Mitochondrial DNA analysis of population structure in the Atlantic croaker, *Micropogonias undulatus* (Perciformes: Sciaenidae)

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Abstract.–Genetic population structure in Atlantic croaker (*Micropogonias undulatus* Linnaeus) was examined by using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA). Juvenile croaker from three U.S. Atlantic localities (Delaware, North Carolina, and Florida) and one Gulf of Mexico locality (Louisiana) were screened to document the magnitude and spatial distribution of mtDNA variation in *M. undulatus*; to evaluate the integrity of Cape Hatteras, North Carolina, as a genetic stock boundary; and to estimate levels of gene flow among Atlantic localities to provide an improved basis for future decisions regarding coastwide management of this fishery resource.

RFLP analysis of the ATPase 6 and D-loop mtDNA regions revealed a total of 15 composite haplotypes in 93 individuals. Monte Carlo simulations revealed no geographic heterogeneity in mtDNA haplotype frequencies among Atlantic localities and no evidence that juveniles collected north and south of Cape Hatteras originated from separate gene pools (net sequence divergence=–0.002%). There was significant heterogeneity between Atlantic and Gulf of Mexico samples, suggesting restricted gene flow between these two regions. Analysis of molecular variance also indicated regional (Atlantic versus Gulf) population structure, but provided no evidence that Cape Hatteras represents a genetic stock boundary. AMOVA indicated relatively high gene flow ($N_e m_{\varphi}$ = 12–23 effective female migrants per generation) among Atlantic localities. These findings are consistent with 1) a single genetic stock of *M. undulatus* on the Atlantic coast and 2) separate, weakly differentiated stocks in the Atlantic and Gulf of Mexico.

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Atlantic croaker (*Micropogonias undulatus* Linnaeus) is an important commercial and recreational fishery species along the U.S. Atlantic and Gulf of Mexico coasts (Chao and Musick, 1977; Mercer[1]). On the Atlantic coast, *M. undulatus* is common in estuarine and coastal waters from Indian River, Florida, to Chesapeake Bay (Nelson et al., 1991; Stone et al., 1994). Current fishery management practices for *M. undulatus* assume a single or "unit" stock for the entire Atlantic coast (Kline and Speir, 1993) despite indications that two stocks may exist. The two-stock hypothesis is based on variation in life history traits and population dynamics of Atlantic croaker occurring north and south of Cape Hatteras, North Carolina. White and Chittenden (1977) reported lower mortality rates, greater longevity, and larger maximum size for *M. undulatus* north of Cape Hatteras. Ross (1988) concluded that two groups with contrasting life histories may overlap in the vicinity of Cape Hatteras, with the northern, mainly offshore group exhibiting greater size-at-age, greater longevity, lower annual mortality, and delayed maturation compared with southern individuals. Geographic variation in biological characteristics of northern and southern *M. undulatus* implies that these groups may respond independently to exploitation and may require management as separate stocks; however, the basis for these differences (i.e. genetic or ecophenotypic) is unclear.

Identification of Atlantic croaker stock structure was listed among the priority research needs by the 1981 Sciaenid Assessment Workshop sponsored by the Atlantic States Marine Fisheries Commission (ASMFC) and National Marine Fisheries Service (Wilk and Austin, 1981). The Atlantic Croaker Fishery Management Plan, prepared under the ASMFC Interstate Fishery Management Program, also recommended research to identify croaker stocks along the Atlantic coast (Mercer[1]). In the present study, polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis

[1] Mercer, L. P. 1987. Fishery management plan for Atlantic croaker. Fisheries Management Rep. 10, Atlantic States Marine Fisheries Commission, Washington, D.C., 90 p.

Table 1

Sampling localities for juvenile Atlantic croaker. *n* is the number of individuals assayed from each locality.

Region and locality	Site	Collection date	*n*	Standard length (mm) mean (SD)	Standard length (mm) range
Atlantic					
Delaware Bay, Delaware (DE)	38°48'N 75°10'W	Oct 94	23	17.1 (1.8)	13–20
Cape Fear River, North Carolina (NC)	33°58'N 78°01'W	May 93	24	16.0 (1.9)	13–19
Indian River, Florida (FL)	28°03'N 80°35'W	Mar 94	22	19.4 (2.2)	16–23
Gulf of Mexico					
Terrebonne Bay, Louisiana (LA)	29°10'N 90°30'W	Jul 96	24	89.4 (4.5)	79–98

(RFLP) were used 1) to document the magnitude and spatial distribution of mtDNA variation in *M. undulatus* from U.S. Atlantic and Gulf of Mexico localities, 2) to evaluate the integrity of Cape Hatteras, North Carolina, as a genetic stock boundary, and 3) to estimate levels of gene flow among Atlantic localities to provide an improved basis for future decisions regarding coastwide management of this fishery resource.

