A molecular genetic investigation of the population structure of Atlantic menhaden (*Brevoortia tyrannus*)

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Abstract—Atlantic menhaden (*Brevoortia tyrannus*), through landings, support one of the largest commercial fisheries in the United States. Recent consolidation of the once coast-wide reduction fishery to waters within and around Chesapeake Bay has raised concerns over the possibility of the loss of unique genetic variation resulting from concentrated fishing pressure. To address this question, we surveyed variation at the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region and seven nuclear microsatellite loci to evaluate stock structure of Atlantic menhaden. Samples were collected from up to three cohorts of Atlantic menhaden at four geographic locations along the U.S. Atlantic coast in 2006 and 2007, and from the closely related Gulf menhaden (*B. patronus*) in the Gulf of Mexico. Genetic divergence between Atlantic menhaden and Gulf menhaden, based on the COI gene region sequences and microsatellite loci, was more characteristic of conspecific populations than separate species. Hierarchical analyses of molecular variance indicated a homogeneous distribution of genetic variation within Atlantic menhaden. No significant variation was found between young-of-the-year menhaden (YOY) collected early and late in the season within Chesapeake Bay, between young-of-the-year and yearling menhaden collected in the Chesapeake Bay during the same year, between YOY and yearling menhaden taken in Chesapeake Bay in successive years, or among combined YOY and yearling Atlantic menhaden collected in both years from the four geographic locations. The genetic connectivity between the regional collections indicates that the concentration of fishing pressure in and around Chesapeake Bay will not result in a significant loss of unique genetic variation.

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Atlantic menhaden (*Brevoortia tyrannus*) is an ecologically and economically important species along the U.S. East Coast. As a filter-feeder and key prey fish, it provides a critical link between primary production and larger piscivorous predators, such as striped bass (*Morone saxatilis*), bluefish (*Pomatomus saltatrix*), and weakfish (*Cynoscion regalis*). The commercial fishery for Atlantic menhaden consists of a small bait fishery and a larger reduction fishery. Of the 20 menhaden reduction plants (where menhaden are "reduced" to meal and oil) that were once operating along the U.S. Atlantic coast, only the Reedville, Virginia, facility is currently active. The concentration of fishing effort for Atlantic menhaden in and around Chesapeake Bay has raised concerns among many environmentalists and sport fishermen about the possibility of "localized depletion" of Atlantic menhaden in the area. A potential consequence of localized depletion could be the loss of unique genetic variation within Atlantic menhaden, if there is spatial partitioning of genetic variation (stock structure) within the species.

Results of previous analyses of the stock structure of Atlantic menhaden have been discordant. Two populations of Atlantic menhaden, one north and the other south of Long Island, New York, were suggested on the basis of vertebral counts and transferrin allele frequencies (Sutherland, 1963; Epperly, 1989). Two populations, one north and one south of Cape Hatteras, North Carolina, have also been proposed. This division was based on the presence of small, sexually mature fish before the arrival of the larger, migrating fish in North Carolina waters and the presence of spawning fish off northern Florida in late winter and early spring (June and Nicholson, 1964). One coast-wide population has been indicated by tag recovery studies (Nicholson, 1978), which have shown that Atlantic menhaden of differing ages and sizes share the overwintering grounds off Cape Hatteras and undergo seasonal migrations and that larger fish travel farther north. The Atlantic States Marine Fisheries Commission currently assesses Atlantic menhaden as a single coast-wide stock.

In addition to the uncertainty regarding the stock structure of Atlantic menhaden, the relationship between Atlantic menhaden and Gulf menhaden (*B. patronus*) is not well understood. The mean values of several of the morphometric and meristic characters are significantly different between the two putative species, although the ranges of variation are coincident (Dahlberg, 1970). Similarly, preliminary genetic analyses indicate limited divergence between the putative species, and therefore the use of larger sample sizes and additional genetic characters have been recommended (Avise et al., 1989; Anderson, 2007).

Table 1

Collection location, date of collection, and number of Atlantic menhaden (*Brevoortia tyrannus*) and Gulf menhaden (*B. patronus*) analyzed in this study and range of sample fork lengths. For all collections outside the Chesapeake Bay, young-of-the-year (YOY) and yearling samples are pooled.

Collection	Location	Date	*n*	Fork length (mm)
New England 2006	Gloucester, MA	Aug. 2006	25	43–58
New England 2007	Gloucester, MA	Aug. 2007	25	34–64
Mid-Atlantic 2006	Pennsville, NJ	Aug. 2006	27	61–110
Mid-Atlantic 2007	Oakwood Beach, NJ	June 2007	25	77–190
Chesapeake Bay YOY 2006	Gloucester Point, VA	May 2006	25	27–67
Chesapeake Bay yearling 2006	Gloucester Point, VA	Aug. 2006	18	120–200
Chesapeake Bay YOY (early) 2007	Gloucester Point, VA	May 2007	25	34–84
Chesapeake Bay YOY (late) 2007	Gloucester Point, VA	Aug. 2007	31	35–99
Chesapeake Bay yearling 2007	Gloucester Point, VA	June 2007	18	114–184
U.S. South Atlantic 2006	Beaufort, SC	Aug. 2006	28	41–113
U.S. South Atlantic 2007	Beaufort, SC	April 2007	41	28–181
Gulf menhaden 2006	Galveston, TX	Oct. 2006	25	not recorded
Gulf menhaden 2007	Cameron, LA	Oct. 2007	25	52–91

In this study, analyses of sequence data from the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region and allele frequencies of seven nuclear microsatellite loci were used to investigate the genetic relationships of Atlantic and Gulf menhaden, the stock structure of Atlantic menhaden, and to evaluate the potential for loss of unique genetic variation resulting from "localized depletion" of Atlantic menhaden within the Chesapeake Bay region.

