

Optimization of a Suite of Microsatellite Markers for *Nocomis leptocephalus* (Bluehead Chub) and Genetic Characterization of Two Populations in South Carolina

Elizabeth L. Cushman¹, Kimberly L. Kanapeckas Métris^{1,2}, Yoichiro Kanno^{3,4}, Kasey C. Pregler^{3,4}, Brandon K. Peoples³, and Tanya L. Darden^{1,*}

Abstract - *Nocomis leptocephalus* (Bluehead Chub) is a minnow native to the southeastern United States that constructs nests used by many freshwater fishes. No microsatellite markers have been published for Bluehead Chub, and information on genetic structure and diversity is sparse. We evaluated microsatellites from other leuciscid species for use with Bluehead Chub and created a panel of markers that has sufficient power for investigations of population structure and can differentiate between Bluehead Chub and *Notropis lutipinnis* (Yellowfin Shiner) eggs. We applied the panel to Bluehead Chub samples from 2 locations in South Carolina, finding these populations are genetically differentiated with high levels of genetic diversity. Our marker panel can improve our understanding of population dynamics of Bluehead Chub and allow for informed conservation recommendations.

Introduction

Freshwater ecosystems represent some of the most diverse and yet most threatened habitats on Earth. Continental North America (north of Mexico) contains the greatest taxonomic richness of temperate freshwater fishes in the world (Page and Burr 1991); this diversity is concentrated in the southeastern portion of the United States where ~47% of freshwater fish species in North America are located (Burr and Mayden 1992, Page and Burr 1991, Warren et al. 1997). However, North American freshwater fishes face numerous threats, including habitat destruction and degradation, overexploitation, introduction of non-native species, and climate change, which can result in range reductions, declines in abundance, and extinction (Allan and Flecker 1993, Arthington et al. 2016, Dudgeon et al. 2006). The American Fisheries Society's Endangered Species Committee reports that 39% of diadromous and freshwater fish species in North America are now imperiled (i.e., vulnerable, threatened, or endangered; Jelks et al. 2008). Therefore, freshwater fishes, particularly in the southeastern US, are a priority for conservation efforts.

The family Leuciscidae (formerly Cyprinidae), the new world minnows (Tan and Armbruster 2018), is the largest and most diverse family of North American

¹South Carolina Department of Natural Resources, Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, SC 29412. ²Department of Genetics and Biochemistry, Poole Agricultural Center, Clemson University, 130 McGinty Court, Clemson, SC 29634. ³Department of Forestry and Environmental Conservation, Clemson University, 261 Lehotsky Hall, Clemson, SC 29634. ⁴Department of Fish, Wildlife, and Conservation Biology, Colorado State University, Fort Collins, CO 80523. *Corresponding author - dardent@dnr.sc.gov.

freshwater fishes (Page and Burr 1991) and contains almost a quarter of the imperiled taxa identified by the Endangered Species Committee (Jelks et al. 2008). One of the most common leuciscids in eastern North America is *Nocomis leptocephalus* (Girard) (Bluehead Chub), a medium-sized (up to 180 mm standard length) minnow native to freshwater rivers and streams in the Atlantic and Gulf slope drainages of the southeastern US (Jenkins and Burkhead 1994, Page and Burr 1991). In the spring and early summer, male chubs build gravel mound nests for spawning (Jenkins and Burkhead 1994, Maurakis et al. 1991). *Nocomis* nests are unique features on the benthoscape, being constructed of a narrow range of gravel sizes (Bolton et al. 2015, Wisenden et al. 2009) in specific current velocities (Maurakis et al. 1992, Peoples et al. 2014), and are used for spawning by at least 35 species of nest-associates (Johnston and Page 1992) that require these nests to varying degrees (Pendleton et al. 2012, Wallin 1992). Although not considered threatened throughout their general distribution, Bluehead Chub and other *Nocomis* species experience anthropomorphic pressures (e.g., alteration of habitat) in certain areas (Mammoliti 2002, Utz 2014), and many obligatory nest-associates, although locally abundant, have narrow geographic ranges and may face imperilment should populations of *Nocomis* decline (Pendleton et al. 2012). Furthermore, chubs often concentrate scarce gravel in sediment-started or silted habitats (McManamay et al. 2010, Peoples et al. 2011), and may facilitate persistence (Peoples et al. 2015) or spread (Buckwalter et al. 2017, Herrington and Popp 2004, Hitt and Roberts 2011, Walser et al. 2000) of associate species in changing environments. Accordingly, chubs, such as the Bluehead Chub, can function as keystone species in sustaining riverine fish assemblages (Marcy et al. 2005, Vives 1990), highlighting the need to gather information on these important freshwater fishes.

There is currently a paucity of data on the population genetics of Bluehead Chub. Genetic diversity and structure have been examined for a variety of leuciscids (Burridge and Gold 2003, Gold et al. 2004, Hanna et al. 2015, McCusker et al. 2014, Ozer and Ashley 2013, Skalski et al. 2008); however, molecular studies involving Bluehead Chub have been restricted to interspecific phylogenetic comparisons (April et al. 2011, Nagle and Simons 2012, Simons and Mayden 1999) or simply distinguishing them from associate species in field studies (Cashner and Bart 2010, 2018; Floyd et al. 2018; Silknetter et al. 2019). Knowledge of genetic structure and diversity provides valuable insight on the evolutionary distinctiveness and overall status of freshwater fish populations that can be used to develop and execute strategies to protect and conserve imperiled species (Vrijenhoek 1998). The levels of genetic variation and degree of connectivity among Bluehead Chub in adjacent locations remain largely unknown. In this respect, a molecular tool would be valuable for the acquisition of population-level data required for the preservation of Bluehead Chub.

