Genetic Diversity and Population Fragmentation of *Chrosomus* sp. cf. *saylori* (Clinch Dace)

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Thesis submitted to the faculty of the Virginia Polytechnic and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Fish and Wildlife Conservation

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November 8, 2019

Blacksburg, Virginia

Keywords: Clinch Dace, fragmentation, genetic differentiation, road crossings

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ABSTRACT

Clinch Dace (*Chrosomus* sp. cf. *saylori*) is a newly recognized species of minnow with a restricted distribution in southwestern Virginia. Field sampling and genetic analysis support the hypothesis that Clinch Dace populations are small and fragmented. Analysis of neutral genetic markers shows that most Clinch Dace populations have undergone past bottleneck events and are being operated upon by random genetic drift. Bayesian cluster analysis showed that three out of the seven populations found in 2017 are distinct, while the other four show signs of more recent admixture. However, F_{st} values among streams were high and analysis of molecular variance indicated differentiation among populations in all streams. These findings support the view that these populations are genetically isolated. Effective populations sizes were low at most sites, enhancing the likelihood of loss of alleles to genetic drift. Low *M*-ratios, non-zero F_{is} values, and high degrees of relatedness among individuals indicate that some inbreeding is taking place. Habitat analysis did not identify variables affecting distribution or abundance of Clinch Dace populations. As the collection sites were targeted near known Clinch Dace occupied sites, it is likely that habitat variables known to impact Clinch Dace, such as conductivity, were within the species' range of tolerance. Results showed that Clinch Dace seem particularly resilient to sedimentation, corroborating earlier work showing a negative relationship between Clinch Dace abundance to sediment size. That is, small sediment size does not seem to have a negative impact on Clinch Dace abundance. Of all sites where Clinch Dace were found, only one culvert at one site was clearly perched and may present a barrier to upstream migration, a possibility which is

supported by the genetic differentiation found among collections above and below that culvert. While this study demonstrates that selectively neutral genetic differentiation has taken place among Clinch Dace populations, it does address any local adaptation that may be taking place which would render translocations a risk for outbreeding depression. The findings of this study can inform conservation management in identifying possible sources of individuals for translocations among populations or for augmentation following captive breeding.

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GENERAL AUDIENCE ABSTRACT

The Clinch Dace is a small, threatened minnow in the Clinch River basin that was unknown until 1999. Since then, research has addressed the biology, life history, and distribution of this fish. This study used data from selectively neutral genetic markers to analyze the population structure and degree of differentiation of Clinch Dace populations. My study sites were targeted at road crossings near known Clinch Dace populations to assess the effect of habitat fragmentation on Clinch Dace populations and to maximize the likelihood that I would collect enough genetic material for analysis. Genetic analyses showed that while there is some admixture among certain populations of Clinch Dace, there is differentiation at neutral genetic markers. This differentiation does not necessarily indicate adaptive variation among populations which could result in outbreeding depression should populations be mixed through translocations, but it is reason to proceed with caution. Road crossings were generally not found to be a cause of further population fragmentation in Clinch Dace, as demonstrated by genetic analysis and statistical analysis. Almost all of the occupied road crossing sites in this study were either embedded, free-flowing culverts that were not perched or small bridges, and these were not deemed to be obvious barriers to fish movement. The only exception was Hart Creek 2, where the culvert is slightly perched and a measure of genetic diversity is high between populations in the upstream and downstream reaches. The results of this study will help to inform managers as to what conservation actions can be taken to improve population viability. One potential management action from this study could be the retrofitting of culverts that have become perched and are acting as barriers to Clinch Dace movement. Another potential conservation strategy is to translocate individuals from large population to small populations. The study determined: 1) which translocations might be acceptable based on the degree of genetic differentiation among populations, and 2) identified potential donor and receiving streams for translocations.

Acknowledgments

This research was funded through a U.S. Fish and Wildlife Service State Wildlife Grant managed through the Virginia Department of Game and Inland Fisheries. All work was in accordance with collection permits issued by Virginia Department of Game and Inland Fisheries and protocol 16-188FIW, approved by the Virginia Tech Institutional Animal Care and Use Committee.

Special thanks to Dr. Donald Orth, Dr. Eric Hallerman, Dr. Dean Stauffer, Miluska Hyde, Sheila Harris, Corbin Hilling, Kaitlin Hanak, and Chanz Hopkins.

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Introduction

The Clinch Dace (*Chrosomus* sp. cf. *saylori*) is a currently undescribed species of minnow (Family Cyprinidae) that was first discovered in Tazewell County, Virginia, in 1999 (Skelton 2007). Clinch Dace was first thought to be a disjunct population of the closely related Laurel Dace (*Chrosomus saylori*). However, meristic and morphological differences between these congeners, such as a longer anal fin base and shallower head in the Clinch Dace, suggest otherwise (White and Orth 2013). Field identification of the Clinch Dace relies on differences in breeding colors from the Laurel Dace; the Clinch Dace has an upper black lateral band that extends all the way to the caudal fin and two yellow spots at the caudal fin base (White and Orth 2013). Official description of the Clinch Dace as a new species is being conducted by Dave Neeley of the Tennessee Aquarium and Conservation Institute.

Surveys conducted since 1999 (White and Orth 2014, Moore and Orth 2017) have found that Clinch Dace occupy 16 streams in eight drainages to the upper Clinch River in Russell and Tazewell counties. In Virginia's Wildlife Action Plan (Virginia Department of Game and Inland Fishes 2015), it is classified as Tier II – Very High Conservation Need. Clinch Dace populations are isolated and have low densities (Moore et al. 2017). White (2014) showed negative correlations between Clinch Dace presence with substrate particle size, stream width, and conductivity. Clinch Dace occupancy was positively correlated with the proportion of forested land in the respective watersheds (Moore et al. 2017). Clinch Dace occupies restricted habitats in isolated patches, and hence is vulnerable to local extirpation or extinction (Lande 1993). Because Clinch Dace occurs in regions affected by coal mining and logging, protective actions are required. Further, it may be necessary to initiate population augmentations through translocations, or captive breeding and stocking, or through facilitation of natural migration by removal of barriers to migration. Before such actions can be planned, decided upon and implemented, managers need detailed information on habitat features, such as locations of perched culverts or stream reaches with elevated conductivity, that could be fragmenting distributions and creating population structure among stream fragments.

Faced with the need to define biologically justifiable management units for *Chrosomus* sp. c.f. *saylori*, the goal of this research was to characterize population genetic structure useful for informing management planning for the imperiled Clinch Dace (*Chrosomus* sp. c.f. *saylori*).

Objectives

Under this overarching goal, the operational objectives of this study were to:

(1) Determine whether disconnected populations of *Chrosomus* sp. cf. *saylori* (Clinch Dace) in the Upper Clinch River Basin represent distinct management or evolutionarily significant units.

Under this objective, the hypotheses to be tested were:

$H_{1\text{-}0}$: Populations of Clinch Dace are genetically homogeneous.

$H_{1\text{-}A}$: Populations of Clinch Dace are genetically distinct.

(2) Determine whether barriers to upstream migration, particularly road crossings, are fragmenting populations of *Chrosomus* sp. cf. *saylori*.

Under this objective, the hypotheses tested were:

$H_{2\text{-}0}$: Road crossings are not fragmenting Clinch Dace populations, and presence and abundance are determined by site-level habitat and community variables.

$H_{2\text{-}A}$: Road crossings are fragmenting Clinch Dace populations and are determining presence and abundance at sites.

Chapter 1: Does genetic diversity indicate that populations of *Chrosomus* sp. cf. *saylori* (Clinch Dace) represent distinct management or evolutionarily significant units?

Introduction

Populations and Units of Conservation. – Any consideration of genetically based units of management must begin with a clear definition of the term "population". Waples and Gaggiotti (2006) offered two definitions for population under two distinct paradigms. Under the ecological paradigm, they defined a population as "a group of individuals of the same species that co-occur in space and time and have an opportunity to *interact* with each other". They defined a population according to the evolutionary paradigm as "a group of individuals of the same species living in close enough proximity that any member of the group can potentially *mate* with any other member". These are but two of many potential definitions for the term population. Natural or anthropogenic habitat fragmentation could produce a metapopulation, that is "a set of discrete populations of the same species, in the same general geographical area, that may exchange individuals through migration, dispersal, or human-mediated movement" (Akcakaya 2006). Two general requirements for metapopulation status are that: (1) "populations are geographically discrete;" and (2) "mixing of individuals between populations is less than within". In my context, Moore (2017) showed that Clinch Dace populations are isolated from each other and occur at low densities. Hence, they may or may not collectively function as a metapopulation.

Applying concepts of populations to management may involve grouping them into units for conservation. Two dominant approaches are to define management units (MUs) and evolutionarily significant units (ESUs). Although universal consensus on defining the criteria for ESUs is lacking, Ryder (1986) first defined ESUs as populations representing significant adaptive variation as evidenced by multiple data sources, such as genetic, life history, and distribution data. Waples (1991) expanded upon the ESU concept and applied it to U.S. Endangered Species Act considerations. Waples (1991) presented two criteria for a population or a group of populations to qualify as an independent ESU: "1) it must be substantially reproductively isolated from other conspecific population units, and 2) it must represent an important component in the evolutionary legacy of the species". He stated that isolation need not be

absolute, but populations should be isolated enough for "evolutionarily important differences to accrue". Four questions are associated with a population's designation as an ESU: 1) is the population genetically distinct from other conspecific populations? 2) does the population occupy unusual or distinctive habitat, 3) does the population show evidence of unusual or distinctive adaptation to its environment? and 4) is the population part of the evolutionary legacy of the species? A "yes" answer to the genetic isolation question and to any of the four evolutionary legacy questions is required for designating a population or group of populations an evolutionarily significant unit. The data necessary to answer these questions include population genetics, phenotypic characteristics, life-history traits, and habitat use characteristics.

Crandall (2000) took a different approach and emphasized the importance of ecological and genetic "exchangeability." He explained that "the central idea of ecological exchangeability is that individuals can be moved between populations and can occupy the same ecological niche or selective regime". That is, individuals from different populations can be exchanged because they would perform the same ecological function in each population. All that is needed for individuals within populations to be genetically exchangeable is demonstration of sufficient gene flow between populations. The management implication of this viewpoint is that adaptive variation will be preserved by conserving non-exchangeable ESUs.

Moritz (1999) took a contrasting view of ESU designation from those discussed above. His perspective was that ESUs should be designated solely on the basis of the degree of genetic differentiation at neutral genetic markers, irrespective of the presence of adaptive variation. His rationale was that conserving genetic diversity allows the process of evolution that gave rise to phenotypic variants to proceed. In this way, Moritz argued, lost variation can be restored by the same evolutionary processes that created it originally.

The designation of ESUs can prove a contentious consideration, as the adaptive variation that most agree is a key component of an ESU can be hard to demonstrate. Identifying management units (MUs) is relatively straightforward, and a single ESU may comprise multiple MUs. Moritz (1999) stated that "management units represent demographically independent populations, i.e., the functional components of the ESU, and are diagnosed as populations showing divergence in allele frequencies at mtDNA and/or

nuclear loci". Palsboll et al. (2007) further stated that population fluctuations in MUs are more dependent on births and deaths within the population than they are on immigration from other populations. If MU status is to be determined by genetic differentiation among populations, the question then becomes how much differentiation is needed to define an MU. Palsboll et al. (2007) defined management units as "populations of conspecific individuals among which the degree of connectivity is sufficiently low so that each population should be monitored and managed separately". Palsboll et al. (2007) suggested that the amount of genetic divergence that corresponds to a dispersal rate of less than ten percent be used as the threshold criterion for designating MUs for target species, as the ten percent threshold has been indicated as corresponding to the point at which populations become demographically correlated (Hastings 1993).

There are many approaches to measuring genetic diversity and assessing the relations of genetic diversity metrics and conservation status. In this thesis, the number of alleles per locus, expected and observed heterozygosities (H_E and H_O), allelic size range, inbreeding coefficient (F_{is}), *M*-ratio, and degree of departure of observed genotype numbers from Hardy-Weinberg expectations provided indications of genetic differences. Low *M*-ratios would indicate that alleles in the population have been lost over time. The fixation index (F_{st}) measures population differentiation due to population structure and ranges between zero and one. A zero value implies complete panmixia, whereas a value of one implies that all genetic variation is explained by the population structure. I calculated F_{st} among reaches above and below road crossings or midpoints where there was no road crossing, as well as for all whole streams to determine how differentiated population segments are from each other and to assess whether road crossings were causing population differentiation. Analyses of Molecular Variance (AMOVA) determines what proportion of the observed genetic variation is due to differences within or among streams. A high proportion of genetic variance occurring among streams would indicate that population genetic differentiation has taken place. High levels of relatedness among individuals in each stream would suggest the occurrence of inbreeding. The so-call inbreeding coefficient (F_{is}) quantifies the departure of observed genotype frequencies observed within populations from those expected under Hardy-Weinberg expectation. These metrics taken together provide a picture of how these populations are genetically differentiated and structured. Knowledge gained

by interpreting patterns of genetic variation will inform consideration of management actions, such as whether translocations are a viable means of augmenting small populations or if captive breeding of individuals from within the stream and stocking should be conducted. Knowing the effective population size, or the number of breeding individuals in a population, will help to determine whether populations are viable and stable or whether management actions might be required to prevent local extinction.

Given that the Clinch Dace populations in each stream are separated by unsuitable downstream habitat and ultimately by the Clinch River itself, it is reasonable to hypothesize that some, if not all, of these populations represent unique management units. To meet the objective of determining management units for Clinch Dace, I tested whether Clinch Dace populations are genetically distinct or genetically homogenous. To do this, I analyzed variation of microsatellite and mitochondrial DNA isolated from Clinch Dace fin-clips collected from across their distribution to determine population genetic structure among Clinch Dace populations. The hypotheses for testing this possibility are as follow:

H_{01}: There is no population genetic differentiation of Clinch Dace among streams, or

H_{A1}: There is population genetic differentiation of Clinch Dace among streams.

H_{02}: There is no population genetic differentiation of Clinch Dace within streams, or

H_{A2}: There is population genetic differentiation of Clinch Dace within streams.

Methods

Study design

Towards the objective of determining whether barriers to upstream migration, particularly road crossings, are further fragmenting populations of *Chrosomus* sp. cf. *saylori*, I employed an observational study design. I collected observational data from different sampling locations or "sites" in streams within the known distribution of Clinch Dace. Most sampling sites were located at road crossings to determine whether they are fragmenting Clinch Dace upstream and downstream populations. Whenever possible, each site consisted of two sampling units. In the case of sites located at road crossings, these sampling units were reaches extending upstream and downstream from a road crossing. In the case of non-road crossing sites,

there was an upstream and a downstream reach relative to an arbitrary point along a stream. The number of sites and the number of sampling units within each site was determined by permission to access a landowner's property, accessibility, proximity of other road crossings, and practical aspects of ability to sample effectively (e.g., the stream is not dry or converted by a beaver dam). I collected and analyzed habitat and fish community data. Such analyses were conducted on all experimental units for which data was collected and tested the hypothesis that Clinch Dace abundance and/or presence is determined by habitat and community factors. To determine the effects of barriers on Clinch Dace populations, I used both sampling reaches in a paired experimental design. Genetic analysis contributed to achievement of this objective, as I used paired experimental units and multiple metrics of genetic divergence to test for genetic differentiation of Clinch Dace collected upstream and downstream of road crossings.

Sampling Methods

To test the hypothesis that road crossings fragment Clinch Dace populations and affect site occupancy and abundance versus the alternative hypothesis that site occupancy and abundance are determined by habitat quality and fish community, I used genetic methods and habitat analyses. Genetic methods included analysis of DNA markers to determine population genetic structure and to estimate effective population sizes among sites. I used correlation and regression analyses to determine the effects of several habitat and fish community metrics on Clinch Dace occupancy and abundance. Most sites sampled for Clinch Dace straddled road crossings to evaluate their impact as barriers to fish movement. I characterized road crossings by type (bridge, box culvert, pipe culvert, etc.), assessed them as possible barriers to movement of Clinch Dace, and measured their dimensions and degree of "perchedness" at all sites.

In the summer of 2017, I surveyed streams at 19 sites near locations known to be inhabited by Clinch Dace based on results of stream surveys conducted by Shannon White in 2011 and 2012 and Michael Moore in 2014 and 2015. Most sites consisted of two sub-reaches, each extending upstream and downstream of the road crossing to determine whether that crossing was fragmenting the Clinch Dace

population. Control sites were not located at road crossings, but still consisted of an upstream and a downstream reach. However, no analyses were conducted on control reaches versus road crossing sites as I could not get permission for enough control sites. Reach lengths were established as 40 times average stream width with a minimum of 200 meters, conditions permitting, as site lengths of approximately 40 times average stream width are adequate in eastern streams to characterize community and associated habitat (Klemm and Lazorchak 1995; Lyons 1992). Not all sub-reaches met this criterion due to such factors as close proximity of other culverts, barriers, confluences with other streams, or lack of landowner permission to access beyond a certain portion of the reach.

Site Selection and Reconnaissance

I identified approximately 80 potential sampling sites using ArcGIS 10.1 and Shannon White's and Michael Moore's shapefiles of Clinch Dace-occupied sites in Russell and Tazewell Counties, Virginia. Due to lack of response of landowners to initial contact via mail, I visited landowners in person to request permission. Difficulties in obtaining landowner permission eliminated most of the potential sites that I identified, and I had to sample where I could receive permission. This did not allow me to specifically target road crossings that may or may not be potential barriers to fish movement. The sampling design was, hence, opportunistic.

