Lessons from two sculpin species in southeastern Minnesota: Species interactions in native populations and reproduction dynamics in reintroduced populations

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Abstract

Cottus cognatus and *Cottus bairdi* are small, benthic, freshwater fish species native to southeastern Minnesota. Current conservation efforts conducted by the Minnesota Department of Natural Resources (MNDNR) have included reintroducing sculpin to rehabilitate the native fish fauna in streams historically impacted by agricultural land-use. These restoration activities have provided diverse opportunities to study aspects of sculpin ecology in southeastern Minnesota.

The MNDNR reintroduction plan did not attempt to differentiate between *C. cognatus* and *C. bairdi*, in part because the two are morphologically similar and difficult to identify in the field. The two species are known to occur syntopically in some areas, and hybridization between the two has been documented. Fish survey data from the Minnesota Pollution Control Agency along with personal observation were used to identify sites in southeast Minnesota with syntopic populations. Fin clips were taken from fish in syntopic populations and genotyped using three species-specific microsatellite DNA markers. Habitat data were collected from syntopic sites to further understand conditions that allow for species co-occurrence. There was no evidence for hybridization in syntopic *C. cognatus* and *C. bairdi* populations in southeastern Minnesota, even though there was no spatial or temporal separation during breeding season. Syntopy occurred only in areas where a 1st- 2nd order spring-fed tributary flowed into a larger stream. Temperatures in syntopic locations were intermediate to temperatures recorded in allopatric locations for both species. The results of this study suggest that differences between the species limit or prevent hybridization in southeastern Minnesota. Sculpin reintroduction efforts should take into account the different preferences of each species to increase success in introduced populations.

Currently, the only species used for reintroductions is *C. cognatus*. Research started in 2005 at the University of Minnesota examined overall ancestry, changes in genetic diversity, and fitness in reintroduced *C. cognatus* populations up to two generations after stocking. New introductions were conducted to examine the initial dynamics in survival and reproduction and how they affect genetic diversity. Two streams were stocked in the fall of 2007 with an equal mix of individuals from two source strains. Fin clip samples were taken from all individuals stocked into each stream, and samples from their offspring were collected the following fall. Parentage analysis requires highly polymorphic genetic markers, and microsatellite loci developed for other *Cottus* species were insufficient for the needs of this study. To fulfill this need, thirteen polymorphic microsatellite loci were developed from *C. cognatus* libraries enriched for tri- and tetranucleotide repeats. These loci had 2 to 22 alleles and observed heterozygosities ranged from 0.36 to 0.86 in a sample of 47 individuals from one population. All parents and offspring from the two reintroduction sites were genotyped at 12 microsatellite loci, 6 from those described above and 6 from an existing set. Parentage analysis revealed small founder numbers, differential strain success, and skewed contributions by individuals. One family in each reintroduction stream was responsible for 21-28% of the offspring genotyped, and there was evidence for polygamy in both streams. Allelic diversity in the offspring was reduced by 35-49%. These findings indicate that the effective population size in reintroduced sculpin populations is likely low, and a large number of fish would have to be stocked to maintain genetic diversity in new populations.

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Prologue

Freshwater sculpin (family Cottidae) are small-bodied, benthic, nongame fish. Sculpin are important members of fish communities and are among the most abundant species present in coldwater assemblages (Quinn and Mundahl 1994, Carter et al. 2004, Gray et al. 2005). Coldwater systems have low species diversity (Vannote et al. 1980), and sculpin participate in many trophic interactions, including complex predator/prey relationships with syntopic trout species (Dineen 1947, Beauchamp 1990, Tabor et al. 2007). Research on sculpin has been limited in the past because morphological and behavioral similarities between species made determination of species identification and distributions difficult (Godkin et al. 1982, Daniels and Moyle 1984, Kinziger et al. 2005). In addition, most sampling methods used for fish were inefficient at capturing benthic vertebrates and, until the advent of the electrofisher, presence and abundance of sculpin in systems was largely unknown or underestimated. The development of more efficient sampling gear and, more recently, the advent of molecular analysis techniques, have allowed researchers to study sculpin populations more closely. Genetic analysis of populations has allowed for clarification of species identities and taxanomic relationships in the sculpin family. Phylogenies reveal that the *Cottus* genus is not a monophyletic group (Kinziger et al. 2005), and taxanomic classifications of sculpin are being modified with new species and subspecies frequently being identified (e.g., Williams and Robins 1970, Daniels and Moyle 1984, Freyhoff et al. 2005).

Sculpin are largely sedentary organisms, with small home ranges and limited dispersal capabilities (Utzinger et al. 1998, Petty and Grossman 2004). The isolated nature of sculpin populations and their high abundance where they occur make them ideal species for testing ecological theories and examining large scale population processes over small geographic areas

(Adams and Schmetterling 2007). Many studies are interested in interactions between divergent sculpin species where they come into contact with one another, whether through anthropogenic disturbance or natural processes. Large scale disturbances, such as dam construction and channel restructuring, have been known to cause hybridization between divergent lineages of sculpin (Zimmerman and Wooten 1981, Nolte 2005). In other cases, where syntopic populations exist without disturbance, sculpin species can remain isolated through a variety of ecological and behavioral mechanisms (e. g., Finger 1982, Matheson et al. 1983, Hansson 1984, White and Harvey 1999, Quist et al. 2004). Close examination of species interactions in both isolated and hybridized sculpin populations can reveal information on mechanisms of speciation and maintenance of genetic integrity. Nolte (2005) identified sculpin hybrids created by two distinct lineages of sculpin in the Rhine River system where the hybrids demonstrated an adaptive advantage and had invaded systems unsuitable for either parent group. However, hybrids were restricted to certain regions due to strong selection against hybrid individuals and genotypes (Nolte 2006). Examination of the stabilized hybrid zones and how they remained isolated from other local sculpin populations revealed a genetic basis for reproductive isolation and found that patterns of genetic introgression were different in two different environments (Nolte 2009). In his body of work, Nolte was able to use sculpin to examine genetic changes that occur in species formation in a natural environment, where genetic and environmental factors both play a role in the development of new species.

The isolated nature and restricted movement patterns of sculpin make them useful for studies on reproduction dynamics in naturally reproducing populations. The ability to sample a large proportion of the population can provide valuable information on population parameters such as effective population sizes and the nature of sexual selection. Fiumera et al. (2002) was able to obtain accurate estimates of census sizes and effective number of breeders in a semi closed sculpin population; information that could be used to examine patterns of genetic drift and rates of mutation in a natural population.

Sculpin also have uses as bioindicators. It has been shown that sculpin are sensitive to changes in habitat quality such as the presence of heavy metals (Maret and MacCoy 2002), temperature (Gray et al. 2005), increased sediment levels (McCormick et al. 2001, Mebane et al. 2003), habitat fragmentation (Fischer and Kummer 2000), and stream obstructions (Utzinger et al. 1998). Because sculpin are sensitive to changes in their environment they have been included as a metric in some indices of biotic integrity (IBI). McCormick et al. (2001) uses the proportion of individuals in the Cottidae family as a metric that is negatively correlated to sediment levels and positively correlated to indices of habitat and watershed quality. Mebane et al. (2003) uses sculpin as an indicator of long term stream quality as the sedentary nature of sculpin means they are more likely to be representative of those conditions than are mobile species.

Conservation interest in sculpin species has increased as researchers become more aware of the diverse ecological roles that sculpin fill in freshwater ecosystems. In 2003 the Minnesota Department of Natural Resources (MNDNR) began a sculpin reintroduction program with the intent of restoring sculpin to their native range in southeastern Minnesota (MNDNR 2003). These restoration activities have provided diverse opportunities to study aspects of sculpin ecology in southeastern Minnesota. In the paragraphs below I provide a brief description of the background and objectives for each chapter of my thesis. Each chapter has been or is intended to be incorporated into manuscripts with co-authors for peer-reviewed journals, and plural personal pronouns are used throughout the thesis.

Chapter 1. Lack of hybridization between syntopic slimy sculpin (*Cottus cognatus*) and mottled sculpin (*Cottus bairdi*) in southeast Minnesota. Co-author: L. M. Miller (Department of Fisheries, Wildlife, and Conservation Biology, University of Minnesota). Target journal not yet specified.

Two sculpin species are native to southeastern Minnesota and the MNDNR reintroduction plan did not differentiate between them, in part due to the difficulty in visual identification of the species (Godkin et al. 1982). It is thought the species are capable of hybridization (Strauss 1986), which adds to the confusion surrounding species identification and distribution. The first chapter of my thesis examines syntopic populations of the two species in southeastern Minnesota to determine if hybridization is occurring and what environmental conditions could allow for species co-occurrence.

Chapter 2. Low founder numbers in reintroduced populations of slimy sculpin (*Cottus cognatus*) in southeastern Minnesota streams. Co-author: L. M. Miller. This chapter represents a piece of a larger project by L. M. Miller that is not yet complete.

The MNDNR reintroduction project creates an ideal situation to examine reproduction dynamics in sculpin populations. The second chapter of my thesis examines initial dynamics of survival and reproduction and how that affects genetic diversity in reintroduced populations.

Chapter 3. Isolation of 13 polymorphic microsatellite loci for slimy sculpin (*Cottus cognatus*). Co-authors: F. K. Barker (Department of Ecology, Evolution, and Behavior, University of Minnesota), D. D. Huff (Department of Fisheries, Wildlife, and Conservation Biology, University of Minnesota), and L. M. Miller. Published in *Conservation Genetics Resources* 1: 429-432, and used in my thesis with the kind permission of Springer Science and Business Media.

Currently available DNA analysis tools developed for sculpin would have had insufficient resolution for the parentage analysis conducted in the second chapter of my thesis. The third chapter deals with the development of the first microsatellite DNA markers for slimy sculpin (*Cottus cognatus*).

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Chapter 1

Lack of hybridization between syntopic slimy sculpin (*Cottus cognatus*) and mottled sculpin (*Cottus bairdi*) in southeast Minnesota

Introduction

Cottus cognatus and *Cottus bairdi* are freshwater sculpin species that inhabit coldwater streams, rivers, and lakes and are broadly sympatric across North America (Koster 1936). Research interest in sculpin species has increased in recent years and they have become a target for ecological, behavioral, and phylogeographic studies (Adams and Schmetterling 2007). Members of the family Cottidae are cryptic and it is often difficult to differentiate between species: both morphological similarities between species and variation within species can complicate identification. Historically, research conducted on syntopic sculpin populations relied on morphology to determine species interactions and potential hybridization (e.g., Nyman and Westin 1968; Godkin et al. 1982; Lyons 1990). Advances in technology have allowed for genetic confirmation of hybridization in syntopic zones (e.g., Strauss 1986; Nolte et al. 2005), eliminating some of the problems encountered with morphometric analysis.

As with many sculpin species, *C. cognatus* and *C. bairdi* are morphologically similar. The only trait that can readily be used to differentiate between the two in the field is pelvic fin ray count (Eddy and Underhill 1957), although this method is not completely reliable as the trait varies within each species (McAllister and Lindsey 1961; Godkin et al. 1982). There are two other characteristics considered diagnostic for the species, presence of palatine teeth and branching of the last anal ray. However, these characters are difficult to distinguish on live specimens in the field, and can also be variable (Godkin et al. 1982). Even though they closely resemble one another physically, previous research suggests they have different levels of tolerance for environmental conditions. *C. bairdi* are known to have a higher thermal lethal limit than *C. cognatus* (Otto and Rice 1977; Kowalski et al. 1978), and it has been observed that *C. bairdi* can tolerate streams with a higher amount of sedimentation than can other coldwater

Cottus species (Quist et al. 2004). Despite these differences, syntopy between the two species has been documented at a small number of sites in the upper Midwest and Pennsylvania (Godkin et al. 1982; Strauss 1986; Lyons 1990). The sympatric range of *C. cognatus* and *C. bairdi* extends into Canada, but currently there is no documentation of syntopic populations north of the Great Lakes.

