Virginia Recreational Fishing Development Fund
Final Report

Project Title: A genetic assessment of the potential for local depletion of Atlantic menhaden (*Brevoortia tyrannus*) within Chesapeake Bay

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Executive Summary:

Atlantic menhaden is an ecologically and economically important species along the U.S. east coast. As a filter-feeder, it provides a critical link between primary production, phytoplankton, and larger piscivorous predators, such as striped bass, bluefish, and weakfish. The species is also the target of one of the largest commercial fisheries in the country. Menhaden are assessed as a single, coastwide stock, and recent assessments indicate that it is not overfished. However, there is very limited population genetics data to support the assumption of a single stock and the recent consolidation of the fishery and intensified harvests within and around Chesapeake Bay have raised concerns over the possibility of 'localized depletion' of the species in this area. In this study, we used rapidly evolving molecular markers to examine Atlantic menhaden stock structure along the U.S. Atlantic coast, specifically to determine the potential for the loss of unique genetic variation resulting from concentrated fishing pressure in and around Chesapeake Bay.

Samples were collected from three cohorts of Atlantic menhaden (2005, 2006, and 2007 year classes), at four geographic locations along the U.S. Atlantic coast (New England, mid-Atlantic, Chesapeake Bay, and U.S. south Atlantic) in 2006 and 2007. We surveyed two independent classes of molecular markers: the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region and six nuclear microsatellite loci. Hierarchical analyses of molecular variance (AMOVA) and examination of pairwise Φ_{ST} (COI) and F_{ST} and R_{ST} estimates (microsatellite loci) indicate high genetic variability and a homogeneous distribution of genetic variation within Atlantic menhaden. The genetic connectivity between New England, mid-Atlantic, Chesapeake Bay, and south Atlantic samples suggests that loss of unique genetic variation due to intensified fishing pressure in Chesapeake Bay is not likely to occur in Atlantic menhaden.

Introduction:

Atlantic menhaden is a member of the New World genus *Brevoortia* Gill 1861 (Pisces: Clupeiformes: Clupeidae). The genus contains six planktivorous species that form large, dense schools in nearshore enviroments. The four North American species are divided into two types: large-scaled and small-scaled menhadens. Large-scaled menhadens are Atlantic menhaden, distributed along the Atlantic coast from Indian River, Florida to Nova Scotia (Whitehead 1985), and gulf menhaden (*B. patronus* Goode 1879), distributed along the Gulf of Mexico coast from Florida Bay to the Gulf of Campeche (Whitehead 1985). Small-scaled menhadens are yellowfin menhaden (*B. smithi* Hildebrand 1941), distributed around the Florida peninsula from Beaufort, North Carolina to Louisiana (Whitehead 1985), and finescale menhaden (*B. gunteri* Hildebrand 1948), distributed from Chandeleur Sound, Louisiana to the Gulf of Campeche (Whitehead 1985). Anderson (2007) used genetic data to validate that large-scaled and small-scaled menhadens comprise two distinct lineages.

Identification of Atlantic menhaden can be particularly difficult because they are morphologically similar to gulf menhaden and are sympatric over part of their range with yellowfin menhaden. When compared with gulf menhaden, Atlantic menhaden are larger, have a less convex body shape, as well as a higher number of predorsal scales, vertebrae, and ventral scutes (Bigelow *et al.* 1963). While 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). When compared with yellowfin menhaden, Atlantic menhaden have larger scales with notably longer

pectinations, pale gray fins (as opposed to golden yellow), and a series of spots behind the large shoulder spot (Bigelow *et al.* 1963).

Of 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 have the most widely distributed clupeoid larvae in the western North Atlantic, occurring from Maine to Mexico, from fresh waters (Kendall and Reintjes 1975) to more than 40 miles offshore (Massmann *et al.* 1961). The northward spring migration begins from the overwintering grounds off Cape Hatteras, N.C. and appears to be triggered by seasonal ocean temperature changes (Reintjes 1969). This migration is age and size dependent with the older, larger fish migrating further north (Dryfoos *et al.* 1973, Quinlan *et al.* 1999). By summer, Atlantic menhaden are distributed from northern Florida to Maine (Ahrenholz 1991). Some spawning occurs in the northern part of the range throughout the summer and continues as the fish migrate southward in September (Rice *et al.* 1999). By November, most of the adults have returned to waters off Cape Hatteras, N.C.. Peak spawning is believed to occur in that region during the winter months (Checkly *et al.* 1988).

Atlantic coast estuaries serve as nursery grounds for larval and juvenile Atlantic menhaden until they join the seasonally migratory adults. Larvae are feeding within six days of fertilization and enter the estuaries and metamorphose between 30 and 90 days later (Checkley *et al.* 1988). Juvenile emigration from the estuarine nursery area is triggered by the onset of sustained low water temperatures, which is coincident with autumnal phytoplankton blooms (Friedland and Haas 1988). Tag recoveries suggest that Atlantic menhaden of differing ages and sizes share the overwintering grounds off Cape

Hatteras (Dryfoos *et al.* 1973). Large juveniles participate in the northward spring migration but few age-1 fish are caught north of Delaware Bay and none are caught north of New Jersey (Nicholson 1972, Kroger and Guthrie 1973). By two years of age, most fish are mature, migrating adults (Higham and Nicholson 1964). The longevity for Atlantic menhaden is estimated at 10-12 years, but few fish have been reported to reach that age (Reintjes 1969).

"The Most Important Fish in the Sea"

Cultural historian H. Bruce Franklin (2007) describes Atlantic menhaden as "the most important fish in the sea" because of their major economic and ecological significance. The commercial fishery for Atlantic menhaden generates over $45 million in annual revenue (Southwick Associates and Loftus 2006). Atlantic menhaden-dependent recreational fisheries are another fiscal powerhouse with $236 million in annual revenue (Southwick Associates and Loftus 2006). Atlantic menhaden also have high ecological value as a forage base and consumer of primary production (Goldsborough 2006).

The Atlantic menhaden commercial fishery is divided into two components: a smaller bait fishery and a larger reduction fishery. Bait fishery landings have been steady, averaging 34,000 metric tons since the mid 1980s, and currently comprise 21% of total Atlantic menhaden landings (ASMFC 2006). Reduction fishery landings, however, have progressively declined from peak landings in the 1950s (>600,000 metric tons) to 146,900 metric tons in 2005 (ASMFC 2006). Of the 20 fish reduction plants once operating along the U.S. Atlantic coast, only the Reedville, Virginia facility is currently active. The Reedville fleet ranges from New Jersey to North Carolina waters but focuses

its efforts within Chesapeake Bay. As a result, the proportion of Atlantic menhaden

reduction landings taken from inside Chesapeake Bay has increased from 47% (1985-

1995 average) to 58% (1996-2004 average) (ASMFC 2005), although the actual removals

from the Bay have decreased by 28% over the same period (ASMFC 2005). Nonetheless,

Atlantic menhaden account for 6% of all U.S. commercial tons landed, making the

fishery the fifth largest in the country, by weight (NMFS 2005).

Atlantic menhaden are a principal component of the diets of many piscivorous

fishes, including striped bass (*Morone saxatilis*), bluefish (*Pomatomus saltatrix*), and

weakfish (*Cynoscion regalis*), which support large recreational fisheries (Hartman and

Brandt 1995). Economically, the value of these recreational fisheries has far surpassed

the value of the traditional commercial Atlantic menhaden fishery. In 2005 alone, the

Atlantic menhaden-dependent recreational fisheries produced $111,507,900 more in

Virginia income than the Atlantic menhaden commercial fishery (Southwick Associates

and Loftus 2006).

In addition to their importance as a prey item, Atlantic menhaden may regulate

water quality. As planktivores, Atlantic menhaden remove plankton from the water

column which may enhance water clarity and mitigate problems associated with

eutrophication (Goldsborough 2006). Durbin and Durbin (1975) suggested that large

schools of adult Atlantic menhaden can significantly impact local phytoplankton and

zooplankton concentrations.

Atlantic Menhaden Stock Structure Analyses

Though the extensive seasonal migrations of Atlantic menhaden would suggest

the species might comprise one homogeneous population, some widely distributed,

migratory, marine fishes exhibit stock structure (e.g. Atlantic herring (*Clupea harengus;* Iles and Sinclair 1982), Atlantic cod (*Gadus morhua*; Campana *et al.* 1999)). Stocks, defined as populations with spatial and temporal integrity, respond to fishing pressure independently (Carvalho and Hauser 1994). They are traditionally differentiated using tagging, life history, and/or morphometric and meristic data (Carvalho and Hauser 1994). Stock structure analyses of Atlantic menhaden have proposed, principally on the basis of meristics and morphometrics, as few as one and as many as three different stocks (June 1958, 1965; Sutherland 1963; June and Nicholson 1964; Nicholson 1972, 1978; Dryfoos *et al.* 1973; Epperly 1989).

Two populations of Atlantic menhaden, one north and the other south of Long Island, N.Y., have been suggested on the basis of vertebral counts and transferrin allele frequencies (June 1958, 1965; Sutherland 1963; Epperly 1989). Sutherland (1963) hypothesized that mean vertebral number differences between juveniles were associated with water temperature at spawning time and that they were indicative of reproductive isolation. June (1965) related the vertebral differences to two discrete groups of Atlantic menhaden spawners, one occurring in cool, Cape Cod and Long Island waters in the spring and the other occurring in the warmer, Long Island and North Carolina waters in the fall. Nicholson (1972), however, refuted claims to a distinct northern population, citing that the vertebral differences are more likely phenotypic plasticity due to environmental factors (i.e. water temperature and spawning time) than heritable characters. Epperly (1989) found allele frequencies differences at the transferrin locus, a genetic character, to parallel vertebral count differences and suggested that Atlantic menhaden comprise at least two stocks.

Based on the presence of two generalized north-south migration tracts, June and Nicholson (1964) proposed two major population components, one occurring north and the other occurring south of Cape Hatteras, N.C.. They found small, sexually mature fish in North Carolina waters in late October and early November, well before the arrival of the larger, sexually mature fish from the north, as well as spawning fish off northern Florida in late winter and early spring (June and Nicholson 1964). Dryfoos *et al.* (1973) and Nicholson (1978) used tag and recapture methodology to follow migrations of Atlantic menhaden. The recoveries indicated that Atlantic menhaden stratify by age and size as they migrate northward from Cape Hatteras, N.C. in spring and return southward in fall, with the older, larger fish migrating further distances (Dryfoos *et al.* 1973, Nicholson 1978). Because of the pattern of tag recoveries, Dryfoos *et al.* (1973) and Nicholson (1978) recommended the Atlantic menhaden resource be considered only one stock. Citing the phenotypic plasticity of Nicholson (1972) and the tag returns of Dryfoos *et al.* (1973) and Nicholson (1978), the Atlantic States Marine Fisheries Commission considers Atlantic menhaden one coastwide stock, although the results of Epperly (1989) suggest that further genetic study is warranted.

