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CONSERVATION GENETICS OF SAUGER IN THE UPPER MISSOURI RIVER DRAINAGE

By

DANIEL MICHAEL BINGHAM

B.S., University of Montana, Missoula, Montana, 2009

Thesis

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CONSERVATION GENETICS OF SAUGER IN THE UPPER MISSOURI RIVER DRAINAGE

Chairperson: Fred W. Allendorf

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Hybridization with non-native walleye may play a substantial role in sauger declines throughout the upper Missouri River drainage of Montana and Wyoming. I identified 11 microsatellite loci to detect interspecific hybridization and describe the genetic population structure of sauger. Two major population groupings of sauger were revealed by principal component analysis of allele frequencies. The first consisted of samples from the Missouri and lower Yellowstone River drainages, which showed no evidence for genetic divergence among each other. The second major grouping contained samples from the Bighorn River drainage and the upper Yellowstone River. Samples from the upper Bighorn River drainage were genetically distinct from downstream samples. The Bighorn and upper Yellowstone River samples had substantially lower heterozygosity and allelic richness than the lower Yellowstone and Missouri River samples. Analysis of simulated data sets suggested that 100% of sauger and walleye and 100% of first and second generation hybrids could be correctly identified using these 11 loci. This indicates that my analysis method has the power to discriminate sauger and walleye and to detect hybridization and introgression. I detected only eighteen hybrids out of 925 individuals analyzed. Hybridization appeared recent, as nearly 50% of the hybrids showed significant evidence for having a non-hybrid ancestor within two generations. Only one hybrid was detected in the Missouri River. All others were found in the Yellowstone River drainage, despite a substantially higher rate of walleye stocking in the Missouri River drainage. Environmental conditions in the Yellowstone River drainage may be more conducive to hybridization, or hybrid and walleye survival. The rarity of hybrids, despite massive walleye stocking, is unexpected. Introgression of walleye genes into native sauger does not appear to be an immediate threat. Nevertheless, the presence of hybrids could still be harmful because their production represents wasted reproductive effort. Given my results, I recommend that (1) the transfer of genetically distinct stocks of sauger not take place; (2) historical levels of gene flow among populations be restored; and (3) the walleye fishery in the upper Missouri River drainage be replaced with a sauger or sterile walleye fishery.

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Chapter 1

Introduction

The human domination of Earth's ecosystems is of growing concern in conservation biology (Vitousek et al. 1997). Most troubling are increasing rates of species introductions, habitat destruction, and habitat fragmentation (Mooney and Cleland 2001). These issues act independently or in conjunction with one another to reduce population viability and rapidly drive species to extinction (Rhymer and Simberloff 1996; Mooney and Cleland 2001). Continued loss of biodiversity is likely as human populations increase.

The establishment of non-native species is a common result of ecological destruction. In the United States, introduced taxa are the main threat to 42% of the species on the threatened or endangered species lists and are estimated to cost $120 billion annually in environmental damage (Pimentel et al. 2005). These and similar issues develop when exotics escape the biological constraints of their native habitat and expand rapidly, leading to loss of ecosystem function, niche displacement, competitive exclusion, **hybridization**[1], and extinction (Rhymer and Simberloff 1996; Gordon 1998; Mooney and Cleland 2001). Rahel (2002) has argued that the replacement of native taxa with nonindigenous species is resulting in the homogenization of biodiversity worldwide.

Aquatic systems have been dramatically impacted by exotics (Rahel 2002; Eby et al. 2006). In the United States, this is primarily due to sport fish stocking (Schade and Bonar 2005). Millions of fish are introduced each year by thousands of stocking events in managed fisheries (Hickley 1993). Introductions for angling have led to an estimated one in four individual fish being non-native in streams throughout the western United States (Schade and Bonar 2005). Competition, predation, and hybridization have facilitated the establishment of non-native and stocked fishes, which has led to reductions, extirpations, and extinctions of native fish populations (reviewed by Eby et al. 2006). Further loss of biodiversity due to negative interactions with non-native fishes is likely due to the growing need for fishery supplementation to meet the demands of anglers and the propensity for non-natives to rapidly expand and establish (Eby et al. 2006).

Genetic variation in fishes, an integral component of aquatic biodiversity, is being lost at an alarming rate, largely due to negative interactions with non-natives (Krueger and May 1991; Leary et al. 1995; Scribner et al. 2001). For example, competition, predation, and hybridization commonly result in the contraction of species effective population sizes, extinction of unique populations and species, and homogenization of gene pools (reviewed by Krueger and May 1991). Freshwater fishes are sensitive to genetic impacts due in part to their breeding patterns and the selective pressures they experience in discrete aquatic environments e.g., homing segregates populations leading to the evolution of population-specific adaptations to local

[1] Hybridization reorganizes genomes at the organismic level and reorganizes gene pools at the population level. Consequently, very precise language is needed to avoid confusion when describing its characteristics. Definitions for words in bold appear in the glossary in Table 1-1.

conditions (Garcia de Leaniz et al. 2007). Despite their importance in species' persistence, genetic impacts on native fishes are an often neglected penalty of non-native establishment.

Here, genetic impacts are defined as alterations to the gene pools of indigenous taxa (modified from Krueger and May 1991). Direct genetic impacts typically result from hybridization—that is, interbreeding between native and introduced species (interspecific) or stocks (intraspecific) (e.g., Allendorf et al. 2001). Indirect genetic impacts include the extinction of genetically distinct stocks, loss of genetic variation, and/or inbreeding (Krueger and May 1991). These impacts are the proximate result of predation, competition, disease, fragmentation, and habitat destruction — processes that ultimately result in reduced effective population sizes and shifts in gene flow and selection regimes (Krueger and May 1991; Lande 1998).

From a conservation perspective, the scope of genetic impacts is of considerable scale. Introduced fish, fragmentation, and habitat destruction have impacted many watersheds in the United States. It is, therefore, critical to understand the means by which genetic impacts result in decreased viability of native fishes. Knowledge of two vital genetic issues have become increasingly important in fishery management: (1) knowledge of the threat that hybridization with introduced fishes poses to natives (Allendorf et al. 2001), and (2) an understanding of the genetic population structure of the species being affected by genetic impacts (Laikre et al. 2005).

Hybridization

Hybridization with introduced species is a common result of non-native establishment and threatens the long-term persistence of many indigenous fishes (Leary et al. 1995). In North America, hybridization has been implicated as a major factor in 38% of fish extinctions during the 20th Century (Miller et al. 1989). Understanding the mechanisms by which hybridization reduces fitness is critical in preventing further loss of species.

The threat of hybridization is dependent upon the survival and fertility of hybrid offspring (for a general consideration of hybrids in conservation see Allendorf et al. 2001). Fertile hybrids can act as a vehicle for genomic **introgression**, resulting in fitness penalties due to intrinsic genetic incompatibility and extrinsic disruptions in local adaptation (e.g., gene-by-environment interactions) (Templeton et al. 1986). Despite even heavy fitness consequences, however, introgression can still spread and result in the formation of **hybrid swarms** (Epifanio and Philipp 2001) in which every individual in the population is a hybrid. Hybrid swarms may be of little or no conservation value (Allendorf et al. 2001). Hybridization that produces sterile offspring is not benign either, as it may result in wasted reproductive energy and a competitive advantage for the invasive species (Leary et al. 1993).

Three factors play a critical role in describing the threat of hybridization in a hybrid swarm: (1) sample size, (2) the number of **diagnostic loci** examined, and (3) the minimum **proportion of admixture** considered acceptable in 'non-hybridized' populations. The relationship between these factors can be expressed by the following equation:

$$\alpha = (1 - p)^{2NX},$$

where p is the proportion of admixture, N is the number of individuals in the sample, X is the number of diagnostic loci, and a is the probability of detecting admixture. By increasing both the number of individuals sampled and the number of diagnostic loci examined, investigators garner more power to detect smaller proportions of admixture within populations. Theoretically, the first two factors (sample size and number of diagnostic loci) can be determined by investigators (e.g., collect more individuals and genotype more loci). The third factor, however, is influenced by answers to both biological (e.g., at what proportion of admixture does **outbreeding depression** affect fitness?) and social questions (e.g., at what proportion of admixture do we legally or morphologically consider a fish to be a hybrid?). In the end, determining the appropriate sample size and number or loci depends heavily on both acceptable proportions of admixture and probabilities of error in detecting hybridization.

The equation described in the previous paragraph only applies to populations that are hybrid swarms. Detecting hybridization in a population that contains a mixture of parental and hybrid genotypes is dependent upon the percentage of hybrids in the population and the level of admixture within hybrids. To detect hybridization in populations with few hybrids that contain low admixture (e.g., later generation backcrosses), many individuals need to be sampled and many loci need to be genotyped (Boecklen and Howard 1997). Quantifying the power of hybrid detection in such cases is complicated. Consequently, frequent sampling needs to be conducted to ensure the absence of hybrids.

Finally, to understand the history of hybridization in populations, interpretation of the data must occur at both the individual and population level (Barton and Gale 1993). This is critical because allele frequencies and **admixture** estimates do not adequately describe the history, pattern, or threat of hybridization (Allendorf et al. 2001). For example, both a hybrid swarm with 50% admixture and another population comprised of all F_1s can be described by the same proportion of admixture: $q = 0.50$. Nevertheless, the patterns of hybridization in these populations are distinct. As a result, hybridization is best described by the distribution of hybrid genotypes and by the amount of gametic disequilibrium among alleles at diagnostic and **informative loci** (Allendorf et al. 2001).

Genetic population structure

The genetic population structure of a species describes the distribution of genetic variation within and among populations. This structure is determined by complex, long-term interactions among four evolutionary forces: mutation, natural selection, genetic drift, and gene flow (Chakraborty and Leimar 1987). Knowledge of the genetic structure of a species and the evolutionary relationships among populations is critical for planning and applying management strategies (Chakraborty and Leimar 1987; Allendorf and Leary 1988; Leary et al. 1993; Laikre et al. 2005).

The genetic population structure of a species can be classified into three basic categories: (1) distinct populations, (2) **isolation-by-distance** (IBD), and (3) no differentiation (Laikre et al. 2005). Each category results from differences in the relative strengths of the evolutionary forces mentioned in the previous paragraph. The category in which the species of interest is classified

determines which management strategies will be most effective in conservation and sustainable use planning (Laikre et al. 2005).

Groups of individuals that are genetically homogenous should represent basic management units (Laikre et al. 2005). This is because such groups that have undergone isolation and selective pressures for many generations may contain locally adaptive genetic variation and/or co-adaptive gene complexes (Templeton et al. 1986). In each category of genetic population structure, it is possible to identify genetically homogenous groups (or relatively homogenous in the case of IBD) of individuals that should constitute these management units (Laikre et al. 2005). Biologists will avoid the loss of genetic resources, unique populations, and adaptive alleles by managing species with regard to their genetic population structure.

When adaptive alleles that are unique to a population or area of relative genetic homogeneity are lost, they are essentially gone forever (Allendorf and Leary 1988). This is because they can be recovered only by mutation, which is highly improbable. It is likely that extinction of unique populations, and therefore loss of alleles, will permanently reduce the ability of populations to make adaptive responses to altered environmental conditions (Allendorf and Leary 1988).

Laikre et al. (2005) have stressed that studies should be designed to provide estimates of the different sources of genetic variation over the geographic area examined — that is, among geographical regions and among presumed populations within regions. To do this, studies using genetic markers must take place. Additionally, sampling at spawning sites during the spawning season is necessary to provide information on the species' true genetic structure (Laikre et al. 2005).

Background

Sauger and walleye

Sauger (*Sander canadensis*) and walleye (*Sander vitreus*) are two cool-water species in the family Percidae that co-occur throughout much of the Mississippi, Great Lakes, and Hudson Bay drainages (Scott and Crossman 1979) (Figure 1-1). The western most distribution of sauger occurs in the upper Missouri River drainage of Montana and Wyoming, which historically did not contain walleye (Scott and Crossman 1979; Lee et al. 1980). Walleye stocking in this drainage began in the 1950s, and they are now abundant as over 40 million are stocked annually in over 60 water bodies in Montana (McMahon and Bennett 1996). Although the prairie lakes and reservoirs in which walleye are stocked generally do not support successful reproduction, stocking pressure is high and walleye have established self sustaining fisheries in some locations (McMahon and Bennett 1996). In fact, in the upper Missouri drainage prior to the 1980s, walleye made up less than 0.001% of the combined sauger-walleye catch, increasing to 30% from 1985 to 2005 (Gardner 2005).

The timing and environmental cues for spawning occasionally overlap between sauger and walleye. Spawning times are variable across latitudes and temperatures (Kerr et al. 1997). They begin in the spring, typically between 3.9° and 6.1°C for sauger and between 6.7° and

8.9°C for walleye (Scott and Crossman 1979). For both species, these temperatures coincide with a two-week period between March and early May (Scott and Crossman 1979; Ickes et al. 1999; Jaeger et al. 2005). Males generally arrive first and are followed by the females, which leave soon after spawning (Scott and Crossman 1979). Reproduction occurs at night in 2-12 feet of water (61-366 cm) (Scott and Crossman 1979; Penkal 1992; Kerr 2008). Females are usually attended by one to six smaller males (Lee et al. 1980). Neither species builds a nest or shows territoriality, but both species show a propensity for natal homing (Scott and Crossman 1979; Penkal 1992; Kerr et al. 1997; Jaeger et al. 2005).

There is limited overlap in spawning habitat between sauger and walleye. Occasionally, both species broadcast their spawn over the same shoals in turbid lakes or large rivers (Scott and Crossman 1979; Lee et al. 1980; Penkal 1992). When such sympatric spawning occurs, it is usually over substrate characterized by clean rubble or gravel (Nelson and Walburg 1977; Penkal 1992). However, overlap in spawning habitat is not typical (Rawson and Scholl 1978; Siegwarth 1993; McMahon 1999; Gangl et al. 2000). For example, in the Missouri and Yellowstone drainages of Montana, sauger recruitment takes place in very specific locations associated with bedrock, boulder substrate, and rocky ledges that are resistant to erosion (Gardner and Stewart 1987; Jaeger et al. 2005; Belgraph and Guy 2008). Sauger generally avoid all other habitat types during spawning (Jaeger et al. 2005). Conversely, walleye in these drainages select for pebble, cobble, or gravel substrate while spawning (Penkal 1992).

Hybridization between sauger and walleye

Sauger and walleye hybridize rarely where they are found in natural sympatry (Billington et al. 1997; White et al. 2005). The resulting offspring are apparently fertile (Hearn 1986) and readily backcross with both parental species (Billington et al. 1988; Leary and Allendorf 1997). Hybridization appears more common, however, where (1) walleye and saugeye (female walleye x male sauger) are introduced, (2) both species occur in altered habitat, and/or (3) spawning sites are limited (reviewed by McMahon 1999). Such conditions are pervasive throughout the native range of sauger, and several investigators have documented loss of genetic integrity of native populations due to hybridization (Riegier et al. 1969; Nelson and Walburg 1977; Leary and Allendorf 1997; Billington et al. 2006).