To date, no microsatellite markers have been published for Bluehead Chub. The objective of our study was to identify microsatellite markers from other leuciscid species that function for Bluehead Chub and to create and test a panel of markers for population genetic research. This molecular tool would have numerous applications from characterizing the genetic diversity and structure of Bluehead Chub

populations to assessing parentage on Bluehead Chub nesting mounds. The ideal panel could also differentiate between Bluehead Chub eggs and those of *Notropis lutipinnis* (Jordan and Brayton) (Yellowfin Shiner), a common and obligatory nest-associate on chub spawning mounds in Georgia and South Carolina (McAuliffe and Bennett 1981; Wallin 1989, 1992). Eggs of the 2 species are difficult to distinguish visually. The results of our study and future investigations will offer valuable information for Bluehead Chub research and conservation efforts in the southeastern United States.

Field-site Description

Our study area included 2 sites, located on Todd Creek (34°45'15"N, 82°48'56"W) and Shoal Creek (34°48'12"N, 82°47'02"W). Todd Creek and Shoal Creek are second-order freshwater streams in Pickens County, located in the Piedmont region of South Carolina. The 2 sampling locations are just 6 km apart from each other in Euclidian distance, and both streams are tributaries to Twelvemile Creek. However, dispersal of riverine fish between Todd Creek and Shoal Creek may be hampered by Lake Hartwell, a man-made reservoir completed in 1963 that is located in the waterway between the streams. Todd Creek and Shoal Creek are mostly forested, but the surrounding area has the potential to become more urbanized as the Piedmont Atlantic is the fastest growing megaregion (i.e., network of connected metropolitan centers and their surrounding areas) in the United States (Ross et al. 2008).

Methods

Microsatellite testing and panel development

We compiled a list of 123 microsatellite markers developed for leuciscid species. From these records, we chose 40 microsatellite markers for testing based on adherence to Hardy–Weinberg equilibrium (HWE) in the published literature. Examined markers originated from several species including *Clinostomus elongatus* (Kirtland) (Redside Dace; Pitcher et al. 2009), *Camptostoma anomalum* (Rafinesque) (Central Stoneroller; Dimsoski et al. 2000), *Dionda episcopa* Girard (Roundnose Minnow; Renshaw et al. 2009), *Rhinichthys osculus* (Girard) ssp. (Santa Ana Speckled Dace; Nunziata et al. 2013), *Notropis mekistochola* Snelson (Cape Fear Shiner; BurrIDGE and Gold 2003, Gold et al. 2004), *Notropis topeka* (Gilbert) (Topeka Shiner; Anderson and Sarver 2008), *Notropis suttkusi* Humphries and Cashner (Rocky Shiner; Schwemm et al. 2014), and *Pimephales promelas* Rafinesque (Fathead Minnow; Bessert and Ortí 2003). We initially tested all 40 microsatellite markers on fin clip samples from 4 Bluehead Chub and 4 Yellowfin Shiner that were collected by backpack electrofishing from Shoal Creek in January 2017 and stored dry on filter paper. Of these, we chose 11 markers based on amplification success and degree of polymorphism (i.e., markers displayed at least 3 alleles) in Bluehead Chub, with select markers amplifying Yellowfin Shiner (Table 1). For simultaneous amplification, we arranged the 11 markers into 3 multiplex groups (Table 1), depending on

fluorescent label and allelic size range, and optimized them with 15 Bluehead Chub and 15 Yellowfin Shiner samples (the original 4 samples plus an additional 11) from Shoal Creek.

Laboratory methodology

All Bluehead Chub and Yellowfin Shiner samples were processed at the Hollings Marine Laboratory in Charleston, SC. We isolated DNA using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) with modifications to the manufacturer's protocol. Briefly, we removed the fin clips from the filter paper, placed them in a digestion solution (145.5 μ l of Promega Nuclei Lysis Solution, 36.36 μ l of 0.5 M EDTA, 14.55 μ l of 20 mg/ml proteinase K, and 3.64 μ l of water), and allowed the fin clips to digest for 3 hours at 55 °C. We mixed the digestion products with 180 μ l of Promega Lysis Buffer, added it to a spin column assembly, and centrifuged the columns at 13,000 rpm for 2 minutes to bind the DNA to the filter in the column. We added Promega SV Wash Solution (containing 99% ethanol) to each spin column and allowed the wash solution to pass through 4 separate times (650 μ l per wash). We centrifuged the columns at 13,000 rpm for 3 minutes to dry the filter. Finally, we added 100 μ l of Promega Nuclease-free Water and centrifuged the column at 13,000 rpm for 1 minute to flush the DNA from the filter. The resulting product was used as a template for the polymerase chain reactions (PCR).

We amplified the 11 optimized microsatellite markers via multiplex PCR in a final volume of 11 μ l with final concentrations of 1X HotMaster Buffer (Quantabio, Beverly, MA), 0.8 mM dNTPs (0.2 mM each), 1.5 mM MgCl₂, 0.4 μ M forward primers (total across markers), 0.4 μ M reverse primers (total across markers), and

Table 1. Microsatellite markers (arranged into 3 multiplex groups) used to genotype Bluehead Chub from South Carolina. L = fluorescent label on forward primer (D2 = black, D3 = green, D4 = blue), PCR (μ M) = primer concentration in the PCR, YFS = whether or not there was amplification in Yellowfin Shiner. †denotes markers that were removed from the microsatellite panel.

