

Genetic assessment of Abrams Creek reintroduction program for the federally threatened yellowfin madtom (*Noturus flavipinnis*), and endangered smoky madtom (*Noturus baileyi*) and Citico darter (*Etheostoma sitikuense*)

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Summary of major findings

1. Low levels of genetic diversity were observed for each species
2. Comparisons of genetic diversity were significantly different between Citico and Abrams Creek populations for each species
3. These differences were attributed to variance in reproductive success (either in the hatchery or wild) and not as having used too few brood for the reintroduction program
4. Simulations indicated that at least four effective migrants per generation are necessary to minimize genetic differentiation between Citico and Abrams Creek populations for each species
5. Low levels of genetic diversity in Citico creek species were attributed to wholesale deforestation of the surrounding watershed in the 1900s and highlight the importance of protecting these species from further genetic and demographic bottlenecks.

INTRODUCTION

The southeastern portion of the United States has been identified as a region of high ichthyofaunal diversity as well as a region that harbors the greatest number of imperiled freshwater fishes (Warren et al. 2000; Jelks et al. 2008);. Of the nearly 700 fish species found in southeastern United States waters, more than 25% are considered imperiled. Disproportionately represented among these imperiled fishes are madtom catfishes of the ictalurid genus *Noturus* and darters of the percid genus *Etheostoma* (Warren et al. 2000; Jelks et al. 2008). While principal causes of freshwater fish imperilment in the southeastern United States are often associated with habitat loss and degradation from impoundments, urbanization, agriculture, deforestation, erosion, and pollution, (Moyle and Leidy 1992), the aforementioned ictalurid and percid genera are often at greater risk due to their specialization for lotic, benthic habitats (Angermeier 1995), and because many are geographically and/or genetically isolated (Warren et al. 2000). One of the most notable and poignant examples of the wholesale loss of a native fish community due to human induced changes was that of Abrams Creek, TN (Fig. 1). In the summer of 1957 coinciding with the closing of Chilhowee Dam, an ichthyocide was applied to 14 mile stretch of the creek in order to create a recreation trout fishery. As a result, at least 20 species of fish were extirpated including the now the federally threatened yellowfin madtom (*Noturus flavipinnis*), and endangered smoky madtom (*N. baileyi*) and Citico darter (*Etheostoma sitikuense*).

Until a relatively recent discovery of *N. baileyi* in Citico Creek , TN (Bauer et al. 1983) and *N. flavipinnis* in the Powell River, TN (Taylor et al. 1971), both madtom species were believed extinct. Currently *N. baileyi* is known from only a 13.8 km portion of Citico Creek but has been reintroduced into Abrams Creek and Tellico River (Fig. 1; Dinkins and Shute 1996; Shute et al. 2005). *N. flavipinnis* was thought to have been wide spread throughout the upper Tennessee River drainage system (Taylor 1969), but now has a disjunct distribution inhabiting portions of Citico Creek and Clinch and Powell

rivers (Etnier and Starnes 1993; Dinkins and Shute 1996). Both *Noturus* species often seek shelter under bedrock crevices making detection and collection difficult (Shute et al. 2005; Davis et al. 2011). Like many other *Noturus* species, *N. flavipinnis* and *N. baileyi* deposit eggs (average clutch size approximates 55 and 36 eggs/nest, respectively) in a cavity and are guarded by the male (Dinkins and Shute 1996). Sexual maturity for both males and females is reached at 1-2 years of age for both species with an average generation time of two for *N. baileyi* and three for *N. flavipinnis* (Etnier and Starnes 1993; Dinkins and Shute 1996). Individuals attain lengths of 68.9mm SL for *N. baileyi* and 96 mm SL for *N. flavipinnis* (Dinkins and Shute 1996).

The federally endangered *E. sitikuense*, is a small (28-64 mm SL) benthic darter species that until recently, was a member of the *Etheosotma percnurum* species complex. It was elevated to species status based on meristic, morphometric, and pigmentation differences among other members of the *E. percnurum* complex (Blanton and Jenkins 2008). The distribution of *E. sitikuense* is confined to a 3.5 km stretch of Citico Creek, TN, but has been reintroduced to Abrams Creek and Tellico River (Shute et al. 2005). Like both madtom species, *E. sitikuense* is nocturnal seeking shelter beneath cobble and bolder substrate. Females lay adhesive eggs (19-44) in nesting cavities created and guarded by males beneath rocks and are capable of producing multiple clutches as suggested by varied egg counts ($n = 23-200$) found on the undersides of rocks (Layman 1991). It is not known when *E. sitikuense* reaches sexual maturity in the wild (sexual maturity of age-1 fish has been observed under hatchery conditions; P Rakes, Conservation Fisheries, Inc, pers. comm.), but appreciable mortality of age 2 adults occurs soon after spawning suggesting that the average generation time is no greater than two. Note that most of the known life history of *E. sitikuense* is from specimens collected from the Little River, TN and that these specimens are now considered as *E. marmopinum* (Blanton and Jenkins 2008). Thus slight variation in the life history may exist between *E. marmopinum* and *E. sitikuense*.

In part due to the rarity of each of these species (both in terms of abundance and distribution), a captive propagation and reintroduction program was initiated by a multi-agency team (in accordance with United States Fish and Wildlife Service recovery plans) in an effort to restore the extirpated Abrams Creek population for each species. Reintroductions began in 1986 for *N. baileyi* and *N. flavipinnis* and 1993 for *E. sitikuense*, and entailed the annual removal of nests from Citico Creek for hatchery grow out and subsequent stocking of offspring in Abrams Creek (see Shute et al 2005 for details). Furthermore, in the spring of 2004, a relicensing settlement agreement for the Tapoco Hydroelectric Project (Federal Energy Regulatory Commission No. 2169) established a fish passage plan for the continued passage of *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* from Citico to Abrams Creek. The rationale by the United States Fish and Wildlife Service was that historic migration occurred between Citico and Abrams populations (prior to the construction of Chilhowee Dam) and that this migration rate should be mimicked and maintained via the reintroduction effort. The objective of this plan, thus, was to move a certain number of each of the targeted fish species' nests (*N. flavipinnis*, *N. baileyi*, and *E. sitikuense*) from Citico Creek to Abrams Creek in an effort to maintain USFWS's goal of one effective genome per generation that was deemed sufficient to obtain genetic mixing between the populations for each species of concern.

Although each species' reintroduction effort appears demographically successful (i.e., the observed occurrence of natural reproduction and multiple age classes; Shute et al. 2005), perceived genetic risks (i.e., loss of genetic diversity due to inbreeding or genetic drift) should be evaluated. For example, the source population generally should have a high degree of genetic diversity and genetic similarity to that of the new or recipient population to offset the potential decrease in average fitness associated with inbreeding and/or a loss of genetic variation (Miller and Kapuscinski 2003). Furthermore, the USFWS recommendation of one effective genome per generation as a measure of fish passage success should be evaluated because this theoretical expectation is based on many simplifying assumptions of which many are routinely violated (Mills and Allendorf 1996; Vucetich and Waite 2000).

Finally, an understanding of past and present processes shaping present levels of genetic variation is also critical to management and conservation planning because information gleaned from conservation genetics can assist in the proper design, implementation, and monitoring of management and conservation strategies for imperiled species. For example, populations or species that have undergone population bottlenecks throughout their evolutionary history may have reduced genetic load (i.e., a reduction in mean fitness of a population resulting from detrimental variation) and be less prone to have inbreeding depression during subsequent population bottlenecks (Hedrick 1994; 2001). As a consequence, such a population may have increased viability and be more likely to recover from near-extinction/extirpation than a population lacking such a history (Hedrick 2001).

The objective of this study was to assess putative genetic risks associated with the Abrams Creek reintroduction effort for *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* as well as provide base-line genetic data for genetic risk assessment and monitoring of each species inhabiting Citico Creek. These objectives were accomplished by 1) estimating and comparing levels of genetic diversity and divergence between Citico and Abrams Creek populations for each species, 2) estimating effective population size for each species, 3) estimating the level of migration necessary to minimize genetic differentiation between Abrams and Citico creek populations for each species, and 4) understanding the processes that have shaped present estimates of genetic diversity in Citico Creek populations for each species. In doing so, the effectiveness of each reintroduction program and the Tapoco Hydroelectric Project fishway passage strategy can be quantitatively evaluated.

MATERIALS AND METHODS

Sampling Design. Tissue collections were conducted by Conservation Fisheries, Inc. via mask and snorkel (Dinkins and Shute 1996). Sampling was performed from 2008-2010, encompassed the known range of each fish species in Citico and Abrams creeks, and entailed noninvasive pelvic or caudal fin collections

(Table 1). All tissue samples were placed in 95% non-denature ethanol and archived at to the United States Fish and Wildlife Service Conservation Genetics Lab in Warm Springs GA. Genomic DNA was extracted from each fin clip using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, California) protocol.