Extensive stocking of walleye and saugeye is probably the most pervasive issue contributing to hybridization between the species. In Montana, Leary and Allendorf (1997) found that hybrids constituted 10% of *Sander* samples in both Fort Peck Reservoir and the Yellowstone River where non-native walleye stocking effort is high (Table 1-2) (Montana Fish, Wildlife & Parks (MFWP) 2009). In a similar study, Billington et al. (2006) found that samples from the Missouri and Yellowstone River drainages consisted of 22% and 10% hybrids, respectively. Graeb (2006) established that samples in Lewis and Clark Lake, South Dakota (where walleye are stocked regularly) contained 21% hybrids. In locations not known to have sauger, Billington et al. (1988) identified 141 walleye using two allozyme loci and found that two of these individuals had sauger mtDNA; they credited this to walleye stocking from locations known to have sauger. Ward (1992) used one diagnostic allozyme locus and discovered that the *Sander* population in Lake Sakakawea, South Dakota consisted of 10% hybrids. Lake Sakakawea has a native population of both sauger and walleye, but the walleye

fishery is regularly supplemented. The use of just one diagnostic allozyme locus, however, undoubtedly makes their estimate conservative. In Normandy Reservoir, Tennessee, where saugeye are stocked extensively, Fiss (1997) found that 75% of individuals in *Sander* samples were hybrids. Likewise, White and Schell (1995) examined samples from the Ohio River and established that saugeye stocking had resulted in a *Sander* population of over 30% post-F_1 hybrids.

Despite pervasive walleye and saugeye stocking across the distribution of sauger, no studies have detected evidence of hybrid swarms, indicating that ecological (pre-mating) or intrinsic (post-mating) barriers to introgression may be present. Acquiring evidence that such barriers to introgression exist, however, has proven difficult due to the availability of only four species diagnostic allozymes. As it stands, introgression appears limited by some mechanism, but its strength and cause are not well understood due to insufficient markers.

Conservation issues

Sauger in the upper Missouri River drainage of Montana and Wyoming persist in only a fraction of their historical range (McMahon and Gardner 2001; Welker et al. 2001). Sauger declines in Montana first became a concern during a region wide drought in the 1980s; however, when precipitation returned to normal in the 1990s, sauger did not return to pre-drought abundances in some areas (McMahon and Gardner 2001). Blame for failed recovery has been placed on loss of habitat and population connectivity due to water development and habitat destruction (McMahon and Gardner 2001). For similar reasons, sauger have declined in Wyoming where they are considered rare or absent in all but one (Bighorn River drainage) of their native river drainages (Welker et al. 2001). Accordingly, sauger are a critically imperiled species of special concern, making them a conservation priority (Carlson 2003).

Hybridization

Hybridization with non-native walleye may be a potentially underestimated factor in sauger declines in the upper Missouri River drainage (Leary and Allendorf 1997; McMahon and Gardner 2001; Billington et al. 2006; Graeb 2006). The impact of hybridization on failed recovery in some locations may be substantial, as hybridization has been documented (Leary and Allendorf 1997; Billington et al. 2006) and conditions favorable to its spread are present: there is overlap with walleye populations during the spawning season (Penkal 1992; Belgraph and Guy 2008); habitat alterations including reservoirs and irrigation infrastructure are common (McMahon and Gardner 2001; Welker et al. 2001); hybrids have been inadvertently stocked as sauger or walleye (Leary and Allendorf 1997; Gardner 2005); and massive walleye stocking occurs within the drainage (Kerr 2008).

The demand for a booming walleye fishery governs much of eastern Montana's reservoir fishery management. In fact, at Fort Peck Reservoir, "rearing walleye…is the facility's top priority" (MFWP 2009). Walleye stocking records for MFWP and Wyoming Game and Fish (WGF) indicate that an average of 45 million are stocked annually in the upper Missouri River drainage (Kerr 2008) (Table 1-2). Over 90% of the walleye stocking in Montana occurs in the Missouri River drainage. The remainder occurs in the Yellowstone River drainage. Alarmingly,

evidence provided by Leary and Allendorf (1997) has indicated that hatchery personnel mistakenly include as much as 5% hybrids in their brood stock collections, suggesting that hybrids are occasionally stocked as walleye.

Walleye stocking also occurs in the Bighorn River drainage of Wyoming. In reservoirs located on the main stem river, walleye stocking occurs in Bighorn Lake and historically took place in Boysen Reservoir (most years between 1952 to 1971 (P. Gerrity, WGF, Lander, WY, personal communication)). Walleye stocking also occurs in Ocean Lake, which is located upstream of Boysen Reservoir, but not on the main stem river. Walleye stocked in Ocean Lake may eventually arrive in Boysen Reservoir through an irrigation return flow canal, but this is probably very rare (P. Gerrity, WGF, Lander, WY, personal communication).

MFWP propagated sauger in the middle Missouri River from 1998 to 2003 (Gardner 2005). The program disbanded, however, partially because of genetic purity concerns. Specifically, several individuals used for spawning in 2003 were identified as definite hybrids using diagnostic allozymes (Gardner 2005). The lots containing these hybrids were discarded; however, given only four diagnostic allozymes were used for screening, hybrids may have been unintentionally released, increasing the possibility for further hybridization in the wild.

Within the upper Missouri River drainage, field studies have detected hybrids in Montana (Leary and Allendorf 1997; Billington et al. 2006), but not in Wyoming (Krueger et al. 1997; Billington et al. 2006). Hybridization appears most common in the Missouri River drainage (samples have consisted of 22% hybrids) and least common in the Yellowstone River drainage (10% hybrids) (Billington et al. 2006). Samples from Montana do not appear to come from hybrid swarms (Leary and Allendorf 1997; Figure 1-2). In contrast, Wyoming is considered a regional stronghold for sauger due to the apparent 'purity' of their stocks. Nevertheless, studies of hybridization in both states have been hampered by two weaknesses: investigators did not objectively target discrete spawning aggregations, and they used, at most, four diagnostic allozyme loci to examine the **dynamics of hybridization** (Krueger et al. 1997; Leary et al. 1997; Billington et al. 2006). Thus, the extent to which hybridization has progressed within breeding groups in the upper Missouri River drainage is not well understood.

Genetic population structure

Little work has taken place on the genetic population structure of sauger across their range. Billington (1996) conducted an RFLP analysis of mtDNA across the native distribution of sauger, walleye, and yellow perch (*Perca flavescens*). Sauger showed very little mtDNA variation with only four haplotypes detected across the species' range, compared to 42 different haplotypes identified in walleye. Although sauger haplotype frequencies among populations showed significant genetic distinction from one another, this study found no geographical structuring. Thus, Billington (1996) suggested that sauger spent the Pleistocene in a single Mississippian glacial refuge.

Little is known about the genetic population structure of sauger in Montana and Wyoming. An allozyme and microsatellite study by Billington et al. (2006) identified the presence of genetically distinct stocks. However, there was a ubiquitous excess of homozygotes

across samples. This is probably the result of a Wahlund effect because individuals were not collected from spawning aggregations and many samples were conglomerates from multiple geographic locations. Thus, an accurate assessment of the true genetic structure of sauger in the upper Missouri River drainage is lacking.

Managers are uncertain of whether genetically distinct stocks of sauger are present in the upper Missouri River drainage. Consequently, they do not know how anthropogenic impacts, non-native competition, predation, and hybridization may affect sauger genetically. Steep population declines in the 1980s, and failed recovery in some locations since then may have led to genetic bottlenecks, and therefore, susceptibility to inbreeding and genetic drift. Competition and predation from non-native fishes may further affect ecological (Eby et al. 2006) and evolutionary (Krueger and May 1991) processes, making knowledge of the genetic population structure of sauger critical for predicting potential outcomes and formulating functional management programs. Finally, inter- and intra-specific hybridization can homogenize the genetic composition of sauger, resulting in outbreeding depression.

Objectives and research questions

To develop a more biologically informed sauger conservation program, a better understanding of their genetic status is needed. The use of allozymes in genetic analysis has two major drawbacks. First, it requires lethal sampling, which is undesirable for a species of special concern. Second, only four diagnostic allozyme loci between sauger and walleye have been identified (Billington et al. 1988), which greatly reduces the power to investigate the dynamics of hybridization. A nonlethal genetic method for hybrid detection, in addition to genetic markers with high allelic diversity, is needed to examine the genetic status of sauger in Montana and Wyoming. The research questions of this study are as follows:

1. What are the patterns of hybridization between sauger and walleye?
    a. How common is hybridization?
    b. Where is hybridization occurring?
    c. What are the patterns of introgression?

2. How is genetic variation distributed within and among sauger populations?

Summary and synthesis

I identified 11 microsatellites that are useful in discriminating sauger, walleye, and their hybrids. Four are diagnostic and the other seven are informative. Analysis of simulated data sets suggested that 100% of sauger and walleye and 100% of F_1, F_2, and first generation backcrosses are correctly identified by program STRUCTURE at $q_i = 0.98$ and 0.02 (q_i is the posterior proportion of an individual's genome with walleye ancestry). This result indicates that my analysis method has the power to (1) discriminate between sauger and walleye, (2) detect interspecific hybrids, and (3) assess levels of introgression.

I examined 925 individuals collected from 22 locations throughout the upper Missouri River drainage. With one exception (Boysen Reservoir, Wyoming), samples represented random

samples of the genus *Sander*. Samples were collected primarily during the spawning season at spawning locations, and therefore, almost undoubtedly represent discrete spawning populations.

Although I found little genetic divergence among samples, two major population groups were revealed by principal component analysis. The first consisted of samples from the Missouri and lower Yellowstone River drainages, which showed no evidence for allele frequency differences among each other. The second major population grouping contained samples from the Bighorn River drainage and the upper Yellowstone River. Within the Bighorn River drainage, samples from upstream locations (Little Wind and Popo Agie Rivers) clustered separately from downstream samples (Bighorn Lake and main stem Bighorn River), indicating the presence of significant genetic structuring. Samples collected from the upper Yellowstone and Bighorn River drainages also displayed reduced genetic variation compared to all other samples. Populations of sauger within these locations are known to be small and isolated, and thus genetic distinctiveness and reduced genetic variation are likely due to genetic drift and lower migration rates rather than long-term isolation.

I detected only eighteen hybrids between sauger and walleye out of 925 individuals analyzed. Hybridization appeared recent, as nearly half of the hybrids showed significant evidence for having a sauger or walleye ancestor within two generations. I found no evidence for hybrid swarms, as hybrids were few and widely scattered across sample locations. Thus, introgression of walleye genes into native sauger does not appear to be an immediate threat. Nevertheless, hybridization could still be harmful to sauger because it represents wasted reproductive effort.

The near absence of hybrids despite massive walleye stocking is unexpected. Surprisingly, nearly all hybrids were found in the Yellowstone River drainage despite a 90% higher rate of stocking in the Missouri River drainage. The Yellowstone is the only major river sampled that does not contain the clear, cold water that is characteristic of tail water habitat. Thus, environmental conditions in the Yellowstone River may be more conducive to hybridization, or to hybrid and walleye survival. Genetic incompatibility could also explain the rarity of hybridization because nearly half (eight of 18) of the sampled hybrids were early generation (e.g., F_1, F_2, BC_1).

Given my results, I conclude that preservation of sauger within the upper Missouri River drainage will require the persistence of many populations in order to retain genetic diversity. I recommend that (1) the transfer of genetically distinct stocks of sauger not take place; (2) historical levels of gene flow among populations be restored; and (3) the walleye fishery in the upper Missouri River drainage be replaced with a native sauger or sterile walleye fishery.

Table 1-1. Glossary. Modified from Allendorf et al. (2001) with additions from Allendorf and Luikart (2007) and Laikre et al. (2005).

Admixture: the production of new genetic combinations in hybrid populations through recombination.

Diagnostic locus: a locus that is fixed or nearly fixed for different alleles in two hybridizing populations.

Dynamics of hybridization: the differential patterns of admixture and introgression that result from the reorganization of genomes at the organismic level and the reorganization of gene pools at the population level.

Hybridization: interbreeding of individuals from genetically distinct populations, regardless of the taxonomic status of the populations.

Hybrid swarm: a population of individuals that all are hybrids by varying numbers of generations of backcrossing with parental types and mating among hybrids.

Informative locus: a locus with substantial allele frequency differences between two hybridizing populations.

Introgression: gene flow between populations whose individuals hybridize.

Isolation-by-distance: a pattern of genetic differentiation in which genetically effective migration among closely located populations is larger than in the case of distinct populations, resulting in successive genetic change over geographic distance.

Outbreeding depression: a reduction in fitness in hybrid individuals relative to the parental types.

Proportion of admixture: the proportion of alleles in a hybrid swarm or individual that comes from each of the hybridizing taxa.

Table 1-2. Number of walleye stocked in Montana by county: 2006-2009. "X" indicates no walleye were stocked that year.

	County	Year			
		2006	**2007**	**2008**	**2009**
1	Bighorn	5,066,072	5,559,793	15,20,672	1,106,337
2	Blaine	13,030	10,168	5,108	X
3	Cascade	5,476	5,023	X	5,565
4	Chouteau	X	X	X	40,068
5	Custer	240	X	X	X
7	Fergus	5,000	X	X	X
8	Garfield	1,000	1,000	121,662	1,000
9	Hill	21,911	115,012	140,716	10,017
10	McCone (Ft. Peck)	X	X	1,941,754	534,380
11	Park	10,000	35,000	8,650	21,000
12	Petroleum	22,000	20,055	20,000	21,528
13	Phillips	19,829	113,909	118,898	8,000
14	Pondera	101,270	X	X	X
15	Richland	40,000	80,000	X	X
16	Rosebud	X	1,000	99,786	1,049
17	Sheridan	752,110	4,937	54,030	50,000
18	Valley (Ft. Peck)	39,302,130	29,186,151	15,824,507	49244171
19	Wibaux	2,000	2,000	2,000	2,000
	Total	45,568,262	35,339,048	18,537,111	51,450,115

Figure 1-1. Historical distribution of sauger and walleye; modified from Scott and Crossman (1979).

