RAINBOW TROUT INVASION AND THE SPREAD OF HYBRIDIZATION WITH NATIVE WESTSLOPE CUTTHROAT TROUT

by

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Chairperson: Fred W. Allendorf

The rate of hybridization has increased dramatically as a result of species introductions and many taxa are threatened with genomic extinction. Consequently, there is a need to refine molecular genetic methods for detecting hybridization in order to understand how introgression spreads. We identified seven diagnostic microsatellite loci for detecting hybridization between native westslope cutthroat trout (*Oncorhynchus clarki lewisi*) and introduced rainbow trout (*O. mykiss*) and compared this method to a Bayesian admixture model. Although the admixture model produced robust estimates of population admixture with diagnostic and nondiagnostic loci, estimates of individual admixture were highly sensitive to the amount of allele frequency divergence at the loci, suggesting that estimates of individual admixture based on nondiagnostic loci should be interpreted with caution. Additionally, we estimated the amount of gene flow among *O. c. lewisi* populations in the North Fork Flathead River and tested whether the pattern of *O. mykiss* invasion in this drainage fit a stepping-stone or continent-island model of migration. Significant genetic divergence was found among *O. c. lewisi* populations ($\theta_{ST} = 0.076$), indicating low straying rates. In contrast, we found higher dispersal rates for hybrids. *Oncorhynchus mykiss* introgression was detected in 17 of 31 sites and declined significantly with fluvial distance from Abbot Creek. Individuals from this site represent a hybrid swarm with a high percent of *O. mykiss* admixture (91.6%) and most (85%) of the *O. mykiss* alleles found among hybridized sites were present in Abbot Creek, indicating that this site is the ultimate source of introgression in the study area. Evidence for stepping-stone dispersal of *O. mykiss* alleles included a significant decline in the proportion of admixture with upstream distance, and the presence of later-generation backcrosses in populations with low levels of *O. mykiss* admixture. However, the spatial distribution of F_1 hybrids indicated that long distance dispersal of individuals with the highest amounts of *O. mykiss* admixture also contributes significantly to the spread of hybridization. Over time, introgression of nonnative alleles in genetically divergent *O. c. lewisi* populations is expected to lead to a loss of inter-population genetic diversity and local adaptation. Management strategies for preserving nonhybridized *O. c. lewisi* populations should attempt to eradicate populations with high levels of *O. mykiss* admixture to reduce further introgression.

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Chapter 1.

Introduction

The introduction and establishment of exotic species has impacted ecosystems worldwide (Mack et al. 2000). Of particular concern are introduced species that have escaped many of the biologic constraints in their native habitat and subsequently expanded in population size and range. These invasions can occur rapidly and are a frequent and increasing cause of species extinctions and local extirpations (Allan and Flecker 1993).

Aquatic systems have been greatly influenced by both intentional and accidental releases of nonnative species (Courtenay and Robins 1975). Many well-intended management programs have irreversibly altered the biotic community and function through the transplanting of nonnative sportfish (eg., northern pike, *Esox lucius*, McMahon and Bennett 1996; brown trout, *Salmo trutta*, Taylor et al. 1984; brook trout, *Salvelinus fontinalis*, Holton 1990). Additionally, countless taxa have been unknowingly translocated via ballast water in ships (eg., zebra mussel, *Dreissena polymorpha*) or by aquarium release (Fuller et al. 1999).

From a conservation perspective, the scope of the invasive species issue is of considerable scale. Introduced fishes have impacted nearly every major watershed in the United States (Courtenay et al. 1984). Furthermore, the rate of introductions has increased dramatically in the last 50 years (Fuller et al. 1999). Once established, the introduced taxa are often impossible to remove from the environment and may become invasive, causing unpredicted effects on native biota.

Hybridization

Hybridization can be a major consequence of species introductions, especially in circumstances where introduced species hybridize with rare or endangered species, threatening their persistence (Rhymer and Simberloff 1996). The genomic extinction of many rare taxa is often a direct result of the anthropogenic introduction of species into novel environments. In North America, species introductions were found to be a factor in 68% of fish extinctions and hybridization with introduced species was linked to 38% of these extinctions (reviewed in Miller et al. 1989).

Hybridization has been proposed as an important mechanism of speciation and can allow for rapid evolutionary change by producing novel recombinant genotypes (Barton and Hewitt 1981; Arnold 1997; Reiseberg et al. 2003). Natural hybridization in fishes is relatively common (Leary et al. 1995) however; rates and patterns of hybridization have been drastically affected by human activities. The effect that anthropogenic hybridization will have on native taxa is a function of the survival and fitness of hybrid offspring. If hybrid offspring are fertile, introgression may occur, resulting in the formation of hybrid swarms (Epifanio and Phillip 2001; Allendorf et al. 2001). However, in cases where hybridization does not result in genetic introgression (eg., sterile progeny), there is still the demographic cost of wasted reproductive effort (Leary et al. 1993).

Hybridization can have a wide range of effects on fitness. Improved fitness (i.e., heterosis or hybrid vigor) may occur as a result of either increased heterozygosity or the production of novel recombinant genotypes (Lewontin and Birch 1966). Although many examples of heterosis exist in nature (eg., Hartl and Clark 1989), the increase in fitness

observed in the F_1 progeny may be lost in subsequent generations due to the breakup of coadapted gene complexes (Dobzhansky 1970). In fact, several authors have argued that increased fitness in the offspring of genetically divergent taxa is the exception rather than the rule (Carson 1975; Leary et al. 1995).

Another potential outcome of hybridization is outbreeding depression. Intrinsic outbreeding depression results from genetic or chromosomal incompatibilities between the hybridizing taxa. For example, epistatic interactions occur when the genotype at one locus controls the phenotypic expression of alleles at other loci. Introgression of foreign alleles is expected to disrupt these coadapted gene complexes resulting in reduced fitness in the hybrid progeny (Dobzhansky 1970; Shields 1982). Evidence for intrinsic outbreeding depression has been demonstrated experimentally in crosses between rainbow trout, *Oncorhynchus mykiss*, and westslope cutthroat trout, *O. c. lewisi*, where hybrid fry exhibit reduced growth and survival (Leary et al. 1995). Similarly, Leberg (1993) demonstrated that offspring produced from mating genetically divergent stocks of mosquitofish, *Gambusia holbrooki,* had reduced growth rate and size compared to control groups. Reduced fitness of hybrid offspring can be a powerful selective force promoting reproductive isolation between the hybridizing taxa (Taylor and Foote 1991).

Alternatively, outbreeding depression may be caused by interactions between genes and the environment (i.e., extrinsic outbreeding depression). Local adaptation is well documented in many plant and animal species and can be disrupted by hybridization, whereby locally adapted gene complexes are lost due to recombination (Templeton 1986). For example, Philipp and Whitt (1991) found that hybrids between northern largemouth bass, *Micropterus salmoides salmoides*, and Florida largemouth bass, *M. s.*

floridanus, had reduced survival and growth when raised in Illinois ponds. Outbreeding depression in hybrids was attributed to reduced tolerance to cold water temperatures.

***Population genetic structure of* O. c. lewisi**

Some authors have proposed that the discrete nature of freshwater habitats may lower genetic variability within populations of fish and increase allele frequency divergence among drainages (Avise and Smith 1977; Gyllensten 1985). In addition, Rieman and Clayton (1997) have suggested that complex life histories and high levels of genetic divergence between drainages may be the evolutionary result of periodic disturbances such as drought and fire. Therefore, the degree of population subdivision is likely to have conservation implications for species (Leary et al. 1993; Knudsen et al. 2002; Spruell et al. 2003).

The distribution of genetic variation within a species is a function of the opposing forces of gene flow and genetic drift. Populations exchanging a greater number of migrants will have higher intrapopulation genetic diversity and reduced differentiation between populations (Allendorf and Phelps 1981). Leary et al. (1988) examined samples from across the range of *O. c. lewisi* and found one-third of the total amount of genetic variation detected at 32 protein loci to be attributable to differences among populations (F_{ST}=0.33). Similarly, Taylor et al. (2003) examined 8 microsatellite loci in 36 *O. c. lewisi* populations from southeastern British Columbia and found extensive population subdivision (F_{ST}=0.32). Results from these studies demonstrate high spawning site fidelity and limited gene flow among populations of *O. c. lewisi*.

Based upon surveys of genetic variation within and among populations of *O. c. lewisi* we conclude that any one population will not represent the range of allelic diversity

contained within the evolutionary lineage of this taxon. Furthermore, highly divergent populations, or populations fixed at some loci for rare alleles, likely possess local adaptations necessary for long-term persistence (Allendorf and Leary 1988; Leary et al. 1995). These findings have important implications for future reintroduction efforts and management of *O. c. lewisi* at the landscape scale. Preservation of this species will therefore require the persistence of many populations in order to retain genetic diversity in *O. c. lewisi* (Allendorf and Leary 1988).

***Hybridization between* O. c. lewisi *and* O. mykiss**

The westslope cutthroat trout, *O. c. lewisi*, is threatened by extinction primarily due to introgressive hybridization with introduced rainbow trout, *O. mykiss*, and Yellowstone cutthroat trout, *O. c. bouvieri* (Allendorf and Leary 1988). Hanzel (1960) published one of the first reports documenting the loss of cutthroat trout populations in areas where *O. mykiss* were introduced and Liknes and Graham (1988) later reported that *O. c. lewisi* have declined to the extent that genetically pure populations occupy only 2.5% of their historic range. Furthermore, habitat degradation has exacerbated declines in *O. c. lewisi* populations by increasing water temperature and stream sediment load and fragmenting migratory populations. These habitat disturbances may make the system more prone to the successful invasion of nonnative *O. mykiss* (Taylor et al. 1984), leading to displacement of native *O. c. lewisi* (Liknes and Graham 1988; Shepard et al. 1997).

Most of the theoretical work on hybrid zones has been based on studies of naturally sympatric populations. However, models used to describe the existence of hybrid zones between naturally sympatric taxa may not accurately explain the maintenance and spread of hybridization between organisms that are brought into contact

by human activity. For example, the widespread introduction of nonnative *O. mykiss* into the range of *O. clarki* subspecies has led to extensive and expanding introgression and the formation of stable hybrid swarms (Forbes and Allendorf 1991*a*; Carmichael et al. 1993; Hitt et al. 2003; Rubidge et al. 2001). In contrast, hybridization between naturally sympatric populations of *O. clarki* and *O. mykiss* is rare, presumably due to evolved reproductive isolation between the two taxa. For example, Hanson (1977) found strong spatial segregation between populations of *O. gairdneri* and *O. c. lewisi* in the Lochsa River basin, Idaho. Furthermore, Campton and Utter (1985) reported low levels of introgressive hybridization between naturally sympatric populations of coastal cutthroat trout, *O. c. clarki*, and steelhead trout, *O. gairdneri*, in tributaries to Puget Sound. The evolution of reproductive isolation between these naturally sympatric trout species suggests selection may be acting against hybrids (Young et al. 2001). Nevertheless, the rapid spread of introgression following anthropogenic secondary contact between *O. c lewisi* and *O. mykiss* remains unexplained.

The rapid spread of introgression between *O. c. lewisi* and introduced trout taxa may be caused by selection, dispersal, or a combination of the two. Several authors have tested for a selective advantage in *O. clarki* x *O. mykiss* hybrids. Although salmonids have a higher degree of developmental compatibility than other taxonomic groups of fishes with similar levels of genetic divergence (Gyllensten et al. 1985; Ferguson et al. 1985, 1988), it is unclear whether *O. c. lewisi* x *O. mykiss* hybrids experience heterotic effects in wild populations. Forbes and Allendorf (1991*a*) found no evidence of natural selection in *O. c. lewisi* x *O. c. bouvieri* hybrid swarms. Similarly, Rubidge et al. (2004) found no evidence for selection in natural populations of *O. c. lewisi* x *O. mykiss* hybrids.

Conversely, hatchery crosses of *O. c. lewisi* and *O. mykiss* resulted in progeny with significantly lower survival and growth rates than parental controls (Allendorf and Leary 1988; Leary et al. 1995). The results of these studies are inconclusive on the matter of hybrid fitness in wild populations and indicate that we cannot rule out the possibility of outbreeding depression or heterosis.

Introgression may spread among individuals despite outbreeding depression in the hybrid progeny. The model of Epifanio and Philipp (2001) provides one explanation for this phenomenon based on the unidirectional nature of hybridization (simplistically, all offspring of hybrid matings will be hybrids). This "ratchet effect" predicts that the genomic extinction of parental taxa can occur despite heavy fitness costs in the hybrid offspring (Huxel 1999; Wolf et al. 2001).

Alternatively, increased dispersal rates in hybrids can cause the spread of introgression. For example, hybridization may prompt invasiveness by disrupting the genetic basis for homing (Ellstrand and Schierenbeck 2000; Hard and Heard 1999; Bams 1976). Native *O. c. lewisi* exhibit significant genetic differentiation over short geographical distances (Leary et al. 1988; Taylor et al. 2003), indicating low levels of straying among populations. In contrast, the rapid spread of hybridization in the Flathead River drainage (Hitt et al. 2003) suggests that hybrids have greater straying rates than native *O. c. lewisi* (Allendorf et al. 2004; 2005). Increased dispersal by hybrids is expected to lead to maladaptation through the breakup of coadapted gene complexes and a reduction in the level of adaptive divergence among populations (Lenormand 2002). Furthermore, behavioral differences between *O. c. lewisi* and hybrids (such as increased dispersal rates) will have implications for determining whether to protect hybridized

populations as *O. c. lewisi* under the Endangered Species Act (USFWS 2003). As a result, there is a need to identify sources and patterns of hybrid invasion in order to make informed decisions regarding the eradication of sources of introgression.