Molecular Methods. Polymerase chain reaction (PCR) amplification conditions for *N. flavipinnis* and *N. baileyi* followed that of previously outlined protocols (Williams and Moyer In press). Primer information for *N. flavipinnis* and *N. baileyi* can be found in Tables 2 and 3, respectively. For *E. sitikuense*, we used a suite of nine microsatellite markers (Table 4) known to amplify in other *Etheostoma* species (Tonniss 2006; Beneteau et al. 2007; Gabel et al. 2008). PCRs were performed in 8 μ L reaction volumes consisting of 30–100 ng of template DNA, 1 \times *Taq* reaction buffer (Applied Biosystems Inc.), 3.25 mM MgCl₂, 0.375 mM of each dNTP, 0.50 μ M of each primer (Tables 2-4), and 0.0875 U *Taq* polymerase (Applied Biosystems, Inc.). PCR conditions for *Ebl1*, *Ebl2*, *Ebl4*, *Ebl6*, *Ebl8* were an initial denaturation at 94 °C (10 min.), followed by a touchdown procedure involving 33 cycles and consisting of denaturing (94 °C, 30 s), annealing, and extension (74 °C, 30 s) cycles, where the initial annealing temperature was initiated at 56 °C (30 s), and decreased by 0.2 °C/cycle. For *Eca11EPA* and *Eca13EPA* PCR conditions were an initial denaturation at 94 °C (10 min.), then 27 cycles each at 94 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s cycles, followed by an extension step at 72 °C for 10 min. Finally, *Esc26b* and *Esc187* PCR conditions were an initial denaturation at 94 °C (10 min.), then 33 cycles each at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s cycles, followed by an extension step at 72 °C for 10 min.

Prior to electrophoresis, 2 μ L of a 1:100 dilution of PCR product was mixed with a 8 μ L solution containing 97% formamide and 3% Genescan LIZ 500 size standard (Applied Biosystems, Inc.).

Microsatellite reactions were visualized with an ABI 3130 genetic analyzer (Applied Biosystems, Inc.) using fluorescently labeled forward primers and analyzed using GeneMapper software v3.7 (Applied Biosystems, Inc.).

Estimation of genetic differentiation. Tests for gametic disequilibrium (all pairs of loci per population, where the population was either Abrams or Citico creeks) and locus conformance to Hardy–Weinberg equilibrium (HWE; for each locus in the population) were implemented using GENEPOP v4.0.10 (Raymond and Rousset 1995) for each species. Significance levels for all simultaneous tests were adjusted using a sequential Bonferroni correction (Rice 1989).

We compared basic estimators of genetic diversity for Abrams and Citico creek populations for each species. Specifically, we tested for homogeneity in average allelic richness, observed heterozygosity, expected heterozygosity, and fixation index between populations. The fixation index along with genetic diversity in the form of per locus observed and expected heterozygosity were calculated using the computer program GenAlEx v6.4 (Peakall and Smouse 2006). The program HP-RARE (Kalinowski 2005) was used to estimate allelic richness. Tests for significance were conducted using the Wilcoxon rank-sum test (Sokal and Rohlf 1995) as implemented in S-Plus v7.0 (Insightful Corporation).

To assess the degree of genetic differentiation between Abrams and Citico creek populations for each species, we first compared per locus genic frequency distributions between populations using the genic differentiation option in GENEPOP v4.0.10 (Raymond and Rousset 1995) with default parameter settings. We also calculated D_{EST} and F_{ST} , which are measures of population differentiation based on genetic polymorphism data (Jost 2008; Meirmans and Hedrick 2011), between creeks using the programs DEMETics (Gerlach et al. 2010) and Arlequin v3.5 (Excoffier and Lischer 2010), respectively. Confidence in D_{EST} was assessed via bootstrap resampling (500 replicates as implemented in DEMETics). Analysis of population structure was performed using a Bayesian-based clustering algorithm implemented in the program STRUCTURE v2.3.3 (Pritchard et al. 2000; Falush et al. 2003). The program STRUCTURE assumed no a priori sampling information; rather, individuals were probabilistically assigned to groups in such a way as to achieve Hardy-Weinberg and gametic equilibria. The program STRUCTURE was run with three independent replicates for K (i.e., distinct populations or gene pools), with K set from

one to eight. The burn-in period was 50,000 replicates followed by 500,000 Monte Carlo simulations run under a model that assumed no admixture and independent allele frequencies.

Estimation of effective population size. The effective population size (N_e) for each population was estimated for each species using the linkage disequilibrium (LD) method (Hill 1981). The measure of LD was that of Burrow's composite measure (Campton 1987) and was estimated for each species using the program LDNe (Waples and Do 2008). Allele frequencies close to zero can affect estimates of N_e (Waples 2006); therefore, we excluded alleles with frequencies less than 0.02 (Waples and Do 2010). Parametric 95% confidence intervals were also calculated using LDNe (Waples and Do 2008; Waples and Do 2010). Note that we first attempted to estimate N_e for each population but our point estimate in each case was infinity -- an indication that sample size was limited). Note also that we sampled multiple cohorts to obtain our estimate of N_e for each species (a violation of LDNe assumptions), but as pointed out by Waples and Do (2010), a reasonable conjecture is that if the number of cohorts represented in a sample is roughly equal to the generation time for each species, then the LD estimate should roughly correspond to N_e for a generation. Until this conjecture is found true, the estimates of N_e for each species should be treated with caution. Note that the generation time approximates a value of two for *N. baileyi* and *E. sitikuense* and three for *N. flavipinnis* (Etnier and Starnes 1993; Dinkins and Shute 1996)

Testing alternate evolutionary scenarios. We tested whether the lack of genetic diversity in Citico Creek species (see below) was attributed to a recent or more historical bottleneck by testing competing hypothesis (Fig. 2) in an approximate Bayesian computation (ABC) framework (Beaumont et al. 2002), as implemented by the program DIY ABC v1.0.1.34beta (Cornuet et al. 2008). This approach was employed to model evolutionary scenarios given a distribution of values for each parameter (discussed below) and summary statistics based on the observed microsatellite data. Summary statistics included average number of alleles, expected heterozygosity, allele size variance across loci, and *M*-index (Garza and Williamson 2001). The ABC method entailed generating simulated data sets (based on the microsatellite

data for each species collected from Citico Creek), selecting simulated data sets closest to observed data set, and estimating posterior distributions of parameters through a local linear regression procedure (Beaumont et al. 2002; Cornuet et al. 2008). In doing so, this approach provided a way to quantitatively compare alternative evolutionary scenarios.

The ABC approach relied on prior knowledge of the following four parameters: ancestral effective population size (N_{ea}), contemporary effective population size (N_e), effective population size during a bottleneck (N_{eb}) and time of bottleneck (T , in generations). The parameter N_{ea} was modeled as having a distribution bounded by 436-2180 for each species. This parameter was unknown for each species; therefore, we chose to model it using a very broad uniform distribution. The upper end of this bound was based on tripling the upper 95% confidence interval estimate of the census size for *N. flavipinnis* in Citico Creek (1453 adults; Dinkins and Shute 1996) and an understanding that the effective size is often 10-50% of the census size (i.e., $1453 \times 3 \times 0.10 \approx 436$; $1453 \times 3 \times 0.50 \approx 2180$; (Palstra and Ruzzante 2008). Our observed distribution for N_e for each species was based on the LDNe 95% confidence interval estimate of this parameter (below). Note that LDNe estimated the upper confidence interval of N_e for *N. flavipinnis* as infinity, so we arbitrarily set the upper bound for the N_e distribution in the DIY ABC analysis as 50% of the current census estimate of 1453 (note we used the upper 95% CI census estimate found by Dinkins and Shute to incorporate any measure of uncertainty). The parameter T took on differing values depending on the evolutionary scenario. For the first two scenarios (A and B; Table 8) we model either a gradual decrease in genetic diversity (scenario A) or an historic (Pleistocene) bottleneck. For these scenarios we chose to set T to 3333 generations for *N. flavipinnis* and 5000 for *E. sitikuense* and *N. baileyi*, which was representative of the end of the most recent glacial event approximately 10,000 years ago (i.e., 10,000 years divided by a three or two year generation time; Etnier and Starnes 1993; Dinkins and Shute 1996). Scenario C was modeled as a bottleneck occurring during the construction of a concrete dam in 1973; thus T was set to 12 generations for *N. flavipinnis* and 18 for

E. sitikuense and *N. baileyi* (2009-1973 = 36 years divided by a two or three year generation time). Finally, scenario D described a more historical event – the intensive land use in 1900 that left much of the region deforested (T set to 36 or 55 depending on generation time). After the initial time of the bottleneck, N_{eb} was assumed to be between a value of 2 and 50 and the duration of the bottleneck was modeled as having a uniform distribution from either [1-4999], [1-3332], [1-17], [1-12], [1-35], or [1-54] generations depending on the evolutionary scenario and generation time of each species.

We simulated 1,000,000 datasets per scenario for each species (via DIY ABC) to produce reference datasets using uniform priors for each parameter (Table 8). Prior information regarding the mutation rate and model for microsatellites was taken as default values in DIY ABC. The posterior distribution of each scenario was estimated using local linear regression on logit transformed data for the 10,000 simulated datasets closest to the observed dataset (Cornuet et al. 2008). The exact posterior probability of each scenario was reliant on the model that generated the posterior probability distribution; therefore, poor model fit could lead to inaccurate estimation of the models posterior distribution and subsequent model choice (Cornuet et al. 2010). As recommended by Cornuet et al. (2010), we employed the model checking function of DIY ABC to assess the goodness-of-fit between each model parameter posterior combination and the observed dataset by using different summary statistics for parameter estimation and model discrimination. The parameter estimation summary statistics used were M -index and allele size variance, while the model discrimination summary statistics were average number of alleles and average expected heterozygosity.

Assessment of one genome per generation strategy. We assessed the effectiveness of the pre-specified management recommendation that one genome per generation be exchanged between populations via coalescent simulations. Ten microsatellite loci were simulated ($n = 100$ simulations) for two populations using SIMCOAL2 v2.1.2 (Laval and Excoffier 2004) to assess the amount of migration necessary to maintain current levels of genetic diversity and minimize population differentiation. We assumed a

diploid model for which two populations diverged either 10, 30, or 50 generations ago. For each divergence date we assumed either a one-way (simulating the movement of genes from Citico to Abrams) or two-way migration model and assessed the degree of expected genetic differentiation (as estimated by F_{ST}) for three differing migration rates (0.00, 0.02, and 0.05). Input values for effective population and sample sizes for each population were 75 and 30 (respectively) and were similar to observed values (see below). For each simulated dataset, F_{ST} was estimated using ARLEQUIN v3.5.