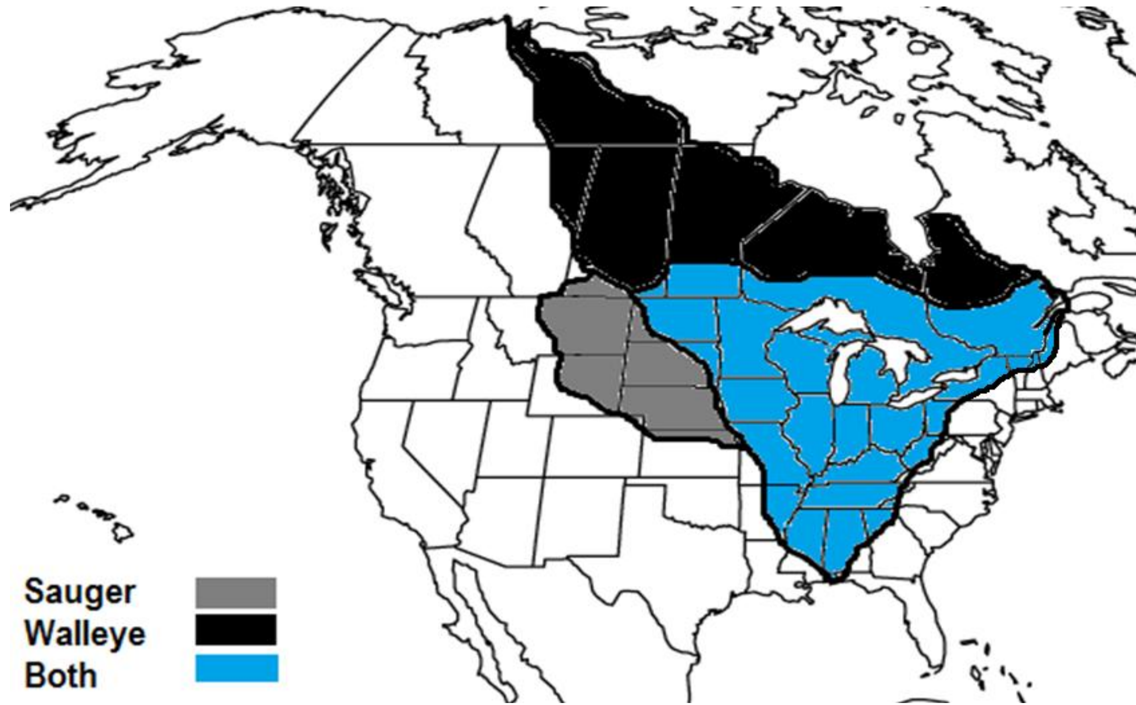
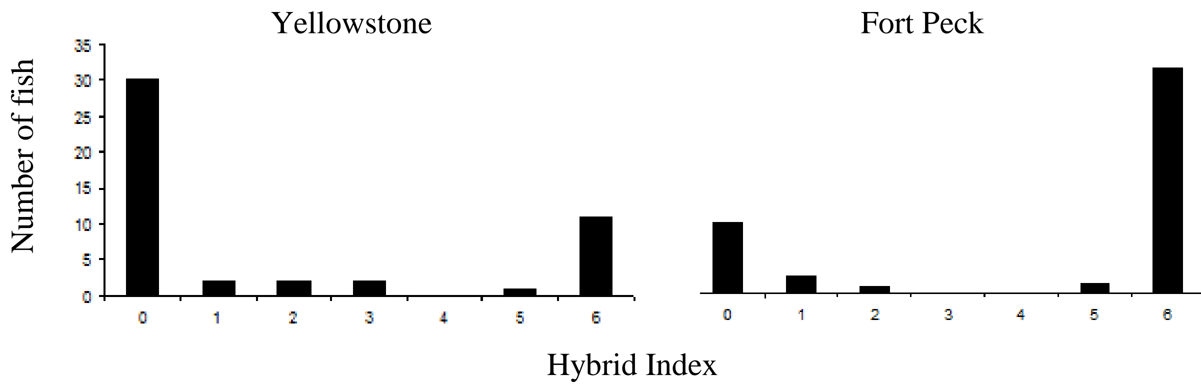


Figure 1-2. Hybrid indices for sauger samples collected from the lower Yellowstone River and Fort Peck Reservoir (Leary and Allendorf 1997).



Chapter 2

Selection of microsatellite loci for detecting hybridization between sauger and walleye using a Bayesian-likelihood model

Abstract

We screened 20 microsatellite loci for usefulness in discriminating sauger, walleye, and their hybrids. Eleven loci reliably amplified in PCR and were optimized in multiplexes. Four of the loci are *diagnostic* (non-overlapping allele sizes between the species) and the other seven are *informative* with substantial allele frequency and size differences between the species. We created simulated data sets in which 100% of sauger and walleye and 100% of F_1, F_2, and first generation backcrosses are correctly identified by program STRUCTURE at $q_i = 0.98$ and 0.02 (q_i is the posterior proportion of an individual's genome with walleye ancestry). This q-value threshold can be considered very conservative because it ensures that hybrid individuals not be classified as parentals. This study shows that assignment tests have the power to (1) discriminate between sauger and walleye, (2) to detect interspecific hybrids, and (3) to assess levels of introgression. Used in conjunction with one another, these diagnostic loci and Bayesian likelihood methods will provide a powerful new analytical framework with which to detect and to describe the dynamics of hybridization between sauger and walleye.

Introduction

Hybridization between individuals of genetically distinct populations is a growing concern in conservation biology (Allendorf et al. 2001). This issue is especially pervasive within freshwater fishes (Leary et al. 1995). In North America, hybridization with introduced species was considered to be the main factor in 38% of fish extinctions during the 20th Century (Miller et al. 1989). Given increasing rates of species introductions, habitat destruction, and habitat fragmentation worldwide, further loss of aquatic biodiversity as a result of hybridization is expected.

Accurate detection of hybrid offspring has a variety of applications in fishery management. For example, non-hybrid individuals are often desired for management programs designed to reestablish populations of extirpated indigenous taxa (Novinger and Rahel 2003). Conversely, hybrids between native and introduced species are at times targeted for removal from populations because they can serve as vehicles for genomic introgression (Allendorf et al. 2001). Given the pervasiveness of artificial supplementation for sport and commercial fishing, biologists often want to know the extent to which hatchery fish contribute genetically to wild populations (Campton 1987; McGinnity et al. 2003; Sanz et al. 2009). On a similar note, estimated proportions of admixture within samples are sometimes used to determine which populations receive legal protection (Allendorf et al. 2004). Whatever the case, accurate classification of the ancestry of an individual or the genetic status of a population has long-term evolutionary, ecological, and at times legal implications.

The advent of protein electrophoresis in the 1960s and of PCR in the 1980s allowed for the discovery of distinctive genetic variation between species, and therefore, the ability to identify individuals of definite hybrid ancestry. When applying these methods, investigators most commonly seek codominant, diagnostic loci for hybrid detection. Diagnostic loci are those that are fixed for different alleles between hybridizing taxa. Such markers allow all genotypes to be distinguished and to assess the probability that parental individuals remain in the population. Additional benefits of diagnostic loci include the ability to determine directionality of hybridization (Scribner et al. 2001) and the ability to identify specific hybrid classes (e.g., F_1, F_2, and backcrosses.) with high power.

Current model-based methods can be used to efficiently identify individuals of hybrid origin. Many different methods are available, but most identify hybrid genotypes based on allele frequency differences between taxa (e.g. BAPS (Corander and Marttinen 2006)) or by minimizing Hardy-Weinberg and linkage disequilibrium within putative populations (e.g., STRUCTURE (Pritchard et al. 2000)). There are three significant advantages of such model-based methods: (1) diagnostic loci between species, populations, and stocks are not necessary; (2) it is possible to use more information from the data compared to approaches that are not explicitly based on genetic models (e.g., Anderson 2009); and (3) many of these programs operate within a Bayesian framework that allows for the use of prior information. As described in Vaha and Primmer (2006), these methods have already been used to identify and discount admixture, to describe hybrid zones, and to detect introgression. For a detailed consideration of model-based methods in hybrid detection see Anderson (2009).

Sauger (*Sander canadensis*) and walleye (*Sander vitreus*) are two congeneric North American perch species, and field studies using diagnostic allozymes and mtDNA have indicated that the species hybridize in nature (e.g., Billington et al. 1988). Their hybrids are apparently fertile (Hearn 1986) and may display heterosis (Malison et al. 1990). Nevertheless, hybridization and introgression appear rare where the species live in native sympatry (Clayton et al. 1973; Todd 1990; Billington et al. 1997). Conversely, hybridization appears more common where (1) walleye and saugeye (female walleye x male sauger) are introduced, (2) both species occur in altered habitat, and/or (3) spawning sites are limited (reviewed by McMahon 1999). Such conditions are pervasive throughout the native range of sauger and several investigations have documented loss of genetic integrity of native populations of sauger (Riegier et al. 1969; Nelson and Walburg 1977; Leary and Allendorf 1997; Billington et al. 2006).

The extent to which introgression threatens sauger has been examined by using, at most, four diagnostic allozymes (occasionally in conjunction with diagnostic mtDNA analysis (e.g., Billington et al. 1988)). This small number of diagnostic loci limits the power of detecting and describing certain characteristics of hybridization that are critical for effective hybrid management. For example, using these four diagnostic loci to examine a sample of 30 individuals from a hybrid swarm containing 1% admixture from walleye leaves investigators with almost a 10% chance of concluding that the population is not a hybrid swarm. Additionally, there is about a 6% chance of concluding that a first generation backcross to sauger is a sauger, which can have serious consequences in supplemental stocking programs. Finally, sauger are considered a species of special concern across much of their native distribution and thus the

lethal sampling required to screen fish at these loci is undesirable. What is needed is a powerful and nonlethal genetic method of detecting hybridization between sauger and walleye.

The objectives of this chapter are to (1) find diagnostic and informative microsatellite loci for hybrid identification between sauger and walleye; and (2) to estimate the power of hybrid detection between the species using simulated hybrid individuals and Bayesian analysis of the multiple locus genotypes in program STRUCTURE (Pritchard et al. 2000).

Materials and methods

Sample collection and DNA extraction

Reference individuals from both species were collected from wild populations to identify distinctive allelic variation at microsatellite loci. To represent the geographic range of the species, reference walleye were obtained from numerous sources, including the Cumberland drainage, Kentucky ($N = 20$); the Muskegon River ($N = 20$) and Lake Gogebic ($N = 10$), Michigan; Lake Mistassini, Quebec ($N = 44$); and Lake Erie ($N = 20$). If samples were collected from areas in which sauger occur, the species identity of each walleye was confirmed by allozyme, microsatellite, and/or mtDNA analysis (R. Leary, Montana Fish, Wildlife & Parks, Helena, MT, personal communication). Reference sauger were collected from the Bighorn River, Wyoming ($N = 50$). Reference sauger were confirmed by morphology (M. Smith, Wyoming Game and Fish, Cody, WY, personal communication) and were collected from locations that had no evidence of introgression from a prior allozyme study (Billington et al. 2006; Leary unpublished data). We randomly selected 40 sauger and walleye reference samples for diagnostic and informative microsatellite analysis.

We extracted DNA from fin clips using a detergent-based cell lysis buffer and ammonium acetate protein precipitation followed by isopropyl alcohol DNA precipitation. DNA was resuspended in 100uL TE buffer, and diluted 1:10 for PCR amplification in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA) using the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, CA). Following the PCR reaction conditions described by the original primer developers, PCR amplification was initially conducted on 20 pairs of percid microsatellite primers (Table 2-1) including those originally developed from walleye: *Svi7*, *Svi2*, *Svi20*, *Svi26* (Eldridge et al. 2002), *Svi* L9, *Svi* L10, and *Svi* L11 (Wirth et al. 1999); yellow perch (*Perca flavescens*): *YP13*, *YP17, YP41*, *YP60*, *YP78, YP80, YP113,* (Li et al. 2007), *Pfla* L1, *Pfla* L2, *Pfla* L4, and *Pfla* L8 (Leclerc et al. 2000); and pike-perch (*Sander lucioperca*): *MSL-1, MSL-2*, and *MSL-9* (Kohlman and Kersten 2008). From these 20 primer pairs, a subset of eleven were used in hybrid analyses (see results; Table 2-2). All multiplex PCR reactions used a total volume of 10ul and followed the QIAGEN Microsatellite protocol. All loci were amplified using a touchdown PCR profile with an initial annealing temperature of 65 or 60°C stepping down -1 or -0.5°C until the bulk of the cycles ran at 45°C (Table 2-2). PCR products were visualized on an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA (ABI)). Allele sizes were determined using the ABI GS600LIZ ladder, (ABI). Electropherogram output was viewed and analyzed using GeneMapper version 3.7 (ABI).

Genetic analysis

Within sauger and walleye, we measured base genetic diversity at each locus as expected heterozygosity (H_e) using FSTAT Ver. 2.9.3 (updated from Goudet 1995). The assumption of the absence of linkage disequilibrium between loci was checked using a log likelihood test (G-test) in which P values were calculated using the Markov Chain algorithm in GENEPOP (Raymond and Rousset 1995) using a dememorization period of 10,000, in 100 batches with 1000 iterations per batch. We tested for allele frequency differences among the species at each locus with log-likelihood-based exact tests (Goudet 1995) using the default parameters for Markov chain tests in GENEPOP. We quantified population differentiation between sauger and walleye in three different ways: (1) F_{ST} (Θ_{ST}; Weir and Cockerham (1984)); (2) R_{ST}, which is an F_{ST} analogue based on microsatellite mutational patterns and allele sizes (Slatkin 1995); and (3) F_{ST}' (Hedrick 2005), a standardized measure of genetic differentiation, which ranges from 0 to 1 for all levels of genetic variation. F_{ST} and R_{ST} were estimated in GENEPOP. An unbiased estimate of F_{ST}' was calculated using the equation described in Meirmans and Hedrick (2011):

$$F'_{ST} = \frac{k(H_T - H_S)}{(kH_T - H_S)(1 - H_S)},$$

where H_T is the total gene diversity, H_S is the mean within population gene diversity, and k is the number of sampled populations, in this case $k = 2$ (sauger and walleye).

Diagnostic and informative microsatellites

We first selected those informative loci that produced easily scorable and reproducible PCR products in both sauger and walleye. We did this by examining the banding patterns of the PCR product on agarose gels following electrophoresis and ethidium bromide staining. Specifically, we eliminated loci that did not produce visible size differences between the species and those that had technical problems, such as failure to amplify in one or both species after two PCRs, complex stutter bands that could potentially complicate the scoring of alleles, or irreproducible banding patterns. For those primer pairs producing acceptable amplification products, our final criterion for selection was successful optimization in multiplex PCR reactions.

Next we distinguished between diagnostic and informative microsatellites. We chose diagnostic loci if they had non-overlapping allele sizes between our walleye and sauger reference samples. We selected informative loci based on the presence of substantial allele frequency differences between the species (e.g., high F_{ST} and bimodal distribution of allele sizes between the species). Although diagnostic loci are more desirable than informative loci, Boyer et al. (submitted) established that Bayesian model-based methods of hybrid detection do not require diagnostic loci for efficient population admixture estimation. Additionally, it is known that power of hybrid detection using Bayesian-likelihood methods increases with the number of loci and the amount of allele frequency divergence between hybridizing taxa (Davies et al. 1999).

Using diagnostic loci, population admixture is estimated as the proportion of diagnostic walleye alleles found among individuals within a sample from the population of interest. Likewise, individual admixture is estimated as the proportion of diagnostic walleye alleles found

within a single fish. For example, first generation hybrids between sauger and walleye will have a proportion of admixture equal to 0.5 and will be heterozygous for alleles from the parental taxa at all diagnostic loci.