I reconnoitered all sites which I received permission to access. The main purposes of reconnaissance were to determine accessibility, sampleability, and reach length. I measured stream width at eleven points along each sub-reach, starting at the road crossing or and extending 200 m upstream or downstream. At non-crossing sites, measurements began at the downstream-most transect of the downstream reach and proceeded upstream to the 200-meter mark. If average width of the first eleven transects exceeded five meters, then additional transects were added until a total sampling distance of 40 X average width was reached. Upstream reaches at non-road crossing sites were measured from the midpoint (200-meter mark as measured from the downstream reach) up to a 400-meter mark. However, I did not have any reaches where average width exceeded five meters. Some reaches did not reach the minimum

200-meter length, due to such factors as inaccessibility, the presence of other road crossings or barriers, and lack of permission to access that portion of the stream. Some sites lacked a downstream or upstream reach altogether.

Fish Sampling

I conducted three-pass electrofishing depletions on most sampleable reaches. One backpack Smith-Root LR 24 backpack electrofisher set to 300 volts was used per three meters of average stream width (Kazyak 2000). All fish were identified to species and counted. Due to time constraints at a few sites, not all fish were counted and a species list is reported. Clinch Dace were measured for total length and a small fin-clip was cut from the upper caudal fin and stored in 95% ethanol. Fin-clips were given a unique identifier corresponding to stream, reach, and fish length, and, in some cases, a photograph was taken. Any Clinch Dace mortalities were preserved in 10% formalin, along with any fish that could not be positively identified in the field. All fish were retained in bins, with Clinch Dace being kept separately from all other fish, until all three electrofishing passes were completed. Total shocking seconds per pass and voltage used were recorded. Their populations in each sub-reach were estimated using three-pass depletion data in Microfish 3 (Van Deventer 1989) and standardized to 100 meters of stream length. All fish sampling was done in accordance with Virginia Tech IACUC protocol FWC 16-188. Not all fin-clips were used for genetic analysis when Clinch Dace were abundant (Table 1).

Table 1. Numbers of Clinch Dace DNA samples for given reaches sampled in 2017 (this study) and 2015 (Moore 2015).

Reach	*N*
Big Lick1 down	22
Big Lick1 up	24
Big Lick 2 down	1
Big Lick 2 up	5
Big Lick 3 down	19
Big Lick 3 up	24
Big Lick 4 down	11
Greasy Creek 1 down	5
Greasy Creek 2 down	1
Hart Creek 1 down	20
Hart Creek 1 up	22
Hart Creek 2 down	21
Hurricane Fork down	20
Hurricane Fork 1 up	20
Lewis Creek 4 up	3
Middle Creek 3 down	8
Middle Creek 3 up	8
Pine Creek IM	16
Pine Creel low	7
Pine Creek mid	3
Pine Creek up	6

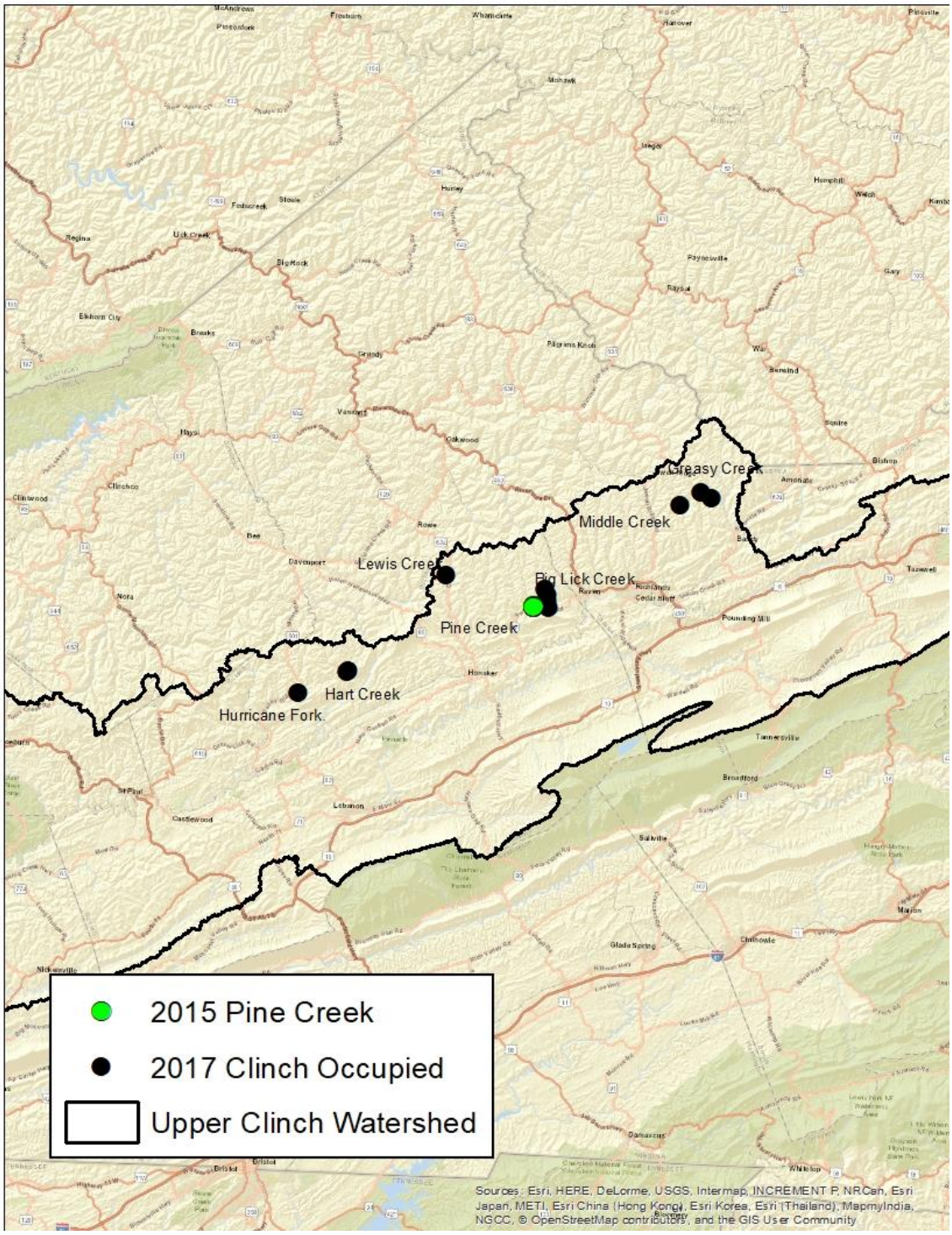

Figure 1. Clinch Dace occupied sites sampled in 2017 by Rebecca Bourquin and in 2015 by Michael Moore.

I extracted DNA from fin-clips using DNeasy Blood and Tissue kits (Qiagen). Concentration and purity of extracted DNA were quantified using a µLite spectrophotometer (BioDrop, Cambridge, UK). I

screened 17 microsatellite loci using primer pairs that were developed to amplify microsatellite DNA in the closely related *Phoxinus phoxinus* (Grenier et al. 2013): *Cto-A-247, LleB-072, LleC-090, Rru4, BLi-84, BLi-98, BLi-153* (Debut et al. 2009a, 2009b, 2010), *CypG9* (Baerwald and May 2004), *LceC1* (Larno et al. 2005), *Lco3* (Turner 2004), *Ppro132* (Bessert and Orti 2003), *Rru4* (Barinova et al. 2004), *Lsou5, Lsou8* (Muenzel et al. 2007), *Rhea20* (Girard and Angers 2006), *NLi-153* (Dimsoski et al. 2000), *CypG30* (Vyskeoilova et al. 2007), and *MFW1* (Crooijmans et al. 1997). Polymerase chain reaction amplification protocols were modified from those outlined by Grenier et al. (2013) as necessary to promote amplification across species. The PCR protocol was as follows: initial denaturation 94º for three minutes; 35 cycles of denaturation at 94º for one minute, annealing at 56º for 45 seconds, and extension at 72º for one minute; and final extension 72º for five minutes. Nine of twelve primer sets successfully amplified Clinch Dace microsatellite DNA for fragment-size analysis (Table 2). A small subset of samples was sent to the Virginia Biocomplexity Institute (Blacksburg, VA) for Sanger sequencing to confirm the structure of microsatellite loci to ascertain that I did indeed amplify the target microsatellite DNA.

Table 2. Clinch Dace microsatellite loci amplified and analyzed for fragment size.

Locus	Primers (5' - 3')	Core Motif	Allele Size Range	Reference
CtoA247	F-6FAM:TGCAAACATATAAACTGAAACAAGG R:GCAGGTATATTCCCAGCC	$(ATC)_7$	160-166	Dubut et al. (2010)
LleC90	F-PET:TCAGACACAACTAACCGACC R:GGCGCTGTCCAGAACTGA	$(TC)_{15}GG(TC)_3$	218-228	Dubut et al. (2009b)
BLI_84	F-6FAM:CATTACTACGGAACCACAT R:GCGAAAAGGAAAGAGACTGA	$(AC)_4N_{24}(CA)_9$	180	Dubut et al. (2009a)
BLI153	F-6FAM:GCACAGCTCTAATCGGTCACT R:TATGGTCAAACACGGGTCAA	$(AC)_{20}$	216-212	Dubut et al. (2009a)
Lco3	F-VIC:GCAGGAGCGAAACCATAAAT R:AAACAGGCAGGACACAAAGG	$(TG)_9$	246-262	Turner et al. (2004)
Lsou8	F-PET:GCGGTGAACAGGCTTAACTC R:TAGGAACGAAGAGCCTGTGG	$(GT)_{17}$	170-176	Muenzel et al. (2007)
Rhca20	F-NED:CTACATCTGCAAGAAAGGC R:CAGTGAGGTATAAAGCAAGG	$(GA)_{17}$	87-91	Girard and Angers (2006)
CypG30	F-VIC:GAAAAACCCTGAGAAATTCAAAAAGA R:GGACAGGTAAATGGATGAGGAGATA	$(TAGA)_7$	280-240	Baerwald and May (2004)
MFW1	F-NED:GTCCAGACTGTCATCAGGAG R:GAGGTGTACACTGAGTCACGC	$(GT)_{14}N_3(GA)_4$	172	Crooijmans et al. (1997; Tong et al. (2005)

Primer pairs found effective at amplifying microsatellite DNA in Clinch Dace were multiplexed to the degree practical. Amplification products were examined on ethidium bromide-stained gels, and those showing clear products in the appropriate size range were sent to Cornell University for DNA fragment-size analysis using an automated DNA sequencer. The program Genemarker (Hulce et al. 2011) was used to score microsatellite fragments.

Data analysis

The program Microchecker (van Oosterhout et al. 2004) was used to test for segregation of null alleles and other PCR artifacts and to calculate the Oosterhout value, which is the frequency of null alleles as estimated by heterozygote deficiency. Departures from Hardy-Weinberg equilibrium and linkage disequilibrium were tested in Arlequin 3.5 (Excoffier et al. 2005) with exact tests using Markov chain with forecasted chain length of 1,000,000 and 100,000 dememorization steps for all loci in all stream reaches. Genetic diversity was quantified in terms of number of alleles per locus (A), allele frequencies, expected and observed heterozygosities (H_E and H_O), and allelic size range (number of repeats, R) for each locus and averaged across all loci for each population. M-ratios were calculated as the number of alleles divided by the allele size range; M-ratios lower than about 0.7 indicate the occurrence of prior genetic bottlenecks (Garza and Williamson 2001). The inbreeding coefficient (F_{is}) in each population was calculated using Fstat (Goudet 1995). All of these analyses were also conducted at the hierarchical level of whole streams, in addition to reaches within streams.

Population differentiation and mixing among sites was assessed using Bayesian cluster analysis implemtented within Structure 2.3.4 (Pritchard et al. 2000), to assess support for different numbers of population clusters (K) and to assign individuals' genotypes to those clusters. I used the admixture model to infer ancestry, with a burn in length of 10,000 and 100,000 Monte Carlo Markov Chain repetitions after the burn-in period. I assessed population differentiation at several levels. First, I ran Structure's admixture model with all samples from all streams with the set number of clusters (K) running from one to ten, with

five replications (Figure 3). On the basis of the results of this run, I applied the algorithm to the four streams where admixture seemed most likely with *K* values from 1 to 5 and five replicate runs (Figure 4). Finally, I ran the admixture model for each stream individually, each with *K* running for one to five and five replicate runs.

I measured the distances between the most downstream Clinch Dace occupied sites between each pair of streams in ArcGIS 10.2.2 (Esri 2012). I plotted pairwise distances between the most downstream occupied site on each stream against F_{st} in a scatterplot and calculated r^2 in order to assess the effects of isolation by distance.

Population-based genetic differentiation methods included calculations of F_{st} (Wright 1965) and Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992). F_{st} was calculated in GENEALEX 6.5 (Peakall and Smouse 2012), and AMOVA was executed in Arlequin 3.5 (Excoffier et al. 2005). AMOVAs partitioned genetic variation within and among streams in Arlequin 3.5 (Excoffier et al. 2005). Effective population sizes (N_e) for all reaches were estimated in NeEstimator v2 (Do et al. 2014) using the linkage disequilibrium method. If some microsatellite alleles seemed to be very rare, they were removed from the analysis to avoid upward bias of estimates (Do et al. 2014). I used the program MLrelate (Kalinowski 2006) to estimate genetic relatedness among individual Clinch Dace within each stream.

Results

Of the 17 primer pairs tested, nine produced amplification products sufficiently clear to be analyzed for fragment size (Table 7). These loci were *Cto-A-247, LleC-090, BLi-84, BLi-153* (Debut et al. 2009a, 2009b, 2010), *Lco3* (Turner 2004), *Lsou8* (Muenzel et al. 2007), *Rhea20* (Girard and Angers 2006), *CypG30* (Vyskeoilova et al. 2007), and *MFW1* (Crooijmans et al. 1997). Sequencing of amplicons for loci *BLI-84, BLI-153, CtoA-247, Lco-3, Lsou-8,* and *MFW-1* all showed one good tract of the repeated core motif as reported by Grenier et al. (2013). Locus *LleC-090* exhibited two tracts of the core motif. The reported core motif could not be found in the sequence data for locus *CypG-30*. Amplification products for

Rhca-20 failed multiple attempts at sequencing and the microsatellite structure of this locus could not be confirmed.

Of 266 samples, 18 samples failed to amplify PCR products. After fragment analysis in GeneMarker (SoftGenetics, State College, PA) was complete, I analyzed data for individual reaches (Table A2) and for whole streams (Table A3) for segregation of null alleles in Microchecker (Van Oosterhout et al. 2004). Microchecker detected three instances of null alleles for reaches and four instances of null alleles in the streams analyzed as a whole. No one locus consistently yielded null alleles (Appendix A, Table 15). Therefore, null alleles were not considered to be an issue and data for no loci were excluded from subsequent analyses. No linkage disequilibrium was found at the reach level at Bonferroni-corrected critical values. Data for two loci in two streams (Hart Creek and Big Lick Creek) showed apparent linkage disequilibrium, which was attributed to chance, and data from these loci were not dropped. The Hardy-Weinberg population structure analyses that I conducted on individual reaches (Table A3) and on streams as a whole (Table 4) did not yield results that would lead to excluding any loci from the analyses. For individual stream reaches, four populations were out of Hardy-Weinberg equilibrium at two loci each at the Bonferroni-corrected critical value. Where data from whole streams were tested for Hardy-Weinberg equilibrium, five loci distributed across three populations were out of equilibrium based on the Bonferroni-adjusted critical values. In no case was there any one locus that was out of equilibrium at all sites, so Hardy-Weinberg equilibrium was not considered a reason to exclude data for any of the nine loci. Given the results of these analyses, I kept data for all nine loci that consistently amplified Clinch Dace DNA in the subsequent analyses. Expected heterozygosity (H_E) and observed heterozygosity (H_O) were reasonably similar in most cases and there were few loci that were out of Hardy-Weinberg equilibrium (Table 4). The number of alleles per locus varied, but some were quite limited and even monomorphic for entire streams (Table 4). Fis values varied widely per locus (Table 4). Bonferroni corrected alpha values in Table 4 vary because the number of loci tested in each stream vary due to some loci being monomorphic in some streams.

For whole streams, 25 of 30 *M*-ratios (Table 4) were lower than the criterion level of 0.70 suggested by Garza and Williamson (2001), suggesting that populations at these sites have undergone genetic

bottlenecks in the recent past. Average inbreeding coefficients (F_{IS}) within collections at the reach scale was -0.01, indicating that localized departures from Hardy-Weinberg expectation were small, arguing against widespread inbreeding. However, some values of F_{is} were high, suggesting localized inbreeding in some reaches, such as Greasy Creek (*P*-value = 0.0003) Middle Creek (*P*-value = 0.038), and Hart Creek (*P*-value = 0.003). Average F_{IS} for collections from whole streams was 0.092, and 17 of 30 F_{IS} values were high for certain loci, suggesting some degree of inbreeding within streams (Table 4). Greasy Creek, Hart Creek, and Middle Creek all had F_{is} values significantly different from zero, indicating inbreeding in those streams (Table 3). F_{is} values may be biased by small samples sizes in some streams.

Table 3. Average F_{is} values for streams and the probability of a random F_{is} value being greater or equal to the observed F_{is}. Significant *P* values are shown in bold font.

Stream	F_{is}	*P*(Random F_{is}≥Observed F_{is})
Big Lick Creek	-0.023	0.747
Greasy Creek	0.212	**0.003**
Hart Creek	0.139	**0.003**
Hurricane Fork	-0.095	0.969
Lewis Creek	0.142	0.681
Middle Creek	0.164	**0.038**
Pine Creek	-0.085	0.896

Table 4. Genetic diversity in Clinch Dace streams. Monomorphic loci not shown. N = number of samples, H_O = observed heterozygosity, H_E = expected heterozygosity, A = number of alleles, Range = range of allele sizes in base pairs, M-ratio = ratio of A to Range with ratios suggestive of a recent bottleneck shown in bold font, F_{is} = inbreeding coefficient, HW= p-values associated with departures from Hardy-Weinberg expectations, Bonferroni alpha = Bonferroni-corrected critical p-value.