Results indicated that a migration rate of 0.02-0.05 (depending on one or two way migration) was necessary to minimize genetic differentiation among populations; however, each model assumed constant population size. To assess the effects of population growth on genetic differentiation, we evaluated three differing growth rates (0.009, 0.09, and 0.18) over 10, 30, and 50 generations using either a one way migration rate of 0.05 or a two way migration rate of 0.02. The program SIMCOAL2 uses a continuous exponential growth model with a growth parameter (r) to simulate growth; thus the growth rates used in our study correspond to discrete growth parameter (λ) values of 1.01, 1.1, and 1.2, respectively.

RESULTS

Estimation of genetic differentiation -- *N. flavipinnis*. A total of 59 *N. flavipinnis* were analyzed using 21 microsatellite markers ($n = 30$ Citico Creek; $n = 29$ Abrams Creek; Tables 1 and 2). For each population, all loci conformed to per locus HWE after sequential Bonferroni corrections (all $P > 0.17$) except *Nfl D146*. Microsatellite marker *Nfl D146* was monomorphic for allele 234 except two individuals from Citico Creek had genotype 246/254. There was no evidence of gametic disequilibrium after sequential Bonferroni correction (all $P > 0.006$; $n = 43$ comparisons per population for an $\alpha = 0.001$).

A comparison of genetic diversity between Citico and Abrams Creek samples (Table 5) showed no significant differences in average allelic richness (1.507 vs. 1.506; $P = 0.90$), average observed

heterozygosity (0.110 vs. 0.106, $P = 0.51$), or average expected heterozygosity (0.111 vs. 0.108, $P = 0.70$). There was also no significant difference in the average fixation index (0.077 vs. 0.045, $P = 0.61$).

Genic differentiation between Citico and Abrams creeks was significant ($P > 0.001$) with three (*Nfl C143*, *Nfl C145* and *Nfl D139*) of 21 loci causing the significance. The value of D_{EST} (averaged across loci) between Citico and Abrams creeks was 0.012 and significantly different ($P = 0.001$) from zero. As above, loci driving this significance were *Nfl C143*, *Nfl C145*, and *Nfl D139*. The value of F_{ST} was 0.018 and not significant ($P = 0.198$). The program STRUCTURE revealed that the most probable number of groups was one, as the proportion of sampled individuals to each sampling site was symmetrical for all K -values 2-4 (data not shown) -- an indication that Abrams and Citico creeks are essentially the same population (Evanno et al. 2005).

Estimation of genetic differentiation -- *N. baileyi*. A total of 87 *N. baileyi* were analyzed using ten microsatellite markers ($n = 64$ Citico Creek; $n = 23$ Abrams Creek; Tables 1 and 3). For Abrams Creek, all loci conformed to per locus HWE after sequential Bonferroni corrections (all $P > 0.07$). Quite the opposite was found for Citico Creek samples – four (*Nfl D109*, *Nfl A12*, *Nfl C120*, and *Nfl C135*) of ten loci deviated significantly from HWE. Deviations from HWE are presumably due to a high degree of relatedness among a large portion of individuals (i.e., 38 captive individuals whose tissues were preserved on the same date were collected after the juveniles had been through several tank moves and no record was kept to be able to elucidate sibships; P. Rakes, CFI., pers. comm.). There was no evidence of gametic disequilibrium after sequential Bonferroni correction (all $P > 0.004$; $n = 28$ comparisons per locality for an $\alpha = 0.002$).

A comparison of genetic diversity between Citico and Abrams Creek samples (Table 6) showed no significant differences in average allelic richness (2.59 vs. 2.08; $P = 0.38$), average observed heterozygosity (0.162 vs. 0.178, $P = 0.63$), or average expected heterozygosity (0.195 vs. 0.177, $P = 0.73$).

While the average fixation index was greater for Citico Creek samples, presumably due sampling related individuals, the disagreement was not significant (0.255 vs. 0.054, $P = 0.08$).

Significant differences ($P > 0.001$) in allelic distributions were found for two of ten loci (*Nfl D109* and *Nfl C120*). The value of D_{EST} (averaged across loci) between Citico and Abrams samples was 0.035 and significantly different ($P = 0.001$) from zero. The value of F_{ST} was 0.060 and significant ($P < 0.001$). As above, the loci driving this significance were *Nfl D109* and *Nfl C120*. The program STRUCTURE revealed that the most probable number of groups was one.

Estimation of genetic differentiation -- *E. sitikuense*. A total of 56 *E. sitikuense* were analyzed using nine microsatellite markers ($n = 25$ Citico Creek; $n = 31$ Abrams Creek; Tables 1 and 4). All loci conformed to per locus HWE for each locality (all $P > 0.08$). There was no evidence of gametic disequilibrium after sequential Bonferroni correction (all $P > 0.01$; $n = 15$ comparisons per locality for an $\alpha = 0.003$).

A comparison of genetic diversity between Citico and Abrams Creek samples (Table 7) showed no significant differences in average allelic richness (2.67 vs. 2.62; $P = 1.00$), average observed heterozygosity (0.246 vs. 0.227, $P = 0.86$), or average expected heterozygosity (0.228 vs. 0.225, $P = 0.93$). There was also no significant difference in the average fixation index (0.066 vs. 0.092, $P = 0.61$).

There were significant differences in allelic distributions between Citico and Abrams samples ($P = 0.005$). Four (*Ebl 6*, *Eca 13EPA*, *Ebl 4*, and *Esc 26B*) of nine loci appeared to be causing the significance. The value of D_{EST} (averaged across loci) between Citico and Abrams samples was 0.025 and significantly different ($P = 0.004$) from zero. The value of F_{ST} was 0.020 and significant ($P = 0.027$). The loci driving this significance were the same as that in the allelic distribution test. The program STRUCTURE revealed that the most probable number of groups was one.

Estimation of effective population size. Estimates of N_e via LDNe for *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* were 75 (15-infinity), 72 (29-691), and 46 (19-291), respectively (95% confidence interval in

parentheses). Note that negative estimates of N_e occur when the genetic results can be explained entirely by sampling error without invoking any genetic drift, so the biological interpretation of a negative value is an N_e of infinity (Waples and Do 2010).

Testing alternate evolutionary scenarios. We were interested in testing whether the observed genetic variation present in Citico Creek populations for each species could be attributed anthropogenic events (e.g., the construction of an impoundment); therefore, we tested four alternative evolutionary scenarios that might explain the observed genetic variation (Table 8, Fig. 2). Scenario D (model depicting deforestation during the 1900s) had the greatest posterior probability for two of three species; however, no scenario produced a significant posterior probability (i.e., >95%) when compared to other competing evolutionary scenarios (Table 8). Our assessment of model misfit indicated that several test quantities had low tail-area probabilities when applied to scenarios A-C for each species (Table 8) casting serious doubts on the adequacy of the tested model-posterior combination. For example, scenario B for *N. flavipinnis* had the greatest posterior probability among competing scenarios; yet, both test quantities (no. alleles and heterozygosity) were significantly different from observed values suggesting that this scenario inadequately explained the observed pattern of genetic diversity found in this species. Finally, scenario D had the greatest posterior probability for each species when comparing it only to scenario C (i.e., recent dam construction vs. 1900 deforestation; Table 8).

Assessment of one genome per generation strategy. Results of computer simulations for one-way migration assuming no population growth in both populations are summarized in Figure 3. As expected with no migration, our two populations diverged significantly at 30 generations and beyond. A similar pattern was observed for a migration rate of 0.02 (Fig 3A). In contrast, a migration rate of 0.05 maintained a value of F_{ST} that was non-significant among 10, 30, and 50 generations (Fig. 3A). Incorporating population growth in our one-way migration model resulted in values of F_{ST} that were less than that of assuming no growth (except for $r = 0.18$ for 30 generation model; Fig 3B). In all cases,

however, the average value of F_{ST} was not significantly different (i.e., overlapping 95% confidence intervals) than the estimated value assuming no growth (Fig 3B).

DISCUSSION

Abrams Creek reintroduction program

Metapopulation theory (Levins 1969) emphasizes the importance of connectivity between seemingly isolated wild populations. No single population may be able to guarantee the long-term survival of a given species, but the combined effect of many populations may be able to do this. In accordance with metapopulation theory, the United States Department of Interior's prescription for fishway pursuant to section 18 of the Federal Power Act for the Tapoco Project (P-2169) adopted a fishway passage strategy for *N. flavipinnis*, *N. baileyi*, and *E. sitikuense*. This strategy included translocation of target species' nests (individuals) from Citico to Abrams Creek with the intent that genetic mixing (i.e., connectivity) between populations would transpire.

If genetic mixing was occurring on a per generation basis, then we would expect a high level of genetic similarity between Citico and Abrams populations for each species. Estimates of genetic diversity (i.e., allelic richness and expected heterozygosity) were similar between each population for each species and suggest that each species' reintroduction program has been successful in the preservation of genetic diversity between source and founding populations. However, these indices provide little information about the degree of differentiation between populations. For example, each population could have the same number of alleles, but the identity of each allele may perhaps be different between the populations indicating significant differentiation between populations. Allelic distribution tests, the measure of D_{EST} or F_{ST} , and STRUCTURE results should provide an understanding of these patterns and provide a better measure of differentiation between populations.

