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Conservation genetic studies of the endangered Cape Fear Shiner, *Notropis mekistocholas* (Teleostei: Cyprinidae)

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Abstract

Genetic variation at ten microsatellite loci and one anonymous-nuclear locus was assayed for three geographic samples of the critically endangered North American cyprinid *Notropis mekistocholas* (Cape Fear shiner). Despite low abundance of this species, there was little suggestion of small population effects; allele diversity and heterozygosity were relatively high, F_{IS} values within samples were non-significant, and genotypes were distributed in frequencies according to Hardy-Weinberg expectations. Genetic divergence among samples was minimal despite the presence of dams, constructed in the early 1900s, that separate the sample sites. This suggests that recent gene flow has been sufficient to inhibit genetic divergence or that gene flow has been reduced but there has been insufficient time for genetic divergence to develop. Tests of heterozygosity excess were non-significant, suggesting that *N. mekistocholas* in the localities sampled have not undergone recent reductions in effective population size. Future studies employing larger sample sizes to provide more robust tests of population structure and temporally separated samples to estimate contemporaneous N_e are warranted.

Introduction

The Cape Fear shiner *Notropis mekistocholas* Snelson (1971) is a small (<70 mm SL) herbivorous cyprinid fish from the east-central Piedmont region in North Carolina. It is the only endemic fish known from the Cape Fear drainage, and by all accounts has a more restricted distribution than any of the other 70 or so described species of North American shiners of genus *Notropis* (Snelson 1971; Mayden 1991). The species was discovered in 1962 in a poorly sampled region of the Cape Fear drainage, and although it is morphologically similar to sympatric *Notropis procne* (swallowtail shiner), surveys of museum collections failed to identify earlier undocumented collections of *N. mekistocholas* (Snelson 1971). While it is possible that *N. mekistocholas* may always have existed in low numbers (Snelson 1971), surveys subsequent to the late 1970s suggested a reduction in abundance and range that was not evident for other sympatric taxa (Pottern and Huish 1985, 1986, 1987). More recent surveys (North Carolina Wildlife Resources Commission 1995) have documented only five extant 'populations' of *N. mekistocholas* within the Cape Fear drainage. The species was federally listed in 1987 as 'endangered' (Federal Register 1987) and designated as 'critically endangered' in 1996 (Hilton-Taylor 2000). Perceived impacts on abundance and distribution of *N. mekistocholas* are dams and hydroelectric plants in the Cape Fear drainage and resultant changes to stream morphology and flow regime (Federal Register 1987; U.S. Fish and Wildlife Service 1988).

In this paper, we report the first genetic analysis of populations of *N. mekistocholas*. The study had three objectives: (i) quantify genetic diversity to assess whether small population effects might be occurring; (ii) determine whether geographic samples of *N. mekistocholas* differed genetically and hence might represent separate management units; and (iii) assess whether sampled populations were increasing

or decreasing in genetic effective population size (N_e). Nuclear-encoded DNA markers were developed and used to meet these objectives.

Materials and methods

Specimens of *N. mekistocholas* were collected by Chittick et al. (2001) from multiple seine hauls at three localities (Figure 1) in the Cape Fear drainage. Ten individuals were collected from the Deep River near the bridge on SR 1456 in Moore County, North Carolina (*SR1456*); 15 individuals were sampled further downstream on the Deep River, adjacent to a bridge on NC 22 and downstream of the dam at High Falls, Moore County (*High Falls*); and 15 individuals were collected at the confluence of the Deep and Rocky rivers, downstream of the two aforementioned sample sites and the dam at Carbonton (*Confluence*). *SR1456* is separated from *High Falls* by the High Falls dam, built sometime before 1935, and *High Falls* is separated from *Confluence* by the Carbonton dam, built in 1922 (Figure 1; D. Rabon, US Fish and Wildlife Service, personal communication). Ages of individuals were not known. Caudal peduncle tissues from all specimens were sent on dry ice to College Station, where they were stored at −80 °C. DNA for polymerase chain reaction (PCR) amplification was extracted from frozen muscle tissue following procedures in Gold and Richardson (1991).