Stream	Locus	N	H_0	H_e	A	Range (bp)	M-ratio	F_{is}	HW	Bonferroni alpha
Big Lick Creek	*CtoA247*	106	0.01887	0.01878	2	4	**0.50**	-0.005	1.0000	0.01
	Lco3	106	0.34906	0.3335	4	8	**0.50**	-0.047	0.0289	0.01
	LleC90	106	0.12264	0.13422	4	8	**0.50**	0.087	0.0543	0.01
	Rhca20	106	0.00943	0.00943	2	4	**0.50**	0	1.0000	0.01
	CypG30	106	0.69811	0.73303	6	24	**0.25**	0.048	0.0016	0.01
	Lsou8	106	0.54717	0.47662	2	6	**0.33**	-0.149	0.1538	0.01
Greasy Creek	*Lco3*	6	0.33333	0.31818	3	12	**0.25**	-0.053	1.0000	0.012
	BLI153	6	0.16667	0.5303	2	2	1.00	0.706	0.1508	0.012
	CypG30	6	0.5	0.74242	5	20	**0.25**	0.348	0.7424	0.012
	Lsou8	6	0.66667	0.48485	2	6	**0.33**	-0.429	1.0000	0.012
Hart Creek	*Lco3*	63	0.26984	0.34171	4	8	**0.50**	0.212	0.0199	0.01
	LleC90	62	0.08065	0.28311	4	10	**0.40**	0.717	0.0000	0.01
	BLI153	63	0.22222	0.22349	2	2	1.00	0.006	1.0000	0.01
	CypG30	62	0.91935	0.75492	7	28	**0.25**	-0.22	0.0001	0.01
	Lsou8	63	0.31746	0.49778	1	6	**0.17**	0.364	0.0051	0.01
Hurricane Fork	*CtoA247*	40	0.05	0.0962	2	4	**0.50**	0.483	0.0757	0.012
	Lco3	40	0.45	0.37975	2	2	1.00	-0.188	0.3987	0.012
	CypG30	40	0.925	0.82342	8	32	**0.25**	-0.125	0.0025	0.012
	Lsou8	40	0.15	0.14051	2	6	**0.33**	-0.068	1.0000	0.012
Lewis Creek	*BLI153*	3	1	0.6	2	4	**0.50**	-1	0.4008	0.025
	CypG30	3	0	0.53333	2	8	**0.25**	1	0.2003	0.025
Middle Creek	*Lco3*	16	0.25	0.31452	2	2	1.00	0.317	0.4334	0.017
	CypG30	16	0.625	0.77419	7	32	**0.22**	0.779	0.4674	0.017
	Lsou8	16	0.1875	0.1754	2	6	**0.33**	0.175	1.0000	0.017
Pine Creek	*Lco3*	32	0.28125	0.30208	4	12	**0.33**	0.07	0.0988	0.008

Table 4 (continued)

	LleC90	31	0.35484	0.29667	2	2	1.00	-0.2	0.5535	0.008
	Rhca20	32	0.25	0.22222	2	4	**0.50**	-0.127	1.0000	0.008
	BLI153	32	0.03125	0.03125	2	2	1.00	0	1.0000	0.008
	CypG30	32	0.875	0.7996	6	24	**0.25**	-0.096	0.0024	0.008
	Lsou8	32	0.03125	0.03125	2	6	**0.33**	0	1.0000	0.008

Fixation index (F_{st}) values among populations compared across streams ranged from a low of 0.037 between Pine Creek and Middle Creek to a high of 0.527 between Middle Creek and Lewis Creek (Table 5). The mean F_{st} value of streams that are rather close together, such as Pine Creek and Big Lick Creek, which are separated by only two stream kilometers, was 0.2, the standard deviation was 0.04, and the standard error was 0.02 (Table A9). The mean F_{st} value for streams that are most distant, such as Greasy Creek and Hurricane Fork, which are separated by 117 kilometers (Table A9), from each other was 0.3, with a standard deviation of 0.12, and a standard error of 0.03. I generated a scatterplot using those distances and F_{st} values to assess whether there was an effect of isolation-by-distance (Figure 2). There was a very weak, non-significant relationship between distance and F_{st}, with a R^2 value of 0.08. This indicates that isolation-by-distance (Wright 1943) was not an important factor in generating population genetic differentiation among Clinch Dace populations.

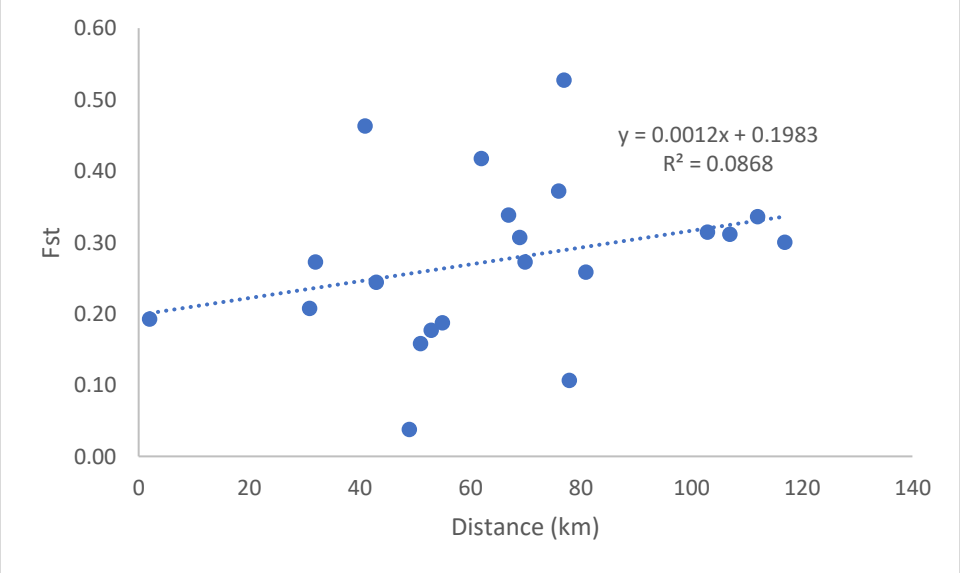

Figure 2. Pairwise comparisons of fixation index (F_{st}) of Clinch Dace populations and the stream distances between them.

Table 5. Pairwise fixation index (F_{st}) values for each Clinch Dace stream.

	Big Lick Creek	Greasy Creek	Hart Creek	Hurricane Fork	Lewis Creek	Middle Creek
Big Lick Creek	-					
Greasy Creek	0.186	-				
Hart Creek	0.305	0.310	-			
Hurricane Fork	0.105	0.299	0.272	-		
Lewis Creek	0.243	0.257	0.416	0.272	-	
Middle Creek	0.157	0.207	0.313	0.335	0.526	-
Pine Creek	0.191	0.175	0.337	0.371	0.462	0.036

Results of Structure analysis of all samples revealed strongest support for there being five clusters (K = 5) among the seven stream populations (Figure 3). The least negative log-likelihood of the five iterations was -1919.5 with a variance of 341.1, and the least negative average log-likelihood was -1923.96, with a variance of 347.5 (Table A5), both corresponding to five clusters. Population 1 (Hurricane Fork), and population 2 (Hart Creek) clustered separately, while those in the remaining five streams seemed to display some degree of admixture. In Structure graphs, the different colors represent different inferred populations. When the colors are mixed and not solid, then that is evidence that admixture has taken place and that the populations are not entirely differentiated. The *y*-axis represents the degree of individual membership in one or more multi-locus clusters.

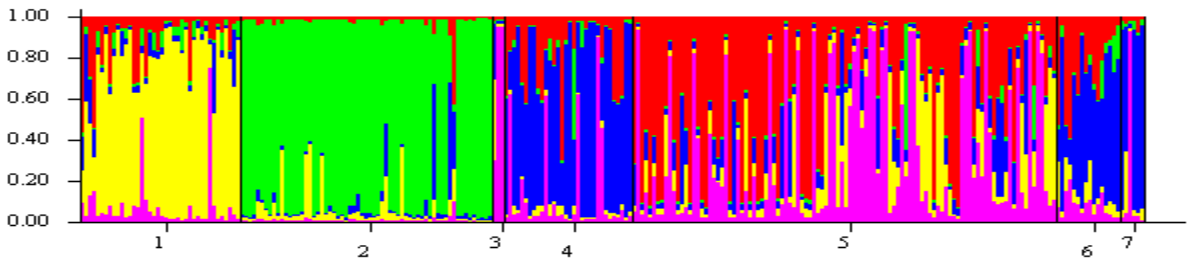

Figure 3. Bar plot of results of Structure Bayesian cluster analysis showing the seven stream populations. 1 = Hurricane Fork, 2= Hart Creek, 3 = Lewis Creek, 4 = Pine Creek, 5 = Big Lick Creek, 6 = Middle Creek, 7 = Greasy Creek. Results indicate the number of multilocus genetic clusters (K) in the metapopulation.

When the four populations showing admixture (Pine Creek, Big Lick Creek, Middle Creek and Greasy Creek) were analyzed as a group, results for *K* varied across the five iterations, but the least negative average log-likelihood, -1133.62, was at *K* = 3 with a variance of 246.9 (Table A6; Figure 4).

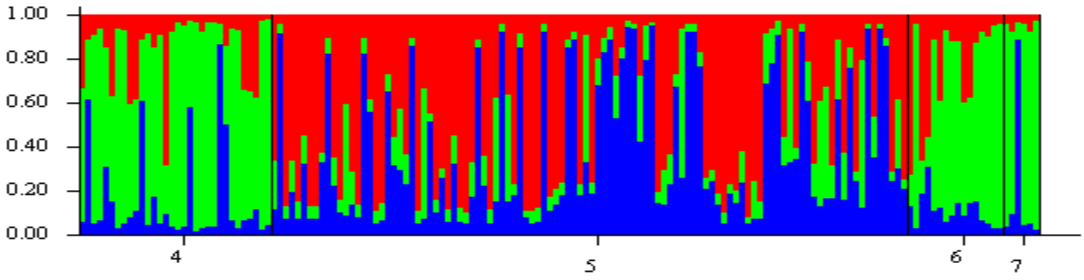

Figure 4. Bar plot of results of Structure Bayesian cluster analysis showing the four populations which displayed admixture in previous analyses. 4 = Pine Creek, 5 = Big Lick Creek, 6 = Middle Creek. 7 = Greasy Creek.

Cluster analysis of populations in whole streams run independently of one another (Table A7) resulted in widely varying highest log-likelihoods across streams and iterations; however, bar plots of streams showed no structure among sites or reaches within particular streams.

Basing definition of populations on the results from application of STRUCTURE, analysis of molecular variance (AMOVA) was conducted: (1) for all streams as wholes independently (Table 6), and (2) with Hart Creek, Hurricane Creek, and Lewis Creek as distinctive, while all other streams were combined into a single population cluster (Table A8). AMOVA results included data from seven loci, as two loci (*BLI84* and *MFW1*) were monomorphic. Results from both AMOVA analyses were largely convergent; there was more variation within populations than among populations, although there was considerable inter-population divergence, indicated by a significant *F*st of 0.259.

AMOVA results showed a considerable amount of variance among populations (25.99%) (Table 6). Most of the rest of the variance was within individuals (72.48%), which is to be expected. There was only a slight amount of variance among individuals within populations (1.54%). This analysis indicated that there is some differentiation taking place among the seven streams.

Table 6. AMOVA results for collection from all streams analyzed separately.

Source of variation	Sum of squares	Variance components	Percentage of variation
Among populations	125.008	0.030259	25.99
Among individuals within populations	227.857	0.01789	1.54
Within individuals	224.5	0.84398	72.48
Total	577.365	1.16446	

Average *F*-statistics over all loci
Fixation index $F_{ST} = 0.259$
Significance tests (1023 permutations)
F_{ST} : P(rand. value > obs. value) = 0.000
P(rand. value = obs. value) = 0.000
P-value = 0.000

Effective population sizes (N_e) for Clinch Dace populations in each stream (Table 7) ranged from the low tens (five streams) to approximately 500 (Hart Creek). Small sample sizes for Lewis Creek and Middle Creek precluded the estimation of N_e for these populations using the linkage disequilibrium method. Indeed, only for Big Lick Creek, which had the largest sample size, was an upper bound for N_e estimated. Estimated effective population sizes may have been influenced by the number of reaches sampled for each stream; for instance, Hurricane Fork had a high population density, but only two reaches were sampled. Clinch Dace were less dense on Big Lick Creek, but seven reaches were sampled. Therefore, there are many more samples from Big Lick Creek than from Hurricane Fork, making calculations of N_e easier for Big Lick Creek than for other large samples.

Table 7. Estimated effective population sizes. *N* is the total number of samples analyzed and N_e is the effective population size.

Stream	*N*	N_e	95% confidence Interval
Hurricane Fork	40	23.5	3.3 - ∞
Hart Creek	63	491.9	27.1 - ∞
Lewis Creek	3	∞	∞ - ∞
Pine Creek	32	60.7	4.0 - ∞
Big Lick Creek	106	40.3	14.1 - 177.9
Middle Creek	16	∞	9.4 - ∞
Greasy Creek	6	58.1	0.5 - ∞

Individuals within steams often showed relatedness, i.e., they were inferred to have full-sibling, half-sibling, or parent-offspring relationships (Figure 5). In Big Lick Creek, which had an estimated effective population size of 106, 19% of individual pairs were parent/offspring, 10% full-siblings and 7% half-siblings. In Greasy Creek, with an N_e of 58, 13% were half-siblings. In Hart Creek, where N_e was 492, 14% full-siblings, 10% half-siblings, and 11% had parent/offspring relationships. In Hurricane Fork, where N_e was 23.5, 1% were half-siblings, 22% had parent/offspring relationships and 10% were full-siblings. In Lewis Creek, with an N of 3 and where N_e could not be estimated, two individuals were full-siblings. At Middle Creek, with an N of 16 and where N_e could not be estimated, 23% had parent/offspring relationships, 6% were half-siblings, and 3-% were full-siblings. In Pine Creek, which had an N_e of 61, 23% were parent/offspring, 6% were half-siblings, and 3% were full-siblings.

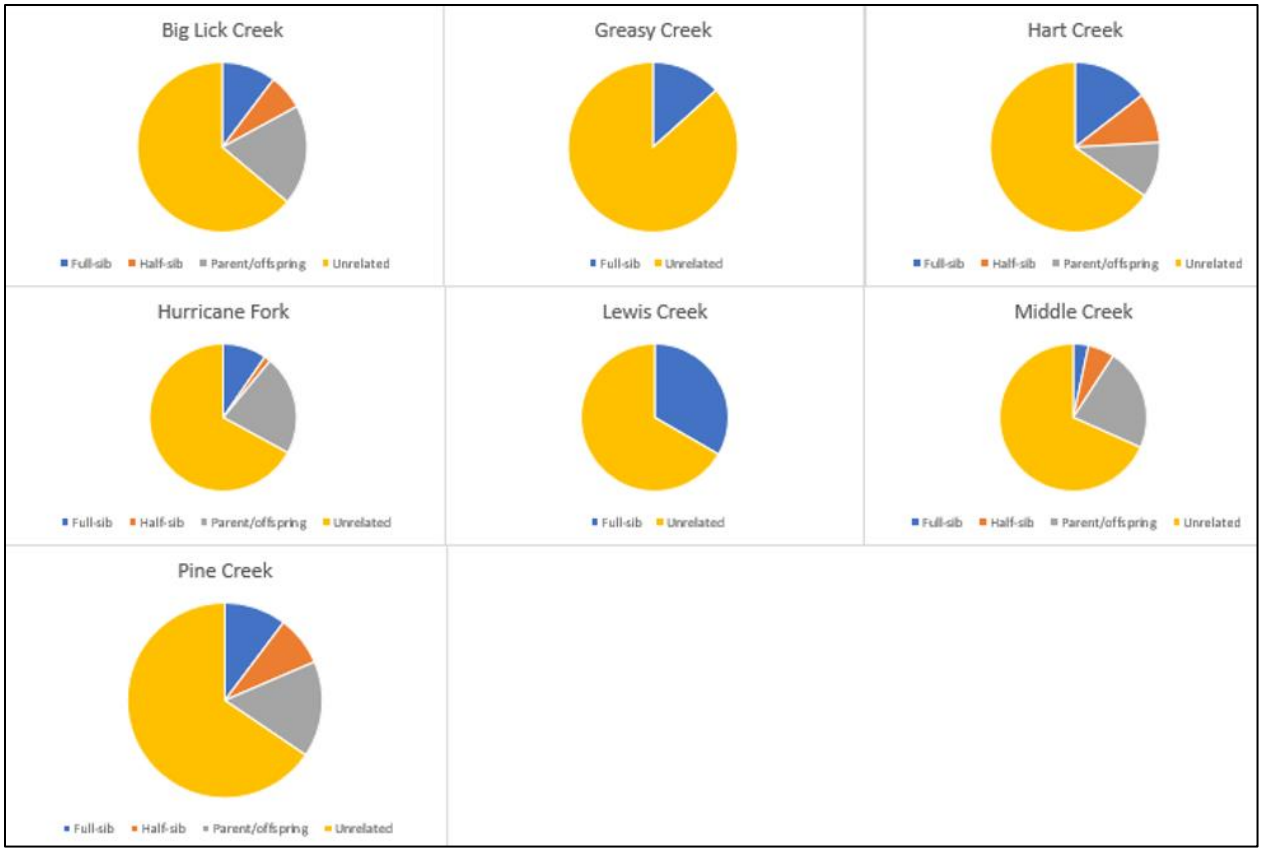

Figure 5. Frequencies of inferred relatedness among individual Clinch Dace in seven streams. Yellow represents unrelated individuals, blue full-siblings, orange half-siblings, and grey parent/offspring relationships.