Using model-based methods that do not explicitly incorporate fixed differences between species, estimating population and individual admixture is more complicated: population admixture is based on an underlying genetic model. We used the Markov chain Monte Carlo (MCMC) model in program STRUCTURE (Pritchard et al. 2000), in which individuals are assigned to either taxa by minimizing both Hardy-Weinberg and gametic disequilibrium. In STRUCTURE, population admixture is calculated as the arithmetic mean of *q*-values for individuals in a population. This is done by estimating the posterior probability of an individual being assigned to either taxa using allele frequency differences between the species adopting a Bayesian approach:

$$Pr(Z, P|X) \approx Pr(Z)Pr(P)Pr(X|Z, P),$$

where Z is the species of origin, P is the unknown allele frequencies in both taxa, and X is the multilocus genotypes of the sampled individuals (Pritchard et al. 2000).

Power of hybrid detection

We assessed the accuracy of parental assignments using STRUCTURE to estimate the proportion of admixture in simulated sauger and walleye by Bayesian analysis of the multiple locus genotypes. We randomly selected 30 sauger and 30 walleye reference samples to simulate 500 each of both species using HYBRIDLAB (Nielsen et al. 2006). HYBRIDLAB generates genotypes by randomly sampling alleles from parental frequency distributions, assuming random mating, neutrality, and linkage disequilibrium. We replicated both species five separate times and used STRUCTURE to evaluate each replicate. In STRUCTURE we used no prior population information, a burn-in period of 10,000, 50,000 batches and the independent allele frequency (*I*) model. These simulations allowed us to establish a *q*-value threshold that no 'pure' sauger or walleye in an empirical sample is expected to cross.

Next, we assessed the accuracy of hybrid detection using STRUCTURE to estimate the proportion of admixture in simulated hybrid individuals. We randomly selected 30 sauger and 30 walleye from our reference samples to generate 500 of each F_1, F_2, and first-generation backcrosses using HYBRIDLAB (Nielsen et al. 2006). Once again, we replicated each hybrid class five separate times. In STRUCTURE, we used the same settings as described above; however, sauger and walleye were excluded from STRUCTURE runs, as Vaha and Primmer (2006) established that hybrid detection efficiency was only minimally affected by whether reference population allele frequency information was included or not.

Results

Diagnostic and informative microsatellites

We discarded nine loci that did not meet our selection criteria (Table 2-1). *MSL-9* and *Pfla* L2 were polymorphic in both species, but we found no allele frequency differences between species. *YP78* did not produce any product after two attempted PCRs. This was not expected as Li et al. (2007) documented that these primers, developed for yellow perch, successfully amplified in both sauger and walleye. *YP13* and *YP17* produced irreproducible stutter bands on agarose gels and were not selected for further optimization. Likewise, these primers had been optimized for yellow perch, and were successfully cross-amplified in both sauger and walleye (Li et al. 2007). Finally, we were unable to optimize *Pfla* L8, *Svi* L11, *Svi* L9, and *YP80* in multiplex PCR reactions.

The remaining eleven loci were optimized in multiplexes (Table 2-2). Genetic summary statistics for these loci are presented in Table 2-3. Overall, genetic divergence between the taxa was high: $F_{ST} = 0.232$; $R_{ST} = 0.583$; $F_{ST}' = 0.941$. Tests of genetic differentiation at all loci were highly significant (log-likelihood based exact test, $P<0.001$). Four of the eleven loci were diagnostic because they had non-overlapping allele sizes between sauger and walleye (Figure 2-1a). The seven remaining loci were informative because they had substantial allele frequency differences between the species (Figure 2-1b). Of these eleven loci, *Svi20* and *Pfla* L1 showed significant evidence for being in pairwise linkage disequilibrium with each other in our sauger reference samples (G-test, $P<0.001$). However, no pairwise locus comparisons were significant within our walleye sample suggesting this result is likely due to sampling error or small effective population size and not due to physical linkage. The later is likely as Bighorn River sauger populations are known to be small and isolated (Krueger et al. 1997).

All three summary statistics provided a distinct quantification of, and perspective on, genetic differentiation between sauger and walleye. Not surprisingly, R_{ST} (with the exception of *MSL-2*) and F_{ST}' were greater than F_{ST} across loci (Table 2-3). This is expected because the upper limit of F_{ST} can never exceed 1-H_S. The eleven loci were highly polymorphic, and estimates of F_{ST} across all loci were essentially at their maximum values (mean 1- $H_S = 0.25$). In contrast to F_{ST}, R_{ST} takes into account the evolutionary distance between microsatellite alleles. As expected, estimates of R_{ST} were much larger at loci with bimodal allele size distributions (e.g., *MSL-1* and *Pfla* L1; Figure 2-1a and 2-1b) and substantially smaller for those with similar allele sizes (e.g., *MSL-2*; Figure 2-1c). This is likely due to the fact that R_{ST} assumes that each mutation changes the base-pair length of an allele by a single repeat unit. Unlike F_{ST} and R_{ST}, F_{ST}' is standardized by the maximum value it can obtain (1-H_S) and also has the advantage of being equal to 1 when populations do not share any alleles in common. Estimates of F_{ST}' revealed substantial genetic differentiation that had otherwise been masked at loci with high polymorphism and/or overlapping allele size ranges (e.g., *MSL-2*; Figure 2-1c). Additionally, F_{ST}' was equal to 1 for all four diagnostic loci.

MSL-2 provides an example of how at a single locus, any one measure of genetic differentiation does not provide a comprehensive description of the genetic variation present between sauger and walleye. Specifically, vastly different proportions of genetic variation

between the species were explained by the three measures at this locus: $F_{ST} = 0.174$, $R_{ST} = 0$, and $F_{ST}' = 0.939$. F_{ST} at *MSL-2* is essentially at its maximum value, as $1\text{-}H_S = 0.197$. This suggests substantial allele frequency divergence is present between the species. However, R_{ST} is equal to zero, which is likely because the size distribution of walleye alleles at this locus is completely encompassed by that of sauger (Figure 2-1c). Finally, the two species share only three alleles out of 22 total in common; consequently, $F_{ST}' = 0.939$.

Simulation results

The ability to differentiate parental from hybrid genotypes was assessed using simulations and Bayesian admixture analyses in STRUCTURE. Summary statistics for the simulated parental and hybrid genotypes appears in Table 2-4. The proportions of individual admixture in the populations of sauger ranged from 0.001 to 0.013 with a mean of 0.001 (SE<0.001). Conversely, walleye ranged from 0.998 to 0.999 with a mean of 0.999 (SE<0.001). F_1 hybrids ranged from 0.298 to 0.668 with a mean of 0.499 (SE = 0.002). F_2 hybrids ($F_1 \text{x} F_1$) ranged from 0.164 to 0.821 with a mean of 0.5 (SE = 0.002). Finally, first generation backcrosses to sauger ranged from 0.106 to 0.573 with a mean of 0.262 (SE = 0.009) whereas first generation backcrosses to walleye ranged from 0.481 to 0.895 with a mean of 0.735 (SE = 0.006).

Our simulation and STRUCTURE analyses revealed that these eleven loci can distinguish 100% all first and second generation hybrids (F_1, F_2, and backcrosses) from parental genotypes. That is, the q_i-value distributions between the simulated hybrids and parental individuals displayed absolute distinction (Figure 2-2). In particular, no hybrid had a q_i-value greater than 0.90 or less than 0.10 and no sauger or walleye had a q_i-value greater than 0.02 or less than 0.99, respectively.

Thus, analyses of the simulated data set suggest that 100% of F_1, F_2, and backcrosses in our empirical data set using all eleven microsatellites and STRUCTURE with $q_i = 0.02$ and 0.98 could have been identified correctly. This threshold can be considered very conservative because it ensures that hybrid individuals not be classified as parentals (Vaha and Primmer 2006).

Discussion

This study identified four diagnostic microsatellite loci between sauger and walleye and demonstrated that assignment tests have the power to (1) discriminate between the species, (2) to detect interspecific hybrids, and (3) to assess levels of introgression. These two methods of hybrid detection provide a powerful new analytical framework with which to detect and to describe the dynamics of hybridization between sauger and walleye.

Unlike allozyme loci in which diagnostic differences between taxa are due to fixation for alternate alleles at a locus, microsatellite loci are usually polymorphic and diagnostic differences are due to non-overlapping allele sizes between species (Boyer et al. submitted). Consequently, differences identified in one geographic area may not be conserved across the entire range of the species (Spruell et al. 2001). In such cases, individuals may be incorrectly identified as hybrids if they display alleles for the congeneric species at what is considered to be a diagnostic locus.

Boyer et al. (2008) described that in a hybrid swarm, alleles diagnostic of both species are expected to be randomly distributed across loci. Thus, if alleles of one species are much more common at a single locus than others, this is likely evidence for a shared allele between the hybridizing taxa and not evidence for hybridization. Our reference walleye more or less represent the geographic distribution of the species. Our sauger references, however, represent only a small fraction of the sauger distribution in North America. Therefore, it is conceivable that both species share alleles at the four loci we found to be diagnostic.

Using diagnostic loci it is possible to describe admixture using a hybrid index (e.g., Muhlfeld et al. 2009). This index ranges from 0 for sauger (no walleye alleles) to 1 for walleye (two walleye alleles at each locus) and is calculated by summing the total number of diagnostic walleye alleles in an individual and dividing by $2X$ where X is the number of diagnostic loci (in this case four). First generation hybrids between sauger and walleye have a hybrid index of 0.5 and are heterozygous for alleles from the parental taxa at all diagnostic loci. It is assumed that fish with a hybrid index of 0.5 that are not heterozygous for alleles from sauger and walleye at all loci are post-F_1 hybrids. This method of describing hybridization has two critical applications: (1) testing for the presence of hybrid swarms and (2) examining the distribution of hybrid genotypes in a sample.

Statistical power to detect admixture in a hybrid swarm is described by the equation $\alpha = (1 - p)^{2NX}$, where p is the proportion of admixture, N is the number of individuals in the sample, and X is the number of diagnostic loci. Therefore, analyzing four diagnostic loci in a sample size of 30, from a hybrid swarm with 1% genetic contribution from walleye, investigators have about a 90% probability of detection. However, on the individual level, confidence in discerning between non-hybridized parental types and later generation backcrosses requires more diagnostic loci (Boecklen and Howard 1997). Consequently the hybrid index likely overestimates parental types and underestimates the number of individuals with low levels of admixture.

The results of our simulation study support the findings of Boyer et al. (submitted) who established that Bayesian, model-based methods of hybrid detection that do not explicitly account for fixed allelic differences between hybridizing taxa can provide reliable estimates of population admixture. The results of our simulation study indicate that hybrids having a sauger or walleye ancestor within two generations can be identified by STRUCTURE 100% of the time using a q-value threshold of 0.02 and 0.98. Other recent hybridization studies (Beaumont et al. 2001; Barilani et al. 2007) have used a q-value threshold of ≤ 0.10 as a cutoff for defining parental populations with an F_{ST} of ~0.20 (F_{ST} for sauger and walleye = 0.22) because of limited power (number of loci ≤ 8).

Vaha and Primmer (2006) described that when estimating hybrid detection efficiency, an additional factor that needs to be considered is the proportion of hybrids in the sample. Using STRUCTURE and simulated genotypes, they noted a 1.8% reduction in efficiency following a drop from 10% hybrids within samples to 1%. Consequently, they warned that in empirical samples with small proportions of admixture, hybrids may be difficult to distinguish. In our study, similar to Barilani et al. (2007), we did not vary the proportion of hybrids in each run of STRUCTURE: hybrids represented either 0% (e.g., in our estimates of q for parentals) or 100%

of each run (e.g., in our estimates of q for hybrids). Therefore, it is plausible that our q-value thresholds of 0.02 and 0.98 will result in a small proportion of misclassified hybrids and/or parental individuals because most studies have demonstrated that natural hybridization between sauger and walleye is rare (Billington et al. 1988; 1997).

Using STRUCTURE and all eleven microsatellite loci provides a more powerful way to detect hybridization than the four diagnostic microsatellites alone because allele frequency divergence at an additional seven loci is accounted for. Nevertheless, we recommend that researchers apply both methods to examine hybridization between the species: diagnostic loci provide a means to identify individuals of definite hybrid origin, not those that are ‘probably’ hybrids. Additionally, as Boyer et al. (submitted) pointed out, another advantage of model-based admixture estimates is the ability to measure variation around point estimates of individual admixture. This is not possible using diagnostic loci, as hybrid indices are direct counts of alleles.

Table 2-1. Twenty microsatellite loci tested for easily scorable and reproducible PCR products and for diagnostic and informative properties between sauger and walleye. Loci appearing below the dashed line were not used in hybrid analyses for reasons appearing under the column labeled "PCR status".

Locus	PCR status	Primer species	Reference
MSL-1	Optimized	Pike perch	Kohlman and Kersten (2008)
MSL-2	Optimized	Pike perch	Kohlman and Kersten (2008)
Pfla L1	Optimized	Yellow perch	LeClerc et al. (2000)
Svi2	Optimized	Walleye	Eldridge et al. (2002)
Svi20	Optimized	Walleye	Eldridge et al. (2002)
Svi26	Optimized	Walleye	Eldridge et al. (2002)
Svi7	Optimized	Walleye	Eldridge et al. (2002)
Svi L10	Optimized	Walleye	Wirth et al. (1999)
YP113	Optimized	Yellow perch	Li et al. (2007)
YP41	Optimized	Yellow perch	Li et al. (2007)
YP60	Optimized	Yellow perch	Li et al. (2007)
MSL-9	No allele frequency differences	Pike perch	Kohlman and Kersten (2008)
Pfla L2	No allele frequency differences	Yellow perch	LeClerc et al. (2000)
Pfla L8	Unable to optimize	Yellow perch	LeClerc et al. (2000)
Svi L11	Unable to optimize	Walleye	Wirth et al. (1999)
Svi L9	Unreliable in sauger	Walleye	Wirth et al. (1999)
YP13	Irreproducible stutter bands	Yellow perch	Li et al. (2007)
YP17	Irreproducible stutter bands	Yellow perch	Li et al. (2007)
YP78	No amplification	Yellow perch	Li et al. (2007)
YP80	Unable to optimize	Yellow perch	Li et al. (2007)

Table 2-2. Multiplex reaction conditions for the eleven microsatellites used for hybrid detection. Primer sequences are those of the original authors (Table 2-1).

Locus	Touchdown annealing temperature (C°)	Final Primer (um)
Multiplex 1		
*Svi7**	65-45	0.050
*Svi2**	65-45	0.050
*YP41**†	65-45	0.050
Multiplex 2		
Pfla L1†	65-45	0.200
YP60	65-45	0.300
Svi20	65-45	0.100
Svi26	65-45	0.075
MSL-2	65-45	0.150
Multiplex 3		
Svi L10	65-45	0.200
YP113†	65-45	0.200
Single reaction		
*MSL-1**	60-50	0.200

Note: *diagnostic, † a 5' tail containing GTGTCTT was added to reverse primer (Brownstein et al. 1996).