Models of hybrid invasion

The spatial distribution of introgressed populations results from the pattern of invasion and the amount of admixture in the migrants. Under a stepping-stone dispersal model (Kimura and Weiss 1964), migration occurs between adjacent populations (Fig. 1-1*a*). This model of genetic invasion predicts a negative correlation between distance from a source population and level of *O. mykiss* admixture, a serial dilution of diagnostic *O. mykiss* alleles from low elevation to higher elevation hybridized sites, and the presence of F_1 hybrids (i.e., gametic disequilibria) in lower elevation sites. In the stepping-stone model, introgression is expected to spread at a slower rate since individual dispersal distance is shorter and neighboring populations tend to have similar amounts of admixture.

Alternatively, a continent-island dispersal model assumes an equal probability of dispersal independent of distance from the source population (Fig. 1-1*b*). This model predicts no spatial autocorrelation for *O. mykiss* admixture, diagnostic *O. mykiss* alleles, or the presence of F_1 hybrids. Under this model, the incidence and proportion of *O. mykiss* admixture among populations are expected to increase at a faster rate than they would by stepping-stone dispersal since migrants disperse from a common source with a high proportion of admixture.

Objectives

Understanding the patterns and mechanisms of biotic invasions is increasingly important for the conservation of native species. In particular, preservation of *O. c. lewisi* within its native range necessitates an understanding of the pattern of invasion in order to inform management actions aimed at eradicating sources of introgression. In part, this will require both the identification of many diagnostic markers for detecting hybridization and a description of how the invasive genome spreads among native populations of *O. c. lewisi*.

This thesis focuses on two important topics concerning the spread of anthropogenic hybridization: molecular genetic detection of admixture, and determining the pattern of invasion. In Chapter 2, I addressed the following objectives:

1) develop diagnostic microsatellite markers for detecting hybridization between *O. c. lewisi* and *O. mykiss*, and

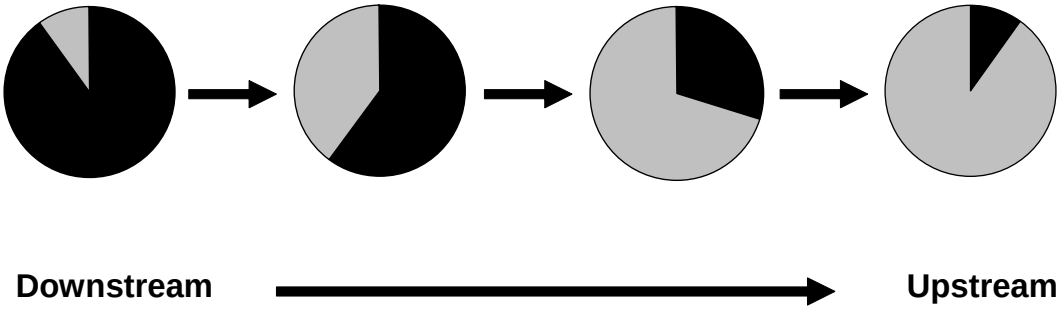
2) evaluate the performance of the Bayesian program STRUCTURE for estimating admixture using diagnostic and nondiagnostic loci.

In Chapter 3, my objectives were the following:

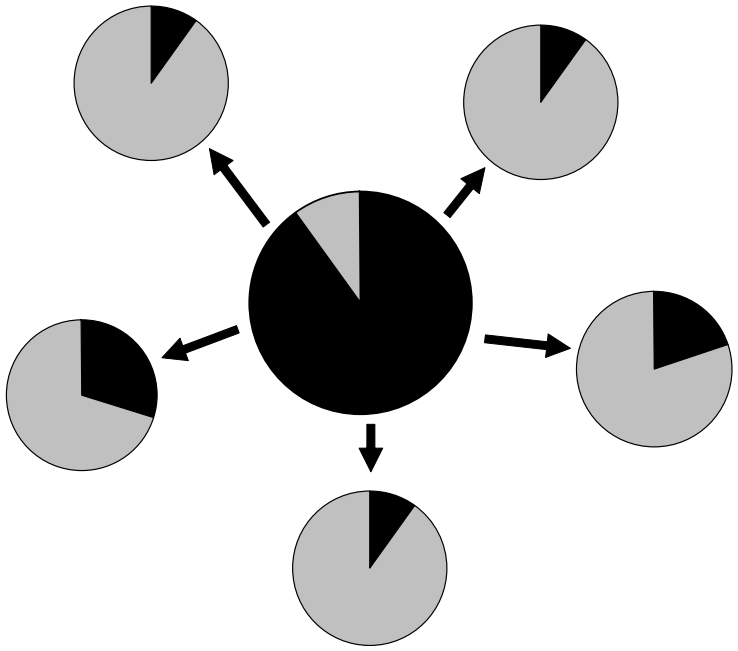
1) estimate the amount of gene flow among populations of *O. c. lewisi* in the North Fork Flathead River drainage, and

2) test whether the spread of hybridization results from stepping-stone (Fig. 1-1*a*) or continent-island patterns (Fig. 1-1*b*) of invasion.

Figure 1-1. *a*) Stepping-stone model of hybrid invasion assumes that the spread of introgression results from gene flow between neighboring populations. Circles represent hybridized populations; areas in black denote proportion of *O. mykiss* admixture. Under this model, F_1 hybrids are found in the furthest downstream populations and later generation backcross hybrids are located at the upstream periphery.



b) Continent-island model where hybridization spreads from a source to satellite subpopulations. This model assumes an equal probability of gene flow between the source and all populations. Differences in proportion of *O. mykiss* admixture are attributed to stochasticity in the invasion process and/or sampling error. Long distance dispersal from a source population with a high proportion of *O. mykiss* admixture results in F_1 hybrids in nonadjacent populations.



Summary and synthesis

I identified seven microsatellite loci that were diagnostic between *O. mykiss* and *O. c. lewisi*. Comparisons between estimates of population and individual admixture using these loci and the admixture model in program STRUCTURE revealed that model estimates of population admixture are robust to the amount of allele frequency divergence (i.e., R_{ST}) at loci. However, the admixture model was highly sensitive to allele frequency divergence when estimating individual admixture. Estimates of individual admixture using nondiagnostic loci had wide probability intervals and varied dramatically for a given hybrid index score from diagnostic loci. These results suggest that model-based estimates of individual admixture should be cautiously interpreted when using nondiagnostic loci.

Populations of *O. c. lewisi* in the North Fork Flathead River drainage exhibit low gene flow and high genetic divergence, even between adjacent sites. In contrast, increased straying by hybrids contributes to the spread of introgression. Spatial patterns of population admixture provided evidence for stepping-stone dispersal and indicated that *O. mykiss* introgression is ultimately spread through upstream invasion from the hybrid swarm in Abbot Creek. However, the spatial distribution of hybrid genotypes indicates that long distance dispersal plays a significant role in the spread of introgression in this drainage. Specifically, populations with a high proportion of *O. mykiss* admixture were located furthest downstream and F_1 hybrids were found in 2 populations over 50 fluvial km upstream from Abbot Creek. These findings indicate that hybrids are much more prone to stray than *O. c. lewisi* and support the argument of Allendorf et al. (2004) that including hybridized populations as *O. c. lewisi* will protect future sources of

introgression and contribute to the spread of hybridization. Furthermore, results suggest that eradicating sources of *O. mykiss* may reduce the spread of introgression.

Hybridization and introgression with introduced species has led to the genomic extinction of taxa worldwide and may be the most underestimated problem in conservation biology (Rhymer and Simberloff 1996). Advances in molecular genetic techniques have greatly improved the capability to identify hybridization and test for effects on fitness. Furthermore, the increased refinement of Bayesian admixture models and assignment tests has allowed for the estimation of admixture proportions without the use of diagnostic loci. These methods represent a major advancement in our ability to identify hybridization and define units for conservation.

Introgression with introduced trout taxa is the primary reason for declines in populations of *O. c. lewisi* (Allendorf and Leary 1988). However, defining formal guidelines for the treatment of hybrids in general, and, specifically, for the protection of nonhybridized *O. c. lewisi* has been extremely difficult. The U. S. Fish and Wildlife Service (2003) concluded that listing *O. c. lewisi* as threatened under the U. S. Endangered Species Act was not warranted because of the widespread distribution of populations that possess phenotypic characteristics of *O. c. lewisi*. The argument for protecting hybridized populations with the morphological attributes of *O. c. lewisi* assumes that hybrids possess similar life history and behavioral characteristics of *O. c. lewisi* (USFWS 2003). The findings of this study and Hitt et al. (2003) indicate that there are significant differences in dispersal behavior in hybrids and emphasize that including hybridized populations in the unit considered for federal protection will likely protect

sources of future introgression, leading to further declines in *O. c. lewisi* (Allendorf et al. 2004, 2005).

Chapter 2.

Comparison between diagnostic loci and a Bayesian likelihood model for estimating genetic admixture in hybrid swarms

Abstract: Rates of hybridization have increased dramatically due to human activity, resulting in the genomic extinction of taxa worldwide. Consequently, methods for detecting admixture are increasingly important tools for the management of taxa threatened by hybridization. We describe seven diagnostic microsatellite loci between rainbow trout, *Oncorhynchus mykiss*, and westslope cutthroat trout, *O. clarki lewisi*, and estimate individual and population admixture in the North Fork Flathead River drainage, Montana. Additionally, we tested for concordance between two methods for estimating admixture: a hybrid index based on diagnostic loci, and a model-based likelihood estimator. Overall, estimates of the admixture coefficient, *q*, from the Bayesian program STRUCTURE were highly correlated with estimates of admixture from diagnostic loci. However, the Bayesian estimates of individual admixture were sensitive to the amount of allele frequency divergence between the hybridizing taxa. For individuals with a given hybrid index score, estimates of *q* and their 95% probability intervals were highly variable when nondiagnostic loci were used in the model. These findings demonstrate that Bayesian admixture models provide reliable estimates of population admixture with relatively small numbers of nondiagnostic loci. However, Bayesian estimates of individual admixture based on nondiagnostic loci should be interpreted with caution. The

diagnostic microsatellite loci described here will be valuable for future studies of *O. mykiss* introgression into native populations of *O. c. lewisi*.

Introduction

The study of genetically admixed populations is of interest in many fields of biology. In conservation biology, much attention has been devoted to identifying and controlling hybridization and introgression between taxa that are brought into secondary contact by human activity (Allendorf et al. 2001). This anthropogenic hybridization can be an important consequence of species translocations, especially in circumstances where invasive species hybridize with rare or endangered taxa, threatening their persistence (Rhymer and Simberloff 1996).

Most commonly, studies of hybridization have used diagnostic loci to detect genetic admixture. Diagnostic loci are those that are fixed or nearly fixed for different alleles in hybridizing taxa (Ayala and Powell 1972). With codominant diagnostic markers such as allozymes and microsatellites all genotypes are distinguishable, making it possible to categorize individuals by the type of hybrid cross and assess the likelihood that some individuals in the population are nonhybridized. This method has been used extensively for describing natural hybrid zones (Szymura and Barton 1986), measuring barriers to genetic exchange between species (Sage et al. 1986), and identifying nonintrogressed populations for conservation efforts (Dowling and Childs 1992).

When diagnostic loci are not available for the hybridizing taxa of interest, model-based approaches may be used to estimate genetic admixture. Several Bayesian likelihood-based models have been developed to identify genotypes of hybrid origin

based on allele frequency differences in the hybridizing taxa (eg., Pritchard et al. 2000; Chikhi et al. 2001; Anderson and Thompson 2002; Falush et al. 2003). Bayesian models such as program STRUCTURE (Pritchard et al. 2000) infer the number of populations (K) within a data set by grouping individuals such that Hardy-Weinberg and linkage disequilibrium are minimized. Individual multilocus genotypes are assigned probabilistically to one or more of the K populations to estimate individual admixture. The performance of these models has been greatly improved by advances in molecular genetic techniques that allow for large numbers of highly polymorphic markers, such as microsatellites, to be assayed. These models have been used extensively to identify admixture and are useful for evaluating the threat to native taxa posed by anthropogenic hybridization (Beaumont et al. 2001, Randi and Lucchini 2002; Hansen 2002).

Despite the widespread use of these models for inferring admixture, no study has investigated how well they perform compared to diagnostic markers. We examine this issue in the context of hybridization between introduced *O. mykiss* and native *O. c. lewisi*, an exemplary case where the identification of hybrids is critical for developing effective management policy (Allendorf et al. 2004). The objectives of this study were to (1) find diagnostic microsatellite loci to identify hybridization between *O. mykiss* and *O. c. lewisi*; and (2) evaluate the performance of the Bayesian program STRUCTURE for estimating admixture using diagnostic and nondiagnostic loci.

Methods

Study taxa

Rainbow trout, *Oncorhynchus mykiss*, and westslope cutthroat trout, *O. clarki lewisi* have evolved predominantly in allopatry throughout their native ranges (Behnke 1992). A small region of sympatry exists between the inland redband subspecies, *O. m. gairdneri*, and *O. c. lewisi*; however, introgression is extremely rare as evidenced by substantial genetic divergence (Nei's $D = 0.130$; Leary et al. 1987) and the presence of many diagnostic allozyme loci between the two taxa (Allendorf and Leary 1988). Conversely, introgression is common where *O. mykiss* and other nonnative trout taxa have been introduced into the range of *O. c. lewisi* and is responsible for major declines in *O. c. lewisi* populations (Allendorf and Leary 1988).