Measures of population differentiation produced somewhat conflicting results. Allelic distribution tests and estimates of D_{EST} or F_{ST} (except for *N. flavipinnis*) indicated significant differentiation between Abrams and Citico creek populations whereas our STRUCTURE result, which assigned individuals to groups in such a way that Hardy Weinberg and genotypic equilibrium were achieved, indicated no significant population structure between samples from Abrams and Citico creeks. The interpretation of significant genetic differentiation must be viewed with caution. While allelic frequency distributions and D_{EST} estimates were found to be significant between populations, the amount of differentiation inferred by D_{EST} was minimal (D_{EST} scales from zero to one with zero being no differentiation and one being complete differentiation) calling in to question whether this level of differentiation is biologically meaningful. Furthermore, the loss of alleles (which was minimal in our study) is potentially much more detrimental to a population than allele frequency differences between populations because lost alleles can be recovered only by migration or mutation. Thus, while genetic differences were observed between Abrams and Citico populations for each species, the fish passage strategy appears to be capturing a large portion of the neutral genetic variation observed in each species inhabiting Citico Creek.

The cause of such genetic differentiation between populations arises due to the sampling phenomenon of genetic drift. Genetic drift is defined as the random changes in allele frequencies of a population between generations due to the binomial sampling of genes during meiosis (Allendorf and Luikart 2007); thus, genetic drift is more pronounced in small populations. Minimizing genetic drift is a primary concern when attempting to reestablish a population because utilizing too few brood stock (termed a founder effect and is a special case of genetic drift; Allendorf and Luikart 2007) or observing large differences in reproductive success among brood stock will cause changes in allele frequencies (if not loss of genetic diversity) between founder and source populations. To avoid potential founder effects, a reasonable estimate of 30-50 individuals has often been recommended to capture most allele

frequencies in the source population (Miller and Kapuscinski 2003); however, often as many as 100-200 parents are recommended in order to capture multiple low frequency alleles (Miller and Kapuscinski 2003; Allendorf and Luikart 2007); in the population. The total number of nests sampled over the years for the reintroduction program has been greater than 30 for each species (approximately 48, 150, and 40 nest for *N. flavipinnis*, *N. baileyi* and *E. sitikuense*; pers. comm. P. Rakes, Conservation Fishes Inc.). These values equate to a minimum of 96, 300 and 80 brood parents (respectively) assuming one female and one male contributed to the nest. Thus sampling effects due to too few brood stock should have been minimized for this reintroduction effort and cannot explain the observed frequency differences between species in Abram and Citico creeks. Yet, many of these collected nests did not produce offspring (pers. comm. P. Rakes, Conservation Fishes Inc); thus greater than expected variance in reproductive success (i.e., larger than binomial variance of family size) may be attributing to the observed differentiation in allele frequencies between Abram and Citico creek populations. In conclusion, while there appeared to be significant genetic differentiation between the source and introduce population, much of the observed differences were likely explained by a greater than expected variance in reproductive success. Future monitoring efforts should therefore focus on assessing the degree of differential reproductive success that might be occurring in the hatchery and if necessary, take steps to equalize family contributions in order to minimize variance in reproductive success (Allendorf 1993).

Genetic variation of Citico Creek species

Genetic variation is important in maintaining the adaptive potential of species/populations and the fitness of individuals to help ensure their survival (Frankel and Soule 1981; Frankham 2005; Laikre 2010): its importance is reflected by the International Union for Conservation and Nature's recognition that genetic diversity is an essential component of biodiversity (McNeely et al. 1990). The observed low level of genetic diversity found in *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* from Citico Creek is an

indication that past processes have greatly influenced current levels. A simple comparison of average number of alleles and observed heterozygosity values summarized in other freshwater fishes (9.1 and 0.54, respectively; (DeWoody and Avise 2000) suggest that species in our study have undergone a past bottleneck event. The low level of heterozygosity found in each species was quite intriguing because heterozygosity is relatively insensitive to the effects of bottlenecks (Allendorf 1986); thus the bottleneck must have been extremely intense and/or have occurred for a long duration. We chose to model differing evolutionary scenarios to help explain this discrepancy. Results indicated that the observed lack of genetic diversity was better explained by contemporary rather than more historic processes. Specifically, only scenario D (i.e., modeled during the 1900s and a period of significant deforestation around Citico Creek) had a relatively high posterior probability of support and had all simulated test quantities equaling that of observed – a pattern consistent for each examined species. In contrast, Pleistocene events or a gradual reduction in genetic diversity since the Pleistocene often had posterior probabilities less than that of scenario D, as well as test quantities that were significantly different from observed values. These findings indicated that anthropogenic events during the 1900s attributed to the lack of genetic variation seen today for *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* from Citico Creek and highlighted the need to protect these species and their respective habitat from future demographic bottlenecks as witnessed in the 1900s.

Management recommendations

Abrams Creek population

Our findings indicated observed allele frequency differences between Citico and Abrams creek populations of *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* that were likely the result of larger than binomial variance of family size occurring either in the hatchery or wild (or both). Minimizing this variance (if it is occurring in the hatchery) can be achieved to some degree by implementing hatchery protocols that attempt to better maintain and monitor equal family contributions prior to release. A

hatchery protocol that rears each family/nest separately is the simplest method to maintain and monitor variance in family size; however, if tank space is a limiting factor then all individuals should be marked in such a way as to distinguish family of origin. When combining families is deemed necessary, family contributions should be equalized but only after significant periods of mortality have passed (often after the critical early life history stages). Families could be combined incrementally as space dictated. Ideally, the numbers of offspring to rear per family should be determined *a priori* based on expected survival rates during incubation and rearing so that a target stocking number is attained with all families contributing equally throughout the entire period of stocking. However, equalization of family sizes ($\pm 5\%$) at stocking does not necessitate reduction of all families to the size of the smallest annual production group. Doing so could unduly compromise the intended demographic benefits of the effort. Instead, offspring from those families that are below the target number will simply be underrepresented and will likely necessitate the rearing of additional families in future years to meet propagation targets. Further, the numbers stocked from other families should not be increased to make up for this shortfall but should be kept as targeted originally. In all cases, hatchery rearing protocols should be assessed and refined so that documentation of individual family sizes upon stocking are recorded to monitor and assess the variance in annual family contribution of hatchery reared individuals. In doing so, genetic drift and the loss of genetic diversity via hatchery reintroductions should be minimized in Abrams Creek species of concern.

The intent of the fish passage strategy adopted by the Federal Energy Regulatory Commission relicensing agreement was to provide genetic mixing (i.e., connectivity) between Abrams and Citico creek for populations of *N. flavipinnis*, *N. baileyi*, and *E. sitikuense*. Upon implementation of the fishway passage strategy, no genetic information existed to quantify the rate of exchange of each focal species between Abrams and Citico; therefore, a target objective of one effective genome (migrant) per generation was established. The one-migrant-per-generation rule has been applied widely to species

conservation plans (Mills and Allendorf 1996); however many of the assumptions of this model are often unrealistic and violated, drawing into question its interpretation and implementation in a conservation context (Vucetich and Waite 2000; Wang 2004). In an effort to more adequately address this rate of exchange between Abrams and Citico populations of concern, we assessed the level of migration necessary to impede significant population divergence over 50 generations (given the current levels of genetic diversity found in our focal species). Our simulation approach revealed that approximately a 5% migration rate per generation (or $0.05 \times$ an N_e of $75 \approx$ four individuals per generation) was necessary to offset the influence of genetic drift over the course of 50 generations (approximately 100-150 years given the species of concern). It is important to note that the estimated value of four migrants per generation (approximately two migrants per year) is the number of individuals that successfully migrate between populations and reproduce, resulting in gene flow. Thus, to successfully achieve the goal of genetic mixing for Citico and Abrams creek populations, the introduction of offspring from more than four nests per generation will be necessary – how much more is dependent on a clearer understanding of average survival rates for hatchery reared individuals inhabiting Abrams Creek and should be an area of future research. Also of importance is a better understanding of N_e for Abrams and Citico creek populations. Current simulations assumed both populations (for each species) had an N_e of 75 because insufficient data were available to estimate N_e for each population. Accuracy of N_e estimation (and subsequent migration simulations) relies on either increasing sample sizes or the number of molecular markers (Waples and Do 2010). The former may be hard to accomplish given the difficulties of sampling these species; however, new genomic approaches may offer a feasible way to increase the number of markers (Hohenlohe et al. 2011). In doing so, a better estimate of the effective number of migrants will be achieved for each species.

Citico Creek population

Noturus baileyi and *E. sitikuense* present a difficult and somewhat unique conundrum – except for the introduced Abrams and Tellico Creek populations, they are only found in Citico Creek (in 13.8 and 9.6 river km stretch, respectively), are in relatively low numbers, exhibit low levels of neutral genetic variation, and this variation has been lacking for at least 50-100 generations. We must be reminded that extinction is a demographic process and protection of these species from human-induced habitat loss and habitat modification should be of high priority. Fortunately, reintroduction efforts in Abrams and Tellico creeks appear successful (i.e., increasing population size and similar levels of genetic diversity; Shute et al 2005, this study), which eases the risk of extinction for the abovementioned species. However, small populations are often vulnerable to random fluctuations in demographic, environmental, and genetic processes -- all of which are not mutually exclusive and can influence the rate of extinction (Gilpin and Soule 1986; Reed 2010). Our study was concerned with assessing the risk of genetic conditions (inbreeding depression, loss of genetic variation, genetic load) likely to influence population persistence. General conservation goals based on genetic considerations are frequently established at an $N_e = 50$ to minimize inbreeding depression and an $N_e = 500$ to maintain sufficient evolutionary potential (Franklin 1980; Franklin and Frankham 1998). The empirical point estimate of N_e for each species in this study approximated the critical threshold level for inbreeding, but it was less than other estimates of N_e for populations of conservation concern (Palstra and Ruzzante 2008). These findings suggest that inbreeding depression could be of immediate importance to the persistence of Citico Creek species that we examined. Yet, the relative risk of inbreeding depression for each species appears lower if we consider the demographic history of the organisms. Our ABC analyses suggest that species inhabiting Citico Creek have been isolated and confined at small population sizes for 50-100 generations. Most detrimental variants of medium and large effect have presumably been purged over the course of 50-100 generations. Such populations in theory should show limited lowered fitness upon inbreeding (Hedrick 2001; Glémin 2003); thus the risk of extinction/extirpation due to inbreeding

depression appears low (note that there still is a risk of inbreeding depression because alleles with minor effects can still persist in the populations). In contrast, the risk of extinction from low genetic diversity and genetic load (i.e., a reduction in mean fitness resulting from detrimental variation for a population when compared to other populations; Hedrick 2001), while not of immediate concern, raises alarms regarding the long-term persistence of these species (Hedrick 2001; Hedrick and Fredrickson 2010; Reed 2010).

