Fragmentation and Genetic Diversity in Clinch Dace Populations

Final Report

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Executive Summary

In 1999 Clinch Dace, *Chrosomus* sp. cf. *saylori*, was discovered in the Tennessee drainage of Virginia. Subsequent sampling of southwest Virginia and portions of Tennessee indicated that Clinch Dace populations are small, fragmented, and of questionable viability. Further, riparian land use and mining pose significant threats to critical habitat. As such, Clinch Dace were listed as a Federal Species of Concern and on Virginia's Wildlife Action Plan as Tier I - Critical Conservation Need. A management plan and species description for Clinch Dace is of utmost importance, but data on distribution and life history are needed before these objectives can be realized.

The objectives of this study were to: 1) Monitor known populations of Clinch Dace to characterize at fine-scale distribution and status. The latter will be addressed in terms of relative abundance. 2) Analyze distribution data using geographic information systems and other approaches to identify habitat and landscape features that isolate Clinch Dace and associated fish populations, 3) Map stream crossings and assess likely barriers to upstream passage and measurements of specific conductance, 4) Screen molecular genetic markers in order to define demographic and any evolutionarily significant units for the species. Molecular genetic variation will be screened at nuclear microsatellite loci to assess levels of molecular genetic variability and population-level differentiation. We will examine the effect of geographic distance on genetic differentiation (Botta et al. 2015). Habitat use will be compared to assess whether genetic differentiation relates to any observed differences in adaptive characters among populations. 5) Use these findings to define demographic and evolutionarily significant units for the species and work toward estimating effective population size and protocols for translocating individuals. We sampled 29 reaches on ten streams for fish with a three-pass depletion method and measured eight habitat variables which might inform conservation actions. We also conducted statistical analyses on six habitat and fish community variables to determine whether habitat rather than fragmentation was influencing Clinch Dace presence and abundance.

We conclude that seven Clinch Dace populations vary in their degree of isolation, with some populations showing signs of recent admixture and others not. Populations with the least admixture, such as Hurricane Fork and Hart Creek in Russell County, may represent distinct management units. However, they are also among the largest populations found in 2017 and may therefore be the best candidates for donor population for translocations. The effects of road crossings in our study area was minimal and most crossings were not obvious barriers to fish passage. Instream habitat metrics that we measured also seemed to have little effect on Clinch Dace presence and abundance.

We recommend that further management actions be taken with an adaptive management approach, as it is not clear from our results that translocations should be ruled out, but rather undertaken initially as a pilot study with follow-up monitoring to determine whether outbreeding depression is taking place as a result of moving locally adapted fish. Stream restoration activities may not be warranted, as the Clinch Dace shows some resilience to habitat degradation, such as sedimentation and lack of woody debris. We only found one culvert on Hart Creek which could be considered for a retrofit and that does seem to be acting as a barrier to Clinch Dace movement. Further research could measure temporal changes in abundance and characterize the relationship between population size and extinction risk and identify minimum viable population thresholds. Further monitoring should include the seven populations characterized as well as nine streams (Hess, Indian, Laurel, Left Fork Coal, Mudlick, Pine, Town Hill, and West Fork Big Creeks).

Introduction

The Clinch River in Virginia contains more aquatic diversity than any other river basin in the state. Unfortunately, many of these species are rare or imperiled due to a landscape that has been highly altered by anthropogenic development (Bernhardt and Palmer 2011). In response to these threats, The Nature Conservancy named the Clinch Basin its number one hotspot for imperiled species in the U.S. The organization reports that 29 species of imperiled or vulnerable freshwater mussels and 19 species of fish call the Clinch River home (Master et al. 1998). It is likely that the list of endemic and imperiled fauna will grow if (or when) a new species of *Chrosomus* dace is officially described.

In 1999, populations of a *Chrosomus* dace were discovered in Mudlick Creek (Skelton 2007), and subsequently in the Indian Creek watershed in Tazewell County, Virginia (Lingenfelser et al. 2004). Up until those discoveries, *Chrosomus* daces were not known from the upper Clinch watershed (Jenkins and Burkhead 1994). Christopher Skelton realized that this population likely represented an undescribed species separate from the closely related Laurel Dace (Skelton 2001). The Laurel Dace is restricted to six streams on Walden Ridge in the upper Tennessee River drainage (Skelton 2007). Skelton based his distinction on variation in nuptial coloration. The new *Chrosomus* taxon had two gold spots on the caudal peduncle that were absent in the Laurel Dace. Since his discovery, researchers have referred to this form as the Clinch Dace *Chrosomus* sp. cf. *saylori*, which remains undescribed, although life history, morphometric and meristic data support its classification as a distinct species (White and Orth 2013b, White and Orth 2014a). Biologists believed the Clinch Dace was endemic to headwaters of Clinch River tributaries north of the mainstem river in Russell and Tazewell counties (White and Orth 2013a), Virginia; however, in 2012, distant and presumably native populations of what appear to be Clinch Dace were discovered in Rugby State Natural Area, Tennessee in the upper Cumberland basin and in two streams in the Emory basin (a tributary to the Clinch River) in Tennessee. These discoveries further complicate the phylogenetic understanding of the genus (Dave Neely, Tennessee Aquarium Conservation Institute, personal communication, 2014).

Most species of *Chrosomus* dace, excluding *C. eos* and *C. neogaeus*, are headwater stream specialists (Moore et al. 2018). For instance, Clinch Dace populations are rarely found in streams larger than third order and four meters in average wetted width (White and Orth 2013a). This headwater ecological niche is uncommon among Virginia stream fishes. Jenkins and Burkhead (1994) name only one other species that is restricted to these types of habitats -- the Rosyside Dace (*Clinostomus funduloides*), which co-occurs and even occasionally may hybridize with Clinch Dace in the Middle and Indian Creek systems in Tazewell Co., Virginia (Michael Moore et al. 2016). This headwater restriction suggests that *Chrosomus* populations are sensitive to piscine predation or competition from cyprinids with similar ecological niches. Clinch Dace are rarely found alongside centrarchid or other piscivorous fishes, other than Creek Chub (*Semotilus atromaculatus*).

The Clinch Dace is considered a Tier I species (critical conservation need) in the Virginia Wildlife Action Plan, endangered by Jelks et al. (2008), and "critically imperiled" by NatureServe (2015), but does not appear on the Virginia or Federal endangered species lists. During sampling conducted from 1999-2007, Skelton located only 16 streams of eight larger tributaries in the upper Clinch basin occupied by Clinch Dace (Skelton 2007). These tributary systems were Big Creek, Coal Creek, Indian Creek, Middle Fork Clinch River/Dumps Creek, Mudlick Creek, Swords Creek, Town Hill Creek, and Weaver Creek. Additional surveys conducted in 2011 and 2012 established Clinch Dace presence at 14 of 60 sites sampled (23%

naïve occupancy, also known as sampling prevalence in the distribution modeling literature) (White and Orth 2014a). These surveys discovered Clinch Dace in only one new stream, namely Jackson Fork, a tributary to Indian Creek in Tazewell County. Nevertheless, ~125 km of unsampled second-and third-order streams remained within the species' presumed range (White 2012). The few populations of Clinch Dace that exist are likely small and fragmented (White and Orth 2012; White and Orth 2014a; Moore et al. 2018). Clinch Dace may occur at lower local densities than any of the other *Chrosomus* daces due to the Clinch Dace's more specialized diet, higher trophic position (White and Orth 2013b), shorter lifespan, and lower fecundity. Clinch Dace sex ratios were female-biased (3:1 female to male), while other congeners have male-biased sex ratios (Hamed et al. 2008; White 2012; White and Orth 2014b). Clinch Dace sex is difficult to distinguish without dissection, although males can develop pearl organs when reproductively active and relative body position of fins changes as females become gravid (White and Orth 2013b). Other *Chrosomus* mature at age 1 and have higher average fecundities than Clinch Dace (Settles and Hoyt 1978; Das and Nelson 1990; Hamed et al. 2008; White and Orth 2014b). Clinch Dace usually reach maturity at age 2 and die following spawning, which reduces the intrinsic population growth rate (White and Orth 2014a).

Observed reproductive timing is similar to that of other *Chrosomus* species. Reproduction may occur from April-July, but the peak period seems to occur in late May and early June. Clinch Dace were observed on Stoneroller (*Campostoma* spp.) or Creek Chub (*Semotilus atromaculatus*) nests on the 23 May and 6 June in two streams in 2011 (White and Orth 2014b), on 4-6 June in 2014 and 2-5 June, 2015 (Hatcher et al. 2017; https://vimeo.com/117837464) The rarity of Clinch Dace is comparable to those of other federally protected *Chrosomus* species. The most closely related taxon, the Laurel Dace, is listed as endangered, and the Blackside Dace (*C. cumberlandensis*) is listed as threatened (Black et al. 2013). Laurel Dace occupy six streams in three stream clusters (George et al. 2015), while Blackside Dace occupy roughly 125 streams in 52 stream clusters or populations (Biggins 1988; Bivens et al. 2013; O' Bara 1990; Floyd 2015; Skelton 2013). Prior to this study, Clinch Dace were known from only 16 streams in eight stream clusters (Skelton 2007; White and Orth 2014a). Past surveys for Clinch Dace characterized broad-scale patterns of presence, which remains an imperative research goal. Temporal sampling replication is needed to quantify detection probability for Clinch Dace (White and Orth 2014a). Conducting surveys with multiple collection gears can inform the design of future monitoring protocols. Additionally, little attention has focused upon individual population units. Population-level research goals should include delineating upstream and downstream population boundaries within streams and estimating population density at particular sites.

The objectives of this study were to: 1) Monitor known populations of Clinch Dace to characterize at fine-scale distribution and status. The latter will be addressed in terms of relative abundance. 2) Analyze distribution data using geographic information systems and other approaches to identify habitat and landscape features that isolate Clinch Dace and associated fish populations, 3) Map stream crossings and assess likely barriers to upstream passage and measurements of specific conductance, 4) Screen molecular genetic markers in order to define demographic and evolutionarily significant units for the species. Molecular genetic variation will be screened at nuclear microsatellite loci to assess levels of molecular genetic variability and population-level differentiation. We will examine the effect of geographic distance on genetic differentiation (Botta et al. 2015). Habitat use will be compared to assess whether genetic differentiation relates to any observed differences in adaptive characters among population. 5) Use these findings to define demographic and evolutionarily significant units for the species and

work toward estimating effective population size and protocols for translocating individuals. To achieve these objectives, we sampled 29 reaches on ten streams for fish with a three-pass depletion method and measured eight habitat variables which might inform conservation actions. We also conducted statistical analyses on six habitat and fish community variables to determine whether habitat rather than fragmentation was influencing Clinch Dace presence and abundance.

Methods

Site Selection

Selected sites were targeted at or near to locations where Clinch Dace were caught by Shannon White or Michael Moore (Moore et al. 2017a, Figure 1). ArcGIS was used to identify 52 potential sites at road crossings and over 30 non-road crossing sites on ten streams (Appendix A, Figure 20; Pine Creek, Hurricane Fork, Jackson Fork, Hess Creek, Harts Creek, Mudlick Creek, Town Hill Creek, Little Town Hill Creek, Lewis Creek, Big Lick Creek, and Middle Creek). Most of these sites cannot be sampled due to lack of permissions, inaccessibility, or other considerations, such as dry conditions. Some sites were added or moved during reconnaissance based on suitability and accessibility. Three sites selected on Middle Creek were identified on location.