Approximately 17 μg of *N. mekistocholas* genomic DNA was used to construct a partial genomic library, using methods employed routinely in our laboratory (Turner et al. 1998). Transformant colonies were screened with $[CA]_{15}$ and $[GA]_{15}$ dinucleotide probes. Inserts from positive colonies were sequenced, and PCR primers designed using PrimerSelect (Lasergene package DNASTAR). PCR reaction composition and electrophoretic conditions followed Heist and Gold (2000), except that 35 cycles and 60 sec extensions were employed. A 2 h final extension at 72 °C was adopted to promote "a-tailing" (Brownstein et al. 1996) when excessive 1 bp 'stutter' banding was observed.

Genetic variability was measured as number of alleles per locus, observed heterozygosity (H_o), and deficiency/excess of heterozygotes; Weir and Cockerham's (1984) f statistic was used to assess the last. Tests of Hardy-Weinberg equilibrium within samples and homogeneity of allele distributions among samples employed Fisher's exact test when there were less than five alleles per locus; otherwise, an unbiased estimate of the exact test statistic was calculated using a Markov chain procedure. All of these analyses were carried out using GENEPOP 3.3 (Raymond and Rousset 1995). Critical significance levels were adjusted for simultaneous tests by using the sequential Bonferroni procedure to obtain type I error rates less than 0.05 (Rice 1989).

Genetic divergence among samples was quantified via F_{ST} (Wright 1951), using FSTAT 2.9.1 (Goudet 1995). A multilocus estimate of F_{ST} was obtained by averaging variance components across loci. One thousand replicates of permutation were used to determine whether estimates of F_{ST} differed significantly from zero. Analysis of molecular variance (AMOVA) after Excoffier et al. (1992) also was used to quantify genetic divergence among samples.

BOTTLENECK 1.2.02 (Piry et al. 1999) was used to test for increasing or decreasing effective population size (N_e). The program calculates the difference between Hardy-Weinberg expected heterozygosity (gene diversity) and expected heterozygosity based on number of alleles and sample size, assuming mutation-drift equilibrium. We used the three models of mutation available in the software package: IAM (infinite allele model), SMM (stepwise mutation model), and TPM. The last is a two-phase model that incorporates 95% single-step mutations and a 12% variance of multiple-step mutations (Di Rienzo et al. 1994).

Results

Inserts from forty positive colonies were sequenced and found to contain a total of 52 microsatellite-like repeats, mostly of the motif $[TG]_n$. Ten of 16 designed primer pairs produced reliably scored products and yielded 11 putative loci. Primers developed for locus *Nme 18A6.158* amplified several products in addition to the target repeat: three of these products (119, 120, and 121 bp) were scored as alleles of an anonymous locus, *Nme 18A6.0*. Microsatellite loci and their repeat sequence, GenBank Accession number of clones used to design PCR primers, annealing temperature, and PCR primer pair are given in Appendix 1. Microsatellites *Nme 4F4.154* and *Nme 4F4.222* were developed from the same clone sequence, as were microsatellites *Nme 24B6.191* and *Nme 24B6.211*.

Summary data for all 11 loci are given in Table 1. Allele frequencies at each locus in each sample may be obtained from JRG. With the exception of