Clupeiform Genetic Stock Structure Analyses

Though little work has been done on Atlantic menhaden, genetic studies have been used to examine stock structure for other clupeids and engraulids. A genetic stock is defined as a reproductively isolated unit that is genetically distinct from other units (Waples 1987). Stocks can be distinguished using a number of different molecular markers including allozymes, restriction fragment length polymorphisms (RFLPs), microsatellites, and direct DNA sequences.

Initially, allozymes were the main marker used to detect genetic stocks in clupeiforms. Allozymes are protein polymorphisms that can be separated primarily by charge using gel electrophoresis. Using allozymes, Vrooman *et al.* (1981) identified three stocks of northern anchovy (*Engraulis mordax*) and Grant and Utter (1984) differentiated Pacific herring (*Clupea pallasi*) between the Bering Sea and the eastern North Pacific. Also with allozymes, Grant (1984) and Jørstad *et al.* (1991), Grant (1985), and Morgan *et al.* (1995) found evidence supporting panmictic populations of Atlantic herring (*C. harengus*), cape anchovy (*E. capensis*), and bay anchovy (*Anchoa mitchilli*), respectively. The discriminatory power of allozymes, however, has a limit because the evolutionary rate of the amino acid sequences of most proteins is very low.

Analysis of DNA, which investigates changes at the nucleotide level, provides a higher level of genetic resolution than analysis of allozymes, which can only investigate charge changes of translated proteins. While proteins are usually under intense selective pressure, many regions of DNA are under limited selective pressure and can mutate with little functional consequence. Originally, restriction fragment length polymorphism (RFLP) analyses were used to study DNA variation. In a RFLP analysis, a restriction enzyme recognizes and breaks (cuts) the DNA strand at a restriction site, generating fragments. RFLP studies can be conducted on the whole mitochondrial genome or an amplified mitochondrial gene region. The fragment(s) resulting from restriction digestion can be separated using gel electrophoresis. Individuals with different RFLP patterns have different genetic sequences. This process can be extended to include a suite of restriction enzymes to effectively survey more of a gene region. Avise *et al.* (1989) used RFLP analysis of the whole mitochondrial genome to investigate genetic

relationships of Atlantic and gulf menhaden, finding two clades that failed to resolve the two species. Bowen and Avise (1990) suggested historical isolation and secondary contact (recent gene flow) between Atlantic and gulf menhaden around the Florida peninsula as an explanation for the paraphyletic, two-clade structure. In another RFLP study of clupeids, Hauser *et al.* (2001) found evidence of genetic differentiation between Baltic and Celtic Sea Atlantic Herring (*C. harengus*) using the mitochondrial *ND3/4* and *ND5/6* regions.

More recently, nuclear microsatellite loci have been favored for intraspecific comparisons of clupeids because of their rapid evolutionary rates. Microsatellites, also known as variable number tandem repeats, are nuclear loci consisting of short sequences (usually 2-5 base pair) that are variably duplicated by slip-strand mispairing. Slip-strand mispairing is a DNA replication error in which a DNA polymerase disassociates from a repeat and incorrectly rebinds to another repeat on a template DNA strand. As a result, copies of the repeat are either added to or deleted from the new strand of DNA. Because of their fast rate of mutation, microsatellites are best suited for studies of recent divergences. The development of suitable microsatellite primers is a time intensive process. Nonetheless, primers have been characterized for a number of clupeids, including allis shad (*Alosa alosa*; Faria *et al.* 2004), American shad (*A. sapidissima*; Julian and Bartron 2007), Atlantic herring (McPherson *et al.* 2001), Pacific herring (O'Connell *et al.* 1998, Miller *et al.* 2001, Olsen *et al.* 2002), Pacific sardine (*S.sagax sagax*; Pereyra *et al.* 2004), and twaite shad (*A. fallax*; Faria *et al.* 2004). O'Connell *et al.* (1998) used microsatellite analysis to confirm the separation of the Bering and Gulf of Alaska stocks of Pacific herring. Similarly, Shaw *et al.* (1999) found significant genetic

structuring between Icelandic summer-spawners, Norwegian spring-spawners, and Norwegian fjord stocks of Atlantic herring. Microsatellite analysis has shown significant genetic differentiation between the donor and recipient populations of American shad (Pamunkey and James River, respectively), as well as the potential for outbreeding depression in the restoration program (Brown *et al.* 2000). Some of the microsatellite primers show interspecies amplification. For example, Anderson (2007) used American shad microsatellite primers to make inferences regarding the relationships of the North American menhadens.

Direct sequencing of mitochondrial genes provides an independent perspective to analysis of nuclear microsatellite loci for high-resolution population comparisons. The mitochondrial genome, with its fast rate of evolution, lack of recombination, and maternal inheritance, has proven to be an appropriate molecular character for analysis of intraspecific genetic structure (Avise *et al.* 1987). Evolutionary rates, however, are not equivalent throughout the mitochondrial genome (Cann *et al.* 1984). The non-coding mitochondrial control region is reported to have a rate of evolution two to five times higher than that of mitochondrial protein-coding genes (Meyer 1993). Of the 13 mitochondrial protein-coding genes, cytochrome *c* oxidase subunit I (COI) is considered the most conserved in fishes (Meyer 1993). Yu *et al.* (2005), using cytochrome *b* and COI, did not detect genetic structure in the Japanese anchovy (*Engraulis japonicus*) between the Yellow and East China seas. Tinti *et al.* (2002) found no evidence for genetic stock structure between European pilchards (*Sardina pilchardus*) from the Adriatic Sea and Ionian Seas using direct sequencing of a cytochrome *b* gene fragment. On a larger scale, Atarhouch *et al.* (2006) identified genetic differentiation in European

pilchards between the Bay of Biscay and Mediterranean Sea using the mitochondrial control region sequence data. Anderson (2007) used mitochondrial control region sequence data to validate the large-scaled and small-scaled designations for North American menhadens but could not resolve the sequences to species.

Direct sequencing of nuclear gene regions has been used to infer stock structure in some fishes, but it has not been reported for any clupeiform. While most protein-coding nuclear genes do not evolve at a rate fast enough to make them useful for intraspecific comparisons, non-coding regions, such as internal transcribed spacer 1 (ITS 1), that have fewer selective constraints and a faster evolutionary rate have been used to investigate population structure (Avise 1987). ITS 1, which separates the 5.8S and 18S ribosomal RNA genes, exhibits high variability and a fast rate of mutation (Jansen *et al.* 2006). For example, Brentro (2006) used ITS 1 sequencing data to determine that four escolar (*Lepidocybium flavobrunneum*), collected in the Atlantic Ocean but assigned to the Pacific Ocean using mitochondrial control region sequencing, were a result of recent rather than historical migration to the Atlantic Ocean.

The genetic basis of stock structure for Atlantic menhaden has not been well studied. With the recent concentration of the Atlantic menhaden reduction fishery in Chesapeake Bay, it is important to understand the stock structure of the species. If Atlantic menhaden exhibit stock structure, there will be spatial partitioning of unique genetic variation. Because a portion of the genetic differentiation among fish populations is considered adaptive (Ryman 1981), loss of unique genetic variation may inhibit stocks from responding to shifting environmental pressures (Higgins and Lynch 2001). This is particularly vital to exploited fishes, such as Atlantic menhaden, because undetected

genetic structure may result in overexploitation of a local population, diminishing overall fishery yield (Carvalho and Hauser 1994).

Project Objectives:

This study utilizes sequence data from the mitochondrial COI gene region and three nuclear microsatellite loci to investigate the temporal and spatial genetic stock structure of Atlantic menhaden and to evaluate the potential for loss of unique genetic variation resulting from 'localized depletion' within the Chesapeake Bay region. We sampled young-of-the-year (YOY) and yearling (age-1) fish over a two year period (2006, 2007) from four regions along the U.S. Atlantic coast (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic). The following null hypotheses are addressed:

$H_{0,1}$: There is no genetic difference between YOY menhaden recruiting to Chesapeake Bay early and late in the season during the same year.

$H_{0,2}$: There is no genetic difference between YOY and yearling menhaden collected in Chesapeake Bay in successive years (following the 2006 year class).

$H_0,3$: There is no genetic difference between YOY and yearling menhaden collected in Chesapeake Bay in the same year.

$H_0,4$: There is no genetic difference among YOY and yearling menhaden (combined) from four geographic regions along the U.S. Atlantic coast.

Materials and Methods:

Sample Collection

YOY and yearling Atlantic menhaden were sampled from throughout the species range in 2006 and 2007. Collections were grouped into four broad geographic regions: New England (Massachusetts, mid-Atlantic (New Jersey), Chesapeake Bay (Virginia), and U.S. south Atlantic (South Carolina) (Figure 1). Atlantic menhaden were identified on the basis of morphological characters by local experts (New England: Gary Nelson, Massachusetts Division of Marine Fisheries; mid-Atlantic: Heather Corbett, New Jersey Division of Fish & Wildlife; Chesapeake Bay: Patrick Lynch or Troy Tuckey, Virginia Institute of Marine Science; U.S. south Atlantic: John Archambault, South Carolina Department of Natural Resources). Voucher specimens were retained from all regions in 2007 to corroborate field identification. Fork length was measured for each individual to estimate age (Higham and Nicholson 1964, Lewis *et al.* 1972, Nicholson 1972) and scale samples were sent to the NOAA Beaufort Lab / NMFS for independent age assessments. Muscle tissue samples were either frozen or stored in DMSO buffer (Seutin *et al.* 1991) at room temperature.

Molecular Markers

For any genetic study, it is important to use a molecular marker with an evolutionary rate appropriate to the genetic question to be addressed (Lannan *et al.* 1989). High levels of genetic variation are often needed to describe stock structure relationships, but too much polymorphism can be problematic. Highly variable regions, for example, may actually underestimate differentiation because of mutational saturation. The highly variable mitochondrial control region is often used for intraspecific studies (McMillan and Palumbi 1997) but J.D. Anderson (personal communication) suggested that it might be too variable within Atlantic menhaden and that a more conserved region may prove

more useful to evaluate population structuring. For that reason, a preliminary study was conducted to survey variation in the highly variable control region and the more conserved cytochrome *c* oxidase subunit I (COI). In addition, genetic variation was screened at the nuclear internal transcribed spacer 1 (ITS-1) and 3 nuclear microsatellite loci.

Extraction and Amplification

Total genomic DNA was extracted from each tissue sample using a Qiagen DNeasy® Tissue Kit (Qiagen) following the manufacturer's protocol. The mitochondrial control region, COI, ITS-1, and six microsatellite loci (Asa-2, Asa-4, Asa-16, Brown *et al*. 2000; and Aa-16, Faria *et al*. 2004), were amplified using the polymerase chain reaction (PCR). The primers, reagents, and PCR parameter optimizations for the sequencing regions are listed in Appendix 1. The microsatellite loci used in this study, along with their GenBank Accession numbers, repeat motifs, and primer sequences are listed in Table 1. The PCR conditions and reagents used for microsatellite amplification are listed in Appendix 2.

For the sequencing reactions, 5µL of each PCR amplification was run on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light to verify that a single fragment of the correct size was amplified. The remaining 5µL of the mitochondrial amplification was purified using column filtration with a QIAquick® PCR purification kit (Qiagen) following the manufacturer's protocol.