Permissions

Over 300 landowners from more than 80 potential sites were identified with property owner shapefiles for ArcGIS provided by Russell County and through the Tazewell County mapping website. These landowners were initially contacted through mail, with stamped reply cards provided in the mailing. As only a small number of reply cards were received, contact by phone was attempted, but it was difficult to acquire phone numbers, and most calls went unanswered. Finally, door-to-door contact was initiated and this proved the most effective method for receiving permissions. Since many of permissions were given verbally, no record exists for most permissions, and landowners are not being identified by name.

Site Reconnaissance and Habitat Sampling

Prior to fish sampling, sites were reconnoitered to determine length and sampling considerations. The ideal site was composed of a reach upstream and a reach downstream with lengths of a maximum 40 times average width or a minimum 200 meters. Many sites do not meet these conditions for several reasons. Culverts may be closer than the minimum distance and some sites become un-sampleable due to physical barriers or lack of permission. Therefore, some reaches were shorter than 200 meters and may lack a downstream reach altogether. For instance, the distance between the culverts at Lewis Creek-2 and Lewis Creek-3 is only 50 meters, so the upstream reach of LC-2 is 50 meters and LC-3 has no downstream reach at all. Average wetted width was calculated from transect measurements spaced 20 meters apart. Average width was also used to determine sampling considerations, as one backpack electrofisher (Smith-Root LR-24) was used for each three meters of wetted width. At each transect, the abundance of woody debris and percent canopy cover was estimated. Depth measurements were taken throughout each reach to determine maximum depth. GPS points were taken at road crossings or at the 200-meter mark of non-road crossing sites. Road crossings were photographed, classified by type, and assessed as possible barriers. Out of 27 sites on ten streams that were reconnoitered, 19 sites

were at road crossings, only six of which were perched culverts that may be barriers to fish movement. The remaining eight were non-crossing sites (Table 1). Sites were numbered from downstream to upstream. For example, Big Lick 1 was the downstream-most site on Big Lick Creek and Big Lick 4 is the upstream-most site.

Table 1. Total number of sites reconnoitered by stream, the total number of those sites that were at road crossings, and the total number of road crossings that had perched culverts.

Stream	Total Number of Sites	Road Crossings	Perched Culverts
Big Lick Creek	4	3	1
Greasy Creek	2	2	1
Harts Creek	2	2	1
Hess Creek	2	1	0
Hurricane Fork	1	1	0
Jackson Fork	3	2	1
Lewis Creek	4	3	0
Middle Creek	3	1	1
Pine Creek	4	4	0
Town Hill Creek	2	1	1
Totals	27	20	6

Fish Sampling

We conducted three pass backpack electrofishing (Smith-Root Model LR-24) depletions at 19 sites on ten streams in Russell and Tazewell counties (Table 2, Figure 1). Eight reconnoitered sites were not sampled because of time constraints or sampling considerations. The upstream reach of Town Hill 1 was not sampled due to revocation of permission. The upstream reach of Greasy Creek 2 was dammed by beavers after reconnaissance and was no longer sampleable for fish. A total of 417 Clinch Dace were collected from eleven sites on six of these streams (Table 3). Clinch Dace were measured by total length and fin-clipped for genetic analysis. Mortalities were preserved on formalin; these samples currently are housed in the Fluvial Fishes Laboratory and will eventually be housed in a museum. Except at Town Hill 2 and Hart 2, all other fish were identified to species and all fish were retained until sampling was complete. At all sites where a barrier was present, either the upstream or downstream reach did not exist or could not be sampled (Table 3). One conductivity measurement (Lamotte 1741) was taken at the downstream most end of every site.

Table 2. Sites sampled for fish in 2017.

Site Name	Locality
Jackson Fork1	Firetower Rd. crossing, Bandy VA
Jackson Fork2	Unnamed road crossing, immediately upstream of JF1, Bandy VA
Hurricane Creek	Sandy Ridge Rd. crossing, Cleveland VA

Big Lick 1	Franks Hollow Rd. upstream of Swords Creek Rd. crossing, Raven VA
Big Lick 2	Franks Hollow Rd. crossing at address 489, Raven VA
Big Lick 3	Elkins Branch Rd. crossing, Raven VA
Big Lick 4	Franks Hollow Rd. crossing, Raven VA
Hess 1	Miller Creek Rd. crossing, Rowe VA
Lewis 2	Drill Rd. crossing, Honaker VA
Lewis 3	Old Drill Rd. crossing, Honaker VA
Lewis 4	Maple Crest Dr., Honaker VA
Town Hill 1	Hill Creek Rd. crossing, Richlands VA
Town Hill 2	Maple Lane crossing, Richlands VA
Greasy Creek 1	Greasy Creek Rd., Bandy
Greasy Creek 2	Forest access road off Greasy Creek Rd., Bandy VA
Middle Creek 3	Hunting Hill Rd., Jewell Ridge VA
Hart Creek 1	Sandy Ridge Rd. crossing, 8 miles west of Honaker VA
Hart Creek 2	Driveway crossing upstream of Hart Creek 1, 8 miles west of Honaker VA
Pine Creek 3	Forest access road crossing off Pine Creek Rd., Raven VA

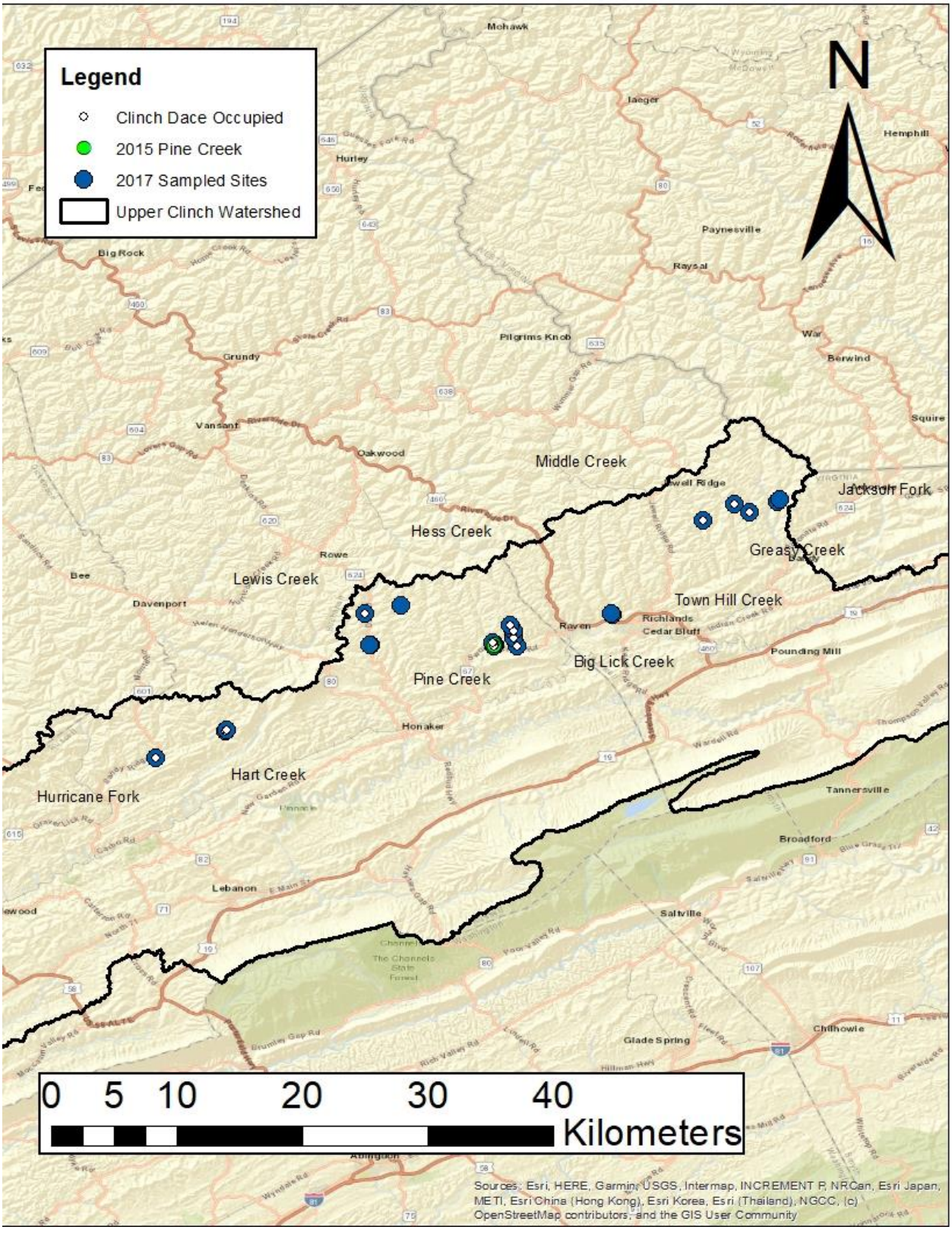

Figure 1. All sites sampled in 2017. Blue dots represent sampled sites, white dots indicate sites where Clinch Dace were found.

Table 3. Sites sampled in 2017, site length (m), and total number of Clinch Dace caught at each site.

Site Name	Site Length	Clinch Dace Abundance
Big Lick1	400	91
Big Lick2	400	6
Big Lick3	400	38
Big Lick4	200	11
Greasy1	400	5
Greasy2	200	1
Harts1	324	132
Harts2	200	43
Hess2	268	0
Hurricane1	400	71
Jackson Fork1	336	0
Jackson Fork2	200	0
Lewis2	235	0
Lewis3	200	0
Lewis4	312	3
Middle3	400	16
Pine3	188	0
Town Hill1	200	0
Town Hill2	200	0
		417

Table 4. Species richness by site. The table indicates whether a potential barrier was present and the number of species caught in the upstream and downstream reaches of each site. NS indicates that the reach was reconnoitered but could not be sampled. NA indicates that that reach did not exist.

Site	Barrier	Upstream	Downstream
Big Lick 1	No	8	9
Big Lick 2	No	5	5
Big Lick 3	No	7	5
Big Lick 4	Yes	NA	5
Greasy 1	No	6	7
Greasy 2	Yes	NS	7
Hart 1	No	5	5
Hart 2	Yes	5	NA
Hess 2	No	4	5
Hurricane 1	No	9	8
Jackson Fork 1	No	6	10
Jackson Fork 2	Yes	8	NA

Lewis 2	No	7	8
Lewis 3	No	9	NA
Lewis 4	No	6	9
Middle 3	Yes	6	6
Pine 3	Yes	7	NA
Town Hill 1	Yes	NS	16
Town Hill 2	No	2	NS

Habitat Analysis

At each transect, we ranked canopy cover as percentage of shaded stream according to the Daubenmire (1968) classes (Table 5) and noted the presence or absence of woody debris. From this, we 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, we calculated the proportion of transects containing woody debris for all transects in each reach.

Table 5: 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, we evaluated culverts or bridges as barriers to fish movement (Table 4). we also measured culvert dimensions, the distance from the bottom of the culvert to the top of the water and to the stream bed, length of the culvert impact zone, and photographed the crossing. Along each reach, we 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 (Lamotte 1741) was taken at the downstream-most point of the site before sampling began.

Habitat and Fish Community Data Analysis

We tallied species richness as the total number of species caught during all three passes. To demonstrate that adequate sampling effort was allocated, we created a scatterplot of species richness vs. total length of each reach sampled. If the relationship was insignificant, then we 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, we 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). Other variables that were measured in the field were intended for use in management actions. Because canopy cover was recorded as ranks, we 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.

We conducted quasipoisson regressions (Table 6) 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. We also conducted logistic regression on Clinch Dace presence/absence (Table 6) Because only one conductivity reading was taken per site and not in every reach, I conducted quasipoisson regressions and logistic regression on Clinch Dace abundance and presence/absence and conductivity for each total site. We also calculated the proportion of fish caught in each reach that was represented by Clinch Dace (Table 9). These tests were used because the data were not normally distributed and dispersion was high.

