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Assessing the Genetic Status and Factors Leading to the Decline of the Roanoke Bass (Ambloplites Cavifrons)

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ASSESSING THE GENETIC STATUS AND FACTORS LEADING TO THE DECLINE OF THE ROANOKE BASS (*AMBLOPLITES CAVIFRONS*)

by

JACKMAN ESCHENROEDER

(Under the direction of James H. Roberts)

ABSTRACT

Although numerous factors have led to the staggering declines in freshwater biodiversity throughout the United States and the world, habitat alteration and introduced species pose some of the greatest challenges to conservation efforts. Learning more about how these two factors lead to the decline of an endemic organism could help prevent the future loss of unique species and the premature conclusion of evolutionary trajectories. Roanoke bass (*Ambloplites cavifrons*) is a sport fish endemic to portions of the Roanoke, Chowan, Tar, and Neuse river basins of North Carolina and Virginia. This species has been in decline for many years, and it is believed that their continued existence is threatened by competition, and potentially hybridization and introgression with their introduced relative, the rock bass (*Ambloplites rupestris*). In addition to interactions with this invasive species, significant alteration of habitat is likely also a contributing factor in the decline of *A. cavifrons*. This study seeks to evaluate the relative contributions of these various factors to the decline of *A. cavifrons*. I utilized a combination of nuclear markers and mitochondrial sequence data to address the question of whether or not the two species are hybridizing in areas of syntopy, and furthermore to determine whether hybrids are fertile and able to breed back with the parental species. In addition, I identified extant populations of *A. cavifrons* throughout their historic range, and evaluated the genetic diversity of

these populations and correlated these values with changes to the landscape in the form of alterations to watershed land use and the construction of impoundments. My results indicate large portions of the historic range of *A. cavifrons* no longer contain the species, and that remaining populations occur at the stream level and exist in isolation from one another. Obtaining this information allows for a better understanding of the current state of this unique species, provides information that may help managers prevent its disappearance from its native range, and affords insight into the interactions of an introduced and a native species in a landscape that has been heavily altered by human activity.

INDEX WORDS: Hybridization, Conservation genetics, Invasive species

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by

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GENERAL INTRODUCTION

North American freshwater systems hold an enormous amount of diversity, and are home to 1,213 known species of fish, representing 8.9% of all freshwater fish on Earth (Burkhead 2012). It is likely that the actual species richness is even higher, as there has been no asymptotic decline in the rate of species description (Burkhead 2012). The considerable amount of biodiversity in freshwater systems arises from the propensity for populations to become isolated within different drainages, as well as through behavioral adaptations, both of which lead to a high degree of endemism, and thus irreplaceability of taxa (Allan and Flecker 1993). The diverse biota in these systems are, however, much less visible to the public than are organisms in terrestrial environments. Because of this, dramatic changes in freshwater systems are not as readily apparent as the loss of habitat and imperilment of charismatic species in tropical rainforests, although the degree of imperilment and extinction is similar between these two ecosystems (Riccardi and Rasmussen 1999). In fact, North American freshwater fauna is experiencing an estimated extinction rate of 4% of total diversity per decade, a rate five times higher than that of North American terrestrial fauna (Riccardi and Rasmussen 1999).

According to Dudgeon *et al.* (2006), there are five major factors leading to the decline of freshwater biodiversity: overexploitation, water pollution, modification of flow, destruction and degradation of habitat, and the prevalence of invasive species. In the broad sense, water pollution and modification of flow are inextricably linked factors contributing to the phenomenon of habitat degradation. Researchers have suggested that habitat degradation and invasive species are the main threats driving imperilment of freshwater fish species in North America (Jelks *et al.* 2008). Evidence suggests these factors are also the main threats driving extinction. For example, physical habitat alteration, which includes impoundments,

channelization, and other human created modifications to streams, was cited as a contributing factor in 73% of North American fish extinctions over the past century, while the effects of introduced species were implicated in 68% (Miller *et al.* 1989).

In most incidences of extinction and imperilment of freshwater fish species, more than one factor is believed to play a role, and it can be difficult to evaluate the relative effects of multiple factors (Allan and Flecker 1993). In many cases, the factor that leads to the imperilment of a species only occurred because it was indirectly catalyzed by another factor. This is often the case with introduced species. Many species introductions fail to result in the establishment of an invasive species, and even those that become established may have no apparent negative effects on native species. However, both North American and exotic introduced species can potentially become invasive and drive the imperilment of native species. It is important to clarify that in strictly biological terms, the word "invasive" is an adjective describing organisms that are capable of colonizing and spreading to new areas (Riccardi and Cohen 2007). The physical alteration of stream systems may make them less resistant to invasive species, thereby placing native species at higher risk of imperilment (Dudgeon *et al.* 2006). Studies indicate abiotic conditions are more important that biotic interactions in determining whether an introduced organism will be successful (Moyle and Light 1996), and researchers have suggested that the most detrimental effect of habitat alteration is the tendency of altered habitats to be more hospitable to invasive fishes (Light and Marchetti 2007). That being said, the introduction of a predator or a strong competitor can result in the extirpation of a native species even when the habitat remains relatively intact (Rahel 2002). Currently, the United States Geological Survey Nonindigenous Aquatic Species website (http://nas.er.usgs.gov) reports 150 established introduced freshwater fish species in the South Atlantic-Gulf Region alone,

including non-exotic invaders translocated from different regions within the US. The presence of such "native-invasives" can be just as indicative of habitat degradation as the presence of exotic invasives, and they can be even more detrimental to native species (Scott and Helfman 2001). It is difficult to determine when habitat modification is and is not necessary to facilitate invasion, but the combination of habitat modification and invasive species is likely to have a greater impact than either factor would alone.

Physical habitat degradation is not limited to the direct alteration of streambeds (e.g., the construction of dams), but also includes numerous land use activities. Activities such as timber harvest, livestock grazing, agriculture, and urbanization are the primary causes of altered flow regimes in many rivers (Poff *et al.* 1997). Studies have shown that deforestation of areas surrounding headwater streams leads to excessive nutrient loss from the surrounding terrestrial system (Allan and Flecker 1993), and the reduction of base flows associated with anthropogenic modifications can compound existing water chemistry problems (Walsh *et al.* 2005). These factors contribute to reductions in suitable habitat that may lead to smaller populations with lower genetic diversity, which means populations will have decreased adaptive potential and will be more subject to the effects of demographic and environmental stochasticity (Frankham *et al.* 2010). This increased risk for population collapse is compounded by the fact that anthropogenic barriers can preclude the movement of individuals from one population into another, thereby preventing demographic or genetic rescue (Caughley 1994).

A prime example of a fish species facing such interwoven threats as those described above is the Roanoke bass, *Ambloplites cavifrons* Cope. This species is an increasingly rare sportfish endemic to portions of the Roanoke, Chowan, Tar, and Neuse drainages in Virginia and North Carolina (Cashner and Jenkins 1982). There are four species within the genus

Ambloplites, and *A. cavifrons* is the only one of these four that naturally occurs east of the Appalachians (Roe *et al.* 2008). Its geographically nearest relative *A. rupestris*, the rock bass, was heavily stocked into the upper Roanoke River from as early as 1898 until 1971 (Cashner and Jenkins 1982). Researchers reported the occurrence of *A. rupestris* in several streams throughout the historic range of *A. cavifrons*, and noted that its establishment in the Roanoke River coincided with a precipitous decline of *A. cavifrons* in that system (Jenkins and Cashner 1983).

The major unknown regarding the interaction between these species is whether *A. rupestris* is replacing *A. cavifrons* through competition, through hybridization, or a combination of both. Furthermore, it is unknown if this potential hybridization is resulting in introgression. Genetic introgression refers to the gene flow between two groups (species, subspecies, populations, evolutionary significant units, etc.) that occurs when hybrids backcross with one or both of the parental types (Rhymer and Simberloff 1996). In short, hybridization occurs on an individual level, while introgression occurs on a gene pool level. Hybridization is not invariably accompanied by introgression, as the hybrid individuals may be sterile or of lower fitness in some cases. As such, the frequency of hybridization between any two species is not predictive of the probability of introgression (Keck and Near 2009). Additionally, if introgression is occurring it may be unidirectional, with hybrids backcrossing with only one of the parental groups (Rhymer and Simberloff 1996). Hybridization between an introduced and a native species has been shown to have severe consequences, and may lead to a rapid extinction, particularly if the native species is competitively inferior (Wolf *et al.* 2001). Ascertaining whether hybridization has played a role in the replacement of *A. cavifrons* will provide insight into processes that led to the success of *A. rupestris* as an invasive species.

Conservation genetic techniques provide a more precise description of processes leading to declines than knowledge of the biology of population growth and the natural history of the species could provide on their own. Estimates of parameters such as effective population size and degree of gene flow can be used to more clearly delineate the factors leading to a decline (DeSalle and Amato 2004). Furthermore, demographic values derived from genetic data can inform management decisions by locating the populations most at risk for future decline or extinction (Luikart *et al.* 2010).

This thesis seeks to address four key objectives : 1) Measuring the contemporary distribution of *A. cavifrons* and the extent of *A. rupestris* invasion, 2) Investigating the nature and outcome of interaction between the two species by evaluating the occurrence of hybridization, introgression, and replacement, 3) Estimating the size and status of, and connectivity between extant *A. cavifrons* populations, and testing hypotheses about the natural and anthropogenic factors driving these parameters, and 4) Drawing general conclusions about the factors most responsible for the ongoing decline of *A. cavifrons*, and to make recommendations for management of the species. The first and second objectives are addressed in Chapter 1, in which the extent of hybridization was investigated through the use of data from 11 nuclear microsatellite markers and one mitochondrial gene in *Ambloplites* individuals collected from streams known to harbor only *A. rupestris*, streams known to harbor only *A. cavifrons*, and streams known to currently and/or historically harbor both species. The third objective is addressed in Chapter 2, in which 19 nuclear microsatellite markers and one mitochondrial gene in *A. cavifrons* individuals collected across the entire range of the species were utilized to evaluate the genetic diversity, effective size, and connectivity of *A. cavifrons* populations. Spatial analyses assessing the geographic attributes of inhabited streams and degree of

fragmentation were also implemented in this chapter. The fourth objective is addressed in General Conclusions, in which I discuss how my analysis may be used to inform the design and implementation of management efforts, and provides data that may be utilized as a baseline in future genetic monitoring studies to evaluate changes and monitor the effect of efforts to conserve this species.

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CHAPTER 1

THE EXTENT OF HYBRIDIZATION AND REPLACEMENT OF ROANOKE BASS (*AMBLOPLITES CAVIFRONS*) BY INTRODUCED ROCK BASS (*A. RUPESTRIS*) IN VIRGINIA

ABSTRACT

Hybridization between native and invasive species can precipitate the decline or loss of the native taxon. Such interspecific hybridization is particularly common among freshwater fishes, which have inherently low resistance to hybridization and have been extensively stocked outside of their native ranges. Roanoke bass (*Ambloplites cavifrons* Cope), a sunfish species (Perciformes:Centrarchidae) endemic to the Roanoke, Chowan, Tar, and Neuse River basins of Virginia and North Carolina, have long been believed to hybridize with introduced rock bass (*Ambloplites rupestris* Rafinesque) in areas of syntopy (Jenkins and Cashner 1983). However, the status of *A. cavifrons* throughout its Virginia range has not been evaluated in over thirty years, and molecular techniques have never been used to evaluate the reproductive interactions between the two *Ambloplites* species. The goals of this study were to update the distribution of extant *A. cavifrons* populations in Virginia and to determine the extent and mechanisms of hybridization between *A. cavifrons* and *A. rupestris*. I utilized 504 *Ambloplites* specimens from sites throughout the historic Virginia range of *A. cavifrons*, as well as from two reference *A. rupestris* sites in the New and James basins of Virginia, and two reference *A. cavifrons* sites, one in the Neuse basin of North Carolina and one in the Chowan Basin of Virginia. Specimens were classified as *A. cavifrons*, *A. rupestris*, or hybrid through the use of genetic classification models based on measured variation at 11 microsatellite DNA markers developed for these species. This

analysis revealed relatively few hybrids (n = 15), but these individuals were widely distributed across six of the eight historical *A. cavifrons* populations evaluated in this study. Furthermore, the majority of identified hybrids were post-F_1, indicating that genetic introgression is occurring between the two species. A complementary analysis of mitochondrial DNA supported the findings of the microsatellite analysis, and did not reveal any historical introgression not detected by nuclear markers, nor any trends in directionality of hybrid matings. My findings indicate that *A. cavifrons* has been completely replaced by *A. rupestris* in the Roanoke, Otter, and Staunton river systems. Additionally, widespread hybridization in the Pigg river system suggests that replacement there is imminent. This represents a substantial decline in the Virginia range of the species. Of the streams assessed, *A. cavifrons* appear to persist only in the Blackwater, upper Falling, Smith, and Nottoway rivers. Most extant *A. cavifrons* populations are immediately adjacent to streams harboring *A. rupestris* and thus are at risk for future invasion and introgression. Eradication of *A. rupestris* from streams where they have become established may be infeasible. Therefore, I recommend that conservation efforts focus on the education of anglers and other citizens about the importance of not transporting *Ambloplites* among waterways. The establishment of refuge populations in streams where neither species currently occurs may also be warranted.

INTRODUCTION

Invasive species are one of the major contributors to the decline in biodiversity of freshwater fish in North America (Jelks *et al.* 2008). An introduced species is considered invasive only if it successfully colonizes and spreads to new areas. Many introductions fail to result in establishment, and even those species that do become established may have no detectable impact on the ecosystem they have colonized (Ricciardi and Cohen 2007). However,

successful invasive species may cause declines in native species through various mechanisms, including competition or predation (Mills *et al.* 2004), spread of disease (Gonzlan *et al.* 2005), and hybridization with the native taxon (Rhymer and Simberloff 1996).

Wolf *et al.* (2001) found that hybridization can lead to rapid extinction of a native species, particularly if the native species is numerically rare, competitively inferior, uses similar habitats, or has weak reproductive isolating barriers against the invader. Fish species often fit into the latter category, as external fertilization, weak behavioral isolating mechanisms, and competition for limited spawning habitat are common (Allendorf and Leary 1988, Scribner *et al.* 2001). Freshwater fishes are also frequently the subject of introductions by managers in an attempt to improve fisheries. Rahel (2000) noted 901 fish introduction events in the contiguous United States since European settlement. Notably, 14 of the top 17 most commonly introduced species in Rahel's (2000) analysis were native to another region of North America (i.e., not exotic). While much emphasis is often placed on the introduction of exotic species from foreign continents, estimates suggest that more than 65% of all introduced freshwater fish in the United States were introduced from a different region within the country (Perry *et al.* 2002). These native invasives may pose an even greater hybridization risk than those from distant continents because they may be pre-adapted to local conditions and thus more likely to establish, and they may be more closely related to the native species and thus less likely to possess reproductive isolating barriers (Perry *et al.* 2002).

Developing an understanding of the reproductive interactions between native and introduced species is pivotal for guiding conservation efforts. Allendorf *et al.* (2001) identified three distinct types of anthropogenically induced hybridization. Distinctions between these types are important because they engender different management options. The first type is

hybridization without introgression, in which case hybridization does not progress beyond the F_1 stage. This may occur when two previously allopatric species become sympatric, either through anthropogenic or natural processes, and can produce viable offspring, but an inability to backcross and/or variable degrees of hybrid fitness prevents introgression from occurring (Keck and Near 2009, Verspoor and Hammar 1991). This results in a detrimental effect to the native species in the form of reduced reproductive potential (Leary *et al.* 1993). In this case, removal of the non-native taxon and management to protect remaining pure populations and to prevent further introduction and spread of the introduced species is the most prudent course of action. The second type is hybridization with introgression, in which case F_1s are fertile and backcross with parental species, eventually resulting in a "hybrid swarm" with few remaining pure individuals (Childs *et al.* 1996, Bettles *et al.* 2005). In this case, management is more difficult and would require that non-native and hybrid-origin individuals be identified (e.g., using genetic methods) and, if possible, removed so that remaining pure individuals can be used to recover the population (Allendorf *et al.* 2001). The final type is complete admixture, in which case no pure populations remain and only an ensemble of admixed population remains. In such a scenario, recovery of the native population would be impossible, as no pure native individuals remain.

Hybridization is frequently reported across various North American freshwater fish taxa (e.g., Allendorf and Leary 1988, Dowling and Childs 1992, Childs *et al.* 1996, Echelle and Echelle 1997, Weigel *et al.* 2002) and there is growing awareness that hybridization can produce fertile offspring capable of introgression (Keck and Near 2009, Bolnick 2009). For example, sunfishes (family Centrarchidae) may be particularly prone to experiencing hybridization, as they appear to evolve post-zygotic isolation more slowly than many other taxa, with hybrid viability declining at a mean rate of only 3.13% per million years (Bolnick and Near 2005). Studies

suggest that estimates of sunfish hybrid viability provide an underestimate of the total post-mating isolation between species, as fertility may decline beyond the F_1 generation (Bolnick 2009). However, available evidence suggests that while intergeneric hybrids are often infertile, intrageneric hybrids are often partially or fully fertile (Bolnick 2009). Not all intrageneric pairs of Centrarchids will produce hybrids that extensively backcross (Travnichek *et al.* 1996, Epifanio *et al.* 1999), but the fact that many pairs are able to produce fertile hybrids suggests that introducing a sunfish species to a system containing congeners, even if they have been separated for millions of years, may result in introgression.

This ability for species to produce viable hybrids, both fertile and infertile, combined with a long history of introductions to systems outside their native ranges (i.e., to support fisheries) makes sunfish species particularly interesting models for studying the processes driving hybridization and introgression, as well as the ecological impact of these phenomena. Their retention of the ability to produce fertile hybrids after many millions of years of separation provides an opportunity to understand what genetic changes lead to differentiation between species (Bolnick 2009), and understanding these patterns may help researchers predict the outcome of reproductive interactions between introduced and native species. Furthermore, there is evidence to suggest that hybridization events are even more common when one of the species is rare and the other is more abundant, a phenomenon which holds true among Centrarchidae (Avise and Saunders 1984). This suggests that sunfish species which are already uncommon could experience heightened imperilment from introduced congeners, potentially leading to a rapid decline. Understanding the effects of invasive species on rare sunfishes could allow for the identification of at-risk populations, the prevention of damaging introductions, and the management of invaded and introgressed populations.

The Roanoke bass (*A. cavifrons*) is a rare Centrarchid at risk of hybridization and introgression with an invasive congener, the rock bass (*A. rupestris*). Of the four species within the genus *Ambloplites*, only *A. cavifrons* is native to the Atlantic Slope, occurring in the Roanoke, Chowan, Tar, and Neuse River basins of Virginia and North Carolina. In contrast, *A. rupestris* are native to the Gulf Slope and Great Lake drainages (Roe *et al.* 2008). These two species lineages are believed to have diverged between 8 and 16 million years ago (Near *et al.* 2005, Roe *et al.* 2008), and had remained allopatric until recent times. However, to provide new fishing opportunities, *A. rupestris* were heavily stocked into streams in the Roanoke basin from as early as 1898 until 1971 (Cashner and Jenkins 1982). A complete record of stocking events is unavailable, as many applicants who were supplied with fish did not record the geographic placement of these fish (Jenkins and Burkhead 1994). However, a general chronology of the introduction and spread of *A. rupestris* begins with introduction of *A. rupestris* from the North Fork Holston River (Tennessee basin) to hatcheries and various tributaries of the New River in 1876 by the US Fish Commission (Cashner and Jenkins 1982). From these sources, *A. rupestris* were introduced into many streams in the historic range of *A. cavifrons* in the late 19th and early 20th centuries, including the Nottoway River in 1895, streams in the middle and upper Roanoke (the Blackwater River in 1898 and Back Creek in 1905), and the Meherrin River in 1921 (Jenkins and Burkhead 1994). However, it was not until the 1940's that Virginia's Front Royal and Buller hatcheries distributed many thousands of *A. rupestris* throughout the Roanoke drainage (Cashner and Jenkins 1982). *A. rupestris* and putative hybrids (based on morphology) began to appear widely in collections from the upper Roanoke by 1952, and collections from that time period suggest the two species were about equally numerous in the upper Roanoke, with replacement by *A. rupestris* occurring gradually between 1945 and 1965 (Cashner and Jenkins

1982). With the exception of a relic population of *A. cavifrons* in Bradshaw Creek (a tributary of the Upper Roanoke) that persisted until at least 1978, morphological studies suggest all *Ambloplites* in the upper Roanoke have been *A. rupestris* since 1963 (Jenkins and Burkhead 1994).

It is possible that abiotic conditions may have precipitated the takeover by *A. rupestris*. The Roanoke River was impounded by five major dams between 1952 and 1964 (Petrimoulx 1983), and this human alteration of the environment may have promoted the success of invading *A. rupestris*. Research suggests that favorable environmental conditions are of paramount importance in determining the success of an invasive species (Moyle and Light 1996), and the presence of impoundments has been demonstrated to be a strong predictor of the presence of introduced species (Johnson *et al.* 2008). The disappearance of *A. cavifrons* from many of the streams in the upper Roanoke basin has been largely attributed to competitive exclusion by *A. rupestris* (Cashner and Jenkins 1982). However, to my knowledge there are no studies demonstrating *A. rupestris* to be competitively superior to *A. cavifrons*. In fact, anecdotal evidence from co-propagation suggests that neither feeding nor reproductive behaviors of *A. cavifrons* are hampered by the presence of *A. rupestris* (Petrimoulx 1983).

Although introduced *A. rupestris* may negatively impact *A. cavifrons* through competition, it is also hypothesized that the two species may hybridize. If so, *A. cavifrons* populations could become genetically introgressed or replaced by *A. rupestris* over time. Cashner and Jenkins (1982) detected putative interspecies hybrids in collections from three tributaries of the upper Roanoke River (North Fork Roanoke, South Fork Roanoke, and Bradshaw Creek) via an analysis using sixteen morphometric and meristic characteristics, but until now no study had used molecular techniques to determine the extent and mechanisms of

hybridization between the species. Although in certain cases the morphology of hybrid individuals is intermediate to the parental species and can be used to identify hybrid individuals (Godbout *et al.* 2009), in some cases there is disagreement between morphology and genetic identity (Gerber *et al.* 2001) or hybrid individuals may not be morphologically intermediate (Allendorf and Leary 1988). Moreover, morphological methods are not useful for discriminating F_1 from post-F_1 individuals. For this reason, the use of nuclear molecular markers is a more reliable method for the identification of hybrid-origin individuals and can be used to further categorize them as F_1, F_2, or backcross individuals (Scribner *et al.* 2001). In addition, analysis of mitochondrial DNA can provide insight into the mating patterns that lead to hybridization by examining whether (1) there is a sex bias in hybrid pairings, (2) if F_1 hybrids are disproportionately backcrossing with the invasive species (Perry *et al.* 2002), and (3) if historical introgression occurred but is now not detectable with nuclear markers due to backcrossing. This information would provide a more nuanced understanding of reproductive interactions than could be elucidated by a morphological analysis.

In this study, a combination of nuclear and mitochondrial DNA markers were used to infer the ancestry of *Ambloplites* individuals collected at sites throughout the historic range of *A. cavifrons*. The questions driving this study were fourfold: First, what is the extent of hybridization among these two *Ambloplites* species in Virginia? Second, if the species have hybridized, is there evidence for genetic introgression? Third, where do "pure" populations of *A. cavifrons* remain? Fourth, given this knowledge, how might *A. cavifrons* be conserved?

METHODS

Sample Collection

Personnel from Virginia Tech and the Virginia Department of Game and Inland Fisheries (VDGIF) collected *Ambloplites* specimens from 30 sites across 13 Virginia watersheds between 2012 and 2014 (Figure 1.1; Table 1.1). Fish were collected by angling or by backpack, barge, or boat electrofisher, depending on the site conditions. The sites were distributed across all of the Virginia watersheds known to historically harbor *A cavifrons*, with the exception of the Meherrin River, which was not sampled due inaccessibility. For genetically pure reference *A. cavifrons*, I used *Ambloplites* specimens collected in 2013 by the North Carolina Wildlife Resource Commission (NCWRC) from the Eno River (Neuse basin) of North Carolina, as well as *Ambloplites* specimens collected from the Nottoway River (Chowan basin) of Virginia. *A. rupestris* are not native to the Neuse basin and have never been observed there. Although *A. rupestris* were stocked into a tributary of the Nottoway River in 1985, they apparently did not establish (Jenkins and Burkhead 1994) and have not been observed since. Therefore, I presume that they did not affect the gene pool in this system. Correspondingly, I collected *Ambloplites* from Toms Creek (New basin) and Craig Creek (James basin) to act as a genetic reference for pure *A. rupestris*. *A. cavifrons* are not native to these basins and have never been observed there. Furthermore, Toms Creek was most likely the source for *A. rupestris* stocked into the Roanoke basin (Cashner and Jenkins 1982, Jenkins and Burkhead 1994), making it a logical choice to represent *A. rupestris*. Upon capture, specimens were euthanized in a 100 mg/L solution of MS-222 (Argent Laboratories, Redmond, Washington, USA). The right pectoral fin was removed with flame-sterilized scissors and placed in either a coin envelope or a vial of 95% ethanol. Each envelope or vial was given a unique identification number and a metal jaw tag with the same

number was attached to the corresponding specimen, which was vouchered in 10% formalin for future morphological study. Tissue samples were stored at -20˚C until DNA extraction.

Laboratory Methods

DNA was extracted from fin clips using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Using libraries developed from *A. rupestris*, I identified 11 microsatellite loci that co-amplified in *A. rupestris* and *A. cavifrons* (Eschenroeder and Roberts 2016). I grouped these loci into three multiplexes for screening samples: Multiplex 1 containing A107, A111, A114, and A115; Multiplex 2 containing A118, A138, and A145; and Multiplex 3 containing A432, A435, A464, and A472. The PCR cycling conditions were identical for all three multiplexes, and were as follows: initial denaturation at 95˚C (120 s), 35 cycles of denaturation at 95˚C (30 s), annealing at 56˚C (30 s), and extension at 72˚C (40 s), followed by a final extension at 72˚C (300 s).

Amplified PCR products were analyzed using an ABI 3500 Genetic Analyzer with a Genescan 500HD LIZ dye size standard (Applied Biosystems) and allele sizes were scored independently by both authors in GeneMapper (GeneMapper v.4.0; Applied Biosystems). In cases of disagreement, the GeneMapper output was discussed until a consensus was reached. All putative hybrids as well as a random sample of 10% of other individuals were genotyped twice to verify that there were no PCR or scoring errors.

Data Analysis

I used two different statistical algorithms to assign individuals to pure species or hybrid categories. First, I utilized the approach described by Pritchard (2000) in the software STRUCTURE 2.3.4 to estimate the proportion of each individual's ancestry that was derived from the *A. cavifrons* genome. Pure *A. cavifrons* should have membership coefficients (Q

values) close to 0, pure *A. rupestris* should have Q values close to 1, and hybrids should have some intermediate value. For my two reference *A. rupestris* (Toms and Craig Creeks) and two reference *A. cavifrons* populations (Eno and Nottoway Rivers), I included prior information about the sample origin in the model, allowing these samples to "train" the model to predict the ancestry of the remaining samples, for which I included no prior data on membership. The model allowed for two parental species (i.e., K=2), background admixture and correlation of allele frequencies between species, and searched parameter space using 10^6 recorded Markov chain Monte Carlo (MCMC) iterations, following a burn-in of 10^5 iterations. Replicate runs of this configuration (data not shown) indicate that this modeling intensity was sufficient to obtain consistent results.

The use of multiple, independent statistical techniques has been shown to increase the confidence of species and hybrid-class assignments (Vähä and Primmer 2006). Therefore, I ran a complementary analysis using Anderson and Thompson's (2002) approach implemented in the software NewHybrids 1.1 (Anderson 2003). Whereas STRUCTURE attempts to determine the proportional ancestry of each individual as a continuous variable, NewHybrids estimates the posterior probabilities that each individual belongs in each of six discrete categories: (1) pure *A. cavifrons*, (2) pure *A. rupestris*, (3) F_1 hybrid, (4) F_2 hybrid, (5) F_1 x *A. cavifrons* backcross, and (6) F_1 x *A. rupestris* backcross. Additional crosses (e.g., F3, backcross x backcross, etc.) were possible, but were not considered in this analysis. The NewHybrids model did not incorporate any prior information regarding the origin of the individuals. I used a Jeffreys-type prior distribution for parental species allele frequencies, and made $5x10^6$ sweeps through the MCMC simulation algorithm following a burn-in of 10^5 sweeps. Replicate runs of this simulation (data not shown) indicate that this modeling intensity was sufficient to achieve consistent results.