The occurrence of individuals displaying intermediate morphologies or a mosaic of diagnostic characteristics in syntopic populations led to questions regarding hybridization in these areas. An early study attributed the variation to hybridization, although it was difficult to distinguish between putative hybrids and single species variants (Godkin et al. 1982). A later study conducted on *C. cognatus* in the Great Lakes region found that individuals with intermediate pelvic fin ray counts only occurred in areas where they were syntopic with *C. bairidi* (Lyons 1990). Strauss (1986) applied genetic techniques in combination with morphometric analysis to confirm hybrids at a syntopic site in Pennsylvania, where some individuals displayed intermediate fin ray counts. Strauss' study used allozymes, which proved useful in detecting hybrids; however, this method requires lethal sampling and immediate ultracold storage of tissue samples. Microsatellite DNA markers have the potential to provide similar information with the advantages of non-lethal sampling, easier sample storage, and, potentially, many more diagnostic markers.

In 2003 the Minnesota Department of Natural Resources (MNDNR) initiated a sculpin reintroduction plan in the Driftless Area Ecoregion of southeast Minnesota that was intended to restore sculpin to their historic range and improve ecosystem integrity (MNDNR 2003). The reintroduction plan did not distinguish between *C. cognatus* and *C. bairdi,* in part due to the difficulty in visual identification of the two species, which was further complicated by the possibility of hybridization. The lack of a reliable method for species identification could have led to situations of syntopy through translocation, which would have unintended consequences for species integrity if hybridization does occur (Allendorf et al. 2001). Coldwater ecosystems tend to be low order systems with very few species (Vannote et al. 1980); therefore, all are important for the overall health and function of the system. Limited information on the ecology of non-game species, such as sculpin, can hinder effective management of ecosystems, therefore there is a need for research that examines non-game members of the fish community.

Here, we report on a genetic survey of sculpin populations in southeast Minnesota, aimed at determining how commonly the two species are syntopic and whether or not hybridization occurs in these syntopic areas. The objectives for the study were threefold: 1) to identify syntopic populations of *C. cognatus* and *C. bairdi*, 2) to use microsatellite analyses to differentiate between species and detect hybridization if it is occurring, and 3) to characterize habitats at syntopic sites to further understand what conditions allow for species co-occurrence and hybridization (if it occurs).

Methods

Study area

Our study was conducted in southeast Minnesota in the Driftless Area Ecoregion, a unique part of Minnesota that was left uncovered by the last glacial advance. The area is known for its karst topography and is characterized by limestone bluffs and steep river valleys. Land use in the Driftless Area is dominated by agriculture, which contributed to severe stream degradation in the past (Waters 1977). Much of the land in steep terrain remains wooded and many of the regions’ headwaters are in these areas. Headwater streams in the Driftless Area are predominantly spring-fed and provide coldwater habitat throughout the year.

Site identification and sampling

The first objective was to identify syntopic sites in southeastern Minnesota. We compiled fish census data of the Minnesota Pollution Control Agency (MPCA) from 2004 and 2009, noting all records of sculpin in the Driftless Area. We then made a list of possible syntopic sites based on the presence of the species in close proximity to one another and previous casual observations by L. Miller (Dept. of Fisheries, Wildlife, and Conservation Biology, University of Minnesota). The MPCA fish survey data were collected during the summer, but we needed to confirm syntopy during the spring breeding seasons. For this purpose, we identified 10 sites to survey in May 2008 with a backpack electrofisher, making preliminary species identifications using pelvic fin ray counts. Three sites were in the Whitewater River watershed and seven sites were in the Root River watershed. We took fin clip samples from fish at each site to be used for genetic confirmation of species identity. Once syntopic sites were identified, we took fin clips from approximately 100 fish at each site and stored the clips in 95% ethanol. We noted the general stream location of fish as they were caught.

In April 2009 we returned to one syntopic site to monitor nest activity and to record fish locations in the stream. Using a backpack electrofisher we surveyed the stream twice (4/9/09 and 4/23/09), proceeding in a haphazard fashion so as not to disturb multiple nests in one area. We noted the location of the fish where they turned and checked the underside of the rocks nearest the fish for signs of a nest. We took note of fish species and number of egg masses, and then picked a few eggs from each mass and fixed them in 95% ethanol. We identified adult individuals using pelvic fin ray count as the method proved reliable for this location based on previous genetic analysis of the population.

Genetic analysis

Our laboratory previously employed a set of eight microsatellite DNA markers to examine genetic structure and population divergence in *C. cognatus* for the sculpin reintroduction plan (L. Miller, unpublished data). Of those eight markers, we found three that differentiated the species—*Cott*ES1 (Nolte et al. 2005), *Cba*14 (Fiumera et al. 2002), and *Cgo*310 (Englebrecht et al. 1999). Surveys for both species revealed that *C. cognatus* were moderately variable, whereas *C. bairdi* was fixed for an allele that did not occur in *C. cognatus*. The size separation between alleles of the two species was large enough to allow resolution using short polyacrylamide gels and ethidium bromide staining. The species specificity of the three markers was verified in surveys of 904 *C. cognatus* individuals from 16 southeastern Minnesota populations, and one population from the Driftless Area Ecoregion in Iowa, as well as 98 *C. bairdi* individuals from 5 populations in southeastern Minnesota.

DNA was extracted from all samples using 300 µL of a 5% Chelex® (Sigma Chemical) solution. Subsamples of tissue were placed in solution, incubated overnight in a 56°C water bath, and then boiled for 8 minutes. All samples were amplified at each of the species-specific loci by the polymerase chain reaction (PCR). Amplifications were carried out in 15 µL reaction volumes consisting of 5 µL DNA template, 1X GoTaq® DNA polymerase buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.33 µM of each primer, 0.5 U GoTaq® DNA polymerase, and water to the final reaction volume. Thermocycler conditions for amplification were a 3 minute denaturation at 95°C, 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and an extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were separated on 8% polyacrylamide gels, stained with ethidium bromide, and then visualized with a UV transilluminator. A digital photo was taken of the gel and allele scores were recorded from the images by hand.

Species distribution at syntopic sites

We used contingency table testing to determine if the proportion of each species was dependent on stream location. All syntopic sites were located at confluences of streams. We compiled fish counts at each syntopic site into a 2x2 contingency table with species as the row variable and stream location (tributary or mainstem) as the column variable. We assessed significance using the G-test of independence, or Fisher's exact test when the expected value of a table cell was less than five (Zar 1996). A Freeman-Tukey transformation was used for multiple comparisons of proportions to determine which individual cells deviated significantly from expectations (Freeman and Tukey 1950).

Habitat data collection

Physical habitat data were collected from two of our syntopic sites as well as two reference sites for each species. We did not measure habitat variables at all four syntopic sites as conditions at two sites made them unsuitable for habitat data collection. One location was part of a large system that would have made accurate measurements difficult. However, this site was within a mile of a syntopic site we included in habitat analysis, and it was likely conditions at both sites were somewhat comparable. The second site excluded from habitat analysis had been impacted by a recent flood, and it is possible that habitats were altered to the extent the site was not representative of syntopic conditions. This site had been identified in MPCA records as a syntopic site prior to the flood, so we knew the flood had not caused the conditions leading to syntopy.

Measurements of habitat variables were taken from cross-sectional transects of the stream reach (Simonson 1993). Habitat variables quantified using transects included substrate composition (Kaufmann et al. 1999), width, depth, and velocity (Lazorchak et al. 1998). Cross-sectional transects were planned so that an equal number of transects were taken across each channel unit type (riffle, glide, pool). Thalweg measurements were taken between transects to further characterize depth profiles within each channel unit. We also estimated sedimentation levels in each channel unit by calculating the proportion of thalweg measurements where sediment was present.

Temperature loggers (HOBO® water temp pro v2) were placed at two of the overlap sites to record temperatures for one year starting in June 2008. Three loggers were placed at each site to capture the full range of conditions across the syntopic zone. In both cases, we found the border between the syntopic zone and *C. cognatus* and placed one logger in the *C. cognatus* only area. We were unable to find the border for *C. bairdi* in the immediate area of the syntopic zones at the two sites, so we placed a logger at Willow Creek in the South Branch Root system to act as a *C. bairdi* reference site. We calculated a 7-day running mean of maximum temperature at each logger location to detect differences in temperature extremes between sites. We focused on summer temperatures as they can be important factors in determining species distributions (Quist et al. 2004; Huff et al. 2005). Summer temperature ranges were defined as the coldest 7-day summer minimum paired with the warmest 7-day summer maximum at each logger location.

Results

Site identification

The MPCA fish survey records identified sculpin at 32 sites in southeastern Minnesota. Of these sites, 18 had only *C. cognatus*, 11 had only *C. bairdi,* and 3 had syntopic populations. Of the 10 sites surveyed during breeding season 1 had only *C. cognatus*, 3 had only *C. bairdi* , 4 had syntopic populations, and 2 had no sculpin (Figure 1). We confirmed syntopy during breeding season at two of the syntopic sites in the fish survey records. During our survey we identified two syntopic sites that were not included in the MPCA fish survey records. Finally, one site identified as syntopic in the MPCA fish survey records had only *C. bairdi* during our survey.

All syntopic sites occurred where a 1st- 2nd order spring-fed tributary flowed into a larger stream. Species overlap occurred at the confluences of Trout Run Creek with the Whitewater River, Forestville Creek and Canfield Creek with the South Branch Root River, and Hemingway Creek with Pine Creek. We collected 89-111 individual fin clips from each syntopic site for genetic analyses.

One site we surveyed in the Whitewater watershed, Crow Spring, contained fish exhibiting intermediate pelvic fin ray counts, but was not one of the sites where we identified syntopy. The fish at this site tended to have fin ray counts consistent with *C. bairdi* but many individuals had pelvic fin ray counts of 4 rays on one side and 3 to 3 ½ rays on the other. Genetic analysis revealed the individuals with intermediate fin ray counts were *C. bairdi*, and there was no evidence they represented hybrid individuals.

Genetic confirmation of species identity and distribution at syntopic sites

Using our three species-specific genetic markers we genotyped a total of 401 individuals from the four syntopic sites. There was no evidence of hybridization at any of the sites. All individuals displayed species-specific alleles at all three loci, with no genotypes indicative of hybrids; however, analysis of species distributions revealed an interesting pattern. At three of the sites *C. bairdi* did not extend into the tributaries, but were constrained to the mainstems. They were present in lower Hemingway Creek, at the confluence to Pine Creek, but in relatively low numbers (Figure 2). A finer scale description of species location within each syntopic site is given in Appendix I.

At all four syntopic sites we found that species proportions were significantly dependent on stream location (G-test or Fisher's exact test, $p<0.05$ [Table 1]). At all four locations, there were more *C. cognatus* and fewer *C. bairdi* in the tributary than expected. There were also fewer *C. cognatus* and more *C. bairdi* in the mainstem than expected. The trend of fewer *C. bairdi* in tributaries was significant at all four locations. At Canfield Creek and South Branch Root River site all trends were significant for both species (Freeman-Tukey tests, $p<0.05$).