Sample collection and DNA extraction

A total of 847 individuals were collected in 2003-2004 from 31 sites in the North Fork Flathead River drainage, Montana. This drainage has historically received transplants of nonnative *O. mykiss* and introgressive hybridization with native *O. c. lewisi* is common and widespread (Hitt et al. 2003; Muhlfeld et al. 2002, 2003). Fin clips were collected nonlethally, stored in 95% ethanol, and DNA was extracted using the Gentra DNA isolation kit (Gentra Systems, Inc.). Microsatellite loci were amplified in an MJ Research PTC-100 thermocycler using fluorescently labeled primers. PCR products were electrophoresed through 7% polyacrylamide gels and visualized using a Hitachi FMBIO-II fluorescent imager. Allele sizes were determined using MapMarkerLOW size standards (Bio Ventures, Inc.) and Hitachi FMBIO-II software (MiraiBio, Inc. 1999).

Previously scored individuals were included on each gel as controls to ensure consistent allele scoring across populations.

Diagnostic microsatellite loci

In contrast to allozyme loci, in which diagnostic differences between taxa are primarily due to fixation for alternate alleles at a locus, microsatellite loci are typically highly polymorphic and diagnostic differences result from non-overlapping allele sizes between taxa. Hybridization in individuals and populations may be identified by the presence of alleles from both parental taxa at diagnostic loci. Furthermore, the type of hybrid cross may be recognized by examining the distribution of alleles at diagnostic loci. For example, F_1 hybrids will be heterozygous for alleles from the parental taxa at all diagnostic loci.

The high mutation rate at microsatellite loci increases the likelihood that two taxa may share an allele at a typically diagnostic locus as a result of mutation rather than hybridization (i.e., homoplasy). However, it is possible to detect homoplasy by examining several diagnostic loci. Hybridization is expected to result in approximately equal rates of introgression throughout the genome. Therefore, a relatively high frequency of a diagnostic allele at a single locus is likely evidence of homoplasy and not hybridization (Forbes and Allendorf 1991*a*). Additionally, we may assess the likelihood that a novel allele (i.e., one that has not been previously identified in either taxon) indicates either hybridization or intraspecific genetic variation based on the number of base-pair repeats. For example, a novel allele at locus *Omm1019* with a base pair size of 166 likely originated in the *O. c. lewisi* genome either due to a dinucleotide insertion mutation at the 164 allele or a dinucleotide deletion at the 168 allele (Fig. 2-1*a*).

Our criteria for selecting diagnostic microsatellite loci were the following: 1) polymerase chain reaction (PCR) amplifications must produce easily scorable and reproducible products, and 2) loci must show non-overlapping allele sizes between the taxa. To test these criteria, we screened PCR primer pairs with reference individuals from hatcheries and wild populations in order to identify a wide range of allelic variation within and between the taxa. Species identity for each reference individual was confirmed using allozyme or PINE analysis (Table 2-1). The Anaconda M012 hatchery strain of *O. c. lewisi* is derived from individuals from several major drainages within this species' native range. The Arlee strain of *O. mykiss* contains a mixture of coastal rainbow trout (*O. m. irideus*) and inland redband trout (*O. m. gairdneri*). Primer sequences and PCR conditions followed those of the original authors (Table 2-2).

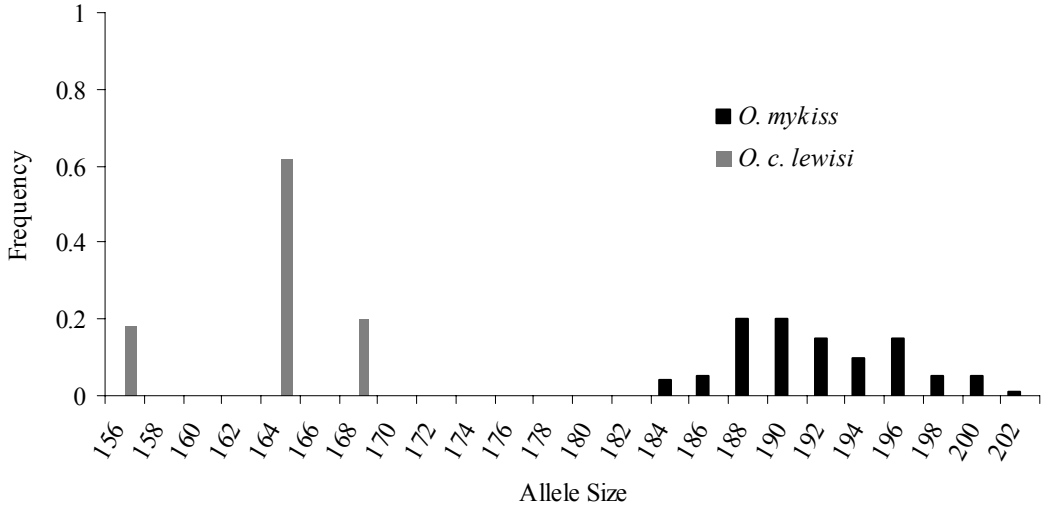
Using diagnostic codominant markers, population admixture is expressed as the proportion of diagnostic *O. mykiss* alleles found among individuals within a population. To estimate individual admixture, we calculated a hybrid index by summing the number of *O. mykiss* alleles at the diagnostic loci. This index ranges from 0 (no *O. mykiss* alleles) to 2N (where N equals the number of diagnostic loci) and is scaled from 0 to 1 by dividing the number of *O. mykiss* alleles found in an individual by 2N.

Table 2-1. Sources and size of reference samples.

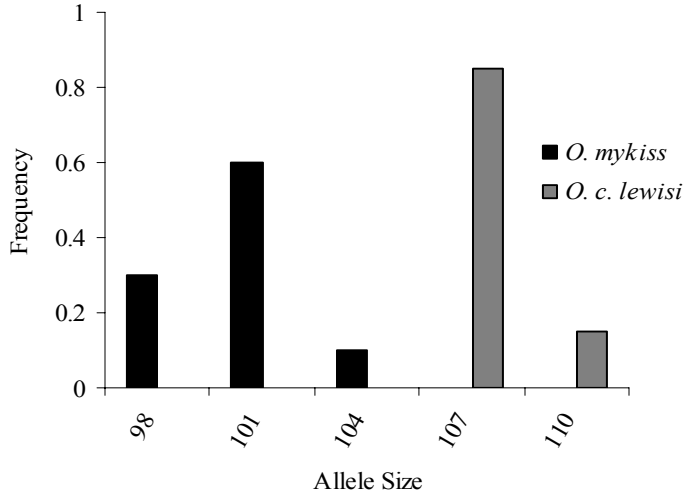
Taxon	Source	Sample size	Hybrid analysis and reference
O. mykiss	Arlee hatchery strain	7	allozymes; R. Leary, unpublished data
O. m. gairdneri	North Fork Callahan Cr., MT	7	allozymes; Knudsen et al. 2002
O. c. lewisi	Anaconda hatchery strain (M012)	30	allozymes; R. Leary, unpublished data
	Danaher Cr., MT	7	PINES; Dunning and Knudsen 2004

Figure 2-1. Frequency histograms showing non-overlapping allele sizes between *O. mykiss* and *O. c. lewisi* at two diagnostic microsatellite loci: a) *Omm1019* and b) *Omm1060*.

a)



b)



Bayesian admixture analysis

We used the admixture model with correlated allele frequencies implemented in program STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) to estimate the proportion of an individual's genome that originated from *O. mykiss* (the statistic *q*). Our methods followed those of Beaumont et al. (2001) where we assumed that the number of parental populations (K) is 2 (i.e., *O. mykiss* and *O. c. lewisi*). Twenty-two individuals with a hybrid index score of 14 were designated as priors in the model and assigned the highest admixture value of $q = 1$ (i.e., we forced the model to consider these individuals as *O. mykiss*). To test the validity of using these individuals as priors, we ran the model without priors and obtained very similar results ($r^2 > 0.99$). We concluded that these individuals are accurate representations of pure *O. mykiss* and present results from the model using priors. Population admixture was calculated as the arithmetic mean of *q* values for individuals in that population.

To test how allele frequency divergence at loci affects the ability of program STRUCTURE to estimate the proportion of admixture in individuals and populations, we ran 3 separate modeling scenarios using multilocus genotypes from the 7 diagnostic loci, 6 nondiagnostic loci, and all 13 loci. We compared these results to estimates of individual and population admixture using diagnostic loci and identified significant correlations using Pearson's correlation coefficient (r^2).

Genetic variation at diagnostic and nondiagnostic loci

The power of Bayesian likelihood tests to detect admixture increases with the number of loci genotyped, the level of heterozygosity, and the amount of allele frequency divergence between the hybridizing taxa (Davies et al. 1999). We calculated expected

heterozygosities for *O. c. lewisi* and hybrid populations in the North Fork Flathead River drainage and estimated allele frequency divergence (i.e., F_{ST} and R_{ST}) between the two hybridizing taxa using FSTAT version 2.9.3 (Goudet 1995).

Both F_{ST} (Wright 1951) and R_{ST} measure population subdivision, however, R_{ST} accounts for variance in allele size at loci undergoing a step-wise mutation model (Slatkin 1995). At loci for which heterozygosity is high, F_{ST} often underestimates divergence since it does not account for the absence of shared alleles between the taxa. Nevertheless, these mutations are important indicators of population subdivision. R_{ST} assumes that each mutation changes the base-pair length of an allele by a single repeat unit. Populations with alleles differing in allele size by only a few steps will have experienced greater and/or more recent gene flow than populations with alleles that differ by many base-pair repeat units. To calculate F_{ST} and R_{ST} for the two taxa, individuals from populations without evidence of hybridization were assumed to represent parental *O. c. lewisi* and individuals with a hybrid index score of 14 represent parental *O. mykiss*.

Results

Microsatellite loci

We found seven microsatellite loci that reliably amplify in PCR and which exhibit non-overlapping allele sizes between *O. mykiss* and *O. c. lewisi* (Fig. 2-1). These loci were used as diagnostic loci between the two taxa. Relatively few alleles occurred at the diagnostic loci in *O. c. lewisi* populations and heterozygosity tended to be low. In contrast, these loci tended to be highly polymorphic in *O. mykiss*, making these markers well suited for studying patterns of *O. mykiss* introgression in native *O. c. lewisi*

populations (Boyer et al. in prep.) Six additional polymorphic, nondiagnostic loci were selected to increase power to detect population subdivision in *O. c. lewisi*.

Estimates of F_{ST} between the two taxa ranged from 0.199 at *Ocl2* to 1 at *Ssa456* and averaged 0.666 over all loci. R_{ST} ranged from 0.161 at *Ocl2* to 1 at *Ssa456* and averaged 0.951 over all loci indicating that strong divergence between *O. c. lewisi* and *O. mykiss* at microsatellite loci is attributable to the accumulation of private alleles. Both F_{ST} and R_{ST} tended to be greater at diagnostic loci (Table 2-2) and, as expected, R_{ST} was considerably greater than F_{ST} at diagnostic loci with high heterozygosity. Expected heterozygosities, number of alleles, and base-pair size ranges for diagnostic loci are shown in Table 2-2.

Population admixture

Estimates of population admixture using diagnostic loci and program STRUCTURE were highly correlated for all three modeling scenarios ($r^2 > 0.98$), indicating that the model produces reliable estimates of population admixture using a relatively small number of nondiagnostic loci. Analysis of variance found no significant difference in mean population admixture between the four estimates ($F_{3, 64} = 0.039$, $P = 0.989$). Although these results were not statistically significant, Bayesian estimates of population admixture were consistently greater than estimates produced using diagnostic loci (86%; 44 of 51 comparisons). This pattern is most pronounced for the modeling scenario with 6 nondiagnostic loci and suggests that individual estimates of q tend to be biased high (Fig. 2-2).

Table 2-2. Summary statistics for diagnostic (above the dotted line) and nondiagnostic loci. F_{ST} and R_{ST} values illustrate allele frequency divergence between parental *O. mykiss* and *O. c. lewisi*. Base-pair size ranges for nondiagnostic loci. Primer sequences and PCR conditions are those of the original authors.