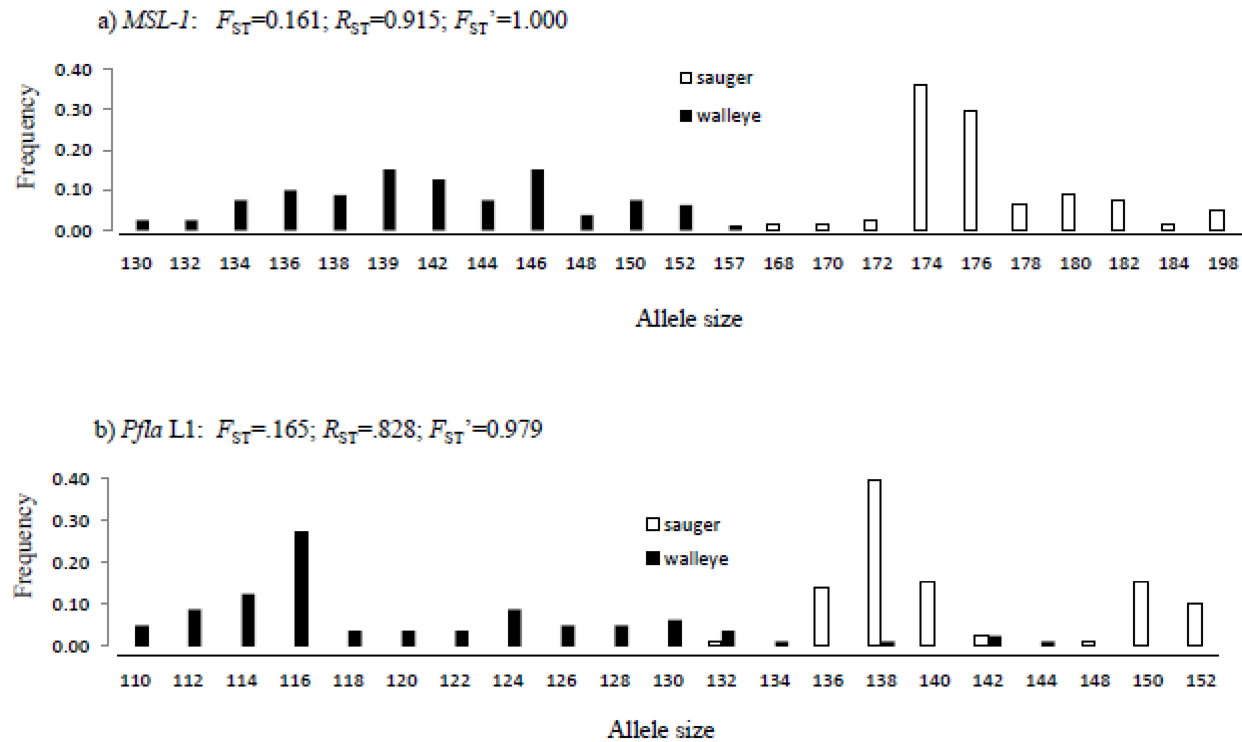
Table 2-3. Genetic summary statistics for sauger and walleye reference samples. Diagnostic loci are above the dashed line and values are sorted by R_{ST} values.

	H_e					Size range (base pairs)	
Locus	sauger	walleye	F_{ST}	F_{ST}'	R_{ST}	sauger	walleye
MSL-1	0.762	0.898	0.161	1.000	0.915	168-198	130-158
Svi2	0.695	0.875	0.291	1.000	0.891	236-258	188-202
Svi7	0.843	0.759	0.189	1.000	0.861	170-210	154-168
YP41	0.000	0.641	0.676	1.000	0.828	172	176-196
YP60	0.588	0.701	0.413	0.971	0.887	190-210	174-194
Pfla L1	0.759	0.798	0.165	0.979	0.828	125-145	103-149
Svi L10	0.867	0.939	0.161	0.923	0.759	186-194	188-246
Svi20	0.799	0.563	0.128	0.837	0.690	167-189	151-181
Svi26	0.734	0.865	0.156	0.914	0.115	151-186	151-188
YP113	0.909	0.945	0.044	0.780	0.110	138-268	138-242
MSL-2	0.802	0.858	0.174	0.939	0.000	140-226	156-196
Mean	0.689	0.804	0.232	0.941	0.583		

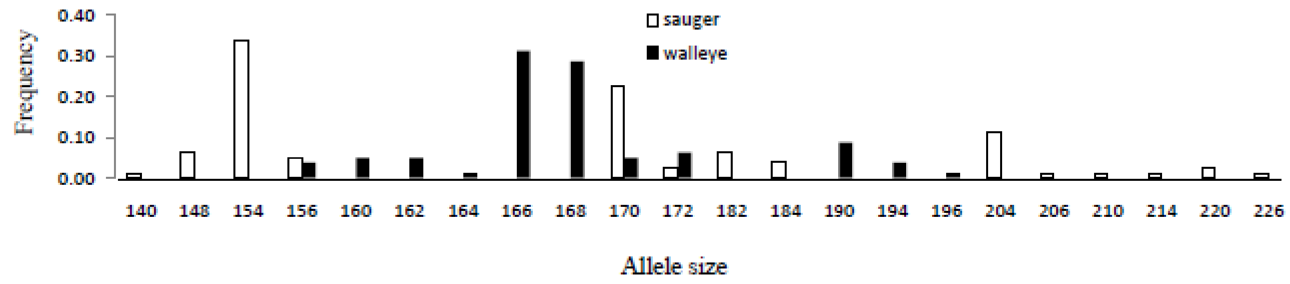
Table 2-4. Summary statistics for the simulated parental and hybrid populations. Values under the columns labeled "*q*" are the average proportions of an individual's genome with walleye ancestry as estimated in STRUCTURE. Values under the columns labeled "min" and "max" are the minimum and maximum q_i-values for individuals within each parental or hybrid simulation replicate. *BCS* = backcross to sauger; *BCW* = backcross to walleye.

Simulation replicate	sauger			*BCS*			F_1			F_2			*BCW*			walleye		
	q	min	max	*q*	min	max	*q*	min	max	*q*	min	max	*q*	min	max	*q*	min	max
1	0.001	0.001	0.013	0.243	0.110	0.550	0.497	0.353	0.640	0.506	0.211	0.770	0.746	0.440	0.890	0.999	0.998	0.999
2	0.001	0.001	0.012	0.240	0.125	0.536	0.505	0.298	0.633	0.495	0.176	0.802	0.749	0.481	0.880	0.999	0.998	0.999
3	0.001	0.001	0.013	0.281	0.106	0.519	0.499	0.331	0.668	0.498	0.179	0.804	0.719	0.427	0.890	0.999	0.997	0.999
4	0.001	0.001	0.013	0.273	0.107	0.523	0.498	0.323	0.657	0.501	0.193	0.821	0.727	0.454	0.890	0.999	0.998	0.999
5	0.001	0.001	0.010	0.275	0.113	0.573	0.504	0.311	0.631	0.498	0.164	0.798	0.732	0.445	0.900	0.999	0.998	0.999
mean (SD)	0.001 (<0.001)			0.262 (0.019)			0.499 (0.003)			0.500 (0.004)			0.735 (0.013)			0.999 (<0.001)		

Figure 2-1. Frequency histograms showing a) non-overlapping allele sizes between sauger and walleye at *MSL-1*; b) partially overlapping allele sizes at *Pfla* L1; and c) the completely encompassed allele size distribution of walleye within sauger at *MSL-2*.



c) *MSL-2*: F_{ST}=0.174; R_{ST}=0.000; F_{ST}'=0.939



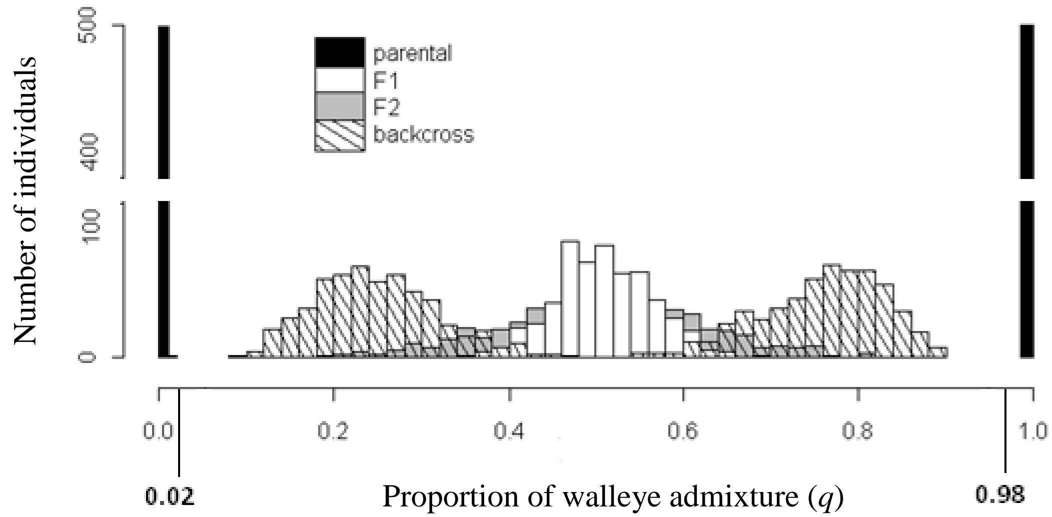


Figure 2-2. Frequencies of simulated parental and hybrid genotypes sorted by *q*-value from the first simulation replicate. The *q*-values represent the probability of an individual being a sauger (*q*-value = 0.0) or walleye (*q*-value = 1.0). The graph portrays the range of frequencies of the STRUCTURE-based *q*-values of the simulated sauger, walleye, F_1, F_2, and backcrossed hybrids generated in HYBRIDLAB. The *q*-value thresholds for hybrid detection of 0.02 and 0.98 are indicated on the graph.

Chapter 3

Genetic population structure of sauger and the pattern of hybridization with non-native walleye in the upper Missouri River drainage

Abstract

We analyzed 11 microsatellite loci to determine the genetic population structure of sauger and describe the pattern of hybridization with widely introduced, non-native walleye in the upper Missouri River drainage. Although we found little genetic divergence among samples, two major population groups were revealed by principal component analysis of allele frequencies. The first consisted of samples from the Missouri and lower Yellowstone River drainages, which showed no evidence for allele frequency differences among themselves. The second major population grouping contained samples from the Bighorn River drainage and the upper Yellowstone River. Within the Bighorn River drainage, samples from upstream locations (Little Wind and Popo Agie Rivers) clustered separately and showed significant allele frequency differentiation from downstream samples (Bighorn Lake and main stem Bighorn River), indicating the presence of genetic structuring. Samples collected from the upper Yellowstone and Bighorn River drainages also displayed reduced genetic variation compared to all other samples. Sauger populations within these locations are known to be small and isolated, and thus genetic distinctiveness and reduced genetic variation are likely due to genetic drift and lower migration rates rather than long-term isolation. We detected only eighteen hybrids between sauger and walleye out of 925 individuals analyzed. Hybridization appeared recent, as nearly half of the hybrids showed significant evidence for having a sauger or walleye ancestor within two generations. We found no evidence for hybrid swarms, as hybrids were few and widely scattered across sample locations. Only one hybrid was detected in the Missouri River, and all others were detected in the largely unaltered Yellowstone River drainage. The near absence of hybrids despite massive walleye stocking is unexpected. Equally surprising is that the majority of hybrids were sampled in the Yellowstone River drainage despite a 90% higher rate of stocking in the Missouri River drainage. This might be due to environmental conditions in the Yellowstone drainage that are more conducive to hybridization, or to hybrid and walleye survival. Introgression of walleye genes into native sauger does not appear to be an immediate threat and might be due to selection against hybrids. Nevertheless, the presence of hybrids could still be harmful because their production represents wasted reproductive effort. Given our results, we recommend that (1) the transfer of genetically distinct stocks of sauger not take place; (2) historical levels of gene flow among populations be restored; and (3) the walleye fishery in the upper Missouri River drainage be replaced with a native sauger or sterile walleye fishery.

Introduction

Management for productive sport fisheries has dramatically altered freshwater ecosystems worldwide (Miller et al. 1989; Hickley 1993; Cowx 1994; Eby et al. 2006). Each year millions of individuals are introduced by thousands of stocking events (Hickley 1993), often without regard for the potential establishment of invasive species (Boyer et al. 2008), the extinction of native taxa (Miller et al. 1989; Rhymer and Simberloff 1996), and the loss of genetic integrity of native fishes (Araki and Schmidt 2010). Such loss of aquatic biodiversity is often the result of

hybridization between native and introduced fish (Leary et al. 1995; Scribner et al. 2001). Given the likelihood of a continued and growing need for supplementation, further loss of species as a result of hybridization is expected.

The threat of anthropogenic hybridization is dependent upon the survival and fertility of hybrid offspring (for a general consideration of hybrids in conservation, see Allendorf et al. 2001). Fertile hybrids can act as a vehicle for genomic introgression, resulting in fitness penalties due to intrinsic genetic incompatibility and extrinsic disruptions in local adaptation (e.g., gene-by-environment interactions) (Templeton et al. 1986). Despite even heavy fitness consequences, however, introgression can still spread and result in the formation of hybrid swarms (Epifanio and Philipp 2001) in which every individual in the population is a hybrid. Hybrid swarms may be of little or no conservation value (Allendorf et al. 2001). Likewise, hybridization that produces sterile offspring is not benign, as it may result in wasted reproductive energy and a competitive advantage for the invasive species (Leary et al. 1993).

Sauger (*Sander canadensis*) are a highly migratory, cool-water fish species in the family Percidae that occur in large, turbid river systems throughout much of the Mississippi, Great Lakes, and Hudson Bay drainages (Scott and Crossman 1979). Historically, sauger were considered to be the most widely distributed percid species in North America (Lionberger 2006); however, populations have declined across a large portion of their range primarily due to habitat destruction and fragmentation (Caruful 1963; Nelson and Walburg 1977; Hesse 1994; Pegg et al. 1997; McMahon and Gardner 2001). A potentially underestimated factor in sauger declines, however, may be introgressive hybridization with the widely introduced, congeneric walleye (*Sander vitreus*).

Despite apparent F_1 fertility (Hearn 1986), allozyme studies have provided evidence that hybridization and introgression are rare where sauger and walleye live together in native sympatry (Clayton et al. 1973; Todd 1990; Billington et al. 1997). Conversely, hybridization appears more common where (1) walleye and saugeye (female walleye x male sauger) are introduced, (2) both species occur in altered habitat, and/or (3) spawning sites are limited (reviewed by McMahon 1999). Such conditions are pervasive throughout the native range of sauger, and several investigators have documented loss of genetic integrity of native populations due to hybridization (Riegier et al. 1969; Nelson and Walburg 1977; Leary and Allendorf 1997; Billington et al. 2006). Curiously, however, no studies have detected evidence of hybrid swarms between walleye and sauger, indicating that ecological (pre-mating) or intrinsic (post-mating) barriers to introgression may be present.

Two barriers potentially limiting the extent to which introgression proceeds include temporal and spatial differences during the spring spawning season and genetic incompatibility. Acquiring evidence that such barriers to introgression exist, however, has proven difficult due to the availability of only four species diagnostic allozymes. Generally, during the spring spawning season, sauger tend to spawn later and in different locations than walleye (Rawson and Scholl 1978; Siegwarth 1993; McMahon 1999; Gangl et al. 2000). However, overlap in timing (Collette et al. 1977) and location (Medlin 1990) is known. Genetic barriers do not seem as obvious. Culture experiments have revealed that when reared in hatchery ponds, second-generation hybrids (F_2 and backcrosses) demonstrate high survival (Hearn 1986). Nevertheless,

post-F_1 hybrids are rare in the wild (Billington et al. 1988; but see Fiss et al. 1997) and their reproductive capabilities have not been studied. As it stands, introgression appears limited by some mechanism, but its strength is not well understood due to insufficient markers.