We found nests of both species concurrently in the mainstem area of Pine Creek near the confluence with Hemingway Creek. During our first nest exploration (4/9/09) we counted 72 individuals. In the mainstem, we identified 30 *C. bairdi* and 22 *C. cognatus*. In the tributary, we identified 20 *C. cognatus*. We found two nests in the tributary, but none in the mainstem. Eggs taken from one nest were genetically analyzed and identified as *C. cognatus*. Eggs from the second nest failed to amplify. During our second exploration and (4/23/09) we counted 37 individuals. In the mainstem, we identified 7 *C. cognatus* and 25 *C. bairdi*. In the tributary, we identified 4 *C. cognatus* and 1 *C. bairdi*. We found 6 nests, 5 in the mainstem and 1 in the tributary. Genetic analysis revealed that one nest in the mainstem was *C. cognatus*, and the other 4 were *C. bairdi.* The nest in the tributary was *C. cognatus*.

Habitat data analyses

The small number of syntopic sites and their correspondence with the confluence of streams led to high variability in physical habitat measurements within syntopic sites. Also, since the mainstems at syntopic sites were at least 3rd order systems, it was not possible to obtain accurate measurements of deep channel sections with our sampling gear. For these reasons we did not draw any conclusions about the relationship of physical habitat variables with syntopic or single-species populations.

Weekly maxima in each stream revealed that the periods of highest temperature divergence between streams occured in the summer months and to a lesser extent in the winter (Figure 3). We lost loggers that were in the mainstem upstream of the confluence at both of the sites, but information was collected for the first summer. The areas with syntopy downstream of the confluence exhibited temperatures intermediate to those in the tributary and in the mainstem upstream of the confluence. Temperatures in the Whitewater system were cooler in the summer than those in the South Branch Root system, indicative of higher groundwater inputs. Summer maxima at the Whitewater overlap site did not exceed 16°C, and the South Branch Root overlap site maxima did not exceed 19°C. The tributaries to these systems, Trout Run Creek and Forestville Creek, did not exceed 14°C and 16°C, respectively.

C. cognatus were present in areas where summer maxima reached 19°C and where summer minima reached 10°C. *C. bairdi* were present in areas where summer maxima reached 21°C and summer minima reached 12°C. No *C. bairdi* were found in areas with a summer minimum less than 11°C

Discussion

We found no evidence for hybridization between syntopic populations of *C. bairdi* and *C. cognatus* in southeastern Minnesota. Previous study of syntopic populations has either suggested or confirmed hybridization and reported individuals with intermediate, or a mosaic of, diagnostic characteristics. Since we found areas of syntopy and individuals with intermediate fin ray counts we expected to find hybrids in our study populations. Hybridization is often associated with disturbance, where conditions are altered enough to break down species barriers (Hubbs 1955). There were no obvious environmental conditions that might have led to hybridization in southeastern Minnesota, such as permanent habitat restructuring (e. g., Zimmerman and Wooten 1981, Nolte et al. 2005). However, sculpin hybridization in Pennsylvania occurred in what appeared to be a relatively undisturbed ecosystem with no unusual conditions (Strauss 1986). Three possible reasons explain why we did not detect hybrids in conditions similar to where they have been reported: 1) hybridization is occurring at low frequencies difficult to detect, 2) hybridization is not occurring due to spatial and temporal separation at the time of spawning, or 3) species differences lead to assortative mating (prezygotic isolation) or reduced hybrid viability (postzygotic isolation) so that no hybrids were present when we sampled. We will address each reason in turn and how our study narrows the possibilities.

The first possibility is that hybridization is occurring, but at low frequencies that would make it difficult to detect. Hybridization does not appear to be common within populations, as Strauss genetically confirmed only 8 hybrids out of 79 individuals (1986). The ability to detect low frequency occurrence of hybrids depends on sample size and the number of molecular markers employed. Based on binomial probabilities, a single species-specific marker and our sample sizes of 89-111 individuals would provide a 99.0-99.7% chance of detecting F_1 hybrids if they represent 5% of the population. Previous morphometric analysis of syntopic populations revealed a degree of variation suggesting the presence of advanced generation hybrids (Godkin et al. 1982; Strauss 1986), which would have a mixture of genotypes, thus a single marker may not detect all hybrid individuals. However, using three species-specific markers provided reasonably high power to detect advanced hybrids, for example, 97% and 88% probability of detecting F_2 and first-generation backcross individuals, respectively. We conclude that it is unlikely hybrids were present at the time of our sampling, as we had high power to detect F_1 hybrids and we used three markers to increase the probability of detecting advanced generation hybrids. Furthermore, there is no genetic evidence confirming advanced generation hybrids as the genotypes acquired by Strauss were all representative of first generation individuals (Strauss 1986).

The second possibility is that hybridization is not occurring because of spatial and temporal separation during breeding season. Hybridization in syntopic populations of cottid species is not well documented. Many studies regarding syntopic populations of sculpin describe species separation due to different microhabitat preferences and competitive interactions (e.g., Finger 1982; Matheson and Brooks 1983). Breeding season overlap between *C. cognatus* and *C. bairdi* is only partly synchronous; *C. cognatus* spawn slightly earlier and at a colder temperature than *C. bairdi* (Downhower et al. 1983; Keeler and Cunjak 2007). However, we found nests of both species at the same time and in the same location, indicating there was not complete spatial and temporal separation during breeding season. The conditions in syntopic zones between *C. cognatus* and *C. bairdi* appear conducive to the production of hybrids as mating behaviors of the two species are the same (Downhower et al. 1983; Fiumera et al. 2002), and we found them together during breeding season, therefore spatiotemporal separation does not explain why we failed to detect hybrids.

We found no hybrids where conditions suggest they should occur, which leads us to the third possibility: species differences led to assortative mating or reduced hybrid viability. Freshwater sculpin have limited mobility, as demonstrated by a study conducted on an established *C. bairdi* population where fish were recorded to move no more than 165 meters over a 45 day period (Petty and Grossman 2004). Genetic analyses of sculpin populations reveal that sculpins have well-developed population structure due to long-term separation from other populations (Meyer et al. 2008). The degree of divergence between populations is sometimes so great that previous taxanomic classifications are called into question. Closer inspection of *Cottus gobio*, a European sculpin species, revealed that what was previously classified as a single species could be divided into eight different species (Freyhof et al. 2005). Strong divergence has been noted in another freshwater fish species, one with a range similar to our cottid species of interest, for which molecular data suggest colonization of distinct geographical regions by fish from different glacial refugia (Bernatchez and Dodson 1991). It is possible that populations of *C. bairdi* and *C. cognatus* in southeastern Minnesota are divergent enough from populations where hybridization has been reported that they have evolved isolating mechanisms specific to this region. Sculpin are known to be highly territorial, competing for food resources and optimal breeding habitat (Petty and Grossman 2007). Species recognition between *C. cognatus* and *C. bairdi* coupled with territorial behaviors could act as a reproductive barrier. Another possibility is that *C. cognatus* and *C. bairdi* are capable of hybridizing but postzygotic isolation results in low viability of offspring that do not survive the egg stage or do not survive to adulthood. Strauss (1986) showed that F_1 hybrids in Pennsylvania are capable of surviving. However, the proportion of hybrids he identified in his sample was significantly lower than one would expect based on random mating between species, which could indicate reduced viability in hybrid individuals. In our streams, the viability could be so reduced that no hybrids survived their first summer to appear in our surveys. In this case, interbreeding is a wasted reproductive effort which could have significant impacts on population persistence (Epifanio and Nielson 2001).

All of the explanations discussed above raise more questions than we have been able to answer with this research. There does not appear to be spatiotemporal separation between species during breeding season, but we still do not know exactly what accounts for the lack of hybrids in these populations. The reasons we have described above are not mutually exclusive, and a combination of factors may be responsible. What we do know, however, is that hybrids are not detected in every place these two species are syntopic. A broader scale study is needed to determine the prevalence of hybridization across the sympatric range of *C. cognatus* and *C. bairdi*.

Based on our observations it is also apparent that syntopy is not a widespread occurrence. This observation agrees with Strauss' (1986) study of natural populations, where he identified only 6 syntopic sites out of 642 locations with *C. cognatus* or *C. bairdi*. The habitat attributes at syntopic sites provide clues as to why syntopic sites are so rare. Intergradation of habitat is known to be important in allowing for species co-occurrence (Hubbs 1955). In Lake Ontario, Godkin et al. (1982) found depth to be a separating factor between species and syntopy occurred in the transition zone between the shallows and deepwater. The syntopic site studied by Strauss (1986) was a confluence of a coldwater tributary to a larger system. Even though there are many sites with sculpin in southeastern Minnesota, we found syntopy only in such confluence areas. Our temperature profiles for these areas are consistent with what one expects to find at a confluence: two different systems merging, where tributaries act to cool the waters in mainstem systems during the summer, creating a localized temperature mixing zone. Where conditions allowed we were able to find the boundary of the syntopic region where we found only *C. bairdi* in the mainstem. It appears that *C. cognatus* are limited to areas where groundwater springs provide coldwater refuges, reinforced by the observation that *C. cognatus* were more prevalent in tributaries than expected. In contrast, *C. bairdi* were rarely found in the tributaries and appeared to be limited by the summer low temperatures, not extending into areas where summer minima were too cold. This finding agrees with that of Quist et al. (2004), who found no *C. bairdi* in systems where the summer minimum was below 10°C. The rarity of syntopy reflects the small number of sites where intermediate habitats are available to existing *C. cognatus* and *C. bairdi* populations.

As long as conditions remain relatively stable in syntopic zones, species barriers may remain intact in southeastern Minnesota. However, climate change is expected to impact coldwater aquatic resources as groundwater temperatures are predicted to rise (Meisner et al. 1988). In a scenario where atmospheric CO_2 doubles, stream temperatures are predicted to rise 2.4 °C to 4.7 °C (Stefan and Sinokrot 1993). Warming water temperatures will decrease the amount of habitat available to coldwater species, limiting their range to groundwater-fed refuges (Meisner 1990). In the case of *C. cognatus* and *C. bairdi*, changing water temperatures might lead to conditions that allow for hybridization. Restricting habitat availability for one species can create a situation where limited access to spawning habitat and fewer members of the same species can lead to hybridization (Allendorf et al. 2001). A drastic change in the temperature regime can also break down species barriers and create hybrid zones where none occurred before (Zimmerman and Wooten 1981).

Warming temperatures could also lead to species displacement—with or without hybridization. Our temperature data indicate that *C. bairdi* are capable of tolerating warmer summer maxima than *C. cognatus*. It is likely that temperatures in the summer will become more extreme, creating unfavorable conditions for *C. cognatus*, whereas *C. bairdi* may be able to inhabit areas previously unsuitable to the species. Historical records indicate that such shifts in species distributions have occurred in southeastern Minnesota in the past. Dineen (1947), in a study of trout and sculpin diet overlap that included two of our syntopic site tributaries, identified only *C. bairdi* in areas currently occupied by only *C. cognatus*. Drought in the 1930's and degraded stream conditions could have led to warmer temperatures, which could explain the presence of *C. bairdi* in this historical record. Limited dispersal capabilities of sculpin species (Utzinger et al. 1998), along with a lack of nearby coldwater tributaries, lead us to believe that *C. cognatus* survived in coldwater refuges within the streams rather than colonizing from elsewhere. The distributions of *C. cognatus* and *C. bairdi* may act as indicators of climate and anthropogenic impacts on coldwater streams as they shift with warming water temperatures.