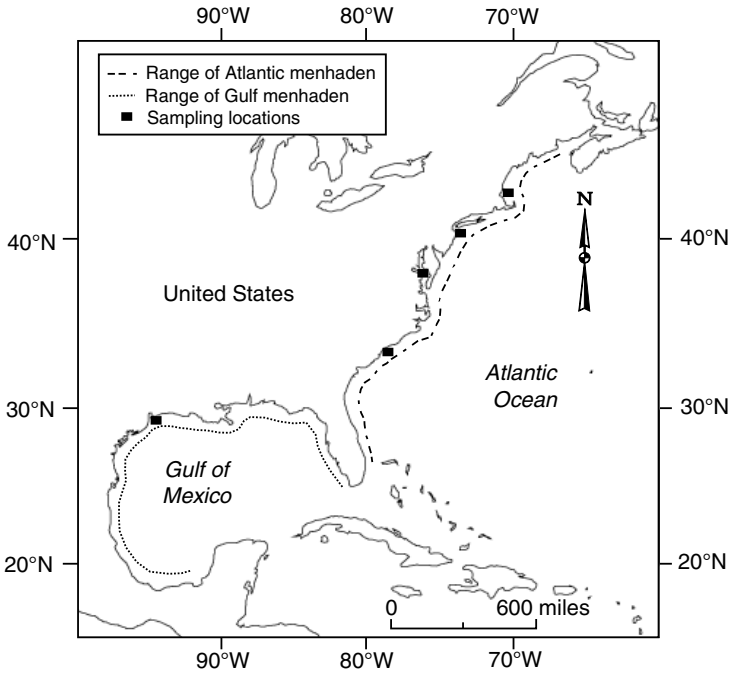

Figure 1

Map of ranges of Atlantic menhaden (*Brevoortia tyrannus*) and Gulf menhaden (*B. patronus*) and the approximate location of sites sampled in 2006–2007 for the analysis of stock structure in Atlantic menhaden.

Materials and methods

Sample collection

Young-of-the-year (YOY) and yearling Atlantic menhaden were sampled from New England (MA), the U.S. mid-Atlantic (NJ), Chesapeake Bay (VA), and the U.S. South Atlantic (SC) in 2006 and 2007, and YOY Gulf menhaden were sampled from the Gulf of Mexico in 2006 and 2007 (Table 1; Fig. 1). For all collections outside the Chesapeake Bay, samples of YOY and yearling Atlantic menhaden were pooled. For Chesapeake Bay collections, scales were aged from a subsample of 20% of individuals taken in 2007 and length was used as a surrogate for the remaining samples (where fish less than 100 mm fork length were considered YOY and fish greater than 100 mm fork length were considered yearling). The younger cohorts were sampled because they are less likely to have migrated far from where they were spawned. Local experts identified menhaden on the basis of morphological characters and capture location. Muscle tissue samples were taken from each individual and either frozen or stored in DMSO buffer (Seutin et al., 1991) at room temperature. Voucher specimens were retained from all U.S. Atlantic

coast regions in 2007 to corroborate field identifications. Some of the identifications were retroactively verified with sequences from a portion of the mtDNA control region, namely haplotypes were compared to those of Anderson (2007).

Total genomic DNA was extracted from each tissue sample by using a Qiagen DNeasy® Tissue Kit (Qiagen, Valencia, CA) and following the manufacturer's protocol. A 459 base-pair (bp) fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region (F: 5′CTTTCGGCTACATGGGAATG3′ and R: 5′AGCCCTAGGAAGTGTTGTGG5′, GenBank accession number DQ867533), a 535-bp fragment of the mitochondrial control region (Pro-F: 5′ CTA CCY CYA ACT CCC AAA GC 3′, [Gray et al., 2008] and Phe-R: 5′ GTA AAG TCA CGA CCA AAC C 3′, [Brendtro et al., 2008], and eight microsatellite loci *Asa*2, *Asa*4, *Asa*16, [Brown et al., 2000]; *Aa*16, [Faria et al., 2004]; *Asa*B020, *Asa*C334, *Asa*D055, [Julian and Bartron, 2007]; *Sar*BH04, [Pereyra et al., 2004]), were amplified in either 5-μL (microsatellites) or 10-µL (COI) reaction volumes using the polymerase chain reaction (PCR) with the conditions outlined in Lynch (2008).

Mitochondrial PCR products were purified for sequencing by using column filtration with a QIAquick® PCR purification kit (Qiagen) following the manufacturer's protocol, and the concentration was measured with a BioMate™ 3 series UV Sspectrophotometer (Thermo Spectronic, Madison, WI). PCR products were prepared for sequencing with the ABI PRISM® BigDye™ Terminator, vers 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) at a 1:8 dilution and sequenced on an 80-cm capillary ABI PRISM® 3130*xl* genetic analyzer (Applied Biosystems). Samples were sequenced in the forward and reverse direction.

The chromatographic curves for each 80-cm capillary sequence were analyzed using Sequencing Analysis Software, vers. 5.2 (Applied Biosystems). All mitochondrial sequences were edited with Sequencher 4.7.2 (Gene Codes Corp., Ann Arbor, MI), variable positions were confirmed visually, and sequences were aligned by using the ClustalW algorithm (Thompson et al., 1994) for multiple alignments in MacVector 9.0.1 (MacVector Inc., Cary, NC).