I complemented this nuclear DNA analysis with an analysis based on mitochondrial DNA, seeking to test whether (1) any individuals deemed pure by the nuclear analysis showed historical introgression in the mitochondrial genome (i.e., nuclear genotype from one species but a mitochondrial haplotype from the other), and whether (2) there was a sex bias in hybrid matings (as indicated by a species bias in the haplotypes of deduced hybrids). Following the assignment of individuals to species/hybrid categories by nuclear DNA, I sequenced mitochondrial DNA from 10 reference *A. rupestris* individuals, 10 reference *A. cavifrons* individuals, all putative hybrids, and at least 4 randomly selected, putative non-hybrids from each non-reference river system. I amplified a portion of the mitochondrial cytochrome B gene of selected specimens using the degenerate primers AmbloCytB-F (5' – RTGRCTTGAAAAACCACCGTTG) and AmbloCytB-R (5' – CYCSRYVTCCRGTTTACAAGAC). PCR reaction mixes were 25µL in total volume, and consisted of 12.5µL GoTaq Mastermix (Promega, Madison, Wisconsin, USA), 0.5µL forward primer, 0.5µL reverse primer, 9.5µL molecular-grade H_20, and 2 µL of template DNA (typical concentration ~40ng/µL). Cycling conditions were as follows: initial denaturation at 95˚C (150 s), 35 cycles of denaturation at 95˚C (60 s), annealing at 58˚C (60 s), and extension at 72˚C (90 s), followed by a final extension at 72˚C (420 s). PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium, Hayward, California, USA). Upon verification of successful amplification, PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, California, USA) and forward and reverse sequenced by Eton Bioscience, Inc. (Research Triangle Park, North Carolina, USA). The sequences were checked and edited in Sequencher® v.5.4. software (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned using the "Clustal" option in MEGA v.6 (Tamura *et al.* 2013).

Deduced haplotypes were assigned to species based on a phylogenetic analysis that also included cytochrome-B sequence data from reference specimens and outgroup taxa, downloaded from the NCBI Genbank database. These sequences included reference *A. rupestris* (AY115978, collected in the Tennessee basin; Roe et al.. 2008) and *A. cavifrons* sequences (AY115980, collected in the Tar basin; Roe et al.. 2008), two sequences each from the congeners *A. constellatus* (EU501081 and EU501085) and *A. ariommus* (EU501112 and EU501117), and two sequences from *Centrarchus macropterus* (AY225666 and AY115982). Alternative models of sequence evolution were evaluated using the "model test" module of MEGA v.6, and the model that minimized the Bayesian Information Criterion was used in subsequent analyses. Based on this model, I conducted a maximum likelihood phylogenetic analysis in MEGA, using a neighbor-joining tree as the initial tree and nearest-neighbor-interchange as the heuristic inference method. Support for tree nodes was based on 1000 bootstrap replicates. I then classified haplotypes to species based on their co-occurrence with reference sequences in species-level clades. I also used MEGA to calculate the pairwise p-distances between haplotypes.

After estimating the nuclear and mitochondrial ancestry of each individual, I removed putative hybrids from the dataset and separately analyzed the genetic diversity of populations of *A. cavifrons* and *A. rupestris*. I grouped individuals into populations based on the distribution of barriers to movement (dams and watershed boundaries). For microsatellite data, I used ARLEQUIN version 3.5 (Excoffier and Lischer 2010) to estimate the average number of alleles (A), expected heterozygosity (H_E), and observed heterozygosity (H_O) across loci. I also tested for Hardy-Weinberg equilibrium for each locus in each population based on 10^5 random permutations, evaluating tests results at an alpha of 0.05. For mitochondrial data, DNAsp v.

5.10.1 (Librado and Rozas 2009) was used to estimate the total number of haplotypes and segregating sites, and the mean and standard deviation of haplotype and nucleotide diversity, for each population. Only species x population combinations with a sample size of at least 3 individuals were analyzed.

RESULTS

In total, I genotyped 417 *Ambloplites* from 30 sites at the 11 nuclear microsatellite loci (Table 1.1). Sample size varied widely among sites due to variation in sampling efficiency and apparent density of fish. I double-genotyped a total of 46 individuals (11% of the total samples), and determined the genotyping error rate to be 0.033. All genotyping errors involved loci appearing to be heterozygotic in one reaction and homozygotic in the other, presumably due to imperfect primer annealing (i.e., sporadic null alleles). I presume that this error rate had little effect on downstream analyses and retained the heterozygotic genotypes for further analyses. Evidence for null alleles was further evaluated by Hardy-Weinberg tests and found to be limited, as described below.

Based on analyses of nuclear markers, most sampled individuals were classified as non-hybrids. The STRUCTURE analysis indicated that most individuals exhibited a membership coefficient close to 0 or 1, indicating that most individuals were either strongly *A. cavifrons* or strongly *A. rupestris*, respectively (Figure 1.2). All individuals in the Toms Creek, Craig Creek, and Roanoke River systems were classified as strongly *A. rupestris*, while all individuals in the Nottoway and Eno river systems were classified as strongly *A. cavifrons*. In each of the other six sampled river systems, there was at least one individual with an intermediate probability representing a potential hybrid. The Pigg river system had the most such individuals (n = 7).

Because STRUCTURE yields membership probability as a continuous variable, it was necessary to establish a probability threshold to allow for conversion of these results into categorical classifications (*A. cavifrons*, *A. rupestris*, or hybrid). All reference *A. rupestris* were assigned to that species with >99% certainty (i.e., $Q > 0.99$), whereas all reference *A. cavifrons* were assigned to that species with >99% certainty (i.e., $Q < 0.01$). I therefore adopted these thresholds for assigning all remaining individuals (i.e., individuals with $Q > 0.99$ were classified as *A. rupestris*, individuals with $Q < 0.01$ were classified as *A. cavifrons*, and all other individuals were classified as hybrids. Based on this threshold, STRUCTURE identified 186 *A. cavifrons*, 216 *A. rupestris*, and 15 hybrids in the dataset. Use of alternative Q thresholds as low as 0.93 and as high as 0.995 had no effect on these results (data not shown).

Results of the NewHybrids analysis supported the findings from the STRUCTURE analysis and provided further information about hybrid classifications. Moreover, the number of individuals classified to species by NewHybrids was relatively insensitive to the selected probability threshold. Across threshold probability values from 0.80 to 0.99, the number of individuals assigned to species varied from 402 to 403, and the number of hybrids varied from 14 to 15. I therefore adopted the same thresholds used in the STRUCTURE analysis ($Q > 0.99$ for *A. rupestris*; $Q < 0.01$ for *A. cavifrons*). Based on this threshold, every individual was classified to the same category (*A. cavifrons*, *A. rupestris*, or hybrid) by both NewHybrids and STRUCTURE. For the 15 deduced hybrids, I presumed that the hybrid category with the highest Bayesian posterior probability was the true hybrid class of that individual. Based on this assumption, I identified one F_1, four F_2s, seven F_1 x *A. cavifrons* backcrosses, and three F_1 x *A. rupestris* backcrosses (Table 1.2). Thus, the data supported the hypothesis that post-F_1 genetic introgression is occurring.

A. rupestris and hybrids were distributed non-randomly across sample sites. As expected, *A. rupestris* reference populations (Toms and Craig creeks) contained only fish assigned as *A. rupestris*, and *A. cavifrons* reference populations (Nottoway and Eno Rivers) contained only fish assigned as *A. cavifrons* (Figure 1.3). Sites in the Smith River system contained predominantly *A. cavifrons*. In both the upper (above Philpott Reservoir) and lower (below Philpott Reservoir) Smith River, only putative *A. cavifrons* were identified, but in Town Creek, a tributary of the lower Smith River, 3 of 27 analyzed individuals were assigned as hybrids (one F_2 and two F_1 x *A. cavifrons* backcrosses, Table 1.3). All three of these hybrids were captured at the same site near the mouth of the creek.

The situation was more complex at sites in the Roanoke basin, where I found trends of species replacement and ongoing hybridization in many systems (Figure 1.4). Among four sites sampled in the Blackwater River, only one hybrid (an F_1 x *A. cavifrons* backcross) was identified; the remaining 23 individuals were all identified as *A. cavifrons*. However, pure *A. cavifrons* were not detected in three other historically occupied river systems. I identified only *A. rupestris* (n = 69) among five sites in the upper Roanoke system, 40 *A. rupestris* and 2 F_1 x *A. rupestris* backcrosses among three sites in the Otter system, and 33 *A. rupestris* and 1 F_1 hybrid in the Staunton River. In the Falling and Pigg River systems, I collected *A. cavifrons*, *A. rupestris*, and hybrids. A dam near the mouth of the Falling River separated a collection of solely *A. cavifrons* upstream (n = 21) from a mix of *A. cavifrons* (n = 5), *A. rupestris* (n = 1), and an F_2 hybrid downstream. There are no such barriers in the sampled reaches of the Pigg River system, and I found a mixture of both species and hybrids in the system. The upper Pigg River site contained only apparent *A. rupestris* (n = 25) and 1 *A. rupestris* x F_1 backcross, the lower Pigg site contained apparent *A. cavifrons* (n =7), *A. rupestris* (n = 1), and 3 hybrids (1 F_2 and 2 *A.*

rupestris x F_1 backcrosses), and the site in Chestnut Creek (a major tributary that enters the Pigg River between the two Pigg sites) contained an even mix of *A. cavifrons* (*n* = 3) and hybrids (1 F_2, 1 *A. rupestris* x F_1 backcross, and 1 *A. cavifrons* x F_1 backcross)

I obtained an 1100bp sequence of the CytB gene for 108 individuals, including the 15 individuals identified as hybrids by the microsatellite analysis. A total of 19 unique haplotypes were identified; 7 of these were distributed across multiple stream systems, whereas 12 were present only in one system (Table 1.4). Phylogenetic analyses of these haplotypes, plus ingroup and outgroup sequences from Genbank, indicated a clear distinction between an *A. cavifrons* clade (which contained haplotypes A through Q plus the reference *A. cavifrons* sequence from Genbank) and an *A. rupestris* clade (which contained haplotypes R through Z plus the reference *A. rupestris* sequence from Genbank) (Figure 1.4). The average p-distance between *A. cavifrons* haplotypes and *A. rupestris* haplotypes was 0.1092 (standard deviation = 0.0014). The node separating these clades had 100% bootstrap support, as did the nodes separating most other species (with the exception of *A. cavifrons* versus *A. constellatus*). Intraspecific topologies had varying levels of bootstrap support, but the evolutionary distance separating haplotypes was low within species relative to the large evolutionary distances between species. For subsequent analyses I considered these clade memberships representative of the matrilineal species ancestry of haplotypes. Based on this assumption, mitochondrial analysis did not reveal any additional hybrids, as all 93 individuals deemed non-hybrid by nuclear analyses bore a mitochondrial DNA haplotype matching the nuclear DNA species assignment. Furthermore, I saw no strong evidence for a species bias in hybrid matings. Among individuals deemed hybrids by the nuclear DNA analysis, 10 possessed *A. cavifrons* haplotypes and 5 possessed *A. rupestris* haplotypes (Table 1.2). Thus, *A. cavifrons* matrilineal ancestry was more common, but this difference was

not statistically significant (Fisher's exact $P = 0.143$). In the Eno, Nottoway, Blackwater, Smith, and upper Falling river systems, I detected only *A. cavifrons* haplotypes, whereas in the Roanoke and Otter river systems and the Toms and Craig creek systems I detected only *A. rupestris* haplotypes, and in the lower Falling, Pigg, and Staunton river systems I observed haplotypes of both species (Table 1.4).

I removed putative hybrids from the dataset and estimated the nuclear and mitochondrial genetic diversity of *A. cavifrons* and *A. rupestris* populations. Only 7 of 143 tests for Hardy-Weinberg disequilibrium were significant at an alpha level of 0.05, and instances of disequilibrium were evenly distributed across markers and populations (Table 1.3), so I retained data from all markers for subsequent analyses. The number of microsatellite alleles per locus and observed (H_O) and expected heterozygosity (H_e) were generally lower in *A. cavifrons* than in *A. rupestris*. Among *A. cavifrons* populations, H_e ranged from 0.136, in the Blackwater River population, to 0.255, in the upper Smith River population. Among *A. rupestris* populations, H_e ranged from 0.376 in the Pigg River population, to 0.454 in the Roanoke River population (Table 1.3). Allele richness followed these same patterns. Based on mitochondrial DNA in *A. cavifrons*, the number of haplotypes and haplotype diversity were highest in the lower Smith River (including Town Creek) and Falling River populations, whereas the highest observed nucleotide diversity was in the Nottoway River population. In contrast, upper Smith and Eno river individuals exhibited no variation at cytochrome B. Among *A. rupestris*, the number of haplotypes was highest in the Staunton River population, and haplotype diversity and nucleotide diversity were highest in the Toms Creek population. The *A. rupestris* population with the lowest diversity was that in the Otter River, which exhibited no variation at cytochrome B (Table 1.5).

DISCUSSION

My findings provide clear genetic evidence of the widespread introduction of *A. rupestris* in streams throughout the Virginia range of *A. cavifrons*. Of the 8 historically-occupied river systems I assessed, 6 contained hybrid individuals. Of the remaining 2, only the Nottoway River harbored a putatively pure population of *A. cavifrons*, whereas the Roanoke River, the first river to be invaded by *A. rupestris*, now apparently contains only *A. rupestris*. I was surprised at the lack of detected hybrids in the Roanoke watershed, despite the fact that the two species are known to have co-occurred and suspected to have hybridized there in the past (Jenkins and Cashner 1983). Indeed, hybrids were relatively rare across all sampled systems, representing only 15 out of 417 individuals. This relative paucity of hybrids could occur for any of three reasons: (1) the species rarely hybridize, (2) hybrids are not fertile or have low fitness, which inhibits backcrossing, or (3) hybrids readily backcross with the more common species, and do so preferentially, eventually resulting in the disappearance of the hybrid "signature" in the genotypes of hybrid descendants. The first scenario would suggest that the displacement I observed has been driven by competition rather than reproductive interactions. Competition for resources between the two species has been posited to occur (Petrimoulx 1983). *A. rupestris* are known to be significantly smaller than *A. cavifrons*, and may have a competitive advantage in resource-limited conditions, allowing them to become more abundant than their native counterpart (Petrimoulx 1983). It is likely that the two species would be in direct competition with one another, as both adult and juvenile *A. rupestris* and *A. cavifrons* have diets high in crayfish (Etnier and Starnes 1993). Additionally, because *A. rupestris* are also known to consume small fishes, including young *Ambloplites* (Scott and Crossman 1973), they may be significant predators to immature *A. cavifrons*, although *A. cavifrons* may similarly depredate

young *A. rupestris.* Although competition may have contributed to displacement, I have no way of retroactively assessing the extent of its role. Taken as a whole, the 3.6% of 416 samples that were identified as hybrids does not seem significant, but if I focus on systems where both species currently co-occur, the sample is comprised of a higher proportion of hybrids (e.g., 16% of individuals collected from the Pigg River system). Furthermore, hybrids were detected in every system where both species currently occur, suggesting that hybridization takes place whenever these species come into contact with one another. As such, I cannot discount the fact that hybridization has played a role in the replacement of *A. cavifrons* with *A. rupestris*. Models suggest that hybridization increases a species' risk of extirpation beyond that presented by competition alone (Wolf *et al.* 2001), and this may well be the case with *A. cavifrons* and *A. rupestris*.

If the second scenario were correct, I would expect a low frequency of post F_1 hybrids. However, 14 of the 15 hybrids were determined to be post-F_1, so the scenario of hybrid infertility seems an unlikely explanation. Rather, results of this study seem to support the third explanation. Of the putative hybrids detected, 67% were classified as backcrossed individuals. Although I could not detect complex hybrid scenarios (e.g., F_3, F_2 x F_2) in the present analysis, the preponderance of high and low Q-value assignments in mixed populations (Figure 1.2) suggests that backcrossing with the more common parental species, not formation of hybrid swarms, has been the dominant pattern for sympatric *A. rupestris* and *A. cavifrons* populations. It is possible for hybrids to be fertile beyond the F_1 generation, but still be inferior competitors for mates or resources and have reduced fitness, causing selection against hybrids to preclude the formation of a hybrid swarm (Bettles *et al.* 2005, Fukui *et al.* 2016). Furthermore, studies suggest that subtle differences in mitochondrial sequences (e.g., single amino acid substitutions)

can lead to hybrid breakdown via the disruption of co-adapted nuclear-mitochondrial gene complexes (Harrison and Burton 2006, Ellison and Burton 2008). My analysis indicates that there is a substantial difference between the mitochondrial sequences of *A. cavifrons* and *A. rupestris*, and I observed no individuals with mismatching nuclear and mitochondrial DNA, suggesting the potential for reduced fitness in advanced hybrids and selection against hybrid swarms. Subsequent studies of experimental crosses would provide a better understanding of hybrid fitness and mate choice of these species in a controlled environment, though our ability to reconstruct historical mating patterns will continue to be hampered by a lack of empirical data on the historic abundance and "propagule pressure" of *A. rupestris*.

Based on previous studies of hybridization in Centrarchidae, it was expected that this study would find these two intrageneric species are producing viable hybrids in areas of sympatry. However, Bolnick (2009) noted that there appeared to be no cases of fertile hybrids between species separated more than 14.6 million years. Estimates of divergence between the lineages of *A. rupestris* and *A. cavifrons* range from 8 (Near *et al.* 2005) to 15.75 million years ago (Roe *et al.* 2008). Given that it appears *A. rupestris* x *A. cavifrons* hybrids are fully fertile and able to backcross, if Roe *et al.*'s (2008) more ancient divergence estimate is correct this may be a unique interaction in that the two species are more anciently-diverged than any other Centrarchids known to produce fertile hybrids.

Under preferential backcrossing (i.e., scenario three above), the genotypic signature of hybridization can be lost relatively quickly. With the 11 microsatellite markers used in this study, beginning with an F_1 individual, three generations of backcrossing with *A. rupestris* would result in a 50% chance of loss of all *A. cavifrons* alleles. After six generations of backcrossing, this becomes a >90% chance. Because *Ambloplites* have a generation time of approximately

four years, the signal of historical hybridization could thus become undetectable after only a few decades. *A. rupestris* are known to have been the dominant species in the upper Roanoke River by the late 1950's (Jenkins and Cashner 1983), which may explain the fact that no hybrid signal was detected in this system. Because this limits the ability of my marker set to differentiate between distant backcross and pure individuals, it is possible that some of the individuals characterized as pure by my analysis are the distant descendants of hybrids. Rapid replacement of the native genome has been recorded in other instances of hybridization between native and invasive species, including instances of replacement of 80% of the native genome within a five-year period (Childs *et al.* 1996). By this logic, the hybrids I detected may have been the products of relatively recent hybridization events, as they likely would not have been detectable after several generations of backcrossing. Continued monitoring of the status of populations in streams undergoing invasion and replacement by *A. rupestris* could provide insight into the processes that led to complete loss of *A. cavifrons* from the upper Roanoke during the first half of the 20th century. For example, the genetic status of *Ambloplites* in systems where invasion is ongoing (e.g., the Pigg River), may be monitored in order to determine if the signal of *A. cavifrons* fades slowly over time or if it disappears rapidly. The former scenario is what would be expected if repeated backcrossing with *A. rupestris* led to replacement of the *A. cavifrons* genome, whereas the latter scenario may indicate that competitive exclusion is a more significant contributor to the loss of *A. cavifrons*.

Because mitochondrial DNA is maternally inherited and does not undergo recombination, it is possible that the signature of a past hybridization event could persist in the mitochondrial genome long after it has vanished from the nuclear genome. Studies have revealed individuals, and in some cases entire populations of fishes that have the nuclear DNA and morphologic

appearance of one species but possess the mitochondrial haplotypes of an entirely different species (Gerber *et al.* 2001, Godbout *et al.* 2009, Keck and Near 2009). However, my analysis of mitochondrial sequences from 108 specimens did not reveal any discrepancies in species assignment or remaining *A. cavifrons* mtDNA in systems where the species otherwise appears lost. This could suggest that populations in areas that have become dominated by *A. rupestris*, such as the Roanoke River, experienced biased backcrossing between hybrids and *A. rupestris* females, leading to a loss of *A. cavifrons* mitochondrial DNA. Of the 15 hybrids, 10 possessed *A. cavifrons* haplotypes and 5 possesed *A. rupestris* haplotypes. This difference is not significant, but many supposedly pure *A. rupestris* individuals in systems that have become dominated by that species may be the products of distant hybridization events, in which case the detection of only *A. rupestris* haplotypes would indicate that hybridization was most frequently the result of *A. cavifrons* male x *A. rupestris* female crosses. Significant bias in the directionality of crossing between native and invasive species has been noted in other systems, and may be the result of females of the native species outbreeding more frequently than females of the invasive species, or males of the invasive species outcompeting males of the native species for mates (Dowling and Childs 1992). A second possibility is that, due to high propagule pressure, introduced *A. rupestris* simply numerically overwhelmed *A. cavifrons* and hybrids, resulting in the chance loss of rarer *A. cavifrons* haplotypes over time. Another alternative is that epistatic interactions between nuclear and mitochondrial DNA result in selective pressure against individuals with heterospecific genomes.

My findings suggest that the continued existence of *A. cavifrons* in the Virginia portion of their historic range is in a precarious state. It seems that the two species can only coexist for a short amount of time, as systems where *A. rupestris* was introduced historically are currently

occupied only by that species, and systems where it was apparently introduced more recently are in the process of losing *A. cavifrons* populations. A notable exception to this trend can be seen in the Blackwater River. *A. rupestris* were stocked into this system, and were detected there as recently as 1963 (Cashner and Jenkins 1982), but with the exception of a single hybrid-origin individual identified by this study, no trace of *A. rupestris* remains. It is unclear what led to this unique outcome in the Blackwater, but it is possible that stocking did not introduce enough *A. rupestris* for the population to reach a critical threshold and begin replacing *A. cavifrons*. Further, construction of Smith Mountain Lake in 1966 may have prevented the movement of *A. rupestris* from the Roanoke River into the Blackwater River and reduced *A. rupestris* propagule pressure there. A dam also appears to be preventing invasion into upper reaches of the Falling River. A dam near the mouth of this river separates a mix of *A. rupestris*, *A. cavifrons*, and hybrid individuals from a population of pure *A. cavifrons* upstream. Although the lack of connectivity associated with these barriers may have negative consequences for upstream *A. cavifrons* populations over time, in the form of isolation and resulting genetic drift, they have prevented the invasion of *A. rupestris*.

Available evidence suggests that the invasion of *A. rupestris* stemmed from multiple, independent introduction events. It is unlikely that *A. rupestris* in the Roanoke River were able to disperse to the Pigg and Otter Rivers, due to the fact that these rivers are separated from the Roanoke River by one and two dams, respectively. Moreover, *A. rupestris* were more common in the upper portion of the Pigg River than in the lower portion, which is opposite of the pattern I would expect if they had dispersed into the mouth of the Pigg River from the Roanoke River. Even lentic reservoir conditions appear to be inhibiting dispersal of *A. rupestris*, given that the species has not dispersed from the Roanoke River to the Blackwater River through Smith

Mountain Lake, into which both rivers flow. The incidence of three hybrid-origin individuals in Town Creek is also somewhat puzzling, given that there has been no recorded stocking of *A. rupestris* in that stream and it is isolated both by impoundments and large distances from streams where *A. rupestris* was stocked. However, the Town Creek *A. cavifrons* population was augmented with approximately 900 hatchery-raised *A. cavifrons* in 1980 (Jenkins and Cashner 1983), and it is possible that *A. rupestris* or hybrid individuals were inadvertently introduced to the system in the process. The *A. rupestris* in the Staunton River could be the result of downstream movement of fish from tributaries of the upper Roanoke that occurred prior to the construction of impoundments along the Roanoke River in the 1950's and 1960's. However, no *A. rupestris* were known from this reach the Staunton River pre-impoundment (Jenkins and Burkhead 1994), so this *A. rupestris* population may have resulted from a post-impoundment introduction. In either case, from the Staunton River, *A. rupestris* could have easily dispersed into the Otter River and into the mouth of the Falling River.

Of Allendorf *et al.*'s (2001) three scenarios of anthropogenically-induced hybridization (hybridization without introgression, widespread introgression, complete admixture), the first and the third are not supported for *A. cavifrons*, as I found backcrossed individuals (rejecting scenario 1), as well as populations containing apparently pure *A. cavifrons* (rejecting scenario 3). My findings are more supportive of Allendorf *et al.*'s (2001) scenario 2, though instead of a hybrid swarm I found the dominant pattern to be backcrossing, such that genetically intermediate individuals were rare. The result is that although *A. cavifrons* persists in the Nottoway, Blackwater, Smith, and upper Falling River systems, it has been mostly or entirely replaced by *A. rupestris* in the upper Roanoke, Otter, and Staunton River systems. Additionally, there appears to be ongoing hybridization and replacement occurring in the Pigg River system, where

many *A. rupestris* and hybrids and few *A. cavifrons* were detected. Jenkins and Cashner (1983) found that *A. cavifrons* had been replaced in the Roanoke River, but predominance of *A. rupestris* in the Otter, Staunton, and Pigg River systems was previously unknown.

Allendorf *et al.*'s (2001) three scenarios provide a useful framework to guide management responses to anthropogenic hybridization. The only scenario under which removal of *A. rupestris* and hybrids would be feasible is if hybrids were sterile and/or failing to backcross (i.e., no introgression occurring), or if the two species exhibited assortative mating and hybrids did not occur. This is not the case, and so alternative management strategies will be required to protect remaining *A. cavifrons* populations and prevent further spread of *A. rupestris*. Put another way, remaining populations must be saved in order to prevent the transition from backcrossing in systems where the species co-occur (scenario 2) to complete genetic admixture across the range of *A. cavifrons* (scenario 3). Unfortunately, based on the trends observed in the Roanoke basin (with the exception of the Blackwater River), following several decades after *A. rupestris* enter a system there may no longer be *A. cavifrons* to use in recovery of the population. Anglers can be responsible for the spread of aquatic invasive organisms, both intentionally and unintentionally (Mills *et al.* 1993). Because preventing introductions is more effective and practical than removal of invaders (Ricciardi and Rasmusen 1998), steps should be taken to promote the education of anglers in the region regarding the status of *A. cavifrons* in Virginia and the importance of not transporting *Ambloplites* from one waterway to another. *A. cavifrons* grow to a considerably larger size than *A. rupestris* (Petrimoulx 1983), and promoting the species as a sport fish may help to stimulate interest in its continued existence in the state. The Virginia Angler Recognition Program does not currently distinguish between the species, but rather considers them both "rock bass" when issuing citations for trophy fish. It is also noteworthy that

impoundments may be playing an important role in preventing the dispersal of *A. rupestris*, for example into the Blackwater River or upper portions of the Falling River. Before removing dams to improve habitat connectivity, managers should investigate the potential for *A. rupestris* to spread into formerly isolated systems containing *A. cavifrons* populations.

In addition to taking steps to prevent the spread of *A. rupestris*, it may also be advisable to establish new populations or augment existing populations of *A. cavifrons*. Researchers studying hybridizing trout species in Western North America often reiterate the dangers inherent in supplementing populations through the stocking of hatchery raised individuals as this can result in high levels of homozygosity and loss of local adaptations (Allendorf and Leary 1988, Dowling and Childs 1992). However, captive stocks of rare fish species have proven invaluable in the recovery of lost wild populations (Echelle and Echelle 1997). The translocation of individuals between existing wild populations is one option that may be used to augment imperiled populations and promote genetic diversity (Dehaan *et al.* 2015). Another possible option that would avoid the potential pitfalls associated with captive rearing and may be a prudent management strategy is the establishment of populations of *A. cavifrons* in unoccupied streams with appropriate habitat in the Roanoke and Chowan basins. This would serve to reduce the risk of species extirpation by providing "refuge" populations that could be used to preserve the genetic diversity of the species.