Individuals with intermediate pelvic fin ray counts in southeastern Minnesota proved to be variants of the *C. cognatus* and *C. bairdi* phenotypes, and not representative of hybrid individuals. The *C. bairdi* population sampled as part of this study had individuals with 3 to 3 ½ fin rays on one side and 4 on the other, whereas a population of *C. cognatus* that was part of a previous genetic study had individuals displaying 3 ½ fin rays on one side and 3 to 3 ½ on the other. In both cases, fish with intermediate fin ray counts occurred alongside fish having typical counts for the species we genetically confirmed at the sites. Although we can not conclude that hybrids would not have intermediate fin ray counts, it is clear that intermediate counts are not an indicator of hybrids in this region.

In conclusion, syntopy between *C. cognatus* and *C. bairdi* is uncommon and hybridization does not occur in every location the two are syntopic. However, alterations to stream condition caused by climate change or anthropogenic activities, such as agricultural land use, could alter species distributions and interactions. The different habitat preferences of the two sculpin species could be a useful tool for managers, as presence of one or the other could be used as a metric in measurements of coldwater stream biotic integrity. Stocking sculpin species in coldwater streams should take into account the different habitat preferences of *C. cognatus* and *C. bairdi*. A targeted approach should be implemented to increase the success of introduced populations by stocking the species better suited to a particular environment.

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Table 1. Distribution of species within each syntopic site. P-values for Hemingway/Pine and Canfield/South Branch Root are from G-tests of independence. P-values for Forestville/South Branch Root and Trout Run/Whitewater are from Fisher's exact tests.

	Species	**Mainstem**	**Tributary**	**p**
Hemingway/Pine	*C.cognatus*	54	27	0.028
	C.bairdi	26	4*	
Forestville/South Branch Root	*C.cognatus*	65	19	0.037
	C.bairdi	17	0*	
Canfield/South Branch Root	*C.cognatus*	5*	24*	0.000
	C.bairdi	71*	0*	
Trout Run/Whitewater	*C.cognatus*	55	17	0.035
	C.bairdi	17	0*	

* denotes significantly contributing cells (Freeman-Tukey test, $p<0.05$)

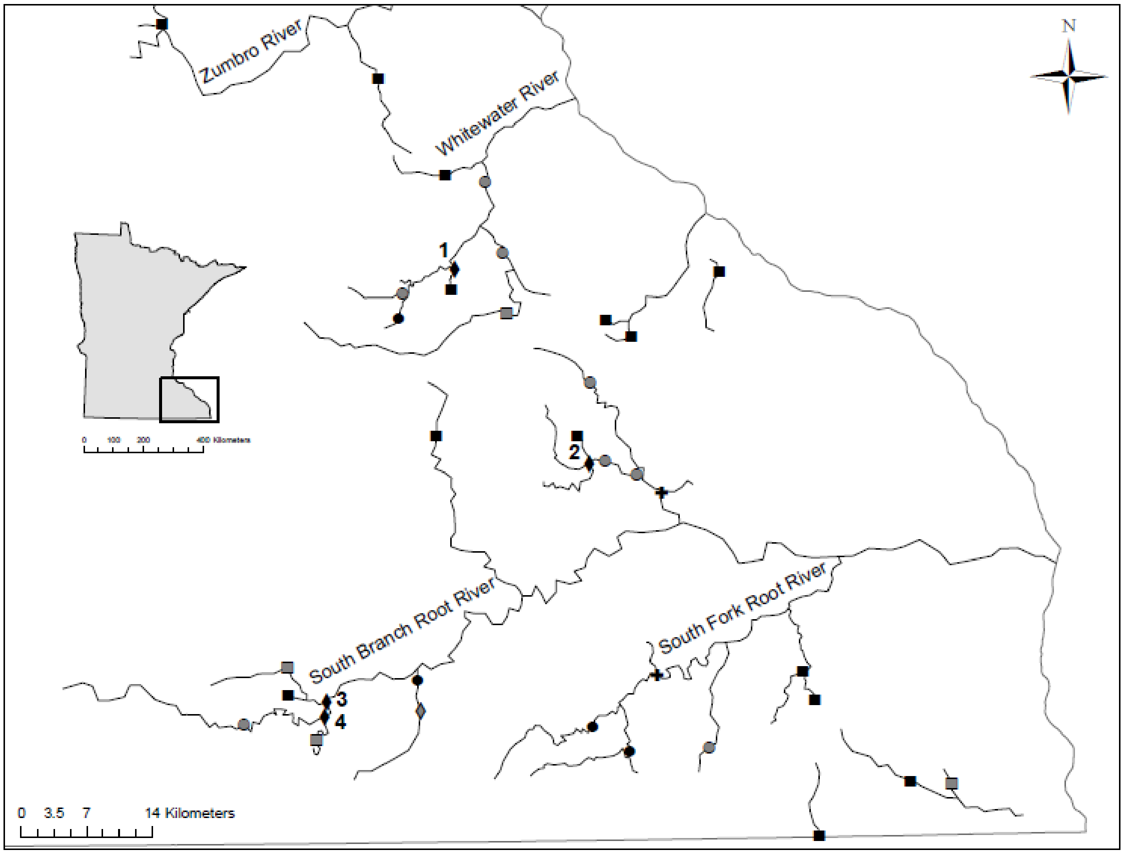


Figure 1. Map showing sculpin species presence in southeastern Minnesota. Squares represent allopatric *C. cognatus* sites, circles are allopatric *C. bairdi* sites, diamonds are syntopic sites, plus signs (+) are sites with no sculpin. Black symbols are sites for which there is genetic confirmation of species identity based on this and previous studies (L. Miller, unpublished data). Gray symbols are sites with visual identification of species only from MPCA surveys. Syntopy occurred in four locations at the confluences of Trout Run Creek with the Whitewater River (**1**), Forestville Creek and Canfield Creek with the South Branch Root River (**2**, **3**), and Hemingway Creek with Pine Creek (**4**). For further geographic reference, a map is provided in Appendix II that supplies stream names for the study area.

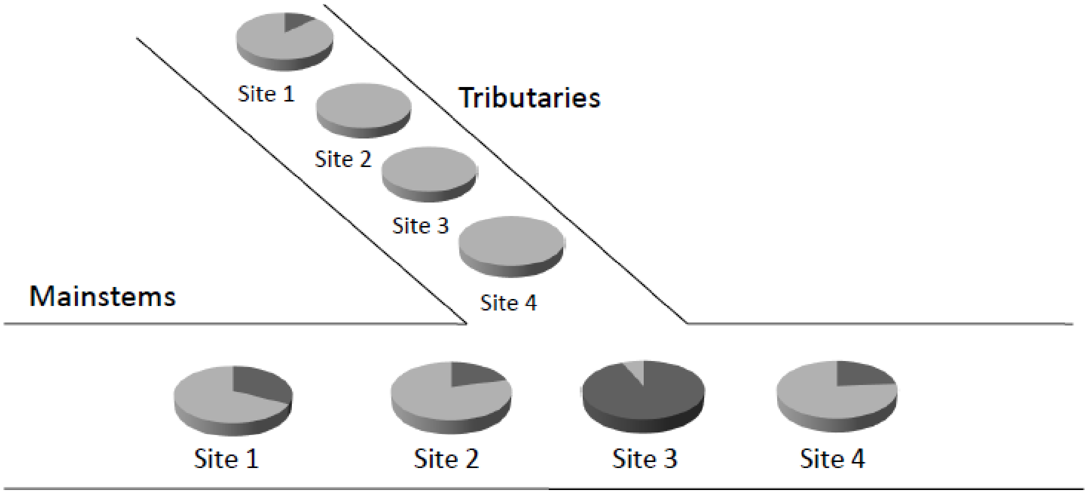


Figure 2. Species distributions at syntopic sites. Light gray indicates the percentage of *C. cognatus* and dark gray is the percentage of *C. bairdi* at each site. Site 1 is Hemingway/Pine, site 2 is Forestville/South Branch Root, site 3 is Canfield/South Branch Root, and site 4 is Trout Run/Whitewater.