Microsatellite loci were amplified by PCR by using locus-specific fluorescent labels with the conditions outlined in Lynch (2008). Following amplification, 1 μL of PCR product for each locus was combined with PCR products from three other unique locus and fluorescent label combinations (4 μL total), 6 μL HiDi formamide (Applied Biosystems), and 0.3 μL 500 Liz Gene Scan Size standard (Applied Biosystems). The reaction mixture was denatured at 95°C for 10 minutes before being separated on a 36-cm capillary ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. The chromatic peaks for each microsatellite locus were scored by GeneMarker AFLP/Genotyping Software, vers. 1.60 (SoftGenetics, State College, PA). Once scored, MicroChecker 2.2.3 (Van Oosterhout et al., 2004) was used to check for the presence of null alleles and evidence of scoring errors. To ensure consistency, 20% of the samples were re-analyzed from the point of DNA extraction through allele scoring.

Genetic analyses

Once aligned, the mitochondrial sequences were characterized in Arlequin 3.11 (Excoffier et al., 2005) to determine the number of haplotypes (N_h), number of polymorphic sites (S), and variable base-pair (bp) locations within a sequence set. Diversity indices, including haplotype diversity (h), nucleotide sequence diversity (π), and mean number of pairwise differences (k) within each collection were also estimated in Arlequin 3.11 (Excoffier et al., 2005). To visualize genetic relationships among mitochondrial sequences, median-joining networks were drawn in Network 4.2.0.1 (Bandelt et al., 1999).

For the microsatellite data, Genepop 3.4 (Raymond and Rousset, 1995) was used to determine observed heterozygosity (H_O) and expected heterozygosity (H_E) and to perform exact tests for deviations of genotypic distributions from the expectations of Hardy-Weinberg equilibrium for each locus at each collection location (10,000 iterations; Guo and Thompson, 1992). Significance levels were adjusted for multiple testing by using a Bonferroni correction (Rice, 1989). Arlequin 3.11 (Excoffier et al., 2005) was used to determine the number of alleles (a), and Microsatellite Analyzer (MSA) (Dieringer and Schlötterer, 2003) was used to determine the allele size range (as). Allelic richness (R_s) was estimated in FSTAT 2.9.3.2 (Goudet, 1995).

Using both mitochondrial COI sequence data (Φ_{ST}) and nuclear microsatellite data (F_{ST}/R_{ST}), we performed a hierarchical analysis of molecular variance (AMOVA) to test for partitioning of variation among defined groups. The groups tested were the following: temporal collections within an age class at a location (e.g., 2007 YOY in Chesapeake Bay sampled early [May] and late [August] in the season), between collections of an age class taken at the same location in different years (e.g., the 2006 year class sampled in Chesapeake Bay as YOY in 2006 and yearling in 2007), between age classes within a region (e.g., YOY and yearling menhaden in Chesapeake Bay in 2007), among Atlantic coast regions both including and excluding the Gulf of Mexico (e.g., New England, mid-Atlantic, Chesapeake Bay, U.S. South Atlantic, and Gulf of Mexico), among COI clades (e.g., "Atlantic only," "ubiquitous," and "anomalous" samples), and between Atlantic and Gulf menhaden. AMOVA calculations based on microsatellite data were analyzed by using both F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995) distance methods. Estimates of population pairwise Φ_{ST} and F_{ST}/R_{ST} were calculated in Arlequin 3.11 (Excoffier et al., 2005) and adjusted for multiple testing with a Bonferroni correction (Rice, 1989).

To assess the statistical power for detecting population differentiation with the applied set of microsatellite markers and sample sizes, a simulation was implemented with POWSIM (Ryman and Palm, 2006), which

Table 2

Sequence statistics of Atlantic menhaden (*Brevoortia tyrannus*) and Gulf menhaden (*B. patronus*) based on cytochorme *c* oxidase subunit I (COI) gene region by location, clade, and across all locations: number of individuals (n), number of haplotypes (N_h), number of polymorphic sites (S), number of transitions (Ts), number of transversions (Tv), haplotype diversity (h), mean nucleotide sequence diversity (π), and mean number of pairwise differences (k) and standard error (SE).

Collection	n	N_h	S	Ts	Tv	h ±SE	π ±SE	k ±SE
New England	50	29	47	47	4	0.940 ±0.0206	0.0258 ±0.0132	11.8 ±5.45
mid-Atlantic	52	32	66	67	5	0.956 ±0.0163	0.0286 ±0.0145	13.1 ±6.01
Chesapeake Bay	117	50	62	63	3	0.932 ±0.0147	0.0267 ±0.0135	12.3 ±5.58
U.S. South Atlantic	69	44	66	67	2	0.954 ±0.0166	0.0295 ±0.0149	13.5 ±6.16
Atlantic menhaden	288	109	97	99	6	0.941 ±0.0084	0.0274 ±0.0137	12.6 ±5.69
Gulf menhaden	50	25	34	33	5	0.879 ±0.0419	0.0071 ±0.0041	3.23 ±1.70
Atlantic + Gulf menhaden	338	124	99	101	7	0.940 ±0.0079	0.0258 ±0.0129	11.8 ±5.37
ubiquitous clade	235	98	76	76	7	0.924 ±0.0138	0.0081 ±0.0045	3.71 ±1.88
Atlantic only clade	100	23	22	22	1	0.732 ±0.0456	0.0036 ±0.0024	1.65 ±0.98

estimates statistical power by testing different combinations of number of samples, sample sizes, number of loci, number of alleles, and allele frequencies for any hypothetical degree of differentiation (F_{ST}). To match the number of collection locations and magnitude of F_{ST} estimates in this study, five hundred replicates were performed on five populations by using Fischer's method with the following combinations of effective population size (N_e) and generations of drift before sampling (t): 10,000: 50, 5000: 25, 1000: 5 (F_{ST}=0.0025); 10,000: 201, 5000: 100.5, 1000: 20.1 (F_{ST}=0.01); 10,000: 1025.8, 5000: 512.9, 1000: 102.6 (F_{ST}=0.05). The hypothetical sample sizes were set to the average across all loci for each sampling location. An additional simulation was performed with t=0, to assess α (type-I) error. The estimate of power was reported as the proportion of significant outcomes (P<0.05).