Discussion

Results of Bayesian cluster analysis using Structure showed that Clinch Dace populations in Hurricane Fork and Hart Creek were the most differentiated from those in other streams, while those in the other five streams showed some degree of admixture. This may be because Hurricane Fork and Hart Creek are the western-most sites and less likely to exchange migrants with other sites. The small sample size (N = 3) for Lewis Creek and the finding that two of the individuals were full siblings weaken the finding that this population is truly genetically distinct. However, the non-significant effect of isolation-by-distance and this degree of admixture among populations in four streams suggests that Clinch Dace can migrate among streams, or could at episodes in the past. Mixing may have occurred at historic times of very low flows where downstream habitats were more suitable for occupancy or passage or during times of high flow when

fish and larvae may have been washed downstream. These results indicate that while there is likely only one evolutionarily significant unit, there may be three management units, consisting of Hurricane Fork, Hart Creek, and the remaining five streams where mixing has taken place in the recent past.

AMOVA results showing over 25 percent of genetic variation among populations indicates considerable genetic divergence among populations. This result, in addition to low *M*-ratios, suggests that genetic drift is operating within the respective gene pools in these streams, leading to differentiation among them. The estimation of relatively small effective population sizes also supports the interpretation that genetic drift is playing a strong role in defining population genetic structure. Non-zero F_{is} values and the high degree of relatedness in these populations indicates that some inbreeding is taking place there. F_{is} values were significantly different from zero for Middle Creek, Greasy Creek and Hart Creek. Inbreeding in Hart Creek seems counter-intuitive, as this was the largest population sampled in 2017. These three lines of evidence - that is, the AMOVA results, low *M*-ratios, and small effective populations sizes - support the inference that isolation is a major force operating upon Clinch Dace populations.

Management implications. – I conducted analyses of population genetic variation and differentiation among seven Clinch Dace populations. The results might inform conservation planning and actions. If genetic variation among Clinch Dace populations is high, then there is a risk of outbreeding depression should there be translocations to augment small populations. If that is the case, then captive breeding and stocking back into the same source populations may be a safe method to augment populations. However, differentiation at neutral genetic markers, such as microsatellites, does not mean that adaptive variation has necessarily taken place. Genetic drift may have operated upon Clinch Dace populations to reduce allelic richness within populations and increase differentiation among populations at neutral loci while natural selection maintained adaptive variation. If neutral variation is considerable, but adaptive variation is absent, then translocations may be safe. However, adaptive management strategies and monitoring should follow any translocations, for either population augmentation or introduction.

Despite relatively large populations in Hart Creek and Hurricane Fork, these may not be suitable as donor populations because of their being so differentiated from other populations. That is, there would

be risk of outbreeding depression if individuals from these streams were added to other Clinch Dace populations. However, it does not rule them out as donor populations from these streams for introductions into streams where Clinch Dace are currently not found. It should be noted again, however, that differentiation at neutral genetic markers does not prove adaptive differentiation. If all the variation that we see in Clinch Dace populations is neutral, then that does not rule out translocations as a population augmentation strategy. Given the fact that these sites all share similar environmental conditions, it is very possible that adaptive differentiation has not taken place. However, when it comes to the definition of evolutionarily significant units, this world view is more in line with that of Crandall (2000), who argues for the necessity of adaptive variation, as opposed to the view of Moritz (1999) who believes that ESU designation should be based on differentiation at neutral genetic markers without regard to adaptation.

Clinch Dace populations are generally small and isolated in separate streams. Random genetic drift seems to have resulted in some differentiation among populations at neutral genetic markers. The small effective population sizes and degree of genetic variation among streams makes it clear that these populations are small and isolated from each other, despite some degree of apparent recent admixture among four or five (Lewis Creek, Big Lick Creek, Pine Creek, Greasy Creek, and Middle Creek) of the streams sampled. This study identifies three sites as possible donor sites (Hurricane Fork, Hart Creek, Big and Lick Creek) and four as possible recipient streams (Lewis Creek, Middle Creek, Pine Creek, and Greasy Creek) for translocations. Translocations may be a viable solution to augment small populations if done with caution and subsequent monitoring.

My sites were targeted near sites where Clinch Dace were found by Shannon White or Michael Moore in 2014 and 2015. Moore's (2018) analysis occupancy of Clinch Dace based on habitat metrics indicated that sites that scored low in habitat ratings sometimes harbored large populations of Clinch Dace. For instance, Hart Creek ranked low in habitat quality, but had an abundant population (Moore 2018). This disconnect between habitat quality and Clinch Dace presence and abundance may make it difficult for managers to choose suitable sites for introductions and prioritize restorations. Current Clinch Dace distribution may be more dependent on legacy effects of large-scale fish kills in the past and previous

mining activities than a lack of instream habitat. Moore (2018) also found a negative relationship between Clinch Dace abundance and substrate size. This is consistent with my findings, as sites such as Hurricane Fork, which has bank-to-bank sediment, had one of the larger populations.

Population viability is dependent on population size and genetic variation. Loss of genetic variation reduces that adaptive potential of a species to deal with a changing environment. The 50/500 rule (Newmark 1986) suggests that for short-term population viability, the minimum effective population size should be at least 50 and for long-term viability that number should by multiplied by a factor of 10. In populations smaller than this, random genetic drift works to eliminate rare, potentially adaptive alleles from the population, rendering the population more vulnerable to extirpation from environmental changes and stochastic events (Hallerman 2003). As all of the Clinch Dace populations that I sampled in 2017 have effective sizes under either 50 or 500, it would seem that all populations are vulnerable to extirpation in either the short-term or the long-term. This problem is compounded by the fact that Clinch Dace populations are fragmented and that contemporary gene flow among populations is restricted, further exacerbating the likelihood of loss of genetic diversity within populations. However, there is a lack of studies estimating minimum effective population size in headwater fishes, and it is not certain that the 50/500 rule applies to Clinch Dace, as they tend to occupy small habitat patches which may naturally support smaller population sizes. This may be the only study estimating effective population size for a *Chrosomus* species. Also, these estimates of effective population size may be biased because of small sample sizes at some streams. As sample sizes increase, the effective population size estimates would increase and confidence intervals would decrease.

While this study demonstrates that there is divergence among populations of Clinch Dace, it does not prove that adaptive variation has arisen among the populations. Adaptive variation may be conserved, while divergence at neutral genetic markers has increased over time due to small population sizes and fragmentation. In the absence of a genomics study assessing adaptive differentiation, it is not possible to definitively prove adaptive differentiation among streams, but an adaptive management strategy with

follow-up monitoring could determine whether any translocations would be leading to outbreeding depression.

Clinch Dace populations are small and fragmented and have lost alleles due to genetic drift. Translocations from larger populations, such as Hart Creek, to smaller populations, such as Middle Creek, may result in added genetic diversity in the receiving populations. However, the risk of outbreeding depression as a result of translocations still exists, so translocations should be undertaken with care and followed up with appropriate monitoring strategies. Hart Creek and Hurricane Fork represent strongholds for this species and could be possible donor populations for population augmentation or introductions. However, using individuals from these streams should be undertaken with caution, as they are the most genetically distinct populations and show the least admixture with other populations. Therefore, the risk of outbreeding depression by using these streams for population augmentation is the greatest. Other populations, with the exception of Lewis Creek which may have water quality issues, are potential recipient sites, as most of them seem to have adequate habitat for Clinch Dace.

Chapter 2: Are barriers to migration, particularly road crossings, fragmenting populations of Clinch Dace?

Introduction

Habitat fragmentation in lotic systems is a serious and growing threat to fish populations worldwide (Perkins and Gido 2012). Landscape changes that fragment populations reduce patch size (and therefore habitat heterogeneity), gene flow, and survival (Whiteley et al. 2013). Barriers to fish movement - such as dams, weirs, and road crossings - prevent upstream migration, potentially causing demographic problems and increasing local extinction risk. Barriers to upstream migration are associated with lower abundance and diversity at sites upstream relative to downstream sites (Nislow et al. 2011, Briggs and Galarowicz 2013; Eisenhour and Floyd 2013)). However, Edge et al. (2017) found that species richness (alpha diversity) correlated more strongly with landscape alteration, and among-site diversity (beta diversity) was correlated with fragmentation and habitat size. By preventing upstream movement, barriers to movement increase likelihood of local extirpation by disturbance events. Where a disturbance event, such as a landslide or chemical spill, occurs and a population is reduced or extirpated, barriers may prevent recolonization from downstream source populations. Even if a population recovers from such an event, it likely would have reduced genetic variation, reducing adaptivity (Guy et al. 2008). Furthermore, by breaking populations into smaller units, fragmentation reduces effective population size (Alo and Turner 2005), making populations more susceptible to random genetic drift and inbreeding depression. Road crossings can fragment fish populations in a variety of ways. Some culverts may become "perched" over time, that is, the bottom of the culvert hangs above the streambed, limiting passage for fish that are not strong leapers (Figure 6).

Figure 6. A culvert that has become perched at Hart Creek site 2.

Road crossings that are not obvious barriers can inhibit fish movement simply by increasing water velocities and altering habitat (Warren and Pardew 1998; Benton et al. 2008; Eisenhour and Floyd 2013; Briggs and Galarowicz 2013). It is therefore reasonable to hypothesize that road crossings in the upper Clinch River basin are further fragmenting Clinch Dace populations that are already fragmented by topography. Fragmentation by road crossings could affect patch occupancy and abundance at sites throughout the species distribution. Alternatively, patch occupancy and population abundance could be determined largely by habitat quality or fish community structure. If fragmentation is indeed taking place within stream segments, then that should be evident from population genetic structure among sites within a stream, as well as low genetically effective population size in reduced habitat patches. If Clinch Dace populations are fragmented by road crossings, then management actions could include retrofitting culverts to make them passable. If habitat factors are determining patch occupancy in Clinch Dace, then stream restorations aimed at improving habitat for Clinch Dace may be considered. In either case, translocations or captive breeding and stocking to augment small populations may be appropriate management strategies.

Should fragmentation be the case, genetic drift, inbreeding, and local adaptation may lead to genetic differentiation among subpopulations within streams. The degree of genetic differentiation has implications for the viability of certain management actions. Translocations could lead to optimal outcrossing and the restoration of lost alleles and diversity. However, if coadapted gene complexes (combinations of alleles at

different loci that come together by chance, increase the fitness of the carrier, and are maintained by selection) have arisen in populations, then outbreeding depression could result from translocations (Templeton et al. 1986).

Traditionally, studies of the effects of habitat fragmentation on fish relied on mark-recapture studies (Warren and Pardew 1998: Benton et al. 2008; Briggs and Galarowicz 2013), which suffer from low recapture rates and typically are carried out on small, reach scales over short time-frames (Nislow et al. 2011). Conservation genetics has revolutionized the way in which we can examine the effects of habitat fragmentation. Firstly, population genetic analysis operates at the landscape scale, and habitat fragmentation is a landscape-level problem. Genetic analysis also can provide insight into whether populations were historically connected before a barrier was constructed or if they were naturally isolated by distance. For instance, Roberts et al. (2013) demonstrated that anthropogenic barriers to migration were creating population structure in Roanoke Logperch *Percina rex* populations, rather than intrinsic limitations to vagility. Fragmentation reduces habitat patch size and the size of the isolated population. Smaller populations are more susceptible to random genetic drift and inbreeding depression, and genetic differentiation can take place across riverine barriers. The effects of fragmenting populations into smaller units is compounded over time. Yamamoto et al. (2004) demonstrated the effects of reduced habitat patch size, and thereby reduced population size, across temporal and spatial gradients. They saw reduced genetic variation and heterozygote deficiency in White-spotted Char (*Salvelinus albopunctatus*) populations above dam sites relative to that at sites below dams.

Genetic diversity may be decreased following disturbance events in fragmented populations. Disturbance events, such as landslides or debris flows, can create habitat, but they also can reduce population size, creating a genetic bottleneck. While a population may recover demographically, without migration from up- or downstream, genetic diversity may not recover. Guy et al. (2008) showed that the landscape disturbance regime interacted with fragmentation to reduce genetic diversity in Coastal Cutthroat Trout (*Oncorhyncus clarkii clarkii*) populations in separate ecoregions characterized by different degrees of fragmentation and contrasting disturbance regimes. Such a degree of fragmentation and the disturbance

regimen may be informative for classifying management units for conservation. These findings reinforce the assertion that the landscape perspective that is accessible through genetic analysis is critical for understanding the effects of habitat fragmentation. Genetic analyses that elucidate patterns of gene flow, population viability, and trends in effective population size can help determine distinct management units and inform prioritization of management units for actions such as population augmentation. Population augmentation can take the form of direct translocations of individuals from large populations to smaller populations or can take place through captive breeding of individuals taken from the receiving population or a larger donor population and stocking of their hatchery-reared progeny. Knowing the degree of genetic variation among populations is of particular interest if population augmentation is considered as a management action, as it may determine whether individuals from one population can be translocated to a receiving population or alternatively whether captive breeding from the managed population and demographic augmentation might be conducted. If populations show significant genetic divergence, then translocation may not be a viable option as it could lead to outbreeding depression.

The purpose of this chapter was to test the hypotheses that: 1) Clinch Dace presence and abundance are determined by habitat and community factors, 2) fragmentation by barriers, such as road crossings, determine Clinch Dace presence and abundance, and 3) that barriers affect genetic divergence.

Methods

Study design

Towards the objective of determining whether barriers to upstream migration, particularly road crossings, are further fragmenting populations of *Chrosomus* sp. cf. *saylori*, I employed an observational study design. I collected observational data from different sampling locations or "sites" in streams within the known distribution of Clinch Dace. Most sampling sites were located at road crossings to determine whether they are fragmenting Clinch Dace populations upstream and downstream. Whenever possible, each site consisted of two sampling units. In the case of sites located at road crossings, these sampling units were reaches extending upstream and downstream from a road crossing. In the case of non-road crossing sites,

there was an upstream and a downstream reach relative to an arbitrary point along a stream. The number of sites and the number of sampling units within each site were determined by permission to access a landowner's property, accessibility, proximity of other road crossings, and practical aspects of ability to sample effectively (e.g., the stream is not dry or converted by a beaver dam). I collected and analyzed habitat and fish community data. Such analyses were conducted on all experimental units for which data were collected and tested the hypothesis that Clinch Dace abundance and/or presence is determined by habitat and community factors. To determine the effects of barriers on Clinch Dace populations, I used both sampling reaches in a paired experimental design. Genetic analysis contributed to achievement of this objective, as I used paired experimental units and multiple metrics of genetic divergence to test for genetic differentiation of Clinch Dace collected upstream and downstream of road crossings.

Sampling Methods

To test the hypothesis that road crossings fragment Clinch Dace populations and affect site occupancy and abundance versus the alternative hypothesis that site occupancy and abundance are determined by habitat quality and fish community, I used genetic methods and habitat analyses. Genetic methods included analysis of DNA marker frequencies to determine population genetic structure and to estimate effective population sizes among sites. I used correlation and regression analyses to determine the effects of several habitat and fish community metrics on Clinch Dace occupancy and abundance. Most sites sampled for Clinch Dace straddled road crossings to evaluate their impact as barriers to fish movement. I characterized road crossings by type (bridge, box culvert, pipe culvert, etc.), assessed them as possible barriers to movement of Clinch Dace, and measured their dimensions and degree of "perchedness" at all sites.

In the summer of 2017, I surveyed streams at 19 sites near locations known to be inhabited by Clinch Dace based on results of stream surveys conducted by Shannon White in 2011 and 2012 and Michael Moore in 2014 and 2015. Most sites consisted of two sub-reaches, each extending upstream and downstream of the road crossing to determine whether that crossing was fragmenting the Clinch Dace

population. Control sites were not located at road crossings, but still consisted of an upstream and a downstream reach. However, no analyses were conducted on control reaches versus road crossing sites as I could not get permission for access to enough control sites. Reach lengths were established as 40 times average stream width with a minimum of 200 meters, conditions permitting, as site lengths of approximately 40 times average stream width are adequate in eastern streams to characterize community and associated habitat (Klemm and Lazorchak 1995; Lyons 1992). Not all sub-reaches met this criterion due to such factors as close proximity of other culverts, barriers, confluences with other streams, or lack of landowner permission to access beyond a certain portion of the reach.

Site Selection and Reconnaissance

I identified approximately 80 potential sites using ArcGIS 10.1 and Shannon White's and Michael Moore's shapefiles of Clinch Dace-occupied sites in Russell and Tazewell Counties, Virginia. Due to lack of response of landowners to initial contact via mail, I visited landowners in person to request permission. Difficulties in obtaining landowner permission eliminated most of the sites that I identified, and I had to sample where I could receive permission. This did not allow me to specifically target certain road crossings that may or may not be potential barriers to fish movement. The sampling design was, hence, opportunistic.

I reconnoitered all sites which I received permission to access. The main purposes of reconnaissance were to determine accessibility, sampleability, and reach length. I measured stream width at eleven points along each sub-reach, starting at the road crossing or and extending 200 m upstream or downstream. At non-crossing sites, measurements began at the downstream-most transect of the downstream reach and proceeded upstream to the 200-meter mark. If average width of the first eleven transects exceeded five meters, then additional transects were added until a total sampling distance of 40 X average width was reached. Upstream reaches at non-road crossing sites were measured from the midpoint (200-meter mark as measured from the downstream reach) up to a 400-meter mark. However, I did not have any reaches where average width exceeded five meters. Some reaches did not reach the minimum 200-meter length, due to such factors as inaccessibility, the presence of other road crossings or barriers, and

lack of permission within that portion of the stream. Some sites lacked a downstream or upstream reach altogether.

Habitat Sampling

At each transect, I ranked canopy cover as percentage of shaded stream according to the Daubenmire (1968) classes (Table 8) and noted the presence or absence of woody debris. From this, I derived average canopy cover in each reach for analysis by taking the midpoint of each rank for each transect and averaging across all transects in the reach. For analysis of woody debris, I calculated the proportion of transects containing woody debris for all transects in each reach.

Table 8: Canopy cover estimation categories.