Nuclear Cloning

Diploid organisms have two copies of the nuclear genome, one from each parent. While both can be amplified simultaneously using PCR, sequencing can only read one

copy at a time. As a result, the ITS-1 PCR products were cloned to separate the two allele fragments before sequencing. One μL of the nuclear product was cloned into a plasmid vector using the TOPO-TA plasmid cloning system (Invitrogen Corporation) prior to sequencing. Fresh PCR product was ligated into the ampicillin resistant TOPO 2.1 plasmid vector with a *lacZ* gene and transformed into competent TOP10 *Escherichia coli* bacterial cells using the manufacturer's One Shot Chemical Transformation Protocol. The *E. coli* cells were then plated on Luria-Bertani (LB) agar plates containing ampicillin (5μg/mL), an antibiotic, and 40μL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40mg/mL), an indicator of *lacZ* expression, and grown up overnight at 37°C. Ampicillin ensured that only *E. coli* cells with the ampicillin resistant vector grew on the plates. The X-gal differentiated the colonies containing the recombinant plasmid (a non-functional *lacZ* gene = white colonies) from colonies without the insert (a functional *lacZ* gene = blue colonies). DNA was extracted from only the recombinant colonies via cell lysis through boil preparation (Sambrook and Russell 2001) and PCR amplified using primers designed to flank the vector insert region. To verify that the insert of the correct size was recovered, 5μL of the cloned PCR amplification was run on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The remaining 5μL of cloned elutant was purified using column filtration with QIAquick® PCR purification kit (Qiagen) following the manufacturer's protocol.

Sequence Analysis

The concentration and quality of each purified ITS-1 cloned PCR product and direct mitochondrial PCR product was measured using a BioMate[TM] 3 Series UV Spectophotometer (Thermo Spectronic) prior to sequencing. Purified direct and cloned

PCR products were prepared for sequencing using the ABI PRISM® Big Dye[TM] Terminator v 3.0 Cycle Sequencing Kit (Applied Biosystems) at a 1:8 dilution of the manufacturer's protocol and subsequently long-run sequenced on an ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems).

The chromatographic curves for each long-run sequence (control region, COI, ITS-1) were analyzed using Sequencing Analysis software v 5.2 (Applied Biosystems), edited using Sequencher 4.7.2 (Gene Codes Corp.), and aligned using the ClustalW algorithm for multiple alignments in MacVector 9.0.1 (MacVector Inc.). Once aligned, the sequences were characterized in Arlequin 3.11 (Excoffier *et al.* 2005) to determine the following: the number of polymorphic sites (S; variable base pair (bp) locations within a sequence set), the number of transitions (Ts; bp mutations from either a purine to a purine (A$\leftrightarrow$G) or a pyrimidine to a pyrimidine (C$\leftrightarrow$T)), the number of transversions (Tv, bp mutations from a purine to a pyrimidine (A or G$\rightarrow$C or T) or a pyrimidine to a purine (C or T$\rightarrow$A or G)), and the number of indels (bp(s) inserted in or deleted from a sequence).

Microsatellite Analysis

Following PCR amplification for the microsatellites, 1µL of PCR product for each locus, associated with a locus-specific fluorescent label, was combined with three other loci PCR products and fluorescent labels (4µL total), 6µL formamide, and 0.3µL 500 Liz Gene Scan Size standard (Applied Biosystems). The reaction mixture was denatured at 95°C for 10 minutes prior to short-run sequencing on an ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol.

The chromatic peaks for each short-run sequence (microsatellite loci) were scored using GeneMarker AFLP/Genotyping Software v 1.60 (SoftGenetics). 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. Genepop v 3.4 (Raymond and Rousset 1995) and Arlequin 3.11 (Excoffier *et al.* 2005) were used to determine the following: allelic diversity (*A*; number of alleles per locus), number of genotypes, observed heterozygosity (H_O), and expected heterozygosity (H_E).

Descriptive Statistics

The amount of sequence variation for each gene region within each collection was estimated in Arlequin 3.11 (Excoffier *et al.* 2005) by the following diversity indices: haplotype diversity (*h*, the probability that two randomly chosen haplotypes (unique genetic sequences within a dataset) are different), nucleotide sequence diversity (π, the mean sequence divergence between two randomly chosen haplotypes), and mean number of pairwise differences (*k*, the mean number of bp differences between two randomly chosen haplotypes).

The amount of microsatellite variation within each collection was estimated in FSTAT v 2.9.3.2 (Goudet 1995) by the following indices: allelic richness (R_s; number of alleles per locus taking into account sample size) and gene diversity (*D*; heterozygosity). To determine if the distribution of microsatellite loci genotypes conformed to the expectations of Hardy-Weinberg equilibrium for each collection location, the probability test, heterozygote deficiency test, and heterozygote excess test were performed in Genepop v 3.4 (Raymond and Rousset 1995).

Genetic Relationships

To initially visualize genetic relationships among aligned mitochondrial and nuclear sequences, unweighted pair group method with arithmetic mean (UPGMA) trees, based on nucleotide base pair similarity matrices, were constructed in MacVector 9.0.1 (MacVector Inc.). The most appropriate nucleotide substitution model for a sequenced region was determined from a series of 56 likelihood ratio tests executed in ModelTest 3.7 (Posada and Crandall 1998). The accumulation of nucleotide substitution is only roughly linear with time shortly after a divergence event (Sullivan and Joyce 2005). The cause of deviation from linearity, however, is not the same in all cases. As a result, a number of different models have been developed to explain the range of deviations. ModelTest 3.7 (Posada and Crandall 1998) matches a dataset with the model that best fits its nucleotide substitution pattern according to two sets of criteria: hierarchical likelihood ratio tests and Akaike's Information Criterion. The resulting model was used in PAUP* 4.0 (Swofford 2000) to produce a neighbor-joining tree of maximum likelihood distances. Support of the internal branches was tested using bootstrap resampling (Felsenstein 1985) with 1,000 replicates.

Additionally, a median-joining network for all COI haplotypes was drawn in Fluxus 4.2.0.1 (Bandelt *et al.* 1999). For intraspecific comparisons, networks more accurately describe genetic relationships than phylogenetic trees because internal nodes are often extant in population level studies (Bryant and Moulton 2002). With a tree, an internal node is a theoretical representation of a common ancestor between sampled taxa. With a network, interior haplotypes are usually the most common haplotypes from which mutations occur. Rarer haplotypes, as a result, are more likely to be related to these common haplotypes than to each other.

Genetic relationships among collection locations based on microsatellite loci data were estimated by constructing neighbor-joining trees using modified Cavalli-Sforza chord distance (D_A; Nei *et al*. 1983) and Nei's standard genetic distance (D_{ST}; Nei 1972) in Microsatellite Analyzer (MSA) (Dieringer *et al*. 2003). D_A is one of the most efficient distance measures for tree topology construction; D_{ST} is considered more suitable than other distance measures for branch length estimation (Sekino and Hara 2001). Support for the internal branches was tested using bootstrap resampling (Felsenstein 1985) with 1,000 replicates.

Genetic Structure

A hierarchical analysis of molecular variance (AMOVA) was used to test for population structure using COI sequencing data and microsatellite loci data. The AMOVA partitions genetic variance at predefined levels (individual, sub-region, region) based on the fixation index. The fixation indices, Φ_{ST} for sequencing and F_{ST} or R_{ST} for microsatellite data, are measures of population subdivision (Excoffier *et al*. 1992). AMOVA calculations based on microsatellite genotypic data were analyzed using two different distance methods: F_{ST}, based on the distribution of number of different alleles (does not consider relationships among alleles), and R_{ST}, based on the distribution and relationship of alleles (sum of squared allele size differences). The R_{ST} algorithm is generally considered the most appropriate measure for microsatellite data (Sekino and Hara 2001). Φ_{ST}, F_{ST}, and R_{ST} were all utilized for this analysis. The AMOVAs, conducted in Arlequin 3.11 (Excoffier *et al*. 2005), partitioned variation across designated groupings: between recruitment times within an age class at a location (2007 YOY menhaden in Chesapeake Bay), years within an age class at a location (the 2006

year class in Chesapeake Bay in 2006 and 2007), between age classes within a region

(eg., YOY and yearling menhaden in Chesapeake Bay in 2007), and among regions (New

England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic). Estimates of population

pairwise Φ_{ST} and F_{ST} were calculated in Arlequin 3.11 (Excoffier *et al.* 2005) as a

measure of genetic distance between individual groupings.

Results:

Evaluation of Variability

To determine which mitochondrial gene region would be appropriate to evaluate

Atlantic menhaden population structure, a preliminary study was undertaken to survey

nucleotide variation in a 535bp fragment of the mitochondrial control region and a 459bp

fragment of cytochrome *c* oxidase subunit I (COI) for 28 individuals. The mitochondrial

control region fragment contained 63 polymorphic sites, 55 transitions, 16 transversions,

and 1 indel (Table 2). Of the 28 menhaden sequenced, the control region produced 27

haplotypes, an overall haplotype diversity (*h*) of 0.9974, and a mean nucleotide sequence

diversity (π) of 0.0326. The COI fragment contained 44 polymorpic sites, 41 transitions,

8 transversions, and no indels (Table 2). Of the 28 menhaden sequenced, COI produced

20 haplotypes, an overall haplotype diversity (*h*) of 0.9603, and a mean nucleotide

sequence diversity (π) of 0.0267. The haplotype diversity estimate for COI is high, but

not as elevated as the control region estimate. Both gene regions revealed similar

patterns of intraspecific relationships (Figure 2), but with the lower haplotype diversity

estimate, the COI sequences were less at risk of mutational saturation than those of the

control region. Mutational saturation curves support this suggestion in that the rate of

transversions approaches that of transitions for the control region data but not for the COI data (Figure 3). Based on this preliminary analysis, COI was considered a more appropriate molecular marker for this study and all samples were surveyed for this gene region.

The COI fragment was sequenced for 289 individuals. The fragment contained 105 polymorphic sites, 97 transitions, 6 transversions, and produced 109 haplotypes (Table 3). Haplotype diversity (h) estimates for the sampling locations ranged from 0.9396 to 0.9563 with an overall (pooled) estimate of 0.9523. Mean nucleotide sequence diversity (π) estimates for sampling locations ranged from 0.0258 to 0.0295, with an overall estimate of 0.0274.

The median-joining network for the 109 COI haplotypes showed two extensive "star-shaped" clusters (clades) connected by an intermediate branch with one minor exterior grouping (Figure 4). Clade I comprised 65% of the pooled samples, while Clade II comprised 34% of the pooled samples. The frequency of the two clades was strongly conserved across samples, with Clade I ranging from 61 – 67%, and Clade II ranging from 34 – 3 6% (Table 4). A chi square analysis of all collections indicated that the two clades were not heterogeneously distributed among the geographic samples.

