridization. In combination with a hybrid monitoring strategy, populations of pure coastal cutthroat and coastal rainbow/steelhead trout, as well as hybrid populations, can be accurately identified and utilized (or avoided) in future broodstock programs.

4) The long-term effects of timber harvesting plays a role in the rising incidence of hybridization. With the extensive knowledge that exists regarding forestry impacts and declining fish populations, there is no doubt that immediate habitat restoration or prevention of habitat loss is essential. The ability to reduce the cumulative nature of forestry impacts, and maintaining habitat at ecologically pristine levels, will aid in the prevention of future hybridization.

Based on my results, I propose the following recommendation for future research designs with the goal of further examining cutthroat/rainbow trout hybrid zone dynamics:

5) Now that hybridization has been shown to be common, the logical next step would be to re-sample known hybrid populations using a complete reach-scale approach. For example, sampling would occur for the entire stream, starting from the confluence (i.e. mouth) and ending at the headwaters. Additionally, streams should be divided into 'reach sections', with sections being fenced off during sampling to minimize fish movement between sections. Utilizing a reach-scale sampling method would identify the spatial distribution of hybrids within the populations. Temporal sampling over several years would provide a better understanding of the stability and/or range expansion (or depletion) of the hybrid zones. This would help in further understanding the effects (or lack thereof) of selection against hybrids.

The abundance of sympatric populations of coastal cutthroat and coastal rainbow/steelhead trout is declining. This study presents evidence that hybridization should not be overlooked as a contributor to their deterioration.

APPENDIX I Diagnostic PCR-RFLP and Length Polymorphism Assays

Locus ID:	Locus	Anealing Temp (°C)	Restriction Enzyme	Fragment Sizes (bp)	Cut Fragments Rainbow/Steelhead (bp)	Cut Fragments Cutthroat (bp)
1	GH2D[2]	55	N/A	1,305/1,100	1,305	1,100
2	GTH II - B[2]	55	*Bgl II*	1,619	1,619	1,050/569
3	IGF - 2[2]	62	*BstN I*	922	922	600/322
4	Ikaros[2]	49	*Hinf I*	813	813	608/205
5	RAG[2]	57	*Dde I*	1,013	600/240/173	600/413
6	TFex 3-5[3]	63	*Nci I*	1,634	917/717	717/487/430
7	GH1D[3]	58	*Mbo I*	1,375	985/390	1,375
8	ND3[1]	53	*Hae III*	320	320	270/50

[1] Docker et al. 2003; [2] Baker et al. 2002; [3] This study

1 – Growth Hormone 2, Intron D
2 – Gonadotropin II β
3 – Insulin-Like Grwth Factor, Intron 2
4 – Ikaros Gene
5 – Recombination Activation Gene
6 – Transferrin, Exons 3-5
7 – Growth Hormone 1, Intron D
8 – Mitochondrial ND3 Subunit

VITA AUCTORIS

Cory Bettles was born in 1974 in Vancouver, British Columbia. He graduated from Athol Murray College of Notre Dame High School, located in Wilcox, Saskatchewan, in 1992. From there he spent time playing junior hockey, until he went on to attend the University of Northern British Columbia (UNBC) where he obtained a Bachelor of Science (BSc.) degree in Biology (minor Biochemistry) in 2000. After graduation from UNBC, he was employed with the British Columbia provincial government for 2 years. He began his Master of Science (MSc.) degree Biology in January 2002 at the University of Windsor and completed his graduate degree in 2004.