Classes	Ranges
1	< 1%
2	1 – 5%
3	6 – 25%
4	26 – 50 %
5	51 – 75 %
6	76 – 95%
7	>95 %

At all road crossing sites, I evaluated culverts or bridges as possible barriers to fish movement. I also measured culvert dimensions, the distance from the bottom of the culvert to the top of the water and to the stream bed, and length of the culvert impact zone, and photographed the crossing. Along each reach, I recorded the type of riparian buffer present, estimated the average width of that buffer, and noted the type of land use adjacent to the buffer zone. The maximum depth in the reach was recorded. At the time of fish sampling, one conductivity reading was taken at the downstream-most point of the site before sampling began.

Fish Sampling

I conducted three-pass electrofishing depletions on most sampleable reaches. One backpack Smith-Root LR 24 backpack electrofisher set to 300 volts was used per three meters of average stream width

(Kazyak 2000). All fish were identified to species and counted. Due to time constraints at a few sites, not all fish were counted and a species list is reported. Clinch Dace were measured for total length and a small fin-clip was cut from the upper caudal fin and stored in 95% ethanol. Fin-clips were given a unique identifier corresponding to stream, reach, and fish length, and, in some cases a photograph was taken. Any Clinch Dace mortalities were preserved in 10% formalin, along with any fish that could not be positively identified in the field. All fish were retained in bins, with Clinch Dace being kept separately from all other fish, until all three electrofishing passes were completed. Total shocking seconds per pass and voltage used were recorded. Their populations in each sub-reach were estimated using three-pass depletion data in Microfish 3 (Van Deventer 1989) and standardized to 100 meters of stream length. All fish sampling was done in accordance with Virginia Tech IACUC protocol FWC 16-188.

Habitat and Fish Community Data Analysis

I tallied species richness as the total number of species caught during all three passes. To demonstrate that adequate sampling effort was allocated, I created a scatterplot of species richness vs. total length of each reach sampled. As the relationship was insignificant ($R^2 = 0.02$), I concluded that sampling effort as recommended by Klemm and Lazorchak (1995) and Lyons (1992) was sufficient to characterize the species assemblage. Population size was estimated by maximum-likelihood methods using Microfish 3 software (Van Deventer and Platts 1989). For each stream reach, I performed logistic regression analysis of Clinch Dace presence/absence as a function of average estimated canopy cover, conductivity, maximum depth, presence of woody debris, fish density, and fish species richness within the catchment upstream of each site in R (R Core Team 2013). Because canopy cover was recorded as ranks, I took the midpoint of each category for each transect and averaged them to give one value of canopy cover for each reach (Daubenmire 1968). As conductivity was measured only once at each site, the sampling unit for conductivity was the site. Fish density was calculated by dividing the total number of fish caught in a reach by the product of the average width and total length of that reach.

I conducted quasi-Poisson regressions (Table 12) in R (R Core Team 2013) on Clinch Dace abundance because the data were not normal and dispersion was high at each reach as a function of average

estimated canopy cover, conductivity, maximum depth, presence of woody debris presence, fish density, and fish species richness. I also conducted logistic regression on Clinch Dace presence/absence. Because only one conductivity reading was taken per site and not in every reach, I conducted quasi-Poisson regressions and logistic regression on Clinch Dace abundance and presence/absence and conductivity for each total site. I also calculated the proportion of fish caught in each reach that was represented by Clinch Dace.

I conducted paired *t*-tests on Clinch Dace abundance on the upstream and downstream reaches of each site. Sites where one reach was not sampled for some reason (e.g., lack of permission, dry conditions) were excluded from this analysis. Some reaches were duplicated in the analysis, as the close proximity of some road crossings caused the upstream reach of one site to essentially be the downstream reach of the next site. Genetic methods, such as comparisons of the fixation index, $F_{st,}$ between upstream and downstream reaches, also were used to assess road crossings as barriers by comparing measures of genetic diversity of upstream reaches of sites to that of their downstream reaches.

Table 9. Experimental variables and the analytic methods. Logistic regression was conducted using Clinch Dace presence/absence data and quasipoisson regression was used with Clinch Dace abundance data.

Variable	Data Analysis
Canopy cover	Logistic regression and quasipoisson regression
Conductivity	Logistic regression and quasipoisson regression
Maximum depth	Logistic regression and quasipoisson regression
Woody debris	Logistic regression and quasipoisson regression
Fish density	Logistic regression and quasipoisson regression
Species richness	Logistic regression and quasipoisson regression
Barriers	Paired *t*-test

Results

Clinch Dace were caught in 17 of 29 sub-reaches and from 11 of 19 sites sampled in 2017 (Figure 7). Some sites lacked an upstream or downstream reach from the road crossing due to barriers or lack of landowner permission. While many reaches were the minimum length of 200 meters, some were shorter

than that due to the presence of barriers or close proximity of other road crossings. Clinch Dace population sizes could not be estimated by three-pass depletion for Hart Creek 2 upstream and Hurricane Fork 1 downstream because time constraints or landowner resistance prohibited multiple passes. Raw counts for Clinch Dace and all other fish caught in each reach are presented in Appendix B. Clinch Dace represented small percentages of fish caught, ranging in the upstream reaches from zero to 7.9% and in downstream reaches from zero to 10.2% of total fish caught at all reaches (Table 10). Sampling effort for all sampled reaches seems to have been sufficient to characterize the populations, as the relationship between richness and reach length was insignificant for $n = 17$ ($R^2 = 0.0196$; p-value = 0.94) (Figure 8). Further evidence that sampling effort was sufficient is that total cumulative richness at 29 out of 30 reaches was achieved in the first of three passes.

Table 10. Percentage (%) of total fish catch represented by Clinch Dace at each reach sampled and for the total site. NS = not sampled, NA = fish not counted, * = perched culvert at site. US = upstream reach. DS = downstream reach. ▪ indicates non-road crossing control site.

Site	% Clinch Dace US	% Clinch Dace DS	Total % Clinch Dace	Total Fish Count	Total Clinch Dace Count
Hess Creek 2	0.0	0.0	0.0	714	0
Lewis Creek2	0.0	0.0	0.0	1075	0
Lewis Creek 3	0.0	0.0	0.0	902	0
Lewis Creek 4	0.5	0.0	0.3	1079	3
Jackson Fork 1	0.0	0.0	0.0	700	0
Jackson Fork 2	0.0	0.0	0.0	337	0
Big Lick Creek 1	3.8	4.3	4.0	2258	91
Big Lick Creek 2	0.9	0.1	0.4	1393	6
Big Lick Creek 3	3.2	2.3	2.4	1569	38
Big Lick Creek 4	NS	1.9	1.9	573	11
Middle Creek 3	7.9	5.0	6.1	262	16
Hurricane Fork 1	4.0	4.7	4.3	1640	71
Greasy Creek 1	0.00	0.3	0.2	2104	5
Greasy Creek▪ 2	NS	0.1	0.1	1281	1
Hart Creek 1	5.1	10.2	8.5	1557	132
Hart Creek 2*	NA	NA	NA	NA	43
Pine Creek 3	0.0	NS	0.0	1491	0
Town Hill Creek 1*	NS	0.0	0.0	3134	0
Town Hill Creek 2	0.0	NA	0.0	NA	0

There were no significant relationships between Clinch Dace presence and canopy cover, conductivity, maximum depth, presence of woody debris, fish density, and species richness (Table 12). All variables except for conductivity were measured at the sub-reach level. However, conductivity and richness are relatively close to an alpha value of 0.5 (0.08 each). These results may be indicative of the known relationship between Clinch Dace presence and conductivity (Moore 2018). Low species richness is likely to be associated with Clinch Dace presence, as Clinch Dace generally occupy small, headwater stretches of stream, which are naturally depauperate in species richness. Sampling larger sites may result in higher recorded richness and fewer to no Clinch Dace found.

Habitat variables examined were canopy cover, conductivity, maximum depth, presence of woody debris, fish density, and species richness. Some data points were missing for some variables, resulting in different sample sizes for different metrics. Conductivity was positively related to Clinch Dace abundance (Table 13) (coefficient = 0.0085; $P = 0.03$). This significant result may be due to an outlier point in the Big Lick Creek site one downstream reach, where both conductivity and census population were relatively large. When I ran the quasi-poisson analysis again in R, the *p*-value decreased to 0.02 and the slope changed from positive to negative.

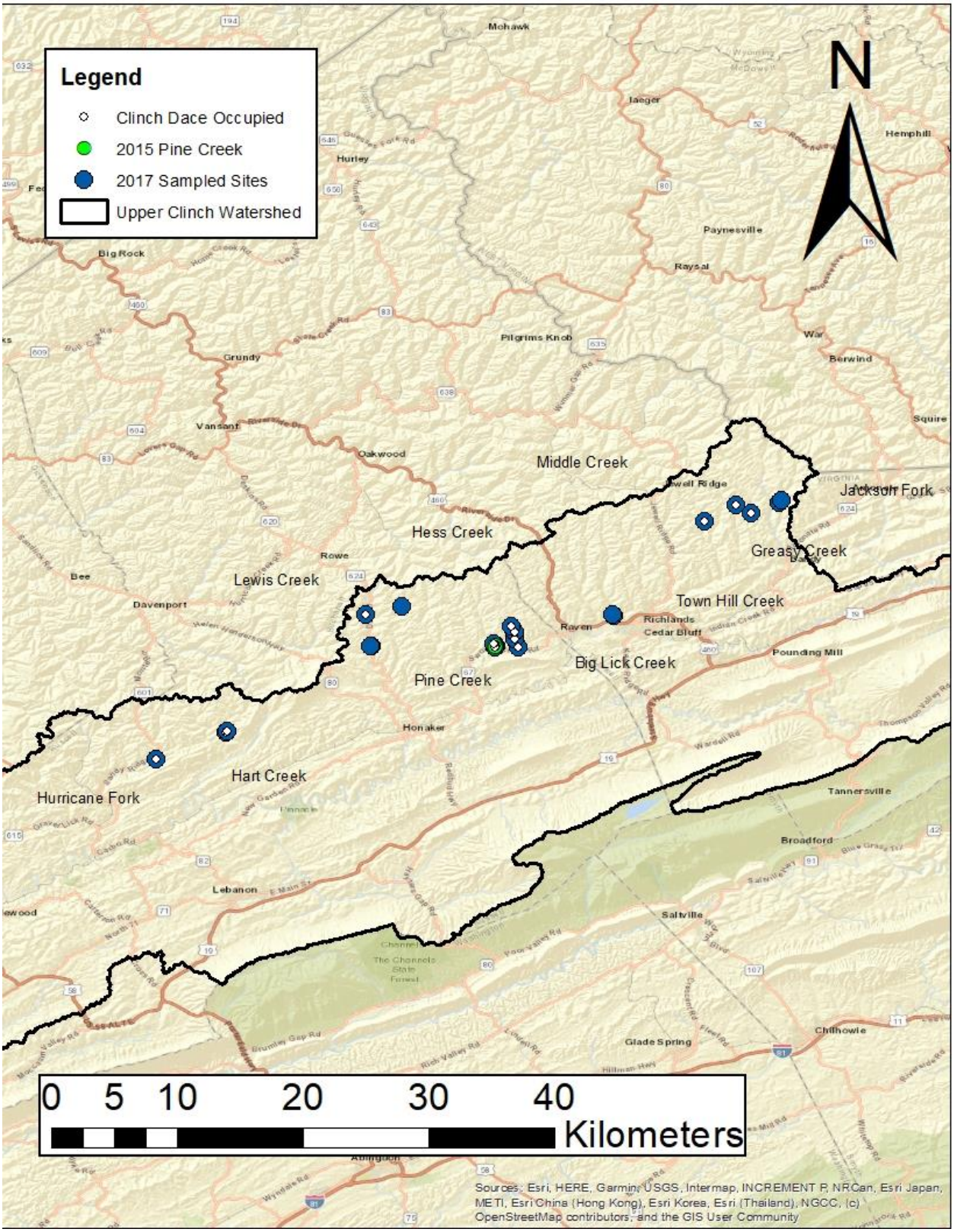

Figure 7. Sites sampled for Clinch Dace in 2017. Blue dots indicate sampled sites and white dots within blue dots indicate sites where Clinch Dace were caught.

Table 11. Clinch Dace population estimates by reach standardized to 100 meters of stream length.

Reach	Total Catch	Pop. Estimate per 100 m	Lower 95% Confidence Interval per 100 m	Upper 95% Confidence Interval per 100 m
Big Lick Creek 1 Downstream	53	29.50	26.50	34.50
Big Lick Creek 1 Upstream	38	19.00	19.00	19.50
Big Lick Creek 2 Downstream	1	0.50	0.50	0.50
Big Lick Creek 2 Upstream	5	4.00	4.00	4.00
Big Lick Creek 3 Downstream	14	7.00	7.00	8.50
Big Lick Creek 3 Upstream	24	12.00	12.00	12.50
Big Lick Creek 4 Downstream	11	6.00	5.50	9.00
Greasy Creek 1 Downstream	5	6.50	2.50	47.50
Greasy Creek 2 Downstream	1	0.50	0.50	0.50
Hart Creek 1 Downstream	106	63.59	54.36	73.33
Hart Creek 1 Upstream	26	22.48	20.16	28.68
Hurricane 1 Upstream	34	17.50	17.00	19.50
Lewis Creek 4 Upstream	3	2.14	2.14	2.14
Middle Creek 3 Downstream	8	4.00	4.00	4.00
Middle Creek 3 Upstream	8	6.50	4.00	20.00

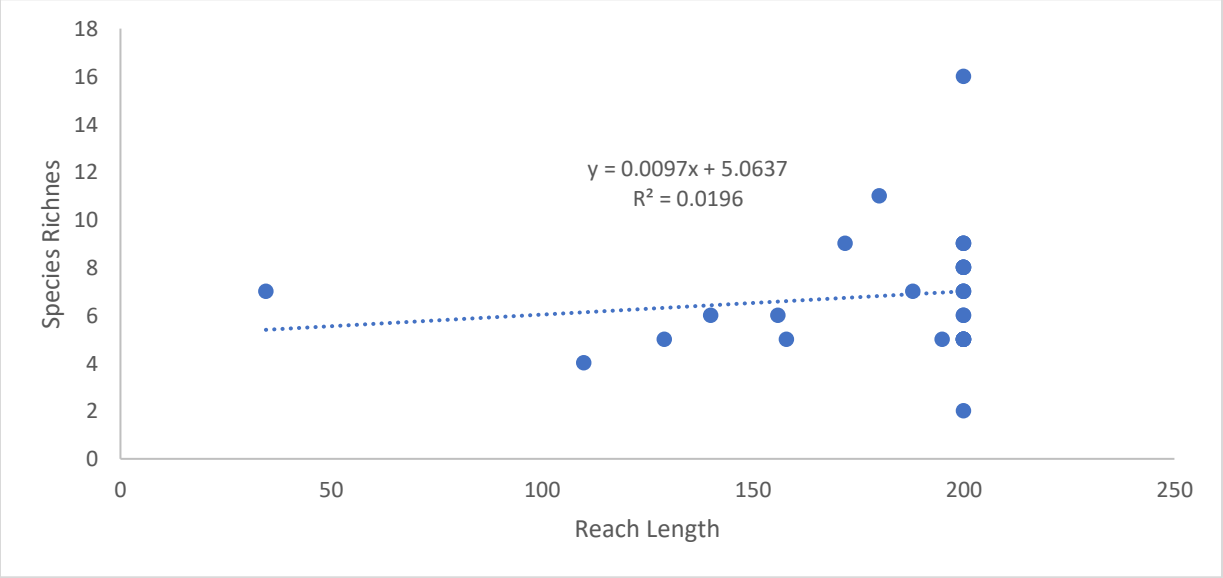

Figure 8. Species richness at all reaches vs. reach length.

Table 12. Logistic regression of Clinch Dace presence/absence and canopy cover, conductivity at site level, maximum depth, presence of woody debris, fish density and species richness.

Variable	*n*	*β*-Estimate	Std. Error	*Z*-value	*P*-value
Canopy cover	27	0.0021	0.023	0.091	0.93
Conductivity	15	0.0114	0.006	1.756	0.08
Max depth	27	-0.0001	0.002	-0.05	0.96
Woody debris	26	2.1208	2.533	0.837	0.40
Fish density	28	0.0122	0.313	0.039	0.97
Richness	30	-0.4193	0.233	-1.8	0.072

Table 13. Quasi-Poisson regression of Clinch Dace abundance and canopy cover, conductivity, maximum depth, woody debris, fish density, and species richness.

Variable	*n*	*β*-Estimate	Std. Error	*t*-value	*p*-value
Canopy cover	27	0.0216	0.014	1.536	0.14
Conductivity	14	0.0085	0.003	2.518	0.03
Max depth	27	0.0006	0.001	0.481	0.63
Woody debris	26	1.6077	1.356	1.177	0.25
Fish density	28	0.3201	0.343	0.932	0.36
Richness	30	-0.1107	0.152	-0.729	0.47

Five of the 19 sites sampled were eliminated from this *t*-test because only one reach could be sampled at those sites. Both Town Hill Creek sites were taken out because I lost permission to sample the overlapping reach connecting the two sites. The upstream reach of Greasy Creek 2 could not be sampled, as it had been dammed by beaver after reconnaissance. The upstream reach of Big Lick 4 was dry at the time of sampling. We also could not get permission to sample downstream of the road crossing at Pine Creek 3. Two of the sites included in the analysis were non-road crossing sites. Only two of the sites used in this analysis were suspected to be barriers to road crossings. Results from these tests showed no significant difference in Clinch Dace abundance between upstream and downstream reaches ($t = 0.83$, 13 df, $p = 0.42$).