To assess the effects of putative barriers, we conducted paired *t*-tests using the upstream and downstream reaches of each site on the dependent variable of Clinch Dace abundance. 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 index of differentation, $F_{st,}$ 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 their downstream reaches.

Table 6. Experimental variables and the data 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

Genetic Methods

To accomplish the objective of testing the effects of road crossings as barriers to migration, most sampling sites were located at road crossings. 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 largely determined by permission to access a landowner's property, accessibility, proximity of other road crossings, and sampling feasibility (e.g., the stream was not dry or converted to a beaver dam). To determine the effects of barriers on Clinch Dace migration , we used both sampling reaches in a paired experimental design. Genetic analysis also contributed to this objective, as we used paired experimental units and multiple metrics of genetic divergence, such as calculations of F_{st} (Wright 1965) and Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) to assess genetic isolation across road crossings. We also tested the hypothesis that Clinch Dace populations are genetically distinct by using a variety of genetic analyses utilizing each experimental unit as well as on whole streams with all sites combined. Multiple measures of genetic variation were used to provide more robust results, as different methods have different strengths and weaknesses and can be used to corroborate each other.

We analyzed 234 Clinch Dace samples collected by Rebecca Bourquin in 2017 and 32 samples collected from Pine Creek by Michael Moore in 2015 (Table 7). These DNA samples were extracted from fin-clips collected in the field. Michael Moore's samples were used because he caught Clinch Dace in Pine Creek and we did not.

Table 7. Numbers of Clinch Dace DNA samples for given reaches sampled in 2017 (this study) and 2015 (Moore 2015).

Reach	*N*
BigLick1down	22
BigLick1up	24
BigLick2down	1
BigLick2up	5
BigLick3down	19
BigLick3up	24
BigLick4down	11
Greasy1down	5
Greasy2down	1
Hart1down	20
Hart1up	22
Hart2down	21
Hurricane1down	20
Hurricane1up	20
Lewis4up	3
Middle3down	8
Middle3up	8
Pine Creek IM	16
Pine Creel low	7
Pine Creek mid	3
Pine Creek up	6

We extracted DNA from fin-clips using DNeasy Blood and Tissue kits (Qiagen Co.). Concentration and purity of extracted DNA were quantified using a µLite spectrophotometer (BioDrop, Cambridge, UK). 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 94ºC for three minutes; 35 cycles of denaturation at 94ºC for one minute, annealing at 56ºC for 45 seconds, and extension at 72ºC for one minute; and final extension 72ºC for five minutes. Nine of twelve primers 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 ensure that we did indeed amplify the target microsatellite DNA.

Table 8. 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 was used to score microsatellite fragments (Hulce et al. 2011).

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 (Appendix B, Table 19. Hardy-Weinberg equilibrium and linkage disequilibrium were tested in Arlequin 3.5 (Excoffier et al. 2005) with exact tests using the Markov chain algorithm with forecasted chain length of 1,000,000 and 100,000 dememorization steps for all loci in all reaches. Genetic diversity was quantified in terms of number of alleles (*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 prior 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 (Table 14; Appendix B, Table 17).

Population differentiation and mixing among sites was assessed using Bayesian clustering analysis using Structure 2.3.4 (Pritchard et al. 2000), to assess support for different numbers of population clusters (*K*). We 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. We assessed population differentiation at several levels. First, we ran Structure's admixture model with all samples from all streams with the number of clusters (*K*) running from one to ten, with five replications (Figure 2). On the basis of the results of this run, we applied the algorithm to the four streams where admixture seemed most likely with *K* values ranging from 1 to 5 and five replicate runs (Figure 3). Finally, we ran the admixture model for each stream individually, each with *K* running from one to five and five replicate runs.

Population-based analytic 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 partitioning genetic variation within and among streams were conducted using Arlequin 3.5 (Excoffier et al. 2005). Effective population sizes (N_e) for all reaches were calculated 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). We used the program MLrelate (Kalinowski 2006) to estimate genetic relatedness among individual Clinch Dace in each stream.

Results

Site Summaries

Big Lick Creek 1. Locality: Next to Franks Hollow Road starting at bridge at Swords Creek Road. Site access: park at old bridge above 200 meter mark at 115 Franks Hollow Road and walk downstream to start of site. This is a not a road crossing site, but there is an old, disused forest access bridge just above the 200 meter mark that is not an impediment to fish movement. Both the upstream and downstream reaches of this site are 200 meters.

Figure 2. Big Lick Creek 1 road crossing, downstream view.

Big Lick Creek 2. Locality: Franks Hollow Road, approximately 3.5 kilometers of Raven, VA. Site access: park off the road at address 489 Franks Hollow Road. The road crossing at Franks Hollow Road is a double pipe culvert that is not perched and not an obvious barrier to fish movement. The left pipe was dry at the time of sampling. Both the upstream and downstream reaches of this site are 200 meters long.

Figure 3. Big Lick Creek 2 road crossing, downstream view.

Big Lick Creek 3. Locality: Franks Hollow Road, approximately 3.5 kilometers west of Raven, VA. Site access: park at Elkins Branch Road pull-off. This road crossing consists of a single pipe culvert that is not perched and is not an obvious barrier to fish movement. Both the upstream and downstream reaches of this site are 200 meters long.

Figure 4. Big Lick Creek road crossing, downstream view.

Big Lick Creek 4. Locality: Franks Hollow Road, approximately 3.2 kilometers west of Raven, VA. Site access: park at pull-off near end of road maintenance and walk downstream to beginning of site. This is a single pipe culvert that is slightly perched and may be a barrier to fish movement during low flows. There is only one reach downstream of the road crossing, as upstream of the culvert was virtually dry.

Figure 5. Big Lick Creek 4 road crossing, downstream view.

Greasy Creek 1. Locality: Greasy Creek Road, north of Bandy. Site access: park at farm road pull-off at the zero-meter mark and hike along the side of the field. This is not a road crossing site. There is a small beaver dam at the 180-meter mark. Both the upstream and downstream reaches are 200 meters long.

Greasy Creek 2. Locality: Greasy Creek Road, north of Bandy. Site access: park at old forest road that is the center of site and hike downstream 200 meters. This is a single pipe culvert that is slightly perched and could be a barrier to fish movement during low flows. There is only a downstream reach on this site, as the upstream reach was turned into an un-sampleable beaver dam between the time of reconnaissance and fish sampling.

Hart Creek 1. Locality: Sandy Ridge Road crossing, approximately 7 kilometers northeast of Cleveland, VA. Site access: park at gravel pull-off south of junction and hike downstream to beginning of site. This is a double box culver that is not perched and is not an obvious barrier to fish movement. In fact, we recaptured a fin clipped Clinch Dace in the upstream reach when we came back to sample upstream. The downstream reach is 200 meters long. The upstream reach is only 130 meters long, as it ends at another culvert, which is the downstream end of the next upstream site, Hart Creek 2.

Figure 6. Hart Creek 1 road crossing, downstream view.

Hart Creek 2. Locality: Private driveway just off Sandy Ridge Road, approximately 7 kilometers northeast of Cleveland, VA. Site access: park at pull-off across the street. This is a large single pipe culvert that is slightly perched and could be a barrier to fish movement during low flows. There is an extremely large and deep plunge pool downstream of the site (the top of Hart Creek 1). There is only a 200 meter long upstream reach, as the downstream end of the culvert is the upstream reach of Hart Creek.

Figure 7. Hart Creek 2 road crossing, downstream view.

Hess Creek 2. Locality: Miller Creek Road crossing, approximately 5.5 kilometers south of Rowe, VA. Site access: park at address 2033 Miller Creek Road and walk downstream to start

of site. This is a bottomless double arch culvert embedded in concrete that is not perched and not an obvious barrier to fish movement. The downstream reach is only 158 meter longs, as it becomes impassable at that point. The upstream reach is only 110 meter long as a fence crosses the stream there.

Figure 8. Hess Creek 2 road crossing, downstream view.

Hurricane Fork 1: Locality: at Sandy Ridge Road, approximately 4 kilometers from Gravel Lick Road junction. Site access: park on side of the road at the crossing. This road crossing is a bridge and is not an obvious barrier to fish movement. Both upstream and downstream reaches are 200 meters long.

Jackson Fork 1. Locality: Firetower Road crossing, Bandy, VA. Site access: heading north on Firetower Road/Hwy 628, take first dirt road on right and park at crossing. This is a double pipe culvert that is not perched and is not an obvious barrier to fish movement. The downstream reach is only 180 meters long and ends at the confluence with Indian Creek. The upstream reach is 156 meters long and ends at the next culvert (Jackson Fork 2).

Figure 9. Jackson Fork 1 road crossing, downstream view.

Jackson Fork 2. Locality: second road crossing on Firetower Road, Bandy, VA. Site access: park at Firetower Road (Jackson Fork 1) and hike gravel road to culvert. This site is contiguous with Jackson Fork 1 and consists of only one reach, upstream of the second culvert.

The culvert is slightly perched and could be a barrier to fish movement during low flows. The upstream reach is 200 meters long. For some extent below the culvert, the channel and banks have been concreted.

Figure 10. Jackson Fork 2 road crossing, downstream view.

Lewis Creek 2. Locality: Old Drill Road crossing, Honaker, VA. Site access: park at intersection of Old Drill Road and Drill Road and walk up driveway to beginning of site. This is a large single pipe culvert that is not perched and is not an obvious barrier to fish movement. The upstream reach is only 35 meters, as it ends at the next culvert, adjacent to Lewis Creek 3. The downstream reach is 200 meters long.

Figure 11. Lewis Creek 2 road crossing, downstream view.

Lewis Creek 3. Locality: Drill Road crossing, Honaker, VA. Site access: park at intersection of Old Drill Road and Drill Road and walk through culvert coming from site Lewis Creek 2. This is a large single pipe culvert that is not perched and is not an obvious barrier to fish movement. There is only one 200 meter long reach, which is upstream of the second culvert at

the end of Lewis Creek 2. Lewis Creek 2 and Lewis Creek 3 are downstream of a row of houses which have sewage drainage directly into the creek. This may be a water quality barrier for Clinch Dace and may make this stream unsuitable as a receiving stream for translocations.

Figure 12. Lewis Creek 3 road crossing, downstream view.

Lewis Creek 4. Locality: Maple Crest Road crossing next to Drill Road, Honaker, VA. Site access: park at Maple Crest Road crossing. This is a single box culvert that is not perched and is not an obvious barrier to fish movement. This site is above several houses that drain sewage directly into the creek. It is the only Lewis Creek site where we caught Clinch Dace. Both upstream and downstream reaches are 200 meters long.

Figure 13. Lewis Creek 4 road crossing, downstream view.

Middle Creek 3. Locality: Hunting Hill Road crossing, approximately 3 kilometers southwest of Jewell Ridge, near junction of Rock Road. Site access: park at pull-off north of Hunting Hill Road crossing. This is a single pipe culvert embedded in a concrete skirt. It is

slightly perched and could be a barrier to fish movement during low flows. Both upstream and downstream reaches are 200 meters long.

Figure 14. Middle Creek 3 road crossing, downstream view.

Pine Creek 3. Locality: Pine Creek Road crossing. Site access: park at forest access road on Pine Creek Road. This is single pipe culvert that is not perched and is not an obvious barrier to fish movement. This is only the reach upstream of the road crossing. The downstream reach could not be sampled due to lack of landowner permission. The upstream reach is only 188 meters long, because it becomes impassable due to a barbed wire fence at the point.