Although low genetic diversity and potential elevated genetic load are of concern, predicting the actual risk is difficult and implementing steps to minimize risk are often contentious. Difficulty and contention arise because one way to increase genetic diversity (other than via the mutational process) and lower the genetic load in a population involves the introduction of unrelated individuals from another population or closely related species (often termed genetic rescue, Hedrick 2001, Hedrick and Fredrickson 2010). Genetic rescue is not the ultimate solution for recovery of endangered species; rather, it provides for a temporary increase in population size with the intent of lowering the probability of extinction and providing time to correct the actual problem(s) associated with endangerment (Hedrick and Fredrickson 2010). Instead, maintaining or increasing the census size and effective population size should minimize risks associated with the loss of genetic diversity and genetic load (Lande 1994; Hedrick and Fredrickson 2010). We, therefore, advocate the need for defining and protecting critical habitat for these species and monitoring of both the census and effective populations size for this population. Monitoring temporal fluctuations in population genetic metrics or other population data generated using molecular markers is an integral tool for the conservation of threatened or endangered species because it can provide for 1) an understanding of the present and historical levels genetic diversity in a population or species (e.g., prior to release of hatchery individuals), 2) an assessment of the alteration of these characteristics (i.e., perhaps due anthropogenic factors), and

3) an evaluation of the biological consequences of management and conservation initiatives (Schwartz et al. 2007; Laikre et al. 2010).

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Table 1. Sample size (N), locality, and sampling information of fishes used to estimate indices genetic diversity.

Species	Drainage	N	Year sampled
<i>Noturus baileyi</i>	Abrams Creek	16	2009
		7	2010
	Citico Creek	48	2008
		13	2010
		3	2010
<i>Noturus flavipinnis</i>	Abrams Creek	4	2009
		25	2010
	Citico Creek	17	2008
		4	2009
		12	2010
<i>Etheostoma sitikuense</i>	Abrams Creek	31	2010
	Citico Creek	25	2010

Table 2. Molecular microsatellite markers used to estimate genetic diversity for *N. flavipinnis*. The abbreviation bp represents base pairs.

Locus	Primer sequence(5'-3')	Repeat Motif	Size Range (bp)	Citation
<i>Nfl</i> A3	F: CCATTTGGTCAACTACCTG R: GAGGGTTAGCATCACAGAAGT	AAC	160	Moyer et al (2011)
<i>Nfl</i> A9	F: TGCCTCCAGTGCTGTAGT R: CGAGCGTATTTTCATTCTTTC	AAC	225	Moyer et al (2011)
<i>Nfl</i> A12	F: GACCTGATTGAGTCAGAATGAC R: AAATTCCTGACACTTAGAG	AAC	241	Moyer et al (2011)
<i>Nfl</i> C1a	F: AAAGCAAAGAGCCGTA AAAAG R: TGACCCTGAAAAGGAGTAAGC	CATC	174	Moyer et al (2011)
<i>Nfl</i> C7	F: TGACCCTGAAAAGGAGTAAGC R: GGTGTGAGGAAACCAGAGAAC	CATC	177	Moyer et al (2011)
<i>Nfl</i> C119	F: ATGCCCTCTGTGTTCTGG R: GAGTGGGTGTGTGTGTGATG	CATC	200	Moyer et al (2011)
<i>Nfl</i> C122	F: CCGTGACACTGAAAGGAAG R: CTGTGATGGTCTATGG	CATC	141-149	Moyer et al (2011)
<i>Nfl</i> C126	F: AGCAGTTCTGTCAAGTGCCTTAG R: ATTCCACATTCCACAATCTACG	CATC	183	Moyer et al (2011)
<i>Nfl</i> C138	F: GGATTGCCTGTAACTCCAAC R: AACCCCTAAGTGCTGATGCTG	CATC	207	Moyer et al (2011)
<i>Nfl</i> C143	F: AATGGAGCAATGGGTGAAAC R: TGATGGGCGTGTCTAAAGTG	CATC	262-266	Moyer et al (2011)
<i>Nfl</i> C145	F: TGACCCTGAAAAGGAGTAAGC R: AAGCAGTCGTTCCCTCACTAG	CATC	236-246	Moyer et al (2011)
<i>Nfl</i> D105	F: CCAGAGCATTAGAAGAGTAGG R: GGAGTTGATCCAATTTGTTG	TAGA	259-263	Moyer et al (2011)
<i>Nfl</i> D109	F: AGTGCACAGACAAAGTTTG R: CCTGGGGATCAATATAGTATC	TAGA	127	Moyer et al (2011)
<i>Nfl</i> D123	F: GCTTTTTGTCCATTTATCTCTG R: GCAACCCTGATTGGATTTC	TAGA	270-274	Moyer et al (2011)
<i>Nfl</i> D129	F: TGCAGTTCAGCTCTTAAAC R: TCCTTGGGGGTAAATGTAA	TAGA	237	Moyer et al (2011)
<i>Nfl</i> D137	F: AGCGCACAAAAATGTACG R: CGGGCTCTAAATACTGTGG	TAGA	235	Moyer et al (2011)
<i>Nfl</i> D138	F: GTAGAAATGCGACACAGACAC R: GACCCTGAAAAGGAGTAAGC	TAGA	250-274	Moyer et al (2011)
<i>Nfl</i> D139	F: ACTGAATGGCAGGCTTAGA R: ACAAGGGCAAGAGGTGAC	TAGA	227-244	Moyer et al (2011)
<i>Nfl</i> D140	F: GTTTGGTCTGTCAAGGTAATC R: TTTATTTGGTGCAGATGTG	TAGA	282	Moyer et al (2011)
<i>Nfl</i> D145	F: ATGGATGGATGGATGGATC R: TCACGTTTACAGAGTGGAACAG	TAGA	255-259	Moyer et al (2011)
<i>Nfl</i> D146	F: TGTGTTTTGTGCGACTACTGTG R: CTTATCAGGGGCTTCTGTCTGT	TAGA	226-234	Moyer et al (2011)

Table 3. Molecular microsatellite markers used to estimate genetic diversity for *N. baileyi*. The abbreviation bp represents base pairs.

Locus	Primer sequence(5'-3')	Repeat Motif	Size Range (bp)	Citation
<i>Nfl</i> A12	F: GACCTGATTGAGTCAGAATGAC R: AAATTCCACTGCACACTTAGAG	AAC	226-246	Moyer et al (2011)
<i>Nfl</i> D109	F: AGTGCACAGACAAAAGTTTG R: CCTGGGGGATCAATATAGTATC	TAGA	112-144	Moyer et al (2011)
<i>Nfl</i> D129	F: TGCAGTTCCAGCTCTTAAAC R: TCCTTGGGGTAAATGTAA	TAGA	254	Moyer et al (2011)
<i>Nfl</i> A10	F: TTGTCGCTGTGGTGATACC R: TTTCTTATTGCCCTCGTG	AAC	160-168	Moyer and Williams unpublished data
<i>Nfl</i> C120	F: GCATCTTCGACATATTGACCT R: CCCTGGCTCTTAATGTATCATG	CATC	205	Moyer and Williams unpublished data
<i>Nfl</i> C135	F: GGCTGTCTTTACCTGTTTACAG R: TCGTCCATAGTGTGTGATTG	CATC	254-270	Moyer and Williams unpublished data
<i>Nfl</i> C142	F: GTGCCCTGTGATGGACTG R: TGCTGGTTGTGCTAAGACG	CATC	273-293	Moyer and Williams unpublished data
<i>Nfl</i> D2	F: ACGGTCTTTCTCAGTGATTG R: ATTACCACAGATTTTCTCAGA	TAGA	105-145	Moyer and Williams unpublished data
<i>Nfl</i> D9	F: CATTAAAGCATGGACGAGTTTA R: GGTTTCCCTACGATGTAGAGC	TAGA	206-214	Moyer and Williams unpublished data
<i>Nfl</i> D120	F: CACCAATTAGCCATTTAGCAG R: CAAGATATGGGTGGGTGTATG	TAGA	145-157	Moyer and Williams unpublished data

Table 4. Molecular microsatellite markers used to estimate genetic diversity for *E. sitikuense*. The abbreviation bp represents base pairs.