Although government introductions have declined as science has demonstrated the potential negative consequences of such actions, illegal movement of fish by private citizens continues to contribute to the introduction of non-native species to freshwater systems (Rahel 2000). Centrarchids continue to be introduced outside of their native range, and understanding the mechanisms of hybridization and replacement may help to prevent the extirpation of native

species. This study will guide the management of *A. cavifrons* populations throughout its historic range by identifying populations where hybridization is ongoing and populations that are particularly at risk for invasion by *A. rupestris*. Ongoing monitoring of extant and newly established refuge populations will serve as a useful model for the management of species experiencing similar threats. For example, monitoring genetic diversity and effective population size during the introduction and establishment of a new refuge population would provide a case study that will demonstrate if this is a viable management option for native species. Additionally, my findings add to a growing knowledge base of the complexity of reproductive interactions between congeneric sunfishes. Additional studies of ongoing hybridization events between these and other congeneric species-pairs (e.g., *Micropterus* species; Koppelman 1994, Barwick *et al.* 2006) would inform general understanding of the commonness of this pattern across taxa. Although *A. rupestris* and *A. cavifrons* are demonstrably capable of producing viable and fertile hybrids, the lack of a hybrid swarm indicates there may be biased mating between hybrid-origin individuals and the more common parental species, a pattern that has contributed to the loss of the genetically distinct *A. cavifrons* from much of its historic range.

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Table 1.1: Origins of genetic samples used in this study.

Basin	Watershed	Stream segment	Latitude	Longitude	*n*	Code
Roanoke	Roanoke River	Roanoke River	37.2683	-80.0253	24	1
	Roanoke River	Roanoke River	37.2823	-80.0941	21	2
	Roanoke River	North Fork Roanoke River	37.2001	-80.3154	11	3
	Roanoke River	North Fork Roanoke River	37.2197	-80.3647	8	4
	Roanoke River	Bradshaw Creek	37.2520	-80.2593	5	5
	Blackwater River	Blackwater River	37.0345	-79.9094	1	6
	Blackwater River	Blackwater River	37.0528	-79.8824	6	7
	Blackwater River	Blackwater River	37.0541	-79.8819	9	8
	Blackwater River	Blackwater River	37.0541	-79.8823	8	9
	Pigg River	Chestnut Creek	36.9063	-79.8010	5	10
	Pigg River	Chestnut Creek	36.9065	-79.8014	1	11
	Pigg River	Lower Pigg River	36.9468	-79.5249	1	12
	Pigg River	Lower Pigg River	36.9402	-79.7674	10	13
	Pigg River	Upper Pigg River	36.9964	-79.8596	26	14
	Otter River	Big Otter River	37.3930	-79.5046	8	15
	Otter River	Little Otter River	37.2768	-79.4350	21	16
	Otter River	North Fork Otter River	37.3923	-79.4534	13	17
	Falling River	Lower Falling River	37.0540	-78.9354	7	18
	Falling River	Upper Falling River	37.1268	-78.9595	21	19
	Staunton River	Staunton River	37.1054	-79.2866	33	20
	Upper Smith River	Upper Smith River	36.8053	-80.2008	10	21
	Lower Smith River	Lower Smith River	36.6138	-79.8226	2	22
	Lower Smith River	Lower Smith River	36.6141	-79.8225	2	23
	Lower Smith River	Town Creek	36.7925	-80.0027	16	24
	Lower Smith River	Town Creek	36.8210	-79.9966	11	25
Chowan	Nottoway River	Nottoway River	36.8477	-77.4934	22	26
	Nottoway River	Nottoway River	36.8590	-77.1898	28	27
New	Toms Creek	Tom's Creek	37.2001	-80.5645	31	28
James	Craig Creek	Craig Creek	37.6084	-79.9921	11	29
Neuse	Eno River	Eno River	36.0751	-79.0076	45	30
Total					**417**	

n = sample size

Table 1.2: Nuclear and mitochondrial characteristics of the 15 *Ambloplites* individuals identified as hybrids by genetic analyses. Structure Q-values indication proportional ancestry from *A. rupestris*. The highest-probability NewHybrids hybrid category assignment for each individual is shown in bold. Haplotypes A through Q and R through Z are putatively *A. cavifrons* and *A. rupestris*, respectively.

		NewHybrids Category Probabilities						
Individual	STRUCTURE Q-value	*A. cavifrons*	*A. rupestris*	F_1	F_2	F_1 x *A. rupestris* backcross	F_1 x *A. cavifrons* backcross	mtDNA Haplotype
Staunton225	0.472	0	0	**0.915**	0.025	0.027	0.033	A
Blackwater131	0.611	0	0	0.169	0.089	**0.742**	0	D
Chestnut086	0.230	0	0	0	0.037	0	**0.963**	U
Chestnut088	0.513	0	0	0	**0.999**	0	0	A
Chestnut298	0.770	0	0	0	0.018	**0.982**	0	L
Lpigg300	0.675	0	0	0	0.053	**0.947**	0	F
Lpigg301	0.566	0	0	0	**0.666**	0.334	0	P
Lpigg304	0.899	0	0.516	0	0.012	**0.473**	0	X
Upigg064	0.716	0	0	0	0.035	**0.965**	0	P
LOtt438	0.145	0.004	0	0	0.016	0	**0.980**	R
NFOtt426	0.333	0	0	0	0.309	0	**0.691**	R
Lfalling466	0.114	0.961	0	0	**0.028**	0	0.011	R
Town347	0.906	0	0.111	0	0	**0.887**	0	P
Town348	0.748	0	0	0	**0.754**	0.246	0	A
Town354	0.905	0	0.124	0	0	**0.874**	0	P

Table 1.3: Estimates of genetic diversity, based on 11 nuclear microsatellite loci, for populations of *A. cavifrons* and *A. rupestris*. Estimators include the number of alleles per locus, expected heterozygosity (H_E), and observed heterozygosity (H_O). H-W tests were evaluated at an alpha level of 0.05.

Species	Population	Sample size	Number of alleles per locus	H_E	H_O	Markers with Hardy-Weinberg $P < 0.05$
A. cavifrons	Blackwater	23	1.5	0.136	0.138	None
	Pigg	4	2.1	0.224	0.250	None
	Falling	26	2.5	0.197	0.217	A472
	Upper Smith	10	2.2	0.255	0.264	None
	Lower Smith/Town Creek	28	2.7	0.236	0.199	A472
	Nottoway	50	3.5	0.243	0.241	None
	Eno	45	2.5	0.214	0.221	A464
A. rupestris	Toms	31	5.0	0.439	0.455	A472
	Craig	11	2.4	0.403	0.438	None
	Roanoke	69	4.6	0.454	0.458	None
	Pigg	32	4.1	0.376	0.378	None
	Otter	40	3.4	0.385	0.402	A114
	Staunton	32	3.5	0.400	0.378	A107, A435

Table 1.4: Distribution of deduced haplotypes by stream. Haplotype letters correspond to Figure 1.4. Haplotypes A through Q are putatively *A. cavifrons*, and haplotypes R through Z are putatively *A. rupestris*.

Site Haplotype	ENO	NOT	BLW	USM	LSM	TWN	UFL	LFL	CHT	PIG	STN	ROA	OTT	TOM	CRG
A	5		1	5	1	3	2	2	1		1				
D			5												
E						2									
F		3								1					
J								1							
K								1							
L									3						
M		1													
P		1				3	3	1		2					
Q					2										
R								1		3	10	9	6		3
S											1				
T											1				
U									1						
V										2				1	
W										3		2		1	2
X								1		5	2			2	
Y											1				
Z														1	
Total	5	5	6	5	3	8	5	7	5	16	16	11	6	5	5

Abbreviations for sample sites are as follows: ENO = Eno River, NOT = Nottoway River, BLW = Blackwater River, USM = Upper Smith River, LSM = Lower Smith River, TWN = Town Creek, UFL = Upper Falling River, LFL = Lower Falling River, CHT = Chestnut Creek, PIG = Lower Pigg River + Upper Pigg River, STN = Staunton River, ROA = Roanoke River + North Fork Roanoke River, OTT = Little Otter + North Fork Otter, TOM = Toms Creek, and CRG = Craig Creek.

Table 1.5: Estimates of mitochondrial sequence diversity for populations of *A. cavifrons* and *A. rupestris* based on a 937 base pair sequence from the cytochrome B gene. The standard deviations of haplotype diversity and nucleotide diversity are displayed in parentheses.

Species	Population	n	Haplotypes	Segregating Sites	Haplotype Diversity	Nucleotide Diversity
A. cavifrons	Blackwater	5	2	1	0.400 (0.237)	0.0004 (0.00024)
	Falling	10	4	4	0.733 (0.101)	0.0015 (0.00044)
	Upper Smith	5	1	0	0	0
	Lower Smith/Town Creek	8	4	3	0.821 (0.101)	0.0014 (0.00028)
	Nottoway	5	3	3	0.700 (0.218)	0.0017 (0.00051)
	Eno	5	1	0	0	0
A. rupestris	Toms	5	4	10	0.900 (0.161)	0.0045 (0.00176)
	Craig	5	2	3	0.600 (0.175)	0.0019 (0.00054)
	Roanoke	11	2	3	0.327 (0.153)	0.0010 (0.00047)
	Pigg	12	4	5	0.803 (0.063)	0.0022 (0.00031)
	Otter	4	1	0	0	0
	Staunton	15	5	8	0.562 (0.143)	0.0020 (0.0006)

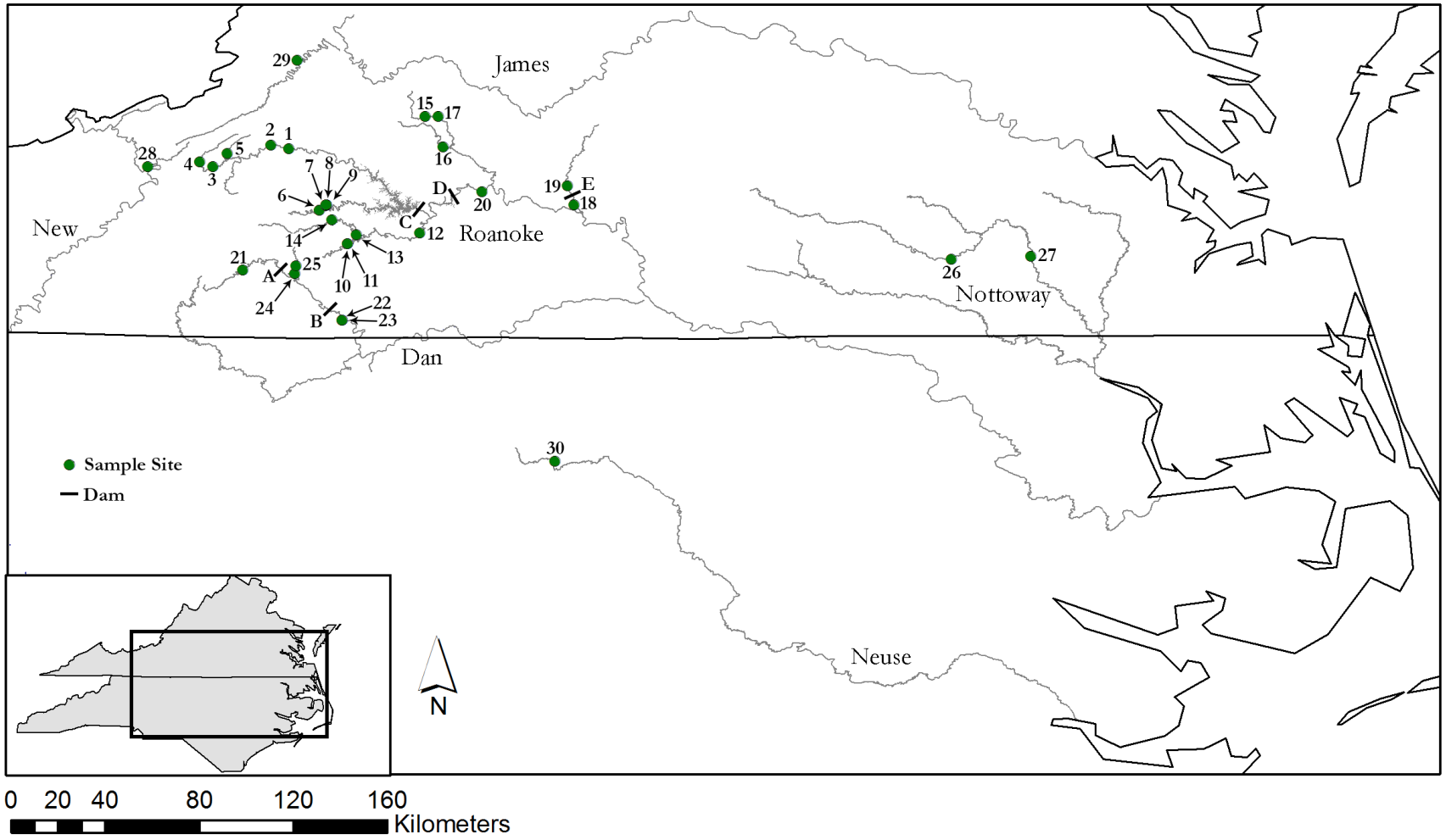


Figure 1.1: Map depicting sample sites throughout the historic Virginia range of *Ambloplites cavifrons*. Numbers correspond to the sites listed in Table 1.1. Several spatially adjacent sites (e.g., 7, 8, and 9) appear as a single point due to the small scale of this map. The five dams depicted are A) Philpott Dam, B) Martinsville Dam, C) Smith Mountain Dam, D) Leesville Dam, and E) Falling River Dam.

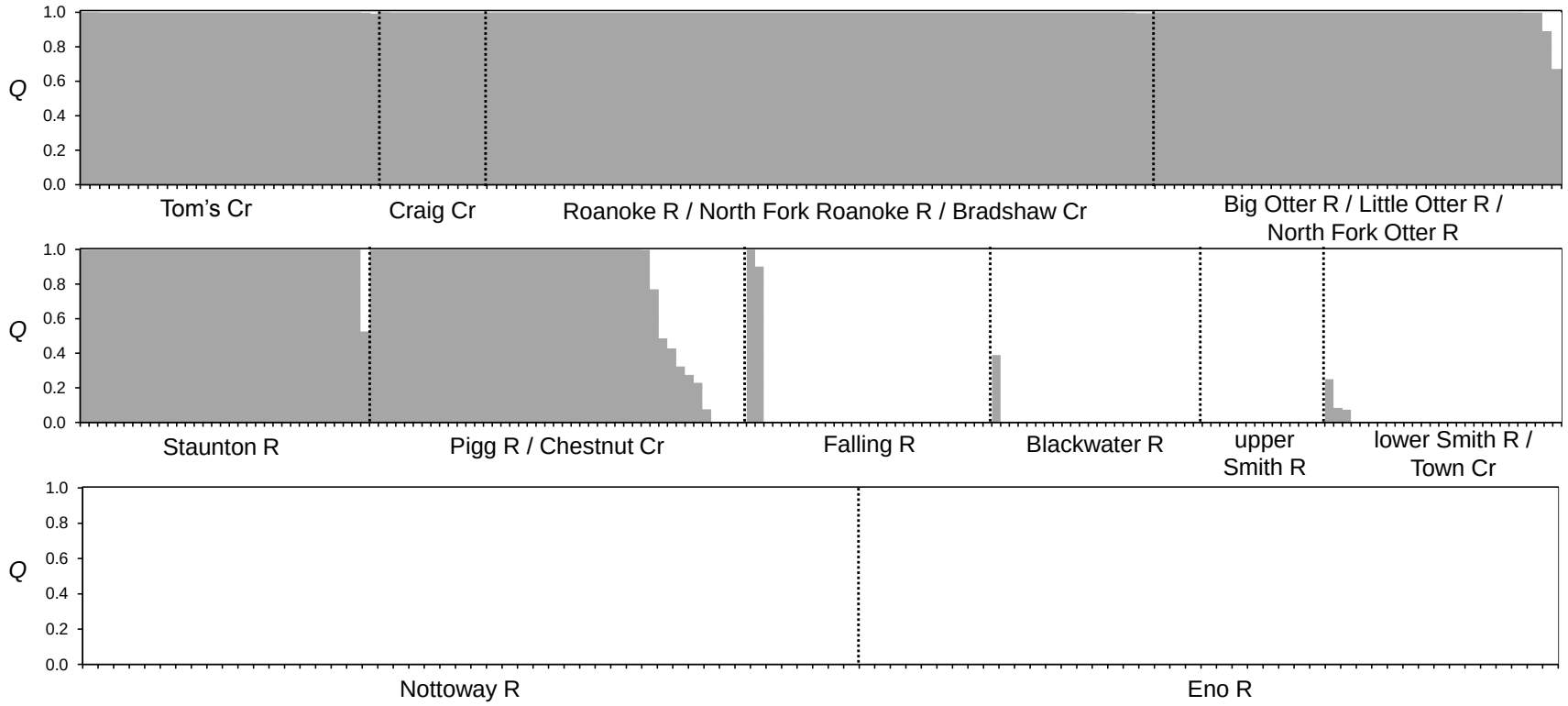
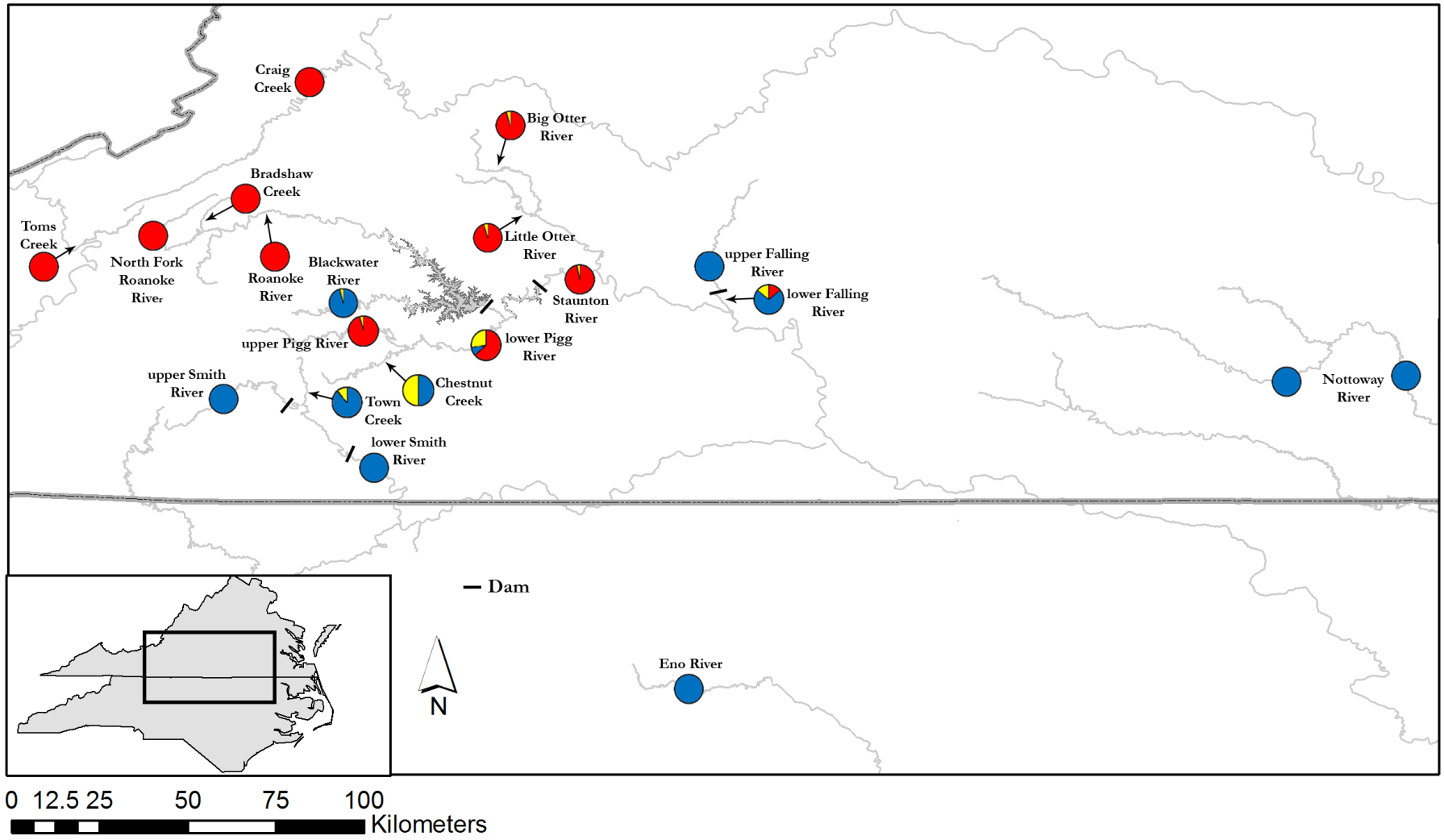


Figure 1.2: Bar plots showing the results of the STRUCTURE analysis. Each vertical bar (n = 417) depicts one individual. The proportion of a bar that is gray or white represents the proportion of that individual's ancestry estimated to have originated from *A. rupestris* or *A. cavifrons*, respectively (i.e., the Q value). Vertical black dotted lines separate river systems.

A)


Craig Creek
Big Otter River
Bradshaw Creek
Toms Creek
North Fork Roanoke River
Roanoke River
Blackwater River
Little Otter River
upper Falling River
lower Falling River
Staunton River
upper Pigg River
lower Pigg River
upper Smith River
Chestnut Creek
Town Creek
lower Smith River
Nottoway River
Eno River
Dam
N
0 12.5 25 50 75 100
Kilometers

B)

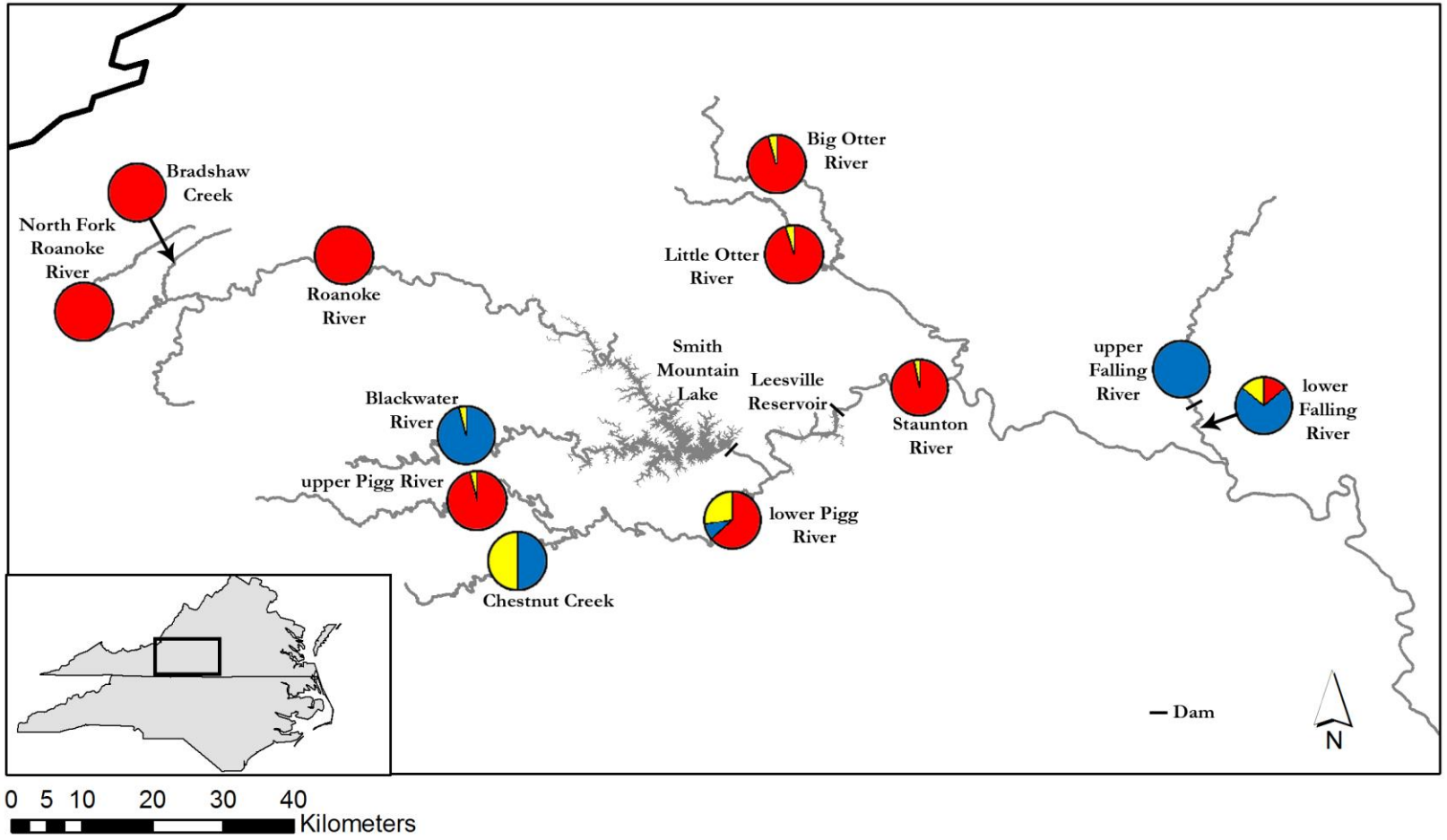


Figure 1.3: A) Map depicting the proportion of parental species across all streams in the study. B) Map depicting the proportion of parental species and hybrids in streams in the Roanoke drainage, where hybridization and replacement are occurring. Genetic identities are derived from the STRUCTURE and NewHybrids analyses. The proportion of *A. cavifrons* is indicated in blue, *A. rupestris* in red, and hybrids in yellow.

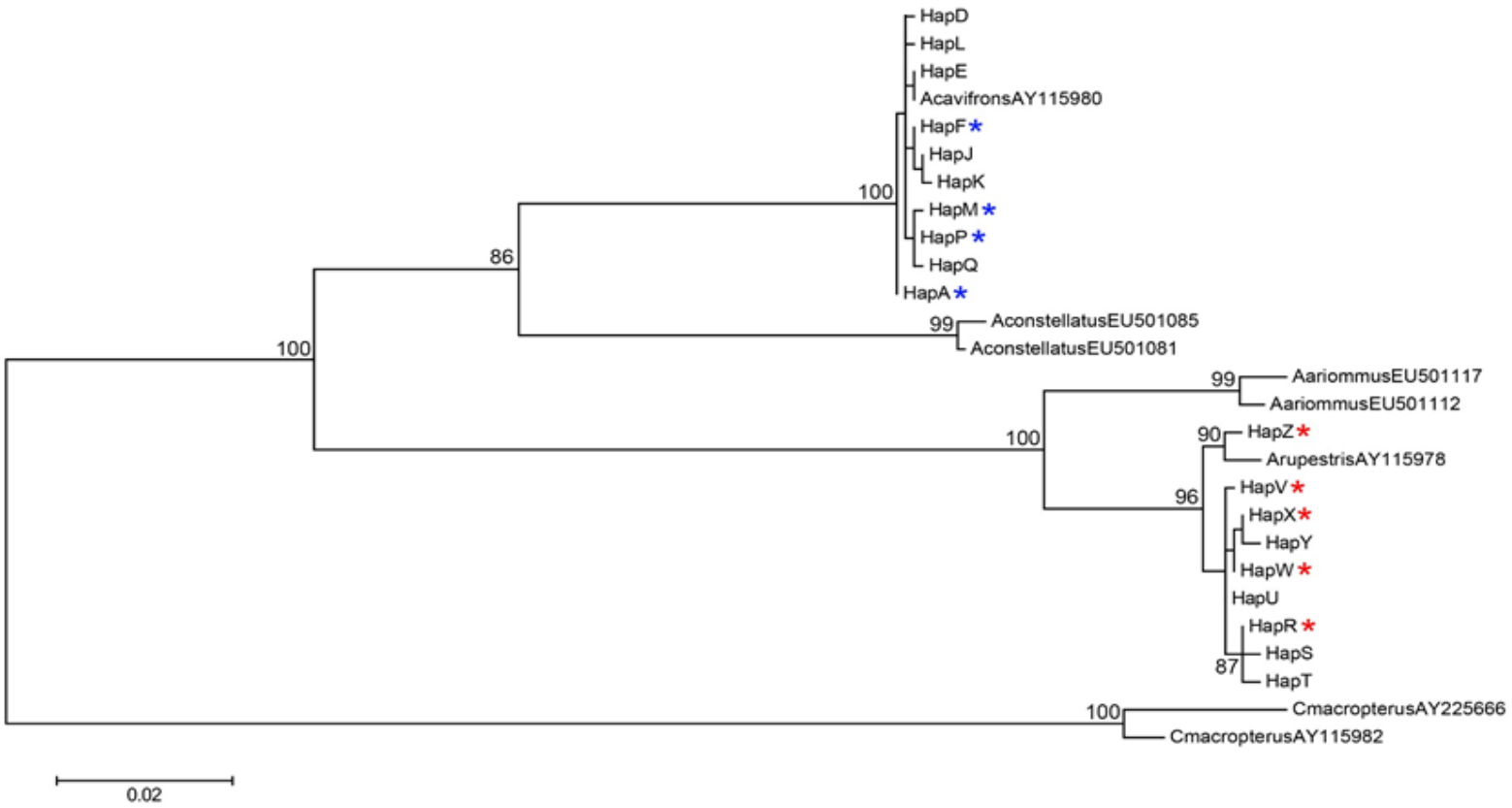


Figure 1.4: Tree displaying inferred phylogenetic relationships among 19 mtDNA haplotypes observed in this study. Sequences from *Ambloplites ariommus*, *A. constellatus*, and *Centrarchus macropterus* obtained from Genbank (accession numbers shown) are also included. A haplotype representative of *A. cavifrons* and a haplotype representative of *A. rupestris* taken from Roe *et al* (2008) were also included for reference. Haplotypes with a blue asterisk are those found in our reference *A. cavifrons* populations (Eno and Nottoway Rivers), and haplotypes with a red asterisk are those found in our reference *A. rupestris* populations (Toms and Craig Creeks). Bootstrap values greater than 80 are displayed at their respective nodes.