Results

The COI fragment was sequenced for 289 Atlantic menhaden and 50 Gulf menhaden. Overall, the fragment contained 99 polymorphic sites (97 in Atlantic menhaden): 5 first codon positions, 1 second codon position, and 91 third codon positions; 101 transitions (99 in Atlantic menhaden), 7 transversions (6 in Atlantic menhaden); and produced 124 haplotypes (109 in Atlantic menhaden) (Table 2). All substitutions were synonymous, resulting in identical amino acid sequences. Haplotype diversity (h) estimates for the Atlantic and Gulf menhaden sampling locations ranged from 0.879 in Gulf menhaden to 0.956 in the U.S. mid-Atlantic, with an overall (pooled) estimate of 0.940 (0.941 for Atlantic menhaden). Mean nucleotide sequence diversity (π) estimates for Atlantic and Gulf menhaden sampling locations ranged from 0.0071 in Gulf menhaden to 0.030 in the U.S. South Atlantic, with an overall (pooled) estimate of 0.026 (0.027 for Atlantic menhaden). The mean number of pairwise differences (k) ranged from 3.2 in Gulf menhaden to 13.5 in the U.S. South Atlantic, with an overall (pooled) estimate of 12.6 for Atlantic menhaden and 11.8 for Atlantic and Gulf menhaden combined.

The median-joining network for the 109 COI Atlantic menhaden haplotypes showed two extensive clusters (clades) separated by 17 substitutions and one minor grouping of three individuals separated by 24 substitutions (Fig. 2). In order to discriminate between the alternate possibilities that either the three individuals were one of the other North American *Brevoortia* species that had been misidentified in the field as Atlantic menhaden, or that there is incomplete lineage sorting at the COI locus, the more rapidly evolving mitochondrial control region was sequenced and compared to those generated by Anderson (2007) for the four species of North American menhaden (GenBank accession numbers EF119342-EF119454). The control region sequences for the three individuals unambiguously clustered with the "ubiquitous large-scaled" menhadens in an unweighted pair group method with arithmetic mean (UPGMA) tree with these sequences, indicating that the field identifications were correct and that the result was likely attributable to incomplete lineage sorting at the COI locus. Examination of the microsatellite genotypes of these three individuals supported this conclusion.

The distribution of the two major COI clades differed among Atlantic and Gulf menhaden. Clade I, the "ubiquitous" clade, comprised all Gulf menhaden samples and 64% of Atlantic menhaden samples. Clade II, the "Atlantic-only" clade, comprised 35% of Atlantic menhaden samples and was not detected in Gulf menhaden (Table 2). A contingency table of the four Atlantic menhaden sampling locations indicated that clades I and II were homogeneously distributed among the U.S. Atlantic Coast sampling locations (χ^2=0.478; $\chi^2_{0.05,3}$; P>0.05).

Eight microsatellite loci, *Aa*16, *Asa*2, *Asa*4, *Asa*16, *Asa*B020, *Asa*D055, *Asa*C334, and *Sar*BH04, were amplified for the Atlantic menhaden and Gulf menhaden

samples. The genotypic distributions of all loci in all samples conformed to the expectations of Hardy-Weinberg equilibrium with the exception of locus *Asa*16 (Table 3). Both the exact test in Genepop and the MicroChecker analysis revealed a significant or nearly significant deficiency of *Asa*16 heterozygotes in all samples, indicating the presence of a null allele. Consequently, this locus was not included in any of the population structure analyses.

For the seven remaining loci, the number of alleles (a) across all Atlantic menhaden samples ranged from 8 at *Aa*16 and *Asa*C334 to 22 at *Asa*B020, and the allelic richness (R_s) ranged from 7.98 at *Aa*16 to 21.8 at *Asa*B020. Allele size ranges were similar for Atlantic menhaden and Gulf menhaden across all seven loci. However, average a and R_s values across all loci were lower in Gulf menhaden as compared to Atlantic menhaden (Table 3).

AMOVAs of the COI haplotype data and microsatellite genotype data were performed to evaluate the temporal and spatial partitioning of genetic variation within Atlantic menhaden (Table 4). No significant differences were detected between early (May) and late (August) collections of YOY Atlantic menhaden in Chesapeake Bay in 2007. Likewise, no differences were detected among year classes of Atlantic menhaden in the Chesapeake Bay based on a comparison of YOY and yearling menhaden collected in Chesapeake Bay in 2006 or a comparison of YOY and yearling menhaden collected in Chesapeake Bay in 2007. Following the same cohort in the same location across years, a comparison of YOY menhaden collected in 2006 with yearling menhaden collected in 2007 in the Chesapeake Bay, did not result in a significant difference based on either the mitochondrial (Φ_{ST}) or the microsatellite (R_{ST}). However, the microsatellite (F_{ST}) AMOVA produced a significant result, where 1.80% (P=0.02) of the variance was attributed to differences within the same cohort in Chesapeake Bay in successive years.