Six sites with putative barriers to Clinch Dace migration were analyzed for *F*st among collections from the upstream and downstream reaches. These sites were Big Lick 1, Big Lick 3, Hart Creek 1, Hart Creek 1/2 (these sites overlap as the distance between culverts is less than 200 meters, so I calculated Fst

for the upstream sub-reach of Hart Creek 1 and the only sub-reach on Hart Creek 2), Hurricane Fork, and Middle Creek. Most *F*st values for sites were low (Table 14), ranging from 0.003 to 0.028. However, two were considerably higher, Middle Creek 3 (*F*st = 0.070) and Hart Creek 2 (*F*st = 0.171). The higher F_{st} value for the Hart Creek 1/2 may be explained by the fact that that culvert is slightly perched with a large, deep scour pool below it. Some fish may be able to cross this culvert in the downstream direction, but upstream migration through this culvert may be impeded. According to the landowner, this culvert is fairly recent, but it degraded quickly after installation. It may represent a barrier to upstream movement in time of normal to low flows, but not a barrier to downstream movement.

Table 14. *F*st values for reaches up- and downstream of putative barriers to migration.

Site	*F*st
Big Lick Creek 1	0.003
Big Lick Creek 3	0.026
Hart Creek 1	0.013
Hart Creek 1/2	0.171
Hurricane Fork 1	0.028
Middle Creek 3	0.070

Discussion

The Clinch Dace may warrant classification as a rare species. Pritt and Frimpong (2009) quantitatively determined the rarity of freshwater fish species according to local population abundance, relative abundance and community-level indices. They found generally that species with small ranges had specific habitat needs. While I did not find as association of Clinch Dace presence and abundance with many habitat variables, their small range may be an indication of habitat specificity. Further research should be conducted to determine Clinch Dace habitat requirements to prioritize conservation areas. Further sampling should be conducted to determine whether road crossings are fragmenting Clinch Dace populations, as this study sampled a relatively small number of sites that did not seem to be obvious barriers to upstream fish movement.

Clinch Dace represent a small proportion of fish abundance in all streams surveyed in 2017. The habitat metrics that I analyzed seem to have little impact upon Clinch Dace presence or abundance. I failed to detect Clinch Dace at apparently habitat-rich sites, such as Jackson Fork, and collected them in abundance at habitat-poor sites, such as Hurricane Fork, which is bank-to-bank sediment with no trees in the riparian zone or wood in the stream. In fact, it seems Clinch Dace mostly inhabited sandy pools, not gravel riffles. While logistic regression showed no relationship between Clinch Dace presence and any of the habitat variables analyzed, quasi-Poisson regression showed a positive relationshiponly between Clinch Dace abundance and conductivity. This is counter to the Moore's (2017) finding of a negative relationship between Clinch Dace abundance and conductivity. However, Moore sampled systems with a wider range of conductivity values than I did. All my sites were targeted near known Clinch Dace populations, so it may be that I did not observe the same relationship because all of my sites were within the conductivity tolerance range for Clinch Dace. It may be that one outlier, Big Lick Creek 1 Downstream, where conductivity was high and abundance very high, may be driving the spurious relationship in my data. When the analysis was re-run without data from site Big Lick Creek 1 Down, the *p*-value decreased to 0.02 and the slope changed from positive to negative. It is possible that because these sites were targeted near sites of known Clinch Dace presence that the range of values for conductivity was within the tolerance for Clinch Dace at present, and that conductivity therefore did not inhibit Clinch Dace presence or abundance at sites where I made collections. It may be that legacy effects of mining that once caused toxicity or higher levels of conductivity eliminated some populations of Clinch Dace. That is, present distribution may reflect the lack of an extirpating event in the past, rather than true habitat preferences. It seems that these instream habitat variables are not currently restricting the distribution of Clinch Dace. Neither does it seem that with one possible exception that barriers are fragmenting Clinch Dace populations in my study area. Paired *t*-tests on Clinch Dace abundance at upstream and downstream reaches revealed no effect of road crossings as barriers to Clinch Dace movement. Furthermore, low *F*st values at most sites where *F*st could be evaluated were low, indicating that road crossings are not causing genetic differentiation among Clinch Dace populations. The exceptions are Middle Creek 3, where the road crossing is not an obvious barrier, and the

upstream reach of Hart Creek 1, and the only reach on Hart Creek 2, where the culvert is slightly perched. Therefore, I do not reject the null hypothesis that road crossings are not fragmenting Clinch Dace populations in general. However, the Hart Creek 2 road crossing may be acting as a barrier to Clinch Dace movement. Eisenhour and Floyd (2013) found that a single road crossing that had become perched dramatically decreased abundance of a similar species, Blackside Dace (*Chrosomus cumberlandensis*), upstream of the culvert since its construction. They theorize that population losses in times of drought, when the upstream reach dries up, cannot be recolonized by individuals from downstream of the culvert, where the population remains strong. These results confirm that culverts, particularly perched culverts, can lead to species extirpation. With the exception of Hart Creek 2, none of my road crossings were perched culverts, and genetic data seem to indicate panmixia upstream and downstream of my road crossings. It appears that within my study sites, road crossings are not acting as barriers to the movement of Clinch Dace.

Chapter 3: Synthesis

Overview

Since its discovery in 1999, numerous sampling events for Clinch Dace have taken place (Table 15). Surveys for Clinch Dace have been conducted by Chris Skelton, Shannon White, Michael Moore, myself, and others. Many of these surveys have detected Clinch Dace, and most showed small populations sizes. Michael Moore delineated 15 candidate conservation areas (CCAs) (Figure 9) based on results of these surveys. Genetic data from my study show the effects of small population size on inbreeding and genetic drift within some of these populations. Some populations have undergone recent admixture, while others are more isolated. However, Clinch Dace were not detected at all historical sites during this study.

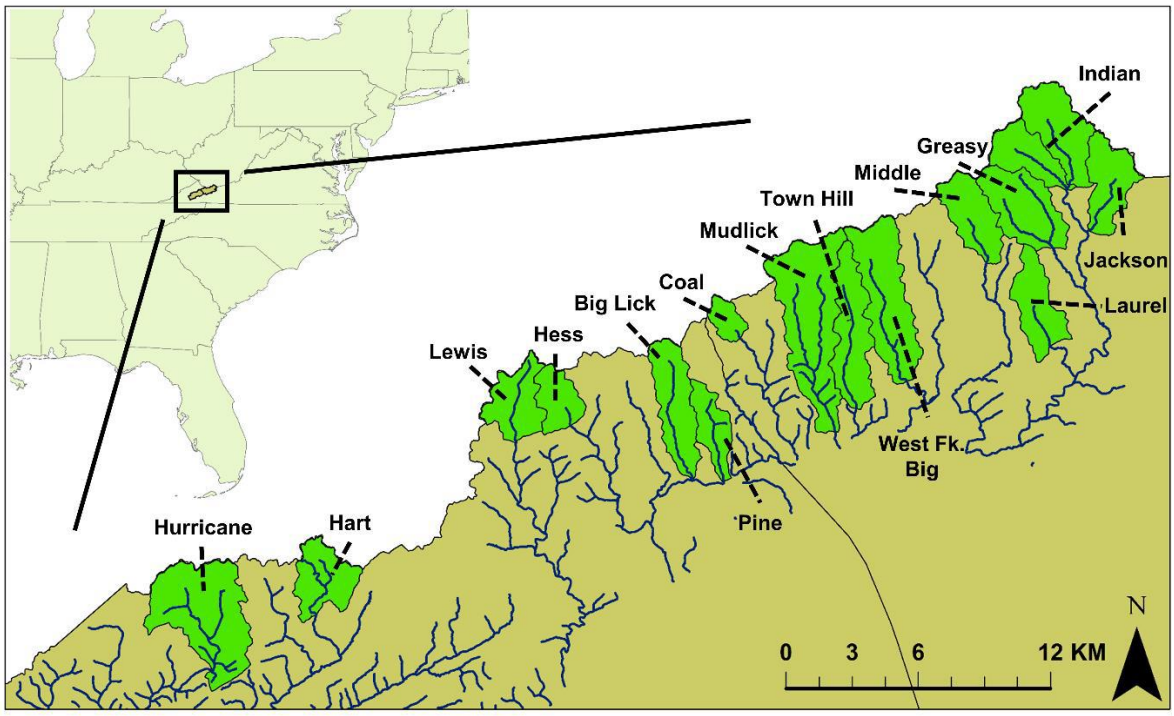

Figure 9**:** Map of study area containing 15 proposed CCAs occupied by Clinch Dace based on prior surveys (Skelton 2007; Coyner unpublished data; White and Orth 2013; White and Orth 2014a, White and Orth 2014b, Hatcher et al. 2017; Moore 2017a; Moore 2017b). Reproduced from Moore (2018).

Table 15. Results of Clinch Dace surveys since 2007. Includes the proportion of sites sampled where Clinch Dace were collected, 95 % confidence interval on proportions, total number of sampling events, average abundances from 2007 to 2015 standardized to 100 meters, average abundances from 2017 standardized to 100 meters, effective population sizes (N_e) from genetics results and 95% confidence interval on N_e. NA = not available.

Stream	Proportion of Surveys Clinch Dace Collected 2007-2017	95 % Confidence Interval on Proportion	Total Sampling Events	Avg. Abundance 2007-2015	Avg. Abundance 2017	N_e	95 % Confidence Interval *N*e
				Moore et al. 2018	This study		
Lewis Creek	**0.14**	0.018 - 0.428	14	N/A	0.4	∞	∞-∞
Jackson Fork	**0.2**	0.005 - 0.716	5	0.92	0	NA	NA
Laurel Fork	**0.4**	0.053 - 0.853	5	NA	NA	NA	NA
West Fork Big Creek	**0.6**	0.147 - 0.947	5	NA	NA	NA	NA
Hess Creek	**0.63**	0.243 - 0.915	8	4.15	0	NA	NA
Indian Creek	**0.67**	0.223 - 0.957	6	NA	0	NA	NA
Middle Creek	**0.7**	0.348 - 0.933	10	40.27	4	∞	9.4 - ∞
Town Hill Creek	**0.73**	0.390 - 0.940	11	3.02	0	NA	NA
Greasy Creek	**0.83**	0.516 - 0.979	12	4.71	0.77	58.1	0.5 - ∞
Mudlick Creek	**0.83**	0.359 - 0.996	6	8.18	NA	NA	NA
Hurricane Fork/Grassy Branch	**0.87**	0.595 - 0.983	15	41.33	17.75	23.5	3.3 - ∞
Big Lick Creek	**0.88**	0.636 - 0.985	17	27.54	10.79	40.3	14.1 - 177.9
Pine Creek	**0.9**	0.555 - 0.997	10	37.94	0	60.7	4.0 –∞
Hart Creek	**1**	0.735 - 1.000	12	49.37	33.4	491.9	27.1 - ∞
Left Coal Creek	**1**	0.478 - 1.000	5	6.35	NA	NA	NA

The results of this study may inform development of management strategies for the species. Moore (2018) showed a positive relationship between Clinch Dace abundance and forest cover. Therefore, one passive management strategy may be simply to preserve or allow regeneration of forested areas. Stream restorations and riparian buffer tree plantings also may be achievable management actions. Translocations from large populations to small, at-risk populations are one management option. However, translocations may not be appropriate where there is significant genetic differentiation among source and recipient populations, as subsequent interbreeding of native and introduced genotypes could lead to outbreeding depression (Miller and Kapuscinski 2003) in the receiving populations. Population genetic analysis showed that Clinch Dace populations are fragmented, and that genetic drift is operating upon them, decreasing diversity within and increasing differentiation among populations. However, microsatellite DNA markers generally document selectively neutral variation, and hence do not address the possibility that that Clinch Dace populations exhibit adaptive genetic differentiation. If there is no adaptive differentiation among populations, then the risk of outbreeding depression following translocation is minimal. Frankham et al. (2011) argued that the risks of outbreeding depression can be predicted and that one of the predictive factors is environmental difference among sites occupied by the respective populations. Frankham et al. (2011) proposed a model predicting the likelihood of outbreeding depression and concluded that concerns regarding the potential consequences of outbreeding depression in recently fragmented populations are likely excessive. They provided a decision-tree to assess whether risk of outbreeding depression is high and thereby determining whether reestablishing gene flow between populations should be avoided or carefully considered:

1. Is the population's taxonomy resolved? If not, resolve. If yes, proceed to question 2.

2. Are there fixed chromosomal differences between the study populations? If yes, keep the populations separated. If no, proceed to question 3.

3. Has gene flow occurred between populations within the last 500 years? If no, keep populations separated. If yes, proceed to question 4.

4. Are there substantial environmental differences between the two population segments? If no, it should be safe to reestablish gene flow. If yes, proceed to question 5.

5. Have populations been separated for more than 20 generations? If no, it should be safe to reestablish gene flow. If yes, evaluate the probability of outbreeding depression in more detail.

In my context, all Clinch Dace populations exist in relatively similar environments that have the same basic, long-term environmental conditions. Without differential selective pressure on the respective populations, it is unlikely that adaptive differentiation has arisen since these populations were last connected (Frankham et al. 2011). Therefore, these results do not rule out the viability of translocations. However, as the risk of outbreeding depression is non-zero, adaptive management strategies should be adopted, and post-translocation monitoring should be employed to ensure that translocations are not resulting in outbreeding depression.

In populations experiencing genetic drift or inbreeding depression, the benefits of genetic rescue from translocations may outweigh the risk of outbreeding depression (Tallmon et al. 2004, Frankham 2015, Whiteley et al. 2015, Hedrick and Garcia-Dorado 2016, Robinson et al. 2017). Pavlova et al. (2017) predicted on the basis of simulations that assisted gene flow through translocations improved long-term viability of small, isolated populations of the Macquarie Perch (*Macquaria australasica*) by reducing inbreeding depression and enhancing genetic variation without resulting in outbreeding depression and improving population viability compared to a do-nothing approach. Ralls et al. (2018) suggested that the risks of outbreeding depression are exaggerated and that outcrossing small populations presents less risk than inaction and suggested that an inbreeding coefficient of 0.1 be adopted as the threshold at which outcrossing of populations should be implemented. Translocations may be undertaken from large populations into small populations, but adaptive management and monitoring will be essential in assuring that appropriate lessons are learned following translocations. If a translocation is tried experimentally and subsequent monitoring indicates a loss of fitness in the hybrid generation, then translocations should be discontinued as a management option for that system. In addition to translocations to augment existing populations of Clinch Dace, translocations can also be used to introduce Clinch Dace into streams in the distribution that are not currently occupied.

An alternative to translocations is captive breeding, where broodstock are removed from a small population, their young reared in captivity and released into the same population. In a 12-year study by Osborne et al. (2012), populations of the fragmented and bottlenecked Rio Grande Silvery Minnow (*Hybognathus amarus*), genetic diversity and effective population size were studied over a long period of introductions of captively breed stock. In this study, both adult individuals and individuals grown from wild-caught eggs were used as broodstock to augment the populations. The authors found that changes in genetic diversity within the Rio Grande Silvery Minnow populations were more strongly influenced by the released of captively bred stock, rather than natural, annual fluctuations of the wild fish. They also concluded that inclusion of fish reared from wild-hatched eggs infused greater genetic diversity into the wild populations, as they likely reflected the influence of more parents than those fish that were captively spawned from fewer individuals. However, captive breeding is not without its risks. While it eliminates the threat of outbreeding depression, it can result in fish that are raised adapting to the hatchery environment and that are poorly suited to life in the wild, a hazard known as domestication selection (Miller and Kapuscinski 2003). Also, captive breeding to augment small populations would mean taking individuals from already at-risk populations, further endangering them. Furthermore, if loss of genetic diversity is a problem in these smaller populations, then releasing inbred hatchery fish could exacerbate genetic drift (Ryman and Laikre 1991) and could lead to subsequent inbreeding depression.

According to the estimated effective population sizes in six of seven streams where Clinch Dace were caught in 2017, populations are too small to be considered viable in the short-term and none are viable over the long-term. Population augmentations may be called for in Lewis Creek, Middle Creek, Big Lick Creek, Greasy Creek, and Hurricane Fork. Census population sizes of Clinch Dace streams sampled between 2007 and 2017 are small enough for population augmentations in Big Lick Creek, Greasy Creek, Hess Creek, Jackson Fork, Left Coal Creek, Lewis Creek, Middle Creek, Pine Creek and Town Hill Creek. These populations could be augmented by either translocations or captive breeding. Evidence of inbreeding in Middle Creek and Greasy Creek support the use of translocations not only to augment the population size, but also to restore genetic diversity and to reduce inbreeding depression. If Clinch Dace populations

are already at carrying capacity, then augmentations may not be helpful. However, given the influence of genetic drift and inbreeding in the population genetic structure of these populations, it is unlikely that they are at carrying capacity in most streams.

Translocations may not only increase the census populations, but also may reverse the effects of genetic drift and inbreeding. Only a small number of individuals would need to be relocated in order to restore genetic diversity lost due to genetic drift. Experimental mixing of healthy populations may be conducted to determine whether outbreeding depression is likely to take place. For example, two of the least admixed and largest populations, Hurricane Fork and Hart Creek, could be mixed and subsequently monitored to determine any negative effects of establishing gene flow among Clinch Dace populations. In particular, introductions of Clinch Dace into currently unoccupied streams within the distribution may help increase the viability of the species without risk of outbreeding depression in any natural populations.

Study Area and Management Recommendations

Although Bayesian cluster analysis indicated that there were five differentiated populations among the Clinch Dace-occupied streams, I propose that there are three management units. According to the Structure output, Hurricane Fork and Hart Creek represent distinct management units, with populations in the rest of the streams being somewhat admixed. The status of Lewis Creek is difficult to discern; while it may be slightly admixed with other streams, it is difficult to draw any conclusions from the three samples that were collected on that stream. Noting that two individuals seemed to be full-siblings, the genetic variation in that population would seem to be very limited.

Hurricane Fork harbors one of the larger Clinch Dace populations that was sampled in 2017, making it a candidate for a donor stream for translocations. However, some differentiation from other populations was detected, which may pose risk of outbreeding depression should individuals from this population be added to other populations. Therefore, population augmentation using Hurricane Fork as a donor stream should be done only with caution. As much of the Hurricane Fork site was unbuffered in the riparian zone and the substrate was bank-to-bank sediment, this population may be resilient to sedimentation and lack of habitat.