Town Hill Creek 1. Locality: Hill Creek Road crossing, approximately 0.5 kilometers north of highway junction and 1.5 air-miles west of Richlands. Site access: pull off after bridge on Maple Lane in front of camper trailer and walk down the road to the beginning of the site. This is a double pipe culvert embedded in concrete that is severely perched and is an obvious barrier to fish movement. We were only able to sample the downstream reach for fish after permission to access the upstream reach was revoked due to landowner dissatisfaction with evident fish mortality from sampling the downstream reach. This site had the highest species richness of any sites sampled in 2017. However, this may be due to the locals stocking the stream just downstream of the culvert with fish caught in other places.

Town Hill Creek 2. Locality: Maple Lane Road crossing, approximately 2.4 kilometers west of Richlands. Site access: park at church and walk down the road to the beginning of the site at the culvert. This is a double box culvert that is not perched and is not an obvious barrier to fish movement. There is only an upstream reach on this site, as the downstream reach is the upstream reach of Town Hill 1 and we lost permission to sample there. Fish were not counted at this site due to time restrictions.

Fish and Habitat Analysis

Clinch Dace were caught in 17 of 29 sub-reaches and from 11 of 19 sites sampled in 2017 (Figure 5). Some sites lacked an upstream or downstream reach from the road crossing due to barriers or lack of 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 observations, ranging in the upstream reaches from zero to 7.92 and in downstream reaches from zero to 10.16, 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 species richness and reach length was insignificant ($R^2 = 0.0196$) (Figure 15).

Table 9. Percentage of total fish catch represented by Clinch Dace at each reach sampled and for the total site. NS=not sampled, NA=not applicable.

Site Name	Upstream	Downstream	Total
Hess 2	0	0	0
Lewis 2	0	0	0
Lewis 3	0	0	0
Lewis 4	0.52	0	0.28
Jackson 1	0	0	0
Jackson 2	0	0	0
Big Lick 1	3.75	4.26	4.03
Big Lick 2	0.86	0.12	0.43
Big Lick 3	3.15	2.34	2.42
Big Lick 4	NS	1.92	1.92
Middle 3	7.92	4.97	6.11
Hurricane 1	3.98	4.71	4.33
Greasy 1	0.38	0.51	0.47
Greasy 2	NS	0.08	0.08
Hart 1	5.06	10.16	8.48
Hart 2	NA	NA	NA
Pine 3	0.00	NS	0.00
Town Hill 1	NS	0.00	0.00
Town Hill 2	0	NA	0.00

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 11). All variables except for conductivity were measured at the sub-reach level. Only one conductivity reading was taken at the downstream-most point of the downstream reach.

Variables examined were 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 reach level. Some data points were missing for some variables. Only conductivity was significantly different among sites with or without Clinch Dace ($p = 0.03$).

Table 10. Clinch Dace population estimates by reach standardized to 100 meters of stream length. Reach level population estimates for Clinch Dace and all other fishes is included in Appendix A. Table 19.

Reach	Standardized Pop. Est.
Big Lick Creek 1 Downstream	30
Big Lick Creek 1 Upstream	19
Big Lick Creek 2 Downstream	1
Big Lick Creek 2 Upstream	4
Big Lick Creek 3 Downstream	7
Big Lick Creek 3 Upstream	12
Big Lick Creek 4 Downstream	6
Greasy Creek 1 Downstream	7
Greasy Creek 2 Downstream	1
Hart Creek 1 Downstream	64
Hart Creek 1 Upstream	22
Hurricane 1 Upstream	18
Lewis Creek 4 Upstream	2
Middle Creek 3 Downstream	4
Middle Creek 3 Upstream	7

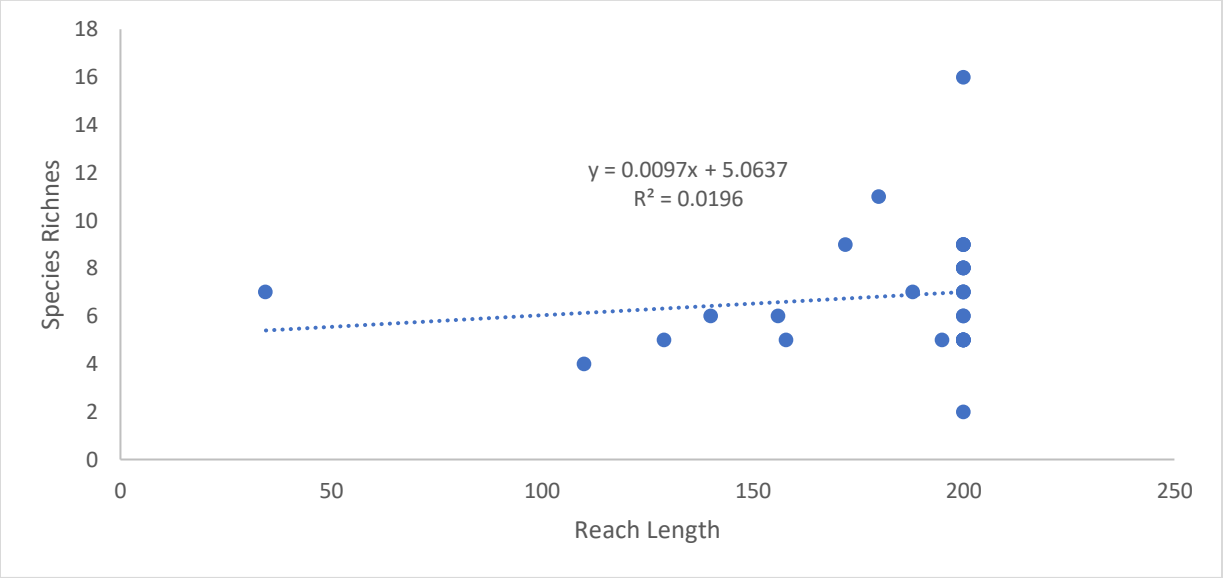

Figure 15. Species richness at all reaches vs. reach length.

Table 11. 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 12. 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

To test the effects of barriers on Clinch Dace abundance, we conducted a paired *t*-test using Clinch Dace abundance values from upstream and downstream reaches, followed by a Student's *t*-test. Five of the 19 sites sampled were eliminated from this analysis because only one reach could be sampled at those sites. Both Town Hill Creek sites were taken out because we 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 beavers after reconnaissance. The upstream reach of Big Lick 4 was dry at the time of sampling. We could not get permission to sample downstream of the road crossing at Pine Creek 3. Two of the sites included in the analysis are non-road crossing sites. Only two of the sites used in this analysis were suspected to be obvious 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, $p = 0.42$).

Results of a genetic assessment of the effects of putative barriers to migration are reported below.

Genetics

Of the 17 PCR primer pairs tested, nine produced amplification products sufficient 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*. *Rhca-20* must be re-sequenced.

We conducted amplification fragment-size analysis on PCR amplified products. Of 266 samples, 18 samples failed to amplify. After fragment analysis in GeneMarker (SoftGenetics, State College, PA) was complete, we analyzed data for individual reaches (Appendix B, Table 20) and for whole streams (Appendix B, Table 21) 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. Therefore, null alleles were not considered to be an issue and no data were excluded from subsequent analyses for that reason. No linkage disequilibrium was found at the reach level at the Bonferroni-corrected critical values. Two loci in two streams (Hart Creek and Big Lick Creek) showed linkage disequilibrium, which was attributed to chance, and data from these loci were not dropped. Hardy-Weinberg population structure analyses that we conducted on individual reaches (Appendix B, Table 22) and on streams as a whole (Table 14) 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 departure from Hardy-Weinberg equilibrium was not considered a reason to exclude data from any of the nine loci. Given the results of these analyses, we retained data for all nine loci that consistently amplified Clinch Dace DNA in the analyses.

For six sites with putative barriers to Clinch Dace migration, we calculated F_{st} among collections from the upstream and downstream reaches (Table 13). 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 we calculated F_{st} 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, ranging from 0.003 to 0.028. However, two were considerably higher, Middle Creek 3 (F_{st} = 0.070) and Hart Creek 1/2 (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.

Table 13. 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

For both individual reaches and whole streams, *m*-ratios (Table 14; Appendix B, Table 22) were generally lower than the criterion level of 0.7 suggested by Garza and Williamson (2001), suggesting that populations at these sites have undergone bottlenecks in the recent past. Average inbreeding coefficients (F_{IS}) within collections at the reach scale was -0.010, 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 and Middle Creek). Mean F_{IS} values for collections from whole streams was 0.092, and were somewhat higher for some loci, again indicating some degree of inbreeding within streams (Appendix B, Table 22).

Table 14. 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, F_{is} = inbreeding coefficient, HW= p-values associated with departures from Hardy-Weinberg expectations, Bonferroni alpha = Bonferroni-corrected critical p-value.

Reach	Locus	N	H_0	H_e	A	Range (bp)	M-ratio	F_{is}	HW	Bonferr-oni alpha
Big Lick Creek	*CtoA247*	106	0.018	0.018	2	4	0.50	-0.005	1.0000	0.01
	Lco3	106	0.349	0.333	4	8	0.50	-0.047	0.0289	0.01
	LleC90	106	0.122	0.134	4	8	0.50	0.087	0.0543	0.01
	Rhca20	106	0.009	0.009	2	4	0.50	0.000	1.0000	0.01
	CypG30	106	0.698	0.733	6	24	0.25	0.048	0.0016	0.01
	Lsou8	106	0.547	0.476	2	6	0.33	-0.149	0.1538	0.01
Greasy Creek	*Lco3*	6	0.333	0.318	3	12	0.25	-0.053	1.0000	0.012
	BLI153	6	0.166	0.530	2	2	1.00	0.706	0.1508	0.012
	CypG30	6	0.500	0.742	5	20	0.25	0.348	0.7424	0.012
	Lsou8	6	0.666	0.484	2	6	0.33	-0.429	1.0000	0.012
Hart Creek	*Lco3*	63	0.269	0.341	4	8	0.50	0.212	0.0199	0.01
	LleC90	62	0.080	0.283	4	10	0.40	0.717	0.0000	0.01
	BLI153	63	0.222	0.223	2	2	1.00	0.006	1.0000	0.01
	CypG30	62	0.919	0.754	7	28	0.25	-0.22	0.0001	0.01
	Lsou8	63	0.317	0.497	1	6	0.17	0.364	0.0051	0.01
Hurricane Fork	*CtoA247*	40	0.050	0.096	2	4	0.50	0.483	0.0757	0.012
	Lco3	40	0.450	0.379	2	2	1.00	-0.188	0.3987	0.012
	CypG30	40	0.925	0.823	8	32	0.25	-0.125	0.0025	0.012
	Lsou8	40	0.150	0.140	2	6	0.33	-0.068	1.0000	0.012
Lewis Creek	*BLI153*	3	1.000	0.600	2	4	0.50	-1.000	0.4008	0.025
	CypG30	3	0.000	0.533	2	8	0.25	1.000	0.2003	0.025
Middle Creek	*Lco3*	16	0.250	0.314	2	2	1.00	0.317	0.4334	0.017
	CypG30	16	0.625	0.774	7	32	0.22	0.779	0.4674	0.017
	Lsou8	16	0.187	0.175	2	6	0.33	0.175	1.0000	0.017
Pine Creek	*Lco3*	32	0.281	0.302	4	12	0.33	0.07	0.0988	0.008

LleC90	31	0.354	0.296	2	2	1.00	-0.200	0.5535	0.008
Rhca20	32	0.250	0.222	2	4	0.50	-0.127	1.0000	0.008
BLI153	32	0.031	0.031	2	2	1.00	0.000	1.0000	0.008
CypG30	32	0.875	0.799	6	24	0.25	-0.096	0.0024	0.008
Lsou8	32	0.031	0.031	2	6	0.33	0.000	1.0000	0.008

F_{st} is a classical metric of genetic variation quantifying the between-population component of departures of genotype frequencies from Hardy-Weinberg expectations. 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 15). These values indicate a range from low to rather high differentiation among Clinch Dace populations. To test for isolation-by-distance, we regressed F_{st} values against geographic distance between the downstream-most occupied sites. There was only a weak, non-significant relationship between geographic distance and F_{st} (R^2 = 0.0868).