CHAPTER 2

INFLUENCES OF LANDSCAPE CHARACTERISTICS ON THE POPULATIONS STRUCTURE AND VIABILITY OF ROANOKE BASS

ABSTRACT

Freshwater fish populations face numerous challenges, as a result of anthropogenic alteration of rivers and watersheds, which can reduce the quality and quantity of habitat and increase habitat fragmentation. Dams act as barriers to fish movement, and along with the watershed urban and agricultural development, may alter flow regimes and in-stream habitat. Streams in the historic range of the Roanoke bass (*Ambloplites cavifrons*) in Virginia and North Carolina have been heavily altered over the past century, through the construction of numerous dams, increases in agricultural production, and urban development. However, the conservation status of *A. cavifrons* has not been recently assessed, and no previous study of genetic diversity and genetic relationships among populations has been conducted, which complicates attempts to conserve this species. I conducted a range-wide conservation genetic study of *A. cavifrons* populations. My objectives were to (1) estimate the spatial scale at which *A. cavifrons* population structure occurs, (2) evaluate the roles of barriers and anthropogenic land use in sculpting genetic diversity and population structure, (3) estimate the degree of connectivity between populations, and (4) estimate effective population size as an index of population viability. I analyzed genetic variation among 331 *A. cavifrons* specimens collected from sites throughout the range of the species. Individuals were genotyped at 19 microsatellite DNA markers; a subset of 64 individuals was further analyzed at the cytochrome B mitochondrial DNA gene. My analysis supported a model of 12 contemporary populations, whose geographic extent approximately corresponded to the stream scale. Genetic structure generally was weak to

nonexistent among sites *within* streams, whereas I found little evidence for connectivity *between* streams. In nearly all cases, population boundaries coincided with the presence of dams. Populations appeared to be isolated from one another, and no migrant individuals were detected. In contrast, weak phylogeographic structuring of mitochondrial haplotypes suggested historically higher connectivity of these now-isolated watersheds. Estimates of effective population size were low (between 40 and 150 individuals) in nearly all populations, and many populations exhibited evidence of past population bottlenecks. Correlation of landscape attributes and genetic diversity statistics suggested positive relationships between genetic diversity and stream size, and negative relationships between genetic diversity and watershed urban and agricultural development. Extant *A. cavifrons* populations are at risk due to isolation, declining stream habitat quality, and negative interactions with invasive Rock bass (see Chapter 1). To counteract these risks and increase population viability, I recommend conservation efforts focus on the translocation of individuals in a manner that mimics historical connectivity of populations. The establishment of refuge populations in streams in the region that do not currently contain the species may also help to reduce the risk of the species' extinction.

INTRODUCTION

Freshwater habitats are easily and frequently fragmented by natural barriers to movement, which isolates populations of aquatic species therein. This propensity for isolation has generated a considerable amount of biodiversity in freshwater systems, as well as a high degree of endemism, and thus irreplaceability of taxa (Allan and Flecker 1993). However, the natural tendency of freshwater habitats towards fragmentation has been artificially inflated by anthropogenic changes to stream systems. Humans have constructed impoundments on virtually every large river in the country, and only 42 rivers remain free flowing for more than 200 km

(Benke 1990). These dams and their accompanying impoundments act as physical barriers to fish movement, and the resulting reservoirs often act as functional barriers to movement of lotic-adapted fishes (Roberts *et al.* 2016). This fragmentation of habitat and the isolation of populations contributes to the large and increasing proportion of freshwater fishes that are imperiled. In fact, physical alteration of stream habitat has been cited in 73% of North American fish extinctions over the past century (Miller *et al.* 1989). Fragmentation may lead to decreases in genetic diversity of isolated populations through the effects of genetic drift (Dehaan *et al.* 2015), increases in the rate of inbreeding (Mills and Allendorf 1996), and prevention of the recolonization of sites that have suffered population declines (Meldgaard *et al.* 2003).

Fragmentation is not solely the result of direct alteration of streambeds (e.g., the construction of dams), but may also be driven by reduced habitat quality, which is often associated with alterations in watershed land use. Unaltered rivers experience a dynamic flow regime, or a pattern in the quantity of stream flow that varies throughout the year, and human land and water use act to alter that pattern. Activities such as timber harvest, livestock grazing, agriculture, and urbanization are the leading causes of altered flow regimes in many rivers (Poff *et al.* 1997). In addition to alteration of flow regimes, the conversion of the watershed to agricultural or urban land often results in increased sedimentation, nutrient enrichment, and contaminant pollution, and may also lead to increases in stream temperature and the loss of large woody debris due to reduced riparian vegetation (Allan 2004). These changes can lead to reductions in the availability of suitable habitat, isolating fish populations in fewer, smaller habitat patches. The phenomenon of freshwater fish imperilment due to the alteration of flow regimes and land use is prevalent in the southeastern United States. In assessing the status of

native southeastern fishes, Etnier (1997) found pollution (including siltation) and anthropogenic alteration of flow contributed to 74% of cases of species imperilment.

Effective management of fishes requires an understanding of population structure, size, and connectivity, and the environmental factors driving these demographic variables. Although traditional mark-recapture techniques can be implemented to obtain estimates of population structure, size, and connectivity, this often requires extensive field work over the course of multiple years (Berry *et al.* 2004). Furthermore, these methods do not lend themselves to demographic study over large geographic extents or long time periods, nor do they provide information about the evolution of genetic diversity within and among populations. Such knowledge provides critical insight into demographic and evolutionary history, population viability, and the processes that lead to the imperilment of a species (DeSalle and Amato 2004).

To establish an understanding of population structure, genetic data can be used to estimate the degree of genetic differentiation between groups of individuals sampled in different locations, and this differentiation can be utilized to cluster individuals into separate populations (Danancher *et al.* 2008, Sønstebø *et al.* 2007). Once populations are delineated, it becomes possible to assess what natural or anthropogenic barriers are correlated or associated with the observed structure (Castric *et al.* 2001, Gomez-Uchida *et al.* 2009, Wofford *et al.* 2005). Additionally, knowledge of population structure permits an investigation of connectivity by testing for individuals with genotypes indicating they originated in a population other than the one from which they were sampled, thereby providing evidence of contemporary dispersal (Hansen *et al.* 2001, Hall *et al.* 2009).

The utility of conservation genetic approaches does not end with the delineation of populations and estimates of connectivity. The characteristics of each population can be

assessed in greater detail, allowing for the identification of those populations that are most at risk. For example, the effective size (N_e) of a population is correlated with its ability to persist (Reed and Frankham 2003), and general guidelines have been established for the minimum N_e necessary for population persistence (Franklin 1980). Molecular techniques may be implemented to estimate the N_e of populations (Luikart *et al.* 2010), thereby providing insight into the relative risk of extirpation faced by each population. In addition to estimates of contemporary N_e, evidence of recent or historic population declines can be obtained via molecular methods (Garza and Williamson 2001).

Finally, molecular techniques can allow for an understanding of the environmental features that have shaped genetics and demography over time. For example, spatial patterns of genetic diversity and estimates of gene flow between populations can be correlated with landscape features that fragment streams, like waterfalls or dams, which can allow for the identification of structures that lead to population isolation and reductions in effective size and/or genetic diversity (Gomez-Uchida 2009, Yamamoto *et al.* 2004). Additionally, patterns in genetic diversity, N_e, and population connectivity can be correlated with spatial characteristics such as drainage area and river distance between populations to determine the effects habitat area and isolation by distance have on the structuring of populations (Castric *et al.* 2001, Yamamoto *et al.* 2004). Analysis of genetic data with an understanding of landscape characteristics may reveal that anthropogenic changes to the landscape have resulted in reductions in the size and connectivity of populations. Such altered population structure can fundamentally disrupt historical demographic and evolutionary processes. Understanding these changes is vital to guiding the conservation of freshwater fish populations, for example by allowing for managed dispersal that mimics historical connectivity (Vrijenhoek 1998).

The Roanoke bass (*Ambloplites cavifrons* Cope) is an increasingly rare sunfish (Perciformes: Centrarchidae) endemic to rivers in southern Virginia and northern North Carolina. The historic range of *A. cavifrons* consists of four drainages: the Tar and Neuse drainages of North Carolina and the Roanoke and Chowan drainages that originate in Virginia and flow into North Carolina (Cashner and Jenkins 1982). The species has one of the smallest native ranges of any sport fish in the eastern United States, and this range has become patchy and diminished in recent history (Jenkins and Burkhead 1994, Chapter 1). Past distributional surveys have indicated that *A. cavifrons* populations are disjunct, as a result of patchiness of suitable habitat (Petrimoulx 1983). *A. cavifrons* prefers areas of continuous flow, firm substrate, and high oxygen content (Cashner and Jenkins 1982), and it appears to be completely intolerant of lacustrine conditions (Jenkins and Cashner 1983).

The patchiness of suitable *A. cavifrons* habitat has likely been exacerbated as a result of changes to streams in the historic range of *A. cavifrons* that have taken place over the past century. The species occurs in a region that is increasingly altered by human activity through ongoing urban development and increases in water consumption. The driving cause behind the alteration of landscapes and riverscapes is the rapid growth in the human population in the southeastern United States, which increased by 84% from 1950 to 1990 (Warren *et al.* 2000). Alterations include the construction of numerous impoundments, which have resulted in habitat fragmentation. For example, the Roanoke River was impounded by the construction of five major dams between 1952 and 1964 (Petrimoulx 1983). These dams and their tailwaters represent physical and functional barriers to in-stream movement of fish. Anthropogenic barriers were found to play a larger role than natural barriers in delimiting populations of Roanoke logperch (*Percina rex*), a benthic darter inhabiting similar stream habitats in this region (Roberts

et al. 2013). A similar pattern may be observed in *A. cavifrons*, however it is important to note that *P. rex* were demonstrated to have remarkably high dispersal (Roberts *et al.* 2016) whereas there is some evidence to suggest that *Ambloplites* may disperse only short distances (based on study of *A. rupestris*; Gatz and Adams 1994). In addition to being barriers to movement, dams act in concert with urban and agricultural development in formerly forested areas to alter the natural flow regime of rivers, which in turn increases sedimentation and pollution and reduces habitat complexity (Poff *et al.* 1997). The range of *A. cavifrons* has been further reduced through the introduction of rock bass (*Ambloplites rupestris* Rafinesque), which apparently has led to the replacement of *A. cavifrons* by *A. rupestris* in several streams (Cashner and Jenkins 1982, Jenkins and Cashner 1983, Petrimoulx 1983, Jenkins and Burkhead 1994, Chapter 1).

If *A. cavifrons* populations exist in isolation from one another, they may face a heightened risk of extirpation. Isolation of populations is often associated with reduced levels of genetic diversity as a result of the increased influence of drift (Dehaan *et al.* 2015), and populations with lower genetic diversity will have reduced adaptive potential (Frankham *et al.* 2010). Furthermore, small, disconnected populations are more subject to stochastic events (both demographic and environmental), and cannot be "rescued" by migrants from another population following a decline (Caughley 1994). On the other hand, a benefit of isolation occurs in the instance of a native species needing protection from an invader, as is the case with *A. cavifrons*. This is an important factor to take into consideration when evaluating the relative costs and benefits of maintaining isolated populations, as improving habitat connectivity has the potential to result in the spread of introduced organisms or the introduction of new invaders (Beisel *et al.* 2015, Dehaan *et al.* 2015). Due to the presence of invasive *A. rupestris* in many streams throughout the Roanoke drainage, and their apparent ability to rapidly displace *A. cavifrons*

(Chapter 1), caution is warranted in the removal of any barriers between streams in that system. Although improving connectivity may benefit populations experiencing decreased genetic diversity due to isolation, the translocation of a small number of individuals may be a better management tool, as this involves less risk of an invader being introduced to a system (Dehaan *et al.* 2015). The use of translocations to supplement streams in which species have been severely reduced or extirpated has been demonstrated to lead to successful population reestablishment. For example, populations of four species of shiner were successfully reestablished in the Pigeon River in North Carolina, an isolated system in the French Broad River drainage, via translocation of individuals from other streams in the drainage (George *et al.* 2009). Translocation carries its own risks, however, namely in the form of outbreeding depression, which is reduced fitness in the offspring of a cross between members of two different populations due to either a disruption in the interactions between genes or between genes and the environment (Edmands 2007). The demographic and evolutionary risks associated with isolation must be weighed against the risk of outbreeding depression prior to carrying out any translocations. Molecular techniques make it possible to evaluate the relative risk of outbreeding depression by allowing for the detection of significant genetic differentiation between populations (Frankham *et al.* 2010).

In this study, I utilized conservation genetic techniques to evaluate the status of extant *A. cavifrons* populations, to help guide the protection and recovery of this species. I used nuclear and mitochondrial DNA analyses to address four main questions. First, what is the contemporary population genetic structure of the species and what maintains population boundaries? I hypothesized that *A. cavifrons* populations exist at the stream scale, and are delineated by a combination of anthropogenic barriers (e.g., dams and reservoirs), natural barriers (e.g., river-basin boundaries), and spatial distance (e.g., isolation by distance).

Alternative hypotheses include *A. cavifrons* occurring in a single panmictic population or as several large populations occurring at the basin scale, or that *A. cavifrons* populations are subdivided within streams and exist at the stream-reach scale. Comparing data from nuclear microsatellite and mitochondrial markers will allow for the disentanglement of historic and contemporary trends in population structure and connectivity. Mitochondrial DNA mutates more slowly than the assessed neutral regions of nuclear DNA (microsatellites) and experiences genetic drift more quickly than nuclear DNA (Ballard and Whitlock 2004). These two factors lead to less standing variation in mitochondrial DNA than there is in nuclear DNA, and therefore it takes longer to reach a new mutation-drift-migration equilibrium following a demographic change. Therefore, analysis of mitochondrial DNA can provide evidence of historic relationships that would have been obscured by time in nuclear DNA.

Second, what is the degree of contemporary gene flow between populations of *A. cavifrons*? Due to the presence of dams in nearly all assessed streams, I hypothesized that there is little to no gene flow occurring between streams, and no gene flow between drainages, which will be evidenced by a high degree of genetic differentiation between individuals collected from different streams. Furthermore, I hypothesized that contemporary dispersal is low, which will be supported by the detection of few or no migrants that have moved between streams. An alternative hypothesis would be high connectivity between populations among drainages and/or between populations within drainages.

Third, what is the contemporary effective size of populations, and do they show signs of decline over time? I predicted that the estimated effective size of extant *A. cavifrons* populations will vary substantially among streams, with those of small streams that are isolated by anthropogenic barriers falling beneath the suggested minimum for long-term adaptive potential

(N_e > 500, Franklin 1980), and those of larger streams with few to no anthropogenic barriers being sufficient for long-term adaptive potential. Furthermore, I predicted that evidence of recent and/or historical decreases (bottlenecks) would only be detected in small, isolated populations, which are more subject to stochastic events leading to population decline. The alternative predictions that all estimated effective population sizes will be similar among all streams and that there will be no evidence of demographic instability are less likely, given that many of the current streams occupied by *A. cavifrons* are low order and isolated, and therefore more subject to stochastic events leading to population declines, and unable to be recolonized following said declines.

Fourth, how have modifications of the riverscape (e.g., dams, reservoirs, and land use changes) affected the genetic diversity of populations? I hypothesized that deforestation and urban and agricultural development would be negatively correlated with measures of genetic diversity, as these changes represent an alteration of the conditions to which *A. cavifrons* is adapted. However, numerous other variables could play a more significant role in the creation of patterns in genetic diversity, including temporal variation in ecological conditions (Castric *et al.* 2002) or the life history attributes of the species (Gomez-Uchida *et al.* 2009). In this case, there may be no apparent correlation between patterns in genetic diversity and contemporary land use or changes in land use.

METHODS

Sample Collection

Personnel from Virginia Tech, the Virginia Department of Game and Inland Fisheries (VDGIF), and the North Carolina Wildlife Resource Commission (NCWRC) collected 568 *Ambloplites* specimens from 45 sites across 17 Virginia and North Carolina watersheds between

2012 and 2016. Fish were collected by angling or by backpack, barge, or boat electrofisher, depending on site conditions. The sites were distributed across all North Carolina and Virginia watersheds known to historically harbor *A cavifrons*, with the exception of the Meherrin River in Virginia, which was not sampled due to difficulties with access. Upon capture, specimens were euthanized in a 100 mg/L solution of MS-222 (Argent Laboratories, Redmond, Washington, USA). The right pectoral fin was removed with flame-sterilized scissors and placed in either a coin envelope or a vial of 95% ethanol. Each envelope or vial was given a unique identification number and a metal jaw tag with the same number was attached to the corresponding specimen, which was vouchered in 10% formalin. Tissue samples were stored at -20˚C until DNA extraction. Based on results from Chapter 1, 331 of these individuals, originating from 31 sites across 12 Virginia and North Carolina watersheds, were determined to be genetically pure *A. cavifrons* (others were *A. rupestris* or hybrids) and were further analyzed for this chapter (Figure 2.1; Table 2.1).

Laboratory Methods

DNA was extracted from fin clips using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Using libraries developed from *A. rupestris*, we previously identified seven microsatellite loci that were polymorphic in *A. cavifrons* (Eschenroeder and Roberts 2016, Chapter 1). To facilitate more powerful genetic analyses of *A. cavifrons*, we partnered with the Savannah River Ecology Laboratory (SREL) to develop an additional panel of microsatellite markers specifically for that species. Using paired-end Illumina sequencing, SREL identified an additional 7453 potential microsatellite loci; from these, I identified 12 that reliably amplified and exhibited polymorphism in *A. cavifrons* samples (Appendix I). The 19 total markers were grouped into five multiplexes, as follows: Multiplex 1

containing A111, A114, A115, and A432; Multiplex 2 containing A145, A464, and A472; Multiplex 3 containing Acav39, Acav22, Acav19, and Acav37; Multiplex 4 containing Acav25, Acav29, Acav17, and Acav31; and Multiplex 5 containing Acav26, Acav21, Acav28, and Acav23 (description of PCR mixes and cycling conditions in Appendix II). Amplified PCR products were analyzed using an ABI 3500 Genetic Analyzer with a Genescan 500HD LIZ dye size standard (Applied Biosystems) and sized in GeneMapper (GeneMapper v.4.0; Applied Biosystems).

In addition to the 19 nuclear markers, I subsequently analyzed variation at the mitochondrial cytochrome B gene across a sample of 2 to 10 individuals (5, in most cases) from each deduced population (64 sequences total) using the degenerate primers AmbloCytB-F (5' – RTGRCTTGAAAAACCACCGTTG) and AmbloCytB-R (5' – CYCSRYVTCCRGTTTACAAGAC). PCR reaction mixes were 25µL in total volume, and consisted of 12.5µL GoTaq Mastermix (Promega, Madison, Wisconsin, USA), 0.5µL forward primer, 0.5µL reverse primer, 9.5µL molecular-grade H_20, and 2 µL of template DNA (typical concentration ~40ng/µL). Cycling conditions were as follows: initial denaturation at 95˚C (150 s), 35 cycles of denaturation at 95˚C (60 s), annealing at 58˚C (60 s), and extension at 72˚C (90 s), followed by a final extension at 72˚C (420 s). PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium, Hayward, California, USA). Upon verification of successful amplification, PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, California, USA) and forward and reverse sequenced by Eton Bioscience, Inc. (Research Triangle Park, North Carolina, USA). The sequences were checked and edited in Sequencher® v.5.4. software (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned using the

"Clustal" option in MEGA v.6 (Tamura *et al.* 2013). All sequences were trimmed to a 973 base-pair fragment that reliably sequenced in all 64 individuals.

Nuclear Data Analysis

Prior to conducting preliminary tests of microsatellite marker suitability, individuals were grouped into 13 tentative "populations" based on the distribution of putative movement barriers (dams and watershed boundaries) between sites (Appendix III). Using ARLEQUIN version 3.5 (Excoffier and Lischer 2010), I tested whether genotype frequencies were at Hardy-Weinberg equilibrium for each locus in each population (10^6 Markov chain Monte Carlo [MCMC] steps following a burn-in of 10^5 steps), and tested for linkage disequilibrium between each pair of loci within each population (10^5 permutations). A sequential Bonferroni correction was used to adjust Hardy-Weinberg and linkage disequilibrium test statistics for multiple comparisons, based on an experiment-wide alpha of 0.05 (Rice 1989).

Following these preliminary tests, I used further analyses to test hypotheses about the spatial scale and drivers of population structure, and grouped individuals into data-informed, *a posteriori* populations. I utilized the Bayesian clustering approach described by Pritchard (2000) implemented in the software STRUCTURE 2.3.4 to estimate the number of hypothetical clusters (K) that give rise to the sample of genotypes. The model allowed for admixture and correlation of allele frequencies among clusters, and searched parameter space using 10^6 recorded MCMC steps following a burn-in of 10^5. Ten replicates were run for each K value from 1 to 20, and log-likelihood values averaged across replicates. An initial analysis of K values 1 to 31 (data not shown) indicated that there was no improvement in log-likelihood of the model beyond a K of 20. The K value with the highest average log-likelihood was considered the best-supported model of *A. cavifrons* population structure.

After the delineation of populations, I used a complementary analysis to assess the prevalence of apparent dispersal between populations. I used STRUCTURE to estimate the posterior probability that each individual's genotype originated from the population where it was collected, versus each of the other sampled populations. An individual whose resident probability was low relative to its immigrant probability was presumed to be a first-generation migrant from the population with the highest probability. For this analysis, *K* was fixed at the estimated true number of populations, as determined by the previous analysis, and capture location was incorporated as a Bayesian prior in the model. The model assumed correlation of allele frequencies among populations, and a liberal prior migrant probability of 0.5, which gave every individual an equal prior probability of being a resident or immigrant. Use of alternative, more-conservative migrant probabilities (< 0.5) produced identical model outcomes (see Results). Three replicate runs were performed, each searching parameter space for 10^6 recorded MCMC steps, following a burn-in of 10^5. All individuals were included in this analysis, except for those captured at sites in Chestnut Creek, lower Pigg River, and lower Smith River (n = 8 individuals total), as these sites were represented by too few individuals to characterize those populations' allele frequencies.

In addition to individual-based STRUCTURE analyses, I used three group-based approaches to further investigate population structure. These analyses were conducted at the site scale, and were based on the 20 sites from which at least 5 individuals were sampled. To visualize population structure and genetic relationships among sites, I calculated Nei's minimum genetic distance (D_m; Nei 1973) between all pairs of sites and used these D_m values to create a neighbor-joining tree in POPULATIONS 1.2.3 (Langella 1999), then visualized the tree in Figtree (Rambaut and Drummond 2009). To estimate the primary spatial scales at which

populations were structured, I conducted an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) in ARLEQUIN. Molecular variation was decomposed into four hierarchical spatial scales: among basins, among streams within basins, among sites within streams, and among individuals within sites. I used permutation tests (10^6 iterations) to assess whether the percentage of variation at each scale was significantly different from zero.

Previous analyses assumed that population structure was discrete, based on the distribution of boundaries to gene flow. I also evaluated the prevalence of continuous or clinal differentiation, by visualizing and testing the relationship between genetic and spatial distance between sites (i.e., isolation by distance; IBD). I used traditional genetic distance methods (pairwise F_{ST}) to determine the magnitude of genetic differentiation among sites. Pairwise F_{ST} values were calculated in ARLEQUIN, and pairwise spatial distances in river kilometers were calculated using network analysis in ArcMap v. 10.4. To evaluate the significance of the IBD relationship, I performed Mantel tests (10^4 permutations) using the ecodist package (Goslee and Urban 2007) in R 3.2.5 (R Core Team 2012). Relationships were tested for all sites together and separately by basin. Although it is within the Roanoke basin, sites in the Dan River sub-basin were treated separately in this and subsequent analyses, as they are separated from the remainder of the Roanoke basin by numerous anthropogenic barriers and substantial river distances. In order to evaluate the role of dams versus stream boundaries in promoting differentiation, I also tested the significance of the IBD relationship separately for: (1) pairs of sites within a stream with no dam separating them, (2) all pairs of sites with no dams separating them, regardless whether they were in the same stream, and (3) pairs of sites separated by a dam. For this analysis, all known, significant manmade structures impounding a stream were considered a dam, regardless of the physical size of the barrier or the size of its associated reservoir (see

Figure 2.1). In addition to Mantel and permutation tests, relationships between spatial and genetic distances were plotted to allow for visual interpretation of differences in slope.

For each of the *a posteriori* populations delineated by the STRUCTURE analysis, I estimated genetic diversity statistics, the effective population size (N_e), and a metric indicative of past population bottlenecks. Observed heterozygosity (H_O), unbiased gene diversity (H_E), allelic richness (A_R; rarified to the minimum sample size of 4 individuals), and inbreeding coefficient (F_{IS}) were estimated in FSTAT 2.9.3.2 (Goudet 2002). Evidence for past bottlenecks was obtained by calculating Garza and Williamson's (2001)*M* statistic (averaged across the 19 loci) for each population in ARLEQUIN, and comparing values to published ranges for stable and bottlenecked populations. The *M* is the ratio of the number of alleles to the size-range of alleles within a population; this metric has been shown to decrease relative to its equilibrium value following a population bottleneck. Estimated M values were compared to the published range of *M*s from populations known to have experienced a decline and the range of *M*s from populations that are known to be stable (Garza and Williamson 2001). To assess the size, potential influence of drift, and potential viability of populations, I estimated N_e using the linkage disequilibrium method implemented in LDN_e v. 1.31 (Waples and Do 2008). I utilized only populations from which at least 20 individuals were sampled. The model implemented by LDNe assumed random mating, non-overlapping generations, and sampling of a single generation. I violated the latter two assumptions, because *A. cavifrons* is iteroparous and I was forced to pool multiple (unknown-age) cohorts for analysis. However, all populations should have been affected similarly by any biases these violations created, and thus the variation in N_e estimates between populations should be proportional to the relative variation in the actual size of the gene pools (Robinson and Moyer 2013). As such, estimates should be construed as relative measures of

approximate gene-pool size that are intermediate to N_e and the effective number of breeders (N_b), rather than exact measures of N_e. In the LDNe approach, rare alleles can have a large effect on estimates (Waples and Do 2008); I estimated mean N_e under three different modeling assumptions, by excluding alleles occurring at a frequency <0.05, <0.02, and <0.01. Ninety-five percent confidence intervals were estimated by jackknifing.

In order to test for correlations between the spatial characteristics and land use patterns of watersheds and genetic diversity variables, I used StreamStats version 3 (water.usgs.gov/osw/streamstats/) to collect values for seven watershed characteristics for each sample site: drainage area, and percent developed area, percent forested area, and percent cropland area in 2001 and 2011. I selected drainage area to serve as a proxy for habitat area, and land cover categories were chosen to evaluate the effect of anthropogenic alteration of the landscape on the genetic diversity of *A. cavifrons* populations. StreamStats derives drainage area through the use of a GIS program that delineates drainage basin boundaries based on elevation data from the USGS 3D Elevation Program. For populations sampled at multiple sites (e.g., four sites in the Tar River), I selected the drainage area estimate from the most-downstream site to represent stream size for that population. StreamStats derives all land cover attributes from the 2001 (Homer *et al.* 2007) and 2011 (Homer *et al.* 2015) National Landcover Databases. For populations sampled at multiple sites, I averaged land cover percentages across all sites for that population. I calculated the percent change in area for each land use category by subtracting the 2011 value from the 2001 value. To test for relationships, I calculated the Pearson correlation coefficient (r) between each of these landscape variables and each of the population genetic diversity statistics (M, H_E, and A_R).for each population. The significance of correlations was tested using one-tailed permutation tests in R (10^4 permutations). Due to the higher sample size

necessary for calculating *M* (Garza and Williamson 2001), only populations with $n \geq 20$ were included in this analysis.