Group	Marker	Repeat motif	L	PCR (μ M)	Reference	YFS
1	RSD53	(AC) ₁₃ (AT) ₄	D2	0.09	Pitcher et al. 2009	No
	Ca5	(TAGA) ₁₅	D3	0.29	Dimsoski et al. 2000	No
	Nme25C8.208	(TG) ₉	D4	0.03	Burridge and Gold 2003	Yes
	Ca10†	(TAGA) ₁₆	D4	-	Dimsoski et al. 2000	-
2	Ca12	(TAGA) ₁₀ (CAGA) ₄ (TAGA) ₂	D2	0.07	Dimsoski et al. 2000	Yes
	Ca11	(TAGA) ₇	D3	0.31	Dimsoski et al. 2000	No
	Nme18A6.158†	(GT) ₁₂	D4	-	Burridge and Gold 2003	-
	Rhos5	ATCT	D4	0.08	Nunziata et al. 2013	No
3	Rhos36	AAAG	D3	0.03	Nunziata et al. 2013	Yes
	Ppro126	(CA) ₁₂	D4	0.02	Bessert and Ortí 2003	Yes
	Ca3	(TAGA) ₁₄	D4	0.40	Dimsoski et al. 2000	Yes

0.03 U/ μ L HotMaster *Taq* DNA polymerase (Quantabio). The forward primers for multiplex reactions were labeled with WellRED fluorescent dyes (Sigma-Aldrich, St. Louis, MO; Table 1). We conducted all PCR amplifications on a Bio-Rad iCycler (Bio-Rad, Hercules, CA) with an initial denaturation of 3 minutes at 94 °C followed by 20 cycles of denaturation at 94 °C for 30 seconds, annealing at 65 °C (decreased by 1 °C every 2 cycles) for 30 seconds, and extension at 72 °C for 40 seconds; another 20 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 40 seconds; and a final extension at 72 °C for 1 hour.

We separated the products from the multiplex reactions by capillary electrophoresis on a Beckman CEQ 8000 (Beckman Coulter, Brea, CA). We mixed the PCR products (2.0 μ l) with 40 μ l of sample-loading solution (0.92% fluorescently labeled 600 base-pair size standard in formamide). We analyzed the results using CEQ Fragment Analysis Software (Beckman Coulter), with all chromatograms scored independently by 2 readers.

Sample collection and statistical analysis

To characterize genetic diversity and differentiation, we applied the optimized marker panel to additional Bluehead Chub samples including 224 individuals from Todd Creek and 335 individuals from Shoal Creek. Bluehead Chub were collected by backpack electrofishing in a 520-m section of Todd Creek in May and June 2016 and a 740-m section of Shoal Creek between January and June 2017. Bluehead Chub varied from 59 to 180 mm in total length at Todd Creek and from 75 to 172 mm in total length at Shoal Creek.

We evaluated departures from HWE, linkage disequilibrium between marker pairs, and the frequency of null alleles segregating at each maker in GENEPOP 4.2 (Rousset 2008). We adjusted significance levels for all simultaneous comparisons using a Bonferroni correction (Holm 1979, Rice 1989). Based on the results of these tests, all further analyses were performed with a final suite of 9 microsatellite markers (see Results). We assessed the power of the marker panel for evaluating structure between locations with the program POWSIM 4.1 (Ryman and Palm 2006) using default parameters for the Markov Chain process with 1000 replicates. We tested an average effective population size of 120 to conform to values inferred in the study streams (see Results). Finally, we assessed the utility of the microsatellite suite for parentage analysis by examining the panel's ability to distinguish between related individuals. We calculated the average parent-pair and identity non-exclusion probabilities for the microsatellite suite in CERVUS 3.0 (Kalinowski et al. 2007). These indices measure, respectively, the probability that a set of markers will (1) match erroneous parents to offspring and (2) will not be able to distinguish between related individuals.

We assessed Bluehead Chub population structure between Todd Creek and Shoal Creek using several measures of differentiation including F_{ST} , R_{ST} , and exact tests for genic distributions (i.e., the "G" statistic), all calculated in GENEPOP with default parameters. We estimated measures of genetic diversity including observed heterozygosity, expected heterozygosity, and inbreeding

coefficients (Weir and Cockerham 1984) in GENEPOP. We determined the number of alleles and rarefied estimates of allelic richness for each marker using the program FSTAT 2.9.3.2 (Goudet 1995). Finally, we found an estimate of contemporary effective population size using the single-sample program LDNE (Waples and Do 2008). Genetic drift generates non-random associations among unlinked markers; LDNE analyzes this linkage disequilibrium to determine contemporary effective population size for a single time point, producing 3 values based on pre-set allele-frequency exclusion criteria. We set allele-frequency exclusion criteria at default values (<0.01 , <0.02 , <0.05), but we only reported the <0.01 allele-frequency exclusion value, as recommended for large sample sizes (>100 samples; Waples and Do 2010). We assumed a random-mating model and chose the “jack-knife on loci” option for the confidence intervals.