Hart Creek was the largest population found in 2017 and is therefore a candidate for a donor population. However, like Hurricane Fork, this population shows some differentiation from others in my study area, so using it as a donor population should be done only with caution and with follow-up monitoring. The culvert separating Hart Creek 1 from Hart Creek 2 is the only culvert found in the 2017 sampling area that may justify a retrofit. If the goal is to simply restore lost alleles to the upstream reach, a simple translocation would be effective. The culvert is slightly perched with a very large, deep scour pool below it and is the stream crossing for the landowner's driveway.

Lewis Creek was one of the smaller populations found in 2017, but it may not be a good candidate for augmentation. I was informed by a local resident that houses along Lewis Creek discharge sewage effluent directly into the creek. This may cause a water quality barrier in the stream. It is worth noting that of the three sites sampled on Lewis Creek, I captured Clinch Dace only from the upstream reach of the upper-most site, which is above the houses discharging effluent. At the point where I caught Clinch Dace, the stream is less than a meter wide with very little available habitat. Adding individuals to this stream may not be advisable unless and until water quality issues are resolved. One potential management action for Lewis Creek could be the subsidization of septic tanks to landowners on the creek to stop the flow of sewage into the creek and improve water quality conditions for Clinch Dace.

Population sizes from the seven reaches on Big Lick Creek varied, but overall they seemed high compared to other streams. Apparent genetic admixture with populations in other creeks in my sample area indicates that Big Lick Creek could be used as a donor stream without particular concern about outbreeding depression. However, it is not certain whether the populations are large enough to support removal of individuals for purposes of translocation, unless reciprocal translocations were employed.

Pine Creek was under-sampled in 2017 due to lack of landowner permissions, and Clinch Dace were not detected there. However, Moore et al, (2018) showed that Clinch Dace do occupy Pine Creek. Further monitoring of this stream could be warranted before translocations. Bayesian cluster analysis showed admixture with other populations in my sample area, indicating that gene flow involving the Pine

Creek populations may have occurred in the past, reducing the risk of outbreeding depression due to any translocations.

The population on Middle Creek is relatively small. I caught only 16 individuals in 400 meters of stream sampled at that site. The stream is narrow and partially buffered where I sampled, and the road crossing is not an obvious barrier. This site could be a potential location for population augmentation. Bayesian cluster analysis of the Clinch Dace from Middle Creek indicates recent admixture with other populations, reducing the risk of outbreeding depression from any translocations. There is nothing apparent that rules out this site as a recipient of translocated individuals.

Greasy Creek is not an obvious candidate as either a donor or recipient stream. The population there is small, but it may not be a good place to add individuals. Greasy Creek 2 is at a road crossing, and between the time that we reconnoitered it and when we came back to sample it, a beaver had built its dam in the culvert, rendering the upstream reach unsampleable. If beaver activity is going to impair habitat for Clinch Dace and hinder monitoring, then this stream should not be considered for further management action. If the beaver were removed from the area, it could make translocations into Greasy Creek feasible.

An alternative to translocations is captive breeding, where broodstock are captively propagated and their progeny stocked into the same stream. This approach eliminates the threat of outbreeding depression. However, captive breeding is not without hazards (Miller and Kapuscinski 2003). It can result in fish that are adapted to the hatchery environment and that are poorly suited to life in the wild. Also, captive breeding to augment small populations would mean taking individuals from already at-risk populations, further endangering them. Furthermore, if loss of genetic diversity is a problem in these smaller populations, then releasing inbred hatchery fish could exacerbate inbreeding depression. Also, captive breeding can result in skewed family sizes, reducing genetic effective population size. Releasing fish thus reared in the hatchery could result in increased genetic drift (Ryman and Laikre 1991).

Future Work

I was unable to get permission to sample any historically occupied streams on lands owned by coal companies in 2017. Future effort should address access to such streams, such as Mudlick Creek, for monitoring and evaluating populations for translocation. The effective population sizes generated by the genetic data from this study could be used to conduct population viability analyses to help prioritize Clinch Dace streams for management actions. To better assess the effect of barriers on viability of Clinch Dace populations, the species' vagility should be examined, as it has been shown that species with lower mobility and that exhibit an isolation-by-distance pattern take longer to manifest the effects of fragmentation by barriers (Coleman 2018). Translocation or captive breeding programs should be followed by monitoring efforts to assess the effectiveness of such programs.

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Appendix A

Table A1. Sequence data from microsatellite loci.

Locus	Sequence
CtoA-247	GAAACAAGGCCTCGGAGATTGAAGTAGGAAAGCATAATCCATGGAAAAA
	GTGTCAAGAGAAGAAAAGATGATTTGTTTTGGTCATCTTAAAATCATCAT
	CATCATCGTATTTTAGACGTTCTCCCAAAGAGTGGGGCT-
Lco-3	ATTCACCACACAGTTTGTGTTTTACAATGTGACAGCTATAAATAAT
	GGCATTGGTGAGCGTTCTGGGTGCATATGCATATGTGTGTGTGTGTGT
	GTGTGTGTGTATGACTGGGCTGTAATGAATGGGAACTACTAAAGCACTGA
	TTTCCTTTTCTCTTTCTTGTGTTTTACGCAGTGAAGACTATCGATTATGG
	CTTAAAGGGGACATTGATTTTACCTT-
LleC-090	AGGGGRGGAGCCACAGTCTCACAGATGA
	GTCCTCTCTCGATTATCATAAACTACCTCCGCCCACCCCACTGTGGATCT
	CTCTCTCTCGCGCTCTCCCCGCGTCTCTC--TCCCCTGTGAAATCAGTTCT
BLI-84	ACATCCATACACACACTGATCGTAAGACAGAGTGACATCACACACACACA
	CAGCAGCAAATCTTGCTCGGTTTGGTGCACCTGGTGCCCGATGTCCCATA
	AGACAAAGAAAGATGAACTCCCTGTCTTATATTGCTTTCTTTCTTTCTCA
	GTCT
BLI-153	CGGTCACTAACATAGCATTTATCTCTAGCTCTTTCTTTATACATTCATCT
	GGTATATAGTCACAAAACACAGACACACACACACACACTTATATGGACCC
	GGTCAGCATGATTTAGGAAAAAATGACATGTTGCTCAAGAGGAGCTGCGA
	TTTGAGCCACGCCGAAATCATGCAACGTCTCTTGACCCG
MFW-1	GGAGCTTCTGTGCTTCAACCTGATGAAATTCAGCCTGCTGTGGAAGAA
	TGTTGCTCTGTGTATGTGTGTGTGTGTGTGTGTTGGGAGAGAGAAATG
	TAATACAGGTAGTTTACAGAGGCTGCATTGTCATGAGCGTG--
CypG-30	AAGCGCAGTCAGGGAAGAWASTGATCTATCTATCTATCTATCTATCTATC
	NTATCTATCTATCTATCTATCTATCTATCTATCCATCCATCC-ATCCATC
	CATCCATCCATCCATCCATCCATCCATCCATCCATCCATCCATCCATCAG
	TCCATCCATCCATCCATCAGTCCA
Lsou-8	AACTCCATATACTTCTCCACCCACGTGTGTGTGTGTGTGGACCCCTGCCT
	ACACACTCTGTCGAGGGCTCTCAGCCCCAACACACTCACAGATAGCCTGA
	TTCACACACTCGTGTACAGAAGCACATTTACACGACAGCCGCCCACAGGCT

Table A2. Inferred occurrence and estimated frequencies of null alleles in samples of Clinch Dace within individual stream reaches

Reach	Locus	Presence of Null Alleles	Oosterhout	*N*
Big Lick 1 down	*CtoA247*	no	0.02	22
	Lco3	no	0.3	22
	LleC90	no	0.1	22
	BLI_84	no	0	22
	Rhca20	no	0	22
	BLI153	no	0	22
	CypG30	no	0.01	22
	MFW1	no	0	22
	Lsou8	no	0.49	22
Big Lick 1 up	*CtoA247*	no	0	24
	Lco3	no	0.24	24
	LleC90	no	0.04	24
	BLI_84	no	0	24
	Rhca20	no	0	24
	BLI153	no	0	24
	CypG30	no	0.1	24
	MFW1	no	0	24
	Lsou8	no	0.11	24
Big Lick 2 down	*CtoA247*	NA		1
	Lco3	NA		1
	LleC90	NA		1
	BLI_84	NA		1
	Rhca20	NA		1
	BLI153	NA		1
	CypG30	NA		1
	MFW1	NA		1
	Lsou8	NA		1
Big Lick 2 up	*CtoA247*	no	0	5
	Lco3	no	0.11	5
	LleC90	no	0.11	5
	BLI_84	no	0	5
	Rhca20	no	0	5
	BLI153	no	0	5
	CypG30	no	0.23	5
	MFW1	no	0	5
	Lsou8	no	0	5
Big Lick 3 down	*CtoA247*	no	0	19
	Lco3	no	0.02	19
	LleC90	no	0.03	19

Table A2 cont.

	BLI_84	no	0	19
	Rhca20	no	0	19
	BLI153	no	0	19
	CypG30	no	0.07	19
	MFW1	no	0	19
	Lsou8	no	0.12	20
Hart Creek 1 up	*CtoA247*	no	0	22
	Lco3	yes	0.21	22
	LleC90	no	0.02	22
	BLI_84	no	0	22
	Rhca20	no	0	22
	BLI153	no	0.14	22
	CypG30	no	0.17	22
	MFW1	no	0	21
	Lsou8	no	0.06	22
Hart Creek 2 down	*CtoA247*	no	0	20
	Lco3	no	0	21
	LleC90	yes	0.38	20
	BLI_84	no	0	21
	Rhca20	no	0	20
	BLI153	no	0.18	21
	CypG30	no	0.08	21
	MFW1	no	0	20
	Lsou8	no	0.15	21
Hurricane Fork 1 down	*CtoA247*	no	0	20
	Lco3	no	0.16	20
	LleC90	no	0	20
	BLI_84	no	0	20
	Rhca20	no	0	20
	BLI153	no	0	20
	CypG30	no	-0.07	20
	MFW1	no	0	18
	Lsou8	no	0.11	20
Hurricane Fork 1 up	*CtoA247*	no	0.15	20
	Lco3	no	0.18	20
	LleC90	no	0	20
	BLI_84	no	0	20
	Rhca20	no	0	20
	BLI153	no	0	20
	CypG30	no	0.09	20
	MFW1	no	0	20

Table A2 cont.

	Lsou8	no	0.05	20
Lewis Creek 4 up	*CtoA247*	NA		3
	Lco3	NA		3
	LleC90	NA		3
	BLI_84	NA		3
	Rhca20	NA		3

Table A2 cont.

	BLI153	NA		3
	CypG30	NA		3
	MFW1	NA		3
	Lsou8	NA		3
Middle Creek 3 down	*CtoA247*	no	0	8
	Lco3	no	0.06	8
	LleC90	no	0	7
	BLI_84	no	0	8
	Rhca20	no	0	8
	BLI153	no	0	8
	CypG30	no	0.03	8
	MFW1	no	0	8
	Lsou8	no	0.21	8
Middle Creek 3 up	*CtoA247*	no	0	8
	Lco3	no	0.06	8
	LleC90	no	0	8
	BLI_84	no	0	8
	Rhca20	no	0	8
	BLI153	no	0	8
	CypG30	no	0.08	8
	MFW1	no	0	8
	Lsou8	no	0	8
Pine Creek IM	*CtoA247*	no	0	16
	Lco3	no	0.17	16
	LleC90	no	0.21	16
	BLI_84	no	0	14
	Rhca20	no	0	16
	BLI153	no	0	16
	CypG30	no	0.13	16
	MFW1	no	0	14
	Lsou8	no	0.03	16
Pine Creek low	*CtoA247*	no	0	7
	Lco3	no	0.15	7
	LleC90	no	0.15	7
	BLI_84	no	0	7

Table A2 cont.

	Rhca20	no	0.62	7
	BLI153	no	0.07	7
	CypG30	no	0.03	7
	MFW1	no	0	7
	Lsou8	no	0	7
Pine Creek mid	*CtoA247*	NA		3
	Lco3	NA		3
	LleC90	NA		3
	BLI_84	NA		3
	Rhca20	NA		3
	BLI153	NA		3
	CypG30	NA		3
	MFW1	NA		3
	Lsou8	NA		3
Pine Creek up	*CtoA247*	no	0	6
	Lco3	no	0.14	6
	LleC90	no	0.37	5
	BLI_84	no	0	6
	Rhca20	no	0.18	6
	BLI153	no	0	6
	CypG30	no	0.23	6
	MFW1	no	0	6
	Lsou8	no	0	6

Table A3. Inferred occurrence and estimated frequencies of null alleles in samples of Clinch Dace within whole streams.

Reach	Locus	Presence of Null Alleles	Oosterhout	*n*
Big Lick Creek	*CtoA247*	no	0	106
	Lco3	no	-0.06	106
	LleC90	no	0.03	106
	BLI_84	no	0	106
	Rhca20	no	0	106
	BLI153	no	0	106
	CypG30	no	0.02	106
	MFW1	no	0	104
	Lsou8	no	0.08	106
Greasy Creek	*CtoA247*	no	0	6
	Lco3	no	0.17	6
	LleC90	no	0	6
	BLI_84	no	0	6
	Rhca20	no	0	6
	BLI153	no	0.28	6
	CypG30	no	0.13	6
	MFW1	no	0	5
	Lsou8	no	0.42	6
Hart Creek	*CtoA247*	no	0	63
	Lco3	yes	0.09	64
	LleC90	yes	0.25	63
	BLI_84	no	0	64
	Rhca20	no	0	63
	BLI153	no	0	64
	CypG30	no	0.13	63
	MFW1	no	0	61
	Lsou8	yes	0.16	64
Hurricane Fork	*CtoA247*	yes	0.12	40
	Lco3	no	0.12	40
	LleC90	no	0	40
	BLI_84	no	0	40
	Rhca20	no	0	40
	BLI153	no	0	40
	CypG30	no	0.07	40
	MFW1	no	0	38
	Lsou8	no	0.08	40
Lewis Creek	*CtoA247*	NA	NA	3
	Lco3	NA	NA	3

Table A3 cont

	LleC90	NA	NA	3
	BLI_84	NA	NA	3
	Rhca20	NA	NA	3
	BLI153	NA	NA	3
	CypG30	NA	NA	3
	MFW1	NA	NA	3
	Lsou8	NA	NA	3
Middle Creek	*CtoA247*	no	0	16
	Lco3	no	0.08	16
	LleC90	no	0	15
	BLI_84	no	0	16
	Rhca20	no	0	16
	BLI153	no	0	16
	CypG30	no	0.09	16
	MFW1	no	0	16
	Lsou8	no	0.1	16
Pine Creek	*CtoA247*	no	0	32
	Lco3	no	0.07	32
	LleC90	no	0.2	31
	BLI_84	no	0	30
	Rhca20	no	0.13	32
	BLI153	no	0.02	32
	CypG30	no	0.07	32
	MFW1	no	0	30
	Lsou8	no	0.02	32

Table A4. Genetic diversity in Clinch Dace samples within stream reaches. Monomorphic loci not shown. N = number of samples, H_0 = observed heterozygosity, H_E = expected heterozygosity, A = number of alleles, Range = range of allele sizes in base pairs, M-ratio = ratio of A to Range, F_{is} = inbreeding coefficient, HW= Hardy-Weinberg p-values, Bonferroni alpha = Bonferroni-corrected critical p-value.