Mitochondrial Analysis

Mitochondrial sequences were obtained for a subset of individuals that were randomly selected from each of the 12 populations identified by the STRUCTURE analysis (see Results), and were grouped by those 12 populations for all subsequent analyses. I sought to obtain a minimum of five sequences from each population, and in cases where sample size fell below this value (e.g., Chestnut Creek) I sequenced all available individuals. Sequence data were analyzed in DNAsp v. 5.10.1 (Librado and Rozas 2009), which was used to obtain estimates of haplotype (Nei and Tajima 1981) and nucleotide (Nei 1987) diversity for each population and overall. Obtaining these values allows for the comparison of genetic diversity among populations, and for the identification of populations with particularly low diversity and reduced adaptive potential. Percent divergence (i.e., p-distance) was calculated between pairs of haplotypes by dividing the number of base-pair changes by the total number of base pairs (973). To infer evolutionary relationships between haplotypes, I used PopART (http://popart.otago.ac.nz) to create a median joining haplotype network. This network was constructed to determine the most parsimonious evolutionary history that is consistent with the observed data, thereby identifying any patterns that may indicate common ancestry among current populations.

RESULTS

Utility of Microsatellite Markers

After Bonferroni correction, Hardy-Weinberg equilibrium was rejected in only one of 309 tests (Appendix III), indicating that null-alleles did not have appreciable influence on allele frequencies. Additionally, only 10 out of 3420 locus pairs showed evidence for linkage

disequilibrium, and these pairs were randomly distributed among loci and sampling locations (Appendix III). These results suggest the sampling efforts were representative of the true allele frequencies, and there is no evidence for strong influences of null alleles or Wahlund effects. I therefore retained all 19 loci for subsequent analyses.

Analysis of Population Structure

Pairwise estimates of F_{ST} among all sample sites with $n \geq 5$ revealed significant variation between basins, as well as between streams within basins (Table 2.3). Insignificant pairwise F_{ST} indicative of panmixia was found among eight site pairs located within the same stream (sites BW1 and BW2, BW2 and BW3, ENO1 and ENO2, ENO2 and ENO3, FISH1 and FISH2, FLAT1 and FLAT2, NOTT1 and NOTT2, and UFALL and LFALL; Table 2.3). The fact that panmixia was found between UFALL and LFALL is noteworthy, given that these two sites are separated by a dam. There was significant differentiation between other site-pairs located within the same stream, despite the lack of a dam or any other known barrier between these sites (sites BW1 and BW3, ENO1 and ENO3, and TOWN1 and TOWN2; Table 2.3). The AMOVA revealed that 10.97% of total genetic variation occurs at the among basin scale, 8.32% of variation occurs at the among stream within basin scale, and only 0.98% of variation occurs among sites within streams (Table 2.4). The preponderance of diversity (79.73%) occurred among individuals within sample sites (Table 2.4). All variance components were found to be significantly greater than zero (all $P < 0.0001$; Table 2.4).

The results of the STRUCTURE analysis of all 331 individuals indicate a model of 10 discrete populations (K=10) had the highest average log-likelihood (Figure 2.2). However, the difference in average likelihood between the K=10 and K=12 models was small, and upon examination of model results, K=12 models contained additional phylogeographic information

not contained in models with K=10. For example, in the K = 10 model, individuals from the Pigg River/Chestnut Creek population cluster were not classified as a separate population, but were grouped with the Smith River and Town Creek populations, and the Swift Creek population clustered with the Fishing Creek population. Thus, I selected the K=12 iteration with the highest log likelihood as the best model of population structure (Figure 2.3). Most of these populations appeared to be separated at the stream scale, with reservoirs and dams acting as boundaries. The only exceptions to this pattern were that (1) lower Smith River individuals clustered together with upper Smith River individuals, despite these areas being separated by Philpott Lake and two sizeable dams, and (2) lower Falling River individuals clustered with individuals from upper Falling River, despite these areas being separated by an unnamed dam near the Virginia State Route 40 bridge. The majority of individuals shared most of their ancestry with other individuals from the same population, although there is some evidence of admixture between sites in the Tar River and Fishing and Swift Creeks (of which only the Tar is isolated by dams and reservoirs).

The site-based neighbor-joining tree supported population memberships indicated by the STRUCTURE analysis, and provided additional information about the magnitude of genetic differentiation among populations (Figure 2.4). The tree was based on 20 sample sites (all sites with $n \geq 5$), and possessed 11 terminal clusters of sites, which reflect the populations identified by the K = 12 model (individuals from sites in lower Smith River, lower Pigg River, and Chestnut Creek were excluded due to low sample sizes). The tree indicates a clear genetic division between sites in Virginia and North Carolina. Moreover, all sites in the Chowan, Dan, Neuse, and Tar drainages clustered together by basin. Only sites in the Roanoke basin did not follow this pattern. Sites in the Blackwater River were genetically distant from other Virginia

sites, and in fact appeared more genetically distant from the other sites in the Roanoke drainage (upper and lower Falling River) than from sites in the Dan and Chowan drainages.

Testing for first-generation migrants in STRUCTURE revealed no individuals assigned with high probability to a different population than the one from which they were collected, despite the assumption of a liberal migration prior of 0.5. Thus, not surprisingly, the same result was obtained with more conservative priors (results not shown). Therefore, it does not appear that our samples contained any individuals who had recently moved from one population to another. Many of the populations are separated by impermeable barriers such as dams and reservoirs, which we hypothesized would preclude dispersal. However even populations not separated by an obvious physical barrier (e.g., Fishing Creek and Swift Creek) had no detectable dispersal events.

Estimation of Population Size, Detection of Bottlenecks, and Assessment of Genetic Diversity

Estimates of mean N_e depended on the lowest allele frequency cutoff used, but the order-of-magnitude and ranking of populations largely stayed the same across these modeling choices (Table 2.5). Waples and Do (2010) suggest the exclusion of alleles occurring at a frequency less than 0.02 yields the most accurate N_e values, so the values derived using a lowest allele frequency of 0.02 may be the best estimates. Regardless the cutoff used, many of the estimates had wide 95% confidence intervals, including an upper limit of infinity. Additionally, the Blackwater River population exhibited a negative mean N_e, which indicates that N_e was inestimable due to either a large true N_e or inadequate sample size. Caution is warranted in the interpretation of estimates for populations with sample sizes less than 30, which can cause an upward bias in N_e (Luikart *et al.* 2010, Waples and Do 2010). This would include Blackwater River (n = 23), Falling River (n = 26), and Town Creek (n = 22). Although the results for these

systems may have experienced a slight upward bias due to small sample size, only that for the Blackwater River population showed evidence of being inestimable (negative N_e), and estimates for the Falling River and Town Creek populations were fairly consistent across multiple allele cutoff values. Of the populations tested, those in the Tar and Nottoway rivers have the largest estimates of N_e, which was expected given that these are the two largest, main-stem systems from which samples were collected. The smallest N_e estimates belonged to the populations in Town Creek and the Eno, Little, and Flat Rivers. Of these streams, Town Creek and the Little and Flat Rivers have the smallest drainage areas of any of the streams in this analysis.

The *M* ratio ranged from 0.657 to 0.843 among populations (Table 2.6). Of these, only the highest (Nottoway River population = 0.843) was within the published range of *M* values (0.823 to 0.926) from populations with known, demographically stable histories (Garza and Williamson 2001). Two populations (those in the Flat and Eno Rivers) had *M* ratios that fell within the published range of *M* values (0.599 to 0.693) from populations known to have experienced a bottleneck (Garza and Williamson 2001). The populations in the Blackwater River and Town Creek are represented by fewer than 25 individuals, which Garza and Williamson (2001) suggested was the minimum sample size at which most alleles present at 2% or above would be detected. Therefore, *M* ratio estimates for the populations in those two systems should be interpreted with caution.

Genetic diversity statistics were calculated for each population identified by the STRUCTURE clustering analysis. Most genetic diversity parameters, including mean number of alleles (*A*), allelic richness (A_R; rarified to a sample size of $n = 4$), and expected heterozygosity (H_E), were found to be lowest in the Blackwater River (2.47, 2.03, and 0.50, respectively; Table 2.2). As the Blackwater River is a system that has been isolated by dams and reservoirs, these

low genetic diversity values may have resulted from the increased effect of drift on that population. A_R and H_E were highest in the Nottoway, Lower Smith, and Pigg River populations (Table 2.2). High genetic diversity in the Nottoway River population may have resulted from a lack of dams and the large drainage area of that system, as higher connectivity and greater habitat area could allow for less genetic drift and higher effective population sizes. However, systems with large drainage areas and no dams did not have universally higher genetic diversity. For example, many populations in small, isolated streams (e.g., Town Creek, Flat River) exhibited higher genetic diversity than the Tar River and Fishing Creek populations, despite the fact that both have very large drainage areas compared to other sites and the fact that Fishing Creek is not isolated by dams. The genetic diversity observed in the Lower Smith and Pigg River populations, which was even higher than that in the Nottoway River population, also fails to support the assumption that small, impounded streams will have lower genetic diversity as both systems are fragmented by dams and have substantially smaller drainage areas than the Nottoway. However, it is possible the high values for these populations could be an artifact of low sample size, as each population was represented by only four individuals.

Isolation by Distance

IBD was significant at large spatial scales, among sites separated by barriers, but generally was not significant at small spatial scales and in the absence of barriers (Figure 2.5). When all sample sites with $n \geq 5$ were considered, the correlation between pairwise F_{ST} and pairwise river distance was found to be positive and significant (190 comparisons, $R = 0.68$, $P = 0.0001$). At the within-basin scale, IBD was significant in the Roanoke (10 comparisons, $R = 0.98$, $P = 0.0340$) and Neuse (15 comparisons, $R = 0.84$, $P = 0.0050$) basins, but not in the Dan sub-basin (3 comparisons, $R = 0.92$, $P = 0.3300$) or the Tar basin (6 comparisons, $R = 0.78$, $P =$

0.0800). The lack of significance in the Dan and Tar basins may be an artifact of small sample size rather than an indication that there is truly no relationship between genetic differentiation and spatial distance. The IBD relationship could not be evaluated in the Chowan basin, as there were only two sample sites in that system. IBD was not significant for sites located within the same stream with no barriers separating them (10 comparisons, R = -0.2668, P = 0.2497), but when sites in Fishing and Swift Creeks (the only sites located in different streams that are not separated by dams) were included, IBD became positive and significant (12 comparisons, R = 0.7855, P = 0.0124). Thus, these two data points had a large influence on the strength of IBD. The IBD relationship for sites with at least one dam separating them was significant (177 comparisons, R = 0.5478, $P < 0.0001$).

Assessment of Mitochondrial Diversity

Among the 64 sequenced *A. cavifrons* individuals, 12 unique haplotypes were identified (Table 2.6). The Falling River population (including upper and lower Falling sites) had the largest sample size of individuals (n = 10) and also the greatest number of haplotypes (k = 4), two of which were unique to that system (Table 2.7). Unique haplotypes also were observed in Fishing and Chestnut creeks and the Blackwater, Nottoway, and lower Smith rivers (Table 2.7). However, based on the median joining haplotype network, all haplotypes had close evolutionary relationships with one another, and were separated by a range of one to five mutation events, or 0.1 to 0.5% divergence (Figure 2.6). There was no clear geographic population structure to the haplotype relationships, and no basin clustered entirely separately from the rest. Populations in three of the five basins (Neuse, Dan, and Roanoke) shared at least one haplotype with populations in all other basins, and populations in the Chowan and Tar basins shared at least one haplotype with all basins but each other (Figure 2.6).

Only 58 of the 64 sampled sequences were considered in the assessment of stream-scale population genetic diversity, as the sample sizes from the Lower Smith River (n = 4) and Chestnut Creek (n = 2) were too small to allow for analysis. The overall haplotype (h) and nucleotide (π) diversity were 0.403 and 0.00064, respectively (Table 2.6). The Town Creek population exhibited the highest h (0.8) and the Nottoway River population exhibited the highest π (0.00165). Populations in the upper Smith and Eno Rivers and Swift Creek all exhibited no variation (Table 2.6). The ranking of populations based on mitochondrial diversity statistics shows some similarities with the ranking of populations based on nuclear diversity statistics, including high genetic diversity in the Nottoway River population and low genetic diversity in the Eno River population. There also were some marked differences, including high relative mitochondrial genetic diversity despite low nuclear genetic diversity in the Blackwater population. In contrast, the upper Smith River population exhibited high nuclear genetic diversity, but it had the lowest mitochondrial genetic diversity.

Comparison of Landscape Characteristics and Estimates of Genetic Diversity

Drainage area and land usage varied considerably among populations, but nearly all populations exhibited increases in percent developed area and decreases in percent forested area and percent cropland between 2001 and 2011, although most changes were less than 1% (Table 2.8). M, H_E, and A_R were all positively correlated with drainage area and percent forested area, and they were all negatively correlated with percent cropland, percent developed area, percent change in cropland, and percent change in developed area (Table 2.9). Tests indicated that the correlations between all genetic diversity variables and drainage area were statistically significant, but the only other significant correlations were between M and percent cropland and between A_R and change in percent cropland (Table 2.9). However, the trends in directionality of

the correlations across all genetic diversity variables suggest a pattern, and the lack of significance may be an artifact of small sample size. Interestingly, *M* had the largest correlations with all of the aforementioned landscape variables with the exception of percent cropland and percent forested area. The strongest correlations were seen between *M*, A_R and drainage area, *M*, A_R, H_E and percent cropland, *M* and change in percent developed area, and *M* and change in percent cropland (Figure 2.7). It should be noted that several of these strong correlations were heavily influenced by outliers. In the case of drainage area the outlier was the Nottoway watershed (which drains an area 1689 km^2 larger than the second largest drainage), and in the case of change in percent developed area the Eno watershed was the outlier with an increase of 0.67%.

DISCUSSION

Spatial Scaling and Determinants of Population Structure

An understanding of how populations are structured is crucial for the interpretation of the patterns driving diversity in a species, as well as for the development of a management or recovery plan. My analysis suggests that the contemporary structure of *A. cavifrons* results from a combination of factors, including the presence of dam, isolation-by-distance, and potentially isolation due to barriers to movement in the form of unsuitable habitat. Analysis of genetic variation among *A. cavifrons* populations indicates the presence of discrete populations that exist at the stream scale, with no detected exchange of individuals between streams. The structuring of populations by stream largely aligns with the presence of dams that separate streams from one another, but there are several notable exceptions. Fishing and Swift Creek were found to be separate populations despite the lack of a dam between them, but swampy, low-gradient habitat in the lower portion of Swift Creek may serve as a functional barrier between the two streams.

The only case in which individuals from multiple streams were assigned to the same population was that of the Pigg River and Chestnut Creek. Although both populations were represented by very low sample sizes (n = 1 and 3, respectively), which precluded a more thorough analysis of differentiation between individuals collected from the two sites, there are no known barriers separating the two streams from one another and populations in this system may be subject to isolation-by-distance rather than isolation due to physical barriers. These streams also grouped together genetically for Roanoke logperch (Roberts et al. 2013). Although there are exceptions, the existence of most populations at the stream scale is supported by both the individual-centered analysis (STRUCTURE) and the group-centered analyses (neighbor-joining tree and AMOVA). The number of populations identified by the STRUCTURE clustering analysis is somewhat equivocal, given the close similarity in the average log-likelihood values of the K = 12 and the K = 10 models. It is possible that this limited ability to identify an unambiguously supported model is due to varying degrees of differentiation between sites. However, the clustering of populations in the K = 12 model matches with watershed and anthropogenic boundaries by delineating most populations at the stream scale, and is supported by the findings of subsequent group-centered analyses.

The populations assessed in the neighbor-joining tree cluster in a manner that reflects the organization of populations in the K = 12 STRUCTURE model. Sites within streams share close connections, and apart from those in the Blackwater and Falling Rivers, sites within drainages cluster more closely than sites between drainages. The results of the AMOVA indicate that most genetic variation occurs among basins, and secondarily among streams within basins, whereas comparably little variation occurs among sites within streams. The variation that does occur among sites within streams is evidenced by F_{ST} values significantly greater than zero among

certain within stream site pairs, and this may be a function of isolation-by-distance. In addition to the spatial distance between sites, patchiness of suitable habitat leading to a discontinuous distribution of *A. cavifrons* (Petrimoulx 1983, Jenkins and Burkhead 1994) may also contribute to population structure within streams. *A. cavifrons* are believed to prefer swift to moderate current and gravel or fine rubble substrate with abundant boulder and/or bedrock cover (Petrimoulx 1983). The watersheds of streams in which internal population structure was detected (the Blackwater and Eno Rivers and Town Creek) have been impacted by increased sedimentation, which may reduce and fragment suitable habitat areas. Patchiness of suitable habitat and spatial distance between sites may act in concert to generate genetic differentiation among *A. cavifrons* within these streams.

Dams appear to function as delimiters of populations, but they do not perfectly explain the differentiation between clusters. All sites that exist in different streams with anthropogenic barriers between them were identified as separate clusters by the STRUCTURE analysis, were on separate branches in the neighbor-joining tree, and exhibited significant pairwise genetic differentiation (F_{ST}). Most between-stream comparisons fell into this category; only Fishing versus Swift creek (Site 31 versus 27, 28, 29, and 30) and Chestnut Creek versus Pigg River (Site 5 versus 6) comparisons lacked anthropogenic barriers (Figure 2.1). On the other hand, there were two cases in which individuals from the same stream, but separated by dams, were assigned to the same population by STRUCTURE: upper and lower Smith River (Site 9 versus 10 and 11) and upper and lower Falling River (Site 7 versus 8) (Figure 2.1). In the STRUCTURE analysis, individuals from lower and upper Smith River were assigned to the same cluster despite separation of these areas by Philpott and Martinsville dams. Low sample size of *A. cavifrons* from lower Smith River ($n = 4$) precludes firm conclusions about the genetic characteristics of

fish in that region, and probably limited STRUCTURE's ability to correctly assign those individuals to a population. However, the sampled lower-Smith fish were in fact significantly differentiated from fish in the upper Smith River (F_{ST} = 0.1408, P < 0.0001) suggesting that dams did promote differentiation that was "missed" by STRUCTURE.

Fish from Town Creek, a tributary that enters the Smith River between Philpott and Martinsville dams, was also found to be genetically distinct from fish from both the upper Smith River (F_{ST} = 0.1382, P < 0.0001) and lower Smith River (F_{ST} = 0.1466; P < 0.0001). Town Creek is separated from the upper Smith by Philpott Dam, and from lower Smith by Martinsville Dam. Similarly, *P. rex* collected upstream and downstream of Philpott Dam were found to be genetically differentiated, suggesting it represents a barrier to movement of fish. However, *P. rex* from above and below Martinsville Dam were found to be genetically indistinguishable, indicating that this dam (which is shorter and impounds a much smaller reservoir) may not represent a barrier to movement of all species (Roberts *et al.* 2013). Furthermore, *P. rex* collected in Town Creek were found to be genetically indistinguishable from those collected from the middle (between Philpott and Martinsville Dams) and lower Smith (below Martinsville Dam) River. No *A. cavifrons* were collected from the middle Smith River, but the differentiation between the lower Smith River and Town Creek populations may be due to *A. cavifrons* stocking in Town Creek, and/or barriers to movement in the form of dams and their tailwaters. In 1980, Town Creek was stocked with 900 hatchery-raised *A. cavifrons* from Buller Hatchery in Virginia, which acquired its stock from North Carolina hatchery stock originating from the Tar and/or Neuse drainages (Jenkins and Cashner 1983). However, significant pairwise F_{ST} values and divergence in the neighbor-joining tree indicate lack of nuclear affinity between the Town Creek population and populations in the Tar and Neuse, which suggests integration of introduced

A. cavifrons may have been minimal. Nonetheless, the hypothesis that individuals of North Carolina origin successfully reproduced and introduced their alleles is supported by the presence of mitochondrial haplotype E in Town Creek, which is a haplotype that otherwise was detected only in streams in the Tar Basin (Table 2.7). Additionally, a cold tailwater from Philpott Reservoir is hypothesized to thermally isolate the Town Creek population from the middle Smith River population (Jenkins and Cashner 1983), which may have prevented individuals from moving out of Town Creek, into the middle Smith River, and subsequently into the lower Smith River, or vice versa.

Genetic differentiation between the upper and lower Falling River sites was weak and non-significant, despite the separation of these areas by an unnamed, approximately 4-m-high mill dam. This may be due to a source-sink dynamic occurring between the two sites. The lower site contained a mix of invasive *A. rupestris*, *A. cavifrons*, and hybrids (Chapter 1), and it is possible that it represents a sink population in which all genetically pure *A. cavifrons* are due to one-way migration from the above-dam site. It has been demonstrated by previous studies that obstructions in the river may allow for unidirectional flow of individuals and alleles from upstream sites (Meeuwig *et al.* 2010, Meldgaard *et al.* 2003, Neraas *et al.* 2001), and this may be the case in the Falling River. Upstream movement appears impossible, as no *A. rupestris* or hybrids have been detected above the dam (Chapter 1).

Population Connectivity and Gene Flow

Although low differentiation among sites within most streams indicates that connectivity typically is high at the stream scale (i.e., spatial extents up to 19 km in most streams, and up to 64 km in the Nottoway River), I found no evidence for contemporary exchange between populations occupying different streams. Not only did the STRUCTURE admixture analysis

indicate that most individuals derived their ancestry from a single ancestral population, but testing for first-generation migrants did not reveal any individuals that originated from a population other than the one from which they were sampled. The STRUCTURE admixture analysis and group-centered analyses indicate that the only pair of streams not separated by a dam (Fishing and Swift Creeks) were genetically distinct, which may be a result of a natural barrier to movement in the form of poor *A. cavifrons* habitat; the lower portion of Swift Creek (downstream of my study site) is swamp-like, with low gradient and an undefined stream channel. All other streams are separated by dams and their associated impoundments, and with only two exceptions (see above) these anthropogenic structures appear to act as impermeable barriers to fish movement. Thus, these now-fragmented environments are poor theatres for understanding the dispersal capabilities of *A. cavifrons*.

Although no studies have specifically assessed the mobility of *A. cavifrons*, an analysis of patterns of Centrarchid movement by Gatz and Adams (1994) found all assessed species (*Lepomis auritus*, *L. macrochirus*, *L. gulosus*, *Micropterus salmoides*, and *Ambloplites rupestris*) to be highly sedentary, with distances between successive captures being < 100 m for approximately two-thirds of all recaptured fishes, and only 6 of 1364 recaptured fish moved more than 10 km. Of the assessed species, *A. rupestris* was found to be the most sedentary (over three years of study, the maximum detected movement of an individual *A. rupestris* was only 600 m; Gatz and Adams 1994). Conventional wisdom suggests freshwater fish rarely move beyond the stream reach boundaries (Gerking 1953), but more recent studies indicate this may not be the case (Roberts *et al.* 2016). Furthermore, studies utilizing mark-recapture techniques may underestimate the extent of fish movement and the frequency with which this movement occurs. Even if sunfishes, and *Ambloplites* in particular, are among the more sedentary of stream

fishes, they may have a stepping-stone pattern of dispersal in which individuals and their associated alleles move from patch to patch of suitable habitat within streams over the course of multiple generations. Although the low genetic differentiation between individuals from sites within streams makes the detection of first-generation migrants more difficult (Berry *et al.* 2004), low differentiation and lack of IBD suggest that stream-scale gene flow is high, whether it occurs over single or multiple generations of movement. Indeed, stream-fish movement may be extensive, a theory supported by the spread of invasive *A. rupestris* in streams where they have been introduced (Chapter 1). Such movement may be what resulted in the detection of individuals of apparently mixed ancestry among the three streams in the Tar Basin (Fishing and Swift Creeks and the Tar River), of which only the Tar is isolated by a dam. Although sites in the Tar River are isolated, the dam was not constructed until 1971 and therefore the Tar River population may have existed in isolation for few enough generations that traces of past connectivity are still evident in microsatellite data. The Pigg and Smith clusters also exhibit some evidence of mixed ancestry in the STRUCTURE plot, but this is may be due to very low sample sizes from the Lower Pigg and Lower Smith Rivers (n = 1 and 4, respectively) confounding the assignment of individuals. In the Pigg River, mixed ancestry may alternatively be an artifact of the introduction of ~900 hatchery-raised *A. cavifrons* following a large chemical spill that eliminated all fish from a 35 km section of the middle Pigg River in 1975 (Jenkins and Cashner 1983). This chemical spill may have caused a population bottleneck, and the subsequent introduction of hatchery-raised individuals could have resulted in the apparent mixed ancestry detected in the Pigg population.

Mitochondrial sequence data indicate a history of connectivity among populations. The mitochondrial genome mutates more slowly than microsatellite markers, but is also more subject

to the effects of drift than the nuclear genome due to its smaller effective size (Ballard and Whitlock 2004). These two factors lead to lower standing variation in mitochondrial DNA, thus it takes longer than nuclear DNA to reach a new mutation-drift-migration equilibrium following a demographic change. Therefore, if mitochondrial haplotypes exhibit a geographic pattern indicating separation of populations, it may indicate that populations have been separated for a long period of time, perhaps predating anthropogenic fragmentation of habitat. There was no apparent geographic pattern in the distribution of *A. cavifrons* mitochondrial haplotypes. In fact, all but two basins shared at least one mitochondrial haplotype with all other basins, suggesting that populations historically experienced higher connectivity. However, it should be noted that nine of the twelve mitochondrial haplotypes were unique to a single basin, and eight were unique to a single stream. It is difficult to definitively determine whether this is a result of contemporary drift or historical lack of gene flow, but a historical lack of gene flow is not likely to have allowed for the numerous haplotypes shared among basins, whereas contemporary drift may have allowed this evidence of past relationships to persist. All haplotypes had a difference of only five or fewer base pairs, indicating divergence among these haplotypes was minimal. These factors suggest that populations may have been separated in recent times (e.g., over the past century) by the construction of dams, as a lack of historical connectivity prior to anthropogenic alteration would be expected to have produced distinct geographic patterns of haplotypes.

Effective Size of Contemporary Populations and Indications of Recent Declines

Effective population size (N_e) is a measure of the effect that drift has on a given population (Charlesworth 2009). Franklin (1980) proposed the general guideline that any population with an $N_e < 50$ may face immediate risks of inbreeding depression and that an $N_e >$

500 may be necessary for a population to maintain genetic diversity in the long term. As no species-specific criterion is available, this represents a rule-of-thumb value against which to compare estimates of the effective size of *A. cavifrons* populations to identify populations that may be at high risk for inbreeding and drift.

My estimation of the effective population sizes through the use of the LD method allows for the ranking of *A. cavifrons* populations in size relative to one another. Although the estimates have wide confidence intervals suggesting low precision, they still provide a means of identifying those populations that are particularly small in comparison to others. The LD method makes several assumptions, including no immigration and non-overlapping generations (Luikart *et al.* 2010). My analysis suggests there is no immigration occurring between sampled *A. cavifrons* populations. However, knowledge of *A. cavifrons*' growth rate (Jenkins and Burkhead 1994) suggests that sampled individuals represent numerous overlapping generations, as they ranged in size from 69 to 234 mm in standard length. The bias caused by a sample that includes individuals from overlapping generations can be difficult to predict (Luikart *et al.* 2010), however simulations suggest that violating this assumption creates only a slight upward bias (i.e., value inflated by less than 10) in estimates of N_e (Waples 2006). Assuming the estimate derived from the model with a lowest allele frequency of 0.02 represents the best balance between the bias associated with the inclusion of rare alleles and the loss of precision associated with the exclusion of rare alleles, as suggested by Waples and Do (2010), only the Tar River population exceeds the long-term threshold of 500 individuals, and the Flat and Little River populations fall below the short-term threshold of 50 individuals. However, given the potential biases in the estimation of N_e values it may be advisable to consider estimates as relative rather than absolute. For example, it is unlikely that the Tar River population is the only population that is viable in

the long term, as analyses of genetic diversity indicate many other populations had higher expected heterozygosity and lower inbreeding than the Tar population. The only population that was inestimable (e.g., had a negative N_e) using a lowest allele frequency of 0.02 was that in the Blackwater River, which may be due to the fact that this population was represented by only 23 individuals, a value that falls below Garza and Williamson's (2001) suggested minimum of 25 individuals necessary for the detection of most alleles occurring at 2% or above (Town Creek also fell below this minimum). Although N_e estimates should probably not be considered in absolute terms, they do provide a method of ranking populations from smallest to largest effective size, which may aid in the identification of populations that may benefit from future translocation efforts, and which populations may serve as the best sources for translocated individuals. For example, those populations identified as being quite small relative to all other populations (e.g., those in the Flat, Little, and Eno Rivers and Town Creek) may be more subject to the loss of heterozygosity through drift. Small amounts of migration (as few as one individual per generation) between subpopulations is known to dramatically reduce the effects of drift (Mills and Allendorf 1996), and thus the introduction of small numbers of individuals from larger populations may preclude loss of diversity in smaller populations by limiting the effects of drift. Furthermore, those populations identified as being of larger effective size, such as those in the Tar and Nottoway Rivers, may serve as reference populations, meaning estimates of demographic parameters in those systems may be used as benchmarks against which other populations may be compared to evaluate population health and stability.