Methods

Juvenile Atlantic croaker were collected from three U.S. Atlantic estuaries (Delaware Bay, Cape Fear River, and Indian River Lagoon) and one Gulf of Mexico estuary (Louisiana) (Table 1). Sampling localities were chosen to represent the northern, central, and southern portions of the species' U.S. Atlantic coast range and to include areas north and south of the hypothesized stock boundary at Cape Hatteras, North Carolina.

Genomic DNA extracts from individual croaker were obtained by using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) and were used in the polymerase chain reaction (Saiki et al., 1988) to amplify two mtDNA regions: the protein-coding ATP synthetase subunit 6 gene (ATPase 6) and the noncoding "D-loop" segment of the control region (Lankford, 1997). ATPase 6 was amplified by using the primers ATPase 6-L and ATPase 6-H (Quattro[2]), which yielded a 705-base-pair (bp) product. Primers L-Thr (5'-3': AGC TCA GCG CCA GAG CGC CGG TCT TGT AAA) and 12SAR-H (5'-3': ATA GTG GGG TAT CTA ATC CCA GTT) were used to amplify a 1540-bp product containing the entire D-loop region, tRNA-Pro and tRNA-Phe genes, and portions of the 12S rRNA and tRNA-Thr genes (Lee et al., 1995). Amplifications were performed in 50 μL reaction volumes according to Kocher et al. (1989). PCR products were digested with twelve restriction endonucleases: *Hinf*I, *Hae*III, *Msp*I, *Pvu*II, *Sau96*I, *Alu*I, *Mse*I, *Rsa*I, *Taq*I, *Dde*I, *Nla*III, and *Hha*I (obtained from New England BioLabs, Beverly, MA) (Lankford, 1997). Variant RFLP patterns were confirmed by means of repeat digestions and electrophoresis on 3% NuSieve agarose gels. Fragment sizes were estimated by using molecular weight markers: pUC18-*Hae*III digest from Sigma-Aldrich Corp., St. Louis, MO, pBR322-*Msp*I digest from New England BioLabs, Beverly, MA, and pBR322-*BstN*I digest from New England BioLabs; and the ANAGEL software program (Mrazek and Spanova, 1992).

Distinctive restriction-fragment patterns were identified by letter codes and subsequently combined to produce composite mtDNA haplotypes for individual fish. Nucleon diversity (h) was calculated for each locality (Nei and Tajima, 1981) with standard errors estimated according to Nei (1987). Nucleotide sequence diversity and nucleotide sequence divergence were calculated with the Restriction Enzyme Analysis Package (REAP, version 4.0) (McElroy et al., 1992). Frequency distributions of composite mtDNA haplotypes were tested for geographic homogeneity by using a Monte Carlo chi-square simulation approach (Roff and Bentzen, 1989) and a total of 10,000 random data resamplings. The integrity of Cape Hatteras as a genetic stock boundary was tested by comparing mtDNA haplotype frequencies north (Dela-

[2] Quattro, J. M. 1996. Department of Biological Sciences, Univ. South Carolina. Personal commun.

ware) and south (North Carolina and Florida) of this locality. Phylogenetic analysis was employed by using the character-state approach to construct a maximally parsimonious network relating individual haplotypes based on the number of restriction-site differences (Avise, 1994). The resulting network was examined for geographic structuring of haplotypes within and among sampling regions.

Population structure in *M. undulatus* was also evaluated by using nested analysis of molecular variance (AMOVA [Excoffier et al., 1992]). AMOVA input consisted of an Euclidean distance matrix containing genetic distance values for all possible pairs of the 15 observed mtDNA haplotypes. Samples were stratified by locality (DE, NC, FL, and LA) and nested within region (Atlantic or Gulf). Total genetic variation was partitioned into three components: within geographic localities, among geographic localities within the Atlantic region, and between regions. Significance of variance components was tested by using 10,000 random permutations to generate null distributions for each variance component. Gene flow among localities was estimated with Wright's (1943) island model, modified for mtDNA: $F_{ST} = 1/2N_e m_{\varphi} + 1$. Calculations employed Φ_{ST} as an approximation of F_{ST}.