			H_S		# Alleles		Base-pair size range		
Locus	F_{ST}	R_{ST}	*O. c. lewisi*	Hybrid	*O. c. lewisi*	Hybrid	*O. c. lewisi*	*O. mykiss*	Citation
Omm1019	0.356	0.966	0.497	0.622	4	15	156-168	174-202	Rexroad et al. 2002
Omm1050	0.784	0.983	0.090	0.292	2	16	230-234	240-360	Rexroad et al. 2002
Omm1060	0.884	0.981	0.061	0.325	2	5	107-110	98-104	Rexroad et al. 2002
Omy0004	0.927	0.999	0	0.284	1	14	76	130-164	Holm and Brusgaard 1998
Sfo8	0.479	0.945	0.361	0.448	4	20	192-204	208-296	Angers et al. 1995
Ssa456	1	1	0	0.568	1	3	159	155-157	Slettan et al. 1995
Ogo8	0.928	0.995	0.033	0.176	2	6	92-94	96-102	Olsen et al. 1998
Omm1037-1	0.362	0.843	0.413	0.209	6	16	131-159	143-205	Rexroad et al. 2002
Omm1037-2	0.997	1	0	0.268	1	3	105	101-105	Rexroad et al. 2002
Ogo5	-	-	0	0.010	1	2	185	182-185	Olsen et al. 1998
Ssa311	0.733	0.767	0.143	0.363	4	13	136-142	130-170	Slettan et al. 1995
Ocl2	0.199	0.161	0.581	0.660	5	13	134-146	110-150	Condrey and Bentzen 1998
Oneu14	0.314	0.308	0.582	0.700	15	19	150-200	150-200	Scribner et al. 1996
Diagnostic loci	0.765	0.979	0.148	0.388					
Nondiagnostic loci	0.521	0.643	0.287	0.368					
Overall	0.666	0.951	0.212	0.379					

Individual admixture

Hybrid index scores and estimates of individual admixture (q) were significantly correlated for all three modeling scenarios, however, correlation between hybrid index scores and q was weakest when the 6 nondiagnostic loci were used in program STRUCTURE (Fig. 2-3). In contrast to the pattern observed with population admixture, the strongest correlation between q and hybrid index scores was not achieved when all 13 loci were used in the model. With nondiagnostic loci there is reduced power to detect gametic disequilibria and departures from Hardy-Weinberg genotypic proportions resulting from uncertainty about which taxa contributed the shared allele.

The width of the 95% probability interval around a point estimate of q is a measure of the reliability of that estimate. Narrow interval widths indicate a greater amount of precision in the estimator. Probability intervals around mean estimates of q were widest when there was roughly even genetic contribution from *O. mykiss* and *O. c. lewisi* (i.e., $q = 0.5$; Fig. 2-4). This pattern was consistent for diagnostic and nondiagnostic loci. However, there was a striking difference in the variability of probability interval widths for a given hybrid index score between diagnostic and nondiagnostic loci. For example, individuals with a hybrid index score of 0 have probability interval widths around q ranging from 0.1 to 0.18 using diagnostic loci (Fig. 2-4*a*). For the same hybrid index score, probability interval widths ranged from 0.1 to 0.65 using nondiagnostic loci (Fig. 2-4*b*), indicating that genotypes at these loci were not consistently assigning to the parental taxa.

Fig. 2-2. Estimates of *q* (using 6 nondiagnostic loci, 7 diagnostic loci, and all 13 loci) and proportion *O. mykiss* admixture using diagnostic loci for 17 hybridized populations in the North Fork Flathead River drainage.

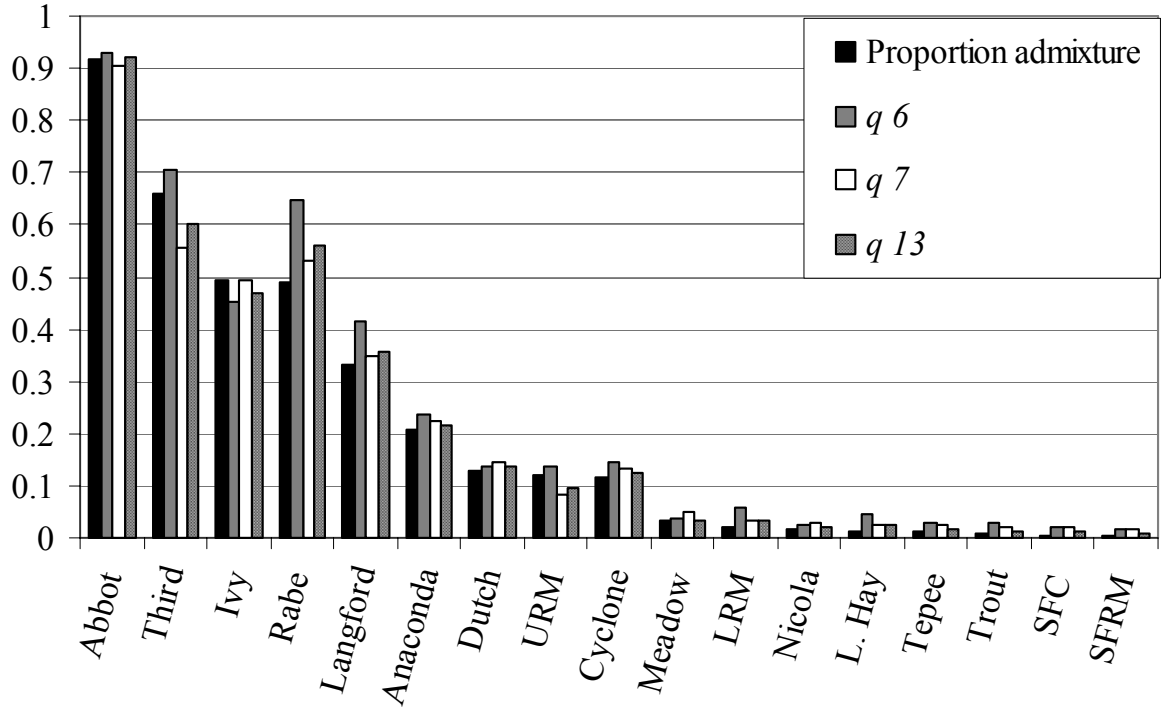
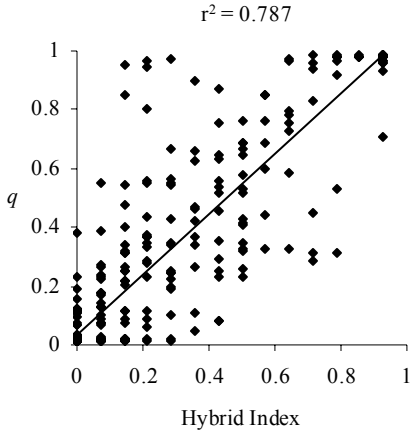
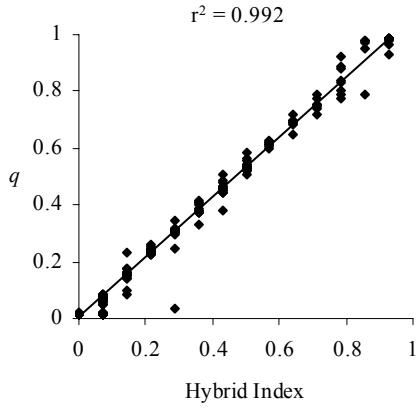


Fig. 2-3. Proportion of *O. mykiss* admixture in individuals (*q*) using (a) 6 nondiagnostic loci, (b) 7 diagnostic loci, and (c) all 13 loci plotted against hybrid index scores. All three correlation coefficients are significant ($P < 0.001$).

(a)



(b)



(c)

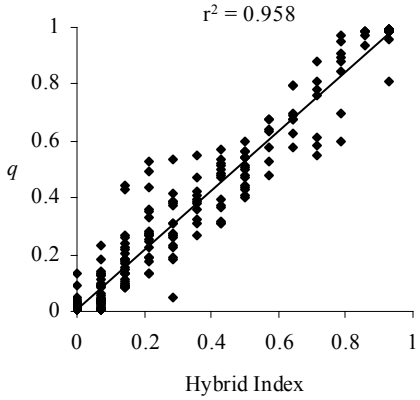
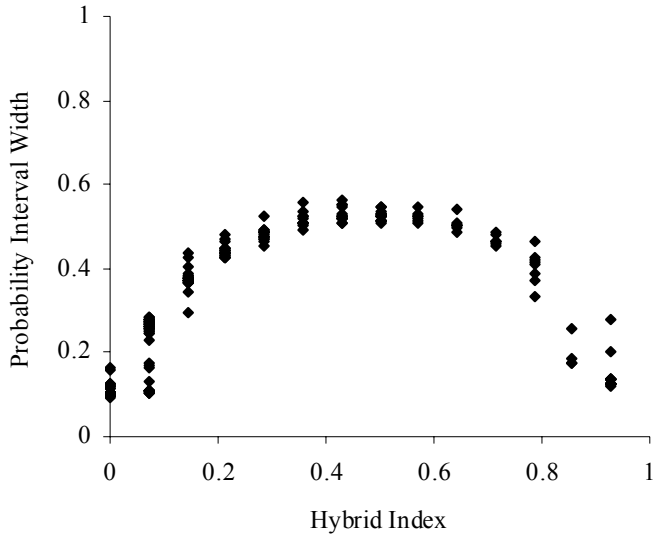
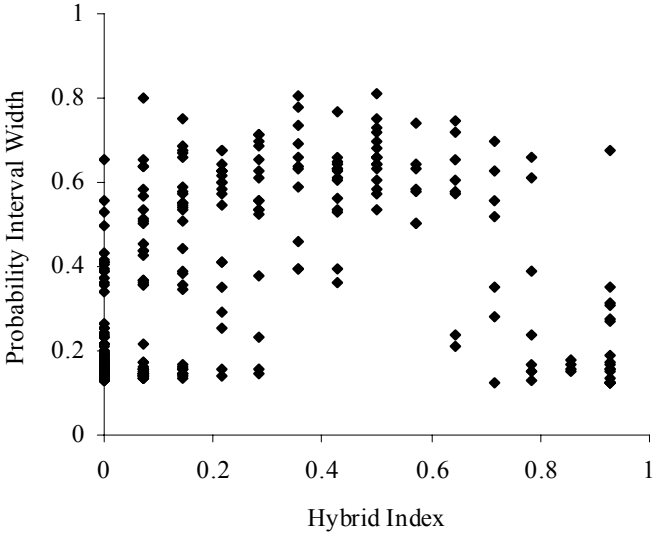


Fig. 2-4. Range of 95% probability interval widths around mean estimates of *q* for a given hybrid index score using (a) diagnostic and (b) nondiagnostic loci. Dots represent individuals and the hybrid index ranges from 0 (pure *O. c. lewisi*) to 1 (pure *O. mykiss*).

(a)



(b)



Discussion

Diagnostic loci

Although diagnostic loci are the most informative and widely used class of molecular marker for identifying hybridization, it is important to note that diagnostic differences may not be conserved across the geographic range of the hybridizing taxa. Microsatellites have qualities desirable for studies of hybridization (codominant Mendelian inheritance of alleles, selective neutrality, and the ability to extract nuclear DNA from nonlethal tissue samples); however, the high level of polymorphism at these loci can make identification of diagnostic differences problematic. Furthermore, high intraspecific genetic variation among *O. c. lewisi* populations (Leary et al. 1988; Allendorf and Leary 1988; Taylor et al. 2003) increases the likelihood that some populations possess rare alleles typically characteristic of *O. mykiss*. We used reference samples of *O. mykiss* and *O. c. lewisi* from native populations and hatchery broodstocks to maximize the likelihood that the diagnostic pattern we observed at the seven loci is consistent across populations for these taxa. Nevertheless, polymorphic loci that have been incorrectly identified as diagnostic could result in false identification of hybrid individuals. Such polymorphic loci should be identifiable since we expect to see *O. mykiss* alleles distributed across most of the diagnostic loci in a hybrid swarm. An appreciable frequency of alleles characteristic of *O. mykiss* at a single locus likely reflects intraspecific genetic variation in *O. c. lewisi* and not evidence of hybridization (Forbes and Allendorf 1991*a*). However, discerning between these two scenarios will not be possible in samples with a small number of individuals.

The statistical power to detect admixture in a hybrid swarm is expressed by the equation $(1-p)^{2NX} = \alpha$, where p equals the percent hybridization, N is the number of individuals in the sample population, X is the number of diagnostic loci, and α is the probability of detection. Thus, in a sample of 30 individuals with 7 diagnostic microsatellite loci there is a greater than 99% chance of detecting as little as 1% genetic contribution from *O. mykiss* in a hybrid swarm with *O. c. lewisi*.

Comparison between diagnostic loci and a Bayesian admixture model

Two major sources of error exist with model-based admixture estimates. First, because we are sampling a finite number of individuals from the greater population, there is sampling error of allele frequencies both temporally and spatially. Second, inter- and intra-population genetic variation in the parental taxa can influence the admixture estimate if this allelic variation is not represented in the sample. Both sources of error may lead to over or underestimates of admixture and should be considered when determining hybridization status for threatened taxa.

Highly concordant estimates of population admixture between diagnostic loci and all three modeling scenarios in program STRUCTURE indicate that Bayesian estimates of population admixture were not exceedingly sensitive to the amount of allele frequency divergence at the loci. Furthermore, there was no significant improvement in the estimate when all 13 loci were used in the model, suggesting that reliable estimates of population admixture may be obtained with a relatively small number of nondiagnostic loci. Future research should determine the threshold level of allele frequency divergence at which Bayesian estimates of admixture have objectionably low power to detect hybridization.

In contrast to the robust estimates of population admixture, estimates of individual admixture (q) varied considerably depending on whether diagnostic or nondiagnostic loci were used in the model. Estimates of q using diagnostic loci were highly concordant with hybrid index scores and 95% probability interval widths around these estimates showed little variability for a given hybrid index score. Conversely, nondiagnostic loci produced estimates of q that were often very different from the hybrid index scores and 95% probability intervals around q were typically wider and more variable for a given hybrid index score. These findings suggest that individual admixture estimates can be highly sensitive to the amount of allele frequency divergence at the loci and caution should be used when interpreting estimates of individual admixture produced from nondiagnostic loci.

Advantages of the admixture model include the ability to use information from nondiagnostic loci to detect hybridization in populations. This is especially valuable in circumstances where diagnostic loci have not been identified for the hybridizing taxa of concern. Another advantage of model estimates of admixture is the capability of placing probability intervals around point estimates of individual admixture, thereby providing an indicator of uncertainty in the estimate. It is not feasible to place probability intervals around hybrid index scores from diagnostic loci.