To assess the evolutionary relationships among COI haplotypes, hierarchical likelihood ratio tests were performed in ModelTest 3.7 (Posada and Crandall 1998). The analysis selected the HKY+I+Γ model (k=parameter estimates=6; Hasegawa *et al.* 1985) and Akaike's Information Criterion selected the K81+I+Γ model (k=4; Kimura 1981) as the most appropriate of the 56 nucleotide substitution models. Because simulation studies have shown that overparameterization is less of a problem than

underparameterization for estimating nucleotide substitution (Huelsenbeck and Rannala 2004), the HKY+I+Γ model was used in this analysis. This model assumes a time-reversible mutational process, a non-uniform distribution of nucleotides, and different rates for transitions and transversions. A neighbor-joining phylogeny using the maximum likelihood distances calculated by the HKY+I+Γ model produced a geographically unresolved structure (Figure 5).

The two genetically divergent clades of haplotypes evident in the mitochondrial COI sequence analysis could be the result of the presence of two sympatric subspecies (or species) or the result of historical isolation with subsequent gene flow. To distinguish between these phylogeographic hypotheses, a 500bp fragment of ITS-1, a biparentally-inherited nuclear marker, was sequenced to provide a nuclear perspective with which to compare with the phylogeographic structure inferred from analysis of COI sequences. Sixty clones from 12 individuals, representative of both mitochondrial clades, were sequenced. The analysis resulted in 81 polymorphic sites, 54 transitions, 23 transversions, and 14 indels (Table 1). The 45 haplotypes have an overall haplotype diversity (h) of 0.979 and a mean nucleotide sequence diversity (π) of 0.0183. The UPGMA tree of the cloned nuclear ITS-1 sequences differed considerably from the distinct two clade mitochondrial structure and revealed no discernable stock structure (Figure 6).

Six microsatellite loci were amplified for the entire dataset (Table 5). Over all samples (data combined), over the six loci, the allelic diversity (A) ranged from 9 to 23 alleles; the allelic richness (R_s) ranged from 6.195 to 16.910, and the gene diversity (D, heterozygosity) ranged from 0.678 to 0.901. Using three separate tests, the genotypic

distribution at each locus was checked for conformance to Hardy-Weinberg equilibrium using the methods of Guo and Thompson (1992). The genotypic distribution of all loci conformed to the expectations of Hardy-Weinberg equilibrium. A seventh locus, Asa 16 was evaluated for use in this study, but in three of the four regions, Asa16 showed statistically significant heterozygote deficiency, suggesting the presence of a null allele (a mutation in a primer binding site that results in non-amplification of an allele) and this locus was not included in the analyses.

Analysis of Molecular Variance

The AMOVAs of COI haplotype data and microsatellite genotype data were performed to evaluate the temporal and spatial genetic stock structure of Atlantic menhaden. The AMOVAs addressed the following null hypotheses:

$H_{0,1}$: *There is no genetic difference between YOY menhaden recruiting to Chesapeake Bay early and late in the season during the same year.*

The mitochondrial (Φ_{ST}) AMOVA between early and late recruiting 2007 YOY in Chesapeake Bay attributed none of the variance to differences in recruitment time (Table 8).

$H_{0,2}$: *There is no genetic difference between YOY and yearling menhaden collected in Chesapeake Bay in successive years (following the 2006 year class).*

The mitochondrial (Φ_{ST}) AMOVA between 2006 YOY and 2007 yearling menhaden collected in Chesapeake Bay attributed none of the variance to differences between the year.

$H_{0,3}$: *There is no genetic difference between YOY and yearling menhaden collected in Chesapeake Bay in the same year.*

The mitochondrial (Φ_{ST}) AMOVAs between YOY and yearling menhaden collected in Chesapeake Bay in 2006 and 2007 attributed none of the variance to differences between the age classes. The microsatellite (F_{ST}) AMOVA that incorporated age (YOY or yearling) and geographic location attributed all of the variance to differences among individuals within collections. There was no signal between year classes (combined). Pairwise comparisons between all yearclass/location combinations resulted in no F_{ST} values that were significantly different from 0.

$H_{0,4}$: There is no genetic difference among YOY and yearling menhaden (combined) from four geographic regions along the U.S. Atlantic coast.

The mitochondrial (Φ_{ST}) AMOVA comparing the four geographic regions along the U.S. Atlantic coast in 2006 and 2007 found none of the variance to be a result of population sub-division between the regions. Pairwise Φ_{ST} estimates replicate these findings, showing no statistically significant differentiation between any pair of regions (Table 7). The nuclear (F_{ST}) AMOVAs found none of the variance to be attributable to regional differences. When tested as separate years at each location, 2 of 28 pairwise comparisons resulted in significant F_{ST} values (New England 2007 vs. Chesapeake Bay 2006 and mid-Atlantic 2007 vs. Chesapeake Bay 2007), but these tests were not significant after correcting for multiple testing.

Principal component analysis of microsatellite allele frequencies also failed to reveal a partitioning of nuclear genetic variation. No discernable structure was evident in plots of samples by year and location (Figure 7), by age (YOY and yearling) and location (Figure 8), or simply by location (Figure 9).

Discussion:

To effectively evaluate Atlantic menhaden stock structure along the Atlantic coast and examine the potential for the loss of unique genetic variation resulting from concentrated fishing pressure in and around Chesapeake Bay, the molecular markers employed need to be variable enough to reveal spatial and temporal partitioning of genetic variation, if it exists. Previous sequence analyses of the mitochondrial COI gene regions of vertebrates have demonstrated it is typically one of, if not the most conserved mitochondrial gene region (Saccone *et al.* 1999; Meyers 1993). However, in a preliminary survey of mitochondrial control region and COI sequences, both regions demonstrated very high variability. Unlike the control region, COI did not seem to be at risk for mutational saturation. High levels of variation of the menhaden mitochondrial genome found in this study are consistent with values reported in other studies of menhaden mtDNA (RFLP analysis, Bowen and Avise 1990; control region sequencing, Anderson 2007). High levels of variation of mtDNA gene regions in other clupeids has also been noted (cytochrome *b* sequencing, Tinti *et al.* 2002; control region sequencing, Atarhouch *et al.* 2006). The microsatellites surveyed in this study, like the mtDNA gene regions, were also highly variable, with heterozygosities for some of the loci approaching 1.0. High levels of microsatellite variation has been reported in other clupeids, and the markers have been used for analyses of population structure (O'Connell *et al.* 1998; Shaw *et al.* 1999; Brown *et al.* 2000; Anderson 2007).

The mitochondrial COI sequence data analysis grouped Atlantic menhaden into two distinct clades, a result that is consistent is consistent with previous studies of menhaden based on RFLP analysis of the whole mtDNA molecule (Avise *et al.* 1989)

and sequence analysis of the mtDNA control region (Anderson 2007). None of these studies were able to resolve the Atlantic and gulf menhaden based on analyses of mtDNA, although Anderson (2007) was able to discriminate among the two species of fine scale menhaden, and the composite Atlantic/gulf menhaden.

This two clade mtDMA structure found in Atlantic/gulf menhaden could be the result of current or historical gene flow. Bowen and Avise (1990) suggested historical isolation and recent gene flow between Atlantic and gulf menhaden around the Florida peninsula as an explanation for the paraphyletic, two-clade structure. Avise (1992), additionally, referenced Florida as a demographic break between Atlantic and Gulf of Mexico congeners of a number of vertebrates (including menhaden) in which southern Atlantic individuals often possessed haplotypes characteristic of the Gulf of Mexico species, but the more northern collections lacked the Gulf influence. Anderson (2007) supported this hypothesis with mitochondrial control region data: four of the eight haplotypes present in the Atlantic menhaden only clade were from the northern-most sampling location (Maine). This study, with a more extensive sampling regime, does not support this hypothesis. A chi-square analysis of all sampling locations indicates that the two mitochondrial clades are equally represented along the U.S. Atlantic coast.

To investigate the origin of the two clade mitochondrial structure in Atlantic menhaden, the nuclear gene region ITS-1 was sequenced for a sub-set of individuals representing both mitochondrial clades. The ITS-1 sequence relationships did not resolve a two clade structure consistent with the mtDNA analyses. In fact, there was no indication of any structure at all. The lack of congruence between mitochondrial and nuclear markers suggests that the two clade mitochondrial structure is most likely a result

of historical isolation as suggested by Bowen and Avise (1990). Similar scenarios in which there is a lack of concordance between the two classes of markers have been reported for several other fishes including the blue marlin, sailfish, swordfish, and bigeye tuna (see Graves and McDowell 2006 and references therein).

ITS-1 sequencing analysis is a costly and time-consuming process and is better suited for phylogenetic studies with a limited number of samples than population structure studies that require the analysis of hundreds of individuals. While the ITS-1 sequencing analysis was useful for making phylogenetic inferences regarding the two mtDNA clades, we felt that analysis of nuclear microsatellite loci would provide a more powerful and cost means to survey population structure in Atlantic menhaden. It is worth noting that all of the nuclear microsatellite loci were consistent with the limited ITS-1 sequence data – none revealed the presence of two clades, characteristic of the mtDNA analyses.

The temporal and spatial genetic stock structure of Atlantic menhaden was statistically analyzed using AMOVAs of COI haplotype data and microsatellite genotype data. None of these AMOVAs produced statistically significant results that would have suggested the partitioning of the genetic variation between cohorts or sampling locations. In the mitochondrial analyses, both AMOVAs and pairwise Φ_{ST} comparisons, gave no indication of geographic stock structuring in Atlantic menhaden. The frequency of the two clades was almost identical across the four sampling locations, and there was no significant heterogeneity in the distribution of the individual haplotypes. These results suggest that there is sufficient gene flow between the regions to maintain a homogeneous mtDNA gene pool, and that the species is not at risk to lose unique genetic mtDNA

variation as the result of localized fishing pressure within the Chesapeake Bay region.

The microsatellite AMOVAs demonstrated no significant partitioning of the microsatellite allele variation. In all cases, the overwhelming majority of variation was represented by the individuals. No significant levels of variance were attributed to differences between early and late recruiting YOY within a year, between year classes at a location within the same year, between the same cohort in a location in different years, or between locations (cohorts separate or combined).

Together, the analyses of nuclear and mitochondrial molecular markers, indicates that there is sufficient gene flow within Atlantic menhaden to maintain a relatively homogeneous distribution of genetic variation. The lack of partitioning of genetic variation in space or time suggests that spatially restricted fishing pressure will not likely remove unique genetic variation. The genetic connectivity of Atlantic menhaden is most likely the result of adult migrations, a spatially and temporally protracted spawning season, and the mixing of eggs, larvae, and early juveniles that occurs during cross shelf transport to the inshore nursery areas.

It is important to realize that gene flow (migration and successful breeding) on the order of individuals per generation is sufficient to prevent significant differences arising between geographically distinct populations (Hartl and Clark 2007). Our genetic data indicate that gene flow sufficient to prevent the accumulation of significant genetic heterogeneity is occurring (individuals per generation), but we cannot determine the magnitude of this exchange above that minimum value. To estimate the time scale on which menhaden may move into locally depleted areas (weeks, months, years) will require other techniques such as tagging, or possibly, otolith microchemistry.

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Tables:

Table 1. Microsatellite loci used in the present study. The fluorescent labels listed refer to the fluorescently labeled T3 (5'
AATTAACCCTCACTAAAGGG) primer utilized.