Results

For statistical analyses with the additional Bluehead Chub samples, most markers adhered to HWE after correction for multiple comparisons ($P > 0.004$). Three markers showed a departure from HWE in just a single location (Ca5 and Ca10 in Todd Creek and RSD53 in Shoal Creek; see Supplemental Table S1, available online at <http://www.eaglehill.us/SENAonline/suppl-files/s19-2-S2570-Darden-s1>, and for Bioone subscribers, at <https://dx.doi.org/10.1656/S2570.s1>), and only 1 marker (Nme18A6.158) was out of HWE in both creeks. A few combinations of markers showed significant linkage disequilibrium in either Todd Creek (Ca11 x Ppro126) or Shoal Creek (Ca12 x Rhos36), but only 1 pair of markers was found to be linked in both locations (Ca5 x Ca10). Although 2 markers displayed a high frequency of possible null alleles at 1 site (RSD53 and Ppro126 in Shoal Creek; see Supplemental Table S1), only 1 marker (Nme18A6.158) showed evidence of possible null alleles at both sites. Since Nme18A6.158 displayed statistical discrepancies in both locations (indicating potential genotyping errors) and Ca10 was shown to be linked to Ca5, these markers were excluded from further consideration, resulting in a final set of 9 microsatellite markers. All subsequent results are based on these 9 markers.

Five microsatellite markers in the final panel amplified in both Yellowfin Shiner and Bluehead Chub; the remaining 4 markers amplified only for Bluehead Chub (Table 1), demonstrating that the microsatellite panel can distinguish between the 2 species. The microsatellite panel provided sufficient power to detect differentiation among locations for Bluehead Chub. The probability of obtaining a significant result ($P < 0.05$) in contingency tests between streams was 1.0 (χ^2) with an $F_{ST} = 0.040$ (or larger) for an average effective population size of 120. The marker suite provided an average parent-pair non-exclusion probability of 7.43^{-10} and average identify non-exclusion probability of 5.34^{-14} , indicating that the possibility of mis-assignment in parentage analysis would be substantially less than 0.01% and that related individuals can be distinguished from one another with confidence.

Bluehead Chub populations were differentiated between Todd Creek and Shoal Creek. Pairwise comparisons between locations were significant for F_{ST} ($F_{ST} =$

0.090, $P = 0.000$), R_{ST} ($R_{ST} = 0.093$, $P = 0.000$), and the “G” statistic ($\chi^2 = \infty$, $df = 8$, $P < 0.0001$). Genetic diversity for Bluehead Chub in both creeks was high. The number of alleles and estimates of rarefied allelic richness varied from 3.0 to 25.0 with an average of 14.8 in Todd Creek and from 6.3 to 36.3 with an average of 17.9 in Shoal Creek (Table 2; also see Supplemental Table S1). Observed heterozygosity varied from 0.500 to 0.929 in Todd Creek and from 0.379 to 0.916 in Shoal Creek, while expected heterozygosity varied from 0.549 to 0.934 in Todd Creek and from 0.368 to 0.933 in Shoal Creek (see Supplemental Table S1). Average observed and expected heterozygosity values were moderately high (>0.70) for both locations (Table 2). Individual sites displayed elevated levels of inbreeding at some markers (Ca5 in Todd Creek, RSD53 and Ca12 in Shoal Creek; see Supplemental Table S1), but none of the markers showed increased inbreeding coefficients in both locations. The overall coefficient of inbreeding, averaged across markers, was very low for Todd Creek and within accepted values for Shoal Creek (Table 2). Point estimates of effective population size for both locations were over 100 individuals (Table 2).

Discussion

We assembled and optimized a set of microsatellite markers that can be used to obtain important information on the genetic characteristics and connectivity of Bluehead Chub populations in the southeastern US and provide a molecular means to distinguish the eggs of Bluehead Chub from those of the nest-associating Yellowfin Shiner. The markers amplify in concurrent multiplex reactions and are polymorphic. All 9 markers were statistically independent in at least 1 collection location, and the majority adhered to HWE and had a low frequency of possible null alleles. Furthermore, our optimized microsatellite suite displayed sufficient power to be reliable for investigations of population structure between localities (Ryman and Palm 2006) and parentage analysis in which paternal and maternal contributions on nesting mounds can be examined. The low non-exclusion probabilities, less than the 0.01–0.0001 probability recommended for codominant makers (Waits et al. 2001), indicate that related individuals can be distinguished from one another

Table 2. Genetic diversity statistics, averaged across 9 microsatellite markers, for Bluehead Chub from 2 streams in South Carolina. n = sample size, A = number of alleles, R = rarefied allelic richness, H_o = observed heterozygosity, H_e = expected heterozygosity, F_{IS} = inbreeding coefficients ($F_{IS} \geq 0.05$ indicates high levels of inbreeding), N_e = effective population size (confidence intervals are shown in parentheses).