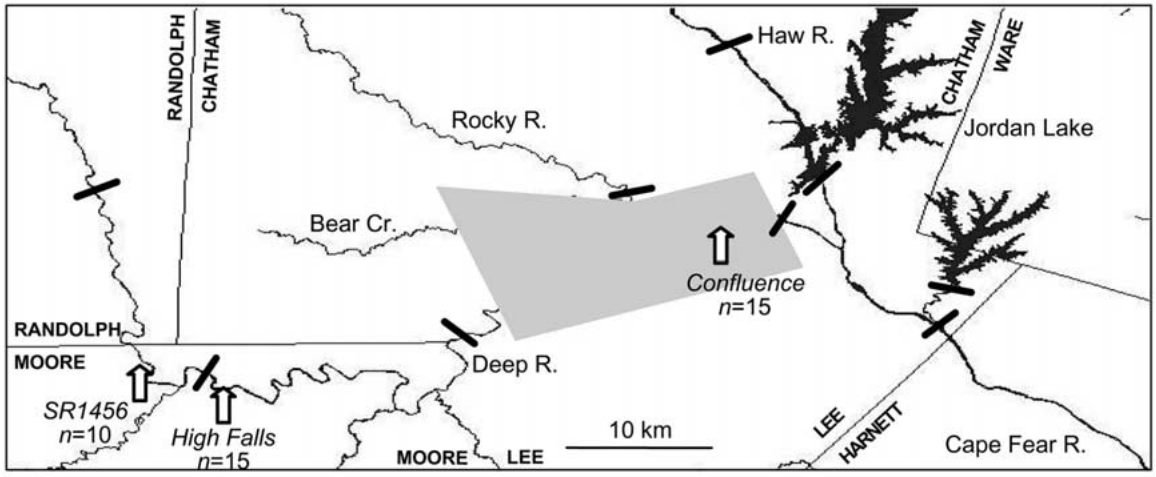

Figure 1. Collection sites (arrows) and sample sizes of *Notropis mekistocholas* from the Cape Fear drainage, North Carolina, USA. Shaded areas indicate "populations" (North Carolina Wildlife Resources Commission 1995), with dark shading representing potentially viable populations and light shading representing populations thought to be vulnerable to extirpation. Bars across rivers represent dams thought to be significant barriers to movement of *N. mekistocholas*. County boundaries are indicated.

Nme 24B6.191 in *Confluence*, all eleven loci were polymorphic in each sample. Allelic diversity per locus ranged from 1–9, with private alleles (14 total) occurring at each locality; observed heterozygosity ranged from ~0.50 to 0.85 at nine loci (Table 1). Three sample-locus combinations (*Nme 4F4.222* in *Confluence*, and *Nme 7B9.190* in *Confluence* and *SR1456*) showed significant heterozygote deficiency ($P_{HW} < 0.05$, $F_{IS} > 0$) before but not following Bonferroni correction. Frequencies of genotypes inferred from 119, 120, and 121 bp products obtained for anonymous locus *Nme 18A6.0* were in Hardy-Weinberg equilibrium, consistent with the hypothesis of three alleles at an anonymous locus.

Significant heterogeneity (exact tests) among samples in allele distribution was observed for *Nme 24B6.211* ($P = 0.046$) and *Nme 4F4.154* ($P = 0.018$); neither was significant following Bonferroni correction. The overall F_{ST} value of 0.003 differed significantly from zero ($P = 0.015$), as did the F_{ST} value (0.007, $P = 0.045$ after Bonferroni correction) for the pairwise comparison of *Confluence* vs. *High Falls*. Analysis of molecular variance illustrated that the majority of genetic variance (>97.5% in each sample) was distributed within rather than among samples; Φ_{ST} values for each locus and for all loci combined were small and none differed significantly from zero.

Tests for heterozygosity (gene diversity) excess or deficiency revealed significant heterozygosity deficiency under a strict stepwise-mutation model for *Confluence* ($P = 0.002$) and *SR1456* ($P = 0.034$). Significant heterozygosity deficiency was not observed under infinite-allele or two-phase (mixed) models, and significant heterozygosity excess was not observed for any sample under any of the mutation models.

Discussion

Our results indicate that small-population effects are not evident in the sampled populations of *N. mekistocholas*. Small-population effects are typically inferred when allele diversity and heterozygosity are low and/or F_{IS} values differ significantly from zero (Ralls et al. 1986; O'Brien et al. 1996). Given the small sample sizes, allele diversity and observed heterozygosity in the populations of *N. mekistocholas* appear commensurate with that observed in other freshwater fishes (DeWoody and Avise 2000). However, as a major concern for small populations of threatened or endangered species is the potential for reduced fitness and accumulation and fixation of slightly deleterious recessive mutations (Frankham 1995; Lynch et al. 1995), continued monitoring of *N. mekistocholas* to detect small population effects is warranted.

One goal of conservation programs is to identify and preserve historical population structure and/or patterns of diversity within and between populations

Table 1. Summary data for 11 nuclear-encoded loci in three geographic samples of *Notropis mekistocholas*: sample size (n), number of alleles (A), with number of private alleles in brackets, observed heterozygosity (H_o), probability (P_{HW}) of deviation from Hardy-Weinberg equilibrium, and F_{IS}.