Calculated *M* ratios suggest that two populations may have experienced a bottleneck (Flat and Eno), and all but one population (the Nottoway) had *M* values below the published range from species with demographically stable histories (Table 2.2). The power of *M* ratios to detect

bottlenecks is greater in the case of severe bottlenecks (98 to 99.9% decline) and bottlenecks that occurred many generations ago (6 to 50), and this power may be limited in the detection of declines that occurred recently (1 to 5 generations ago) and of less severe declines (60 to 98%) (Peery *et al.* 2012). Therefore, it is possible the *M* values for *A. cavifrons* populations underestimate the true number of populations that have experienced declines. Additionally, *M* ratios are believed to drop following a rapid decline, whereas a gradual decline may not be detected by this method (Garza and Williamson 2001). Detection of bottlenecks in certain populations (Blackwater River and Town Creek) may also have been hampered by low sample sizes, as low numbers of samples could have led to certain alleles going undetected by chance. Because the calculation of *M* is based on the number of alleles in a population, it is positively correlated with N_e and H_e, but it provides additional information that those parameters do not. For example, the Flat River had the lowest estimated N_e, but the fact that it also had the lowest *M* value indicates that this low N_e may be the product of a bottleneck. If the *M* value were higher, it might be suggested that the Flat River effective population size had always been small.

It is somewhat unclear what is driving bottlenecks in the systems where *M* values indicate they may have occurred. The fact that most populations have *M* values below the level expected in a demographically stable population suggest all have experienced fluctuations in population size, although the extent of these fluctuations is not estimable from this analysis. The comparison of past effective population sizes with contemporary estimates to assess changes over time would require the analysis of historic samples. Although such historic samples are not available, future analysis of the *A. cavifrons* populations assessed in this study could provide insight into the temporal stability of extant populations (Palstra and Ruzzante 2010). Additionally, temporally replicated samples would allow for estimation of effective population

size through the analysis of temporal change in allele frequencies, which is the most widely used and well evaluated method of N_e estimation (Luikart *et al.* 2010). Ongoing genetic monitoring of populations would improve understanding of the demographic and evolutionary processes affecting populations, and allow for an evaluation of the effects of any management efforts (Schwartz *et al.* 2007).

Correlation of Genetic Diversity of A. cavifrons Populations with Modifications to Watershed Landscape

The trends between genetic variables and watershed land use supported my hypothesis that human alteration of watershed landscape would be correlated with decreases in genetic diversity of *A. cavifrons* populations. This may be a result of the fact that deforestation, agriculture, and urbanization are the primary causes of altered flow regimes and reduced habitat availability in many rivers (Poff *et al.* 1997), and can contribute to alterations the nutrient content and water chemistry of streams (Allan and Flecker 1993, Walsh *et al.* 2005). Although patterns were observed across all land use variables, cropland area had the most significant correlations across all genetic variables. This does not necessarily indicate that agricultural practices have a more significant effect on *A. cavifrons* populations, but rather may be a result of the fact that cropland represents a much larger portion of watershed area than does urban development in all assessed watersheds. Additionally, all land use types exhibited some correlation with one another, and thus relationships of genetic variables to all three were to be expected. Patterns in correlation between genetic variables and *percent change* in watershed land use were less pronounced. This may be because land-use change was measured over a ten-year period (2001 to 2011), and likely represent only a very small fraction of the changes that have taken place over the past century, and relatively few bass generations (generation time is

likely ~3 years; Jenkins and Burkhead 1994). In fact, the land-use change in most watersheds was slight, averaging about 1.5% loss of forest cover. However, some watersheds experienced more significant changes. For example, the Town Creek watershed experienced a loss of forest cover more than twice the average (~3.8%). Additionally, the Eno River watershed experienced an increase in developed area more than twice as large as any other watershed (~0.7%), and it contains nearly twice the developed area of any other watershed (~14.1%). These larger changes to land use may have contributed to the effective sizes of the Town Creek and Eno River populations, which were found to be among the smallest of all assessed populations, and to the evidence of a population bottleneck in the Eno River population. If I were to assess the relationship between population size and diversity and land use changes over a broader time span that encompassed the substantial growth of the human population in the region (i.e., a span beginning prior to 1950), it is possible correlations would have been detected. Additionally, the analysis of landscape characteristics at the stream reach scale rather than the watershed scale could provide a more targeted, detailed insight into the riparian areas, and may allow for the correlation of specific riparian alterations with small effective size and decreased genetic diversity of resident *A. cavifrons* populations.

Implications for Conservation and Management

Populations of freshwater fish that exist in isolation have the propensity to experience declines in genetic diversity due to the increased influence of genetic drift (Wofford *et al.* 2005). Maintaining genetic diversity is important to ensure long-term persistence, as populations with higher levels of genetic diversity will likely be better able to adapt to changing environmental conditions (Reed and Frankham 2003). *A. cavifrons* currently exists in a mosaic of fragmented populations, and my analysis suggests that at least some of these populations may be incapable

of maintaining long-term genetic variability. A lack of connectivity between these populations means those that decline cannot be rescued by migrants from other populations, and thus the fate of each will be decided by local environmental conditions (Danancher *et al.* 2008).

Anthropogenic alteration of the environment in the range of *A. cavifrons* began with European settlement of the region in the 1700's (Jenkins and Burkhead 1994) and became more severe with the construction of numerous dams and reservoirs over the past century (Jenkins and Cashner 1983). The suite of changes associated with the construction of dams and the conversion of forested watersheds into cropland and urban centers, including alterations to the natural flow regime, increased sedimentation, and pollution (Poff *et al.* 1997, Walsh *et al.* 2005), acts to alter the ecosystem to which stream biota are adapted. These alterations result in reductions in the quantity and quality of habitat, and can contribute to decreases in species abundance and extent. However, anthropogenic effects are not limited to chronic impacts, and can include such stochastic events as pollution-induced fish kills. Indeed, streams inhabited by *A. cavifrons* have experienced both chronic pollution and major fish kills from chemical discharge, including a spill in the Pigg River in 1975 that killed all fish in a 35 km segment of the stream (Jenkins and Cashner 1983). Sudden population declines from stochastic events may result in population bottlenecks, and associated demographic instability and reductions in genetic diversity.

Connectivity could be improved through the removal of anthropogenic barriers to *A. cavifrons* dispersal. However, this tactic increases the risk of additional spread of invasive *A. rupestris* (Chapter 1). Alternatively, the translocation of individuals between streams could be carried out in a pattern that mimics historical connectivity (Dehaan *et al.* 2012). This would help to promote the genetic diversity of isolated populations, and could potentially aid in the recovery

populations that have suffered declines. The introductions of individuals from other populations existing in isolation carries some risk of outbreeding depression, which may result from the disruption of coadapted gene complexes and local adaptations (Edmands 2007, George *et al.* 2009). However, studies suggest the risk of outbreeding depression only becomes elevated after populations have been existing in complete isolation from one another for more than 500 years, and that the probability of outbreeding depression occurring is much less than the probability of population extirpation due to loss of genetic diversity and inbreeding depression (Frankham *et al.* 2010). The risk of outbreeding depression seems particularly low in *A. cavifrons* given the empirical evidence that the mixing of fish from the Tar drainage of North Carolina with fish native to Town Creek in the Dan drainage of Virginia did not result in any detectable population declines. However, nuclear genotypes provide little evidence that the fish introduced to Town Creek integrated, and the stocking of *A. cavifrons* into the Pigg River did not result in any detectable increases in abundance in that system (Jenkins and Cashner 1983). Decreased performance, survival, and reproduction have been associated with fish propagated in a hatchery environment (Vrijenhoek 1998), and these factors may have limited the success of individuals stocked into Town Creek and the Pigg River. It is also possible that native fish selectively mated with one another and avoided introduced individuals. More thorough post-stocking monitoring studies are necessary to evaluate the ability of introduced individuals to successfully integrate into the population. The translocation of fish among streams within a basin could mimic the effects of historic connectivity and would decrease the effect of genetic drift and preclude the loss of genetic diversity. This may be particularly important for populations that have experienced declines and/or have effective population sizes relative to other populations. More specifically, populations in the three streams in the Neuse basin may benefit from translocation

efforts as they were found to have the smallest effective sizes of all assessed populations, and there is evidence that two of the three (those in the Eno and Flat Rivers) have experienced bottlenecks. Town Creek had the next smallest effective size, and the translocation of individuals from other streams in the Dan basin (upper and lower Smith River) may also be warranted, although the collection of more samples from sites in the Smith to allow for estimates of effective size and the detection of recent declines is advisable. Although the relative effective size of the Blackwater River population could not be determined, the low genetic diversity in this system suggests it too may benefit from translocations of individuals, which could be collected from the upper Falling River (the only other non-introgressed *A. cavifrons* population in the Roanoke basin). Although the effective population size in the Tar River appears to be the largest of all those assessed, and the effective size of the Fishing Creek population is also quite high relative to other populations, the collection of more samples from Swift Creek to allow for assessment of effective population size may reveal that the population in this system would benefit from translocations. The Chowan basin population appears to have a relatively large effective population size and a high amount of genetic diversity, and the lack of barriers in the Nottoway River preclude the need for translocations. Populations with small effective sizes and those that have experienced declines will have decreased genetic diversity and be more susceptible to the effects of drift and inbreeding depression. In addition to the supplementation of existing populations, there are systems not currently inhabited by *Ambloplites* that appear to have suitable habitat, such as the Mayo and Bannister Rivers in the Dan basin of Virginia. The establishment of new *A. cavifrons* populations in these systems, which exist in drainages that already contain the species, would help to reduce the likelihood of species extinction, and would

help preclude the loss of the species from the Virginia portion of its range, which has been greatly diminished by invasive *A. rupestris* (Chapter 1).

A synergistic approach to conserving genes, species, and ecosystems is essential for the success of any conservation efforts (Bowen 1999). For efforts to boost genetic diversity and the effective size of populations to be effective, the factors causing a loss of genetic diversity must be identified and addressed (Caro and Laurenson 1994). If a catastrophic event or acute point-source pollution has led to population declines then habitat restoration efforts may not be necessary, but if declines are driven by anthropogenic alterations to streams and their watersheds, then improved land use practices may be necessary to restore favorable conditions (George *et al.* 2009). My study detected indications of demographic instability in most populations as well as evidence of bottlenecks in some populations, and the correlation of decreases in genetic diversity to deforestation and increases in cropland and urban development suggest that land use practices have contributed to a gradual decline of *A. cavifrons* populations. As such, direct habitat restoration efforts may be necessary to improve quantity and quality of habitat. The preference of *A. cavifrons* for rocky habitat and swift flows (Petrimoulx 1983) and apparent intolerance of slow flowing and high sediment areas (Cashner and Jenkins 1982, Jenkins and Cashner 1983) suggest efforts to mitigate anthropogenic alterations that cause reductions in flow and increases in runoff may result in improved habitat suitability. Thorough assessments of the contemporary quantity and quality of *A. cavifrons* habitat are warranted, as knowledge of these parameters would help to guide restoration efforts to increase the suitability of streams for the species, to indicate if streams with diminished populations can support supplementation with translocated individuals, and to determine if currently uninhabited streams could support new populations.

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APPENDICES

Appendix I: A description of SREL primer development methods.

Appendix II: A description of PCR multiplex mixes and cycling conditions

Appendix III: Grouping of individuals in tentative populations and tests for Hardy-Weinberg and linkage disequilibrium

Table 2.1: Locations of 31 sites where *A. cavifrons* were sampled for this study. The number (*n*) of individuals sampled and genotyped per site varied based on fish density and capture efficiency.

Basin	Watershed	Stream segment	Latitude	Longitude	*n*	Map Code	Site Code
Roanoke	Blackwater	Blackwater River	37.0528	-79.8824	6	1	BW1
	Blackwater	Blackwater River	37.0541	-79.8819	9	2	BW3
	Blackwater	Blackwater River	37.0541	-79.8823	7	3	BW2
	Blackwater	Blackwater River	37.0345	-79.9094	1	4	BW4
	Pigg	Chestnut Creek	36.9063	-79.8010	3	5	CHEST
	Pigg	Lower Pigg River	36.9468	-79.5249	1	6	PIGG
	Falling	Lower Falling River	37.0540	-78.9354	5	7	LFALL
	Falling	Upper Falling River	37.1268	-78.9595	21	8	UFALL
	Smith	Upper Smith River	36.8053	-80.2008	10	9	USMITH
	Smith	Lower Smith River	36.6138	-79.8226	2	10	LSMITH1
	Smith	Lower Smith River	36.6141	-79.8225	2	11	LSMITH2
	Smith	Town Creek	36.8210	-79.9966	10	12	TOWN1
	Smith	Town Creek	36.7925	-80.0027	12	13	TOWN2
Chowan	Nottoway	Nottoway River	36.8477	-77.4934	18	14	NOTT1
	Nottoway	Nottoway River	36.8590	-77.1898	28	15	NOTT2
Neuse	Eno	Eno River	36.0751	-79.0076	15	16	ENO1
	Eno	Eno River	36.0757	-79.0708	9	17	ENO2
	Eno	Eno River	36.0568	-78.9784	18	18	ENO3
	Eno	Eno River	36.0723	-78.9357	3	19	ENO4
	Little	Little River	36.1378	-78.9371	33	20	LITTLE
	Flat	Flat River	36.1957	-78.8781	37	21	FLAT1
	Flat	Flat River	36.2357	-78.9001	12	22	FLAT2
Tar	Tar	Tar River	36.1907	-78.5577	24	23	TAR1
	Tar	Tar River	36.1840	-78.5369	1	24	TAR2
	Tar	Tar River	36.1932	-78.5758	2	25	TAR3
	Tar	Tar River	36.1839	-78.5419	3	26	TAR4
	Fishing	Fishing Creek	36.1448	-77.8215	9	27	FISH1
	Fishing	Fishing Creek	36.1411	-77.8174	21	28	FISH2
	Fishing	Fishing Creek	36.1515	-77.7537	2	29	FISH3
	Fishing	Fishing Creek	36.1498	-77.8917	2	30	FISH4
	Swift	Swift Creek	36.0741	-77.8691	5	31	SWIFT
Total					**331**		

Table 2.2: Microsatellite genetic diversity statistics for each inferred *A. cavifrons* population, averaged across 19 loci (with standard deviations in parentheses). Statistics included sample size (*n*), number of alleles per locus (*A*), allele richness per locus standardized to a sample size of 4 individuals (A_R), the inbreeding coefficient (F_{IS}), observed (H_O) and expected heterozygosity (H_E), and the ratio of allele richness to allele size-range (*M*). The Pigg population included Chestnut Creek and Lower Pigg River samples, which clustered together in the STRUCTURE analysis. *M* was not calculated for the Pigg River, Smith River, or Swift Creek populations due to low sample size

Population	*n*	*A*	A_R	F_{IS}	H_O	H_E	*M*
Blackwater	23	2.47 (1.19)	2.03 (0.75)	-0.07 (0.21)	0.54 (0.19)	0.50 (0.15)	0.749 (0.2205)
Pigg	4	3.74 (1.89)	3.74 (1.89)	-0.04 (0.22)	0.78 (0.25)	0.76 (0.19)	–
Falling	26	4.26 (2.59)	2.88 (1.33)	-0.05 (0.10)	0.63 (0.27)	0.60 (0.26)	0.708 (0.2323)
Upper Smith	10	3.26 (1.74)	2.89 (1.47)	-0.07 (0.20)	0.69 (0.27)	0.65 (0.22)	–
Lower Smith	4	4.11 (2.40)	2.71 (1.26)	-0.07 (0.17)	0.73 (0.28)	0.69 (0.23)	–
Town	22	4.42 (2.58)	2.89 (1.32)	0.06 (0.21)	0.58 (0.22)	0.61 (0.22)	0.750 (0.1994)
Nottoway	46	6.42 (3.75)	3.40 (1.41)	-0.02 (0.07)	0.68 (0.21)	0.66 (0.21)	0.843 (0.1690)
Eno	45	4.89 (2.71)	2.66 (1.01)	0.04 (0.13)	0.49 (0.26)	0.51 (0.24)	0.693 (0.2063)
Little	33	4.63 (2.76)	2.88 (1.35)	0.00 (0.10)	0.55 (0.27)	0.55 (0.27)	0.738 (0.1614)
Flat	49	4.11 (2.38)	2.84 (1.16)	0.05 (0.16)	0.60 (0.21)	0.63 (0.18)	0.657 (0.1812)
Tar	30	5.00 (3.20)	2.95 (1.46)	0.10 (0.13)	0.52 (0.26)	0.57 (0.28)	0.773 (0.2048)
Fishing	34	5.21 (3.52)	3.10 (1.60)	0.04 (0.10)	0.57 (0.30)	0.59 (0.30)	0.811 (0.8106)
Swift	5	2.68 (1.34)	2.53 (1.20)	-0.17 (0.29)	0.70 (0.30)	0.61 (0.20)	–

n = sample size, *A* = number of alleles, A_R = allelic richness, F_{IS} = inbreeding coefficient, H_O = observed heterozygosity, and H_E = expected heterozygosity, *M* = Garza and Williamson's (2001) M ratio

Table 2.3: Microsatellite genetic differentiation between pairs of sites with $n \geq 5$. Pairwise F_{ST} estimates are below the diagonal, and the corresponding P values (based on 10^4 random permutations) are above the diagonal.

Site	BW1	BW2	BW3	ENO1	ENO2	ENO3	FISH1	FISH2	FLAT1	FLAT2	LFALL	LITTLE	NOTT1	NOTT2	SWIFT	TAR1	TOWN1	TOWN2	UFALL	USMITH
BW1	–	0.2269	0.0492	0	0.0002	0	0.0001	0	0	0.0001	0.0022	0	0	0	0.0016	0	0	0.0001	0	0.0001
BW2	0.0137	–	0.3002	0	0	0	0.0002	0	0	0	0.0008	0	0	0	0.0005	0	0	0	0	0
BW3	0.0336	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ENO1	0.4012	0.3731	0.4052	–	0.0808	0.0025	0	0	0	0	0.0001	0	0	0	0.0003	0	0	0	0	0
ENO2	0.4152	0.3903	0.4235	0.0184	–	0.2530	0	0	0	0	0.0003	0	0	0	0.0004	0	0	0	0	0
ENO3	0.3522	0.3249	0.3582	0.0278	0.0072	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FISH1	0.2889	0.2476	0.2917	0.2000	0.2112	0.1486	–	0.2683	0	0	0.0002	0	0	0	0.0003	0	0	0	0	0.0001
FISH2	0.2976	0.2527	0.2992	0.2437	0.2613	0.1968	0.0054	–	0	0	0	0	0	0	0	0	0	0	0	0
FLAT1	0.2413	0.2322	0.2504	0.1876	0.1887	0.1534	0.1058	0.1215	–	0.0649	0	0	0	0	0	0	0	0	0	0
FLAT2	0.2899	0.2812	0.3040	0.1828	0.1729	0.1379	0.0993	0.1278	0.0139	–	0.0001	0	0	0	0.0001	0	0	0	0	0
LFALL	0.2136	0.2081	0.2541	0.2937	0.3058	0.2577	0.1601	0.1938	0.1638	0.1913	–	0	0	0	0.0071	0	0.0003	0.0002	0.3563	0.0003
LITTLE	0.2950	0.2820	0.3116	0.2325	0.2193	0.1747	0.1389	0.1411	0.1026	0.0963	0.2021	–	0	0	0	0	0	0	0	0
NOTT1	0.1984	0.1723	0.1988	0.2584	0.2763	0.2229	0.1496	0.1667	0.1517	0.1907	0.1138	0.1847	–	0.0762	0	0	0	0	0	0
NOTT2	0.1885	0.1569	0.1978	0.2259	0.2285	0.2001	0.1593	0.1822	0.1494	0.1786	0.1282	0.1837	0.0060	–	0	0	0	0	0	0
SWIFT	0.3816	0.3448	0.3887	0.2715	0.2829	0.2239	0.0742	0.0682	0.1382	0.1399	0.2107	0.1735	0.1932	0.1784	–	0	0.0004	0.0002	0	0.0005
TAR1	0.3235	0.2812	0.3241	0.2541	0.2619	0.2045	0.0689	0.0854	0.1591	0.1451	0.2083	0.1825	0.1990	0.1997	0.1311	–	0	0	0	0
TOWN1	0.2404	0.2150	0.2534	0.3135	0.3052	0.2705	0.2087	0.2347	0.1538	0.2123	0.1421	0.2182	0.1220	0.1220	0.2706	0.2682	–	0.0066	0	0
TOWN2	0.2386	0.2117	0.2543	0.3135	0.3035	0.2597	0.2230	0.2393	0.1686	0.2174	0.1294	0.2102	0.1252	0.1287	0.2746	0.2717	0.0404	–	0	0
UFALL	0.2386	0.2132	0.2580	0.2997	0.3073	0.2612	0.1697	0.1864	0.1780	0.2096	0.0007	0.2055	0.1213	0.1391	0.2015	0.2000	0.1417	0.1561	–	0
USMITH	0.2355	0.1787	0.2435	0.3607	0.3506	0.3153	0.2429	0.2514	0.2148	0.2710	0.1457	0.2480	0.1301	0.1198	0.2749	0.2919	0.1157	0.1468	0.1424	–

Table 2.4: AMOVA partition of total microsatellite genetic variation among four hierarchical scales. The statistical significance of each component scale was based on 10^4 random permutations.

Source of Variation	Degrees of freedom	Molecular variance	Percentage of variation	*P*
Among Basins	4	0.64	10.97	0.0000
Among Streams within Basins	6	0.48	8.32	0.0000
Among Sites within Streams	9	0.06	0.98	0.0000
Among Individuals within Sites	600	4.63	79.73	0.0000
Total	**619**	**5.81**	**100**	

Table 2.5: Linkage-disequilibrium-based estimates of the mean and 95% confidence limits (CLs) of effective population size (N_e) for each population with $n \geq 20$ sampled individuals. Estimates are presented for three different modeling choices, based on exclusion of rare alleles that occurred with frequencies < 0.05, 0.02, or 0.01. Negative N_e values indicate an N_e estimate indistinguishable from infinity, due to large true size, small sample size, or both. The low precision of this analysis led to infinitely large upper confidence limits in certain populations.

		Lowest Allele Frequency Used								
		0.05			**0.02**			**0.01**		
Population	***n***	N_e	**Lower CL**	**Upper CL**	N_e	**Lower CL**	**Upper CL**	N_e	**Lower CL**	**Upper CL**
Blackwater	23	168.3	19.4	infinite	-353.7	32.9	infinite	-353.7	-32.9	infinite
Falling	26	70.2	31.3	infinite	133.3	50.9	infinite	117.6	52.3	infinite
Town	22	40.2	18.8	374.8	79.8	34.6	infinite	79.8	34.6	infinite
Nottoway	46	196.4	89.1	infinite	436.9	159.1	infinite	-3778.2	306.4	infinite
Eno	45	67.6	31.7	428.7	77.4	37.8	421.3	83.2	44.2	298.5
Little	33	31.4	19.4	60.3	43.2	27.5	81.4	57.1	34.2	130.9
Flat	49	35.6	19.7	81.9	41.9	22.5	107.9	53.9	27.1	189.7
Tar	30	-502.5	138.2	infinite	3709.0	118.9	infinite	281.9	91.8	infinite
Fishing	34	99.5	52.0	458.7	161.2	161.2	infinite	134.1	70	717.1

Table 2.6: Estimates of mitochondrial genetic diversity for populations of *A. cavifrons*. Standard deviations are displayed in parentheses.

Population	*n*	Haplotypes	Private haplotypes	Segregating sites	Haplotype diversity	Nucleotide Diversity
Blackwater	5	2	1	1	0.400000	0.00041
Falling	10	4	2	4	0.733000	0.00149
Upper Smith	5	1	0	0	0	0
Town	5	3	0	2	0.800000	0.00103
Nottoway	5	3	2	3	0.700000	0.00165
Eno	5	1	0	0	0	0
Flat	5	2	0	1	0.400000	0.00041
Little	5	2	0	1	0.400000	0.00041
Tar	5	2	0	1	0.400000	0.00041
Fishing	5	2	1	2	0.600000	0.00123
Swift	5	1	0	0	0	0
				Average	0.403000 (0.283000)	0.00064 (0.00058)
				Pooled	0.725000	0.000109

Table 2.7: Frequency of the 12 deduced mitochondrial haplotypes (lettered A through Q) by stream system. Haplotype letters correspond to Figure 2.6, as well as tables and figures in Chapter 1.

	North Carolina						Virginia							
Basin	**Neuse**			**Tar**			**Chowan**	**Roanoke**			**Dan**			
Stream	**ENO**	**FLT**	**LIT**	**TAR**	**FIS**	**SFT**	**NOT**	**BLW**	**UFL**	**LFL**	**CHT**	**USM**	**LSM**	**TWN**
Haplotype														
A	5	4	4	4				1	2	2		5	1	2
B		1	1											
C					2									
D								4						
E				1	3	5								2
F							3							
J										1				
K										1				
L											2			
M							1							
P							1		3	1				1
Q													1	
Total	5	5	5	5	5	5	5	5	5	5	2	5	2	5

Abbreviations for sample sites are as follows: ENO = Eno River, FLT = Flat River, LIT = Little River, TAR = Tar River, FISH = Fishing Creek, SFT = Swift River, NOT = Nottoway River, BLW = Blackwater River, USM = Upper Smith River, LSM = Lower Smith River, TWN = Town Creek, UFL = Upper Falling River, LFL = Lower Falling River, and CHT = Chestnut Creek

Table 2.8: Watershed landscape characteristics of each stream that was represented by 20 or more sampled individuals. Drainage area was calculated for the most downstream site in each stream, and all other values were averaged across all sites in each stream. Percent developed area, percent forested area, and percent cropland refer to values from the 2011 National Landcover Database (Homer *et al* 2015). Change in percent developed area, percent forested area, and percent cropland area represent the difference between values calculated from the 2001 (Homer *et al* 2007) and 2011 (Homer *et al* 2015) National Landcover Databases.

Stream	Drainage Area (km^2)	% Developed Area	% Cropland	% Forested Area	Change in % Developed Area	Change in % Cropland	Change in % Forested Area
Blackwater	282.309	4.015	27.933	66.123	0.100	-0.308	-0.685
Falling	593.108	4.435	29.170	60.445	0.100	-1.095	-1.880
Town	84.175	3.105	9.160	82.315	0.135	-0.180	-3.840
Nottoway	2926.689	4.915	15.525	66.255	0.075	-1.100	-1.470
Eno	339.289	14.050	20.667	59.958	0.674	-0.353	-1.017
Little	199.429	5.940	27.806	60.681	-0.002	-0.107	-0.397
Flat	383.319	7.665	29.245	55.997	0.322	-0.2475	-1.495
Tar	574.978	6.838	22.193	59.034	0.188	-0.666	-0.627
Fishing	1238.015	4.753	13.127	71.477	0.014	-1.049	1.932

Table 2.9: Correlation between genetic diversity statistics and landscape variables for the 9 populations with at least 20 sampled individuals. Pearson's correlation coefficients (*r*) are shown; absolute values greater than 0.5 are highlighted in bold. *P* values for the correlations between genetic diversity statistics and landscape variables are displayed above the diagonal. Percent developed area, percent forested area, and percent cropland values are derived from the 2011 National Landcover Database (Homer *et al* 2015). Percent change values represent the difference between the 2001 (Homer *et al* 2007) and 2011 (Homer *et al* 2015) National Landcover Databases.