In order to determine the threat that hybridization with widely stocked non-native walleye poses to native sauger, we examined the spatial distribution of hybrid genotypes in the upper Missouri River drainage of Montana and Wyoming. Sauger in this location are a critically imperiled species of special concern (Carlson 2003) and are thought to persist in only a fraction of their historical range (McMahon and Gardner 2001; Welker et al. 2001). Significant sauger declines have resulted from loss of habitat and population connectivity due to water development and habitat destruction (McMahon and Gardner 2001). The impact of hybridization, however, may also be substantial as its presence has been documented (Leary and Allendorf 1997; Billington et al. 2006) and conditions favorable to its spread are present: there is widespread overlap with walleye populations during the spawning season (Penkal 1992; Belgraph and Guy 2008), habitat alterations including reservoirs and irrigation infrastructure are common (McMahon and Gardner 2001; Welker et al. 2001), hybrids have been inadvertently stocked as sauger and walleye (Leary and Allendorf 1997; Gardner 2005), and massive walleye stocking occurs within the drainage (Kerr 2008).

Walleye stocking records for Montana Fish Wildlife & Parks (MFWP) and Wyoming Game and Fish (WGF) indicate that an average of 45 million are stocked annually in the upper Missouri River drainage (Kerr 2008). Over 90% of the walleye stocking in Montana occurs in the Missouri River drainage. The remainder occurs in the Yellowstone River drainage. Alarmingly, evidence provided by Leary and Allendorf (1997) has indicated that hatchery personnel in Montana mistakenly may include 5% hybrids in their brood stock collections, suggesting that hybrids are occasionally stocked as walleye. In reservoirs impounding the main stem Bighorn River in Wyoming, walleye stocking occurs in Bighorn Lake and historically took place in Boysen Reservoir (most years between 1952 to 1971 (P. Gerrity, WGF, Lander, WY, personal communication)). Walleye stocking in the Bighorn River drainage also occurs in Ocean Lake, which is located upstream of Boysen Reservoir, but not on the main stem river. Walleye stocked in Ocean Lake may eventually end up in Boysen Reservoir through an irrigation return flow canal, but this is probably very rare (P. Gerrity, WGF, Lander, WY, personal communication).

Within the upper Missouri River drainage, hybrids have been detected in Montana (Leary and Allendorf 1997; Billington et al. 2006), but not in Wyoming (Krueger et al. 1997; Billington et al. 2006). Hybridization appears most common in the lower Yellowstone drainage (samples have consisted of 22% hybrids) and least common the Missouri River drainage (10% hybrids) (Billington et al. 2006). In contrast, Wyoming is considered a regional stronghold for sauger due to the apparent 'purity' of their stocks. Nevertheless, studies of hybridization in both states have been hampered by two weaknesses: investigators did not purposefully target discrete spawning aggregations, and they used, at most, four diagnostic allozyme loci to examine the dynamics of hybridization (Krueger et al. 1997; Leary et al. 1997; Billington et al. 2006). Thus, the extent to which hybridization has progressed within breeding groups in the upper Missouri River drainage is not well understood.

The objectives of this study were to describe the genetic population structure of sauger and determine the pattern of hybridization with non-native walleye in the upper Missouri River drainage of Montana. To describe the pattern of hybridization we asked three questions: How common is hybridization? Where is hybridization occurring? What are the patterns of genomic introgression?

Materials and methods

Study area

The upper Missouri River drainage originates in western Montana and Wyoming and forms a major portion of the greater Mississippi river basin, encompassing approximately 190,000 km^2. The drainage can be broken down into three subsidiary river basins — the Missouri, Yellowstone, and Bighorn. Our study area included these three main stem rivers and several tributaries and reservoirs (Figure 1-1).

The upper Missouri River drainage represents the western extent of the native distribution of sauger in North America. Sauger stocks within the drainage may be of increased conservation value due to their peripheral location in the species' range (Lesica and Allendorf 1995). In fact, Scott and Crossman (1979) have suggested that sauger within the drainage should be classified as a distinct subspecies (*S.c. boreum*) due to their atypical morphology (e.g., distinctive spotting and numbers of pectoral and dorsal rays).

Sample collection

MFWP and WGF personnel sampled 21 locations throughout the upper Missouri River drainage during April 2009 to October 2010 (*N* = 925, Table 3-1). Samples were collected by electrofishing or trammel netting. MFWP and WGF personnel randomly sampled individuals that morphologically represented the genus *Sander* (e.g., sauger, walleye, and their hybrids). An exception to this sampling method was sample 19 (Boysen Reservoir) in which only individuals that morphologically resembled sauger were collected. A small piece of fin tissue was excised and stored dry or in 95% ethanol. In Montana, 713 out of 724 total individuals were collected during or within seven days of the spawning aggregation time period (March 15-May 15; Jaeger et al. 2005). Therefore, a majority of our samples from Montana almost undoubtedly represent individuals collected from discrete spawning groups. Collection of the 213 individuals from the Bighorn drainage of Wyoming ranged in time period from April to October. Thus, there is a possibility they contained individuals from multiple spawning groups.

Controls—Individuals from both species were collected from wild populations to identify distinctive allelic variation at microsatellite loci. The original source of stocked walleye in the upper Missouri River drainage is unknown, and previous walleye brood from Montana's state hatchery at Ft. Peck have contained hybrids (Leary and Allendorf 1997). Therefore, reference walleye were obtained from numerous sources to represent the geographic range of walleye including the Cumberland drainage, Kentucky (*N* = 20); the Muskegon River (*N* = 20) and Lake Gogebic (*N* = 10), Michigan; Lake Mistassini, Quebec (*N* = 44); and Lake Erie (*N* = 20). Sauger controls were collected from the Bighorn River, Wyoming (*N* = 50) to represent populations

native to the upper Missouri River drainage. The species identity of each walleye and sauger was confirmed by morphology, allozyme, microsatellite, and/or mtDNA analysis (L. Bernatchez, Université Laval Québec, Québec City, Québec; K. Scribner, Michigan State University, East Lansing, MI; M. White; Ohio University, Athens, OH; R. Leary, MFWP, Helena, MT, personal communications) if samples were collected from areas in which sauger occur. We randomly selected 40 walleye: Lake Erie (16), Lake Gogebic (7), Muskegon River (10), and Lake Mistassini (7); and sauger as reference samples and examined these individuals for potentially diagnostic microsatellite markers.

Genetic markers

We performed microsatellite PCR amplification on our reference samples to identify markers useful for species and hybrid identification (see Chapter 2). Our criteria for selecting loci were (1) PCR amplifications that produce easily scorable and reproducible products; (2) loci that show non-overlapping allele sizes between our walleye and sauger controls (diagnostic); or (3) loci that display substantial allele frequency differences between the species (informative). Diagnostic loci can be used to identify individuals of definite hybrid origin and to help identify hybrid categories (e.g., F_1, backcross). Both informative and diagnostic loci can be used to identify admixture using model-based methods that mimic the inheritance of genes and the sampling of individuals (e.g., Pritchard et al. 2000). We searched for our criteria on an initial suite of 20 pairs of microsatellite primers including those originally developed from walleye: *Svi7*, *Svi2*, *Svi20*, *Svi26* (Eldridge et al. 2002), *Svi* L9*, Svi* L10, and *Svi* L11 (Wirth et al. 1999); yellow perch (*Perca flavescens*): *YP13*, *YP17, YP41*, *YP60*, *YP78, YP80, YP113,* (Li et al. 2007), *Pfla* L1, *Pfla* L2, *Pfla* L4*,* and *Pfla* L8 (Leclerc et al. 2000); and pike-perch (*Sander lucioperca*): *MSL-1, MSL-2*, and *MSL-9* (Kohlman and Kersten 2008).

Genetic analysis

We extracted DNA from fin clips using a detergent-based cell lysis buffer and ammonium acetate protein precipitation followed by isopropyl alcohol DNA precipitation. DNA was resuspended in 100uL TE buffer, and diluted 1:10 for PCR amplification in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA) using the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, CA). All multiplex PCR reactions (Table 3-2) used a total volume of 10ul and followed the QIAGEN Microsatellite protocol.

From our initial suite of 20, we amplified eleven microsatellite loci that met our diagnostic and informative criteria described above (Table 3-2): *Svi7*, *Svi2*, *Svi20*, *Svi26*, *Svi* L10, *YP41*, *YP60*, *YP113, Pfla* L1, *MSL-2*, and *MSL-1*. All loci were amplified using a touchdown PCR profile with an initial annealing temperature of 65 or 60°C stepping down -1 or -0.5°C until the bulk of the cycles ran at 45°C (Table 3-2). PCR products were visualized on an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA (ABI)). Allele sizes were determined using the ABI GS600LIZ ladder, (ABI). Electropherogram output was viewed and analyzed using GeneMapper version 3.7 (ABI).

Genetic variation—We measured base genetic diversity as expected heterozygosity (H_e) and allelic richness (R_A) using FSTAT Ver. 2.9.3 (updated from Goudet 1995). We tested for

departure from Hardy-Weinberg proportions using exact tests in which *P* values were calculated using the Markov Chain algorithm of Guo and Thompson (1992). We quantified departures of observed and expected heterozygosity using Wright's (1951) F_{IS} in FSTAT. We tested for allele frequency differences among samples with log-likelihood-based exact tests (Goudet 1995) using the default parameters for Markov chain tests in GENEPOP (Raymond and Rousset 1995). After removing samples that contained fewer than ten individuals (samples 14, 15, and 19), we quantified pairwise population differentiation using F_{ST} (Θ_{ST} ; Weir and Cockerham (1984)) in FSTAT and visualized these differences using principal component analysis of the pairwise covariance matrix of allele frequencies using GenAlEx Ver. 6 (Peakall and Smouse 2006).

Hybridization—We used the Bayesian clustering model in STRUCTURE Ver. 2.3.3 (Pritchard et al. 2000; Falush et al. 2003) to identify admixture based on genotypes at eleven microsatellite loci. STRUCTURE gives a *q*-value for each individual, which represents the proportion of an individual's genotype that was derived from walleye ($q = 1$). We used a burn-in period of 10,000, 50,000 batches and the admixture and *I* model. We forced the model to recognize only two populations ($k = 2$; i.e., sauger and walleye) and included the 40 sauger and 40 walleye used in diagnostic microsatellite identification as controls to assess the accuracy of parental assignments (e.g., Schwartz and Beheregaray 2008). However, we did not designate these individuals as priors, as Vaha and Primmer (2006) showed that the inclusion of priors minimally affects Bayesian-based assignment when the F_{ST} between taxa is ≥ 0.12 (F_{ST} between our sauger and walleye controls = 0.22). STRUCTURE was run five separate times and gave very similar results (maximum standard deviation = 0.002). Population admixture proportions obtained from program STRUCTURE (*q*) were highly correlated ($r^2 > 0.99$) with admixture estimates from the four diagnostic loci; however, estimates of *q* from STRUCTURE account for allele frequency differences at an additional seven informative loci.

If hybridization has not progressed to a hybrid swarm, it may still be possible to identify non-hybrid individuals for conservation (Allendorf et al. 2001). To test for recent hybridization, we followed a similar procedure to that of Schwartz and Beheregaray (2008), and ran STRUCTURE a second time defining each fish as belonging to one of the two species based on their q_i-value from the first run of STRUCTURE. That is, individuals with q_i-values < 0.5 were grouped with sauger and >0.5 were grouped with walleye. We used this prior information to run STRUCTURE ($K = 2$) with the generation option set to 2, and other settings as described above. This specifically tested each individual for having an ancestor of the other species in the past two generations (Pritchard et al. 2000), thus indicating a recent hybridization event. Following a significant test ($P<0.05$) for ancestry within two generations, we used genotypic patterns at the four species diagnostic loci to place hybrids into specific hybrid classes (e.g., F_1, F_2, or backcross). For example, a hybrid with a q_i-value ≈ 0.25 that also shows significant evidence for having a walleye ancestor in the past two generations would be considered a first generation backcross to sauger if it was homozygous for sauger alleles at some diagnostic loci and heterozygous for both species' alleles at other diagnostic loci.

Results

Genetic variation within and among samples

Genetic summary statistics for sauger are presented in Table 3-1. All eleven loci were polymorphic. Expected heterozygosity across samples averaged 0.758 and ranged from 0.697 in the Bighorn River (sample 18) to 0.787 in the Tongue River (sample 13). Mean allelic richness was 6.0 and ranged from 4.87 in the Bighorn River to 6.44 in the Tongue River. Genotypes from individuals at Fallon (sample 9), Calypso Bridge (sample 10), and Bighorn Lake (sample 17) did not conform to Hardy-Weinberg proportions (HWP) after correcting for multiple tests (P<a' = 0.05/11 = 0.004), with an apparent deficit of heterozygotes at all sites. No single locus contained genotypes that were consistently out of HWP across populations.

Samples from the Bighorn River drainage and the upper Yellowstone River (samples 16 - 21) showed reduced genetic variation compared to samples from the Missouri and lower Yellowstone River drainages (Figure 3-2). Within the Bighorn River drainage and upper Yellowstone River, median expected heterozygosity was 0.720 and allelic richness was 5.410, compared to 0.773 (Wilcoxon rank-sum test; W = 21, $P < 0.001$) and 6.265 (W = 21, $P < 0.001$) within the Missouri and lower Yellowstone River drainages. Additionally, the *YP41* locus was monomorphic within the Bighorn River drainage and the upper Yellowstone River.

Genetic differentiation among samples was low (global $F_{ST} = 0.009$, 95% CI 0.006-0.013) (Table 3-3). Nevertheless, those from the Bighorn River drainage and the upper Yellowstone River showed several significant pairwise tests of differentiation among each other and all other samples (log-likelihood based exact test, $P<0.05/11 = 0.0045$). Allele frequency divergence among these populations, though significant, was low; the maximum pairwise F_{ST} value was 0.039 between samples 13 and 17. Interestingly, samples collected from the Missouri and lower Yellowstone River drainages showed no significant pairwise tests of genetic differentiation ($P>0.05/11 = 0.0045$) despite over 800 river km of separation.

Three major population groupings are evident in the plot of the first two components of the PCA (Figure 3-3). The first component (PC1), which accounts for 63% of the variation, clearly clusters the Missouri and lower Yellowstone River samples and also separates them from samples collected in the Bighorn and upper Yellowstone River drainages. PC2, which accounts for 19% of the total variation, separates the Little Wind and Popo Agie samples from those collected in the Bighorn River and Bighorn Lake. The PCA analysis also indicates that sample 16 from the upper Yellowstone River is genetically more similar to samples from the Bighorn drainage than to samples from the lower Yellowstone drainage. This lends evidence to the presence of a genetic break between sauger located upstream and downstream of the confluence of the Bighorn and Yellowstone Rivers.