Locus, repeat type	*Confluence* (n = 15)	*High Falls* (n = 15)	*SR1456* (n = 10)
Nme 4F4.154, $[GT]_6GA[GT]_4$			
A	4 [1]	4	4
H_o	0.600	0.867	0.500
P_{HW}	0.895	0.815	1.000
F_{IS}	0.008	−0.170	0.022
Nme 4F4.222, $[GT]_2CT[GT]_9$			
A	7 [1]	6	6
H_o	0.467	0.667	0.800
P_{HW}	0.040	0.273	0.673
F_{IS}	0.263	0.100	0.014
Nme 7B9.190, $[CA]_{12}$			
A	9 [1]	6	6
H_o	0.600	0.600	0.600
P_{HW}	0.034	0.146	0.024
F_{IS}	0.298	0.261	0.280
Nme 18A6.158, $[GT]_{12}$			
A	8 [1]	7	7 [1]
H_o	0.733	0.800	1.000
P_{HW}	0.088	0.079	0.861
F_{IS}	0.099	−0.047	−0.250
Nme 18A6.0, unknown			
A	3	3	2
H_o	0.467	0.467	0.600
P_{HW}	0.386	0.200	1.000
F_{IS}	0.148	0.317	−0.149
Nme 18C2.178, $[TG]_{15}$			
A	5 [1]	7 [3]	6 [1]
H_o	0.533	0.800	0.700
P_{HW}	0.145	0.555	0.514
F_{IS}	0.200	−0.057	0.008
Nme 24B6.191, $[AG]_6TGAC[AG]_6$			
A	1	2	2
H_o	0.000	0.133	0.200
P_{HW}	–	1.000	1.000
F_{IS}	–	−0.037	−0.059
Nme 24B6.211, $[CA]_{10}$			
A	5 [1]	9 [1]	8
H_o	0.600	0.600	0.700
P_{HW}	0.556	0.352	0.223
F_{IS}	0.128	0.060	0.067
Nme 25C8.208, $[TG]_9$			
A	9 [1]	9 [1]	9
H_o	0.867	0.867	0.800
P_{HW}	0.663	0.534	0.754
F_{IS}	−0.040	0.027	0.020
Nme 26G8.96, $[GT]_7$			
A	3	3	3
H_o	0.400	0.600	0.400
P_{HW}	0.437	1.000	1.000
F_{IS}	0.172	−0.172	0.153
Nme 30D2.120, $[GA]_8[CA]_6$			
A	2	2	3
H_o	0.133	0.133	0.300
P_{HW}	1.000	1.000	0.306
F_{IS}	−0.037	−0.037	0.169

of an endangered species (Vrijenhoek 1994). With respect to *N. mekistocholas*, genetic data obtained in this study are compatible with the hypothesis that historical gene flow among Cape Fear shiners inhabiting the localities sampled was generally sufficient to preclude extensive genetic divergence. Exact tests of homogeneity of allele distributions at all 11 loci were non-significant following Bonferroni correction, and none of the Φ_{ST} values obtained from AMOVA differed significantly from zero. Reduced gene flow among present-day *N. mekistocholas* may be suggested by significant F_{ST} values in the among-sample comparison and in the pairwise comparison between *High Falls* and *Confluence*. The dam between *Confluence* and *High Falls* has been in existence since the 1920s and the distance in stream miles between *High Falls* and *Confluence* is greater than that between *SR1456* and *High Falls*. Alternatively, one also might expect to find significant differences in allele distribution between *SR1456* and *Confluence*, given that *SR1456* is upstream of *High Falls* and hence further distant from *Confluence*. The inability to discriminate *SR1456* from *Confluence* (or from *High Falls*) may be due to the high sampling variance resulting from the small sample size (n = 10) for *SR1456*. It also could be that the effective population size at *Confluence* is considerably smaller than that at *SR1456* or *High Falls*, given that the amount of time required for genetic divergence to develop between separated populations depends largely on the number of elapsed generations and effective population size (Neigel and Avise 1986). Regardless, our data are compatible with the hypothesis that either recent gene flow has been sufficient to inhibit genetic divergence or that there has been insufficient time since the construction of dams in the Cape Fear drainage for substantial genetic divergence to develop.

We employed the 'heterozygosity excess' test of Cornuet and Luikart (1996) to assess whether the three populations of *N. mekistocholas* sampled were recently 'bottlenecked' (had undergone a reduction in genetic effective population size). Since the 1970s, reductions in abundance (census size) of *N. mekistocholas* at various localities in the Cape Fear drainage have been documented (Federal Register 1987), and concerns about effective population size (or analogous parameters) are typically raised in reference to management planning for threatened and/or endangered species (Waples 1990; Nunney and Elam 1994; Vrijenhoek 1996). None of the tests for heterozygosity excess were significant in any of the samples

Appendix 1. Repeat sequence, GenBank accession, annealing temperature, and PCR primers for nuclear-encoded loci of *Notropis mekistocholas*. Locus names correspond to the species in which they were characterized, clone identification, and length of the product amplified from the plasmid vector.