Locus	GenBank Accession no.	Repeat motif [reported size]	Fluorescent Label	Primer sequence (5'-3')	Annealing temp	Reference
Aa16	AY617110	$(CA)_4AA(CA)_3AA(CA)_8$ [145-159]	PET	F: TTG ACC GAG CGC AAA CTG R: TGA CAC TGA CTC ATC ATG C	55.7^{o}C	Faria et al. 2004
Asa2	AF039657	$(TTC)_{13}$ [73-133]	6-FAM	F: CAT TAC TCC AAG TTG CTT TTA TTT R: GAG ATG ACA GAA GAA TTG AAG AGA	48.6^{o}C	Brown et al. 2000
Asa4	AF039658	$(ACC)_3(AAC)_{12}(AGC)_6$ [124-172]	NED	F: GAA GAC AAT ACA GTA ATA AAC C R: GCG GGA GGC CAG ACA TA	53.8^{o}C	Brown et al. 2000
AsaB020	EF014990	$(GAT)_{15}$ [114-147]	6-FAM	F: GCA TTA TGA TGG TCA TGT GTA TG R: GAA ATC CTA TGT CTT GGA ATG G	53.8^{o}C	Julian and Bartron 2007
AsaC334	EF014995	$(GTAT)_{17}$ [102-178]	PET	F: ATG GTT ATG TGG GCT CTT TAT G R: GTT CAT CCT GCC AGA TCT AAG G	48.0^{o}C	Julian and Bartron 2007
AsaD055	EF015001	$(CTAT)_{10}$ [231-279]	NED	F: CTC TTT CAC AGG GAT CAA AGT C R: CAA GCA TGT TTA AAT AGG AGG C	48.0^{o}C	Julian and Bartron 2007

Table 2. Summary table of population statistics of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), yellowfin menhaden (*B. smithi*), and finescale menhaden (*B. gunteri*) based on an initial survey of sequence data from 28 individuals for control region and cytochorme *c* oxidase subunit I (COI) and from 60 clones for 12 individuals for internal transcribed spacer 1 (ITS-1) sequence data: number of individuals (*n*), number of haplotypes (N_h), number of polymorphic sites (*S*), number of transitions (Ts), number of transversions (Tv), number of insertions or deletions (indels), haplotype diversity (*h*), mean nucleotide sequence diversity (π), and mean number of pairwise differences (*k*).

Gene Region	*n*	N_h	*S*	Ts	Tv	indels	*h* ± SE	π ± SE	*k* ± SE
Control Region	28	27	63	55	16	1	0.997 ± 0.0104	0.0326 ± 0.0166	18.984 ± 8.668
COI	28	20	44	41	8	0	0.960 ± 0.0241	0.0267 ± 0.0141	9.436 ± 4.464
ITS-1	60	45	81	54	23	14	0.979 ± 0.0110	0.0183 ± 0.0094	10.207 ± 4.726

Table 3. Summary table of population statistics of Atlantic menhaden (*Brevoortia tyrannus*) based on all cytochrome *c* oxidase subunit I (COI) sequence data by region and overall samples: 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*).

	n	N_h	*S*	Ts	Tv	*h* ± SE	π ± SE	*k* ± SE
New England	50	29	47	47	4	0.9396 ± 0.0206	0.0258 ± 0.0132	11.8376 ± 5.4481
mid-Atlantic	52	32	66	65	5	0.9563 ± 0.0163	0.0286 ± 0.0145	13.1448 ± 6.0120
Chesapeake Bay	118	50	62	62	3	0.9323 ± 0.0144	0.0267 ± 0.0134	12.2395 ± 5.5682
U.S. south Atlantic	69	44	66	66	2	0.9535 ± 0.0166	0.0295 ± 0.0149	13.5388 ± 6.1581
Overall	289	109	105	97	6	0.9523 ± 0.0062	0.0274 ± 0.0137	12.5640 ± 5.6842

Table 4. Distribution and frequency of the two major cytochorme *c* oxidase subunit I (COI) sequence clades of Atlantic menhaden (*Brevoortia tyrannus*) in the New England, mid-Atlantic, Chesapeake Bay, and U.S. south Atlantic samples).

Clade:	I	II	other	Total
New England	32 (64%)	17 (34%)	1 (2%)	50
mid-Atlantic	34 (65%)	18 (35%)	0	52
Chesapeake Bay	79 (67%)	39 (33%)	0	118
U.S. South Atlantic	42 (61%)	25 (36%) *24*	2 (3%)	69
Total	187 (65%)	99 (34%)	3 (1%)	289

Table 5. Summary statistics for microsatellite loci: (n), alleleic diversity (A), allelic richness (R_s), gene diversity (D).

Locus	Repeat motif	GenBank accession		New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic	all
						Population:		
Asa-2	$(TTC)_{13}$	AF039657	n	33	36	93	40	202
			A	7	8	10	7	13
			R_s	5.98	6.56	6.76	6.13	6.37
			D	0.622	0.717	0.68	0.647	0.667
Asa-4	$(ACC)_3(AAC)_{12}(AGC)_6$	AF039658	n	26	35	78	39	178
			A	8	11	13	11	115
			R_s	7.54	9.03	9.46	8.86	8.77
			D	0.793	0.819	0.852	0.836	0.835
Aa16	(CA)4AA(CA)3AA(CA)8	AY617110	n	29	35	92	41	197
			A	5	5	6	6	6
			R_s	4.99	5.07	4.63	4.79	4.82
			D	0.691	0.684	0.656	0.59	0.655
AsaB020	$(GAT)_{15}$	EF014990	n	22	35	98	40	195
			A	13	17	19	19	21
			R_s	13	15.55	14.56	12.68	13.38
			D	0.91	0.916	0.899	0.884	0.902
AsaC334	(GTAT)17	EF014995	n	32	29	100	39	200
			A	7	4	8	6	9
			R_s	6.28	4.93	3.76	5.51	5.29
			D	0.664	0.576	0.446	0.651	0.584
AsaD055	(CTAT)10	EF015001	n	28	25	94	38	185
			A	12	13	18	15	20
			R_s	11.72	13.98	12.61	13.69	13.43
			D	0.909	0.92	0.911	0.911	0.913

Table 6. Modifed Cavalli-Sforza chord distances (D_A; Nei *et al.*, 1983) (above diagonal) and Nei's standard genetic distance (D_{ST}; Nei, 1972) (below diagonal) for Atlantic menhaden (*Brevoortia tyrannus*) based on three microsatellite loci by region.

	New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic
New England		0.028	0.02	0.019
mid-Atlantic	0.083		0.016	0.017
Chesapeake Bay	0.061	0.052		0.012
U.S. south Atlantic	0.045	0.054	0.039	

Table 7. Estimates of pairwise Φ_{ST} (below diagonal) and respective p-values (above diagonal) between regions of Atlantic menhaden (*Brevoortia tyrannus*) based on cytochorme *c* oxidase subunit I (COI) sequence data after 10,000 permutations. Bolded *p*-values indicate significance after sequential Bonferroni correction (initial α = 0.05 / 3 = 0.017).

	New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic
New England		0.85754	0.78933	0.71993
mid-Atlantic	-0.01417		0.96307	0.72240
Chesapeake Bay	-0.01001	-0.01120		0.43431
U.S. south Atlantic	-0.01075	-0.00991	-0.00299	

Figures:
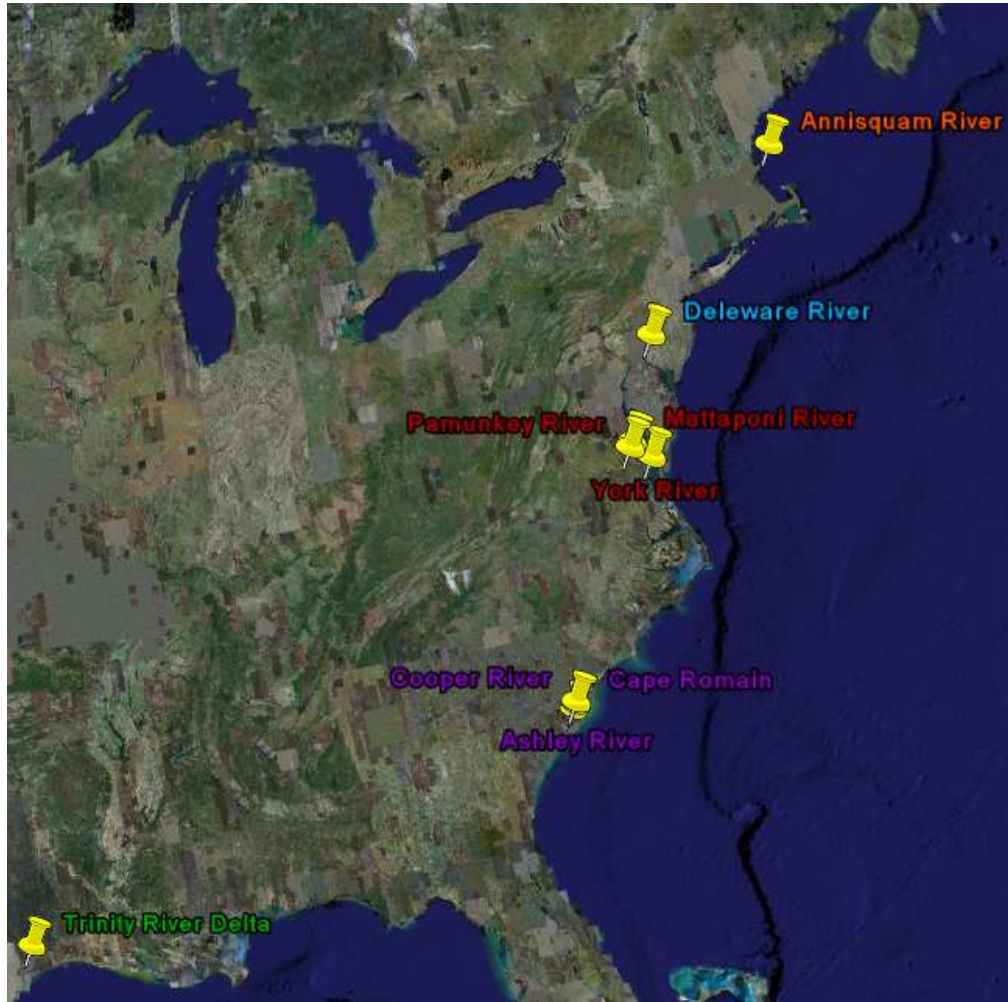
Figure 1. Map of sampling locations of Atlantic menhaden (*Brevoortia tyrannus*) in the Atlantic Ocean and gulf menhaden (*B. patronus*) in the Gulf of Mexico.
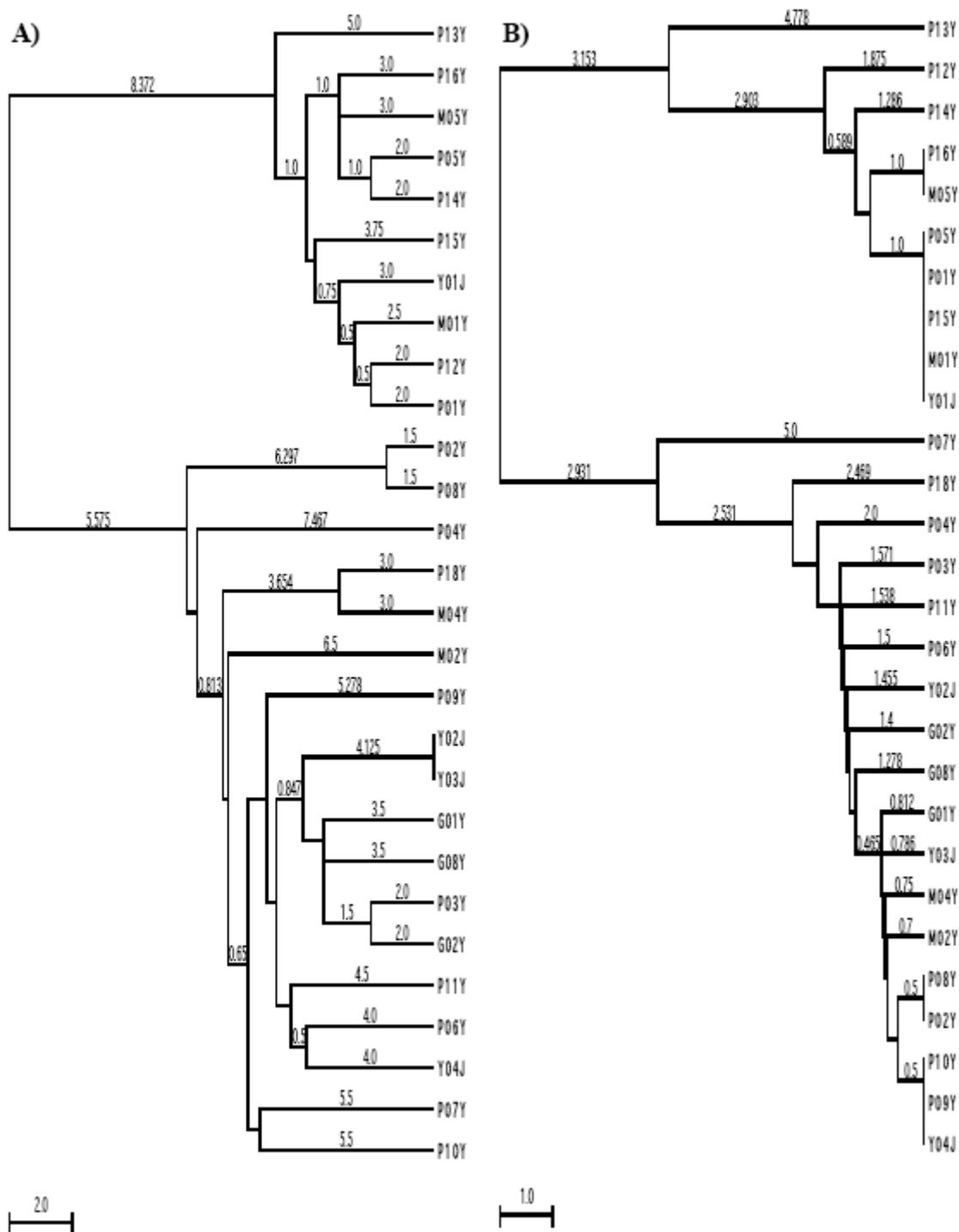
Figure 2. Unweighted pair group method with arithmetic mean (UPGMA) trees of a preliminary survey of Atlantic menhaden (*Brevoortia tyrannus*, *n*=25) and gulf menhaden (*B. patronus*, *n*=3) **A)** mitochondrial control region sequences and **B)** cytochrome *c* oxidase subunit I sequences. The samples are coded by location (M=Mattoponi, P=Pamunkey, Y=York, G=Gulf of Mexico) and age (J=yearling, Y=YOY). Divergence of clades is given as an absolute number of base pair differences.