Samples of YOY and yearling menhaden (combined) from four geographic regions along the U.S. Atlantic Coast (New England, mid-Atlantic, Chesapeake Bay, and U.S. South Atlantic) were compared to test for evidence of spatial partitioning of genetic variation. Only the AMOVA based on the microsatellite F_{ST} was significant, attributing 0.58% (P<0.001) of variation to sampling location. No pairwise comparisons of Φ_{ST}, F_{ST} or R_{ST} revealed statistically significant variation between any two sampling regions of Atlantic menhaden after a Bonferroni correction (Table 5).

All pairwise comparisons between Atlantic menhaden and Gulf menhaden collections revealed statistically significant variation. The mitochondrial (Φ_{ST}) AMOVA between Atlantic and Gulf menhaden attributed 18.2% (P<0.001) of the variance to differences between putative species. Likewise, in the microsatellite comparison, the F_{ST} and R_{ST} AMOVAs attributed 11.5% (P<0.001) and 38.75% (P<0.001) of variance to variation between Gulf and Atlantic menhaden samples.

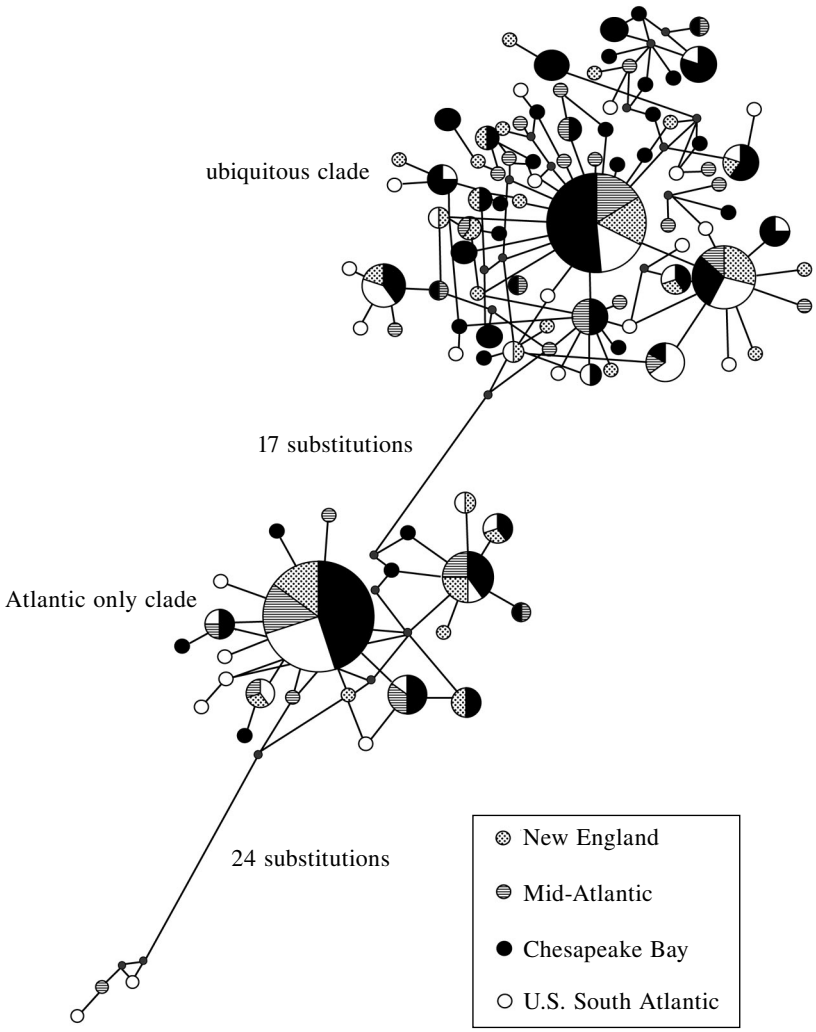

Figure 2

Median-joining network of 109 Atlantic menhaden (*Brevoortia tyrannus*, n=289) cytochrome *c* oxidase subunit I (COI) haplotypes. The observed haplotypes are sized according to frequency and coded by area. Sample sizes: New England (n=50), mid-Atlantic (n=53), Chesapeake Bay (n=117), U.S. South Atlantic, (n=69). The hypothesized intermediate haplotypes are denoted by the smallest circles shaded dark gray.

Table 3

Summary statistics for Atlantic menhaden (*Brevoortia tyrannus*) and Gulf menhaden (*B. patronus*) collections by locus. Number of individuals (*n*), number of alleles (*a*), allele size range (*as*), allelic richness (R_s), observed heterozygosity (H_O), expected heterozygosity (H_E), and probability of conformance with Hardy-Weinberg Expectations (HWE). ** indicates significant deviation from the expectations of HWE after correction for multiple tests (initial α=0.05, k=8).