	Genetic Diversity Statistics			**Landscape Attributes**						
	M	***A_R***	***H_E***	**Drainage Area**	**% Developed**	**% Forested**	**% Cropland**	**Change in % Developed**	**Change in % Forested**	**Change in % Cropland**
M	–			0.010	0.104	0.107	0.046	0.053	0.209	0.060
A_R	0.428	–		0.007	0.322	0.443	0.092	0.205	0.465	0.047
H_E	0.205	**0.809**	–	0.038	0.169	0.340	0.200	0.225	0.241	0.117
Drainage Area	**0.721**	**0.531**	0.342	–						
% Developed	-0.443	-0.165	-0.440	-0.116	–					
% Forested	0.346	0.294	0.487	-0.079	**-0.550**	–				
% Cropland	**-0.528**	**-0.558**	**-0.509**	-0.219	0.252	**-0.852**	–			
Change in % Developed	**-0.572**	-0.207	-0.255	-0.255	**0.884**	-0.270	0.068	–		
Change in % Forested	0.313	0.005	-0.263	0.201	0.143	-0.252	0.116	-0.177	–	
Change in % Cropland	**-0.540**	-0.381	-0.165	**-0.735**	0.151	0.190	0.039	0.285	-0.328	–

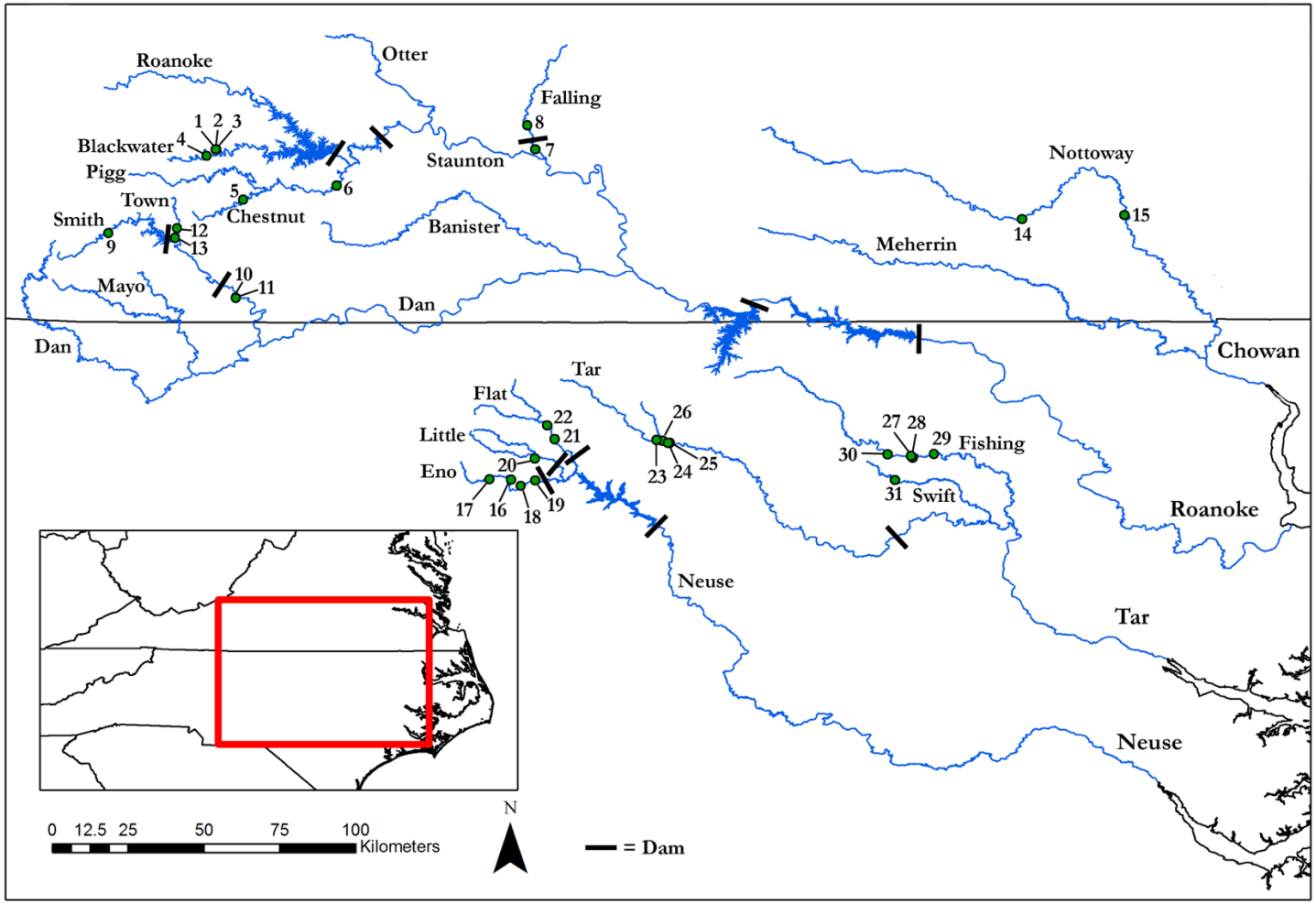


Figure 2.1: Map depicting sample sites throughout the historic range of *Ambloplites cavifrons* in Virginia and North Carolina. Numbers correspond to the sites listed in Table 2.1. Several spatially adjacent sites (e.g., 1, 2, and 3) appear as a single point due to the scale of this map. Solid black bars represent all dams that exist between sample sites.

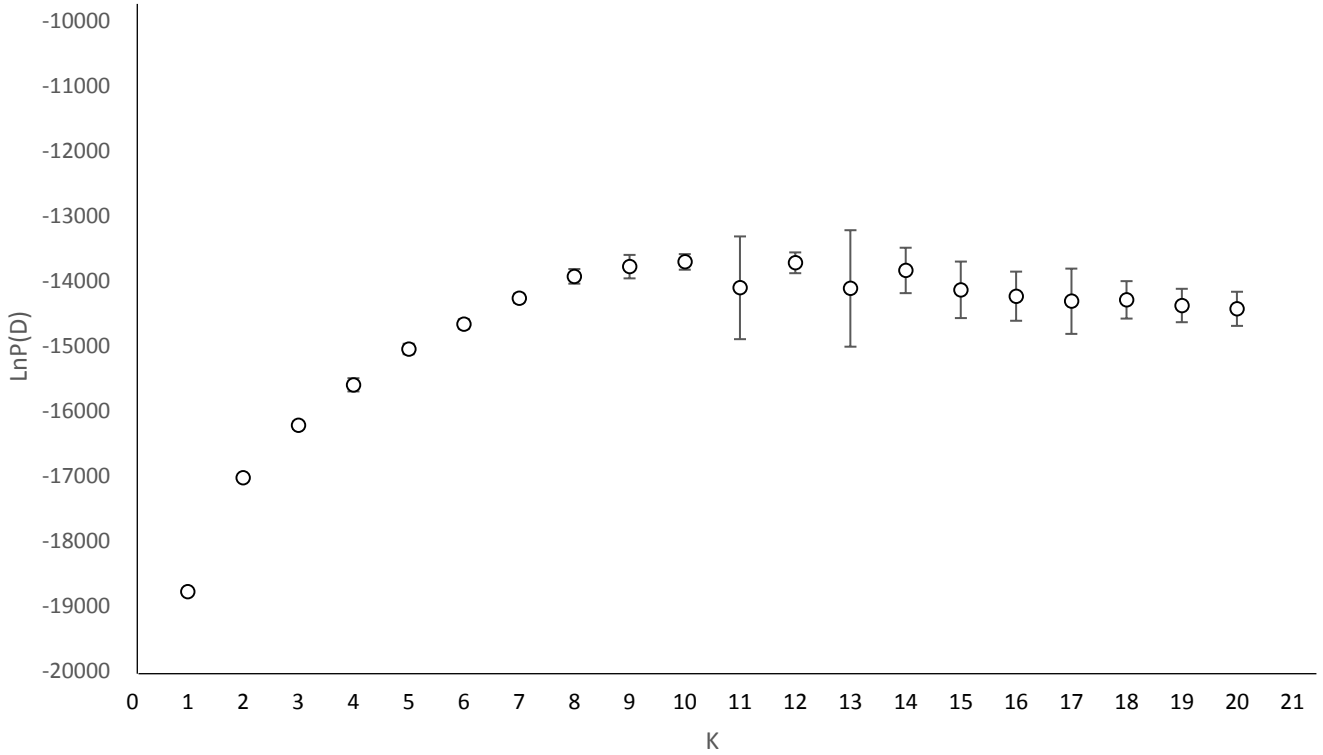


Figure 2.2: Average *K* log likelihood values from STRUCTURE simulation. Error bars represent standard deviation across ten replicate model runs.

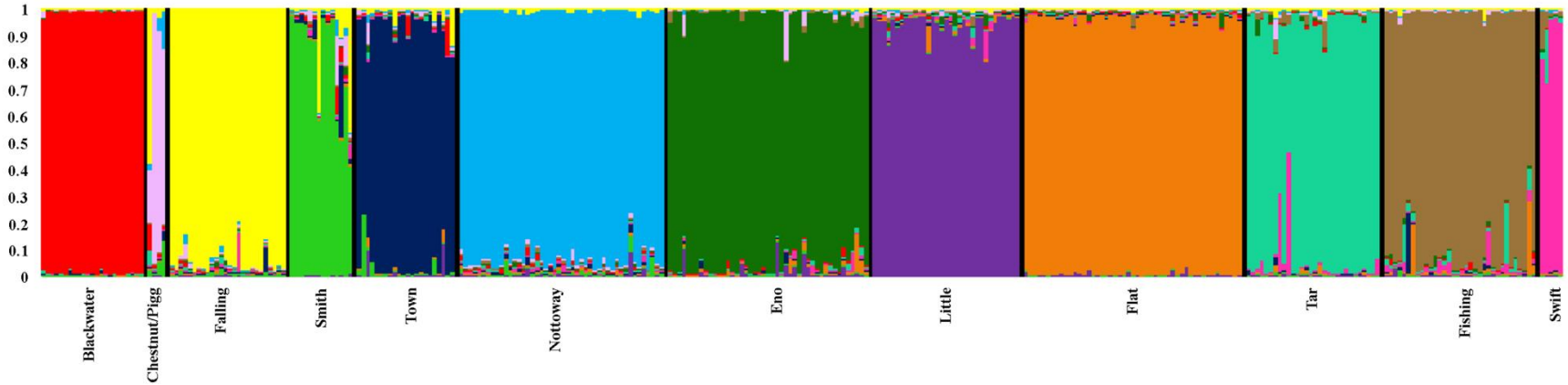


Figure 2.3: Plot of the results from the STRUCTURE *K*=12 iteration with the highest log likelihood.

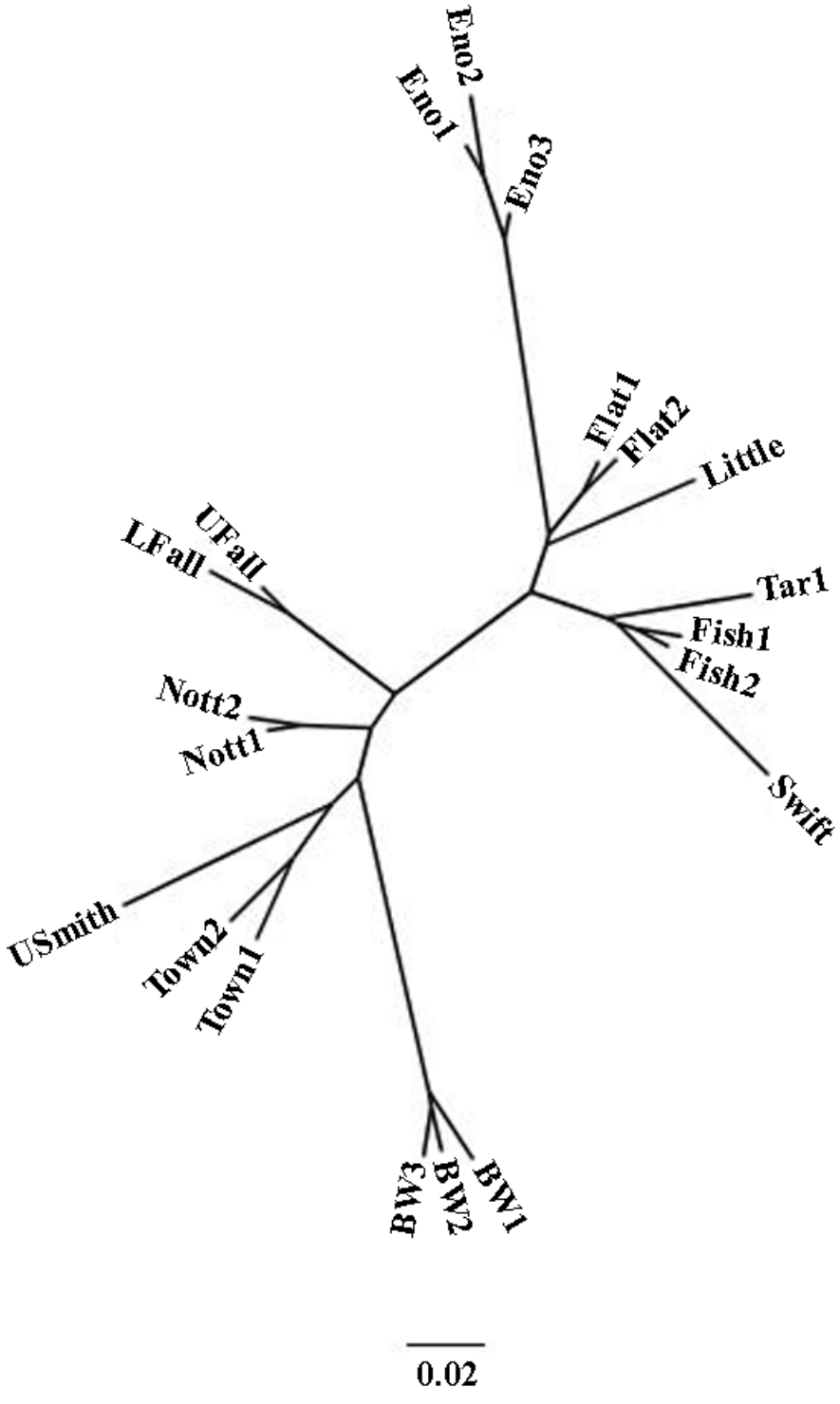
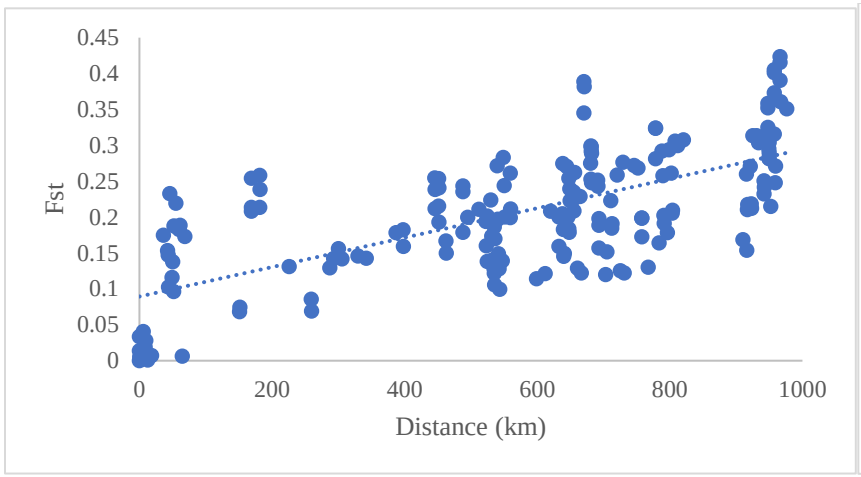
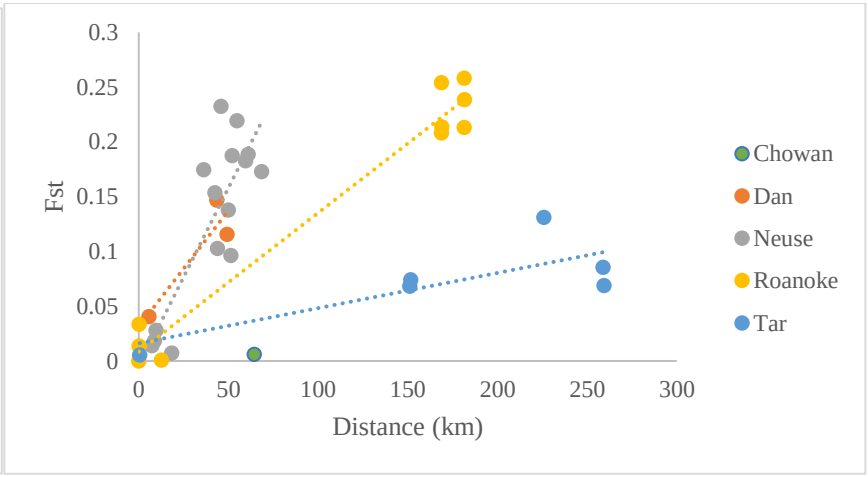


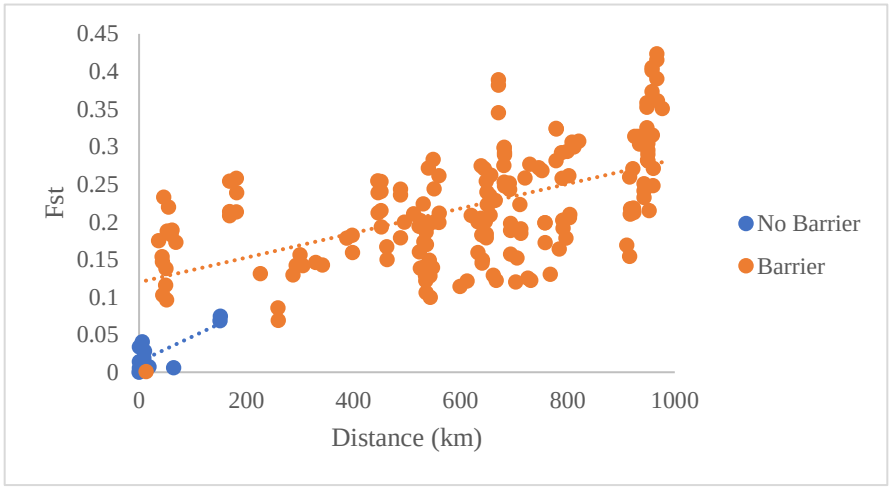
Figure 2.4: Neighbor-joining tree based on a matrix of pairwise Nei's D_m values among sites with $n \geq 5$ individuals.



A



B



C

Figure 2.5: The relationship between F_{ST} and spatial (hydrologic) distance between pairs of sites, for (A) all site-pairs, (B) site-pairs within the same basin, and (C) site-pairs with versus without an intervening barrier between them. Linear trend lines are shown for illustration purposes; relationships were formally tested by Mantel tests (see text). Only sites with n ≥ 5 individuals were used in these analyses.

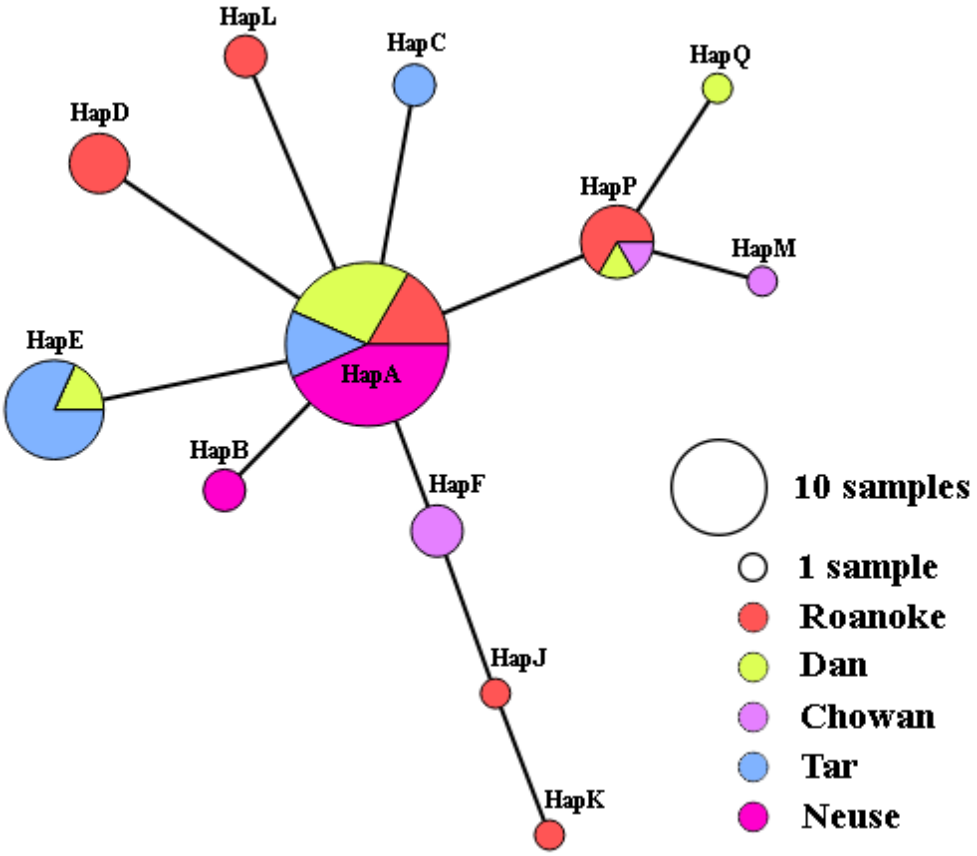
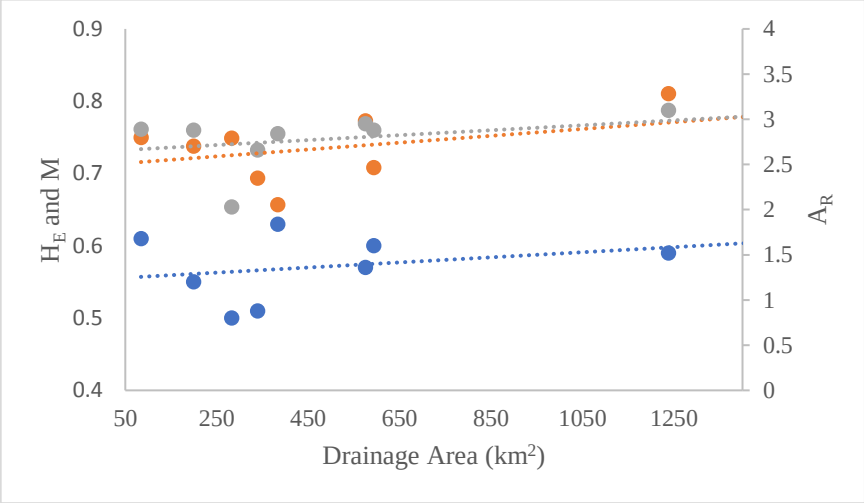
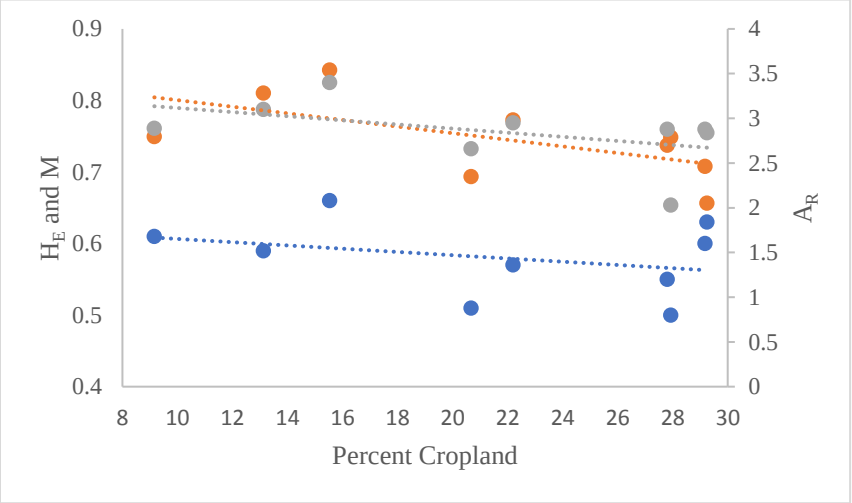


Figure 2.6: Median joining haplotype network depicting relationships among the 12 deduced *A. cavifrons* mitochondrial haplotypes (indicated by lettered circles). Each line segment indicates a single hypothesized mutation event between haplotypes. Circle size indicates the number of individuals and circle color indicates basin of origin.

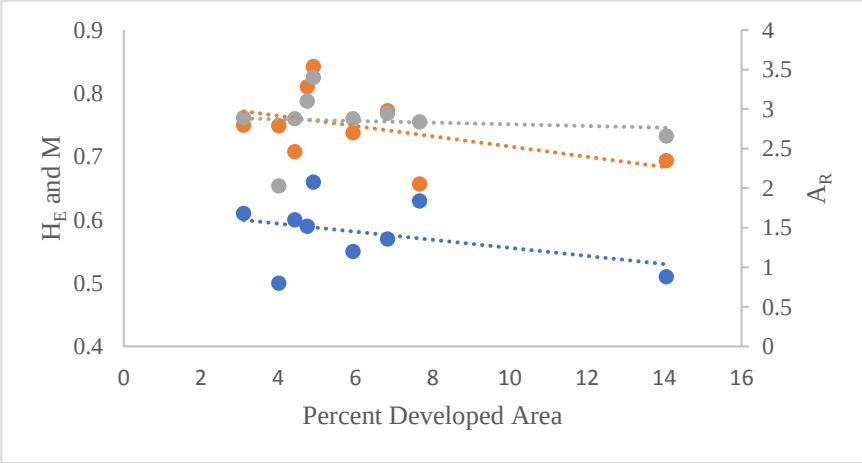
A


0.9
0.8
0.7
0.6
0.5
0.4
H_E and M
4
3.5
3
2.5
2
1.5
1
0.5
0
A_R
50
250
450
650
850
1050
1250
Drainage Area (km^2)

B


0.9
0.8
0.7
0.6
0.5
0.4
H_E and M
4
3.5
3
2.5
2
1.5
1
0.5
0
A_R
8
10
12
14
16
18
20
22
24
26
28
30
Percent Cropland

C


0.9
0.8
0.7
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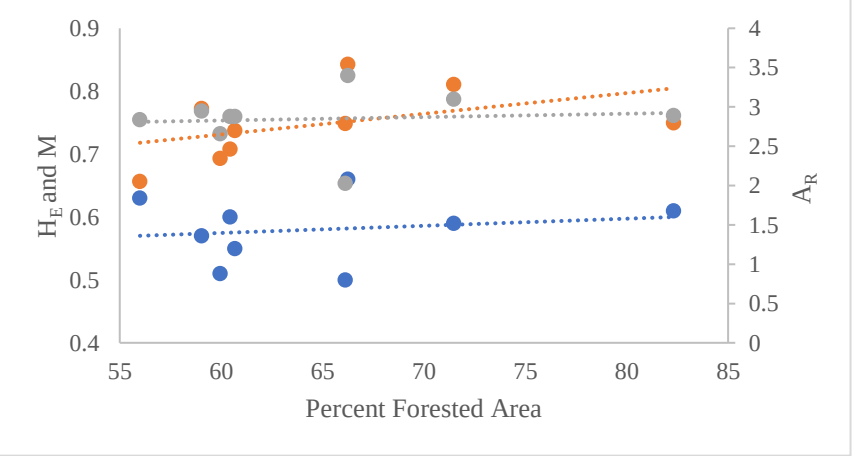
H_E and M
4
3.5
3
2.5
2
1.5
1
0.5
0
A_R
0
2
4
6
8
10
12
14
16
Percent Developed Area

D


0.9
0.8
0.7
0.6
0.5
0.4
H_E and M
4
3.5
3
2.5
2
1.5
1
0.5
0
A_R
55
60
65
70
75
80
85
Percent Forested Area

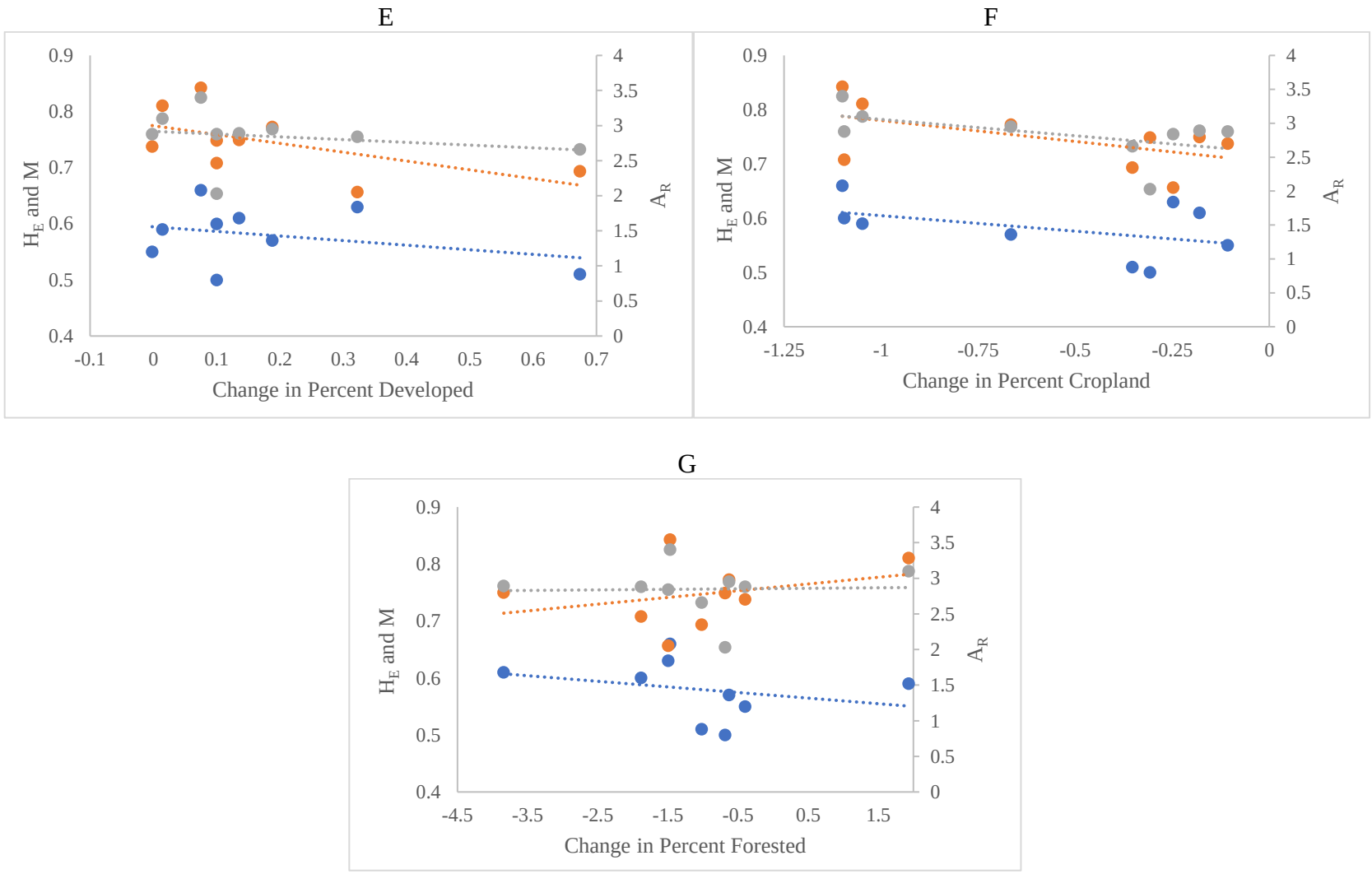


Figure 2.7: Plots depicting the relationships genetic response variables and watershed characteristics. H_e values are displayed in blue, M values are displayed in orange, and A_R values are displayed in gray. Plots depict the relationship between H_e, A_R, M and A) drainage area, B) percent cropland, C) percent developed area, D) percent forested area, E) change in percent developed area, F) change in percent cropland, and G) change in percent forested area.