Statistic	Todd Creek	Shoal Creek
n	224	335
A	15	19
R	14.8	17.9
H_o	0.769	0.705
H_e	0.766	0.740
F_{IS}	-0.004	0.047
N_e	121 (104–142)	155 (127–193)

and offspring correctly matched to parents with a high degree of confidence. Finally, the first multiplex group of our microsatellite panel provides a diagnostic tool for the molecular identification of Bluehead Chub and Yellowfin Shiner eggs, which can be difficult to differentiate visually (Silknetter et al. 2019). Only 1 of the markers in the first group amplifies in Yellowfin Shiner versus all 3 for Bluehead Chub, providing not only a means to distinguish between the species but a positive internal control for all reactions. The tool has been successfully employed for differentiating Bluehead Chub and Yellowfin Shiner eggs on Bluehead Chub nests (Silknetter et al. 2019). It should be noted that the diagnostic capabilities of our microsatellite suite have not been investigated in other leuciscids, and the functionality of this marker panel for additional nest-associating species is unknown. Therefore, this panel may be best applied in areas where Yellowfin Shiner comprise all or the majority of nest-associates on Bluehead Chub spawning mounds.

To evaluate the utility of the marker set in population genetic analyses, we examined genetic structure and diversity of Bluehead Chub in Todd Creek and Shoal Creek in South Carolina. Although they are located in the same major drainage, these creeks have no direct connection to one another and are separated by a man-made reservoir (Lake Hartwell). Even though Bluehead Chub have been known to move more than 1000 m in distance (Albanese et al. 2003), the reservoir likely forms a barrier which impedes the migration and gene flow of riverine species like Bluehead Chub (Luttrell et al. 1999, Matthews and Marsh-Matthews 2007, Pelicice et al. 2014, Roberts et al. 2013). As such, we found Todd Creek and Shoal Creek individuals to be genetically distinct from one another, indicating that Bluehead Chub in these areas form unique populations. A species' ability to respond to environmental variability may be negatively affected by elevated levels of inbreeding and reduced genetic diversity (Frankham 2005, Frankham et al. 2002, Keller and Waller 2002, Reed and Frankham 2003). Similarly, reduced effective population size may decrease the overall fitness and sustainability of a population, making effective population size an important measure in conservation biology (Gilpin and Soulé 1986, Newman and Pilson 1997). Both Todd Creek and Shoal Creek populations were documented to contain moderately high levels of genetic diversity and low levels of inbreeding, as evidenced by their allelic richness values, degree of heterozygosity, and inbreeding coefficients. Furthermore, the levels of genetic variation found in our study are comparable to those in other investigations of North American leuciscids (Gold et al. 2004, McCracken et al. 2014, Osborne et al. 2013, Ozer and Ashley 2013, Pitcher et al. 2009, Skalski et al. 2008). Effective population sizes for both Todd Creek and Shoal Creek were estimated at just over 100 individuals for each location. These values for effective size are similar to estimates from other North American leuciscids (Alò and Turner 2005, Osborne et al. 2013) and are above the minimum value of 50 recommended to prevent significant inbreeding and maintain short-term fitness of a population (Franklin 1980). Although effective population size estimates generated by LDNE may be affected by overlapping generations which can bias estimates downward from the true effective size (Waples et al. 2014), given the short life span of Bluehead Chub (~4 years; Tracey 2009), our

effective population size estimates would likely be minimally biased by overlapping generations.

In conclusion, we compiled a suite of 9 microsatellite markers for Bluehead Chub. Using this new tool, we found that Bluehead Chub may form localized populations, as suggested by the genetic differentiation between Bluehead Chub from 2 creeks in South Carolina. Consequently, conservation efforts for Bluehead Chub will need to account for the possibility of widespread differentiation throughout the range of this species. Moreover, the high levels of genetic variation and low levels of inbreeding, in combination with the modest effective population size estimates, indicate that Bluehead Chub in Todd Creek and Shoal Creek are genetically diverse. However, estimates of genetic variation, inbreeding, and effective population size should continue to be investigated for Bluehead Chub in these streams and other freshwater systems as anthropomorphic activities may impact these regions in the future. The results of our study and other inquiries offer valuable information for the effective preservation of Bluehead Chub and the nest associates that depend on Bluehead Chub in the southeastern United States.

Acknowledgments

This project was supported by the Creative Inquiry Program, Clemson University, and the Warner College of Natural Resources at Colorado State University. Reference to trade names does not imply endorsement by any of the agencies mentioned herein. This is publication number 821 from the Marine Resources Research Institute, SCDNR.

Literature Cited

- Albanese, B., P.L. Angermeier, and C. Gowan. 2003. Designing mark–recapture studies to reduce effects of distance weighting on movement distance distributions of stream fishes. *Transactions of the American Fisheries Society* 132:925–939.
- Allan, J.D., and A.S. Flecker. 1993. Biodiversity conservation in running waters. *BioScience* 43:32–43.
- Alò, D., and T.F. Turner. 2005. Effects of habitat fragmentation on effective population size in the endangered Rio Grande Silvery Minnow. *Conservation Biology* 19:1138–1148.
- Anderson, C.M., and S.K. Sarver. 2008. Development of polymorphic microsatellite loci for the endangered Topeka Shiner, *Notropis topeka*. *Molecular Ecology Resources* 8:311–313.
- April, J., R.L. Mayden, R.H. Hanner, and L. Bernatchez. 2011. Genetic calibration of species diversity among North America’s freshwater fishes. *Proceedings of the National Academy of Sciences* 108:10602–10607.
- Arthington, A.H., N.K. Dulvy, W. Gladstone, and I.J. Winfield. 2016. Fish conservation in freshwater and marine realms: Status, threats, and management. *Aquatic Conservation* 26:838–857.
- Bessert, M.L., and G. Ortí. 2003. Microsatellite loci for paternity analysis in the Fathead Minnow, *Pimephales promelas* (Teleostei: Cyprinidae). *Molecular Ecology Resources* 3:532–534.
- Bolton, C., B.K. Peoples, and E.A. Frimpong. 2015. Recognizing gape limitation and inter-annual variability in Bluehead Chub nesting microhabitat use in a small Virginia stream. *Journal of Freshwater Ecology* 30:503–511.