Locus	Primer Sequence (5'-3')	Repeat Motif	Size Range (bp)	Citation
<i>Ebl 1</i>	F: CCCTTTCGTAACCCTTTTCA R: GGGACCAGATGCTGTGAGAT	(CA) ₁₂	237-258	Beneteau et al. (2007)
<i>Ebl 2</i>	F: TGGTGCGACTGAACAAGAAC R: TACCACAACCACCTGCATTG	(AC) ₂₈	150	Beneteau et al. (2007)
<i>Ebl 4</i>	F:TGTGACTGATATTTTGCTGCTG R:TGCATATCAAGATTCCCATTG	(TATC) ₇ GT(TCTA) ₇	156-164	Beneteau et al. (2007)
<i>Ebl 6</i>	F: TATCATCCCATCGTCTGTCG R: TGGCCCAAACAACAAGCTG	(GT) ₂₂	244-280	Beneteau et al. (2007)
<i>Ebl 8</i>	F:ACAGGTATTAGGGCATTTAGCA R:CGTTCAAGTGGCATCAGAGA	(CA) ₇ CG(CA) ₃ CG(CA) ₅	138-154	Beneteau et al. (2007)
<i>Eca 11EPA</i>	F: CGGGCCAGGTTGTTTAAAGT R: GCAGAAGCACAGGAAAGCACCCCTCAA	(GATA) ₁₆	180-198	Tonnis (2006)
<i>Eca 13EPA</i>	F: CAGAAGCCCAAGAATGGTA R: TGTGTAAGTATATTTTGCTGCTG	(TAGA) ₁₇	182-190	Tonnis (2006)
<i>Esc 26B</i>	F:CAATGCGCCACATTGAGAAGG R:GCACAACATATGTCGTTAAGCTCC	(TAGA) ₂₇	192-232	Gabel et al. (2008)
<i>Esc 187</i>	F:ATCGGCCAGCCCTACTCTG R:GGTGATCAGTCTGGACCACAGC	(GTCT) ₁₃	182-200	Gabel et al. (2008)

Table 5. Comparison of population genetic parameters for sampled *N. flavipinnis* in Citico and Abrams creeks, TN. Abbreviations *N*, *Ar*, *Ho*, *He* and *F* represent the number of samples genotyped, allelic richness, observed heterozygosity, expected heterozygosity and fixation index. Note that average values between localities were not significantly different from one another.

<i>Locality</i>	<i>Locus</i>	<i>N</i>	<i>Ar</i>	<i>Ho</i>	<i>He</i>	<i>F</i>
Citico Creek	<i>Nfl A9</i>	30	1.000	0.000	0.000	
	<i>Nfl C122</i>	30	1.000	0.000	0.000	
	<i>Nfl C119</i>	30	1.000	0.000	0.000	
	<i>Nfl C143</i>	30	2.793	0.310	0.392	0.208
	<i>Nfl C1a</i>	30	1.000	0.000	0.000	
	<i>Nfl C7</i>	30	1.000	0.000	0.000	
	<i>Nf D105</i>	30	1.960	0.069	0.067	-0.036
	<i>Nfl D123</i>	30	2.000	0.154	0.204	0.246
	<i>Nfl D129</i>	30	2.000	0.000	0.000	
	<i>Nfl D139</i>	30	2.000	0.567	0.495	-0.145
	<i>Nfl D145</i>	30	2.000	0.310	0.383	0.191
	<i>Nfl C126</i>	30	1.000	0.000	0.000	
	<i>Nfl D138</i>	30	1.000	0.000	0.000	
	<i>NflC145</i>	30	2.000	0.667	0.444	-0.500
	<i>Nfl A12</i>	30	1.000	0.000	0.000	
	<i>Nfl D140</i>	30	1.000	0.000	0.000	
	<i>Nfl D146</i>	30	2.897	0.067	0.127	0.474
	<i>Nfl C138</i>	30	2.000	0.179	0.219	0.184
	<i>Nfl D137</i>	30	1.000	0.000	0.000	
	<i>Nfl A3</i>	30	1.000	0.000	0.000	
<i>Nfl D109</i>	30	1.000	0.000	0.000		
	Average		1.507	0.110	0.110	0.077
Abrams Creek	<i>Nfl A9</i>	29	1.000	0.000	0.000	
	<i>Nfl C122</i>	29	1.793	0.034	0.034	-0.018
	<i>Nfl C119</i>	29	1.000	0.000	0.000	
	<i>Nfl C143</i>	29	2.000	0.172	0.158	-0.094
	<i>Nfl C1a</i>	29	1.000	0.000	0.000	
	<i>Nfl C7</i>	29	1.000	0.000	0.000	
	<i>Nf D105</i>	29	2.000	0.143	0.191	0.253
	<i>Nfl D123</i>	29	2.000	0.208	0.187	-0.116
	<i>Nfl D129</i>	29	1.000	0.000	0.000	
	<i>Nfl D139</i>	29	2.000	0.308	0.393	0.218
	<i>Nfl D145</i>	29	2.000	0.429	0.436	0.018
	<i>Nfl C126</i>	29	1.000	0.000	0.000	
	<i>Nfl D138</i>	29	1.000	0.000	0.000	
	<i>NflC145</i>	29	2.000	0.130	0.122	-0.070
	<i>Nfl A12</i>	29	1.000	0.000	0.000	
	<i>Nfl D140</i>	29	1.000	0.000	0.000	
	<i>Nfl D146</i>	29	1.980	0.074	0.071	-0.038
	<i>Nfl C138</i>	29	2.821	0.429	0.418	-0.026
	<i>Nfl D137</i>	29	1.000	0.000	0.000	
	<i>Nfl A3</i>	29	1.000	0.000	0.000	
<i>Nfl D109</i>	29	1.000	0.000	0.000		
	Average		1.506	0.106	0.108	0.045

Table 6. Comparison of population genetic parameters for sampled *N. baileyi* in Citico and Abrams creeks, TN. Abbreviations *N*, *Ar*, *Ho*, *He* and *F* represent the number of samples genotyped, allelic richness, observed heterozygosity, expected heterozygosity and fixation index. Note that average values between localities were not significantly different from one another.

<i>Locality</i>	<i>Locus</i>	<i>N</i>	<i>Ar</i>	<i>Ho</i>	<i>He</i>	<i>F</i>
Citico Creek	<i>Nfl D109</i>	64	8.527	0.844	0.820	-0.029
	<i>Nfl A10</i>	64	1.571	0.033	0.032	-0.017
	<i>Nfl A12</i>	64	2.859	0.050	0.142	0.647
	<i>Nfl C142</i>	64	1.333	0.016	0.016	-0.008
	<i>Nfl D129</i>	64	1.000	0.000	0.000	
	<i>Nfl D120</i>	64	2.999	0.367	0.508	0.278
	<i>Nfl C135</i>	64	1.890	0.016	0.047	0.660
	<i>Nfl D2</i>	64	2.284	0.071	0.102	0.301
	<i>Nfl D9</i>	64	2.388	0.222	0.281	0.208
	<i>Nfl C120</i>	64	1.000	0.000	0.000	
	Average			2.585	0.161	0.194
Abrams Creek	<i>Nfl D109</i>	23	6.907	0.682	0.704	0.031
	<i>Nfl A10</i>	23	1.000	0.000	0.000	
	<i>Nfl A12</i>	23	2.000	0.045	0.127	0.642
	<i>Nfl C142</i>	23	2.000	0.136	0.127	-0.073
	<i>Nfl D129</i>	23	1.000	0.000	0.000	
	<i>Nfl D120</i>	23	2.000	0.478	0.405	-0.179
	<i>Nfl C135</i>	23	1.000	0.000	0.000	
	<i>Nfl D2</i>	23	1.913	0.043	0.043	-0.022
	<i>Nfl D9</i>	23	2.000	0.391	0.364	-0.075
	<i>Nfl C120</i>	23	1.000	0.000	0.000	
	Average			2.082	0.177	0.176

Table 7. Comparison of population genetic parameters for sampled *E. sitikuense* in Citico and Abrams creeks, TN. Abbreviations *N*, *Ar*, *Ho*, *He* and *F* represent the number of samples genotyped, allelic richness, observed heterozygosity, expected heterozygosity and fixation index. Note that average values between localities were not significantly different from one another.

<i>Locality</i>	<i>Locus</i>	<i>N</i>	<i>Ar</i>	<i>Ho</i>	<i>He</i>	<i>F</i>
Citico Creek	<i>Ebl 1</i>	25	1.000	0.000	0.000	
	<i>Ebl 6</i>	25	2.000	0.217	0.194	-0.122
	<i>Ebl 2</i>	25	1.000	0.000	0.000	
	<i>Eca 11EPA</i>	25	2.000	0.083	0.080	-0.043
	<i>Eca 13EPA</i>	25	3.000	0.095	0.172	0.447
	<i>Esc 187</i>	25	2.000	0.083	0.080	-0.043
	<i>Ebl 8</i>	25	1.000	0.040	0.113	0.645
	<i>Ebl 4</i>	25	3.000	0.857	0.635	-0.350
	<i>Esc 26B</i>	25	9.000	0.840	0.782	-0.074
	Average	25	2.667	0.246	0.228	0.066
Abrams Creek	<i>Ebl 1</i>	31	1.800	0.033	0.033	-0.017
	<i>Ebl 6</i>	31	1.000	0.000	0.000	
	<i>Ebl 2</i>	31	1.000	0.000	0.000	
	<i>Eca 11EPA</i>	31	2.855	0.143	0.135	-0.062
	<i>Eca 13EPA</i>	31	2.000	0.357	0.299	-0.194
	<i>Esc 187</i>	31	1.994	0.133	0.124	-0.071
	<i>Ebl 8</i>	31	2.942	0.000	0.062	1.000
	<i>Ebl 4</i>	31	3.000	0.667	0.651	-0.024
	<i>Esc 26B</i>	31	6.940	0.710	0.716	0.009
	Average		2.615	0.227	0.225	0.092

Table 8. Prior uniform distributions, posterior probabilities, and summary statistics for coalescent models used to compare competing evolutionary scenarios. Each scenario (A-D) was comprised of four parameters: ancestral effective population size (N_{ea}), contemporary effective population size (N_e), effective population size during the bottleneck (N_{eb}) and time of bottleneck (T , in generations). Each parameter was sampled from a uniform distribution with lower and upper bounds indicated in brackets (refer to Material and Methods for details about each uniform distribution). Also reported is the posterior probability (Posterior) for each evolutionary scenario along with summary statistics (average number of alleles, $No. alleles$; expected heterozygosity, He ; allele size variance across loci, $Var.$; and M -index, MG) used to assess the goodness-of-fit between each model parameter posterior combination and the observed dataset. Test quantities (x), which corresponded to the summary statistics, were interpreted as the probability ($X_{simulated} < X_{observed}$); therefore, values greater than 0.95 and less than 0.05 were considered significant, and denoted with an asterisk.