	Locus								
Sample	*Aa*16	*Asa*2	*Asa*4	*Asa*16	*Asa*B020	*Asa*C334	*Asa*D055	*Sar*BH04	Average across loci
New England									
n	47	44	44	45	47	49	44	40	45
a	7	6	9	16	13	8	15	12	10.75
as	139–155	210–237	168–204	247–292	142–184	118–146	254–314	179–221	
Rs	4.98	5.64	8.42	13.2	12.64	7.14	14.24	11.47	9.72
Ho	0.638	0.614	0.75	0.66	0.787	0.531	0.75	0.675	0.68
HE	0.709	0.6	0.722	0.809	0.894	0.593	0.907	0.72	0.74
HWE				**					
mid-Atlantic									
n	51	50	47	50	49	51	36	49	47.88
a	5	8	12	17	19	5	14	12	11.5
as	139–155	213–234	162–222	223–286	130–193	122–138	254–326	179–221	
Rs	4.07	7.15	11.0	14.4	17.75	4.41	14	10.8	10.45
HO	0.588	0.54	0.809	0.808	0.898	0.431	0.917	0.694	0.71
HE	0.625	0.659	0.839	0.877	0.902	0.436	0.917	0.759	0.75
HWE									
Chesapeake Bay									
n	116	113	101	110	115	113	112	110	111.25
a	7	9	12	21	20	7	18	13	13.38
as	139–155	210–237	162–198	223–298	130–196	122–146	238–334	179–221	
Rs	4.4	6.89	9.58	21	15.8	5.75	15.4	9.14	11
HO	0.586	0.584	0.752	0.773	0.904	0.593	0.893	0.627	0.71
HE	0.629	0.649	0.825	0.917	0.888	0.638	0.912	0.738	0.77
HWE				**					
U.S. South Atlantic									
n	53	68	53	69	66	63	63	56	61.38
a	7	7	12	18	19	6	17	12	12.25
as	139–157	216–237	165–201	223–289	130–187	118–138	238–326	179–221	
Rs	4.7	6.89	10.51	12.8	15.94	5.07	15.96	11.16	10.38
HO	0.691	0.662	0.698	0.797	0.833	0.556	0.905	0.625	0.72
HE	0.712	0.657	0.802	0.9	0.887	0.57	0.923	0.802	0.78
HWE									
All Atlantic									
n	268	276	246	274	278	277	256	256	266.38
a	8	10	18	23	22	8	20	15	15.5
as	139–157	210–237	162–222	223–298	130–196	118–146	238–334	179–221	
Rs	7.98	9.93	17.8	23	21.8	7.99	20	15	15.44
HO	0.621	0.6	0.751	0.785	0.867	0.543	0.875	0.647	0.71
HE	0.664	0.643	0.81	0.911	0.893	0.583	0.915	0.76	0.77
HWE				**					
Gulf menhaden									
n	49	49	44	45	49	49	45	44	46.75
a	9	10	10	15	19	9	18	12	12.75
as	135–161	210–237	165–198	223–298	130–196	118–154	258–330	169–215	
Rs	4.69	8.65	9.27	12.8	17.88	8.73	16.9	10.87	11.22
HO	0.449	0.653	0.682	0.7	0.896	0.898	0.864	0.4	0.69
HE	0.448	0.641	0.79	0.812	0.939	0.855	0.912	0.442	0.73
HWE									

Table 4

Analysis of molecular variance (AMOVA) among Atlantic menhaden (*Brevoortia tyrannus*) and Gulf menhaden (*B. patronus*) based on cytochorme *c* oxidase subunit I (COI) sequence data and seven microsatellite loci (*Aa*16, *Asa*2, *Asa*4, *Asa*B020, *Asa*D055, *Asa*C334, *Sar*BH04) grouped by collection time for Chesapeake Bay samples; by age class (following the 2006 year class) for Chesapeake Bay samples; by age class for Chesapeake Bay in 2006 and 2007; by region for all Atlantic coast samples (New England, mid-Atlantic, Chesapeake Bay, and U.S. South Atlantic); by region for large-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. South Atlantic, and Gulf menhaden); by cytochrome *c* oxidase subunit I clade for large-scale samples ("Atlantic only" clade, "ubiquitous" clade, anomalous samples); and by putative species (Atlantic and Gulf menhaden). The distance methods used were pairwise differences (Φ_{ST}), number of different alleles (F_{ST}), and sum of squared allele size differences (R_{ST}); all with 10,000 permutations. Bolded *P*-values indicate significance ($P<0.05$) after Bonferroni correction (initial $\alpha=0.05/2=0.025$).

	Φ-statistics	*P*	F_{ST}	*P*	R_{ST}	*P*
Grouped by collection time						
between collection times	−0.0195	0.587	0.00096	0.398	0.0155	0.171
Grouped by age class (in successive years)						
between years	−0.0364	0.88	0.0294	0.047	−0.00103	0.436
Grouped by age class (within 2006)						
between age classes	−0.0203	0.552	−0.007	0.732	−0.0087	0.52
Grouped by age class (within 2007)						
between age classes	−0.0033	0.382	0.0099	0.066	0.0233	0.114
Grouped by region (Atlantic coast only)						
among regions	−0.0089	0.920	0.0037	0.041	−0.0053	0.902
Grouped by region (large-scaled menhaden combined)						
among regions	0.0605	<0.0001	0.0489	<0.0001	0.0403	<0.0001
Grouped by COI clade						
among clades	0.8659	<0.0001	0.00379	0.068	0.0059	0.148
Grouped by putative species (Atlantic and Gulf menhaden)						
between species	0.176	<0.0001	0.128	<0.0001	0.102	<0.0001
among regions within a species	−0.00822	1	0.0043	0.0205	−0.00511	0.872

Table 5

Estimates of pairwise Φ_{ST}, above diagonal (*), and pairwise F_{ST} (and R_{ST}), below diagonal, between regional collections of Atlantic menhaden (*Brevoortia tyrannus*) and Gulf menhaden (*B. patronus*) based on cytochorme *c* oxidase subunit I (COI) sequence data and seven microsatellite loci, respectively. Bolded values indicate significance after correction for multiple tests ($\alpha=0.05$, $k=3$ for comparisons within Atlantic menhaden).