Reach	Locus	N	H_0	H_e	A	Range (bp)	M-ratio	F_{is}	HW	Bonferroni alpha
Big Lick 1 Down	*CtoA247*	22	0.045	0.045	2	4	0.5	0	1	0.01
	Lco3	22	0.5	0.384	2	6	0.333	-0.313	0.269	0.01
	LleC90	22	0.182	0.169	2	2	1	-0.077	1	0.01
	CypG30	22	0.636	0.661	4	24	0.167	0.038	0.224	0.01
	Lsou8	22	0.864	0.511	2	6	0.333	-0.72	0.002	0.01
Big Lick 1 up	*Lco3*	24	0.417	0.337	2	6	0.333	-0.243	0.54	0.013
	LleC90	24	0.083	0.082	2	4	0.5	-0.011	1	0.013
	CypG30	24	0.833	0.738	4	24	0.167	-0.132	0.09	0.013
	Lsou8	24	0.583	0.496	2	6	0.333	-0.179	0.434	0.013
Big Lick 2 up	*Lco3*	5	0.2	0.2	2	8	0.25	0	1	0.016
	LleC90	5	0.2	0.2	2	2	1	0	1	0.016
	CypG30	5	1	0.8	4	24	0.167	-0.29	1	0.016
Big Lick 3 down	*Lco3*	19	0.421	0.472	3	8	0.375	0.111	0.042	0.013
	LleC90	19	0.053	0.053	2	2	1	0	1	0.013
	CypG30	19	0.632	0.743	5	24	0.208	0.153	0.168	0.013
	Lsou8	19	0.632	0.478	2	6	0.333	-0.333	0.317	0.013
Big Lick 3 up	*CtoA247*	24	0.042	0.042	2	4	0.5	0	1	0.008
	Lco3	24	0.25	0.231	4	8	0.5	-0.082	1	0.008
	LleC90	24	0.125	0.121	3	8	0.375	-0.03	1	0.008
	Rhca20	24	0.042	0.042	2	4	0.5	0	1	0.008
	CypG30	24	0.708	0.766	5	24	0.208	0.077	0.571	0.008
	Lsou8	24	0.417	0.507	2	6	0.333	0.181	0.433	0.008
Big Lick 4 down	*Lco3*	11	0.091	0.091	2	2	1	0	1	0.013
	LleC90	11	0.091	0.255	3	8	0.375	0.655	0.047	0.013
	CypG30	11	0.455	0.736	5	24	0.208	0.394	0.077	0.013

Table A4 cont

	Lsou8	11	0.273	0.247	2	6	0.333	-0.111	1	0.013
Greasy Creek 1 down	*Lco3*	5	0.2	0.2	2	12	0.167		1	0.013
	BLI153	5	0.2	0.556	2	2	1		0.365	0.013
	CypG30	5	0.4	0.756	5	20	0.25		0.05	0.013
	Lsou8	5	0.8	0.533	2	6	0.333		0.429	0.013
Hart Creek 1 down	*Lco3*	20	0.25	0.296	2	6	0.333	0.159	0.467	0.01
	LleC90	20	0.15	0.142	2	2	1	-0.056	1	0.01
	BLI153	20	0.25	0.224	2	2	1	-0.118	1	0.01
	CypG30	19	0.947	0.762	5	20	0.25	-0.251	0.047	0.01
	Lsou8	20	0.15	0.224	2	6	0.333	0.337	0.246	0.01
Hart Creek 1 up	*Lco3*	22	0.091	0.241	2	6	0.333	0.628	0.025	0.01
	LleC90	22	0.045	0.045	2	2	1	0	1	0.01
	BLI153	22	0.091	0.169	2	2	1	0.468	0.138	0.01
	CypG30	22	1	0.795	7	28	0.25	-0.266	0.084	0.01
	Lsou8	22	0.5	0.46	2	6	0.333	-0.09	1	0.01
Hart Creek 2 down	*Lco3*	21	0.476	0.483	4	8	0.5	0.015	0.222	0.01
	LleC90	20	0.05	0.512	3	8	0.375	0.905	0	0.01
	BLI153	21	0.333	0.285	2	2	1	-0.176	1	0.01
	CypG30	21	0.81	0.725	5	20	0.25	-0.12	0.373	0.01
	Lsou8	21	0.286	0.251	2	6	0.333	-0.143	1	0.01
Hurricane Fork 1 down	*Lco3*	20	0.3	0.262	2	2	1	-0.152	1	0.016
	CypG30	20	0.9	0.814	8	32	0.25	-0.109	0.011	0.016
	Lsou8	20	0.2	0.185	2	6	0.333	-0.086	1	0.016
Hurricane Fork 1 up	*CtoA247*	20	0.1	0.185	2	4	0.5	0.465	0.153	0.013
	Lco3	20	0.6	0.467	2	2	1	-0.295	0.328	0.013
	CypG30	20	0.95	0.829	8	32	0.25	-0.15	0.344	0.013
	Lsou8	20	0.1	0.097	2	6	0.333	-0.027	1	0.013

Table A4 cont.

Lewis Creek 4 up	*BLI153*	3	1	0.6	2	4	0.5	-1	0.401	0.025
	CypG30	3	0	0.533	2	8	0.25	1	0.2	0.025
Middle Creek 3 down	*Lco3*	8	0.125	0.125	2	2	1	0	1	0.016
	CypG30	8	0.625	0.725	5	24	0.208	0.146	0.424	0.016
	Lsou8	8	0.375	0.325	2	6	0.333	-0.167	1	0.016
Middle Creek 3 up	*Lco3*	8	0.375	0.458	2	2	1	0.192	1	0.025
	CypG30	8	0.625	0.8	6	20	0.3	0.231	0.298	0.025
Pine Creek IM	*Lco3*	16	0.313	0.28	3	8	0.375	-0.119	1	0.013
	LleC90	16	0.375	0.315	2	2	1	-0.2	1	0.013
	CypG30	16	0.938	0.784	5	24	0.208	-0.203	0.18	0.013
	Lsou8	16	0.063	0.063	2	6	0.333	0	1	0.013
Pine Creek low	*Lco3*	7	0.286	0.385	3	6	0.5	0.273	0.234	0.01
	LleC90	7	0.286	0.264	2	2	1	-0.091	1	0.01
	Rhca20	7	0.857	0.527	2	2	1	-0.714	0.16	0.01
	BLI153	7	0.143	0.143	2	2	1	0	1	0.01
	CypG30	7	0.714	0.846	5	24	0.208	0.167	0.09	0.01
Pine Creek mid	*CypG30*	3	0.667	0.8	4	24	0.167	0.2	0.603	0.05
Pine Creek up	*Lco3*	6	0.333	0.439	3	8	0.375	0.259	0.276	0.013
	LleC90	5	0.6	0.467	2	2	1	-0.333	1	0.013
	Rhca20	6	0.333	0.303	2	2	1	-0.111	1	0.013
	CypG30	6	1	0.803	5	16	0.313	-0.277	1	0.013

Table A5. Results of STRUCTURE Bayesian cluster analysis of multilocus genotypes at nine microsatellite loci in Clinch Dace populations. Ln*P*(*D*) = log probability of the given data given *K*, where *K* = a set number of clusters. Results presented here are the mean Ln*P*(*D*) from five iterations. Each run was performed with a burn-in of 10,000 and 100,000 MCMC cycles.

K	Ln *P*(*D*)	Var[Ln*P*(*D*)]
1	-2388.4	14.02
2	-2065.32	115.06
3	-2008.48	196.3
4	-1987.36	322.98
5	-1923.96	347.5
6	-1979.26	489.06
7	-2027.78	642.56
8	-2038.16	716.86
9	-2155.38	950.74
10	-2227.72	1103.92

Table A6. Results of STRUCTURE Bayesian cluster analysis of multilocus genotypes at nine microsatellite loci in the four Clinch Dace populations that showed admixture in previous analysis. Ln*P*(*D*) = log probability of the given data given *K*, where *K* = a set number of clusters. Results presented here are the mean Ln*P*(*D*) from five iterations. Each run was performed with a burn-in of 10,000 and 100,000 MCMC cycles.

K	Ln*P*(*D*)	var[Ln*P*(*D*)]
1	-1173.16	11.44
2	-1167.08	176.38
3	-1133.62	246.9
4	-1236	465.6
5	-1267.42	527.6

Table A7. Results of STRUCTURE Bayesian cluster analysis of multilocus genotypes at nine microsatellite loci in seven populations of Clinch Dace run independently. Ln$P(D)$ = log probability of the given data given K, where K = a set number of clusters. Results presented here are the mean Ln$P(D)$ from five iterations. Each run was performed with a burn-in of 10,000 and 100,000 MCMC cycles.

Stream	K	Ln$P(D)$	var[Ln$P(D)$]
Hurricane Fork	1	-236.3	4.6
	2	-237.96	9.28
	3	-236.82	7.04
	4	-237.14	8.22
	5	-237.28	7.48
Hart Creek	1	-484.2	7.16
	2	-507.22	115.24
	3	-561	191.88
	4	-685.08	475.02
	5	-643.48	460
Lewis Creek	1	-8.98	0.58
	2	-9	0.54
	3	-8.96	0.42
	4	-9.12	0.56
	5	-9.78	0.82
Pine Creek	1	-215.02	6.08
	2	-215.44	8.26
	3	-215.22	6.9
	4	-215.5	7.64
	5	-215.1	7.8
Big Lick Creek	1	-671.32	7.32
	2	-796.5	289.84
	3	-806.64	301.56
	4	-810.92	317.38
	5	-825.46	406.96
Middle Creek	1	-81.78	3.26
	2	-82.44	4.28
	4	-81.88	3.92
	5	-81.66	4.16
Greasy Creek	1	-44.66	2.9
	2	-44.56	4.34
	3	-43.92	3.56
	4	-44.32	4.12
	5	-43.68	2.68

Table A8. AMOVA results with Hart Creek, Hurricane Fork, and Lewis Creek analyzed independently and all other streams which showed admixture in Bayesian cluster analysis combined.

Sources of variation	Sum of squares	Variance components	Percentage of variation
Among populations	94.077	0.30725	25.028
Within populations	484.657	0.92038	74.971
Total	578.734	1.22764	

Average *F*-Statistics over all loci
Fixation Indices
F_{ST} : 0.25028
Significance tests (1023 permutations)
F_{ST} : *P*(rand. value > obs. value) = 0.000
P(rand. value = obs. value) = 0.000
P-value = 0.000

Table A9. Pairwise comparisons between the downstream most occupied Clinch Dace sites and distance in kilometers and F_{st};

		Distance (km)	F_{st}
Big Lick Creek	Greasy Creek	55	0.19
Big Lick Creek	Hart Creek	69	0.31
Big Lick Creek	Hurricane Fork	78	0.11
Big Lick Creek	Lewis Creek	43	0.24
Big Lick Creek	Middle Creek	51	0.16
Big Lick Creek	Pine Creek	2	0.19
Greasy Creek	Hart Creek	107	0.31
Greasy Creek	Hurricane Fork	117	0.30
Greasy Creek	Lewis Creek	81	0.26
Greasy Creek	Middle Creek	31	0.21
Greasy Creek	Pine Creek	53	0.18
Hart Creek	Hurricane Fork	32	0.27
Hart Creek	Lewis Creek	62	0.42
Hart Creek	Middle Creek	103	0.31
Hart Creek	Pine Creek	67	0.34
Hurricane Fork	Lewis Creek	70	0.27
Hurricane Fork	Middle Creek	112	0.34
Hurricane Fork	Pine Creek	76	0.37
Lewis Creek	Middle Creek	77	0.53
Lewis Creek	Pine Creek	41	0.46
Middle Creek	Pine Creek	49	0.04

Appendix B

Table B1. Population estimates from Microfish 3 using three-pass depletion data.

Reach and site length	Species	Total Catch	Pop. Estimate std	Lower 95% Confidence Interval std	Upper 95% Confidence Interval std
Big Lick Creek 1 DS (200 m)	Blacknose dace	832	444	432	456
	Clinch dace	53	30	27	35
	Creek chub	76	39	38	40
	Fantail darter	115	88	58	122
	Largescale stoneroller	165	85	83	88
	Snubnose darter	2	1	1	8
	Striped shiner	1	1	1	1
	White sucker	1	1	1	1
Big Lick Creek 1 US (200 m)	Blacknose dace	689	359	351	
	Clinch dace	38	19	19	20
	Creek chub	112	58	56	60
	Fantail darter	72	43	36	51
	Fathead minnow	1	1	1	1
	Largescale stoneroller	92	85	46	56
	Striped shiner	3	2	2	3
	White sucker	6	3	3	5
Big Lick Creek 2 DS (200 m)	Blacknose dace	632	323	316	
	Clinch dace	1	1	1	1
	Creek chub	14	7	7	8
	Fantail darter	112	63	56	70
	Largescale stoneroller	53	27	27	27
Big Lick Creek 2 US (200 m)	Blacknose dace	427	320	320	
	Clinch dace	5	4	4	4
	Creek chub	40	30	30	30
	Fantail darter	55	41	41	41

Table B1 cont.

	Largescale stoneroller	54	41	41	41
Big Lick Creek 3 DS (200 m)	Blacknose dace	525	286	274	
	Clinch dace	14	7	7	9
	Creek chub	114	58	57	59
	Fantail darter	60	33	30	38
	Largescale stoneroller	93	47	47	49
Big Lick Creek 3 US (200 m)	Blacknose dace	502	254	251	
	Clinch dace	24	12	12	13
	Creek chub	118	64	59	69
	Fantail darter	59	48	30	77
	Largescale stoneroller	58	34	29	41
	Striped shiner	1	1	1	1
	White sucker	1	1	1	1
Big Lick Creek 4 DS (200 m)	Blacknose dace	409	213	207	
	Clinch dace	11	6	6	9
	Creek chub	79	40	40	42
	Fantail darter	66	54	33	87
	Largescale stoneroller	8	4	4	5
Greasy Creek 1 DS (200 m)	Blacknose dace	323	167	163	
	Creek chub	79	40	40	42
	Fantail darter	7	4	4	8
	Largescale stoneroller	141	74	71	77
	Rosyside dace	150	76	75	78
	White sucker	26	13	13	14
Greasy Creek 2 DS (200 m)	Blacknose dace	387	214	202	
	Clinch dace	1	1	1	1

Table B1 cont.

	Creek chub	302	160	153	166
	Fantail darter	33	19	17	23
	Largescale stoneroller	185	97	93	101
	Rosyside dace	308	163	157	170
	White sucker	65	35	33	39
Hart Creek 1 DS (195 m)	Blacknose dace	437	227	224	
	Clinch dace	106	64	54	73
	Creek chub	222	115	114	118
	Fantail darter	134	94	70	119
	Largescale stoneroller	144	76	74	79
Hart Creek 1 US (129 m)	Blacknose dace	272	227	216	
	Clinch dace	26	22	20	29
	Creek chub	52	41	40	43
	Fantail darter	93	79	72	88
	Largescale stoneroller	71	68	55	85
Hess Creek 2 DS (158 m)	Blacknose dace	360	184	181	
	Creek chub	50	26	25	29
	Fantail darter	21	12	11	15
	Largescale stoneroller	22	11	11	12
	White sucker	2	1	1	1
Hess Creek 2 US (110 m)	Blacknose dace	171	158	155	
	Creek chub	19	17	17	18
	Fantail darter	20	18	18	20
	Largescale stoneroller	49	47	45	22
Hurricane 1 US (200 m)	Bluegill	3	2	2	
	Bluntnose minnow	11	6	6	6

Table B1 cont.

	Clinch dace	34	18	17	20
	Creek chub	257	132	129	135
	Fantail darter	146	77	73	81
	Largescale stoneroller	376	188	188	189
	Pumpkinseed	1	1	1	1
	Rock bass	9	5	4	6
	White sucker	17	9	9	9
Jackson Fork 1 DS (180 m)	Blacknose dace	196	112	109	
	Creek chub	25	14	14	14
	Fantail darter	72	44	40	51
	Greenside darter	1	1	1	1
	Largescale stoneroller	13	7	7	8
	Pumpkinseed	1	1	1	1
	Rock bass	2	1	1	4
	Rosyside dace	22	14	12	18
	Striped shiner	3	2	2	2
	White suckers	3	2	2	3
Jackson Fork 1 US (156 m)	Blacknose dace	243	163	157	
	Creek chub	21	13	13	15
	Fantail darter	75	72	72	72
	Largescale stoneroller	8	5	5	6
	Redbreast sunfish	1	1	1	1
	Rosyside dace	14	11	9	18
Jackson Fork 2 US (200 m)	Blacknose dace	259	131	130	
	Creek chub	24	12	12	13
	Fantail darter	37	27	19	42
	Largescale stoneroller	3	2	2	2

Table B1 cont.

	Northern hogsucker	1	1	1	1
	Rock bass	8	7	4	20
	Rosyside dace	2	1	1	1
	White sucker	3	2	2	2
Lewis Creek 2 DS (200 m)	Blacknose dace	520	273	265	
	Creek chub	36	18	18	19
	Fantail darter	36	54	41	66
	Largescale stoneroller	86	123	118	128
	Northern hogsucker	5	3	3	3
	Rock bass	4	2	2	5
	Snubnose darter	18	10	9	12
	Striped shiner	26	13	13	14
Lewis Creek 2 US (35 m)	Blacknose dace	94	296	272	
	Creek chub	8	23	23	26
	Fantail darter	15	43	43	49
	Largescale stoneroller	20	58	58	64
	Rock bass	2	6	6	20
	Snubnose darter	2	6	6	43
	Striped shiner	5	17	14	43
Lewis Creek 3 US (200 m)	Blacknose dace	431	222	216	
	Creek chub	72	36	36	37
	Fantail darter	62	33	31	36
	Rock bass	10	5	5	6
	Snubnose darter	8	4	4	5
	Striped shiner	83	44	42	47
	White sucker	5	3	3	4
Lewis Creek 4 DS (172 m)	Blacknose dace	275	160	163	
	Brown trout	1	1	1	1

Table B1 cont.

	Creek chub	109	66	63	70
	Fantail darter	35	24	20	33
	Largescale stoneroller	50	30	29	33
	Rock bass	6	3	3	4
	Snubnose darter	26	27	15	62
	Striped shiner	1	1	1	1
	White sucker	3	3	2	19
Lewis Creek 4 US (140 m)	Blacknose dace	297	219	213	
	Clinch Dace	3	2	2	2
	Creek chub	52	39	37	42
	Fantail darter	52	40	37	46
	Largescale stoneroller	167	124	119	129
	Snubnose darter	2	1	1	1
Middle Creek 3 DS (200 m)	Blacknose dace	89	45	45	
	Clinch dace	8	4	4	4
	Creek chub	23	12	12	13
	Fantail darter	3	2	2	2
	Largescale stoneroller	10	5	5	6
	Rosyside dace	28	14	13	15
Middle Creek 3 US (200 m)	Blacknose dace	44	23	22	
	Clinch dace	8	7	4	20
	Creek chub	32	16	16	17
	Largescale stoneroller	4	2	2	3
	Rosyside dace	13	7	7	8
Pine Creek 3 US (188 m)	Blacknose dace	913	503	495	
	Creek chub	91	50	48	53
	Fantail darter	112	60	60	71

Table B1 cont.

	Highlands shiner	32	21	17	28
	Largescale stoneroller	339	186	128	138
	Striped shiner	3	2	2	2
	Warpaint shiner	1	1	1	1
Town Hill Creek 1 DS (200 m)	Big eye chub	37	19	19	
	Blacknose dace	1666	879	865	894
	Bluntnose minnow	6	3	3	5
	Cottus sp.	253	149	134	164
	Creek chub	10	7	5	16
	Fantail darter	193	135	104	165
	Greenside darter	4	2	2	5
	Highland shiner	3	2	2	2
	Largescale stoneroller	762	403	393	413
	Northern hogsucker	76	40	38	43
	Sawfin shiner	4	2	2	3
	Snubnose darter	31	17	16	20
	Telescope shiner	56	28	28	29
	Tennessee shiner	17	9	9	9
	Warpaint shiner	15	9	8	14
	White sucker	1	1	1	1