Table 15. F_{st} values among Clinch Dace populations.

	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 on all samples revealed strongest support for there being five clusters (K = 5) of multilocus genotypes among the seven stream populations (Figure 16). The least negative log-likelihood among 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 (Appendix B, Table 23), both corresponding to five clusters. Population 1 (Hurricane Fork), population 2 (Hart Creek), and population 3 (Lewis Creek) clustered separately, while populations in the remaining four streams displayed some degree of apparent admixture. In STRUCTURE output histograms, the different colors represent different inferred populations. Individual genotypes shown as narrow vertical bars may be proportionally assigned to more than one cluster. When the colors for any one population are mixed, as for populations 4-7 below, then that is evidence that admixture has taken place and that the populations are not entirely differentiated.

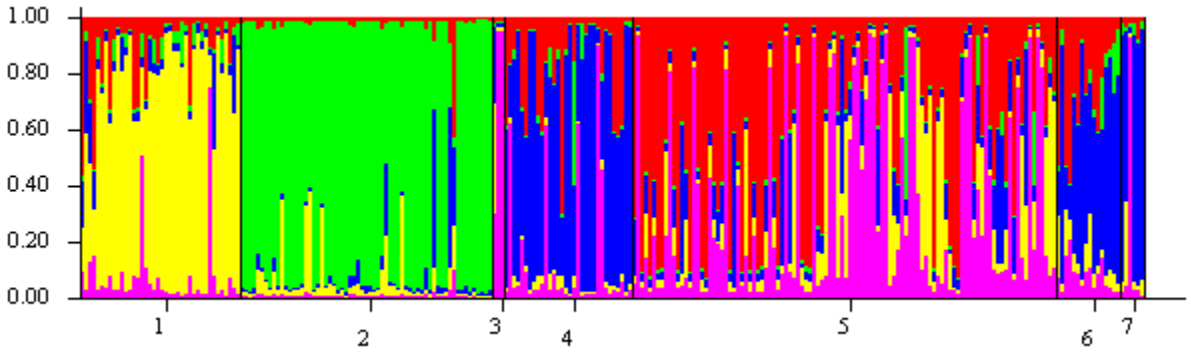

Figure 16. Bar plot of results of STRUCTURE-based Bayesian assignment of individual multilocus genotypes (vertical bars) to K = 5 clusters. The vertical axis shows the proportionate assignment to each of the clusters. The seven stream populations are: 1 = Hurricane Fork, 2= Hart Creek, 3 = Lewis Creek, 4 = Pine Creek, 5 = Big Lick Creek, 6 = Middle Creek, 7 = Greasy Creek.

A follow-up analysis was done to focus upon the four populations showing admixture (Pine Creek, Big Lick Creek, Middle Creek and Greasy Creek). The results for K varied across the five iterations, but the best-supported value of K (i.e., that with the least negative average log-likelihood, -1133.62), was at K = 3 with a variance of 246.9 (Appendix B, Table 24; Figure 17), indicating weak differentiation among Pine, Middle and Greasy creeks (shown in green below), and Big Lick Creek (with mildly differentiated red and blue components).

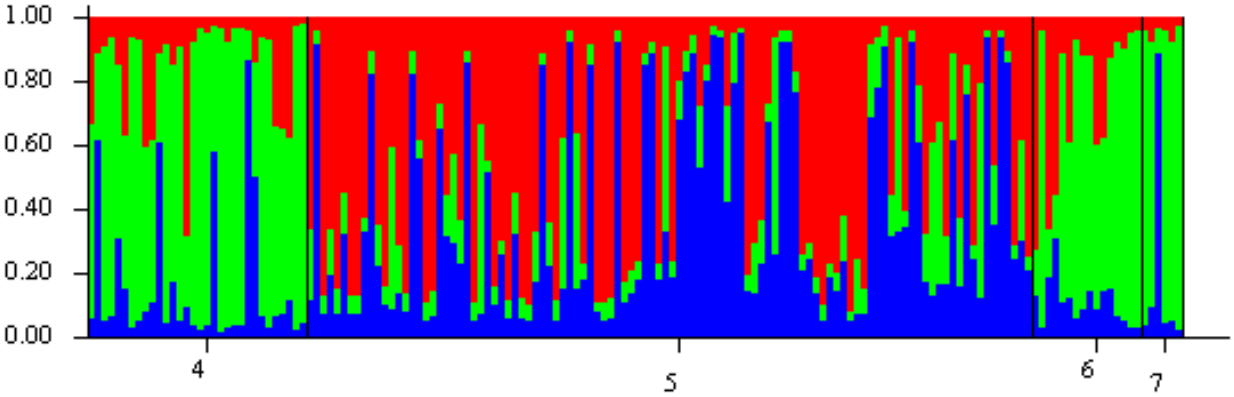

Figure 17. Bar plot of results of STRUCTURE-based Bayesian cluster analysis assignment of individual multilocus genotypes (vertical bars) to K = 3 clusters. This analysis focused upon the four populations which displayed admixture in previous analyses: 4 = Pine Creek, 5 = Big Lick Creek, 6 = Middle Creek. 7 = Greasy Creek.

Cluster analysis of whole streams run independently of one another (Appendix B, Table 25) 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.

Based on the results from application of STRUCTURE, analysis of molecular variance (AMOVA) was conducted for all streams as wholes independently (Table 16) and with Hart Creek, Hurricane Creek, and Lewis Creek held separate while all other streams were combined into a single population cluster (Appendix B, Table 26) in accordance with Bayesian cluster analysis. 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 divergence, indicated by a significant F_{st} value of 0.259.

Table 16. AMOVA results for collection from all streams analyzed separately.

Source of variation	Sum of squares	Variance components	Percentage of variation
Among populations	125.081	0.30325	25.92
Within populations	453.653	0.86654	74.07
Total	578.734	1.16980	

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 17) 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 even other large samples.

Table 17. Estimated effective population sizes. *N* is the total number of samples analyzed and N_e is the estimated 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 18). In Big Lick Creek, which had an estimated effective population size of 106, 19% of pairings 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% were 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 N_e could not be estimated, two individuals were full-siblings. At Middle Creek, with a *N* of 16 and where N_e could not be estimated, 23% were 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.

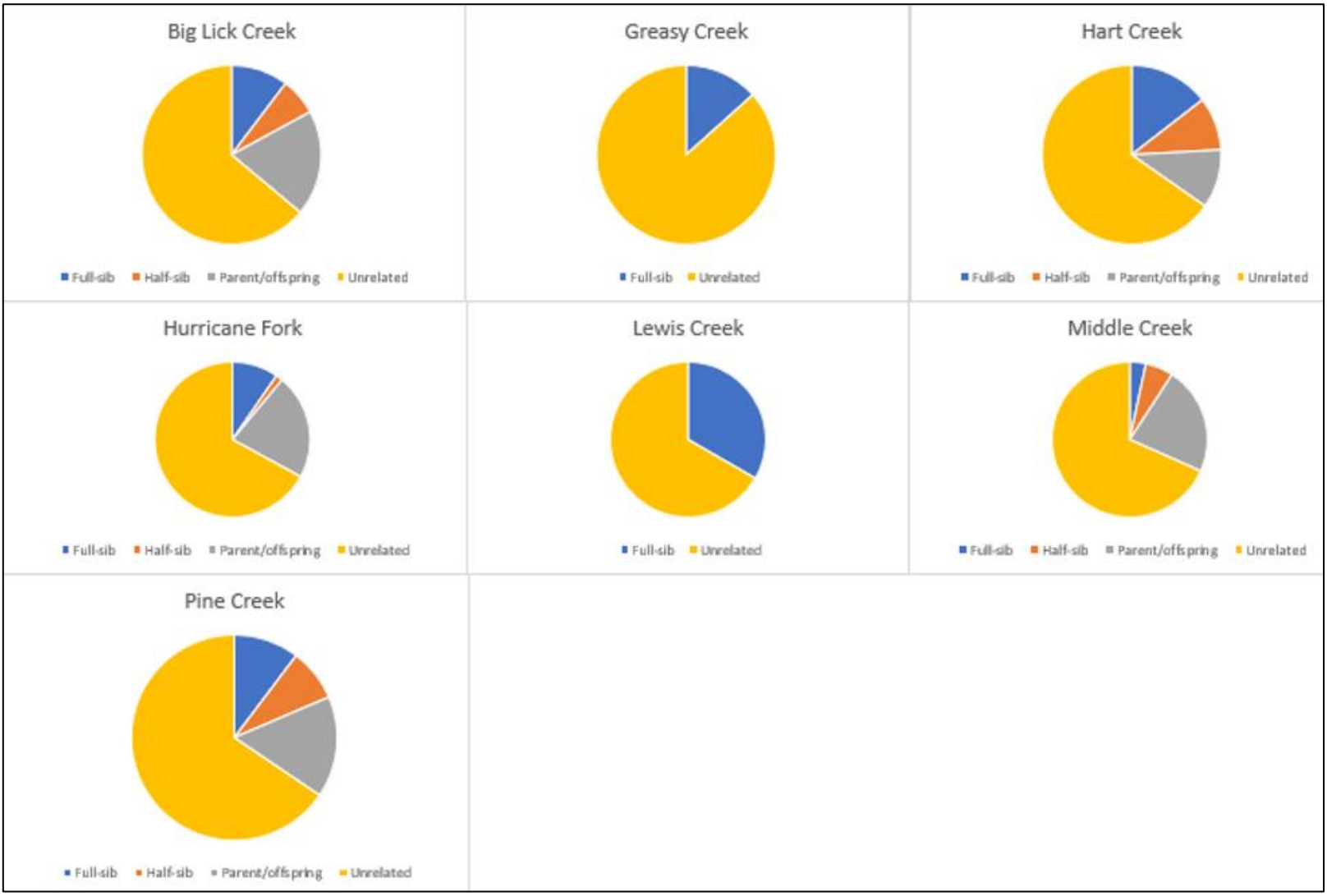

Figure 18. Frequencies of inferred relatedness among individual Clinch Dace in seven streams. Yellow represents unrelated individuals, blue full-siblings, orange half-siblings, and grey parent/offspring relationships.

Discussion

Clinch Dace represented a small portion of fish abundance in all streams surveyed in 2017. The habitat metrics that we analyzed seem to have little influence upon Clinch Dace presence or abundance. We 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 relationship only between Clinch Dace abundance and conductivity. This is counter to the Moore's (2017a) finding of a negative relationship between Clinch Dace and conductivity. Michael Moore in 2015 sampled a wider range of conductivity values than we did. It may be that one outlier, Big Lick Creek 1 Down downstream, where conductivity was high and abundance very high, may have driven the spurious relationship. When the analysis was run without data from site Big Lick Creek 1 Down, the *p*-value was 0.170. It also is possible that because these sites were targeted near sites of known Clinch Dace presence that the range of values for conductivity was within tolerance for Clinch Dace at present and that conductivity at the sites sampled in our study therefore did not inhibit Clinch Dace presence or abundance. It also may be that legacy effects of mining that once caused higher levels of conductivity eliminated some populations of Clinch Dace. In any case, it seems that these instream habitat variables are not currently what is restricting Clinch Dace distribution. Neither does it seem that barriers are fragmenting Clinch Dace populations. 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 appreciable genetic differentiation among Clinch Dace populations. The exceptions were 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, we 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.