A)

B)

C)
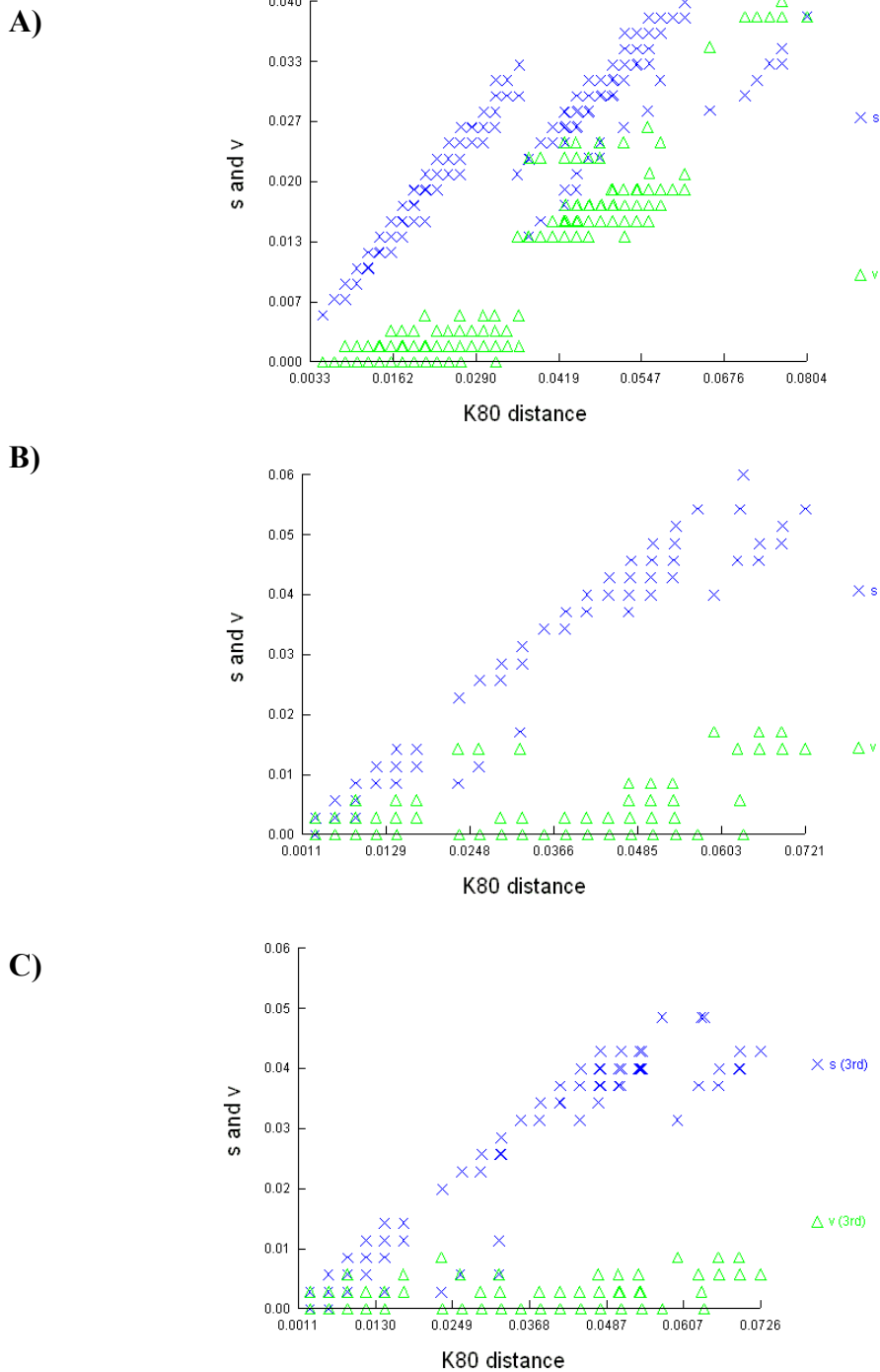
Figure 3. Nucleotide substitution patterns observed in pairwise comparisons of an initial survey of *Brevoortia* mitochondrial **A)** control region sequences overall, **B)** cytochrome *c* oxidase subunit I (COI) sequences overall, and **C)** COI third codon positions. The x-axis represents the Kimura (1980) distances between sequences; the y-axis represents the rate of transitions (blue X's) and transversions (green triangles).
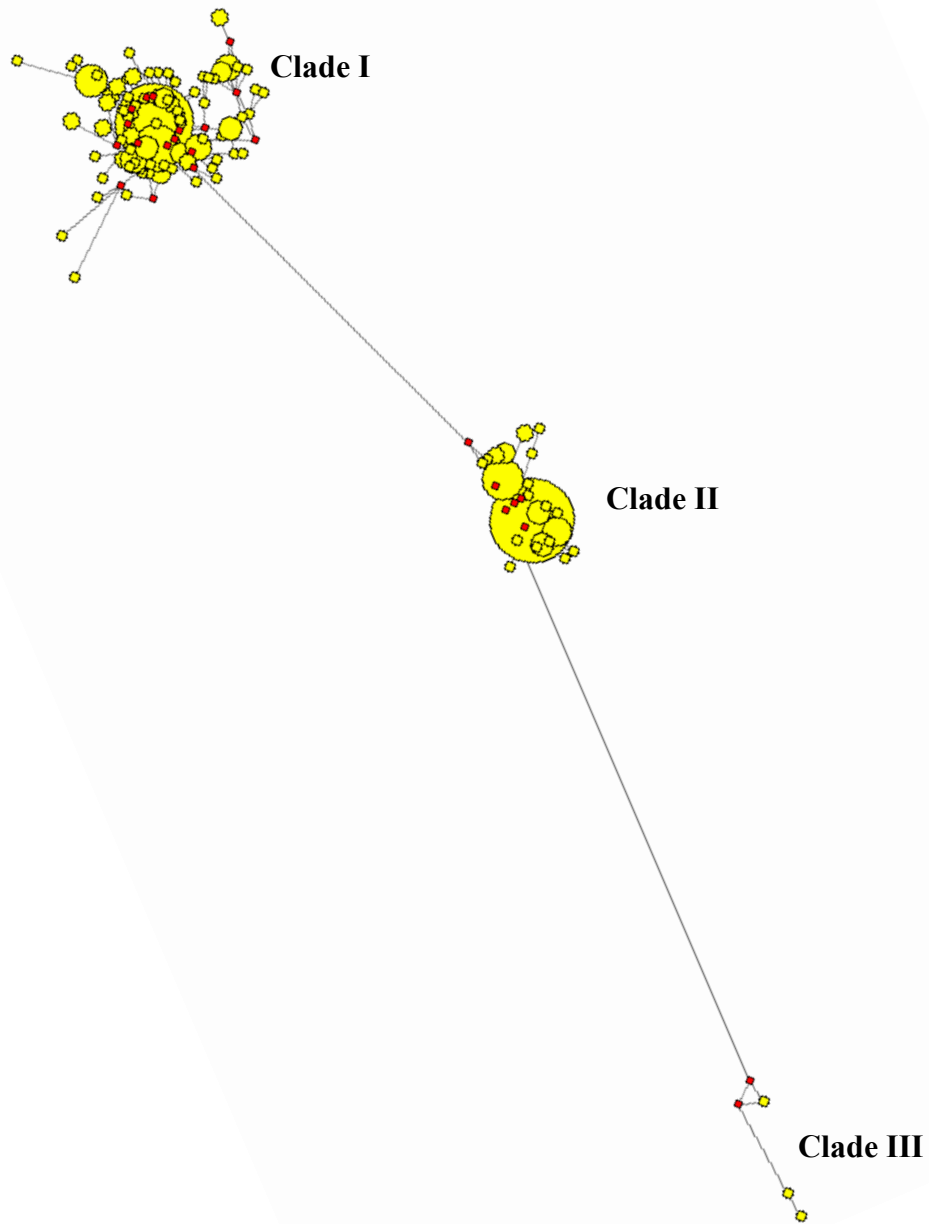
Figure 4. Median-joining network of haplotypes showing distinct cytochrome *c* oxidase subunit I (COI) clades of Atlantic menhaden (*Brevoortia tyrannus*). The observed haplotypes are displayed in yellow and sized according to frequency. The hypothesized intermediate haplotypes are displayed in red.
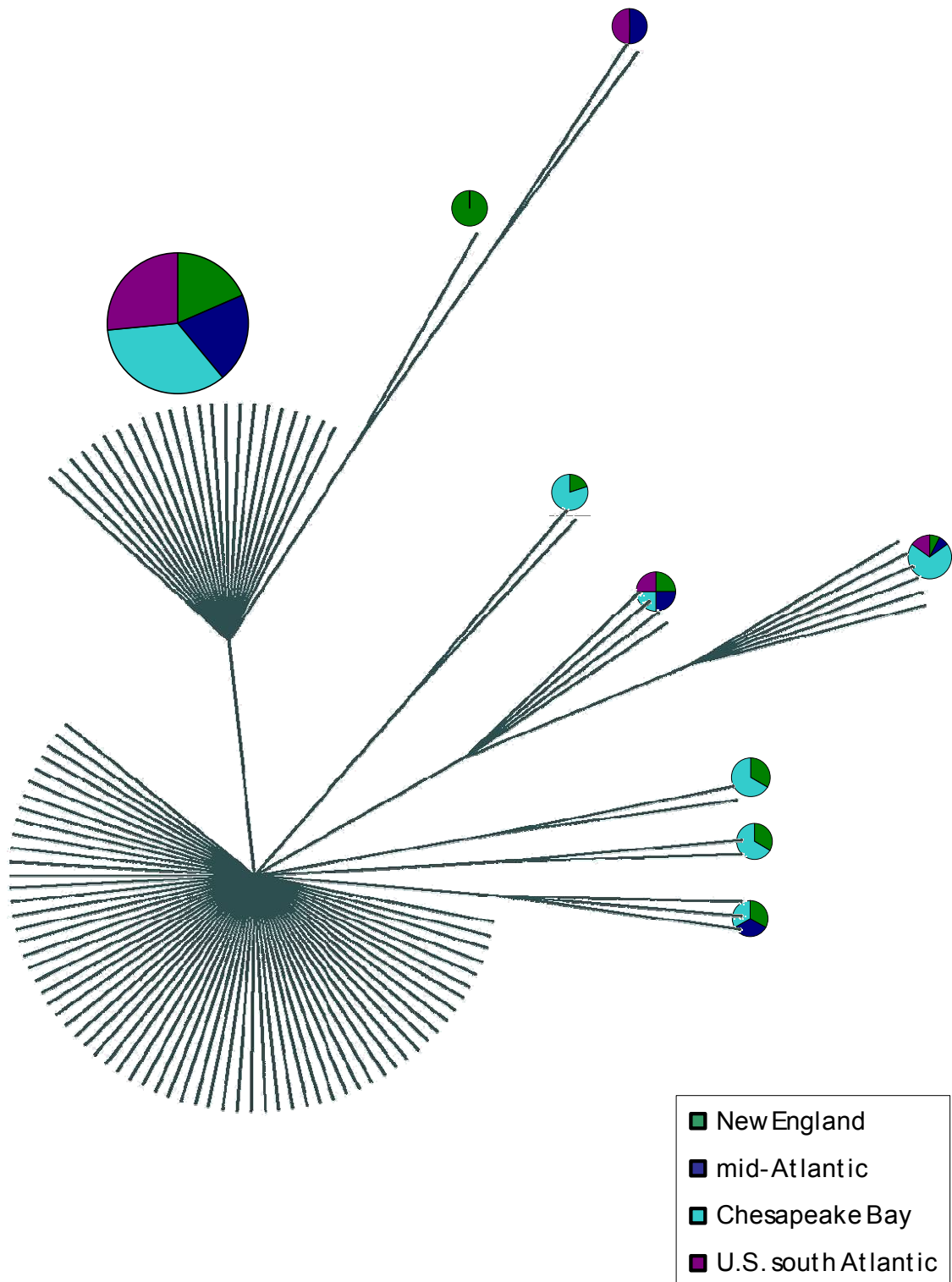
Figure 5. Neighbor-joining tree of Atlantic menhaden (*Brevoortia tyrannus*) cytochrome *c* oxidase subunit I (COI) haplotypes using maximum likelihood distances calculated by the HKY + I + Γ model. Unresolved haplotypes polytomies are coded by regional proportion.