Hybridization

In general, hybridization was rare: 18 of 925 individuals were hybrids, 848 were sauger, and 61 were walleye. The q_i-values for hybrids ranged from 0.026 to 0.517 with a maximum standard deviation of 0.002. Hybrids were identified by STRUCTURE in ten of 21 samples (Table 3-1).

Hybridization was most common in the Yellowstone River drainage, which contained seventeen of eighteen hybrids. Within the Yellowstone River drainage, samples 7, 8, 11, 15, and 16 contained one hybrid each; whereas, samples 6 and 10 contained two, sample 13 contained three, and sample 9 contained five hybrids. The Missouri River drainage contained only one hybrid, in sample 2 about 80 km upstream of Ft. Peck Reservoir. No evidence for hybridization was found in the Bighorn River drainage.

Walleye were found in three of 21 samples (Table 3-1). No walleye were sampled in the Missouri River drainage; however, only those individuals that morphologically resembled sauger were collected. The mouth of the Tongue River (sample 13) in the Yellowstone River drainage contained seven walleye, the highest number in the drainage. The only other walleye in the Yellowstone River drainage was sampled at Forsyth (sample 15). In the Bighorn River drainage, Bighorn Lake (sample 17) contained 50 walleye and was the only sample in the entire data set to contain more walleye than sauger.

We used the second run of STRUCTURE to identify the specific genealogical classes (e.g., F_1, F_2, backcrosses) of recently hybridized individuals (Table 3-4). Three F_1s were discovered ($P < 0.001$), one each in samples 6, 8, and 13. These individuals had q_i-values = 0.492, 0.517 and 0.501 and were heterozygous for walleye and sauger alleles at all four diagnostic loci. One F_2 (F_1xF_1) was identified ($P<0.001$) in sample 13. This individual had a q_i-value = 0.472 and was heterozygous for walleye and sauger alleles at three diagnostic loci but homozygous for sauger alleles at one diagnostic locus. Four first generation backcrosses to sauger were identified ($P<0.001$), one in sample 2, one in sample 6, and two in sample 9. These fish had q_i-values between 0.103 and 0.235 and were homozygous for sauger alleles at some diagnostic loci and heterozygous for sauger and walleye alleles at the other diagnostic loci.

The remaining ten hybrids did not show significant evidence for having a non-hybrid ancestor in the past two generations ($P>0.05$). These individuals all contained q_i-values that ranged from 0.026 to 0.098 indicating they are likely later generation backcrosses to sauger. No hybrids were identified as backcrosses towards walleye.

Discussion

Hybridization with widely introduced walleye has been proposed as a threat facing native stocks of sauger (White and Schell 1995; Leary and Allendorf 1997; White et al. 2005; Billington et al. 2006). Of particular concern is loss of unique behavioral, genetic, and ecological adaptations due to the introgression of genes from walleye. Previous investigations have provided evidence that rates of hybridization and introgression increase where walleye or saugeye are stocked, where both species co-occur in altered environments, where spawning sites are limited, and/or where sauger numbers fall to low levels (reviewed by McMahon 1999). Here, we present evidence that contrasts this paradigm. Our Bayesian analysis of multiple-locus microsatellite genotypes revealed a near absence of hybridization between sauger and widely stocked, non-native walleye despite the presence of conditions considered favorable to hybridization. Below we discuss potential reasons for the apparent rarity of hybridization.

Different spawning locations

Limited overlap in spawning habitat between sauger and walleye likely contribute to the apparent rarity of hybridization. For example, in the Missouri and Yellowstone drainages, sauger recruitment occurs in very specific locations associated with bedrock, boulder substrate, and rocky ledges that are resistant to erosion (Gardner and Stewart 1987; Jaeger et al. 2005; Belgraph and Guy 2008). Sauger generally avoid all other habitat types during spawning (Jaeger et al. 2005). Conversely, walleye in these drainages typically select for pebble, cobble, or gravel substrate while spawning (Penkal 1992).

Besides preferring different habitat, the two species are found spawning in different geographic locations. For example, in the Wyoming section of Bighorn Lake (sample 17), walleye spawning is known to take place near the confluence of the Bighorn River, whereas sauger are more often seen spawning at multiple upstream sites as far as 65 km from this area (M. Smith, WGF, Cody, WY personal communication). Similarly, in the Missouri River below Fort Peck Dam, sauger spawn in the Milk River, the lower Missouri , and likely downstream of Fort Peck Dam, but walleye are not common in any of these areas during the spawning season (T. Haddix, MFWP, Helena, MT personal communication). Above Ft. Peck Dam in the Missouri River drainage, sauger often spawn in the Marias River (Gardner and Stewart 1988; Penkal 1992; McMahon 1999; but see Gangl et al. 2000). Although walleye occasionally spawn here, walleye spawning is more common near Fred Robinson Bridge on the main stem Missouri (W. Gardner, MFWP, Helena, MT personal communication), in Highwood and Belt Creeks, and in the Big Dry arm of Ft. Peck Reservoir (Colby and Hunter 1989). In the Yellowstone River drainage, sauger recruitment most commonly occurs in the main stem river from below the mouth of the Tongue River, continuously, to below Intake diversion (Jaeger et al. 2005). Historically, spawning was also common in the Tongue and Powder Rivers (Penkal 1992). Conversely, walleye do not commonly spawn in the Yellowstone River upstream of Intake diversion in locations of known sauger spawning (Penkal 1992). Recently, however, walleye have been seen spawning in the Tongue River (M. Backes, MFWP, Helena, MT personal communication).

Investigations outside of our study area have also shown that where both species are found together, there is little overlap in spawning habitat (Rawson and Scholl 1978; Siegwarth 1993; McMahon 1999; Gangl et al. 2000). Segregation seems apparent during the spawning season in the upper Missouri river drainage, but it is probably not absolute. For example, our samples from spawning aggregations in the Tongue River (sample 13) and near the mouth of the Powder River (samples 9, 10, and 11) contained several hybrids and ripe walleye. Likewise, both species occasionally spawn in close proximity throughout the main stem Yellowstone River (Penkal 1992; Jaeger et al. 2005), and spawning migrations are of the same direction, distance, and timing in the Missouri River (Belgraph and Guy 2008).

Walleye spawning habitat within the upper Missouri River drainage may be of low quality. Within the Bighorn River and Boysen Reservoir, Wyoming, Krueger et al. (1997) found no evidence for hybridization and attributed this to poor walleye reproduction. Likewise, natural walleye recruitment is also rare in Bighorn Lake, Wyoming (M. Smith, WGF, Cody, WY personal communication) and our study found no hybridization in this location. In the

Yellowstone River drainage, anecdotal evidence suggests that spawning habitat may be a limiting factor for walleye recruitment (M. Ruggles, MFWP, Helena, MT personal communication). The fact that walleye historically had access to the upper Missouri River drainage but did not establish (Hoagstrom and Berry 2010) lends further evidence to the argument that sufficient spawning habitat is not present.

Selection and genetic incompatibility

Natural selection against walleye and hybrids could also be a factor in infrequent hybridization. Hybrids were rare or absent in the Missouri and Bighorn samples, respectively, but seventeen hybrids were found in the Yellowstone River drainage. Of these three rivers, the Yellowstone is the only one that does not contain clear, cold water characteristic of tail water habitat. In a study examining walleye and hybrid ecology and performance in Ohio, Johnson et al. (1988) reported that walleye typically have not produced good tail water fisheries. The study also found that hybrids have diet (e.g., benthic forage fishes) and habitat preferences (e.g., finer substrates and turbidity) more typical of sauger. Thus, the Yellowstone drainage may simply provide better walleye and hybrid habitat. In similar fashion, it is also plausible that walleye and hybrids are traveling in search of suitable habitat, which the Yellowstone drainage may provide. This seems reasonable as both species have been recorded as making long seasonal migrations in our study area (250 to 300 km) (Jaeger et al. 2005; Belgraph and Guy 2008).

The fact that nearly half (eight of 18) of our sampled hybrids were early generation (e.g., F_1, F_2, BC_1) despite over 60 years of walleye stocking indicates that genetic incompatibility between the species may be present. Although survival and reproduction of post-F_1 hybrids has been minimally studied, there is published evidence supporting genetic barriers. For instance, under intensive culture conditions, a study by Malison et al. (1990) demonstrated that hybrids of walleye females and sauger males showed significantly greater weight gain, length gain, and condition factors than the reciprocal hybrid, not accounting for maternal effects. Literature on post-F_1 hybrid fertility and performance, however, is scarce. Hearn (1986) found that second-generation hybrids (F_2 and backcrosses) survived for at least 16 months after stocking in a managed hatchery pond in Kentucky, but reproduction and long-term survival was not documented. Likewise, in an observational field study, Fiss et al. (1997) found that F_1 hybrids were reproducing with themselves and walleye but the extent to which backcrossed and F_2 hybrids survived and reproduced was not recorded. A majority of the hybrids in our study (ten of eighteen) were post second-generation lending evidence that recombinant hybrids are to some extent capable of reproducing.

Absence of 'saugeye' stocking

The highest frequency of hybridization between sauger and walleye reported in the literature occurs where female walleye X male sauger hybrids (saugeye) are stocked (White and Schell 1995; Fiss et al. 1997; White et al. 2005). This is likely because saugeye generally do not show reduced fertility or fitness. Furthermore, they are artificially propagated, so any natural barriers to reproduction between the species are avoided. Saugeye are not intentionally stocked in Montana and Wyoming, which may be another reason why hybridization is apparently infrequent.

Threat of hybridization

Studies have shown that introgression can proceed towards the formation of hybrid swarms even in the presence of severe fitness penalties in hybrid offspring (Epifanio and Philipp 2001; Muhlfeld et al. 2009). Therefore, given enough time and walleye propagule pressure, introgression may eventually result in hybrid swarms in the upper Missouri River drainage. From a short term perspective, however, the threat that hybridization poses for sauger may be wasted reproductive effort rather than genetic introgression. Leary et al. (1993) described that this situation is similar to heterozygote disadvantage whereby hybrids have a fitness value near zero and parentals a relative fitness near one. They explain that the more numerous species will have an advantage because less of their total reproductive effort will be wasted in hybrid production. In the upper Missouri River drainage, walleye reproduction is independent of the species' abundance because walleye are stocked. Thus, when hybridization does occur the wasted reproduction is more harmful to sauger, potentially giving walleye a competitive advantage.

Genetic population structure

Our PCA and pairwise F_{ST} analyses revealed genetic divergence among sauger populations. In particular, populations from the Bighorn River drainage and the upper Yellowstone River displayed significant allele frequency differences among each other and all other samples. Within the Bighorn River drainage, samples from upstream locations (e.g., the Little Wind (sample 20) and Popo Agie (21)) clustered together, as did samples from downstream locations (e.g., Bighorn Lake (17) and the mainstem Bighorn River (18)). This likely indicates limited gene flow among populations located in these areas. Curiously, sample 16, from the upper Yellowstone River clusters more closely to samples located upstream in the Bighorn River drainage than to any other samples. Biologically, this makes little sense and may be an artifact of sampling error. Whatever the case, our results indicate the presence of genetic structuring and divergence of sauger populations located above the confluence of the Bighorn and Yellowstone Rivers.

Further evidence for relative isolation of sauger populations from the Bighorn River drainage and upper Yellowstone Rivers is provided by our finding of reduced genetic variation (allelic richness (R_A) and expected heterozygosity (H_e)) within these populations. This likely indicates reduced gene flow with downstream populations and/or genetic drift within populations. Both explanations are plausible, as samples from the Bighorn River drainage were collected from populations known to be isolated from the other sample sites by a significant stretch that is thought to not contain sauger (Billington et al. 2006). Regarding genetic drift, sauger populations from the Bighorn River drainage are known to be relatively small and isolated (Krueger et al. 1997; Welker et al. 2001).

Sauger from the Missouri and lower Yellowstone River drainages appear to come from one genetically panmictic population. This is an unexpected result, as over such great distances (~800 river km), river fishes are typically distributed into genetically distinct populations (Chakraborty and Leimar 1987; Ward et al. 1994). However, sauger are known to make spawning migrations as far as 300 km (Jaeger et al. 2005), indicating a propensity for genetic

mixing over long distances. The apparent lack of structure raises concern because Ft. Peck Dam represents a complete barrier to upstream fish migration. Thus, historically high levels of gene flow from the lower Missouri and Yellowstone Rivers to the Missouri River above Ft. Peck dam are probably now absent.

Microsatellites are presumably neutral so inference on adaptive differentiation is entirely speculative. Regardless, our finding of genetic distinctiveness of sauger in upstream locations may increase the likelihood of local adaptation and increased conservation value. For example, samples 20 and 21 from the Little Wind and Popo Agie Rivers do not come from typical sauger habitat. In particular, sauger habitat in these locations is high in elevation (1,435 m to over 1,500 m), contains steep gradients, and is characterized by relatively clearer, colder discharge (Amadio et al. 2005). It is known that sauger biomass is positively associated with the availability of deep, low-gradient pools and high summer water temperature and turbidity (e.g., Amadio et al. 2005). Such features are presumably limiting in these locations and thus environmental selective pressures may be strong.

Similarly, the absence of genetic divergence at neutral microsatellites among populations in the Missouri and lower Yellowstone River drainages of Montana does not necessarily indicate the absence of adaptive differences among populations. It is possible that historically high numbers of successful spawners (e.g., large N_e) reduced the effects of genetic drift, thereby slowing the rate at which separate populations diverge genetically at neutral markers.

In summary, based on our survey of genetic variation within and among populations of sauger, we conclude that any one population will not represent the range of genetic diversity contained within the evolutionary lineage of this species. Preservation of the species will, therefore, require the persistence of many populations in order to retain genetic diversity of sauger. Given the presence of genetically distinct sauger populations, stock transfer is not recommended. Managers might also consider prioritizing conservation of both the upper Yellowstone and Bighorn populations due to their genetic distinctiveness.