The results of this study may help inform management strategies. Translocations from large populations to small, at-risk populations are a possible intervention. However, translocations may not be a viable option where there is significant genetic differentiation among donor and recipient populations, as that could lead to outbreeding depression in the receiving population. Furthermore, the few donor populations have small effective population sizes. Population genetic analysis showed that Clinch Dace populations are fragmented and that genetic drift is operating upon them to decrease diversity within and increase diversity among populations. However, microsatellite markers document selectively neutral variation and this inference does not speak to the possibility that that Clinch Dace populations exhibit adaptive genetic variation. If there is no adaptive variation among populations, then the risk of outbreeding depression as a result of translocations is minimal. Frankham et al. (2011) wrote that the risk 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 of 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 risk of when outbreeding depression risks are high and hence when reestablishing gene flow between populations should be avoided and/or carefully considered (Figure 19).

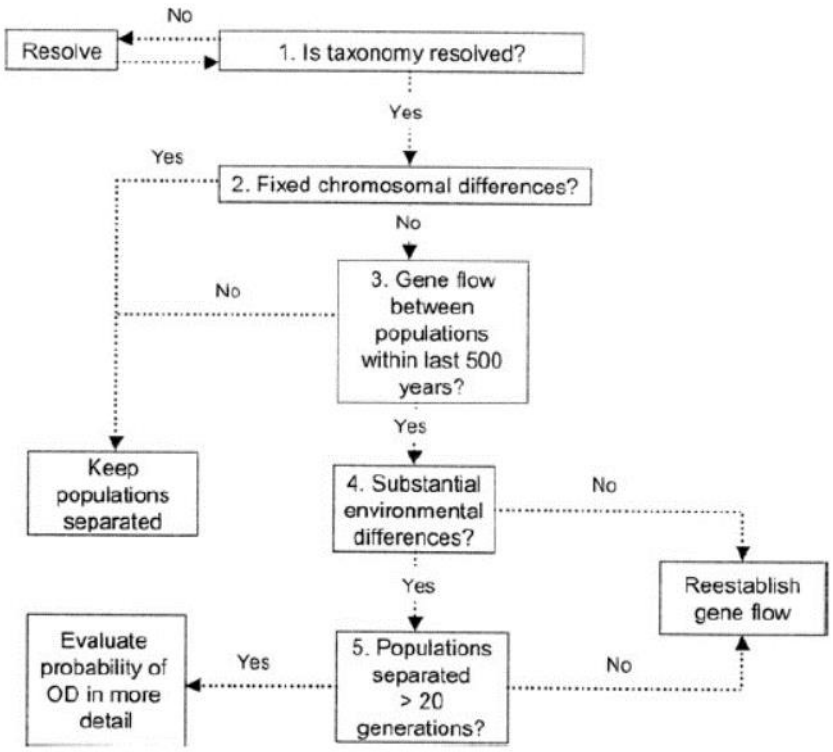

Figure 19. Decision tree for determining the probability of outbreeding depression (OD) between two populations (Frankham et al. 2011).

All Clinch Dace populations exist in relatively close, 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. Therefore, our results do not rule out the viability of translocations if donor populations can first be increased in size. The risk of outbreeding depression is non-zero and we recommend adaptive management strategies and post-translocation monitoring 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). Translocations may be undertaken from large populations into nearby small or absent populations, but adaptive management and monitoring will be essential in assuring that minimum damage is done by 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. In addition to translocations to augment existing populations of Clinch Dace, translocations also can be used to introduce Clinch Dace into streams in the distribution not currently occupied. Augmentation of small populations or establishing new populations via stocking would depend on further development of captive propagation techniques. Limited work on the endangered Laurel Dace resulted in hatchery production in 2019 (Tennessee Aquarium 2019). However, demographic augmentation was deemed ineffective at reducing extinction risk for populations of Roanoke logperch (Roberts et al. 2016). Rather, habitat improvements to increase habitat may have a greater restorative effect.

Population genetic structuring. – Results of Bayesian cluster analysis in Structure showed that Clinch Dace populations in Hurricane Fork, Hart Creek, and Lewis Creek are the most differentiated from those in other streams, while those in the other four streams showed some degree of admixture. The genetic structure is similar to conspecific Laurel Dace, which occurs in multiple small streams but had two management units (Strange and Skelton 2005).

Within-population genetic processes. – AMOVA results of over 25 percent of genetic variation taking place among populations indicates considerable genetic divergence among populations and 74 percent is within 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 inference that genetic drift is playing a strong role in defining genetic population structure. Non-zero F_{is} values and the high degree of relatedness in these populations indicates that some inbreeding is taking place. All these lines of evidence support the inference that isolation is a major force operating upon Clinch Dace populations.

Hence, Clinch Dace populations are generally small, isolated and threatened by human disturbance. 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 undebatable that these populations are small and isolated from each other, despite some degree of admixture among four of the streams sampled. This study identifies sites as possible recipient and donor streams for translocations. It does not prove that adaptive variation among streams is a concern. Translocations may be a viable solution to augment small populations if done with caution and monitoring. There is nothing to suggest that introductions of Clinch Dace into new streams within their distribution would be problematic.

Population viability is dependent on population size and heterozygosity. Loss of heterozygosity reduces that adaptive potential of a species to deal with a changing environment. The 50/500 rule 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 (Franklin 1980). In populations smaller than this, random genetic drift and inbreeding work to eliminate 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 we sampled in 2017 are 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 gene flow among populations is restricted, further exacerbating the loss of genetic diversity within populations.

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 diversity at neutral genetic markers has increased over time due to small population sizes and fragmentation. It may not be possible to definitively prove adaptive variation among streams, but an adaptive management strategy with follow-up monitoring could determine if translocations are leading to outbreeding depression.

Clinch Dace populations are small and fragmented and have lost alleles due to genetic drift. Some also may have been subject to inbreeding. 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. 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 (George et al. 2009). Hart Creek and Hurricane Fork represent strongholds for this species and could be possible donor population 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 amount of 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.

Study Area and Management Recommendations

Life-history characters and low, patchy abundance of the Clinch Dace populations is suggestive of heavy yearly losses of reproductive effort presumably from both emigration and mortality. This reproductive strategy likely results in extremely large variation in reproductive success among individuals and spawning locations. We could identify no concordance in relative abundance from past surveys by White and Orth (2013a), Moore et al. (2017a) and this study. From life-history theory, we can expect that fecundity and juvenile survival are critical parameters for persistence of short-lived fishes, such as the Clinch Dace (Wisdom et al. 2000). Consequently, what remnant populations that exist today are small and highly demographically variable populations. Loss of genetic diversity and concomitant accumulations of deleterious alleles would be expected over ecological time-scales if effective population sizes drop below 100 (Luikart et al. 1998). All but one of the seven estimates of effective population size were below this threshold (Table 17).

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 in nearby streams. Some differentiation was detected in this study, which may be increasing the risk of outbreeding depression by adding individuals from this population to other populations. Population augmentation using Hurricane Fork as a donor stream should be done with caution and follow-up monitoring. As much of the Hurricane Fork site was not buffered in the riparian zone and the substrate was bank-to-bank sediment, this site indicates a certain amount of resilience of Clinch Dace to sedimentation and lack of habitat.

Hart Creek was the largest population found in 2017 and is therefore a candidate for a donor population. Similar to Hurricane Fork, this population shows some differentiation from others in this study, 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 (i.e., genetic augmentation) would be effective. The culvert is slightly perched with a very large, deep scour pool below it and is the crossing for the landowner's drive-way.

Lewis Creek was one of the smaller populations found in 2017, but it may not be a good candidate for augmentation. We were informed by a local resident that houses along Lewis Creek expel 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, we captured Clinch Dace only from the upstream reach of the upper-most site, which is above the houses discharging effluent. At the point where we 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.

Population sizes from the seven reaches on Big Lick Creek vary, but overall they seemed fairly high. Here is where past information will be supportive of apparent genetic admixture with other creeks in this study, which indicates that Big Lick Creek could be used as a donor stream without particular concern about outbreeding depression. Additional sampling would be necessary to determine if populations are large enough to support removal of individuals for translocation purposes.

Pine Creek was under-sampled in 2017 due to lack of landowner permissions, and Clinch Dace were not detected there. Previous sampling documented Clinch Dace in Pine Creek (Moore et al. 2018). Further monitoring of this stream could be warranted before use for translocations. Bayesian cluster analysis showed admixture with other streams in this study, indicating that perhaps gene flow involving the Pine Creek populations has occurred in the past, reducing any risk of outbreeding depression due to translocations.

The population on Middle Creek is relatively small. We caught 16 individuals in 400 meters of stream sampled at that site. The stream is narrow and partially buffered where 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 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.

Another alternative to translocations is captive breeding, where the stock is bred from the same stream into which they will be released. This approach eliminates the threat of outbreeding depression. However, captive breeding is not without problems. Sometimes it can result in fish that are raised adapting to the hatchery environment and that are poorly suited to life in the wild, also 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 and inbreeding depression.

We emphasize that differentiation at neutral genetic markers does not mean that adaptive variation also has taken place. If neutral variation is considerable, but adaptive variation is absent then translocations may be safe. Adaptive management strategies and monitoring should follow any translocations, for either population augmentation or introduction.

Management implications. – The combination of surveys of Clinch Dace habitat, populations, occupancy, and genetic relatedness provides a synthesis that will be useful for management decisions (Table 18). Despite relatively large populations in Hart Creek and Hurricane Fork, these streams may not be suitable as donor streams because of their being so

differentiated from other streams. This increases the 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 streams for introductions into streams where Clinch Dace are currently not found. It should be noted again that variation at neutral genetic markers does not prove adaptive variation. 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 relatively similar environmental conditions, it is very possible that adaptive variation has not taken place.

Table 18. Population sizes, threats, and potential management actions for Clinch Dace occupied streams.

Reach	Total Catch	Pop. Estimate for 100 meters	Lower 95% Confidence Interval	Upper 95% Confidence Interval	Management Action	Threats
Big Lick Creek 1 Downstream	53	29.50	26.50	34.50	None	Habitat
Big Lick Creek 1 Upstream	38	19.00	19.00	19.50	None	Habitat
Big Lick Creek 2 Downstream	1	0.50	0.50	0.50	None	Habitat
Big Lick Creek 2 Upstream	5	4.00	4.00	4.00	None	Habitat
Big Lick Creek 3 Downstream	14	7.00	7.00	8.50	None	Habitat
Big Lick Creek 3 Upstream	24	12.00	12.00	12.50	None	Habitat
Big Lick Creek 4 Downstream	11	6.00	5.50	9.00	None	Habitat
Greasy Creek 1 Downstream	5	6.50	2.50	47.50	Recipient	Beaver impoundment
Greasy Creek 2 Downstream	1	0.50	0.50	0.50	Recipient	Beaver impoundment
Hart Creek 1 Downstream	106	63.59	54.36	73.33	Donor	none
Hart Creek 1 Upstream	26	22.48	20.16	28.68	Culvert retrofit	Barrier
Hurricane 1 Upstream	34	17.50	17.00	19.50	Donor	Habitat
Lewis Creek 4 Upstream	3	2.14	2.14	2.14	Recipient	Water Quality
Middle Creek 3 Downstream	8	4.00	4.00	4.00	Recipient	Habitat
Middle Creek 3 Upstream	8	6.50	4.00	20.00	Recipient	Habitat

All study sites for this study were targeted near sites where Clinch Dace were found by Shannon White or Michael Moore in 2014 and 2015 (Moore 2017a). Moore et al. (2018) tried to predict occupancy of Clinch Dace based on habitat metrics. He found that often sites that scored low in habitat ratings harbored large populations of Clinch Dace. For instance, Hart Creek ranked low in habitat quality, but had an abundant population (Moore et al. 2018). This disconnect between habitat quality and Clinch Dace presence and abundance may make it difficult for managers to locate suitable sites for introductions and prioritize restorations. 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 et al. (2018) also found a negative relationship between Clinch Dace abundance and substrate size. This is consistent with our findings, as sites such as Hurricane Fork, which is bank-to-bank fine sediment, had one of the larger populations.