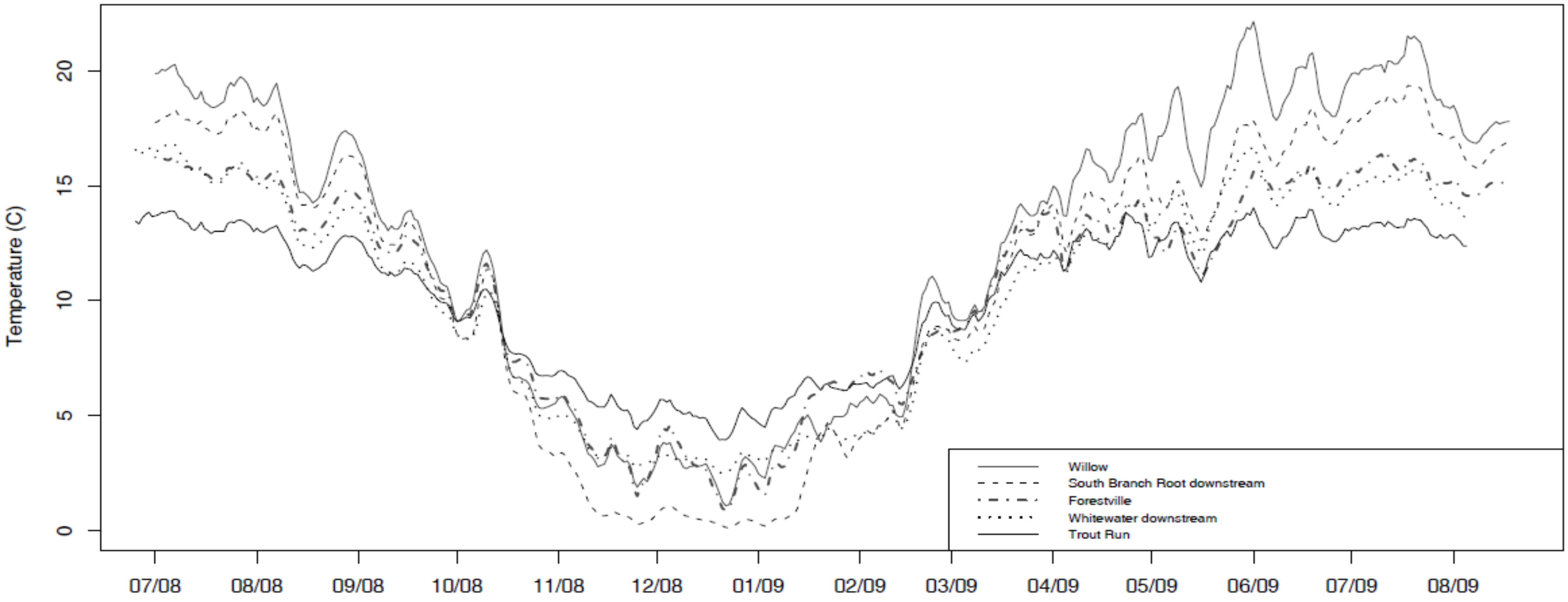
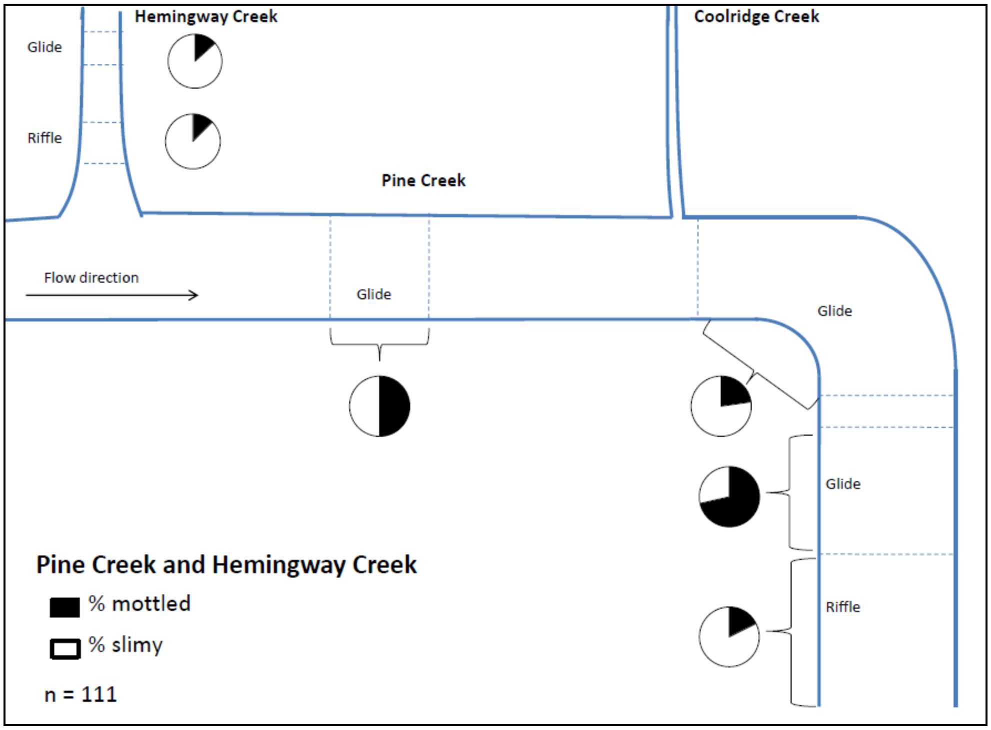
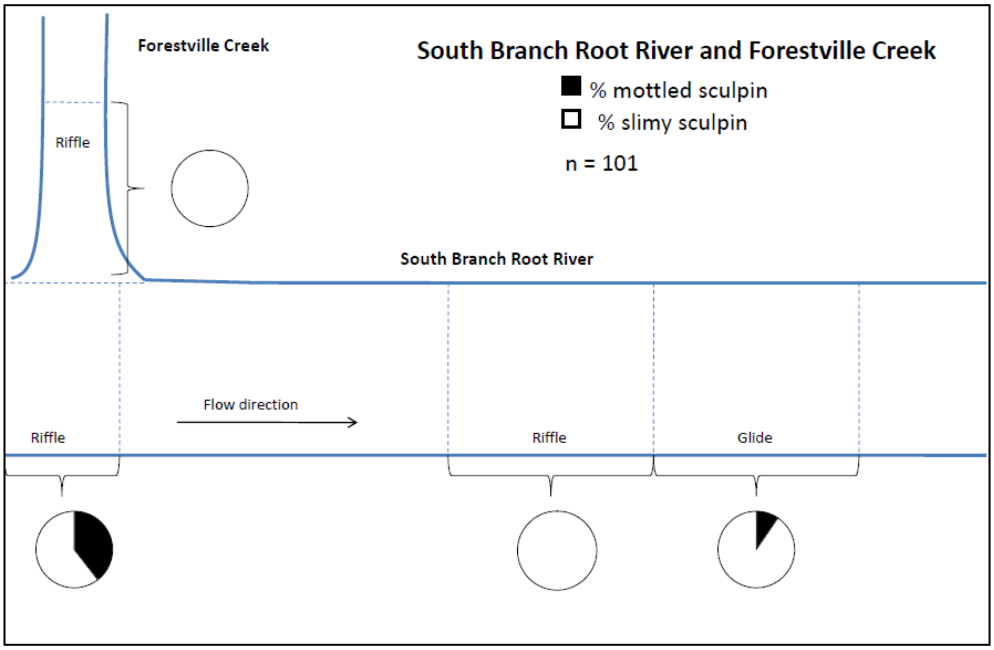
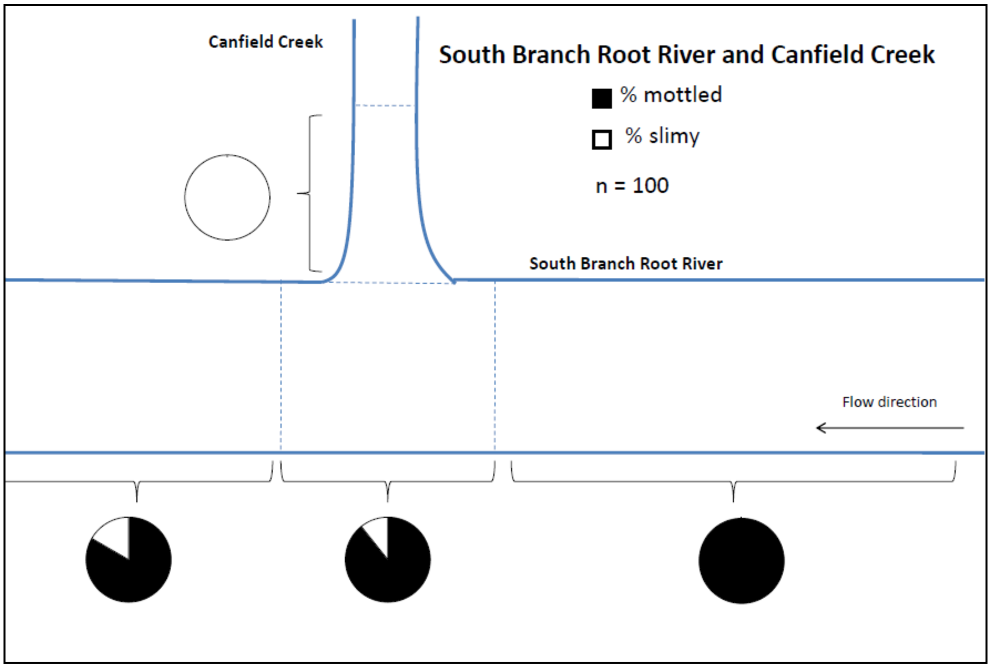


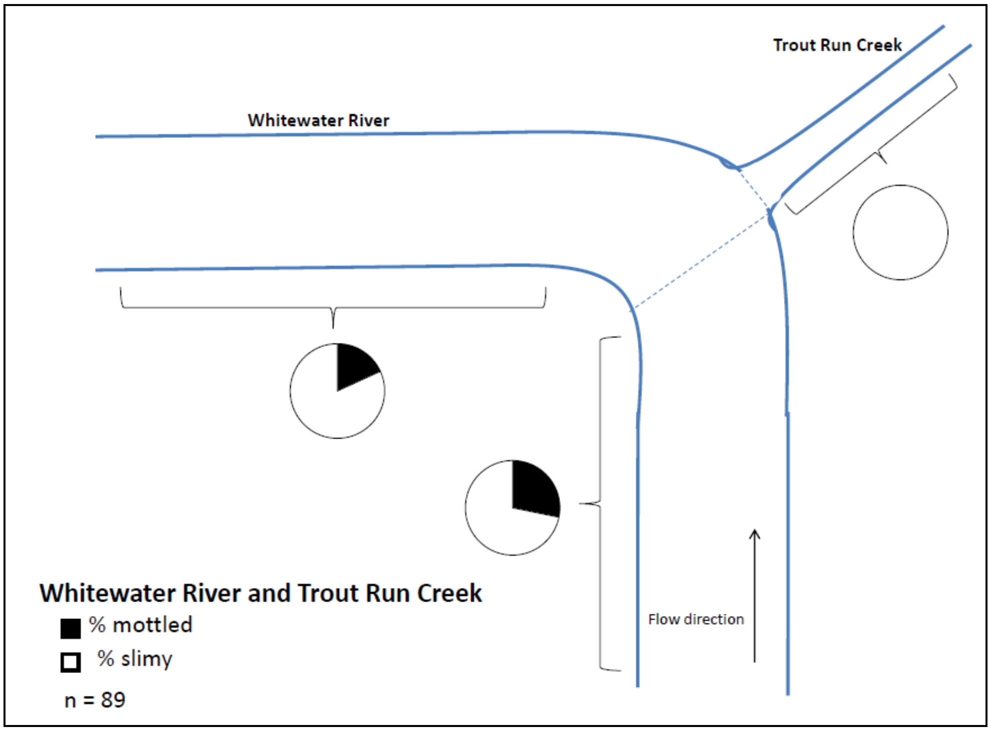
Figure 3. Seven day running mean of maxima at syntopic and reference sites. Forestville and Trout Run are tributaries of syntopic sites that function as *C. cognatus* reference sites and Willow is the *C. bairdi* reference site. South Branch Root downstream and Whitewater downstream are temperatures from the mainstem of syntopic sites downstream of the confluence.

Appendix I. Diagrams representing fine scale species distributions within each syntopic site.

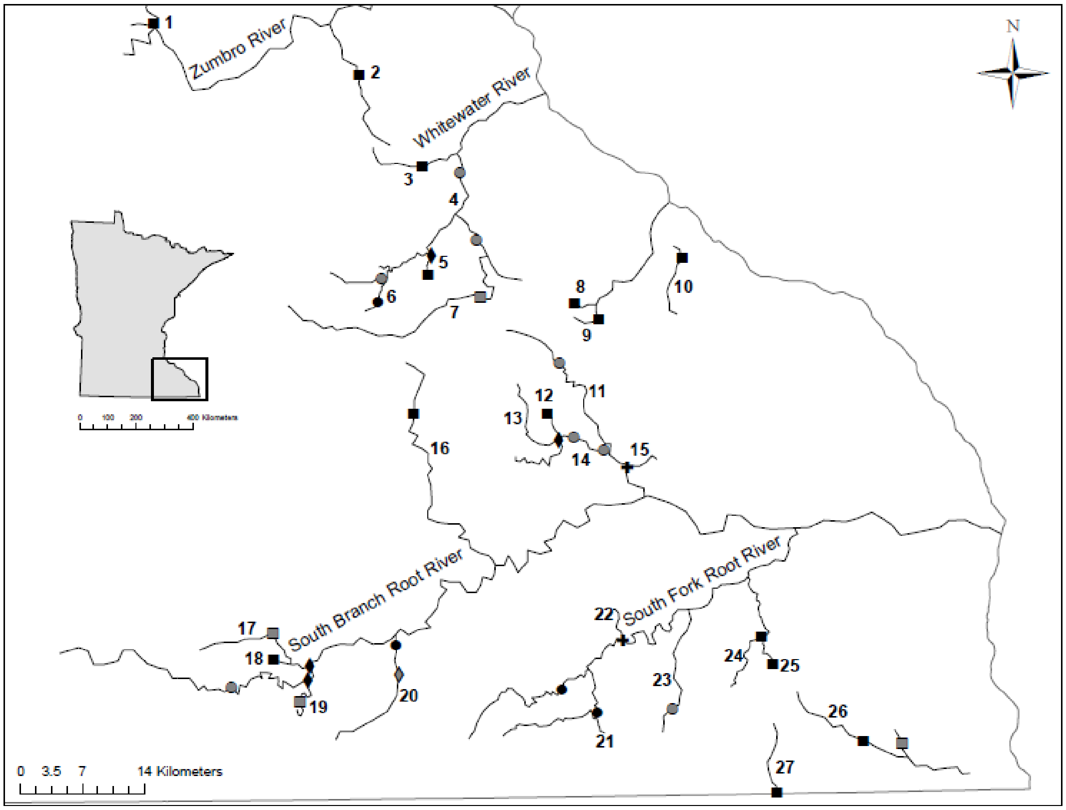





Canfield Creek
South Branch Root River and Canfield Creek
■ % mottled
□ % slimy
n = 100
South Branch Root River
Flow direction


Trout Run Creek
Whitewater River
Whitewater River and Trout Run Creek
■ % mottled
□ % slimy
n = 89
Flow direction

Appendix II. Map of study area with streams identified by number. Stream names are provided in the table below the map.