Implications for studies of hybridization

Hybridization and introgression with introduced species has led to the genomic extinction of taxa worldwide and may be the most underestimated problem in conservation biology (Rhymer and Simberloff 1996). Our underestimation of the threat that hybridization poses to native taxa results from both uncertainty in the fitness of

hybrid progeny and problems associated with detecting introgression. For example, effects on fitness in hybrid progeny may range from heterosis to severe outbreeding depression (Templeton 1986), and in many cases it is not possible to identify hybrids based on morphology alone (eg., Leary et al. 1996; Weigel et al. 2002; Haig et al. 2004). Therefore, it is likely that the incidence of hybridization and genomic extinction may be greatly underestimated.

Advances in molecular genetic techniques have greatly improved the ability to identify hybridization. Furthermore, newly developed statistical models are useful for estimating admixture proportions when diagnostic loci are not available for the hybridizing taxa of interest. The performance of these models will continue to improve as the number of loci genotyped and the differences in allele frequency among parental types increase (Davies et al. 1999).

The diagnostic microsatellite loci described here add to the available molecular markers for detecting hybridization. The ability to obtain DNA nonlethally, the relatively high level of polymorphism, and the codominant inheritance pattern make these markers well suited for describing introgression in individuals and populations. Future research should aim to identify diagnostic microsatellite loci for additional trout taxa in order to better understand the threat of introgression to native *O. c. lewisi*.

CHAPTER 3.

Invasion and introgression of non-native rainbow trout genes in native westslope cutthroat trout populations

Abstract: We used diagnostic microsatellite loci and Bayesian admixture analysis to determine the source and invasion pattern of hybrids between native westslope cutthroat trout, *Oncorhynchus clarki lewisi*, and introduced rainbow trout, *O. mykiss*, in the North Fork Flathead River drainage, Montana, U.S.A. *Oncorhynchus mykiss* introgression was detected in 17 of 31 sites and the proportion of *O. mykiss* admixture within hybridized sites showed a significant negative correlation with upstream distance from Abbot Creek. Individuals from this site represent a hybrid swarm with a high proportion of *O. mykiss* admixture (91.6%) and most (85%) of the *O. mykiss* alleles found among hybridized sites were present in Abbot Creek. These results indicate that this site is the ultimate source of introgression in the study area. Evidence for stepping-stone dispersal of *O. mykiss* alleles includes the presence of later-generation backcrosses in populations with low levels of *O. mykiss* admixture. However, the spatial distribution of F_1 hybrids indicates that long distance dispersal of individuals with the highest amounts of *O. mykiss* admixture also contributes significantly to the spread of hybridization. Migration from Abbot Creek is much greater than that expected for *O. c. lewisi*. Introgression of *O. mykiss* alleles in genetically divergent *O. c. lewisi* populations is expected to lead to a loss of inter-population genetic diversity and local adaptation. Management strategies for preserving

nonhybridized *O. c. lewisi* populations should attempt to eradicate populations with high levels of *O. mykiss* admixture in order to reduce further introgression.

Introduction

Invasive species are an increasing threat to biodiversity (Mack et al. 2000; Allen and Flecker 1993). An often underestimated consequence of biotic invasions is the loss of locally adapted genotypes through introgressive hybridization (Rhymer and Simberloff 1996; O'Brien and Mayr 1991). This phenomenon has been well documented in aquatic communities where species introductions are common and widespread (Leary et al. 1995; Miller et al. 1989). Understanding the pattern of invasion and subsequent introgression between introduced and native populations is essential for conserving native fish taxa.

Westslope cutthroat trout, *Oncorhynchus clarki lewisi*, are threatened with genomic extinction due to introgressive hybridization with introduced rainbow trout, *O. mykiss*, and Yellowstone cutthroat trout, *O. c. bouvieri* (Allendorf and Leary 1988). *O. c. lewisi* is genetically highly divergent from other subspecies of cutthroat trout and introgression with other trout taxa leads to the formation of hybrid swarms and the breakup of evolved genotypic combinations (Allendorf and Leary 1988; Allendorf et al. 2004). Consequently, *O. c. lewisi* were petitioned for federal listing as a threatened species (USFWS 2002) and is recognized as a species of special concern by state agencies in its native range.

Most invasive taxa possess some fitness advantage over their native congeners, enabling them to increase in number and range. However, when native and invasive taxa hybridize, the invasive genome may spread despite severe fitness penalties in the hybrid

progeny (Epifanio and Philipp 2001; Wolf et al. 2001). Several authors have tested for selection in *O. c. lewisi* x *O. mykiss* hybrids. Leary et al. (1984) demonstrated that F_1 hybrids between *O. c. lewisi* and *O. mykiss* had reduced growth and survival in a common hatchery environment. However, other studies of *O. mykiss* x *O. clarki* spp. hybrids in the wild (Forbes and Allendorf 1991*a*; Rubidge and Taylor 2004) have failed to find evidence for selection in hybrids, suggesting that these taxa may have minimal genetic incompatibilities.

Alternatively, increased dispersal rates can cause the spread of invasive species. For example, hybridization may prompt invasiveness by disrupting the genetic basis for homing (Ellstrand and Schierenbeck 2000; Hard and Heard 1999; Bams 1976). Native *O. c. lewisi* exhibit significant genetic differentiation over short geographical distances (Leary et al. 1988; Taylor et al. 2003), indicating a low straying rates among populations. In contrast, the rapid spread of hybridization in the Flathead River drainage (Hitt et al. 2003) suggests that hybrids have greater straying rates than native *O. c. lewisi* (Allendorf et al. 2004; 2005). However, some authors have questioned this statement (Campton and Kaeding 2005). High levels of gene flow among populations can lead to genetic homogenization and reduced levels of adaptive divergence among populations (Lenormand 2002). Therefore, understanding the dispersal pattern of hybrids is vital for informing conservation efforts aimed at identifying and eradicating potential sources of introgression.

Biological invasion models have highlighted the importance of short and long distance dispersal in the spread of introduced taxa (Courtenay and Robins 1975). *Oncorhynchus mykiss* introgression may spread via a stepping-stone model in which

invasion is facilitated by dispersal between neighboring populations (Kimura and Weiss 1964). Alternatively, the rapid spread of *O. mykiss* introgression may fit a continent-island model of invasion, whereby hybridization spreads from a common source to satellite populations. The demographic and genetic consequences of invasion under these two models are likely to differ and these differences will have important implications for determining the rate at which hybridization spreads.

We tested the pattern of hybrid invasion by examining the spatial distribution of hybrid genotypes in a region of secondary contact, the North Fork Flathead River drainage, Montana. The objectives of this study were the following: (1) to estimate the amount of gene flow among native populations of *O. c. lewisi* in the North Fork Flathead River, Montana, and (2) to test whether the spread of hybridization is the result of stepping-stone or continent-island patterns of invasion.

Methods

Study area

The Flathead River drainage originates in southwest British Columbia and northwest Montana, encompassing approximately 18400 km^2. The drainage includes the North Fork, Middle Fork, South Fork, and mainstem Flathead Rivers comprising a major portion of the headwaters of the Columbia River basin. The study area includes tributaries to the North Fork Flathead River, a fifth order stream, flowing approximately 160 km south to its confluence with the Middle Fork. The North Fork Flathead River drains an area roughly 4000 km^2, forming the western boundary of Waterton-Glacier International Peace Park (Fig. 3-1).

The Flathead River basin is considered a regional stronghold for *O. c. lewisi* (Liknes and Graham 1988), although long-term persistence of this species is uncertain due to widespread hybridization with introduced *O. mykiss* and, to somewhat a lesser extent, *O. c. bouvieri* (Sage 1993; Hitt et al. 2003; R. Leary, unpublished data). Montana Department of Fish, Wildlife, and Parks (MDFWP) stocking records indicate that over 20 million *O. mykiss* individuals were stocked in the lower elevations of the Flathead River drainage (i.e., Flathead Lake and mainstem Flathead River) beginning in the late 1800's and continuing until 1969. MDFWP shows no record of *O. mykiss* stocking in the North Fork Flathead River. However, unintentional introductions of *O. mykiss* likely occurred from Sekokini Springs, a privately owned *O. mykiss* hatchery. Furthermore, anecdotal evidence indicates that approximately 70000 *O. mykiss* individuals may have been illegally released in the lower reach of the North Fork Flathead River in 1997 when Sekokini Springs ceased operations (B. Marotz, 490 N. Meridian Road, Kalispell, MT 59901, personal communication). Both the MDFWP stockings and the illegal release were predominantly the Arlee strain of *O. mykiss*. Stocking records also indicate the release of *O. c. bouvieri* in several small headwater lakes in the North Fork drainage, including Red Meadow Lake (near site 21; Fig. 3-1).

Fig. 3-1. Study area location (star) and sample site identification. Sample site codes correspond to Table 3-1.

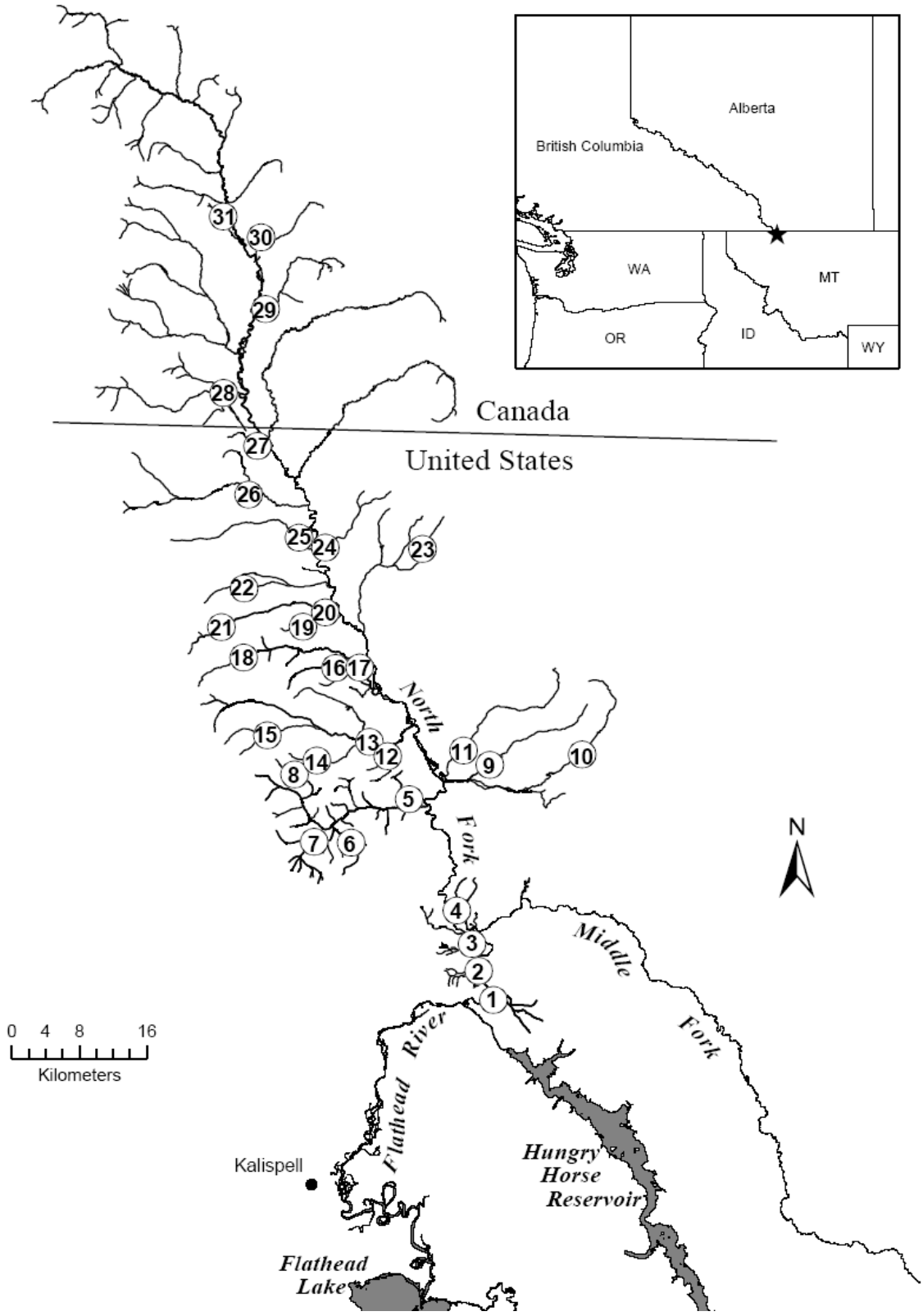


Table 3-1. Sample site information. Sites are coded in approximate order of ascending upstream distance.