- Buckwalter, J.D., E.A. Frimpong, P.L. Angermeier, and J.N. Barney. 2017. Seventy years of stream fish collections reveal invasions and native range contractions in an Appalachian (USA) watershed. *Diversity and Distributions* 24:219–232.
- Burr, B.M., and R.L. Mayden. 1992. Phylogenetics and North American freshwater fishes. Pp. 18–75, *In* R.L. Mayden (Ed.). *Systematics, Historical Ecology, and North American Freshwater Fishes*. Stanford University Press, Stanford, CA. 969 pp.
- Burrige, C.P., and J.R. Gold. 2003. Conservation genetic studies of the endangered Cape Fear Shiner, *Notropis mekistocholas* (Teleostei: Cyprinidae). *Conservation Genetics* 4:219–225.
- Cashner, M.F., and H.L. Bart. 2010. Reproductive ecology of nest associates: Use of RFLPs to identify cyprinid eggs. *Copeia* 2010:554–557.
- Cashner, M.F., and H.L. Bart. 2018. Spawning community and egg deposition for three southeastern nest-associate minnows. *Southeastern Naturalist* 17:43–55.
- Dimoski, P., G.P. Toth, and M.J. Bagley. 2000. Microsatellite characterization in Central Stoneroller, *Campostoma anomalum* (Pisces: Cyprinidae). *Molecular Ecology* 9:2187–2189.
- Dudgeon, D., A.H. Arthington, M.O. Gessner, Z.I. Kawabata, D.J. Knowler, C. Lévêque, R.J. Naiman, A.H. Prieur-Richard, D. Soto, M.L.S. Stiassny, and C.A. Sullivan. 2006. Freshwater biodiversity: Importance, threats, status, and conservation challenges. *Biological Reviews* 81:163–182.
- Floyd, S.P., B.K. Peoples, and E.A. Frimpong. 2018. Disentangling reproductive interactions among communal spawning minnows using reproductive condition and visual observations. *American Midland Naturalist* 179:166–179.
- Frankham, R. 2005. Genetics and extinction. *Biological Conservation* 126:131–140.
- Frankham, R., J.D. Ballou, and D.A. Briscoe. 2002. *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, UK. 617 pp.
- Franklin, I.R. 1980. Evolutionary change in small populations. Pp. 135–149, *In* M.E. Soulé, and B.A. Wilcox (Eds.). *Conservation Biology: An Evolutionary-Ecological Perspective*. Sinauer Associates, Sunderland, MA. 395 pp.
- Gilpin, M.E., and M.E. Soulé. 1986. Minimum viable populations: Processes of species extinction. Pp. 19–34, *In* M.E. Soulé (Ed.). *Conservation Biology: The Science of Scarcity and Diversity*. Sinauer Associates, Sunderland, MA. 584 pp.
- Gold, J.R., E. Saillant, C.P. Burrige, A. Blanchard, and J.C. Patton. 2004. Population structure and effective size in critically endangered Cape Fear Shiners *Notropis mekistocholas*. *Southeastern Naturalist* 3:89–102.
- Goudet, J. 1995. FSTAT (version 1.2): A computer program to calculate *F*-statistics. *Journal of Heredity* 86:485–486.
- Hanna, A.H., E.W. Carson, G.P. Garrett, and J.R. Gold. 2015. Conservation genetics of six species of genus *Dionda* (Cyprinidae) in the southwestern United States. *Monographs of the Western North American Naturalist* 8:1–25.
- Herrington, S.J., and K.J. Popp. 2004. Observations on the reproductive behavior of the nonindigenous Rough Shiner, *Notropis baileyi*, in the Chattahoochee River system. *Southeastern Naturalist* 3:267–276.
- Hitt, N.P., and J.H. Roberts. 2011. Hierarchical spatial structure of stream fish colonization and extinction. *Oikos* 121:127–137.
- Holm, S. 1979. A simple sequential rejective multiple test procedure. *Scandinavian Journal of Statistics* 6:65–70.
- Jelks, H.J., S.J. Walsh, N.M. Burkhead, S. Contreras-Balderas, E. Díaz-Pardo, D.A. Hendrickson, J. Lyons, N.E. Mandrak, et al. 2008. Conservation status of imperiled North American freshwater and diadromous fishes. *Fisheries* 33:372–407.