		Scenario			
		A	B	C	D
<i>N. baileyi</i>	N_{ea}	[450-2180]	[450-2180]	[450-2180]	[450-2180]
	N_{eb}	[2-50]	[2-50]	[2-50]	[2-50]
	T	5000	5000	18	55
	N_e	[29-691]	[29-691]	[29-691]	[29-691]
	$No. alleles$	0.778	0.749	0.905	0.907
	He	0.295	0.301	0.270	0.413
	$Var.$	0.661	0.692	0.428	0.574
	MG	0.042*	0.032*	0.146	0.130
	Posterior ABCD	0.245	0.291	0.118	0.346
	Posterior CD			0.255	0.745
<i>N. flavipinnis</i>	N_{ea}	[450-2180]	[450-2180]	[450-2180]	[450-2180]
	N_{eb}	[2-50]	[2-50]	[2-50]	[2-50]
	T	3333	3333	12	36
	N_e	[15-750]	[15-750]	[15-750]	[15-750]
	$No. alleles$	0.007*	0.008*	0.063	0.072
	He	0.008*	0.008*	0.020*	0.060
	$Var.$	0.460	0.510	0.324	0.347
	MG	0.375	0.354	0.648	0.477
	Posterior ABCD	0.2935	0.333	0.134	0.239
	Posterior CD			0.357	0.643
<i>E. sitikuense</i>	N_{ea}	[450-2180]	[450-2180]	[450-2180]	[450-2180]
	N_{eb}	[2-50]	[2-50]	[2-50]	[2-50]
	T	5000	5000	18	55
	N_e	[19-291]	[19-291]	[19-291]	[19-291]
	$No. alleles$	0.813	0.783	0.806	0.865
	He	0.479	0.431	0.164	0.231
	$Var.$	0.950*	0.952*	0.712	0.730
	MG	0.015*	0.008*	0.120	0.110
	Posterior ABCD	0.078	0.086	0.330	0.506
	Posterior CD			0.397	0.603

Figure 1. Map of the middle Little Tennessee River system.

Figure 2. Depiction of evolutionary scenarios used for DIYABC simulations. A) Scenario A depicting a gradual loss of genetic diversity over time. In this scenario we identified T1 as the initial generation time for the gradual loss. For this scenario T1 was set to 5000 or 3333 generations depending on the species. Parameters N_{ea} and N_e were the ancestral and contemporary effective population size. B) Scenarios B-D depicting a bottleneck in genetic diversity. In these scenarios, T1 was the initial start of the bottleneck (see Table 8) and from T1 to T2, we modeled the effective population size during the bottleneck (N_{eb}) as a uniform distribution of [2-50]. T2 was modeled as a uniform distribution having a lower bound of 1 and an upper bound of T1-1. Finally, from T2 to 0, N_e was modeled as a uniform distribution with an upper and lower bound representing the 95% confidence intervals estimated by the program LDNe.

Figure 3. Simulation results for the amount of migration necessary to minimize population differentiation between Citico and Abrams creek populations of *N. flavipinnis*, *N. baileyi*, and *E. sitikuense* over a time span of 50 generations. All simulations assumed a one-way migration model from Citico Creek to Abrams Creek and are based on 10 microsatellite markers. Bars around each point estimate of F_{ST} represent 95% confidence intervals. A) Simulation results using three differing migration rates of 0.00 (▲), 0.02 (■), or 0.05 (●) assuming a constant effective population size of 75. B) Simulation results for a continuous exponential growth model assuming a 0.05 migration rate at four differing growth rates of 0.00 (✕), 0.009 (▲), 0.09 (●), 0.18 (■).

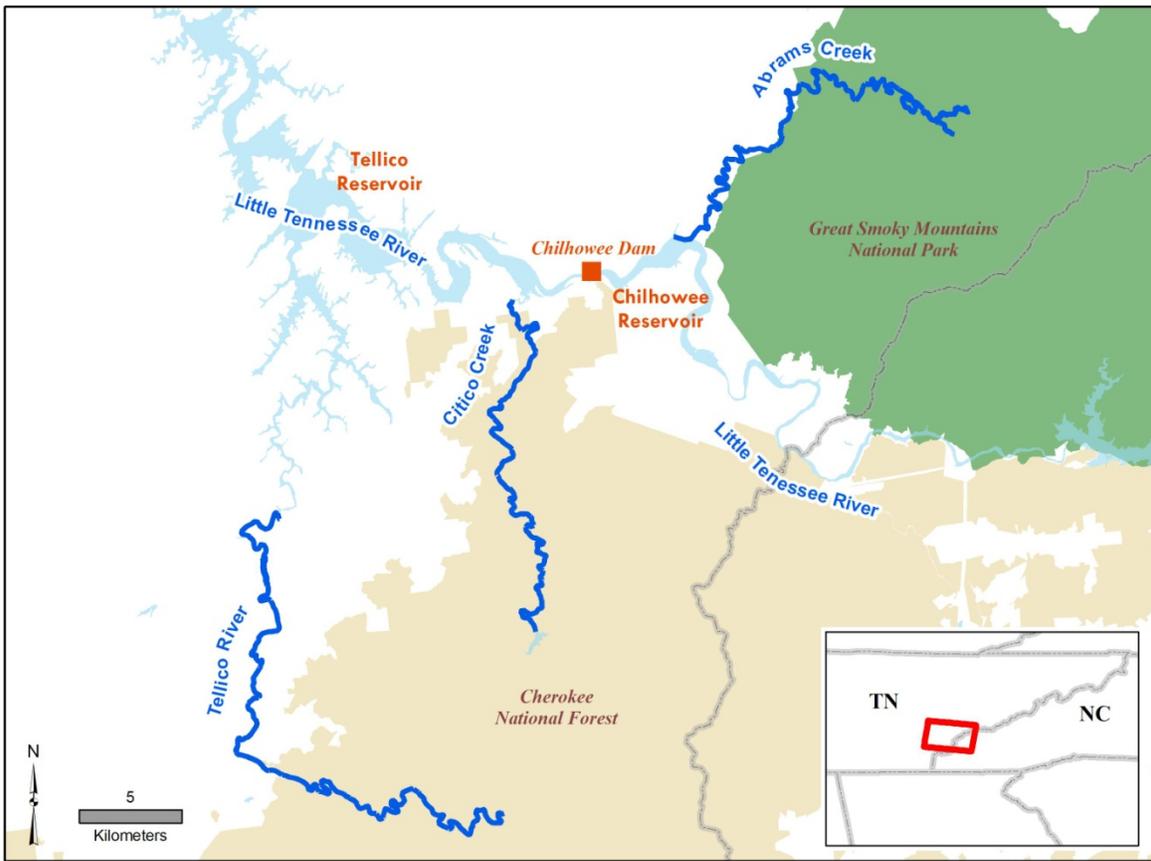


Figure 1.

A)

 N_{ea}
 N_e



B)

 N_{ea}
 N_{eb}
 N_e

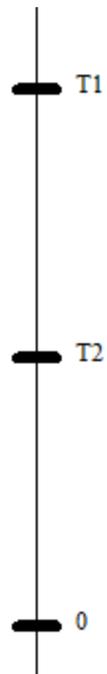
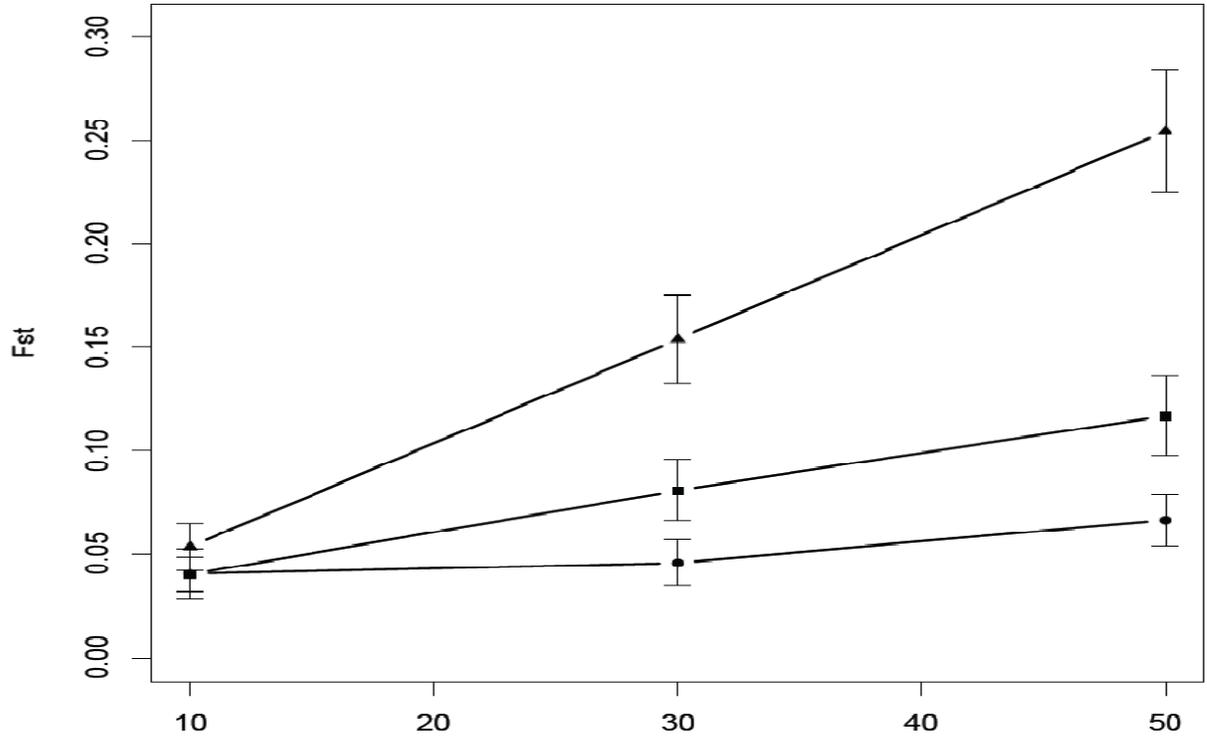


Figure 2.