Site code	Site name	Sample size	Latitude	Longitude	Sample Year
1	Abbot	34	48.395	-114.041	2004
2	Ivy	20	48.426	-114.068	2004
3	Rabe	30	48.454	-114.081	2004
4	Third	19	48.490	-114.108	2004
5	Langford	30	48.609	-114.196	2004
6	Skookoleel	20	48.557	-114.285	2004
7	Nicola	32	48.556	-114.345	2004
8	Kletomus	32	48.628	-114.383	2004
9	Dutch	32	48.657	-114.070	2004
10	Trout	42	48.669	-113.923	2004
11	Anaconda	31	48.666	-114.113	2004
12	Meadow	25	48.655	-114.235	2004
13	Cyclone	24	48.669	-114.266	2004
14	Deadhorse	22	48.645	-114.348	2004
15	South Fork Coal	26	48.670	-114.431	2004
16	Moran	30	48.747	-114.328	2004
17	Lower Hay	25	48.795	-114.289	2003
18	Upper Hay	24	48.752	-114.476	2003
19	South Fork Red Meadow	26	48.797	-114.376	2004
20	Lower Red Meadow	23	48.808	-114.350	2003
21	Upper Red Meadow	24	48.989	-114.481	2003
22	Moose	30	48.828	-114.483	2004
23	Akokala	32	48.884	-114.199	2004
24	Ford	30	48.878	-114.356	2004
25	Tepee	32	48.887	-114.401	2004
26	Ketchikan	31	48.942	-114.486	2004
27	Colts	25	48.878	-114.356	2003
28	Burnham	25	49.040	-114.536	2003
29	Commerce	25	49.135	-114.477	2003
30	Middlepass	25	49.212	-114.492	2003
31	Parker	20	49.232	-114.556	2003

Previous studies (Hitt et al. 2003; Muhlfeld et al. 2003, 2004) have documented high levels of *O. mykiss* admixture in Abbot Creek (site 1; Fig. 3-1) and a decline in the magnitude of introgression with increasing upstream distance from this site (Hitt et al. 2003). Furthermore, radio telemetry studies, migrant-trapping data, and redd count surveys have reported high densities of *O. c. lewisi* x *O. mykiss* hybrids in this spawning tributary. These findings prompted fisheries biologists to implement a hybrid removal program in this tributary, beginning in 2002, in an effort to eradicate sources of introgression in the upper Flathead River drainage (Muhlfeld et al. 2003, 2004). An accurate description of how introgression spreads is critical for informing future hybrid eradication efforts and for providing a sound biological rationale for removing wild fish from a popular recreational fishery.

Sample collection and DNA extraction

We sampled 31 tributaries to the North Fork Flathead River during 2003 and 2004 (N=847, Table 3-1). Fish were captured by electrofishing or angling (sites 10 and 23) in stream reaches ranging from 250m to 1km to minimize sampling of related individuals. Total length was recorded and a portion of fin tissue was excised and stored in 95% ethanol.

DNA was extracted using the Gentra DNA isolation kit (Gentra Systems, Inc.) and microsatellite loci were amplified in an MJ Research PTC-100 thermocycler using fluorescently labeled primers. PCR products were electrophoresed through 7% polyacrylamide gels and visualized using a Hitachi FMBIO-II fluorescent imager. Allele sizes were determined using MapMarkerLOW size standards (Bio Ventures, Inc.) and

Hitachi FMBIO-II software (MiraiBio, Inc. 1999). Previously scored individuals were included on each gel as controls to ensure consistent allele scoring across populations.

Genetic variation within and among populations

Levels of intra- and inter-population genetic diversity (θ_{IS} and θ_{ST}; Weir and Cockerham 1984) were assessed using programs GENEPOP version 3.1 (Raymond and Rousset 1995) and FSTAT version 2.9.3 (Goudet 1995). Additionally, R_{ST} was calculated to take into account differences in allele size among *O. c. lewisi* populations (Slatkin 1995). Deviations from Hardy-Weinberg proportions were examined using exact tests where P-values were estimated using the Markov Chain algorithm of Guo and Thompson (1992). Genotypic differentiation at each locus and between all *O. c. lewisi* population pairs was assessed with log-likelihood (G)-based exact tests (Goudet et al. 1996) using the default parameters for Markov chain tests in program GENEPOP. Sequential Bonferroni adjustments (Rice 1989) were used to assess statistical significance for simultaneous tests with an initial α level of 0.05.

Mantel tests in FSTAT were used to test for isolation by distance (Wright 1943) among *O. c. lewisi* populations using F_{ST} as a measure of genetic distance and fluvial distance as a measure of geographic distance. Following a significant Mantel test result, we performed a Mantel test on the residuals from the fitted regression line against fluvial distance. At migration-drift equilibrium, residuals are expected to increase with distance as drift becomes the dominant force in determining allele frequency divergence.

Individual and population admixture

It is important to examine genetic data at both the individual and population level to understand how hybridization spreads among populations. Diagnostic microsatellite

loci exhibit codominant Mendelian inheritance, allowing for both the estimation of population level admixture and a description of the distribution of hybrid genotypes among individuals in a population. Furthermore, microsatellites allow for nonlethal DNA sampling and typically exhibit high levels of polymorphism, making this class of genetic marker well suited for evaluating dispersal and patterns of introgression in hybridizing taxa.

We estimated *O. mykiss* admixture in individuals by calculating a hybrid index using 7 diagnostic microsatellite loci (Boyer et al. in prep.). Individuals with no *O. mykiss* alleles have a hybrid index of zero and individuals with two *O. mykiss* alleles at each of the seven diagnostic loci have a hybrid index of fourteen. First generation hybrids between *O. mykiss* and *O. c. lewisi* have a hybrid index of seven and are heterozygous for alleles from the parental taxa at all loci.

We used the admixture model in program STRUCTURE (Pritchard et al. 2000; Chikhi et al. 2001) to estimate population level admixture based on genotypes at all 13 microsatellite loci. This is a Bayesian, Markov chain Monte Carlo (MCMC)-based model that estimates individual admixture proportions (q) and partitions individuals into K groups by minimizing Hardy-Weinberg and gametic disequilibrium within groups. For this study, K was assumed to be 2 and 22 priors (i.e., individuals with a hybrid index score of 14 *O. mykiss* alleles) were used to inform the model to allow for more accurate estimation of individual admixture proportions (q) and their 95% probability intervals (Boyer et al. in prep.). Program STRUCTURE (q) and diagnostic loci produced highly correlated estimates of population level *O. mykiss* admixture; however, estimates of q

account for allele frequency differences between taxa at an additional 6 nondiagnostic loci (Boyer et al. in prep.).

High mutation rates in microsatellites and the high degree of intraspecific genetic variation among *O. c. lewisi* populations increase the likelihood that some populations may possess rare alleles typically characteristic of *O. mykiss* (i.e., homoplasy). We differentiated between homoplasy in *O. c. lewisi* populations and low levels of admixture by examining the number and distribution of diagnostic *O. mykiss* alleles across loci. An appreciable frequency of alleles characteristic of *O. mykiss* at a single locus likely reflects intraspecific genetic variation in *O. c. lewisi* and not evidence of hybridization (Forbes and Allendorf 1991*a*). Alternatively, the presence of more than one diagnostic *O. mykiss* allele in a sample site was assumed to indicate hybridization.

Random mating and gametic equilibrium

From a conservation perspective, it is important to discern between panmictic hybrid swarms and recently hybridized populations. In the latter case, individuals from the native parental taxon still exist and hybrids may reduce further introgression of the invasive genome (Allendorf et al. 2001). Tests for gametic disequilibria (i.e., nonrandom association of genotypes between loci) in hybridized populations were used to discern between hybrid swarms and recent introgression (Forbes and Allendorf 1991*a*). When genetically distinct taxa interbreed, gametic disequilibrium will initially be high. For unlinked loci, disequilibrium will decay by one half each generation under Hardy-Weinberg conditions. The presence of gametic disequilibrium in hybridized populations suggests one or more of the following: (*i*) random mating between the parental types has not been established and that the hybridization event is relatively recent, (*ii*) the loci are

linked, and/or (*iii*) selection is acting on certain genotypic combinations. Alternatively, the absence of statistically significant gametic disequilibrium indicates either recent introgression by post-F_2 individuals (and low power of detection) or that hybridization has been ongoing for many generations. We evaluated gametic disequilibria for all pairwise diagnostic locus comparisons within populations using the Markov Chain method implemented in GENEPOP and assessed significance using sequential Bonferroni-adjusted error rates ($\alpha' = 0.05$/number of pairwise comparisons within a sample; Rice 1989).

We employed an additional method to describe the distribution of hybrid genotypes within a population. We tested for a random distribution of *O. mykiss* alleles among individuals (i.e., hybrid swarm) by comparing the observed frequency of hybrid index scores in a population to a binomial probability distribution with equivalent mean proportion admixture. Significant departures from expected values were assessed with a chi-square test ($\alpha = 0.05$).

Invasion pattern of hybrids

We tested between two *a priori* dispersal models to determine the pattern of *O. mykiss* introgression within the study area. The spatial pattern of introgression is a function of both the type of dispersal and the amount of *O. mykiss* admixture in the migrants. Under a stepping-stone dispersal model (Kimura and Weiss 1964), migration occurs between adjacent populations. This model of genetic invasion predicts a negative correlation between distance from a source population and level of *O. mykiss* admixture, a serial dilution of diagnostic *O. mykiss* alleles from low elevation to higher elevation hybridized sites, and the presence of F_1 hybrids (i.e., gametic disequilibria) in lower

elevation sites. In the stepping-stone model, introgression is expected to spread at a slower rate since individual dispersal distance is shorter and neighboring populations tend to have similar levels of admixture.

Alternatively, a continent-island model assumes an equal probability of dispersal independent of distance from the source population. This model predicts no spatial autocorrelation for *O. mykiss* admixture, diagnostic *O. mykiss* alleles, or the presence of F_1 hybrids. Under this model, the incidence and proportion of *O. mykiss* admixture among populations are expected to increase at a faster rate than they would by stepping-stone dispersal since migrants disperse from a common source with a high proportion of admixture.

Straight-line distances may inaccurately characterize spatial processes in riverine environments. Therefore, fluvial measurements were used to calculate distance between all sample sites using ArcView® Spatial Analyst (ESRI, Redlands, Calif.). Correlations between fluvial distance and proportion of *O. mykiss* introgression were assessed using Pearson's correlation coefficient.

Results

O. c. lewisi *population genetic structure*

No evidence of *O. mykiss* introgression was detected in 14 of 31 sites. Furthermore, power to detect as little as 1% *O. mykiss* introgression was at least 94% and generally exceeded 97% (Boecklen and Howard 1997). Genetic variation within *O. c. lewisi* populations was relatively low. Four of 13 loci analyzed were monomorphic in *O. c. lewisi* populations (Table 3-2) and heterozygosities within populations ranged from

0.188 (site 6) to 0.234 (site 29). Genotypic frequencies generally conformed to expected Hardy-Weinberg proportions. The 8 significant deviations ($P < 0.05$) resulted from excesses and deficiencies of heterozygotes and were close to the amount expected to exceed $\alpha = 0.05$ level by chance alone. Skookoleel Creek (site 6) was the only site that showed a significant overall departure from expected Hardy-Weinberg genotypic proportions ($\alpha = 0.05$). Inbreeding or the presence of multiple populations within the sample may account for the observed deficit of heterozygotes in this population. Based on the relatively low abundance of individuals at this site, we consider the former scenario to be more probable. After correcting for multiple tests (Rice 1989) only Burnham Creek (site 28) deviated from expected Hardy-Weinberg proportions with a significant excess of heterozygotes at *Sfo8*. Small effective population size can cause a significant excess of heterozygotes.

Significant genetic divergence exists between *O. c. lewisi* populations in the North Fork Flathead drainage. Log-likelihood (G)-based exact tests for population differentiation showed significant differences in genotypic frequencies at each of the 9 polymorphic loci ($P < 0.01$) and over all loci combined (Table 3-2; $\theta_{ST} = 0.076$; $\alpha' = 0.05/9 = 0.0056$). Under an island model of migration, a θ_{ST} of 0.076 is equivalent to slightly more than 2 migrants per generation. All pairwise tests for population differentiation were significant at the $\alpha = 0.05$ level and 81 of 91 pair-wise comparisons were significant after correcting for multiple comparisons ($\alpha' = 0.05/9 = 0.0056$). Estimates of R_{ST} were similar to F_{ST}, indicating that most of genetic variance among populations was due to differences in allele frequency and not private alleles.

Mantel tests showed evidence for isolation by distance (i.e., stepping-stone migration) among *O. c. lewisi* populations (Fig. 3-2; $P < 0.001$), but residuals from the fitted regression line showed no correlation with distance ($r^2 = 0.000$). The lack of increase in residuals with distance indicates that *O. c. lewisi* are not in migration-drift equilibrium. This result may be due to recent colonization of streams in the study area following the retreat of glaciation or caused by complex migration patterns that do not follow a simple stepping-stone or island model.