Method: UPGMA; Bootstrap (1000 reps); tie breaking = Systematic
Distance: Absolute (# differences)
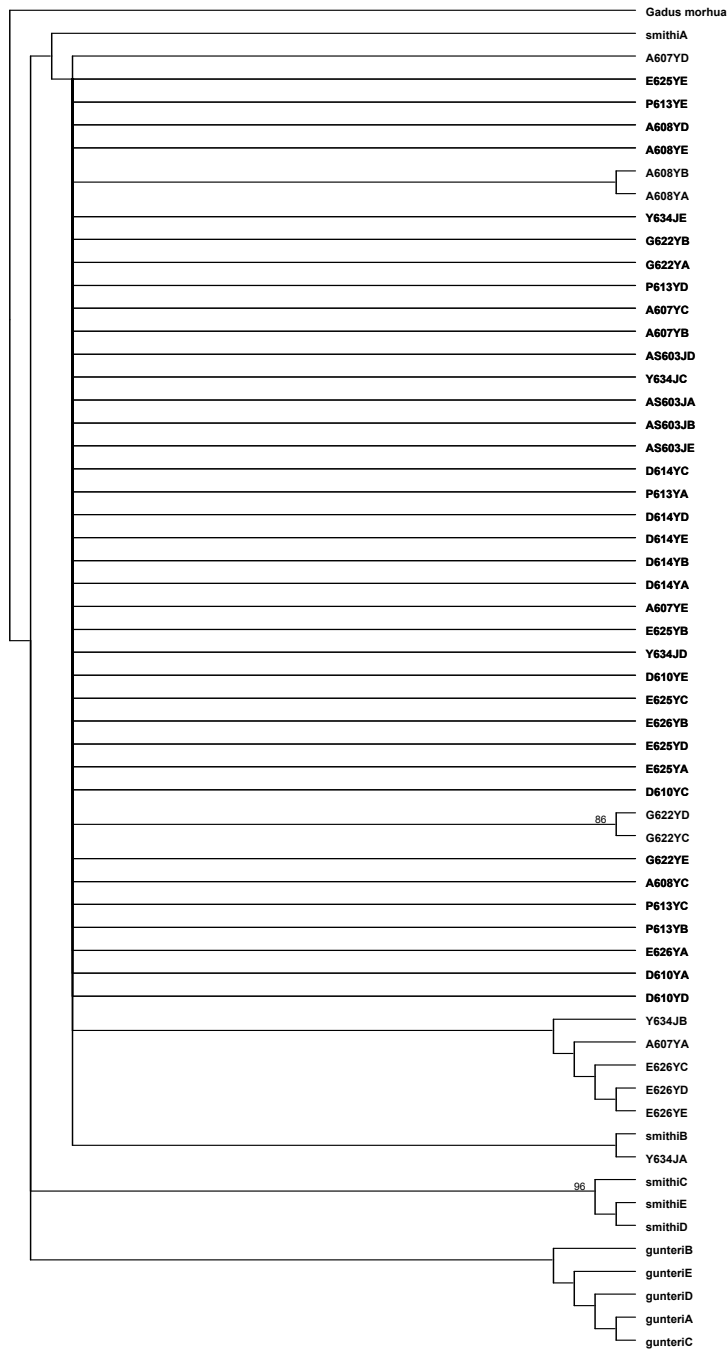Gaps distributed proportionally

Figure 6. UPGMA tree of *Brevoortia* internal transcribed spacer 1 sequences (5 clones/individual; 12 individuals). The samples are coded by location (New England = A, Mid Atlantic = D; Chesapeake Bay = P, Y; Southern Atlantic = AS, E; gulf menhaden = G; yellowfin menhaden = smi; finescale menhaden = gun), age (J = yearling, Y = YOY), and clone number (A-E). Divergence of clades is given as absolute number of base pair differences. Nodal support was assessed with 1,000 bootstrap replicates and the numbers of below each data bipartion indicate bootstrap support (if >50%).