A)



B)

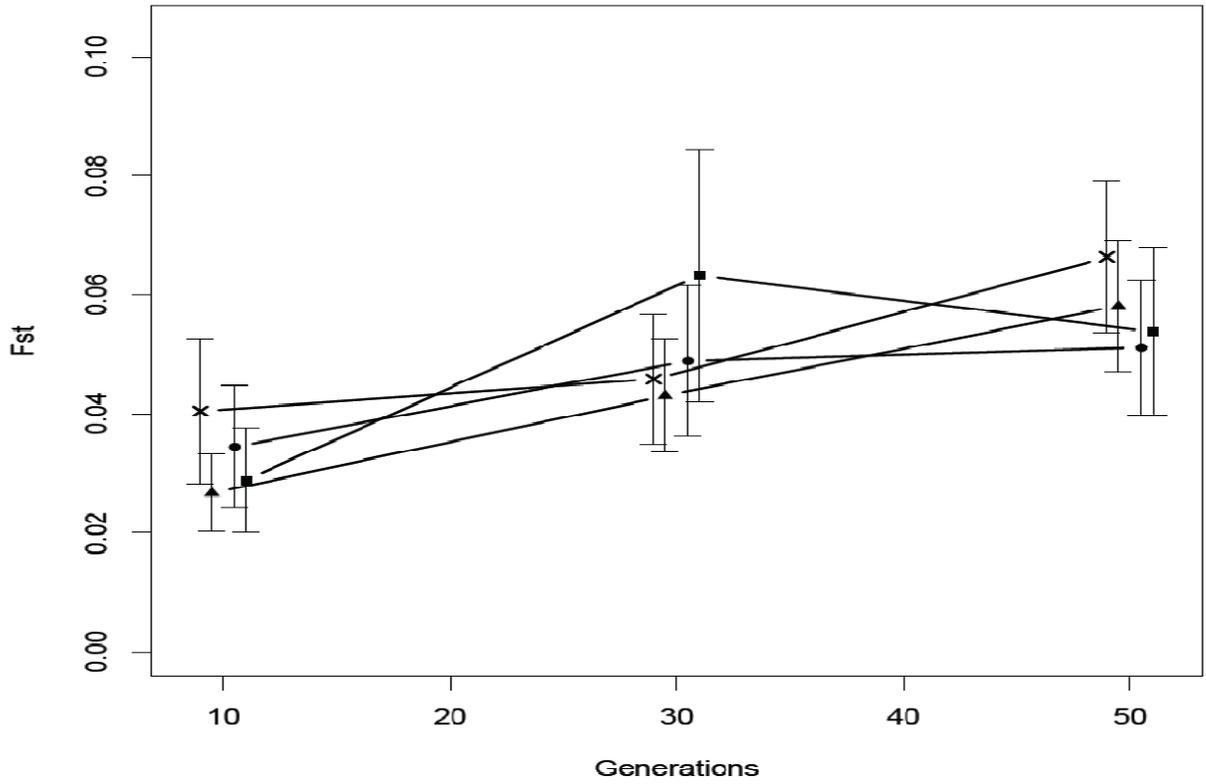


Figure 3.

Appendix A. *Noturus flavipinnis* microsatellite allele frequencies by population.

Locus	Allele/n	Citico Creek	Abrams Creek
<i>Nfl A9</i>	209	0.000	0.000
	225	1.000	1.000
	229	0.000	0.000
	237	0.000	0.000
	253	0.000	0.000
<i>Nfl C122</i>	141	1.000	0.983
	145	0.000	0.000
	149	0.000	0.017
<i>Nfl C119</i>	196	0.000	0.000
	200	1.000	1.000
	210	0.000	0.000
<i>Nfl C143</i>	246	0.000	0.000
	258	0.017	0.000
	262	0.741	0.914
	266	0.241	0.086
<i>Nfl C1a</i>	174	1.000	1.000
<i>Nfl C7</i>	177	1.000	1.000
<i>Nf D105</i>	259	0.966	0.893
	263	0.034	0.107
<i>Nfl D123</i>	262	0.000	0.000
	270	0.885	0.896
	274	0.115	0.104
	282	0.000	0.000
<i>Nfl D129</i>	233	0.000	0.000
	237	0.875	1.000
	244	0.000	0.000
	258	0.125	0.000
<i>Nfl D139</i>	227	0.450	0.731
	244	0.550	0.269
<i>Nfl D145</i>	255	0.259	0.321
	259	0.741	0.679
	263	0.000	0.000
	271	0.000	0.000
	275	0.000	0.000
<i>Nfl C126</i>	167	0.000	0.000

	183	1.000	1.000
	187	0.000	0.000
	191	0.000	0.000
<i>Nfl D138</i>			
	207	1.000	1.000
<i>NflC145</i>			
	236	0.667	0.935
	246	0.333	0.065
	250	0.000	0.000
<i>Nfl A12</i>			
	241	1.000	1.000
<i>Nfl D140</i>			
	234	0.000	0.000
	242	0.000	0.000
	246	0.000	0.000
	254	0.000	0.000
	262	0.000	0.000
	274	0.000	0.000
	282	1.000	1.000
<i>Nfl D146</i>			
	218	0.000	0.000
	226	0.000	0.037
	234	0.933	0.963
	246	0.033	0.000
	254	0.033	0.000
<i>Nfl C138</i>			
	250	0.875	0.714
	270	0.000	0.018
	274	0.125	0.268
	314	0.000	0.000
<i>Nfl D137</i>			
	235	1.000	1.000
<i>Nfl A3</i>			
	160	1.000	1.000
<i>Nfl D109</i>			
	95	0.000	0.000
	107	0.000	0.000
	111	0.000	0.000
	119	0.000	0.000
	127	1.000	1.000
	131	0.000	0.000

Appendix B. *Noturus baileyi* microsatellite allele frequencies by population.

Locus	Allele/n	Citico Creek	Abrams Creek
<i>Nfl D109</i>			
	112	0.055	0.045
	116	0.141	0.023
	120	0.094	0.455
	124	0.094	0.045
	128	0.336	0.227
	132	0.078	0.000
	136	0.047	0.000
	140	0.133	0.182
	144	0.023	0.023
<i>Nfl A10</i>			
	160	0.984	1.000
	168	0.016	0.000
<i>Nfl A12</i>			
	226	0.008	0.000
	234	0.050	0.068
	242	0.017	0.000
	246	0.925	0.932
<i>Nfl C142</i>			
	273	0.008	0.068
	293	0.992	0.932
<i>Nfl D129</i>			
	254	1.000	1.000
<i>Nfl D120</i>			
	145	0.158	0.000
	153	0.658	0.717
	157	0.183	0.283
<i>Nfl C135</i>			
	254	0.976	1.000
	258	0.016	0.000
	270	0.008	0.000
<i>Nfl D2</i>			
	105	0.000	0.022
	125	0.946	0.978
	133	0.000	0.000
	137	0.045	0.000
	145	0.009	0.000
<i>Nfl D9</i>			
	206	0.009	0.000
	210	0.833	0.761
	214	0.157	0.239
<i>Nfl C120</i>			
	205	1.000	1.000

Appendix C. *Etheostoma sitikuense* microsatellite allele frequencies by population.

Locus	Allele/n	Citico Creek	Abrams Creek
<i>Ebl 1</i>	234	0.000	0.017
	258	1.000	0.983
<i>Ebl 6</i>	244	0.109	0.000
	280	0.891	1.000
<i>Ebl 2</i>	150	1.000	1.000
<i>Eca 11EPA</i>	180	0.000	0.018
	182	0.042	0.054
	198	0.958	0.929
<i>Eca 13EPA</i>	182	0.905	0.821
	186	0.000	0.018
	190	0.095	0.161
<i>Esc 187</i>	182	0.042	0.067
	200	0.958	0.933
<i>Ebl 8</i>	138	0.000	0.032
	142	0.060	0.000
	154	0.940	0.968
<i>Ebl 4</i>	156	0.190	0.300
	160	0.429	0.433
	164	0.381	0.267
<i>Esc 26B</i>	192	0.400	0.484
	196	0.060	0.065
	200	0.040	0.016
	204	0.060	0.000
	208	0.120	0.065
	212	0.040	0.081
	216	0.060	0.065
	228	0.060	0.161
232	0.160	0.065	