Collection	New England	mid-Atlantic	Chesapeake Bay	U.S. South Atlantic	Gulf
New England	*	−0.006	−0.004	−0.005	**0.032**
mid-Atlantic	0.004 (0.000)	*	−0.001	−0.004	**0.027**
Chesapeake Bay	0.004 (−0.011)	0.009 (−0.000)	*	−0.000	**0.048**
U.S. South Atlantic	−0.006 (−0.012)	0.010 (0.001)	0.003 (−0.005)	*	**0.029**
Gulf menhaden	**0.106 (0.080)**	**0.157 (0.091)**	**0.130 (0.100)**	**0.106 (0.073)**	*

The POWSIM analysis showed that 94.2%, 93.8%, and 92.6% of the tests where the N_e:*t* combination led to $F_{ST}=0.0025$ (10,000: 50, 5000: 25, 1000: 5, respectively), 100% of the tests where the N_e:*t* combination led to $F_{ST}=0.01$ (10,000: 201, 5000: 100.5, 1000: 20.1), and 100% of the tests where the N_e:*t* combination led to $F_{ST}=0.05$ (10,000: 1025.8, 5000: 512.9, 1000: 102.6) were statistically significant, indicating that there was sufficient statistical power to detect population differences with the set of microsatellite markers and sample sizes used in this study.

Discussion

The mitochondrial and nuclear markers employed in this analysis revealed considerable variation within Atlantic menhaden. The seven microsatellite loci surveyed were highly variable and the number of alleles per locus and average heterozygosities ($a=5$–21, $H_{exp}=0.435$–0.924) were within the range of variation reported for other clupeids ($a=1$–56, $H_{exp}=0.066$–0.98, Brown et al., 2000; McPherson et al., 2001; Olsen et al., 2002; Faria et al., 2004; Anderson and McDonald, 2007; Volk et al., 2007).

Typically, one would not consider using the mitochondrial COI gene region for an analysis of stock structure because this locus tends to be highly conserved in most organisms, exhibiting low levels of intraspecific variation (Meyer, 1993). However, previous studies have documented very high levels of variation throughout the Atlantic menhaden mitochondrial genome (Avise et al., 1989; Bowen and Avise, 1990; Anderson, 2007), and in a preliminary analysis of various menhaden mitochondrial gene regions, we found COI to be sufficiently variable for an analysis of stock structure. The COI genetic diversity in Atlantic menhaden (π=2.74%) is an order of magnitude higher than the average within-species divergence reported for other fishes. For example, Ward et al. (2005) reported an average π of 0.39% for Australian marine fishes, and Hubert et al. (2008) reported a π of 0.302% for Canadian freshwater fishes.

There were significant differences in the distribution of COI haploytpes and microsatellite allele frequencies between Atlantic and Gulf menhaden, although both classes of markers indicate the two species are very closely related (F_{ST}=0.104). These results are consistent with those of Anderson (2007) who surveyed variation at four microsatellite loci, estimating an F_{ST} of 0.110 between Atlantic and Gulf menhaden. These F_{ST} values are more typical of differences between populations than species. For comparison, F_{ST} values between genetically distinct stocks of clupeid fishes based on microsatellites range from 0.002 to 0.226 (Shaw et al., 1999; Sugaya et al., 2008), and are approximately one-fourth of the F_{ST} values between other pairs of North American menhadens (0.355–0.488; Anderson, 2007).

A low level of genetic divergence between Atlantic and Gulf menhaden was also noted for the mitochondrial COI gene region (Φ_{ST}=0.178)—a result consistent with a previous restriction fragment length polymorphism (RFLP) analysis of the mitochondrial genome (Avise et al., 1989) and sequence analysis of the control region (Anderson, 2007) of these two species. In the present study, we did not find a single COI nucleotide position that distinguished Atlantic from Gulf menhaden. In a survey of 207 fishes, including several congeners, Ward et al. (2005) reported that all had different COI sequences. Furthermore, mean nucleotide differences between closely related species were 25 times higher (on average) than differences within species. In the present study, however, the nucleotide sequence diversity for Atlantic and Gulf menhaden combined (π=0.0258) was less than that for Atlantic menhaden alone (π=0.0274).

When compared with Gulf menhaden, Atlantic menhaden are generally larger, have a less convex body shape, and have a higher number of predorsal scales, vertebrae, and ventral scutes (Bigelow et al., 1963). Although the mean values of some of the morphometric and meristic characters are significantly different between the two species, the ranges of variation are coincident (Dahlberg, 1970). Although Atlantic menhaden and Gulf menhaden are morphologically similar, their geographic ranges are not believed to overlap (Bigelow et al., 1963). Thus, the species are typically distinguished by capture location. The high level of genetic and morphological similarity of Atlantic and Gulf menhaden raises concern over the validity of the two species. A thorough morphological and genetic analysis of the same individuals will be required to resolve this problem.

In the present analysis of Atlantic and Gulf menhaden mitochondrial COI gene region sequences, two distinct mitochondrial clades were noted, one of which was found only in Atlantic menhaden, and the other in both Atlantic and Gulf menhaden. These results are similar to those found in a previous RFLP analysis of the whole mitochondrial genome (Avise et al., 1989) and in a sequence analysis of the control region (Anderson, 2007). Avise (1992) hypothesized that the separation of two mitochondrial clades between the Atlantic Ocean and the Gulf of Mexico was a result of historical isolation of Atlantic and Gulf menhaden by the Florida peninsula during times of cooler water temperatures and subsequent unidirectional gene flow during geologically recent times. Anderson (2007) postulated that the distribution of these two clades in Atlantic menhaden supported very recent gene flow between Atlantic and Gulf menhaden because the highest frequency of "Atlantic-only" haplotypes occurred in the northernmost Atlantic menhaden sampling location. However, the purported geographic cline in the distribution of the "Atlantic-only" clade haplotypes was only qualitatively addressed and was based on a small sample size (n=37) of Atlantic menhaden. In the present study, the more extensive sampling regime for Atlantic menhaden along the U.S. Atlantic coast (n=289) refutes Anderson's (2007) hypothesis, because a chi-square analysis of the presence of the two clades among Atlantic coast sampling locations did not indicate a heterogeneous distribution.