Results

RFLP analysis of the ATPase 6 and D-loop regions revealed a total of 68 restriction sites. The average individual was scored for 62 sites and 264 nucleotide positions representing approximately 12% of the ATPase 6 and D-loop regions combined, or 1.6% of the entire mitochondrial genome. A total of 15 composite haplotypes was detected in *M. undulatus* (Table 2). Of the 93 individuals surveyed, 67 (72%) shared the same composite genotype (haplotype 1). Genetic distance among haplotypes averaged 0.17% for the pooled sample, with the most divergent haplotypes (10 and 12) differing from the common haplotype by only 0.75% and 1.0%, respectively.

Nucleon diversity (h) averaged 0.47 ±0.01 (mean ±SE) for the pooled sample, and ranged from h = 0.25 ±0.12 in DE to h = 0.62 ±0.12 in LA. Nucleotide sequence diversity also varied geographically, ranging from π = 0.07% in DE to π = 0.32% in LA. Net sequence divergence (p) among localities was low, indicating that most of the observed mtDNA variation occurred within localities. Average sequence divergence for the pooled sample was p = 0.004%, with comparisons among Atlantic localities yielding slightly lower divergence values than comparisons involving Gulf and Atlantic samples. Croaker collected north versus south of Cape Hatteras were genetically indistinguishable (p=–0.002%) whereas nucleotide divergence between regions (pooled Atlantic vs. Gulf) was 0.005%.

Table 2

Geographic distribution (see Table 1 for specific locations) of composite mtDNA haplotypes among Atlantic croaker samples from Atlantic and Gulf of Mexico localities. Letters denote mtDNA fragment patterns (lowercase = ATPase 6; uppercase = D-loop) produced by digestion of PCR products with the following polymorphic restriction endonucleases: ATPase 6: *Pvu*II, *Alu*I, *Dde*I; D-loop: *Hinf*I, *Hae*III, *Msp*I, *Mse*I, *Taq*I, *Nla*III, *Hha*I.

Composite mtDNA haplotype	Haplotype frequency				
	DE	NC	FL	LA	Total
1. aaaAAAAAAA	20	16	16	15	67
2. bbaAAAAAAA	1	1	0	2	4
3. aaaAAAACAA	1	1	2	0	4
4. aaaAAAABAA	1	3	0	0	4
5. aaaAAAAAAB	0	1	0	0	1
6. aaaDAAAAAA	0	1	0	0	1
7. aaaABAAAAA	0	1	0	1	2
8. aaaBAAAAAA	0	0	3	0	3
9. aaaAAABAAA	0	0	1	0	1
10. aaaAAAABBC	0	0	0	1	1
11. aaaAAAAABA	0	0	0	1	1
12. aaaBBAAAAC	0	0	0	1	1
13. aaaAABAAAA	0	0	0	1	1
14. aabAAAAABA	0	0	0	1	1
15. aaaAAAAAAC	0	0	0	1	1
Totals	23	24	22	24	93

The common mtDNA genotype (haplotype 1) was numerically dominant at all localities (Table 2). The remaining 14 haplotypes occurred at low (<10%) frequencies. Monte Carlo tests for homogeneity revealed no significant heterogeneity in mtDNA haplotype frequency distributions within the SAB (NC vs. FL, χ^2=11.3, p=0.140) or among all Atlantic localities (p=0.148). When NC and FL samples were pooled and compared to the DE sample, there was no indication of heterogeneity among samples collected north and south of Cape Hatteras (χ^2=4.6, p=0.906). To further test for population structure within the Atlantic region, an additional 20 specimens from each Atlantic locality were screened for D-loop variation with the polymorphic enzymes *Taq*I and *Hha*I. Neither *Taq*I (χ^2=3.45, p=0.562) nor *Hha*I (χ^2=1.95, p=0.682) haplotype frequencies exhibited significant heterogeneity among Atlantic localities.

Given the lack of heterogeneity within the Atlantic region, composite haplotype frequencies were pooled across Atlantic localities and compared with the Gulf of Mexico sample. Monte Carlo simulations

Table 3

Hierarchical nested analysis of molecular variance on genetic distance between Atlantic croaker mtDNA haplotypes.