Hybrid populations

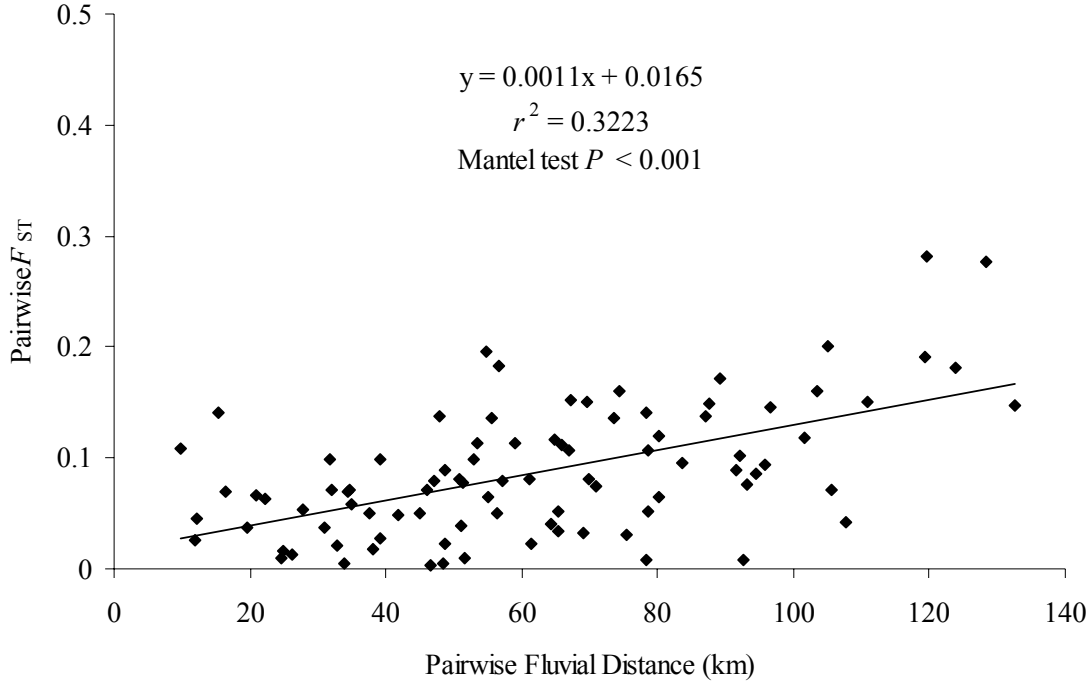
Hybridization was detected in 17 of 31 sample sites and percent *O. mykiss* admixture ranged from 0.27 to 91.6 (Table 3-3). Of the 14 nonhybridized populations, none showed evidence of homoplasy. The occasional presence of an *O. mykiss* allele at a typically diagnostic locus is not uncommon among *O. c. lewisi* populations (Allendorf and Leary 1988; Allendorf et al. 2004). Nevertheless, the lack of evidence for homoplasy increases our certainty in classifying these 14 populations as nonhybridized *O. c. lewisi*.

In addition to *O. mykiss* introgression, we detected alleles that likely indicate *O. c. bouvieri* introgression in the Upper Red Meadow site. Three alleles at *Omy0004* were not found at any other site in the study area and are 26 base pairs larger than the next smallest allele, suggesting they are not *O. c. lewisi* or *O. mykiss* polymorphisms. Furthermore, PINES genetic analysis (Spruell et al. 2001; Kanda et al. 2002) confirmed *O. c. bouvieri* introgression in previous samples collected near this site (Muhlfeld et al. 2003).

Table 3-2. Distribution of genetic diversity in *O. c. lewisi* populations. Loci above the dotted line are diagnostic. Values for F_{IS} and F_{ST} are jackknifed mean and (std. error). Significant values after sequential Bonferroni corrections are marked with an asterisk.

Locus	H_S	F_{IS}	F_{ST}	R_{ST}	# Alleles
Omm1019	0.497	-0.070 (0.035)	*0.072 (0.021)	0.053	4
Omm1050	0.090	-0.040 (0.078)	*0.040 (0.019)	0.038	2
Omm1060	0.061	-0.078 (0.015)	*0.038 (0.014)	0.040	2
Sfo8	0.361	0.010 (0.077)	*0.088 (0.029)	0.091	4
Ogo8	0.033	-0.037 (0.015)	*0.021 (0.012)	0.010	2
Omm1037-1	0.413	-0.076 (0.043)	*0.131 (0.056)	0.137	6
Ssa311	0.143	-0.070 (0.037)	*0.076 (0.044)	0.105	4
Ocl2	0.581	0.002 (0.050)	*0.047 (0.017)	0.062	5
Oneu14	0.582	0.020 (0.052)	*0.072 (0.023)	0.092	15
Overall	0.212	-0.024 (0.019)	*0.076 (0.012)	0.094	

Figure 3-2. Isolation by distance analyses for *O. c. lewisi* populations in the North Fork Flathead River drainage. Pairwise F_{ST} (θ_{ST}) is plotted against pairwise fluvial distance for all populations.



All 13 loci were polymorphic in the hybridized populations (Table 3-4) and heterozygosities within populations ranged from 0.101 (site 10) to 0.648 (site 3). Exact tests for conformity to Hardy-Weinberg proportions revealed a significant deficit of heterozygotes in 9 of 17 (53%) sample sites when analyzing across loci within populations (Table 3-5). Within loci, 28 of 32 (88%) significant departures from Hardy-Weinberg proportions resulted from a heterozygote deficiency (Table 3-5). Significant deficits of heterozygotes in hybridized sites likely indicate the presence of multiple, nonrandomly mating populations within the site. Alternatively, the significant deficit of heterozygotes at *Oneu14* in 6 sample sites (including Abbot Creek) suggests that a null allele may be responsible for the departures from expected Hardy-Weinberg proportions.

Pair-wise tests for gametic disequilibrium at the 7 diagnostic loci within sample sites rejected the null hypothesis of independence in 38 of 357 comparisons ($\alpha' = 0.05/21$ pairwise comparisons within a site = 0.0024). Five of 17 hybridized sites displayed significant gametic disequilibria indicating that hybridization is relatively recent in these populations (Table 3-3). Conversely, Abbot Creek lacked significant gametic disequilibrium, indicating that the hybridization event is relatively old and associations between alleles among loci have largely decayed.

Chi-square tests revealed a nonrandom distribution of *O. mykiss* alleles among individuals in 9 of 17 hybridized sites (Table 3-3), suggesting that these populations are not yet hybrid swarms. However, Abbot Creek appears to be a hybrid swarm since the distribution of hybrid genotypes conformed to binomial expectations. This test confirmed the results of the gametic disequilibrium test and identified nonrandom mating in 4 additional populations. We expect this test to be more sensitive to detecting

nonrandom mating than the pairwise locus test for gametic disequilibria because it tests the distribution of *O. mykiss* alleles across all 7 diagnostic loci simultaneously.

Nevertheless, the power to detect significant departures from binomial genotypic expectations is low in populations with small amounts of *O. mykiss* introgression and failure to reject the null hypothesis of random distribution of *O. mykiss* alleles among individuals does not necessarily indicate that the population represents a hybrid swarm.

Gene flow and genetic drift should have an equivalent affect on all loci. However, selection is expected to affect loci at different intensities. If selection favors alleles from either parental taxon, we would expect diagnostic loci to show higher levels of allele frequency divergence (i.e., F_{ST}) than nondiagnostic loci. Both diagnostic and nondiagnostic loci showed similar levels of genotypic differentiation in hybridized populations (Table 3-4), suggesting that selection on recombinant genotypes may be relatively weak or may act episodically.

Table 3-3. Percent admixture from *O. mykiss* at sample sites. *D* is the number of pair-wise diagnostic locus comparisons in significant gametic disequilibria ($\alpha' = 0.05/21 = 0.0024$). Hybridized populations with a nonrandom association of *O. mykiss* alleles among individuals are marked with an asterisk (*). * = $P < 0.05$; *** = $P < 0.001$. † denotes probable genetic contribution from Yellowstone cutthroat trout (*O. c. bouvieri*).

Code	Site	N	% *O. mykiss*	*D*
1	Abbot	35	91.6	0
2	Ivy	20	49.3	3 ***
3	Rabe	30	49.1	6 ***
4	Third	19	65.8	0 ***
5	Langford	30	33.1	6 ***
7	Nicola	32	1.8	0 *
9	Dutch	32	13.0	0
10	Trout	42	1.0	0
11	Anaconda	31	20.6	21 ***
12	Meadow	25	3.5	0 ***
13	Cyclone	24	11.6	2 ***
15	S.Fk. Coal	26	0.6	0
17	Lower Hay	25	1.4	0
19	S.Fk. Red Mdw.	26	0.3	0
20	Lower Red Mdw.	23	2.2	0
21	Upper Red. Mdw.	24	12.2†	0 ***
25	Tepee	32	1.3	0

Table 3-4. Expected heterozygosities and *F*-statistics for hybridized populations. Values for F_{IS} and F_{ST} are jackknifed mean and (std. error). Number of alleles includes both *O. c. lewisi* and *O. mykiss* alleles. Base-pair size ranges are reported for diagnostic loci above the dotted line. Values judged to be statistically significant after sequential Bonferroni corrections are shown with an asterisk.

					Base-pair size range	
Locus	H_S	F_{IS}	F_{ST}	# Alleles	*O. c. lewisi*	*O. mykiss*
Omm1019	0.622	0.018 (0.025)	*0.212 (0.102)	15	156-168	174-202
Omm1050	0.292	0.045 (0.036)	*0.275 (0.110)	16	230-234	240-360
Omm1060	0.325	0.039 (0.033)	*0.293 (0.105)	5	107-110	98-104
Omy0004	0.284	0.015 (0.041)	*0.295 (0.093)	14	76	130-164
Sfo8	0.448	0.031 (0.028)	*0.221 (0.059)	20	192-204	208-296
Ssa456	0.209	0.089 (0.066)	*0.398 (0.140)	3	159	155-157
Ogo8	0.268	0.137 (0.057)	*0.341 (0.110)	6	92-94	96-102
Omm1037-1	0.568	-0.035 (0.023)	*0.148 (0.049)	16		
Omm1037-2	0.176	0.356 (0.111)	*0.560 (0.187)	3		
Ogo5	0.010	-0.025 (0.012)	*0.020 (0.013)	2		
Ssa311	0.363	0.091 (0.046)	*'0.260 (0.075)	13		
Ocl2	0.660	0.128 (0.060)	*0.134 (0.030)	13		
Oneu14	0.700	0.145 (0.046)	*0.155 (0.051)	19		
Overall	0.379	0.076 (0.024)	*0.230 (0.028)			

Table 3-5. F_{IS} values and deviations from Hardy-Weinberg proportions in hybridized populations.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

			F_{IS}												
Code	Site	N	*Omm1019*	*Omm1050*	*Omm1060*	*Omy0004*	*Sfo8*	*Omm1037-1*	*Omm1037-2*	*Ssa456*	*Ogo8*	*Ogo5*	*Ssa311*	*Ocl2*	*Oneu14*	All
1	Abbot	34	0.053	0.022	0.017	0.031*	-0.024	-0.032	-	0.294	0.135	-	-0.018**	-0.059	0.576***	0.074***
2	Ivy	20	0.194***	0.329***	-0.043	-0.140	0.174*	0.064*	0.321	0.281	0.287**	-0.027	0.133**	0.231	0.227	0.164***
3	Rabe	30	-0.012	-0.027	0.092	-0.094	-0.021*	-0.075	0.740***	0.131	-0.032	-0.018	-0.041	0.078*	0.131*	0.050***
4	Third	19	0.132	0.097	0.236	0.157*	0.207**	0.088	0.297	-0.053	0.452*	-	0.412**	0.564***	0.271*	0.240***
5	Langford	30	0.000	0.060	0.082	0.172	0.057	-0.067	0.114	-0.139	0.068	-	0.105	0.135	0.428***	0.092*
7	Nicola	32	-0.169	-	0.076	-	-	-0.084	-	-	-0.033	-	-0.038	-0.146	0.095	-0.043
9	Dutch	32	0.028	0.129	-0.192	-0.139	-0.022	0.003	-0.088	0.441	-0.054	-	0.119	0.370***	0.028	0.064*
10	Trout	42	-	-	-	-	-0.006	-	-0.025	-	-0.006	-	-	-0.060*	-0.026	-0.038
11	Anaconda	31	-0.048	-0.020	0.085	0.092	-0.071	-0.036	0.437	-0.111	0.286	-	0.129	0.451***	0.184	0.116***
12	Meadow	25	0.160	-0.120	-0.029	-0.011	-0.091	-0.158	-	-0.021	-0.045	-	-0.091	0.170	-0.150	-0.031
13	Cyclone	24	-0.245	-0.040	-0.192	-0.066	-0.025	0.107	0.418	-0.179	-0.100	-	0.189	0.007	0.145	0.009
15	S.Fk. Coal	26	0.025	-0.064	-0.047	-	-	0.096	-	-	1*	-	0.048	0.530*	0.263**	0.208**
17	Lower Hay	25	0.096	-	-0.029	-	0.145	-0.062	-	-	-0.011	-	-	-0.253	-0.016	-0.04
19	S.Fk. Red Mdw.	26	-0.028	-0.020	-0.042	-	-0.084	0.194	-	-	-	-	-	-0.171	-0.078	-0.061
20	Lower Red Mdw.	23	0.155	-0.031	-0.086	-	0.197	-0.341	-	-	-	-	0.148	-0.244*	0.050	-0.037
21	Upper Red. Mdw.	24	0.057	-0.011	0.292	0.229	0.188*	-0.025	0.284	0.130	0.367**	-	0.209	0.395***	0.063	0.173***
25	Tepee	32	-0.045	-	-	-	0.068	-0.123	-	-	-0.051	-	-0.038	0.186*	0.229*	0.063

Spatial patterns of invasion

Populations that were initially invaded by parental *O. mykiss* are likely to be hybrid swarms with a high proportion of admixture. Abbot Creek had the greatest proportion of *O. mykiss* admixture (91.6%) among populations in the study area and genotypes among individuals at this site largely conformed to Hardy-Weinberg proportions. Furthermore, only nine of 58 (15.5%) diagnostic *O. mykiss* alleles found among hybridized populations were absent from the Abbot Creek sample. These results indicate that Abbot Creek likely serves as the ultimate source of *O. mykiss* introgression in the North Fork Flathead River drainage. The nine *O. mykiss* alleles not found in Abbot Creek occurred in hybridized populations within 65 fluvial km from this site and generally occurred at low frequencies. In only one instance did an *O. mykiss* allele not found in Abbot Creek occur in another site at a frequency greater than 0.1 (*Omm1050* * 328 in Third Creek; frequency = 0.18), suggesting that these alleles may be rare and were lost from the Abbot Creek population following removal of adult *O. mykiss*. Nevertheless, these results indicate that Abbot Creek serves as a source of *O. mykiss* introgression in this drainage.