Figure 7. Principal component analysis of Atlantic menhaden microsatellite allelic frequencies. Menhaden are grouped by sampling location and year of collection. New England 06=white, 07=bright blue; mid-Atlantic 06=pink, 07=blue; Chesapeake Bay 06=dark blue, 07=red; South Carolina 06=yellow, 07=grey.
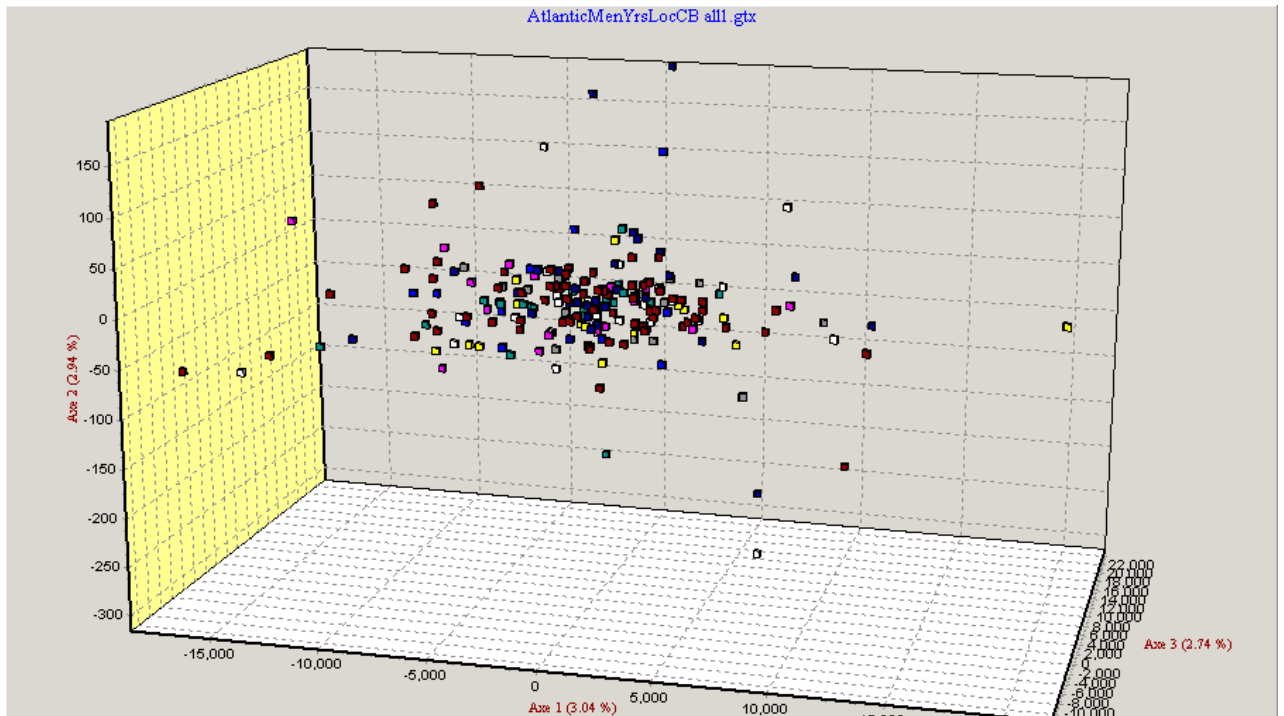
Figure 8. Principal component analysis of Atlantic menhaden microsatellite allelic frequencies. Menhaden are grouped by sampling location and by life history stage; young-of-year (YOY) or yearling. YOY: grey=Chesapeake Bay, white=mid-Atlantic. Yearling: New England=yellow, Chesapeake Bay=pink, South Carolina=blue.
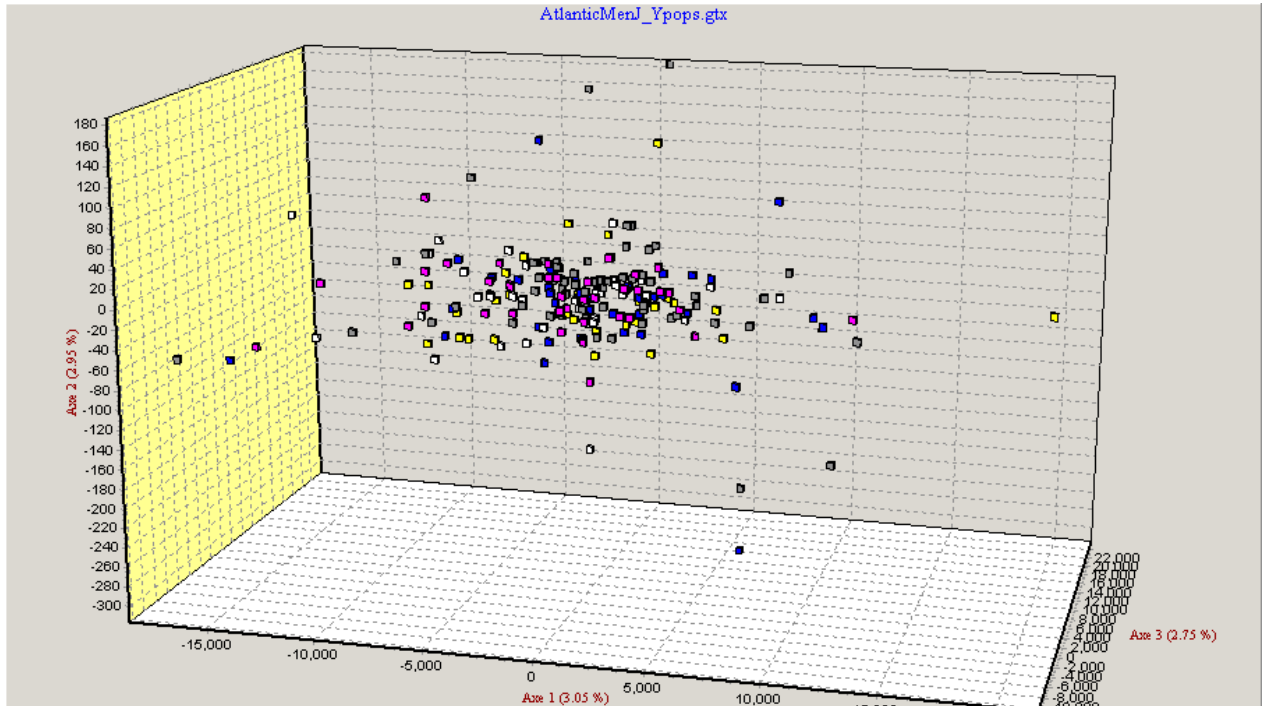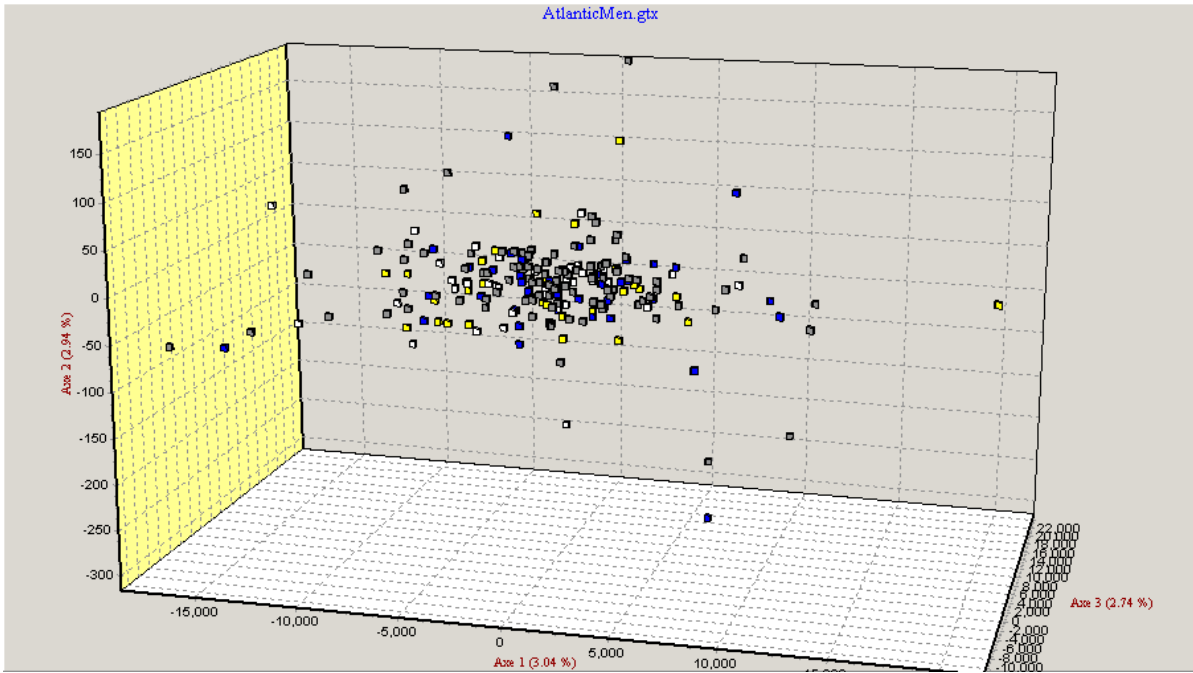
Figure 9. Principal component analysis of Atlantic menhaden microsatellite allelic frequencies. Menhaden grouped by sampling location. Yellow=New England, Blue=South Carolina, Grey=Chesapeake Bay, White=mid-Atlantic.

Appendix 1. Primer sequences, reagents, and PCR parameter optimizations used in the present study for mitochondrial and nuclear amplification of *Brevoortia* specimens.

Control Region

Pro-F: 5' CTA CCY CYA ACT CCC AAA GC 3' (K. Gray, unpublished)
Phe-R: 5' GTA AAG TCA CGA CCA AAC C 3' (K. Brendtro, unpublished)

For each 10μL PCR reaction,

       7.65μL sterile, filtered H_2O
       1μL 10X PCR Buffer plus magnesium (Qiagen)
       0.2μL 10mM dNTPs (Qiagen)
       0.05μL of each 100 μM forward and reverse primer
       0.05μL *Taq*, DNA polymerase (Qiagen)

1 cycle	94^oC initial denaturation for 4 minutes
36 cycles	94^oC denaturation for 1 minute
	54^oC annealing for 1 minute
	72^oC extension for 2 minutes
1 cycle	72^oC extension for 5 minutes
Hold	4^oC

COI

MenCOIF: 5' CTT TCG GCT ACA TGG GAA TG 3' (B. Tarbox, unpublished)
MenCOIR: 5' AGC CCT AGG AAG TGT TGT GG 3' (B. Tarbox, unpublished)

For each 10μL PCR reaction,

       7.25μL sterile, filtered H_2O
       1μL 10X PCR Buffer plus magnesium (Qiagen)
       0.2μL 10mM dNTPs (Qiagen)
       0.4μL BSA (bovine serum albumin; 1mg/mL)
       0.05μL of each 100 μM forward and reverse primer
       0.05μL *Taq*, DNA polymerase (Qiagen)

1 cycle	94^oC initial denaturation for 4 minutes
36 cycles	94^oC denaturation for 1 minute
	49.1^oC annealing for 1 minute
	72^oC extension for 2 minutes
1 cycle	72^oC extension for 5 minutes
Hold	4^oC

ITS 1

ITS-1: 5' GAG GAA GTA AAA GTC GTA ACA AGG 3' (K. Johnson, unpublished)
5.8SR1: 5' ATT CAC ATT AGT TCT CGC AGC TA 3' (K. Johnson, unpublished)

For each 10μL PCR reaction,

       7.25μL sterile, filtered H_2O
       1μL 10X PCR Buffer plus magnesium (Qiagen)
       0.2μL 10mM dNTPs (Qiagen)

0.4µL BSA (bovine serum albumin; 1mg/mL)
0.05µL of each 100 µM forward and reverse primer
0.05µL *Taq*, DNA polymerase (Qiagen)

1 cycle	94°C initial denaturation for 4 minutes
36 cycles	94°C denaturation for 1 minute
	64.5^{o}C annealing for 1 minute
	72°C extension for 2 minutes
1 cycle	72°C extension for 5 minutes
Hold	4°C

Appendix 2. PCR conditions and reagents used in the present study for microsatellite amplification of *Brevoortia* specimens. Primers and annealing temperatures specific to each locus are listed in Appendix 7.

For each 5µL PCR reaction,
2.78µL sterile, filtered H$_2$O
1 µL BSA (bovine serum albumin; 1mg/mL)
0.5µL 10X PCR Buffer without MgCl$_2$ (Invitrogen)
0.15µL 1.5mM Mg+ (Invitrogen)
0.1µL 10mM dNTPs (Qiagen)
0.01875µL 10µM T3 tailed forward primer
0.075µL 10µM reverse primer
0.05µL 10µM fluorescent label
0.025µL *Platinum Taq*, DNA polymerase (Invitrogen)

1 cycle	94°C initial denaturation for 3 minutes
36 cycles	94°C denaturation for 45 seconds
	variable ^{o}C annealing for 45 seconds
	72°C extension for 45 seconds
1 cycle	72°C extension for 7 minutes
Hold	4°C