GENERAL CONCLUSIONS

I carried out this study to enhance and update the state of knowledge of *Ambloplites cavifrons* and to interpret this information in order to provide recommendations for conservation and management efforts, as well as to contribute to the fields of invasion biology and freshwater fish ecology. The main goals of my thesis were 1) to measure the contemporary distribution of *A. cavifrons* and the extent of *A. rupestris* invasion, 2) to investigate the nature and outcome of interaction between the two species by evaluating the occurrence of hybridization, introgression, and replacement, 3) to estimate the size and status of, and connectivity between extant *A. cavifrons* populations, and to test hypotheses about the natural and anthropogenic factors driving these parameters, and 4) to draw general conclusions about the factors most responsible for the ongoing decline of *A. cavifrons* and make recommendations for management of the species. In the subsequent paragraphs I will summarize my findings in regards to each of these objectives.

Extent of invasion and reproductive interactions between A. cavifrons and A. rupestris

Earlier research suggested that *A. cavifrons* and *A. rupestris* readily hybridize in streams where they co-occur (Jenkins and Cashner 1983), and *A. rupestris* was known to have replaced *A. cavifrons* in streams where it was stocked throughout the course of the twentieth century (Cashner and Jenkins 1982). Prior to this thesis, the status of *A. cavifrons* throughout its native range had not been assessed in over thirty years and molecular approaches had never been utilized to assess the extent, impact, and mechanisms of hybridization between the two species. Allendorf *et al.* (2001) identified three potential outcomes to anthropogenically induced hybridization: hybridization without introgression, resulting in reduced reproductive potential; hybridization with introgression, resulting in the formation of a hybrid swarm; or complete genetic admixture, in which no genetically pure populations remain. It was expected that the

case of *A. cavifrons* and *A. rupestris* would fit into one of these three categories, but in actuality, the findings suggest a scenario that does not align with any of these expected outcomes.

The findings did not match the first scenario of hybridization without introgression because all but one of the hybrids identified in my study were post-F_1. This suggests that interspecies hybrids are fertile and able to backcross with parental species. The production of fertile hybrids often result in the formation of hybrid swarms (Childs *et al.* 1996, Bettles *et al.* 2005), but my study found no evidence that this was occurring as a result of *A. cavifrons* X *A. rupestris* hybridization. This finding causes the second scenario to also be unsupported. Instead, most individuals appeared to be strongly one species or the other (i.e., the hybrid index was low), and neither nuclear nor mitochondrial DNA analysis revealed evidence for *A. cavifrons* introgression into the genome of *A. rupestris* in the streams in the former range of *A. cavifrons* that are now completely dominated by *A. rupestris*. The presence of extant populations of genetically pure *A. cavifrons* indicates that the third scenario of complete genetic admixture also has not occurred.

My findings suggest the reproductive interactions between *A. cavifrons* and *A. rupestris* have resulted in a scenario that is intermediate to those predicted by Allendorf *et al.* (2001), suggesting that this framework could be refined and expanded to include additional situations. My study found that these species are capable of producing fertile hybrids, there is evidence for introgression, but something has precluded the formation of a hybrid swarm. This may be because hybrids are inferior competitors for mates or resources and do not have high enough fitness to produce a hybrid swarm (Fukui *et al.* 2016). Alternatively, the formation of a hybrid swarm may have been precluded by high propagule pressure of *A. rupestris* in certain streams through repeated stocking over the course of nearly 100 years which may have resulted in the

introduction of enough individuals to numerically overwhelm *A. cavifrons*. Unfortunately, there is no available information on the exact number and location of *A. rupestris* stocking events (Jenkins and Burkhead 1994), so an accurate estimate of the propagule pressure of *A. rupestris* is impossible to obtain. Future studies focused on modeling could provide insight into the propagule pressure and selection differentials that may have produced observed patterns.

I found that *A. rupestris* have completely replaced *A. cavifrons* in much of the Roanoke drainage, except for the Blackwater and upper Falling Rivers. Elsewhere in the drainage, *A. rupestris* appear to be expanding their range and are in the process of replacing *A. cavifrons* in the Pigg River system. Hybridization between *A. cavifrons* and *A. rupestris* represents a unique pattern that does not fit with the outcome expected for two species that are able to produce fertile hybrids. There is no clear evidence that hybridization is the main driver behind the replacement of *A. cavifrons*, but the detection of hybrids in all streams where both species currently occur suggests that at minimum it leads to wasted *A. cavifrons* reproductive effort. The findings of this study contribute to the understanding of the complexity of reproductive interactions between Centrarchid species. They also reveal that hybridization can lead to the production of fertile hybrids and the loss of a native population without the formation of a hybrid swarm or complete genetic admixture. The commonness of this pattern is unknown, and assessing the patterns of hybridization between other species of Centrarchid fishes may reveal the extent to which it contributes to the imperilment and extirpation of populations.

Characteristics and structure of A. cavifrons populations and assessment of causative factors

Anthropogenic alteration of rivers often results in the fragmentation of freshwater fish populations. This fragmentation is accompanied by a suite of problems including reduced genetic diversity (Dehaan *et al.* 2015), increased rates of inbreeding (Mills and Allendorf 1996),

and the prevention of recolonization by migrants from other populations (Meldgaard *et al.* 2003). A population experiencing these effects faces a higher risk for extirpation as a result of demographic and/or environmental stochasticity. The streams inhabited by *A. cavifrons* have been heavily fragmented by the construction of dams (Roberts *et al.* 2013), and changes in watershed land use have likely reduced the quantity and quality of habitat (Allan 2004).

My analysis suggests *A. cavifrons* populations occur at the stream scale. Clustering results, low pairwise genetic differentiation, and a lack of isolation by distance among continuous stream segments indicate high gene flow occurring at that scale. Tests for migrants, significant pairwise genetic differentiation, and evidence for isolation by distance indicate there is little or no exchange of individuals between populations. Individuals clustered together in populations by stream location, and population boundaries aligned with the presence of in-stream barriers in nearly all cases. Prior to anthropogenic alteration of the environment the grain of population structure may have been larger and limited by natural barriers (e.g., watershed boundaries) rather than dams, but my ability to investigate this is limited by the small number of between stream comparisons lacking anthropogenic barriers. Apart from Swift and Fishing creeks, and Pigg River and Chestnut Creek (the latter comparison lacking sufficient sample-size for analysis), all sampled streams are separated from other streams by one or more dams. Thus, streams tended to form isolated populations; I failed to detect any first-generation migrants among these populations and nearly all individuals were found to derive the majority of their ancestry from the population in which they were sampled. Isolation by distance was found to be significant when assessing comparisons between all sample sites and was also significant in three of the five sampled river basins. The relationship between genetic and spatial distance may be inflated by the presence of barriers, but a paucity of streams lacking barriers between them

makes it difficult to ascertain the degree to which dams affect this relationship. Patchiness of suitable habitat may also play a role in precluding the exchange of individuals between streams, as studies suggest *Ambloplites* may move only relatively short distances (Gatz and Adams 1994).

My findings support the hypothesis that extant *A. cavifrons* populations currently exist in isolation from one another and exhibit significant genetic differentiation that corresponds with biogeography, but a lack of spatial patterns in the distribution of mitochondrial haplotypes suggests historical connectivity of populations. The current delineation of populations at the stream level suggests that existing patterns are an artifact of human activity. One benefit to this human-induced isolation is that it prevents the in-stream movement of invasive *A. rupestris*, which is likely responsible for the persistence of extant *A. cavifrons* populations existing above dams in otherwise *A. rupestris* dominated systems.

In estimating the effective size of *A. cavifrons* populations I found that all but the Tar River population fell below the suggested minimum of 500 for long-term persistence, and the Little and Flat River populations fell below 50 indicating an immediate risk of drift and inbreeding (Franklin 1980). Although my estimates of N_e are perhaps best considered in relative rather than absolute terms, populations identified as being considerably smaller than others may face a heightened risk of extirpation. Aside from the Nottoway River population, all assessed populations had *M* ratios below the expected range for species with stable demographic histories (Garza and Williamson 2001). This suggests that many of the *A. cavifrons* populations have experienced population bottlenecks, and because their isolation precluded demographic rescue by migrants from other populations, these declines resulted in decreases in genetic diversity. Correlations of watershed characteristics and genetic diversity statistics found increases in developed area and decreases in forested area were negatively related to genetic diversity. This

correlation may be a result of decreased habitat suitability due to the alterations to flow and increases in sedimentation associated with anthropogenic alteration of watershed landscapes.

Causes of decline and recommendations for conservation and management

My analyses indicate most *A. cavifrons* populations have a small effective size and unstable demographic histories and that these factors combined with isolation from one another suggests a heightened risk of extirpation. Invasive *A. rupestris* also pose a particular threat to *A. cavifrons* populations existing in streams that are in close proximity to *A. rupestris*-dominated streams. *A. rupestris* currently dominate or nearly dominate many of the streams to which they have been introduced. Introduction of the invader to streams containing *A. cavifrons* populations could result in a complete loss of the native species. Methods to remove invasive fish from streams are often infeasible (Pipas and Bulow 2001) and/or pose risks to other aquatic biota residing in the system (Echelle *et al.* 1997), and thus alternative strategies to removal will be necessary in the case of *A. rupestris*. I recommend an approach that focuses on limiting the spread of *A. rupestris* to new systems through the education of the public and the promotion of *A. cavifrons* as a unique and valuable sportfish. A concerted education campaign should be initiated to discourage anglers from moving *Ambloplites* from one system to another and should include the placement of signage that aids in species identification in areas where risk of introduction is high (e.g., at boat ramps and public fishing areas in invaded and uninvaded streams that are in close proximity to one another). The recognition of *A. cavifrons* and *A. rupestris* as separate species under Virginia's Angler Recognition Program would also help to promote identification of the native species and promotion of its larger size making it the more valuable of the two in terms of trophy fishing. Government stocking of *A. rupestris* has fortunately not taken place in Virginia since 1971 (Jenkins and Cashner 1983), but preventing

the spread of this invasive species by private citizens will continue to be of importance in ensuring the persistence of extant *A. cavifrons* populations.

Translocations of freshwater fish from streams containing larger, more genetically diverse populations to streams with severely reduced or extirpated populations has been demonstrated to be an effective method of population recovery or reestablishment (George *et al.* 2009). My study found that many populations of *A. cavifrons* are small and isolated placing them at higher risk of extirpation due to environmental stochasticity and preventing demographic rescue by individuals migrating from other populations. Small population size also contributes to decreases in genetic diversity leading to reduced adaptive potential. Streams that were found to contain the smallest effective population sizes of all those assessed (the Eno, Little and Flat Rivers and Town Creek) may benefit from the introduction of individuals from streams that were found to contain the largest effective population sizes (the Tar and Nottoway Rivers). These translocations would bolster the effective size and genetic diversity of at risk populations. If translocations are carried out, it will be necessary to establish a genetic monitoring protocol with temporal replicate sampling to evaluate the effects of these management actions (Schwartz *et al.* 2007).

State management agencies should consider the establishment of new populations of *A. cavifrons* to serve as additional refuges from invading *A. rupestris* and preclude the loss of this species from the Virginia portion of its range. Although many of the streams in the Roanoke drainage have become dominated by *A. rupestris*, there are certain streams in the drainage that appear to have suitable habitat yet contain few or no *Ambloplites*. These include Cub Creek and Roanoke Creek in the middle Roanoke basin. There are also several streams with apparently suitable habitat that are devoid of *Ambloplites* in the Dan basin, including the Banister, Sandy,

and Upper Dan Rivers. Although both forks of the Mayo River have been found to contain *A. rupestris*, populations there do not appear to be abundant or widespread (Roberts 2012) indicating that portions of this system may also be able to support new *A. cavifrons* populations. The establishment of new *A. cavifrons* populations would serve to decrease the likelihood of extirpation from the state of Virginia due to the spread of the invasive *A. rupestris*.

The genetic data collected and analyzed for this thesis provides insight into the relative risk faced by extant populations allowing for the identification of populations that may benefit from supplementation and for the prioritization of management efforts (Table 3.1). The data also provide a baseline against which future assessments following management actions may be compared. The ongoing genetic monitoring of *A. cavifrons* populations will afford insights into effectiveness of management strategies for freshwater fish species facing challenges from reproductively-compatible invasive species and anthropogenic habitat fragmentation and alteration.

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Table 3.1 – Summary of findings and management recommendations by system.

Population	Physiographic Region	Drainage Area	Known *A. rupestris* stocking event	*Ambloplites* currently present in population	Most significant threats to *A. cavifrons*	Recommendations
Upper Roanoke	Ridge and Valley	Not assessed	Yes – first introduced in 1905	*A. rupestris*	N/A – *A. cavifrons* population extirpated	Prevent spread of invasive *A. rupestris* inhabiting this system via education efforts
Otter	Piedmont	Not assessed	Yes	*A. rupestris* and hybrids	N/A – *A. cavifrons* population extirpated	Prevent spread of invasive *A. rupestris* inhabiting this system via education efforts
Staunton	Piedmont	Not assessed	No	*A. rupestris* and hybrids	N/A – *A. cavifrons* population extirpated	Prevent spread of invasive *A. rupestris* inhabiting this system via education efforts
Blackwater	Piedmont	282.31 km^2	Yes – first introduced in 1898	*A. cavifrons* and hybrids	Decreased genetic diversity due to isolation by reservoir	Translocate individuals from other *A. cavifrons* populations in the Roanoke Basin (e.g., upper Falling) to promote genetic diversity and mimic historic connectivity
Falling	Piedmont	593.11 km^2	No	*A. cavifrons* in upper portion; *A. rupestris*, *A. cavifrons*, and hybrids in lower portion	Decreased genetic diversity due to isolation by dam, and proximity to population of invading *A. rupestris*	Prevent spread of invasive *A. rupestris* by keeping dam in place and educating anglers; translocate individuals from other *A. cavifrons* populations in the Roanoke Basin to promote genetic diversity and mimic historical connectivity.
Pigg	Piedmont	Not assessed	No	*A. cavifrons*, *A. rupestris*, and hybrids	Ongoing hybridization and replacement by *A. rupestris*	*A. cavifrons* population will likely be completely replaced by *A. rupestris*; limit spread of invasive species through education efforts
Smith	Piedmont	Not assessed	Yes	*A. cavifrons* and hybrids	Isolation by dams and reservoirs	Translocate individuals from other streams in the Dan Basin (e.g., Town Creek) to promote genetic diversity and mimic historical connectivity
Town	Piedmont	84.18 km^2	No	*A. cavifrons* and hybrids	Isolation by dams and reservoirs	Translocate individuals from other streams in the Dan Basin to promote genetic diversity and mimic historical connectivity
Nottoway	Coastal Plain	2926.69 km^2	Yes	*A. cavifrons*	No immediate threats	Allow river to remain unimpounded, continue to monitor genetic diversity
Eno	Piedmont	339.29 km^2	No	*A. cavifrons*	Extensive urban development and isolation by dams	Translocate individuals from other streams in the Neuse Basin (e.g., Little and Flat) to promote genetic diversity and mimic historical connectivity
Little	Piedmont	199.43 km^2	No	*A. cavifrons*	Extensive urban development and isolation by dams	Translocate individuals from other streams in the Neuse Basin to promote genetic diversity and mimic historical connectivity
Flat	Piedmont	383.32 km^2	No	*A. cavifrons*	Extensive urban development and isolation by dams	Translocate individuals from other streams in the Neuse Basin to promote genetic diversity and mimic historical connectivity
Tar	Piedmont/Coastal Plain	574.98 km^2	No	*A. cavifrons*	No immediate threats	Use as a source of individuals for translocation into other streams in the Tar Basin (e.g., Fishing and Swift Creeks)
Fishing	Piedmont	1238.02 km^2	No	*A. cavifrons*	Isolation by dams and reservoirs	Translocate individuals from other streams in the Tar Basin to promote genetic diversity and mimic historical connectivity
Swift	Piedmont	Not assessed	No	*A. cavifrons*	Isolation by dams and reservoirs	Translocate individuals from other streams in the Tar Basin to promote genetic diversity and mimic historical connectivity

APPENDICES

APPENDIX I: *Development of novel polymorphic microsatellite loci for use in Roanoke bass (Ambloplites cavifrons) population analysis*

Source DNA was acquired from *A. cavifrons* individuals collected from the Eno River (Neuse drainage) in Orange County, North Carolina. Fish were collected by angling in May of 2014. A pectoral fin clip was taken from each individual, and placed in 95% ethanol for storage. From fin clips, DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols, and quantified using a Qubit Fluorometer (Thermo Fischer Scientific, Waltham, Washington, USA). We randomly selected three high-DNA-yielding (i.e., $\geq$ 50ng/µL) *A. cavifrons* individuals to pool for microsatellite library development. We sent extracted DNA samples to the Savannah River Ecology Laboratory (SREL, Jackson, South Carolina, USA) for library development. The laboratory utilized the methods described in O'Bryhim *et al* (2012), described briefly here. An Illumina paired-end shotgun library was prepared by shearing 1µg of DNA using a Covaris S220 (Covaris, Woburn, Massachusetts, USA) and following the standard protocol of the Illumina TruSeq DNA Library Kit (Illumina, San Diego, California, USA) and using a multiplex identifier adaptor index. The resulting library was pooled with those from other species and sequencing was conducted on an Illumina HiSeq with 100 base pair paired-end reads. The program PAL_FINDER_v0.02.03 (Castoe *et al* 2012) was utilized to analyze the resulting reads and extract those that contained di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. Once PAL_FINDER_v0.02.03 identified positive reads, they were batched to a local installation of the program Primer3 version 2.0.0 (Untergasser *et al* 2012) for primer design. Loci for which the primer sequences only occurred once or twice in the 5 million reads were selected in order to avoid issues with the copy number

of the primer sequence in the genome. Of the 7454 loci identified, 40 that met this criterion were screened in *A. cavifrons*.

Unlabeled primer sets for these 40 loci were synthesized (Eton Bioscience Inc., Research Triangle Park, North Carolina, USA) and tested for reliable PCR amplification in three *A. cavifrons* individuals. PCR reaction mixes (25µL total) consisted of 12.5µL GoTaq Green Master Mix (Promega, Madison, Wisconsin, USA), 1µL forward primer, 1µL reverse primer, 8.5µL diH_2O, and 2 µL template DNA at stock concentration (typically 10-50 ng•μL^{-1} on a Qubit fluorimeter). PCR cycling conditions were as follows: initial denaturation at 95˚C (120 s), followed by 35 cycles of denaturation at 95˚C (30 s), annealing at 58˚C (30 s), and extension at 72˚C (40 s), followed by a final extension at 72˚C (300 s). PCR prodcuts were visualized on a 2% agarose gel stained with GelRed (Biotium, Hayward, California, USA). Thirty of the primers reliably produced single, clear bands in all three individuals, and were subsequently tested for polymorphism in 30 individuals by utilizing a fluorescently-labeled M13 tail primer. PCR reaction mixes (25.5µL total) consisted of 12.5µL GoTaq Green Master Mix, 1µL forward primer, 1µL reverse primer, 0.5µL M13 tail primer, 8.5µL diH_2O, and 2 µL template DNA at stock concentration (typically 10-50 ng/µL on a Qubit fluorimeter). PCR cycling conditions were as follows: initial denaturation at 95˚C (300 s), 10 cycles of denaturation at 95˚C (30 s), annealing at 57˚C (60 s), and extension at 72˚C (40 s), followed by 27 cycles of denaturation at 95˚C (30 s), annealing at 56˚C (30 s), and extension at 72˚C (40 s), followed by a final extension at 72˚C (600 s). Amplified PCR products were sized using an ABI 3500 Genetic Analyzer with a Genescan 500HD LIZ dye size standard (Applied Biosystems, Foster City, California, USA). Twelve of the thirty loci were found to be polymorphic: Acav17, Acav19, Acav21, Acav22, Acav23, Acav25, Acav26, Acav28, Acav29, Acav31, Acav37, and Acav39. I genotyped 30 *A.*

cavifrons collected from the Eno River at these loci utilizing the M13 tail protocol described above. Fragment sizes were visualized in Genemapper (Applied Biosystems) and manually converted into alleles. Following allele scoring, Arlequin version 3.5 (Excoffier and Lischer 2010) was used to estimate diversity statistics (number of alleles, observed heterozygosity, and expected heterozygosity; Table A1) and test whether each locus was at Hardy-Weinberg equilibrium within the assessed population (10^6 MCMC steps, following a burn-in of 10^5 steps). No marker was found to be out of equilibrium (i.e., all p-values >0.05).

I subsequently grouped these 12 loci into multiplexes and, along with the seven loci described in Chapter 1 as polymorphic in *A. cavifrons* (A111, A114, A115, A145, A432, A464, and A472), used 19 loci to conduct population genetic analyses for Chapter 2.

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Table A1: Diversity statistics for the additional 12 microsatellite markers developed for Chapter 2. Number of alleles per locus (*A*), observed heterozygosity (H_O), and expected heterozygosity (H_E) values are based on the initial screening of 30 individuals from the Eno River with the M13 labeling protocol. No loci were found to be out of Hardy-Weinberg equilibrium (i.e., all $P > 0.05$, based on 10^4 random permutations).

Locus	*A*	H_O	H_E	Hardy-Weinberg *P*
Acav17	7	0.767	0.767	0.961
Acav19	2	0.200	0.235	0.414
Acav21	7	0.600	0.718	0.408
Acav22	3	0.333	0.362	0.412
Acav23	7	0.667	0.750	0.534
Acav25	3	0.367	0.508	0.125
Acav26	6	0.300	0.355	0.208
Acav28	5	0.633	0.640	0.082
Acav29	3	0.367	0.420	0.519
Acav31	7	0.733	0.700	0.500
Acav37	8	0.700	0.792	0.155
Acav39	2	0.467	0.488	1.000

APPENDIX II: *PCR multiplex recipes and cycling conditions*

PCR reaction mixes were 25µL in total volume. Each contained 12.5µL GoTaq Green Mastermix (Promega, Madison, Wisconsin, USA), but the proportions of molecular-grade H_2O and forward and reverse primers (which were all at a concentration of 10µM) varied by multiplex. Multiplex 1 consisted of 2.5µL molecular-grade H_2O, and 1µL of forward primer and 1µL reverse primer for markers A111, A114, A115, and A432. Multiplex 2 consisted of 6.5µL molecular-grade H_2O, 0.5µL forward and 0.5µL reverse primers for markers A145 and A472, and 1µL forward and 1µL reverse primers for marker A464. Multiplex 3 consisted of 3µL molecular-grade H_2O, 0.25µL forward and 0.25µL reverse primer for marker Acav22, 0.5µL and 0.5µL reverse primer for markers Acav19 and Acav39, 1µL forward and 1µL reverse primers for marker ACav37. Multiplex 4 consisted of 3.5µL molecular-grade H_2O, 0.5µL forward and 0.5µL reverse primer for markers Acav17, Acav25, and Acav29, and 1µL forward and 1µL reverse primer for marker Acav31. Multiplex 5 consisted of 1.5µL molecular-grade H_2O, 0.5µL forward and 0.5µL reverse primer for markers Acav26 and Acav28, and 1µL forward and 1µL reverse primer for markers Acav21 and Acav23. PCR cycling conditions were identical for all five multiplexes, and were as follows: initial denaturation at 95˚C (120 s), 35 cycles of denaturation at 95˚C (30 s), annealing at 58˚C (30 s), and extension at 72˚C (40 s), followed by a final extension at 72˚C (300 s).

APPENDIX III: *Grouping of individuals in tentative populations and tests for Hardy-Weinberg and linkage disequilibrium*

Testing the microsatellite markers for evidence of Hardy-Weinberg and linkage disequilibrium required an initial clustering of individuals into tentative populations. This clustering was performed based on the presence of anthropogenic barriers (dams and reservoirs). Individuals sampled from different sites were grouped together if no barriers separated the sites, and individuals sampled from sites with barriers between them were grouped separately. This resulted in 13 tentative populations: the Blackwater, Pigg, Falling, Upper Smith, Lower Smith, Town, Nottoway, Eno, Little, Flat, Tar, Fishing, and Swift. The Pigg cluster represents individuals collected from both Chestnut Creek (n = 3) and the Lower Pigg River (n = 1), but all other clusters consist of individuals collected from a single stream.

I used ARLEQUIN to test for Hardy-Weinberg and linkage disequilibrium at each locus in each of the aforementioned populations, and a sequential Bonferroni correction was applied to adjust values for multiple comparisons. Of 309 tests for Hardy-Weinberg disequilibrium, only a single test was found to be significant: locus A472 in site TOWN2 (site codes correspond to those listed in Table 2.1). Of 3420 tests for linkage disequilibrium between locus pairs, only 10 were found to be significant: Acav39 and Acav31 at site ENO1; Acav39 and Acav31 at site ENO3; Acav39 and Acav31, and Acav17 and Acav26 at site FLAT1; Acav39 and Acav31 at site LITTLE; Acav31 and Acav21, and Acav31 and Acav23 at site NOTT1; Acav39 and Acav31 and Acav31 and Acav23 at site TOWN1; and Acav39 and Acav31 at site UFALL.