Spatial patterns of population admixture are consistent with a stepping-stone dispersal model. Similar to the findings of Hitt et al. (2003), the proportion of *O. mykiss* admixture showed a strong negative correlation with fluvial distance from Abbot Creek ($P < 0.001$; Fig. 3-3) indicating that introgression is spreading in an upstream direction. Sites with low levels of admixture were located on the periphery and contained individuals classified as later generation backcrosses based on their multilocus genotypes. This finding suggests that either: (1) straying is occurring from populations with low to

moderate levels of *O. mykiss* admixture or (2) long distance dispersal from Abbot Creek has occurred in the past followed by several generations of random mating.

We also found evidence of long distance dispersal of individuals with a high proportion of admixture. The presence of F_1 hybrids in the study area indicates recent invasion of relatively pure *O. mykiss* individuals into *O. c. lewisi* populations. Within the study area, 22 individuals were identified as parental *O. mykiss* (i.e., hybrid index score of 14). Sixteen (73%) of these individuals were found in Abbot Creek (site 1), 5 (23%) were found in Third Creek (site 4), and one (4%) was found in Ivy Creek (site 2). We detected the presence of four F_1's in Anaconda Creek, one F_1 in Ivy Creek, and one F_1 in Third Creek (Fig. 3-4). Matings between *O. c. lewisi* and individuals with a hybrid index score of 11, 12 or 13 (Abbot Cr. N = 16; Ivy Cr. N = 6; Third Cr, N = 5; Rabe Cr. N = 2) can produce offspring that are heterozygous for *O. mykiss* and *O. c. lewisi* alleles at six of seven diagnostic loci. Two of these individuals were found in Cyclone Creek, one in Ivy Creek, and one in Anaconda Creek (Fig. 3-4). Within these populations, no F_1 individuals were siblings and none possessed diagnostic *O. c. lewisi* alleles not found within the population in which they were collected. One of the F_1 individuals in Anaconda Creek possessed an *O. mykiss* allele not found in Abbot Creek (*Omy0004* * 158). Third Creek was the only other population with this allele (frequency = 0.079).

The spatial distribution of hybrid genotypes indicates that long distance dispersal plays a significant role in the spread of introgression in this drainage. Specifically, populations with a high proportion of *O. mykiss* admixture were located furthest downstream and F_1 hybrids were found in 2 populations over 50 km upstream from Abbot Creek. The fact that 65% of individuals with a hybrid index score of 11 or greater

Fig. 3-3. Proportion of population *O. mykiss* admixture (*q*) determined by Bayesian analysis plotted against fluvial distance (km) from Abbot Creek. Quadratic regression line was fit using the least squares method.

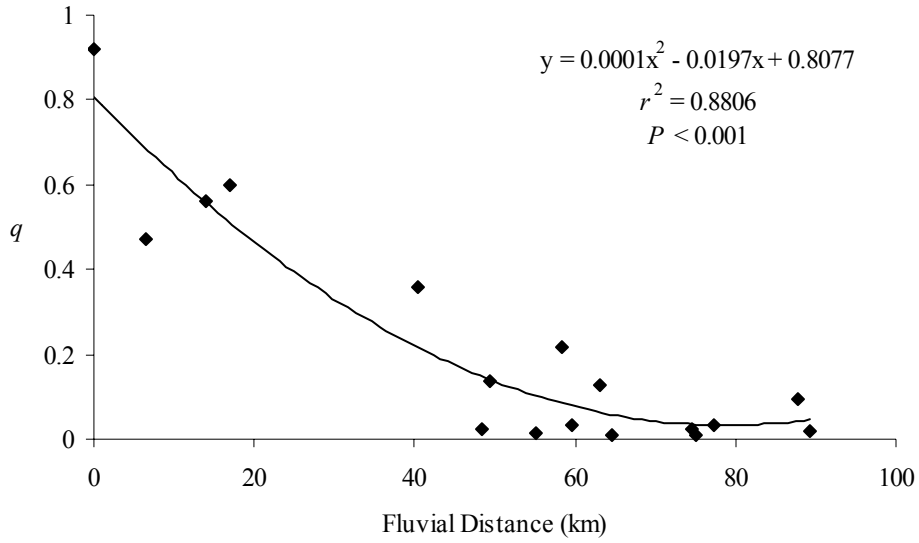
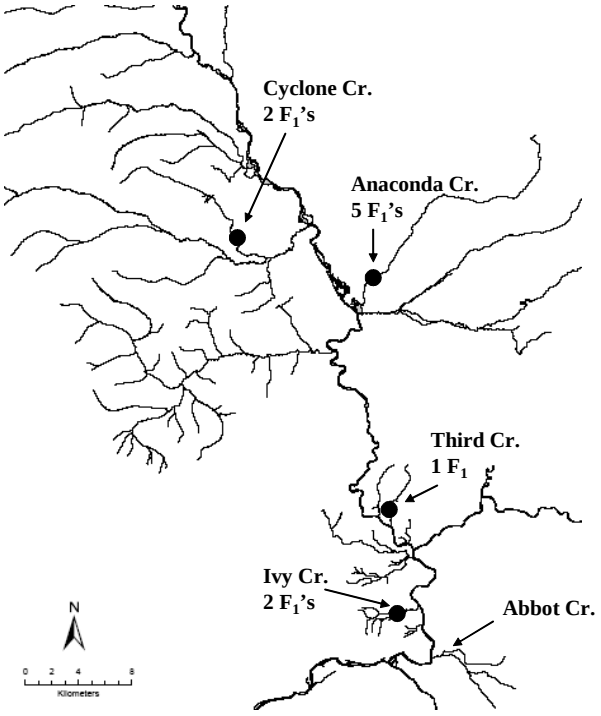


Fig. 3-4. Distribution of F_1 hybrids in the study area. Ivy, Third, and Anaconda Creeks contain F_1 hybrids that are heterozygous for alleles from the parental taxon at all 7 diagnostic loci.



were found in Abbot Creek, suggests that the F_1 hybrids were likely the result of invasion from this population.

Discussion

The location and proportion of *O. mykiss* admixture documented in this study are nearly identical to the findings of Hitt et al. (2003), implying high concordance between microsatellites and PINES for hybrid detection. Similar to PINES, microsatellites allow for nonlethal sampling techniques and the extraction of DNA from small or degraded tissue samples. However, microsatellites have the advantage of codominant Mendelian inheritance and a typically high level of polymorphism, making them ideal for understanding patterns of invasion and hybridization. With microsatellites all genotypes are distinguishable, allowing individuals to be identified by the type of hybrid cross. Furthermore, differences in allele frequencies can be used to draw inferences on selection and dispersal in hybrid zones. Future research should aim to identify diagnostic microsatellite loci for other western trout taxa.

The apparent success of hybrids between *O. c. lewisi* and *O. mykiss* is somewhat of a paradox. Empirical and theoretical studies suggest that hybrids between these taxa experience outbreeding depression (Leary et al. 1995; Templeton 1986). However, hybridized populations are common and introgression seems to be spreading rapidly (Hitt et al. 2003; Rubidge and Taylor 2005), suggesting that extrinsic selection on recombinant genotypes may be relatively weak or imposed episodically (Weins 1977). These results suggest that increased straying by hybrids could influence the spread of hybridization. Our data support this hypothesis and emphasize the importance of dispersal in the spread of hybridization.

O. c. lewisi *population genetic structure*

Significant genotypic divergence among *O. c. lewisi* populations in the North Fork Flathead drainage is consistent with previous studies (Leary et al. 1988; Allendorf and Leary 1988; Taylor et al. 2003) and indicates that dispersal among *O. c. lewisi* populations is relatively rare. Assuming an island model of migration, an overall θ_{ST} of 0.076 is equivalent to slightly more than 2 migrants per generation from any population. However, the assumption of island model migration may not be valid due to evidence of isolation by distance among *O. c. lewisi* populations in the North Fork Flathead drainage. Therefore, 2 migrants per generation is likely an underestimate of the straying rate for native *O. c. lewisi* in this drainage. Nevertheless, the amount of dispersal from Abbot Creek is much greater than we would expect if hybrids had similar dispersal behavior to *O. c. lewisi*.

The spread of introgression among *O. c. lewisi* populations is of conservation concern for several reasons. An increased level of inter-population gene flow from hybrids is expected to homogenize the genetic variability found among populations of *O. c. lewisi*. The geographic range of *O. c. lewisi* is the largest of all cutthroat trout subspecies and many populations contain rare alleles that likely are important for local adaptation. The continued introduction of nonnative trout taxa leads to the homogenization of genetic variation and a reduction in both biodiversity and adaptive potential (Allendorf and Leary 1988).

Another consequence of hybridization is outbreeding depression, due to both the breakup of coadapted gene complexes (Dobzhansky 1970; Carson 1975) and the loss of locally adapted populations (Templeton 1986; Philipp and Whitt 1991). The apparent

success of hybrids between *O. c. lewisi* and *O. mykiss* does not necessarily indicate a lack of outbreeding depression, as fitness consequences may not be readily apparent. Some adaptations found in local populations may only be important during severe environmental disturbances (Weins 1977; Rieman and Clayton 1997).

Invasion pattern of hybrids

Abbot Creek was identified as a major source of introgression in this drainage. The extremely high amount of admixture and lack of allelic associations among loci and individuals indicates that the hybridization event is relatively old and that *O. mykiss* may have initially invaded this site prior to upstream expansion. Furthermore, the proportion of *O. mykiss* admixture in hybridized sites declined significantly with increasing upstream distance from Abbot Creek, suggesting that this population serves as the ultimate source of *O. mykiss* introgression in the North Fork Flathead River. These results are concordant with radio telemetry studies of fluvial hybrid fish that have documented a high incidence of spawning at this site (Muhlfeld et al. 2003, 2004).

The spatial distribution of population admixture and individual hybrid genotypes indicate that both stepping-stone and long distance dispersal contribute to the spread of introgression in the North Fork Flathead River drainage. The distribution of F_1 hybrids in the study area is consistent with a continent-island model of *O. mykiss* invasion. Sixty five percent of the individuals sampled with hybrid index scores of 11 or greater were found in Abbot Creek and only one of 10 F_1 individuals found among hybridized sites had a diagnostic *O. mykiss* allele not present in Abbot Creek. This individual was sampled in Anaconda Creek and had the *Omy0004* * 158 allele found in Third Creek at a frequency of 0.079. The presence of F_1 hybrids in Ivy Creek, Third Creek, Anaconda

Creek, and Cyclone Creek demonstrates that these populations are receiving recent immigrants from one or more source populations with a high percentage *O. mykiss* admixture. Based on these findings we conclude that long distance dispersal from Abbot Creek has contributed substantially to the advancement of introgression in this drainage.

The illegal release of an estimated 70,000 *O. mykiss* individuals in 1997 appears to have played a significant role in the establishment of *O. mykiss* and subsequent spread of introgression in this drainage. In 1984, Huston (1988) sampled 13 sites in the North Fork Flathead drainage and found low levels of introgression in Langford Creek (near site 5) and Moose Creek (near site 22). In this study hybridization was not detected in Moose Creek, however, *O. mykiss* admixture in Langford Creek has increased from 2% in 1984 to 33.1% in 2004. This rapid increase in the spread of *O. mykiss* introgression highlights the importance of propagule pressure in determining the success of biological invasions.

Conservation implications

MDFWP's management plan for restoring native O. c. lewisi includes eradication of nonnative salmonid populations to reduce the threat of introgression. The pattern of introgression found in the North Fork Flathead River drainage indicates that dispersal of individuals with a high degree of O. mykiss admixture from one or more source populations can be a major factor in the spread of hybridization. Our findings suggest that eradication of hybrids from known sources can potentially be an effective management strategy for reducing further introgression.

However, hybrid eradication as a conservation strategy is not without limitations. Eradication of sources of introgression from which the individuals represent a hybrid

swarm is much more straightforward than removal of hybrid individuals from a recently invaded population. The latter scenario will typically require field identification of hybrids. The reliance on morphology for hybrid identification assumes that hybrid phenotypes are intermediate to that of the parental taxa, although this is not always the case (Campton 1987). Furthermore, not all morphological variation has a genetic basis. For example, current Montana fishing regulations in the Flathead River basin call for catch and release of *O. c. lewisi* and define this taxon morphologically by the presence of a red slash on the underside of the throat. However, genetically pure *O. mykiss* in the Kootenai River drainage and elsewhere commonly have red throat slashes.

Protection of *O. c. lewisi* under the Endangered Species Act has been controversial, primarily due to the debate over whether or not to include hybridized populations as *O. c. lewisi* in the unit considered for listing. The principal concern over including hybridized populations as *O. c. lewisi* is the threat of protecting sources of future introgression (Allendorf et al. 2004). The rapid spread of introgression (Hitt et al. 2003) and the pattern of hybrid invasion described in this study suggest that hybrids have dramatically different dispersal behavior than *O. c. lewisi*. In the absence of physical barriers to dispersal the spread of *O. mykiss* introgression will culminate in the genomic extinction of *O. c. lewisi*.

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