Variance component	df	Φ statistic	Variance	% total	*P*
Among regions (Atlantic vs. Gulf)	1	Φ_{CT} = 0.046	0.021	4.6	<0.01
Among localities within Atlantic	2	Φ_{SC} < 0.001	<0.001	<0.1	0.10
Within localities	89	Φ_{ST} = 0.046	0.431	95.4	0.03

revealed significant heterogeneity between Atlantic and Gulf samples (χ^2=24.36, p=0.020) (Table 2). Parsimony analysis indicated that geographically segregated haplotypes were generally closely related (1–2 restriction site changes) to the common haplotype (Fig. 1). AMOVA revealed that the majority (95.4%) of mtDNA variation in Atlantic croaker occurred within samples (Table 3). A significant portion (4.6%) was attributable to regional differences (Atlantic versus Gulf, P<0.01) but variation within the Atlantic region was unstructured (p=0.10) and accounted for <0.1% of the total genetic variance. Φ_{ST} values indicated a lack of geographic structure and relatively high gene flow among Atlantic localities. Gene flow among Atlantic localities was estimated as 23.4 (DE and NC), 16.8 (NC and FL), and 12.0 (DE and FL) effective female migrants per generation.

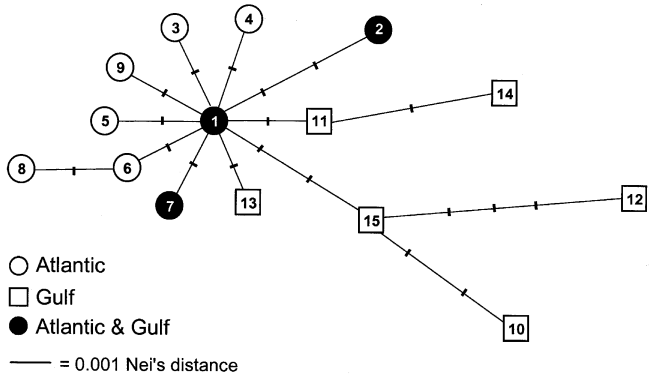

Figure 1

Parsimony network relating 15 mtDNA haplotypes observed in Atlantic croaker. Hatch marks drawn through each branch indicate the inferred number of restriction site differences among haplotypes. Branch lengths denote Nei's genetic distance between haplotypes (note scale).

Discussion

MtDNA analysis provided no evidence that *M. undulatus* is subdivided by Cape Hatteras into discrete genetic stocks. Frequency- and distance-based analyses both suggested a single, panmictic population of croaker on the U.S. Atlantic coast. Low levels of mtDNA divergence among Atlantic localities, although not statistically significant, were more consistent with a pattern of semi-isolation by distance rather than marked subdivision by Cape Hatteras. For example, AMOVA revealed that the two Atlantic localities exhibiting the least genetic divergence were DE and NC (Φ_{ST}=0.021; p=0.89). NC and FL samples were slightly more divergent but not significantly so (Φ_{ST}=0.029; p= 0.180). Lack of population structure was perhaps best indicated by the lack of differentiation between DE and FL samples (Φ_{ST}=0.040; p=0.084), two locations representing the distributional limits of this species' range on the U.S. Atlantic coast.

Kornfield and Bogdanowicz (1987) inferred patterns of gene flow from distributions of unique mtDNA haplotypes and their presumed precursors, predicting that under restricted gene flow, unique haplotypes should occur in the same population as their precursors. This was the case for *M. undulatus* at the regional (i.e. Atlantic versus Gulf) level, where unique Gulf haplotypes 10, 12, and 14 each had likely precursors that were confined to the Gulf region. Within the Atlantic region, however, unique haplotypes and their likely precursors occurred at disparate localities. The occurrence of rare haplotypes at more than one Atlantic locality also implies that genotypes arising at one locality spread rapidly to other localities within the Atlantic. Combined with low levels of nucleotide divergence, these observations support the hypothesis of contemporary gene flow among Atlantic localities. Gene flow estimates based upon Wright's island model are consistent with panmixia: values ranged from $N_e m_{\varphi}$=12.0–23.4, well above the theoretical lower limit of $N_e m_{\varphi}$= 1 sufficient to preclude genetic divergence of populations by random drift (Slatkin, 1985).

Previous studies have reported geographic variation in life history traits and population dynamics of Atlantic croaker found north and south of Cape Hatteras (White and Chittenden, 1977; Ross, 1988), suggesting that two groups of croaker with contrasting life histories overlap in the Cape Hatteras region, with a mainly offshore group north of Cape Hatteras which displays greater size-at-age, greater longevity, lower annual mortality, and delayed maturation in relation to croaker south of Cape Hatteras. A more recent study by Barbieri et al. (1994a) in the Chesapeake Bay concluded that life history variation in *M. undulatus* was ephemeral and that the two-stock hypothesis should be re-evaluated. The present mtDNA analysis supports previous suggestions by Ross (1988) and White and Chittenden (1977) that life history variation, when present, has an ecophenotypic basis.