Locus	Repeat sequence	GenBank accession	Annealing temperature	PCR primer sequences
Nme 4F4.154	$[GT]_6GA[GT]_4$	AF532579	59	tgttctcagtgttggcaaaataaaa* tgaatcagctggtggaggtg
Nme 4F4.222	$[GT]_2CT[GT]_9$	AF532579	59	cacctccaccagctgattcagagtctc* agcatccggcccacgagcag
Nme 7B9.190	$[CA]_{12}$	AF532580	59	tcttggatgacattggggtgag ctggtgtgaatattgtggagagcat*
Nme 18A6.158	$[GT]_{12}$	AF532581	42	agggccaattactgaaca gaatgaaacgtccagatga*
Nme 18A6.0	Unknown	–	42	Same as *Nme 18A6.158*
Nme 18C2.178	$[TG]_{15}$	AF532582	59	tcaaaccctacagacagcaagact* tttctcaggggctccaacaag
Nme 24B6.191	$[AG]_6TGAC[AG]_6$	AF532583	59	ttgcaggggaaacatacc* gaatgggccgttactctc
Nme 24B6.211	$[CA]_{10}$	AF532583	59	cggacaggtgtgatggaatg accctgtggctgtgaacga*
Nme 25C8.208	$[TG]_9$	AF532584	59	aaaaaggcctcccagtgc* aattatatgtcggtgaccagattg
Nme 26G8.96	$[GT]_7$	AF532585	62	acacggctctgttgaatac gcgtgcgaatgttgaga*
Nme 30D2.120	$[GA]_8[CA]_6$	AF532586	59	tcacagtcggagaaaacctaaag* tcaggctcaccccagacc

*Labeled primer.

of *N. mekistocholas* under any of three different mutation models. Significant heterozygosity deficiency in the samples from *Confluence* and *SR1456*, alternatively, was detected but only under a stepwise-mutation model.

Although absence of detectable heterozygosity excess suggests that *N. mekistocholas* in these localities have not undergone recent reductions in effective population size, there are caveats to this inference. First, the approach employed can fail to identify past bottlenecks if the mutation-genetic drift equilibrium has been restored (Cornuet and Luikart 1996). This would not be unexpected for highly variable markers such as microsatellites (Luikart and Cornuet 1998). Second, the approach assumes that samples are representative of well-defined populations with no immigration from outside to inflate estimates of heterozygosity based on allele diversity (Cornuet and Luikart 1996; Luikart and Cornuet 1998). While dams and impoundments in the Cape Fear drainage might seem to preclude movement among seemingly isolated populations of *N. mekistocholas*, the near genetic homogeneity of the samples assayed and the possibility of 'dam-crossing' events (Federal Register 1987) are not inconsistent with the notion that periodic (albeit infrequent) immigration among 'isolated' populations in the Cape Fear drainage occurs. A final caveat regards the number of genetic markers and sample sizes employed in our study. While analyzing more (independent) markers has greater power benefit than sampling more genomes (Cornuet and Luikart 1996), high statistical power is achieved only when there are at least ten polymorphic genetic markers and from 20–30 individuals in each sample (Luikart and Cornuet 1998). While the number of polymorphic markers used in our study appears

suitable, the sample sizes at each locality were less than optimal.

The heterozygosity deficiency observed for the samples from *Confluence* and *SR1456* may be viewed positively from a conservation perspective, as it may reflect recent increases in effective population size at these localities. However, heterozygosity deficiency also may result from other factors, most notably the arrival via immigration of distinct alleles from other populations (Cornuet and Luikart 1996). Another possibility may be that the microsatellite loci used in the study are evolving only under the stepwise-mutation model and exhibiting heterozygosity deficiency because of the opening and closing of 'gaps' in an otherwise continuous allele distribution (Cornuet and Luikart 1996).

A final point to note regards the genetic data obtained in this study relative to the ongoing captive breeding program (Augspurger et al. 1999) for *N. mekistocholas*. This program was initiated with 30 individuals collected from the Rocky and Deep rivers (Augspurger et al. 1999). Assuming that allele frequencies observed in this study (and based on 40 total individuals) are representative of the entire species, a random sample of 30 individuals would be unlikely ($P < 0.05$) to contain an allele present in the wild at frequency of less than 0.067. Thirty-four such alleles were identified in this study, meaning that the captive population of *N. mekistocholas* likely does not contain the allelic diversity found in wild populations.

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