Population structure

Stock structure analyses of Atlantic menhaden along the U.S. Atlantic coast have indicated as few as one and as many as three different stocks based on spawning time, spawning location, and migration tracks (Sutherland, 1963; June and Nicholson, 1964; Nicholson, 1978; Epperly, 1989). We analyzed the distribution of allelic variation of rapidly evolving molecular characters to evaluate population structure of Atlantic menhaden. The resulting AMOVAs did not reveal any significant portion of molecular variance was due to variation between the following group comparisons: YOY menhaden collected in Chesapeake Bay early and late in the season during the same year; YOY and yearling menhaden collected in Chesapeake Bay in successive years (following the 2006 year class); YOY and yearling menhaden collected in Chesapeake Bay in the same year (comparing 2005–2006, 2006–2007 year classes); and YOY and yearling menhaden (combined) from the four geographic regions along the U.S. Atlantic coast (New England, mid-Atlantic, Chesapeake Bay, and U.S. South Atlantic). The POWSIM analysis indicates that the sample sizes and

suite of microsatellite markers used in this study were sufficient for detecting even weak levels of differentiation ($F_{ST} \geq 0.0025$).

Although none of the five COI Φ_{ST} AMOVAs or five microsatellite R_{ST} AMOVAs were significant, two of the five microsatellite F_{ST} AMOVAs showed a small but statistically significant partitioning of genetic variation between YOY and yearling menhaden collected in Chesapeake Bay in successive years (following the 2006 year class, 1.80%, $P=0.0176$) and YOY and yearling menhaden (combined) from the four geographic regions along the U.S. Atlantic coast (0.575%, $P=0.0000$).

The pairwise comparisons between sample locations corroborate the Φ_{ST} and R_{ST} AMOVA results. No pairwise comparison revealed a statistically significant difference between any two of the four geographic regions of Atlantic menhaden after Bonferroni correction. These findings support the hypothesis that the significant results from the F_{ST} AMOVAs were a result of random processes and not biologically meaningful (for a discussion see Waples, 1998). The collective results indicate no significant partitioning of genetic variation between the sampling regions of Atlantic menhaden, and the null hypothesis that Atlantic menhaden comprise a single stock along the U.S. Atlantic coast cannot be rejected.

The lack of statistically significant genetic differences among Atlantic menhaden sampling regions is consistent with the life history traits of the species. Of all the North American *Brevoortia*, Atlantic menhaden undertake the longest coastal migrations and have the most temporally and geographically protracted spawning season (Whitehead, 1985). Atlantic menhaden are batch spawners, spawning multiple times during a year. Additionally, Atlantic menhaden larvae are found in waters from Maine to Mexico and are the most widely distributed larvae of any clupeoid in the western North Atlantic; (Kendall and Reintjes, 1975). The larvae can take up to 90 days to cross the continental shelf and are affected by along-shore transport, coastal storms, freshwater discharge from estuaries, and wind-forcing (Checkley et al., 1988). Menhaden also undergo an ontogenetic shift in migration, where larger fish migrate farthest north (Dryfoos et al., 1973). These characteristics appear to keep Atlantic menhaden—and their gene pool—well mixed.

Population structure has not been found in genetic analyses of other clupeids including Atlantic herring (*Clupea harengus*) (Grant, 1984), twaite shad (*Alosa fallax*) (Volk et al., 2007), and European pilchard (*Sardina pilchardus*) (Gonzalez and Zardoya, 2007). In contrast, some clupeid species exhibit significant stock structure, often attributed to the presence of geographic barriers or temporal reproductive isolation. These include Pacific herring (*Clupea pallasi*) stocks in the eastern North Pacific and Bering Sea (Grant and Utter, 1984), in the Bering Sea and Gulf of Alaska separated by the Alaska Peninsula (O'Connell et al., 1998), and from Honshu and Hokkaido Islands (Sugaya et al., 2008). Shaw et al. (1999) also found significant genetic structuring between Icelandic summer-spawners, Norwegian spring-spawners, and Norwegian fjord stocks of Atlantic herring.

Implications for management

Loss of unique genetic variation due to fishing pressure, habitat degradation, and hatchery stocking has been reported for Pacific cod (*Gadus macrocephalus*), leopard darter (*Percina panterina*), Japanese flounder (*Paralichthys olivaceus*), and American shad (*Alosa sapidissima*) (Grant and Stahl, 1988; Echelle et al., 1999; Brown et al., 2000; Sekino et al., 2003), and there is concern that a concentration of fishing effort in and around Chesapeake Bay could result in the loss of unique genetic variation in Atlantic menhaden. In this study we have demonstrated high genetic variability and a homogeneous distribution of genetic variation within Atlantic menhaden from four sampling locations along the U.S. Atlantic coast—a result consistent with the current management practice that recognizes a single stock of Atlantic menhaden. The apparent genetic connectivity between New England, mid-Atlantic, Chesapeake Bay, and U.S. South Atlantic samples indicates that loss of unique genetic variation due to the consolidation of fishing pressure in Chesapeake Bay is not likely. Our analysis of mitochondrial and nuclear loci revealed significant allele frequency differences between Atlantic and Gulf menhaden, supporting independent management of these resources. However, the small magnitude of these differences found in this and previous studies would indicate that a reevaluation of the specific status of the two putative species, based on analyses of morphological and genetic characters, is warranted.

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