ID #	Stream name	ID #	Stream name
1	Cold Spring Brook	15	Schueler Creek
2	West Indian Creek	16	Trout Run
3	Beaver Creek	17	Tributary to Forestville Creek
4	Middle Fork Whitewater River	18	Forestville Creek
5	Trout Run Creek	19	Canfield Creek
6	Crow Spring	20	Willow Creek
7	South Fork Whitewater River	21	Wisel Creek
8	Peterson Creek	22	Maple Creek
9	Garvin Brook	23	Riceford Creek
10	Gilmore Creek	24	West Beaver Creek
11	Rush Creek	25	East Beaver Creek
12	Coolridge Creek	26	Winnebago Creek
13	Hemingway Creek	27	Bee Creek
14	Pine Creek		

Chapter 2

Low founder numbers in reintroduced populations of slimy sculpin (*Cottus cognatus*) in southeastern Minnesota streams

Introduction

Sculpin (family Cottidae) are benthic fish native to coldwater streams in southeastern Minnesota. These non-game fish are ecologically important in coldwater ecosystems and provide a potential high energy food source for large trout (Dineen 1947). In the late 1800's southeastern Minnesota was heavily developed for agriculture and poor land use practices presumably led to the degradation of many of the area's streams (Waters 1977). High levels of sedimentation as well as warming water temperatures caused by nutrient run-off and denuded stream banks created conditions unsuitable for sculpin, which were likely extirpated from many streams. Efforts to restore habitat integrity and to mitigate impacts of land management practices improved conditions enough that many streams support coldwater species once again. Sculpin are not highly mobile and, as such, have limited dispersal capabilities and are easily deterred by stream obstructions (Utzinger et al. 1998). In 2003 the Minnesota Department of Natural Resources (MNDNR) started a reintroduction program aimed at restoring sculpin populations throughout their native range in southeastern Minnesota (MNDNR 2003). There are two species of sculpin in southeastern Minnesota, slimy sculpin (*Cottus cognatus*) and mottled sculpin (*Cottus bairdi*). The reintroduction program stated that no attempt would be made at treating these species separately, in part because it is difficult to tell them apart in the field. However, all source streams used for reintroductions were subsequently verified to have only *C. cognatus*. Potential recipient streams were identified based on the absence of sculpin in the system and the suitability of the coldwater habitat. Three source streams were used for the reintroductions, and a mixture of the strains was used to stock each stream. Approximately 150 fish were stocked at each event, and streams were stocked annually for one to three years.

In 2005 research began at the University of Minnesota to study the overall ancestry, changes in genetic diversity, and fitness in reintroduced sculpin populations (Huff 2010). Results of this work showed that strains contributed differently to the overall ancestry, that the genetic diversity potentially achievable by mixing the three source strains was not attained, and fitness-related traits significantly declined in second generation descendents of the founders. The patterns of genetic diversity observed in reintroduced populations could be attributable to differential overwinter survival of stocked fish from different strains, variation in reproductive success among survivors, or viability of offspring based on ancestry. Since Huff began his research of reintroduced populations one to two generations after stocking events, and he did not have samples of the founding individuals, he was unable to determine which mechanisms contributed to the changes in diversity he observed. We continued the study of sculpin reintroductions by examining the dynamics of survival and reproduction during the first year of population establishment and how they affect genetic diversity.

One main objective for reintroductions is to ensure sufficient numbers are stocked to establish genetic diversity that can allow for long term persistence of a population. Bottlenecks created by small effective population sizes (N_e) can lead to a loss of genetic information and subsequent loss of fitness (Allendorf and Luikart 2007). Franklin (1980) created the 50/500 rule which can be used as a rough guideline for N_e required to sustain populations over the short term (N_e = 50) or the long term (N_e = 500). In naturally reproducing populations N_e is often less than census size because of nonrandom family sizes and unequal sex ratios (Allendorf and Luikart 2007); therefore excess fish may need to be stocked to achieve N_e goals.

Here, we use microsatellite DNA genotyping and parentage assignment techniques to follow reproduction dynamics in reintroduced populations of sculpin in the first year after stocking. Our objectives were as follows: 1) to determine the number of offspring contributed by each stocked founder fish in its first year of spawning, 2) to determine relative reproductive success of source strains, and 3) to determine genetic diversity of offspring as compared to the stocked fish.

Methods

We used two source strains to provide stock for reintroductions conducted in the fall of 2007: Garvin Brook and Cold Spring Brook. Fish from source streams were collected by electroshocking the day of the stocking event. We weighed and measured each individual and took a fin clip for genetic analysis and preserved the clip in 95% ethanol. Ages of captured fish were unknown, but we avoided young-of-year fish to ensure that the fish stocked were sexually mature (Huff 2010). Once data were recorded and fin clips collected; fish were sorted into two groups, one for each intended reintroduction stream, where the proportions of each source strain were approximately equal.

The two streams selected for reintroduction were Rock Creek and Hay Creek, both spring-fed streams in the Mississippi River drainage. Both streams had been used for reintroductions as part of the original reintroduction plan. We chose to use new reaches in streams previously used for sculpin reintroductions because we knew sculpin did well in those systems, and research conducted on the original introductions provided a control for how source strains performed in each stream (Huff 2010). The reaches selected for the fall 2007 stocking were considered a sufficient distance from original sites to prevent mixing of populations based on current knowledge of sculpin dispersal distances, and the prospective sites were electroshocked with a backpack electrofisher on the day of stocking to test for the presence of sculpin. Rock Creek was stocked with a total of 66 individuals, 30 from Garvin Brook and 36 from Cold Spring Brook. Hay Creek was stocked with a total of 74 individuals, 36 from Garvin Brook and 38 from Cold Spring Brook.

In the fall of 2008 we returned to Rock and Hay Creeks and electroshocked each stream to collect the offspring of our stocked fish. We started shocking downstream of the stocking site to find the downstream boundary of the population and shocked upstream until no fish were collected. Our target number of offspring to collect was 250 individuals, to ensure representation of small families in our samples. We captured 363 offspring at Rock Creek and 330 offspring from Hay Creek. We took fin clips from every young-of-year fish captured and fixed the tissue in 95% ethanol.

DNA was extracted from all fin clips with 5% Chelex® (Sigma Chemical) solution. Subsamples of tissue were placed in solution, incubated overnight at 56 °C, and then boiled for 8 minutes. We amplified DNA at 12 polymorphic microsatellite DNA markers using the polymerase chain reaction (PCR). The markers used for this study were *Cgo*18, *Cgo*42, *Cgo*310, *Cgo*1033 (Englebrecht et al. 1999), *Cba*14 (Fiumera et al. 2002), *Cott*ES1 (Nolte et al. 2005), *Cco*01, *Cco*09, *Cco*10, *Cco*14, *Cco*15, and *Cco*17 (Fujishin et al. 2009). PCR reactions were performed in 15 µl volumes consisting of 5 µl DNA template, 1X GoTaq® DNA polymerase buffer, 3.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.15-0.5 µM of each primer, 0.5 U GoTaq® DNA polymerase (Promega), and water to the final reaction volume. Thermocycler conditions for amplification were a 3 minute denaturation at 95°C, 35 cycles of 95°C for 30 seconds, 50 °C or 55 °C for 30 seconds, and an extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were then pooled by individual and analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems). Alleles were scored at each locus using GeneMapper 4.1 software (Applied Biosystems). Once genotypes were obtained for all individuals, we used the program Cervus 3.0.3 (Kalinowski et al. 2007) for parentage analysis. Although Cervus uses likelihoods incorporating mutation probabilities to assign parentage, we included only those assignments with zero mismatches for this analysis. We calculated exclusion probabilities for each of our source strain populations to determine the probability of being able to uniquely determine parentage of individual fish. Exclusion probabilities for the Cold Spring Brook strain using the 12 microsatellite markers were 97.8% for a single parent and >99.9% for a parent pair. Exclusion probabilities for the Garvin Brook strain were 98.1% for a single parent and >99.9% for a parent pair.

Common measures of diversity within populations are allelic diversity and heterozygosity. We calculated allelic diversity for the stocked fish, treating them as one population. We then calculated allelic diversity for the offspring that assigned to two stocked parents to observe any changes in diversity between the stocked fish and their offspring. We did not use heterozygosity as a measure of diversity. Heterozygosity is less sensitive to changes in diversity due to founder effects, as up to 75% of heterozygosity can be retained in populations that go through the most extreme bottleneck of only one mating pair (Stockwell et al. 1996, Allendorf and Luikart 2007). In addition, our reintroduced populations would be biased towards high levels of heterozygosity due to the mixing of two strains and their unique alleles and allele frequencies.

Results

Initial parentage analysis revealed the presence of immigrants from original reintroductions at both of the new reintroduction sites, therefore not all offspring captured were attributable to our stocked individuals. However, we discovered that a higher proportion of offspring assigned to stocked individuals from fish collected upstream of the stocking site. We genotyped and analyzed 92 and 112 offspring collected from the upstream sections of Rock Creek and Hay Creek, respectively. The results in the remainder of this paper deal only with the fish genotyped from the upstream sections.

In Rock Creek, we had 38% assignment of genotyped offspring to stocked parent pairs. That percentage increased to 51% when offspring that assigned to a single parent were included (i.e., one stocked parent and one unknown immigrant parent). In Hay Creek, we had 40% assignment of genotyped offspring to stocked parent pairs. That percentage increased to 51% when single parent assignments were included.

In Rock Creek we identified five families, and in Hay Creek we identified six families, where both parents were stocked individuals (Figure 1). In some cases, we were able to identify males because there were individuals that contributed to more than one family. In both streams, we found two males that each mated with two different females. In addition, in both streams one of the males that mated with two females was the male responsible for the largest family. The large family in each stream represented the majority of the offspring assigned to two parents, 74% and 52% in Rock and Hay Creek, respectively. However, due to the presence of immigrant offspring these families were responsible for only 28% and 21% of all offspring genotyped. All families in Rock Creek were crosses between Garvin and Cold Spring strains. No pure strain crosses of either Garvin or Cold Spring were found. In Hay Creek, five families were pure strain crosses of the Cold Spring strain and two families were crosses between Garvin and Cold Spring individuals (Figure 1).