E.L. Cushman, K.L. Kanapeckas Métris, Y. Kanno, K.C. Pregler, B.K. Peoples, and T.L. Darden

- Jenkins, R.E., and N.M. Burkhead. 1994. *Freshwater Fishes of Virginia*. American Fisheries Society, Bethesda, MD. 1079 pp.
- Johnston, C.E., and L.M. Page. 1992. The evolution of complex reproductive strategies in North American minnows (Cyprinidae). Pp. 600–621, *In* R.L. Mayden (Ed.). *Systematics, Historical Ecology, and North American Freshwater Fishes*. Stanford University Press, Stanford, CA. 969 pp.
- Kalinowski, S.T., M.L. Taper, and T.C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16:1099–1106.
- Keller, L.F., and D.M. Waller. 2002. Inbreeding effects in wild populations. *Trends in Ecology and Evolution* 17:230–241.
- Luttrell, G.R., A.A. Echelle, W.L. Fisher, and D.J. Eisenhour. 1999. Declining status of two species of the *Macrhybopsis aestivalis* complex (Teleostei: Cyprinidae) in the Arkansas River Basin and related effects of reservoirs as barriers to dispersal. *Copeia* 1999:981–989.
- Mammoliti, C.S. 2002. The effects of small watershed impoundments on native stream fishes: A focus on the Topeka Shiner and Hornyhead Chub. *Transactions of the Kansas Academy of Science* 105:219–231.
- Marcy, B.C., D.E. Fletcher, F.D. Martin, M.H. Paller, and M.J.M. Reichert. 2005. *Fishes of the Middle Savannah River Basin: With Emphasis on the Savannah River Site*. University of Georgia Press, Athens, GA. 480 pp.
- Matthews, W.J., and E. Marsh-Matthews. 2007. Extirpation of Red Shiner in direct tributaries of Lake Texoma (Oklahoma–Texas): A cautionary case history from a fragmented river-reservoir system. *Transactions of the American Fisheries Society* 136:1041–1062.
- Maurakis, E.G., W.S. Woolcott, and M.H. Sabaj. 1991. Reproductive-behavioral phylogenetics of *Nocomis* species-groups. *American Midland Naturalist* 126:103–110.
- Maurakis, E.G., W.S. Woolcott, and M.H. Sabaj. 1992. Water currents in spawning areas of pebble nests of *Nocomis leptcephalus* (Pisces: Cyprinidae). *Proceedings of the Southeastern Fishes Council* 25:1–3.
- McAuliffe, J.R., and D.H. Bennett. 1981. Observations on the spawning habits of the Yellowfin Shiner, *Notropis lutipinnis*. *Journal of the Elisha Mitchell Scientific Society* 97:200–203.
- McCracken, G.R., H.T. Brewis, M.C. McBride, R. Perry, D. Keefe, and D.E. Ruzzante. 2014. Development and characterization of 36 novel microsatellite markers for Lake Chub (*Couesius plumbeus*) using Illumina paired-end sequencing. *Conservation Genetics Resources* 6:873–876.
- McCusker, M.R., N.E. Mandrak, B. Egeh, and N.R. Lovejoy. 2014. Population structure and conservation genetic assessment of the endangered Pugnose Shiner, *Notropis anogenus*. *Conservation Genetics* 15:343–353.
- McManamay, R.A., D.J. Orth, C.A. Dolloff, and M.A. Cantrell. 2010. Gravel addition as a habitat restoration technique for tailwaters. *North American Journal of Fisheries Management* 30:1238–1257.
- Nagle, B.C., and A.M. Simons. 2012. Rapid diversification in the North American minnow genus *Nocomis*. *Molecular Phylogenetics and Evolution* 63:639–649.
- Newman, D., and D. Pilson. 1997. Increased probability of extinction due to decreased genetic effective population size: Experimental populations of *Clarkia pulchella*. *Evolution* 51:354–362.

- Nunziata, S.O., S.L. Lance, K.L. Jones, S.A. Nerkowski, and A.E. Metcalf. 2013. Development and characterization of twenty-three microsatellite markers for the freshwater minnow Santa Ana Speckled Dace (*Rhinichthys osculus* spp., Cyprinidae) using paired-end Illumina shotgun sequencing. *Conservation Genetics Resources* 5:145–148.
- Osborne, M.J., T.A. Diver, and T.F. Turner. 2013. Introduced populations as genetic reservoirs for imperiled species: A case study of the Arkansas River Shiner (*Notropis girardi*). *Conservation Genetics* 14:637–647.
- Ozer, F., and M.V. Ashley. 2013. Genetic evaluation of remnant and translocated shiners, *Notropis heterodon* and *Notropis heterolepis*. *Journal of Fish Biology* 82:1281–1296.
- Page, L.M., and B.M. Burr. 1991. *A Field Guide to Freshwater Fishes: North America, North of Mexico*. Houghton Mifflin Company, Boston, MA. 432 pp.
- Pellicice, F.M., P.S. Pompeu, and A.A. Agostinho. 2014. Large reservoirs as ecological barriers to downstream movements of neotropical migratory fish. *Fish and Fisheries* 16:697–715.
- Pendleton, R.M., J.J. Pritt, B.K. Peoples, and E.A. Frimpong. 2012. The strength of *Nocomis* nest association contributes to patterns of rarity and commonness among New River, Virginia, cyprinids. *American Midland Naturalist* 168:202–212.
- Peoples, B.K., M.B. Tainer, and E.A. Frimpong. 2011. Bluehead Chub nesting activity: A potential mechanism of population persistence in degraded stream habitats. *Environmental Biology of Fishes* 90:379–391.
- Peoples, B.K., R.A. McManamay, D.J. Orth, and E.A. Frimpong. 2014. Nesting-habitat use by River Chubs in a hydrologically variable Appalachian tailwater. *Ecology of Freshwater Fish* 23:283–293.
- Peoples, B.K., L.A. Blanc, and E.A. Frimpong. 2015. Lotic cyprinid communities can be structured as nest webs and predicted by the stress-gradient hypothesis. *Journal of Animal Ecology* 84:1666–1677.
- Pitcher, T.E., C.L. Beneteau, R.P. Walter, C.C. Wilson, N.E. Mandrak, and D.D. Heath. 2009. Isolation and characterization of microsatellite loci in the Redside Dace, *Clinostomus elongates*. *Conservation Genetics Resources* 1:381–383.
- Reed, D.H., and R. Frankham. 2003. Correlation between fitness and genetic diversity. *Conservation Biology* 17:230–237.
- Renshaw, M.A., E.W. Carson, A.H. Hanna, C.E. Rexroad III, T.J. Krabbenhoft, and J.R. Gold. 2009. Microsatellite markers for species of the genus *Dionda* (Cyprinidae) from the American southwest. *Conservation Genetics* 10:1569–1575.
- Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* 43:223–225.
- Roberts, J.H., P.L. Angermeier, and E.M. Hallerman. 2013. Distance, dams, and drift: What structures populations of an endangered, benthic stream fish? *Freshwater Biology* 58:2050–2064.
- Ross, C., J. Barringer, J. Yang, M. Woo, J. Doyle, and H. West. 2008. Megaregions: Literature review of the implications for US infrastructure investment and transportation planning. Final report. US Department of Transportation, Federal Highway System, Washington, DC. 95 pp.
- Rousset, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8:103–106.
- Ryman, N., and S. Palm. 2006. POWSIM: A computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology Notes* 6:600–602.
- Schwemm, M.R., A.A. Echelle, and R.A. Van Den Bussche. 2014. Isolation and characterization of 10 polymorphic microsatellite markers for the Ouachita Highlands endemic *Notropis suttkusi* (Teleostei: Cyprinidae). *Conservation Genetics Resources* 6:209–210.