We recommend that further management actions be taken with an adaptive management approach, as it is not clear from our results that translocations should be ruled out, but rather undertaken initially as a pilot study with follow-up monitoring to determine whether outbreeding depression is apparent as a result of moving locally adapted fish. Stream restoration activities may not be warranted, as the Clinch Dace shows some resilience to habitat degradation, such as sedimentation and lack of woody debris. We only found one culvert on Hart Creek which could be considered for a retrofit and that does seem to be acting as a barrier to Clinch Dace movement. A long-term monitoring program should be implemented to monitor for changes in abundance in response to changing environmental conditions or management actions, as well as continued efforts to get permission to sample sites on lands owned by coal companies. 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.

Research and Monitoring Recommendations. — We characterized seven populations of Clinch Dace across its known range. Within the occupied streams, the species is patchily distributed and vulnerable to climatic variation and human disturbances in the watershed. Population size is presumably a strong influence on extinction risk (Reed et al. 2003). Further research could measure temporal changes in abundance and characterize the relationship between population size and extinction risk and identify minimum viable population thresholds. This type of assessment is called population viability analysis (Gilpin and Soule´ 1986; Burgman et al.1993), and has become an effective tool to predict the likely fate of populations based on the balance between birth, death, reproduction, immigration and emigration. This line of research should accompany attempts to establish an ark population and examine possible benefits of alternative augmentation strategies to decreasing risks of extinction.

All the sampled populations of Clinch Dace were possible only by permission of landowners. This study represents the eleventh sampling effort that collected Clinch Dace since 1999. Due to lack of access, we were unable to sample sites on Hess, Indian, Laurel, Left Fork Coal, Mudlick, Pine, Town Hill, and West Fork Big Creeks. Future monitoring should include efforts to access sites on these nine streams in addition to those sampled in this study. Considering the rarity and fragility of Clinch Dace, we recommend the use of a series of baited, mesh minnow traps to estimate site occupancy as well as relative abundance in five streams that have highest populations. Sampling should commence after spawning season, preferably early

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Appendix A

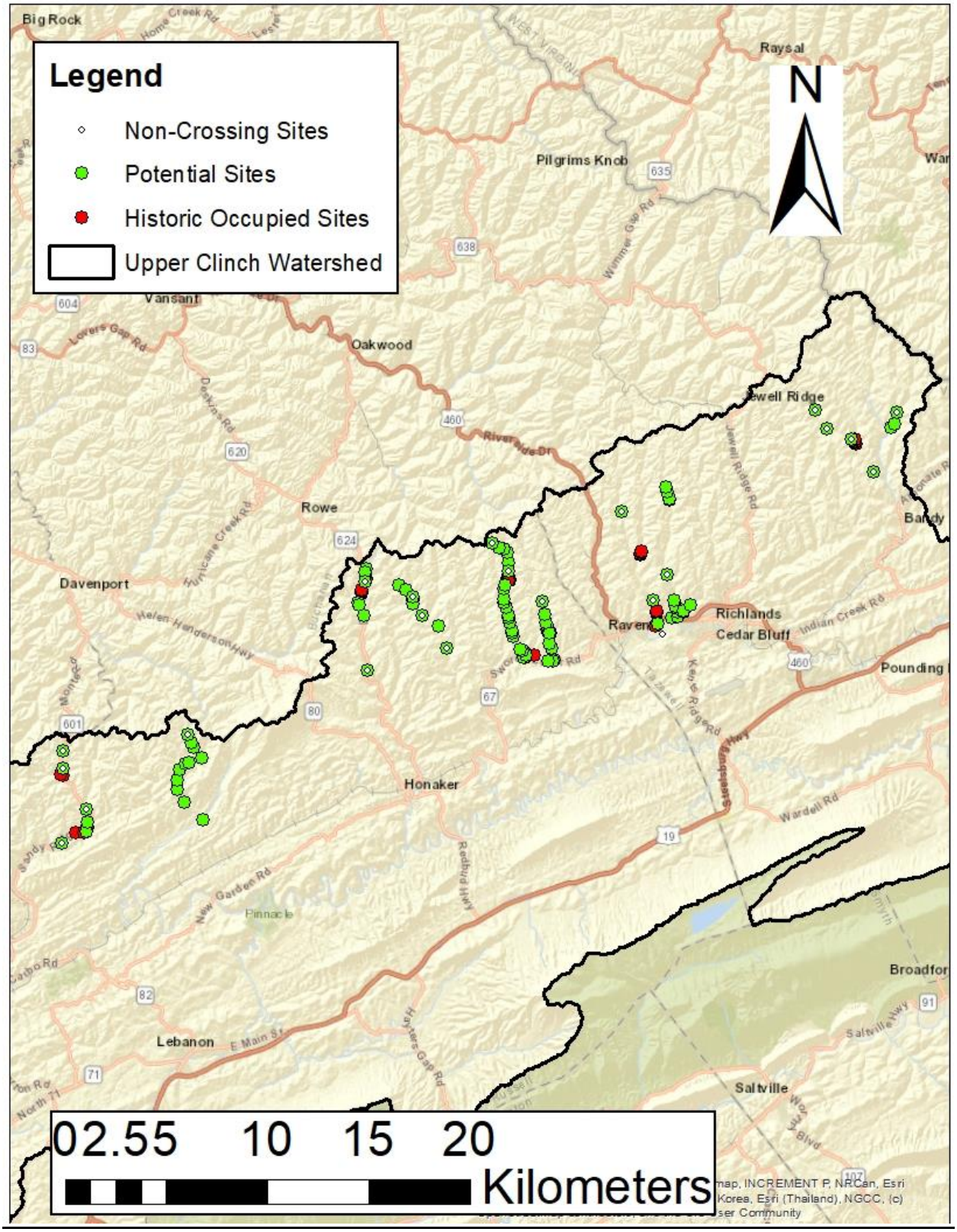

Figure 20. Potential study sites considered for inclusion.

Appendix B

Table 19. 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 Downstream (200 m)	Western 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 Upstream (200 m)	Western Blacknose Dace	689	359	351	366
	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 Downstream (200 m)	Western Blacknose Dace	632	323	316	328
	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 Upstream (200 m)	Western Blacknose Dace	427	320	320	320
	Clinch Dace	5	4	4	4

	Creek Chub	40	30	30	30
	Fantail Darter	55	41	41	41
	Largescale Stoneroller	54	41	41	41
Big Lick Creek 3 Downstream (200 m)	Western Blacknose Dace	525	286	274	298
	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 Upstream (200 m)	Western Blacknose Dace	502	254	251	257
	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 Downstream (200 m)	Western Blacknose Dace	409	213	207	219
	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 Downstream (200 m)	Western Blacknose Dace	323	167	163	172
	Clinch Dace	5	7	3	48
	Creek Chub	93	50	47	55
	Fantail Darter	18	9	9	10
	Largescale Stoneroller	166	86	83	89
	Rosyside Dace	154	85	78	92
	White Sucker	25	13	13	14

Greasy Creek 1 Upstream (200 m)	Western Blacknose Dace	138	72	69	75
	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 Downstream (200 m)	Western Blacknose Dace	387	214	202	225
	Clinch Dace	1	1	1	1
	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 Downstream (195 m)	Western Blacknose Dace	437	227	224	230
	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 Upstream (129 m)	Western Blacknose Dace	272	227	216	239
	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 Downstream (158 m)	Western Blacknose Dace	360	184	181	188
	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 Upstream (110 m)	Western Blacknose Dace	171	158	155	162
	Creek Chub	19	17	17	18
	Fantail Darter	20	18	18	20
	Largescale Stoneroller	49	47	45	22
Hurricane 1 Upstream (200 m)	Bluegill	3	2	2	2
	Bluntnose Minnow	11	6	6	6
	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 Downstream (180 m)	Western Blacknose Dace	196	112	109	115
	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 Sucker	3	2	2	3
Jackson Fork 1 Upstream (156 m)	Western Blacknose Dace	243	163	157	170
	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 Upstream (200 m)	Western Blacknose Dace	259	131	130	133
	Creek Chub	24	12	12	13
	Fantail Darter	37	27	19	42
	Largescale Stoneroller	3	2	2	2
	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 Downstream (200 m)	Western Blacknose Dace	520	273	265	280
	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 Upstream (35 m)	Western Blacknose Dace	94	296	272	325
	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 Upstream (200 m)	Western Blacknose Dace	431	222	216	226
	Creek Chub	72	36	36	37

	Fantail Darter	62	33	31	36
	Largescale Stoneroller	223	114	112	117
	Northern Hogsucker	8	4	4	5
	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 Downstream (172 m)	Western Blacknose Dace	275	160	163	181
	Brown Trout	1	1	1	1
	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 Upstream (140 m)	Western Blacknose Dace	297	219	213	224
	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 Downstream (200 m)	Western Blacknose Dace	89	45	45	47
	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 Upstream (200 m)	Western Blacknose Dace	44	23	22	24
	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 Upstream (188 m)	Western Blacknose Dace	913	503	495	512
	Creek Chub	91	50	48	53
	Fantail Darter	112	60	60	71
	Highland 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 Downstream (200 m)	Bigeye Chub	37	19	19	20
	Western 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

Appendix C

Table 20. 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	-0.023	22
	Lco3	no	-0.2929	22
	LleC90	no	-0.0955	22
	BLI_84	no	0	22
	Rhca20	no	0	22
	BLI153	no	0	22
	CypG30	no	0.0115	22
	MFW1	no	0	22
	Lsou8	no	-0.4853	22
Big Lick 1 up	*CtoA247*	no	0	24
	Lco3	no	-0.2362	24
	LleC90	no	-0.0421	24
	BLI_84	no	0	24
	Rhca20	no	0	24
	BLI153	no	0	24
	CypG30	no	-0.0891	24
	MFW1	no	0	24
	Lsou8	no	-0.1064	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	-0.1056	5
	LleC90	no	-0.1056	5
	BLI_84	no	0	5
	Rhca20	no	0	5
	BLI153	no	0	5
	CypG30	no	-0.229	5
	MFW1	no	0	5
	Lsou8	no	0	5
Big Lick 3 down	*CtoA247*	no	0	19