A review of *M. undulatus* life history characteristics lends support to the panmixia hypothesis for the Atlantic region and provides several mechanisms by which trans-Hatteras gene flow may occur. Mark–recapture studies indicate extensive movement by *M. undulatus* along the U.S. Atlantic coast and thus potential for gene flow by means of adult migration (Pearson, 1932; Haven, 1959; Bearden, 1964; DeVries[3]). Larval dispersal could augment gene flow. Atlantic croaker larvae are abundant at outer-continental shelf locations (Govoni and Pietrafesa, 1994) and may spend more than 60 days in shelf waters before reaching estuaries (Warlen, 1980). Offshore spawning combined with an extended pelagic larval stage in shelf waters could provide ample opportunity for larval dispersal among Atlantic localities. Interestingly, spring recruits are occasionally reported in the mid-Atlantic Bight (MAB) estuaries as late as May and June (Haven, 1957; Chao and Musick, 1977) despite reports that spawning ceases in this area by late December (Morse, 1980; Barbieri et al., 1994b). Spring recruits to MAB estuaries may therefore be transported to mid-Atlantic estuaries from spawning sites south of Cape Hatteras. Thorrold et al. (1997) found no significant differences in otolith chemistry of juvenile croaker from the Neuse River, North Carolina, and the Elizabeth River, Virginia, suggesting that immigrants to estuaries north and south of Cape Hatteras may originate from the same spawning area. The reproductive strategy of *M. undulatus* may also facilitate gene flow. Atlantic croaker are multiple spawners with indeterminate fecundity (Barbieri et al., 1994b). Because spawning occurs during a southerly coastal migration during fall, females could commence spawning within the MAB and spawn multiple times during their southward migration, effectively distributing maternal half-siblings (mtDNA clones) over an extensive geographic range.

MtDNA heterogeneity between Atlantic and Gulf of Mexico localities suggests that these regions support separate populations of *M. undulatus*. The observed genetic break is consistent with a contemporary range discontinuity in southern Florida, where *M. undulatus* seldom occur south of Indian River on the Atlantic coast and are rarely encountered south of Tampa Bay on the Gulf coast (Nelson et al., 1991; Pattillo et al., 1997). Average sequence divergence between Atlantic and Gulf haplotypes (p=0.005%) was relatively low, however, suggesting that the phylogenetic break between Atlantic and Gulf stocks may be less pronounced in *M. undulatus* than in other marine fishes (Bowen and Avise, 1990).

This study represents the first attempt to identify population genetic structure in *M. undulatus* using DNA-level markers. The technique employed in our study proved sufficiently powerful to detect regional (Atlantic vs. Gulf of Mexico) population structure, but did not reveal structured genetic stocks of croaker within the Atlantic. The integrity of Cape Hatteras as a genetic stock boundary could be tested further by using fine-scale markers such as microsatellites, which have revealed genetic differentiation among fish populations that exhibit little mtDNA divergence. Alternatively, higher-resolution mutation-screening analysis or direct sequencing of the mitochondrial D-loop region might provide greater resolution of geographic structure than the RFLP technique employed here (Ong et al., 1996; Stabile et al., 1996).

Although mtDNA analysis did not indicate discrete genetic stocks of croaker within the Atlantic region, harvest stocks (see Gauldie, 1988) worthy of management consideration may exist. Because low levels of gene flow may produce mtDNA homogeneity between otherwise self-recruiting stocks, mtDNA is incapable of distinguishing between low (1%) and moderate (50%) amounts of mixing (Carvalho and Hauser, 1995). Delineation of harvest stocks will thus require more precise estimates of mixing than those obtainable from mtDNA analysis. Mark–recapture studies designed to quantify the level of adult migration across Cape Hatteras, combined with otolith-based microchemical analyses to examine larval dispersal patterns, could provide valuable information on the level of mixing between the MAB and South

[3] DeVries, D. A. 1986. Inshore Atlantic croaker tagging study. *In* J. L. Ross, D. A. DeVries, J. H. Hawkins III, and J. B. Sullivan, Assessment of North Carolina commercial finfisheries, p. 331–394. Completion report for Project 2-386-R, North Carolina Department of Natural Resources and Community Development, Div. Marine Fisheries, Morehead City, NC.

Atlantic Bight (SAB) areas and clarify the extent to which *M. undulatus* in these regions constitute self-recruiting groups.

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