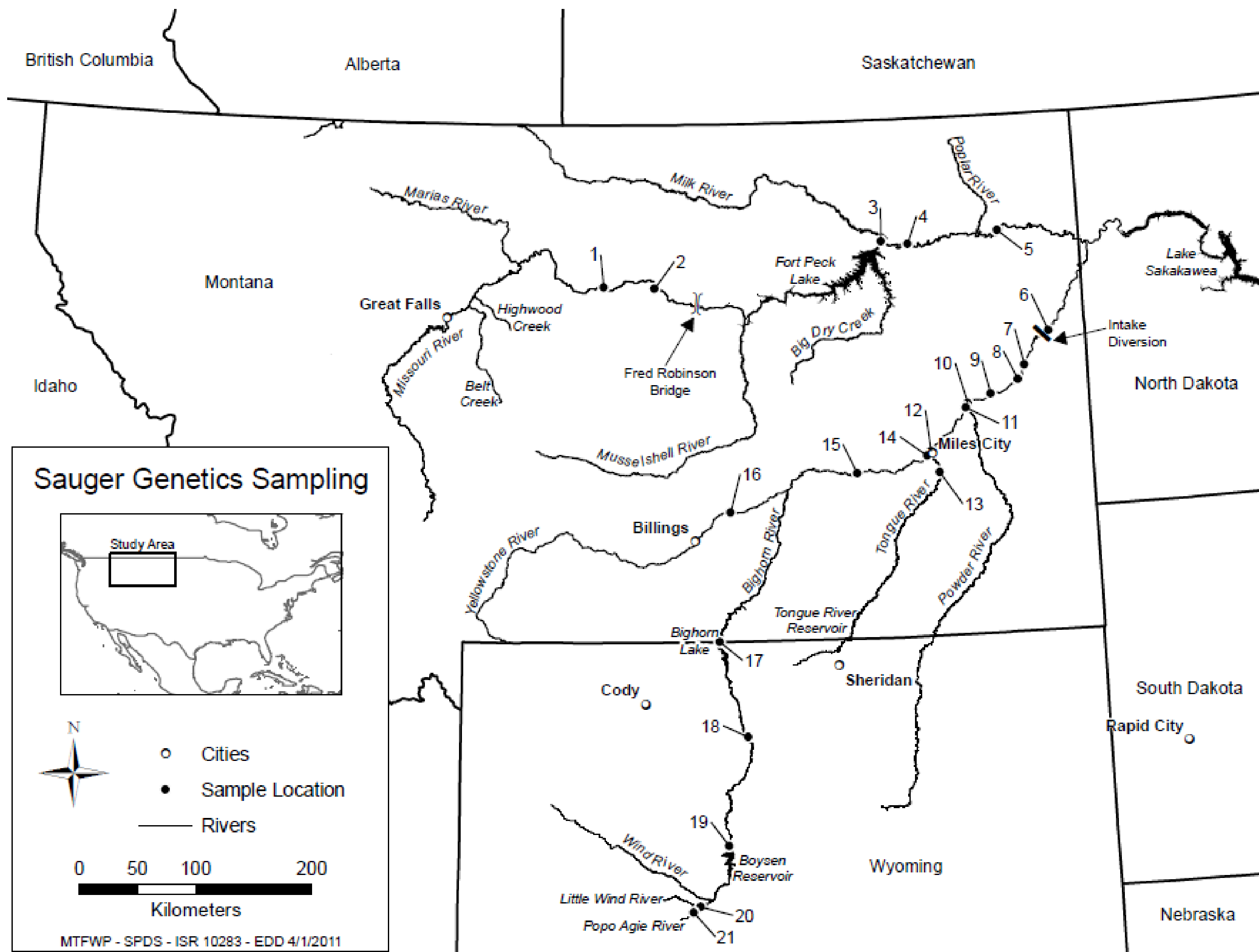


Figure 3-1. Upper Missouri River drainage and approximate sample locations in Montana and Wyoming. See Table 3-1 for sample codes.

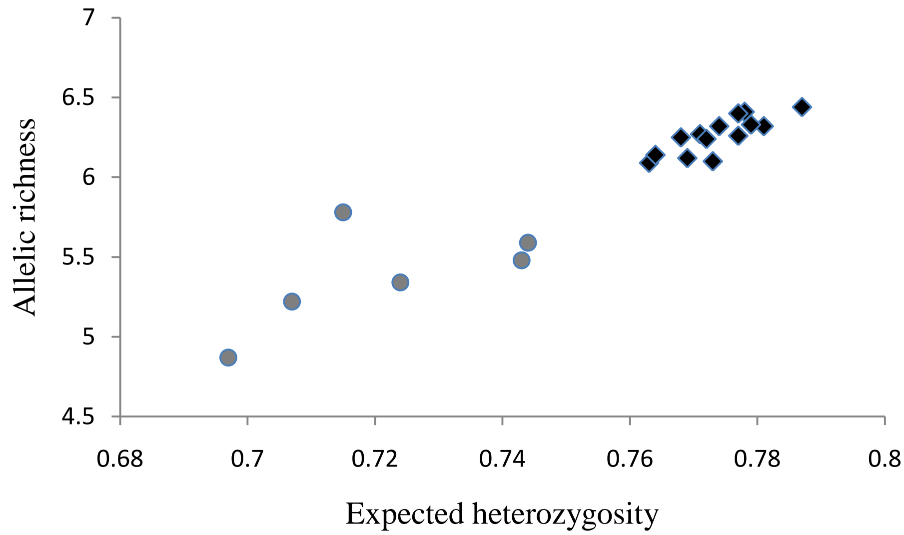


Figure 3-2. Scatter plot of allelic richness and expected heterozygosity within sauger populations. Gray circles represent sauger samples from the Bighorn River drainage and the upper Yellowstone River and black diamonds represent those from the Missouri and lower Yellowstone.

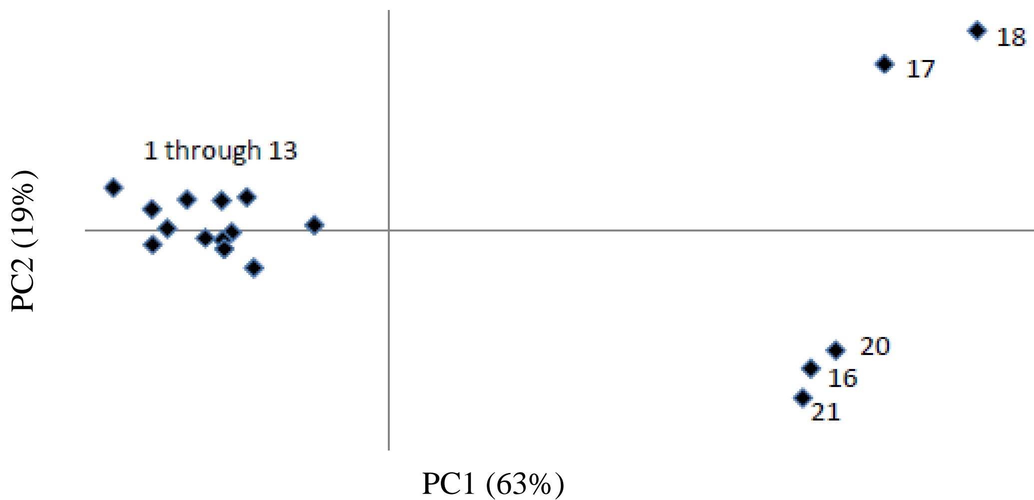


Figure 3-3. Plot of first two principal component scores derived from allele frequencies at eleven microsatellite loci. Diamonds represent individual samples.

Table 3-1. Map codes, and genetic and hybrid analysis summary statistics. Samples from the Bighorn River drainage appear below the dashed line. See Table 3-4 for individual hybrid classes. *N* is the total sample size including sauger, walleye, and hybrids.

Map code #	Samples	*N*	Allelic richness	H_E	F_{IS}	Number of sauger	Number of walleye	Number of hybrids
1	Upper Missouri	11	6.25	0.768	-0.021	11	0	0
2	above Ft. Peck	47	6.09	0.763	0.021	46	0	1
3	Milk River	30	6.32	0.781	-0.005	30	0	0
4	below Ft. Peck	34	6.33	0.779	0.016	34	0	0
5	lower Missouri	30	6.32	0.774	0.036	30	0	0
6	Intake	37	6.41	0.778	0.022	35	0	2
7	Glendive	99	6.26	0.777	0.019	98	0	1
8	Sand Creek	11	6.12	0.769	0.007	10	0	1
9	Fallon	82	6.27	0.771	0.043***	77	0	5
10	Calypso Bridge	215	6.40	0.777	0.025***	213	0	2
11	Crooked Creek	33	6.24	0.772	0.026	32	0	1
12	Miles City	30	6.14	0.764	0.012	30	0	0
13	Tongue River	28	6.44	0.787	0.018	18	7	3
14	Ft. Keogh	7	6.10	0.773	0.091*	7	0	0
15	Forsyth	5	-	-	-	3	1	1
16	upper Yellowstone	53	5.59	0.744	0.032*	52	0	1
17	Bighorn Lake	76	5.78	0.715	0.041**	23	53	0
18	Bighorn	40	4.87	0.697	-0.031	40	0	0
19	Boysen Reservoir	7	5.22	0.707	-0.148	7	0	0
20	Little Wind	25	5.48	0.743	0.014	25	0	0
21	Popo Agie	25	5.34	0.724	0.001	25	0	0
	Total/mean	925	6.0	0.758	0.021	846	61	18

Note: Significance: α = *<0.05, **<0.01, *** <0.001.

Table 3-2. Summary information and multiplex reaction conditions for eleven microsatellites. Primer sequences are those of the original authors.

Locus	F_{ST}	R_{ST}	F_{ST}'	Size range sauger (bp)	Size range walleye (bp)	Touchdown annealing temperature (C°)	Final Primer (um)	Reference
Multiplex 1								
*Svi7**	0.185	0.861	1.000	170-210	154-172	65-45	0.050	Eldridge et al. 2002
*Svi2**	0.281	0.891	1.000	236-258	188-202	65-45	0.050	Eldridge et al. 2002
*YP41**†	0.644	0.828	1.000	172	176-196	65-45	0.050	Li et al. 2007
Multiplex 2								
Pfla L1†	0.175	0.817	0.979	125-145	103-149	65-45	0.200	Leclerc et al. 2000
YP60	0.397	0.873	0.971	190-210	174-194	65-45	0.300	Li et al. 2007
Svi20	0.122	0.632	0.837	167-189	151-181	65-45	0.100	Eldridge et al. 2002
Svi26	0.151	0.176	0.914	151-186	151-188	65-45	0.075	Eldridge et al. 2002
MSL-2	0.187	0.000	0.938	140-226	156-196	65-45	0.150	Kohlman and Kersten 2008
Multiplex 3								
Svi L10	0.157	0.750	0.923	186-194	188-246	65-45	0.200	Wirth et al. 1999
YP113†	0.04	0.079	0.780	138-268	138-242	65-45	0.200	Li et al. 2007
Single reaction								
*MSL-1**	0.156	0.915	1.000	168-198	130-158	60-50	0.200	Kohlman and Kersten 2008
Mean	0.223	0.576	0.941					

Note: * diagnostic, † a 5' tail containing GTGTCTT was added to reverse primer (Brownstein et al. 1996).

Table 3-3. Multilocus pairwise F_{ST} estimates between sauger samples in the upper Missouri River drainage. Sample locations are given in Table 3-1. F_{ST} values in bold italics are significant at $P < 0.0045$ (Bonferroni correction).

	Sample																
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	16	17	18	20
2	0.001																
3	0.008	0.002															
4	-0.005	-0.001	-0.003														
5	-0.002	0.002	0.002	-0.001													
6	-0.006	-0.002	-0.001	-0.002	-0.003												
7	-0.004	0.002	0.003	-0.002	-0.001	-0.001											
8	-0.005	-0.008	-0.003	-0.006	-0.003	-0.006	-0.003										
9	-0.006	0.000	0.002	-0.002	-0.003	-0.004	-0.001	-0.004									
10	-0.001	0.000	0.000	-0.002	0.000	-0.002	0.000	-0.005	0.000								
11	-0.004	-0.004	0.001	-0.001	-0.004	-0.001	0.001	-0.008	-0.001	0.000							
12	-0.005	0.002	0.003	-0.001	-0.001	-0.001	0.000	-0.003	-0.001	0.000	0.002						
13	0.004	-0.002	-0.004	0.000	-0.003	-0.003	0.001	-0.012	-0.002	-0.002	-0.007	0.000					
16	***0.016***	***0.017***	***0.025***	***0.017***	***0.029***	***0.021***	***0.021***	***0.022***	***0.022***	***0.020***	***0.021***	***0.026***	***0.023***				
17	***0.030***	***0.033***	***0.039***	***0.032***	***0.038***	***0.033***	***0.034***	***0.038***	***0.031***	***0.033***	***0.035***	***0.029***	***0.039***	***0.025***			
18	***0.018***	***0.025***	***0.032***	***0.023***	***0.030***	***0.023***	***0.025***	***0.022***	***0.021***	***0.024***	***0.024***	***0.024***	***0.023***	***0.015***	0.001		
20	***0.012***	***0.015***	***0.022***	***0.019***	***0.027***	***0.019***	***0.018***	***0.022***	***0.019***	***0.016***	***0.019***	***0.020***	***0.020***	0.002	***0.017***	***0.013***	
21	***0.018***	***0.017***	***0.027***	***0.021***	***0.028***	***0.026***	***0.021***	***0.025***	***0.022***	***0.020***	***0.018***	***0.019***	***0.024***	0.006	***0.024***	***0.021***	-0.002

Table 3-4. Eighteen hybrids sorted by sample location (see Table 3-1). Columns labeled first and second indicate the probabilities of being a first (F_1) or second (F_2 or backcross) generation hybrid based on the second run of STRUCTURE with asterisks under the column labeled "*P*" indicating the significance level. The column labeled "Hybrid class" is the most likely hybrid class (e.g., F_1, F_2, or backcross) based on the genotypic pattern at four diagnostic loci. Diagnostic walleye alleles appear in bold italics.

		Hybrid generation				Diagnostic loci							
Sample location	q_i	First	Second	*P*	Hybrid class	*Svi7*		*Svi2*		*YP41*		*MSL-1*	
2	0.235	0	1	***	*BCS*	***162***	188	240	256	172	***187***	174	186
6	0.517	0.986	0.014	***	F_1	***158***	182	***202***	248	172	***187***	***142***	184
6	0.103	0	0.5	*	*BCS*	***158***	182	254	254	172	172	0	0
7	0.086	0	0.001	NS	*BCS+*	184	186	244	244	***183***	***191***	170	178
8	0.492	0.487	0.513	***	F_1	***164***	186	***194***	240	172	***183***	***138***	174
9	0.209	0	0.982	***	*BCS*	170	180	***190***	258	172	***183***	178	178
9	0.166	0	0.995	***	*BCS*	***164***	170	244	254	172	172	***139***	170
9	0.072	0	0.017	NS	*BCS+*	170	182	244	254	172	172	174	174
9	0.047	0	0.012	NS	*BCS+*	174	184	240	244	172	172	***146***	174
9	0.028	0	0.009	NS	*BCS+*	176	210	244	244	172	172	168	174
10	0.058	0	0	NS	*BCS+*	188	188	236	242	172	172	***138***	176
10	0.026	0	0	NS	*BCS+*	174	182	***188***	254	172	172	194	194
11	0.098	0	0.002	NS	*BCS+*	170	210	242	244	***183***	***187***	172	182
13	0.501	0.986	0.014	***	F_1	***164***	170	***188***	256	172	***183***	***142***	174
13	0.472	0.446	0.554	***	F_2	***162***	170	234	242	172	***183***	***139***	192
13	0.089	0	0.006	NS	*BCS+*	170	190	244	258	***187***	***191***	176	180
15	0.041	0	0.041	NS	*BCS+*	170	170	244	256	172	172	176	176
16	0.031	0	0.027	NS	*BCS+*	182	182	244	256	172	172	174	184

Note: $F_2 = F_1$ x F_1, *BCS* = F_1 x sauger, and *BCS+*=later generation backcross to sauger. Significance: *α <0.05, *** <0.001.

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