- Silknetter, S., Y. Kanno, K.L. Kanapeckas Métris, E. Cushman, T.L. Darden, and B.K. Peoples. 2019. Mutualism or parasitism: Partner abundance affects host fitness in a fish reproductive interaction. *Freshwater Biology* 64:175–182.
- Simons, A.M., and R.L. Mayden. 1999. Phylogenetic relationships of North American cyprinids and assessment of homology of the open posterior myodome. *Copeia* 1999:13–21.
- Skalski, G.T., J.B. Landis, M.J. Grose, and S.P. Hudman, 2008. Genetic structure of Creek Chub, a headwater minnow, in an impounded river system. *Transactions of the American Fisheries Society* 137:962–975.
- Tan, M., and J.W. Armbruster. 2018. Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei: Ostariophysii). *Zootaxa* 4476:6–39.
- Tracy, B.H. 2009. Bluehead Chub, *Nocomis leptocephalus* (Girard 1856), the first species of freshwater fish scientifically described from North Carolina. *American Currents* 38:2–8.
- Utz, R.M. 2014. Are we overlooking landscape-scale threats to common freshwater fishes? *Fisheries* 39:294–297.
- Vives, S.P. 1990. Nesting ecology and behavior of Hornyhead Chub, *Nocomis biguttatus*, a keystone species in Allequash Creek, Wisconsin. *American Midland Naturalist* 124:46–56.
- Vrijenhoek, R.C. 1998. Conservation genetics of freshwater fish. *Journal of Fish Biology* 53:394–412.
- Waits, L.P., G. Luikart, and P. Taberlet. 2001. Estimating the probability of identity among genotypes in natural populations: Cautions and guidelines. *Molecular Ecology* 10:249–256.
- Wallin, J.E. 1989. Bluehead Chub (*Nocomis leptocephalus*) nests used by Yellowfin Shiners (*Notropis lutipinnis*). *Copeia* 1989:1077–1080.
- Wallin, J.E. 1992. The symbiotic nest association of Yellowfin Shiners, *Notropis lutipinnis*, and Bluehead Chubs, *Nocomis leptocephalus*. *Environmental Biology of Fishes* 33:287–292.
- Walser, C.A., B. Falterman, and H.L. Bart. 2000. Impact of introduced Rough Shiner (*Notropis baileyi*) on the native fish community in the Chattahoochee River system. *American Midland Naturalist* 144:393–405.
- Waples, R.S., and C. Do. 2008. LDNE: A program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources* 8:753–756.
- Waples, R.S., and C. Do. 2010. Linkage disequilibrium estimates of contemporary N_e using highly variable genetic markers: A largely untapped resource for applied conservation and evolution. *Evolutionary Applications* 3:244–262.
- Waples, R.S., T. Antao, and G. Luikart. 2014. Effects of overlapping generations on linkage disequilibrium estimates of effective population size. *Genetics* 197:769–780.
- Warren, M.L., P.L. Angermeier, B.M. Burr, and W.R. Haag. 1997. Decline of a diverse fish fauna: Patterns of imperilment and protection in the southeastern United States. Pp. 105–164, *In* G.W. Benz, and D.E. Collins (Eds.). *Aquatic Fauna in Peril: The Southeastern Perspective*. Lenz Design and Communications, Decatur, GA. 554 pp.
- Weir, B.S., and C.C. Cockerham. 1984. Estimating F -statistics for the analysis of population structure. *Evolution* 38:1358–1370.
- Wisenden, B.D., A. Unruh, A. Morantes, S. Bury, B. Curry, R. Driscoll, M. Hussein, and S. Markegard. 2009. Functional constraints on nest characteristics of pebble mounds of breeding male Hornyhead Chubs, *Nocomis biguttatus*. *Journal of Fish Biology* 75:1577–1585.