Total founder numbers in Rock Creek and Hay Creek were 14 and 19, respectively, resulting in only 21% and 26% of stocked individuals contributing offspring in the respective streams (Table 1). In Rock Creek, 33% of the Garvin individuals stocked and 11% of the Cold Spring individuals stocked contributed offspring in our samples. In Hay Creek, we saw the opposite pattern where 14% of the Garvin individuals stocked and 37% of the Cold Spring individuals stocked contributed offspring. This differential contribution of source strains is apparent in the overall ancestry of offspring that assigned to either a parent pair or a single parent. In Hay Creek, Cold Spring Brook ancestry was predominant (95%), whereas Garvin Brook ancestry was more prevalent in Rock Creek (Figure 2).

Allelic diversity was severely reduced in offspring at both reintroduction locations. The stocked individuals at Rock Creek had an average of 11.0 alleles per locus, whereas offspring had an average of 5.6 alleles per locus, representing a 49% loss of allelic diversity. In Hay Creek, we observed the same pattern; stocked individuals had an average of 10.6 alleles per locus, whereas the offspring had an average of 6.9 alleles per locus, representing a 35% loss of allelic diversity. A comparison of allelic diversity between stocked populations (those fish stocked into Rock Creek versus the fish stocked into Hay Creek) revealed a 4% difference in the number of alleles. The individuals stocked into Hay Creek did not have 9 alleles present in the Rock Creek fish, but had 4 alleles that Rock Creek fish did not; a total difference of 5 alleles.

Discussion

Our results revealed severely reduced genetic diversity in the offspring of our stocked individuals. In Rock and Hay Creeks there was evidence that a series of events could have led to this result. First, differential success of each source strain curtailed the overall success of reintroduced fish since each source did not contribute equally to the next generation and the full genetic potential of stocked individuals was not realized. Second, we found a very small number of founders, which could have been exacerbated by differential strain success. Third, nonrandom family sizes led to an unequal contribution by reintroduced individuals which would make N_e much lower than the number of founders. All three factors acted as a bottleneck to limit the amount of genetic diversity passed on to the next generation. The negative impacts of small N_e are well documented and can include accelerated rates of genetic drift, loss of allelic diversity that can reduce adaptive capabilities of a population, and an increased rate of inbreeding that could cause deleterious alleles to be expressed (Allendorf and Luikart 2007). The reasons for the reductions in genetic diversity in our reintroduced populations of *C. cognatus* need to be understood to prevent genetic bottlenecks and to increase the chance of population persistence.

We found evidence for differential strain success in reintroduction streams, as a large percentage of fish from one strain did not contribute in each of the streams. Often in reintroduction biology the use of multiple strains is considered a positive strategy, as a mixture has the potential of increasing genetic diversity and adaptive potential in new populations (Lesica and Allendorf 1999). However, this strategy is not encouraged for species known to have large genetic differences between populations, as mixing could lead to outbreeding depression in the new population (Stockwell and Leberg 2002). Sculpin are not highly mobile animals and research has revealed that they have well-developed population structure (Meyer et al. 2008). Each population could have local adaptations that would contribute to the success or failure of a particular strain in a new environment. We found that the Cold Spring Brook strain was more reproductively successful in Hay Creek than the Garvin Brook strain. This finding is in agreement with that of Huff (2010) where he found Hay Creek to be the only sculpin reintroduction stream of nine locations where the Cold Spring Brook strain was present in numbers higher than expected based on stocking proportions. In Rock Creek, we observed that the Garvin Brook strain was more successful than the Cold Spring Brook strain which is also in agreement with the findings of Huff (2010). Cold Spring Brook fish could be better adapted to the conditions in Hay Creek, leading them to succeed over the Garvin Brook fish; whereas Garvin Brook fish may be suited to the Rock Creek environment. However, in Rock Creek, all parental pairs of stocked fish represented a cross between strains, which could affect the longevity of the population, especially if strain differences have a negative impact on fitness in advanced generations. There is evidence that strains do better in conditions similar to those found in their respective source streams (Huff 2010), and Leberg (1993) postulated that matching environmental conditions between sources and reintroductions sites could be more important than managing the genetic background of reintroduced individuals. A reintroduction plan that chooses source populations based on environmental conditions could increase the chance of reintroduction success.

Matching environments may increase the chance of reintroduction success, but the amount of genetic information available should be sufficient to allow for population persistence. The small number of *C. cognatus* we used for reintroductions could have led to small N_e. The difference in allelic diversity between the two groups of stocked fish demonstrated that our sample size was not large enough to capture the full extent of genetic information available in the source population. Our small sample sizes increased the probability of a random skew in sex ratios. Unequal sex ratios can cause bottlenecks in a population, as the limited sex disproportionately contributes genetic information to the next generation (Allendorf and Luikart 2007). Identification of sex for live sculpin is difficult, therefore the most practical approach to this problem would be to ensure sample size was large enough to be representative of the source population, as there tends to be equal sex ratios in stable sculpin populations in southeastern Minnesota (D. Huff, Dept. of Fisheries, Wildlife, and Conservation Biology, University of Minnesota, unpublished data).

A third contributor to small N_e is nonrandom family size. Skewed contributions of individuals in naturally reproducing fish populations is well documented and has been demonstrated in reproduction studies for Nile tilapia, *Oreochromis niloticus* (Fessehaye et al. 2006), black sea bream, *Acanthopagrus schlegelii* (Jeong et al. 2007), and sole, *Solea solea* (Blonk et al. 2009). The reproductive ecology of sculpin could contribute to skewed contribution by individuals, as they are polygamous (Fiumera et al. 2002), demonstrate size dependent mate selection (Downhower and Brown 1980), and are often highly territorial and competitive (Petty and Grossman 2007). Large males are also more likely to mate with multiple females than are their smaller counterparts (Brown 1981), which can lead to one male being responsible for a high number of offspring, as we observed.

Unfortunately, our observations are confounded by the presence of immigrants. We do not have a clear picture of what happens to diversity in small, transplanted populations because of the genetic input from immigrants. Our analysis found individuals from the newly stocked population mating with those fish already established. We did not genotype all offspring captured, so we do not know how many stocked individuals mated with immigrants and the percentage of offspring that were attributable to established individuals only. However, we have retained all offspring samples and they could be genotyped in the future to further understand the contribution of stocked individuals when placed in a location where a population already exists. Competition with the established *C. cognatus* could have led to fewer contributions by the stocked individuals, which could have led to our low founder numbers. We may have also missed some founders due to the small offspring sample size we genotyped in this study. We initially captured offspring in excess of our target number. After detecting the problem of immigrants, we determined genotyping all of our offspring samples was inefficient. Had we known so many immigrants were present, especially in lower reaches, we could have increased sampling intensity in upper reaches and increased the number of assigned offspring, perhaps detecting new small family groups. Although the immigrants were problematic for the aim of our study, we can learn from the fact that our stocked individuals mated with immigrant individuals. This intermating can increase genetic diversity, which could make supplementary stocking an effective way to mitigate founder effects and perhaps prevent long-term consequences of bottlenecking.

Additional research is needed to see if the patterns we observed occur in isolated reintroduction populations. Sampling an isolated reintroduction site over multiple years could also provide valuable information on the lifetime reproductive contribution of stocked individuals. In addition, a single strain could be stocked and a similar study to this one conducted to isolate the impact of reproductive behavior on genetic diversity, without the complication of strain effects.

The MNDNR *C. cognatus* reintroduction project can be used to fill a gap in the field of reintroduction biology by allowing for an experimental approach to the study of reintroductions, rather than a retrospective one, as suggested by Seddon et al. (2007). Sculpin allow for a degree of manipulation not possible in endangered populations and have shared reproductive traits with some endangered minnow species (family Cyprinidae), such as the Topeka shiner, *Notropis Topeka* (Stark et al. 2002). There are 139 fish species listed as either threatened or endangered, over half of which are cyprinids (USFWS 2010), and the lessons we learn from sculpin reintroductions may apply to recovery efforts for other species. Furthermore, sculpin also share reproductive traits with centrarchids, which include commonly stocked game fish. The preliminary results of our work demonstrate that naturally reproducing reintroduced populations of *C. cognatus* could be susceptible to genetic bottlenecks based on their reproductive traits. Fiumera et al. (2002) found that effective number of breeders in another freshwater sculpin species was an order of magnitude smaller than the number of potential breeders. Therefore, large numbers of *C. cognatus* would have to be introduced to mitigate the effects of nonrandom family sizes and mate selection, and differential success of strains in each stream may be indicative of a need to match the environment of the source stream to recipient streams to increase the chance of reintroduction success. Additional work on reintroduction sites will further illuminate the population dynamics that led to our observations.

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Table 1. Number of individuals stocked versus the number of individuals that contributed offspring in each recipient stream by strain.

	Stocked	Parents in a pair	Single parents	Total founders
Rock Creek				
Garvin	30	4	6	10
Cold Spring	36	4	0	4
Hay Creek				
Garvin	36	1	4	5
Cold Spring	38	9	5	14

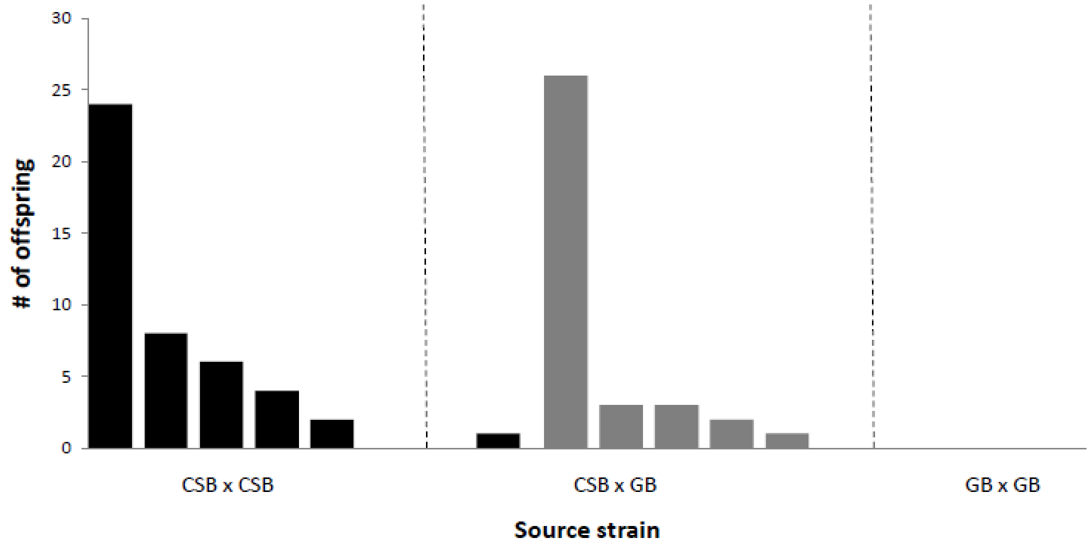

Figure 1. Number of families in reintroduced populations where both parents were stocked individuals. Each histogram bar represents one family. Black bars represent families in Hay Creek and gray bars represent families in Rock Creek. Strain of each mating pair is indicated on the x-axis, and the number of offspring attributable to each family is indicated on the y-axis. CSBxCSB represents a cross between two Cold Spring Brook individuals, CSBxGB is a cross between one Cold Spring Brook and one Garvin Brook individual, and GBxGB is a cross between two Garvin Brook individuals.