	Lco3	no	0.0232	19
	LleC90	no	-0.0267	19
	BLI_84	no	0	19
	Rhca20	no	0	19
	BLI153	no	0	19
	CypG30	no	0.0726	19
	MFW1	no	0	19
	Lsou8	no	-0.2086	19
Big Lick 3 up	*CtoA247*	no	-0.0211	24
	Lco3	no	-0.1292	24
	LleC90	no	-0.0636	24
	BLI_84	no	0	24
	Rhca20	no	-0.0211	24
	BLI153	no	0	24
	CypG30	no	0.0291	24
	MFW1	no	0	23
	Lsou8	no	0.0774	24
Big Lick 4 down	*CtoA247*	no	0	12
	Lco3	no	-0.0465	12
	LleC90	yes	0.2084	12
	BLI_84	no	0	12
	Rhca20	no	0	12
	BLI153	no	0	12
	CypG30	yes	0.1766	12
	MFW1	no	0	12
	Lsou8	no	-0.1472	12
Greasy Creek 1 down	*CtoA247*	no	0	5
	Lco3	no	-0.1056	5
	LleC90	no	0	5
	BLI_84	no	0	5
	Rhca20	no	0	5
	BLI153	no	0.2649	5
	CypG30	no	0.2102	5
	MFW1	no	0	4
	Lsou8	no	-0.5528	5
Greasy Creek 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
Hart Creek 1 down	*CtoA247*	no	0	20
	Lco3	no	0.0603	20
	LleC90	no	-0.078	20
	BLI_84	no	0	20
	Rhca20	no	0	20
	BLI153	no	-0.134	20
	CypG30	no	-0.1613	19
	MFW1	no	0	19
	Lsou8	no	0.118	20
Hart Creek 1 up	*CtoA247*	no	0	22
	Lco3	yes	0.206	22
	LleC90	no	-0.023	22
	BLI_84	no	0	22
	Rhca20	no	0	22
	BLI153	no	0.1425	22
	CypG30	no	-0.1693	22
	MFW1	no	0	21
	Lsou8	no	-0.0589	22
Hart Creek 2 down	*CtoA247*	no	0	20
	Lco3	no	-0.0029	21
	LleC90	yes	0.3817	20
	BLI_84	no	0	21
	Rhca20	no	0	20
	BLI153	no	-0.1835	21
	CypG30	no	-0.0832	21
	MFW1	no	0	20
	Lsou8	no	-0.1548	21
Hurricane Fork 1 down	*CtoA247*	no	0	20
	Lco3	no	-0.1633	20
	LleC90	no	0	20
	BLI_84	no	0	20
	Rhca20	no	0	20
	BLI153	no	0	20
	CypG30	no	-0.0685	20
	MFW1	no	0	18
	Lsou8	no	-0.1056	20
Hurricane Fork 1 up	*CtoA247*	no	0.1456	20
	Lco3	no	-0.1848	20
	LleC90	no	0	20

	BLI_84	no	0	20
	Rhca20	no	0	20
	BLI153	no	0	20
	CypG30	no	-0.0907	20
	MFW1	no	0	20
	Lsou8	no	-0.0513	20
Lewis Creek 4 up	*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
Middle Creek 3 down	*CtoA247*	no	0	8
	Lco3	no	-0.0646	8
	LleC90	no	0	7
	BLI_84	no	0	8
	Rhca20	no	0	8
	BLI153	no	0	8
	CypG30	no	0.0335	8
	MFW1	no	0	8
	Lsou8	no	-0.2094	8
Middle Creek 3 up	*CtoA247*	no	0	8
	Lco3	no	0.0607	8
	LleC90	no	0	8
	BLI_84	no	0	8
	Rhca20	no	0	8
	BLI153	no	0	8
	CypG30	no	0.0752	8
	MFW1	no	0	8
	Lsou8	no	0	8
Pine Creek IM	*CtoA247*	no	0	16
	Lco3	no	-0.1657	16
	LleC90	no	-0.2094	16
	BLI_84	no	0	14
	Rhca20	no	0	16
	BLI153	no	0	16
	CypG30	no	-0.1277	16
	MFW1	no	0	14
	Lsou8	no	-0.0318	16

Pine Creek low	*CtoA247*	no	0	7
	Lco3	no	0.1489	7
	LleC90	no	-0.1548	7
	BLI_84	no	0	7
	Rhca20	no	-0.622	7
	BLI153	no	-0.0742	7
	CypG30	no	0.0267	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	0.1376	6
	LleC90	no	-0.3675	5
	BLI_84	no	0	6
	Rhca20	no	-0.1835	6
	BLI153	no	0	6
	CypG30	no	-0.2294	6
	MFW1	no	0	6
	Lsou8	no	0	6

Table 21. 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	-0.0047	106
	Lco3	no	-0.056	106
	LleC90	no	0.0296	106
	BLI_84	no	0	106
	Rhca20	no	-0.0047	106
	BLI153	no	0	106
	CypG30	no	0.0207	106
	MFW1	no	0	104
	Lsou8	no	-0.0808	106
Greasy Creek	*CtoA247*	no	0	6
	Lco3	no	-0.1743	6
	LleC90	no	0	6
	BLI_84	no	0	6
	Rhca20	no	0	6
	BLI153	no	0.2845	6
	CypG30	no	0.1325	6
	MFW1	no	0	5
	Lsou8	no	-0.4226	6
Hart Creek	*CtoA247*	no	0	63
	Lco3	yes	0.0948	64
	LleC90	yes	0.2516	63
	BLI_84	no	0	64
	Rhca20	no	0	63
	BLI153	no	-0.0011	64
	CypG30	no	-0.1346	63
	MFW1	no	0	61
	Lsou8	yes	0.1645	64
Hurricane Fork	*CtoA247*	yes	0.1199	40
	Lco3	no	-0.1173	40
	LleC90	no	0	40
	BLI_84	no	0	40
	Rhca20	no	0	40
	BLI153	no	0	40
	CypG30	no	-0.0705	40
	MFW1	no	0	38
	Lsou8	no	-0.078	40
Lewis Creek	*CtoA247*	NA	NA	3
	Lco3	NA	NA	3
	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	0.0792	16
	LleC90	no	0	15
	BLI_84	no	0	16
	Rhca20	no	0	16
	BLI153	no	0	16
	CypG30	no	0.0861	16
	MFW1	no	0	16
	Lsou8	no	-0.0986	16
Pine Creek	*CtoA247*	no	0	32
	Lco3	no	0.0685	32
	LleC90	no	-0.1968	31
	BLI_84	no	0	30
	Rhca20	no	-0.134	32
	BLI153	no	-0.0157	32
	CypG30	no	-0.0708	32
	MFW1	no	0	30
	Lsou8	no	-0.0157	32

Table 22. 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, Bonferoni 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	0.500	0.000	1.000	0.010
	Lco3	22	0.500	0.384	2	6	0.333	-0.313	0.269	0.010
	LleC90	22	0.182	0.169	2	2	1.000	-0.077	1.000	0.010
	CypG30	22	0.636	0.661	4	24	0.167	0.038	0.224	0.010
	Lsou8	22	0.864	0.511	2	6	0.333	-0.720	0.002	0.010
Big Lick 1 up	*Lco3*	24	0.417	0.337	2	6	0.333	-0.243	0.540	0.013
	LleC90	24	0.083	0.082	2	4	0.500	-0.011	1.000	0.013
	CypG30	24	0.833	0.738	4	24	0.167	-0.132	0.090	0.013
	Lsou8	24	0.583	0.496	2	6	0.333	-0.179	0.434	0.013
Big Lick 2 up	*Lco3*	5	0.200	0.200	2	8	0.250	0.000	1.000	0.016
	LleC90	5	0.200	0.200	2	2	1.000	0.000	1.000	0.016
	CypG30	5	1.000	0.800	4	24	0.167	-0.290	1.000	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	1.000	0.000	1.000	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	0.500	0.000	1.000	0.008
	Lco3	24	0.250	0.231	4	8	0.500	-0.082	1.000	0.008
	LleC90	24	0.125	0.121	3	8	0.375	-0.030	1.000	0.008
	Rhca20	24	0.042	0.042	2	4	0.500	0.000	1.000	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	1.000	0.000	1.000	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
	Lsou8	11	0.273	0.247	2	6	0.333	-0.111	1.000	0.013

Greasy Creek 1 down	*Lco3*	5	0.200	0.200	2	12	0.167		1.000	0.013
	BLI153	5	0.200	0.556	2	2	1.000		0.365	0.013
	CypG30	5	0.400	0.756	5	20	0.250		0.050	0.013
	Lsou8	5	0.800	0.533	2	6	0.333		0.429	0.013
Hart Creek 1 down	*Lco3*	20	0.250	0.296	2	6	0.333	0.159	0.467	0.010
	LleC90	20	0.150	0.142	2	2	1.000	-0.056	1.000	0.010
	BLI153	20	0.250	0.224	2	2	1.000	-0.118	1.000	0.010
	CypG30	19	0.947	0.762	5	20	0.250	-0.251	0.047	0.010
	Lsou8	20	0.150	0.224	2	6	0.333	0.337	0.246	0.010
Hart Creek 1 up	*Lco3*	22	0.091	0.241	2	6	0.333	0.628	0.025	0.010
	LleC90	22	0.045	0.045	2	2	1.000	0.000	1.000	0.010
	BLI153	22	0.091	0.169	2	2	1.000	0.468	0.138	0.010
	CypG30	22	1.000	0.795	7	28	0.250	-0.266	0.084	0.010
	Lsou8	22	0.500	0.460	2	6	0.333	-0.090	1.000	0.010
Hart Creek 2 down	*Lco3*	21	0.476	0.483	4	8	0.500	0.015	0.222	0.010
	LleC90	20	0.050	0.512	3	8	0.375	0.905	0.000	0.010
	BLI153	21	0.333	0.285	2	2	1.000	-0.176	1.000	0.010
	CypG30	21	0.810	0.725	5	20	0.250	-0.120	0.373	0.010
	Lsou8	21	0.286	0.251	2	6	0.333	-0.143	1.000	0.010
Hurricane Fork 1 down	*Lco3*	20	0.300	0.262	2	2	1.000	-0.152	1.000	0.016
	CypG30	20	0.900	0.814	8	32	0.250	-0.109	0.011	0.016
	Lsou8	20	0.200	0.185	2	6	0.333	-0.086	1.000	0.016
Hurricane Fork 1 up	*CtoA247*	20	0.100	0.185	2	4	0.500	0.465	0.153	0.013
	Lco3	20	0.600	0.467	2	2	1.000	-0.295	0.328	0.013
	CypG30	20	0.950	0.829	8	32	0.250	-0.150	0.344	0.013
	Lsou8	20	0.100	0.097	2	6	0.333	-0.027	1.000	0.013
Lewis Creek 4 up	*BLI153*	3	1.000	0.600	2	4	0.500	-1.000	0.401	0.025
	CypG30	3	0.000	0.533	2	8	0.250	1.000	0.200	0.025
Middle Creek 3 down	*Lco3*	8	0.125	0.125	2	2	1.000	0.000	1.000	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	1.000	0.016
Middle Creek 3 up	*Lco3*	8	0.375	0.458	2	2	1.000	0.192	1.000	0.025
	CypG30	8	0.625	0.800	6	20	0.300	0.231	0.298	0.025
Pine Creek IM	*Lco3*	16	0.313	0.280	3	8	0.375	-0.119	1.000	0.013
	LleC90	16	0.375	0.315	2	2	1.000	-0.200	1.000	0.013
	CypG30	16	0.938	0.784	5	24	0.208	-0.203	0.180	0.013
	Lsou8	16	0.063	0.063	2	6	0.333	0.000	1.000	0.013
Pine Creek low	*Lco3*	7	0.286	0.385	3	6	0.500	0.273	0.234	0.010
	LleC90	7	0.286	0.264	2	2	1.000	-0.091	1.000	0.010
	Rhca20	7	0.857	0.527	2	2	1.000	-0.714	0.160	0.010
	BLI153	7	0.143	0.143	2	2	1.000	0.000	1.000	0.010
	CypG30	7	0.714	0.846	5	24	0.208	0.167	0.090	0.010
Pine Creek mid	*CypG30*	3	0.667	0.800	4	24	0.167	0.200	0.603	0.050
Pine Creek up	*Lco3*	6	0.333	0.439	3	8	0.375	0.259	0.276	0.013
	LleC90	5	0.600	0.467	2	2	1.000	-0.333	1.000	0.013
	Rhca20	6	0.333	0.303	2	2	1.000	-0.111	1.000	0.013
	CypG30	6	1.000	0.803	5	16	0.313	-0.277	1.000	0.013

Table 23. 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 24. 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 25. 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
	3	-82.12	4.16
	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 26. 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