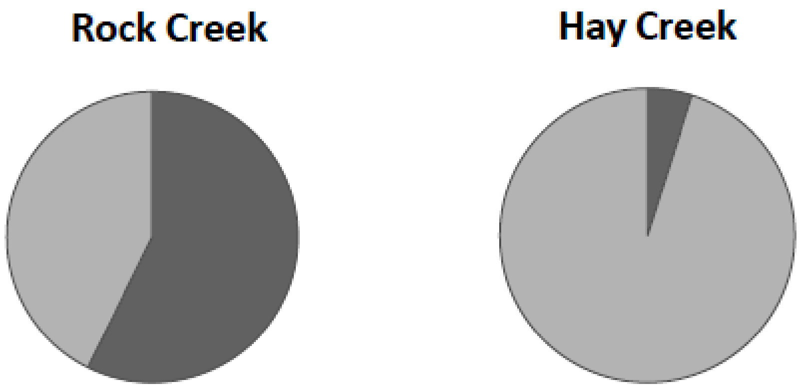

Figure 2. Overall ancestry in offspring for each reintroduction stream. Light gray represents the Cold Spring Brook strain, and dark gray represents the Garvin Brook Strain. Ancestry in Rock Creek was 43% Cold Spring Brook and 57% Garvin Brook. Ancestry in Hay Creek was 95% Cold Spring Brook and 5% Garvin Brook.

Chapter 3

Isolation of 13 polymorphic microsatellite loci for slimy sculpin (*Cottus cognatus*)

Slimy sculpins (*Cottus cognatus*) are small, freshwater, benthic fish widely distributed across Northern North America and a small area in Eastern Siberia. Their sedentary nature renders *Cottus* species ideal for population studies, and research on multiple species has revealed well-developed population structure (Meyer et al. 2008, Englbrecht et al. 2000). *Cottus* species have recently been indicated as ideal study organisms for investigating the early stages of speciation (Sweigart 2009) and there has been considerable interest in the behavior, ecology, phylogeography and conservation of these species (Adams and Schmetterling 2007). Current conservation efforts in Minnesota (USA) have included reintroducing *C. cognatus* into streams historically impacted by heavy agricultural land-use in order to rehabilitate the native fish fauna. Restoration activities have given researchers the opportunity to examine the consequences of local adaptation on the fitness of restored populations and aspects of genetic diversity indicators when multiple source populations are mixed. Currently available genetic markers are sufficient to differentiate *C. cognatus* populations used as sources (L Miller, unpublished data); however, these microsatellite loci were developed for other *Cottus* species (Nolte et al. 2005; Fiumera et al. 2002; Englebrecht et al. 1999) and generally had low variation within *C. cognatus* populations. Our goal was to develop additional highly-polymorphic loci to aid in parentage assignment for a study of factors that contribute to reproductive success at reintroduction sites.

Genomic DNA was extracted from *C. cognatus* organ tissue using a DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions. We constructed libraries enriched for microsatellite repeats using a protocol developed by Glenn and Schable (2005). To summarize, we digested the genomic DNA with *BstU I* and *Xmn I* (New England Biolabs) then ligated SuperSNX linkers (Forward: 5'GTTTAAGGCCTAGCTAGCAGAATC; Reverse: 5'GATTCTGCTAGCTAGGCCTTAAACAAAA) to the digested fragments. We hybridized our DNA with a mixture of biotinylated oligonucleotide probes [$(AGAT)_8$; $(AAGT)_8$; $(ACAT)_8$; $(ACCT)_6$; $(ACAG)_6$; $(ATC)_8$; $(AAC)_6$; $(AAG)_8$; $(ACT)_{12}$; $(AGC)_6$] under high stringency conditions (52°C). Hybridized sequences were captured using Dynabeads® M-280 Streptavidin (Invitrogen), and then recovered through the polymerase chain reaction (PCR) with SuperSNX forward primer.

We used a pGEM®-T vector system (Promega) to clone the PCR-amplified enriched DNA pool. After plating and incubation on Ampicillin/X-Gal/IPTG LB agarose, putative transformants were identified by blue-white screening and 672 colonies were picked and PCR amplified. Amplified inserts were sized on 1% TBE agarose gels, and fragments larger than 550 base pairs (indicating inserts large enough to contain a repeat and flanking sequence for primer design) were identified. We submitted PCR products from 130 colonies to the BioMedical Genomics Center (University of Minnesota, St. Paul, MN) for sequencing using BigDye v3.1 chemistry and an ABI 3730xl DNA Analyzer (Applied Biosystems). Although a large percentage of sequences contained repeats, many were duplicated or had short or poor quality flanking sequence so they were unusable for primer design. We designed primer pairs from 19 sequences using the program Primer3 v. 0.4.0 (Rozen and Skaletsky 2000). Amplification with our primers yielded 13 unique, polymorphic microsatellite loci (Table 1). Two loci (*Cco04* and *Cco08*) were scorable through separation on 8% acrylamide gels followed by ethidium bromide staining and visualization on a UV light table. We obtained fluorescently labeled forward primers for six loci and attached M13 tails to the forward primer of the remaining five loci according to Boutin-Ganache et al. (2001).

All loci were characterized using 47 *C. cognatus* from a single population. We extracted DNA from fin clips fixed in ethanol by mixing each with 300 µL of 5% Chelex® (Sigma Chemical) solution, incubating samples overnight in a water bath (56°C) then boiling for 8 minutes.

Amplifications for *Cco04* and *Cco08* as well as the fluorescently labeled loci were performed in 15 µL reaction volumes consisting of 5 µL DNA template, 5X GoTaq® DNA polymerase buffer with 1.5 mM $MgCl_2$ (Promega), 0.2 mM each dNTP, 0.33 µM of each primer, 0.5 U GoTaq® DNA polymerase, and water to the final reaction volume. In some cases additional $MgCl_2$ was needed up to a final concentration of 2.5 mM. Amplifications using the M13 tailed primers were carried out in 15 µl reactions according to Boutin-Ganache et al. (2001) with the exception that the molar ratio between the M13 labeled primer and the M13-tailed forward primer was 4:1 instead of 15:1. Primer concentrations were as follows: 0.4 µM M13 labeled primer, 0.4 µM reverse primer, and 0.10 µM M13-tailed forward primer. Concentrations of all other reagents agreed with those conditions used for the other eight loci. Thermocycler conditions for amplification were a 3 minute denaturation at 95°C, 35 cycles of 95°C for 30 seconds, primer annealing temperature (Table 1) for 30 seconds, and an extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products from the labeled and M13-tailed loci were resolved on an ABI 3730xl DNA Analyzer, and alleles sized using Genotyper 2.5 and LIZ 500 size standards (Applied Biosystems).

Our 13 microsatellite loci had 2 to 22 alleles and observed heterozygosities ranging from 0.36 to 0.86 (Table 1). We used the program HW-Quick Check (Kalinowski 2006) to calculate observed and expected heterozygosities and to detect deviations from Hardy-Weinberg Equilibrium (HWE). One locus (*Cco 07*) deviated from HWE, with evidence for a null allele according to the program MicroChecker (Oosterhout et al. 2004). We found no evidence of linkage disequilibrium after Bonferroni correction using GENEPOP v4 (Raymond and Rousset 1995).

We also amplified all loci using seven individuals from two populations of mottled sculpin (*Cottus bairdi*), the only other *Cottus* species found in our study region. We used the same PCR conditions applied to *C. cognatus* except for an annealing temperature of 50°C for all loci. Eight of the loci were variable for *C. bairdi*, four loci appeared fixed, and one did not amplify (Table 2). Several of the loci have non-overlapping allele size ranges, at least for these few samples, and may prove useful for genetically confirming the identity of these morphologically similar species.

These new polymorphic microsatellite loci will be useful for investigations of *C. cognatus* population structure and processes. The amplification success of these markers on another species demonstrates that they may also be useful for research on other *Cottus* species.

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Table 1. Characteristics of 13 microsatellite loci for *Cottus cognatus* (Beaver Creek, Minnesota, USA; n = 47). Shown are repeat motifs, primers, annealing temperatures (T_A), number of alleles per locus (N_A), observed and expected heterozygosities (Ho and He, respectively), and accession numbers for clone sequences.

Locus	Repeat motif	Primer sequences (5'-3')	Size range (bp)	T_A (°C)	N_A	H_o	H_e	Genbank Accession no.
Cco01	(AAG)$_{13}$(AAC)$_2$(AAG)$_8$AAC(AAG)$_7$	F:AAAGTTGTGAAAAGACATAATGG R:CCAAGGGGATTAATAAAGTATACC	243-327	50°C	13	0.77	0.77	GQ341572
Cco02	(AAG)$_{14}$	F:TTCTTGTTCTCCGTCTTGAGC R:CCCATCTTCTCCTCCTGTCC	263-278^a	50°C	5	0.68	0.66	GQ341573
Cco04	(AAG)$_{15}$	F:GGGTTGAATTTGCAATCTGG R:TTAATTTCAGGCAGGATCACG	79-160	46°C	4	0.72	0.70	GQ341574
Cco07	(CT)$_{33}$	F:ATTCTCTTCGGTATACACGG R:AATCAACCAAACCCTGCTGG	204-260^a	55°C	12	0.67	0.86*	GQ341575
Cco08	(AAG)$_7$	F:TTGCAAACTTCAGACAGTAAAGC R:GCTGAGAATCCAGGAAGGAG	98-110	55°C	3	0.59	0.60	GQ341576
Cco09	(AAG)$_{24}$	F:GGAGAAGAACACAGTAAACAAATTC R:TCTTCGTTGGCGGTTTTAAG	252-330	55°C	22	0.80	0.82	GQ341577
Cco10	(AAG)$_{18}$	F:GACCCTTGCCCTGAATCG R:AGCTCTGAACCGCCACAC	122-185	55°C	9	0.68	0.78	GQ341578
Cco11	(TG)$_{16}$	F:GCAGGAGGAACACGAAGATG R:CTCAAGGAACTACACACACATGC	220-232^a	55°C	2	0.41	0.36	GQ341579
Cco13	(ATG)$_{11}$	F:CCTGGAATTTCACCAAGGTC R:TCACAACAAAGCCAGAGGAC	246-258^a	55°C	5	0.48	0.47	GQ341580
Cco14	(ACAG)$_7$	F:CATAAAACCTGTGGCTTTGG R:GACGCTCTGCTGGAGAGATG	167-191	55°C	6	0.72	0.78	GQ341581
Cco15	(AAG)$_{15}$	F:TTGGCACATTGTGGAGACTG R:TGGAAAAGTTGTTCTTGTAACG	164-227	55°C	11	0.83	0.86	GQ341582
Cco16	(AC)$_9$n14(ACACACAT)$_5$n4(ACAT)$_{16}$	F:GTTGCTTCCTCTTTGTGGTTG R:TCACACAAAAGACCTACAAGGAC	220-260^a	50°C	8	0.72	0.79	GQ341583
Cco17	(AAG)$_{30}$	F:TCGTCTTGGAAATGGAAAGC R:CATGTCAGCAGGATATCACGTC	108-126	55°C	6	0.79	0.81	GQ341584

[a] Loci amplified with an M13-tailed primer. Product size is reduced by approximately 18 base pairs if fluorescently labeled primers are used.

*Deviates from Hardy-Weinberg Equilibrium

Table 2. Amplification of 13 *Cottus cognatus* microsatellite loci for seven *Cottus bairdi*.

Locus	Amplified	Product size range
Cco01	yes	270-300
Cco02	yes	160-166
Cco04	no	na
Cco07	yes	172-224
Cco08	yes	80
Cco09	yes	210-249
Cco10	yes	120
Cco11	yes	204
Cco13	yes	255-264
Cco14	yes	264
Cco15	yes	171-180
Cco16	yes	260-300
Cco17	yes	99-132

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