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A Molecular Analysis of Atlantic Menhaden (Brevoortia tyrannus) Stock Structure

Abigail J. Lynch
College of William and Mary - Virginia Institute of Marine Science

A Molecular Analysis of Atlantic Menhaden (*Brevoortia tyrannus*) Stock Structure

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Master of Science

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by

Abigail J. Lynch

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of

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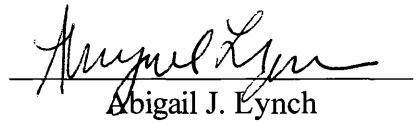

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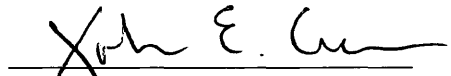
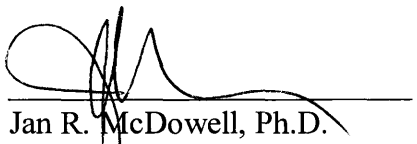


John E. Graves, Ph.D.
Committee Chairman/Advisor



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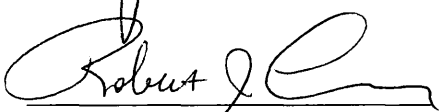
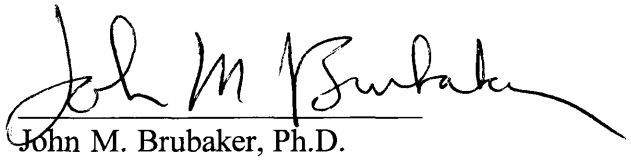


Robert J. Latour, Ph.D.



John M. Brubaker, Ph.D.

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A MOLECULAR ANALYSIS OF ATLANTIC MENHADEN (*BREVOORTIA TYRANNUS*) STOCK STRUCTURE

ABSTRACT

Atlantic menhaden 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, 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. Additionally, the recent consolidation of the fishery and localization of harvests within and around Chesapeake Bay have raised concerns over the possibility of 'localized depletion' of the species in this area. This study 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 up to 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. Two independent classes of molecular markers were surveyed: the mitochondrial cytochrome *c* oxidase subunit I (COI) gene region and seven nuclear microsatellite loci. All markers revealed considerable genetic variation. Hierarchical analyses of molecular variance (AMOVA) and examination of pairwise Φ_{ST}, F_{ST}, and R_{ST} estimates indicate a homogeneous distribution of genetic variation within Atlantic menhaden (all region AMOVAs: Φ_{ST} = -0.00873, F_{ST} = 0.00515 (F_{ST} method), F_{ST} = -0.00666 (R_{ST} method); p>0.05). The genetic connectivity between the regional collections suggests that concentrated fishing pressure in and around Chesapeake Bay will not result in a significant loss of unique genetic variation.

INTRODUCTION

Atlantic menhaden (*Brevoortia tyrannus* Latrobe 1802) is a member of the New World genus *Brevoortia* Gill 1861 (Clupeiformes: Clupeidae: Alosinae). The genus contains five planktivorous species that form large, dense schools in nearshore environments. The four North American menhadens are divided into two groups: large-scaled and small-scaled. The large-scaled group includes Atlantic menhaden, distributed along the Atlantic coast from Indian River, Florida to Nova Scotia, Canada (Whitehead 1985), and gulf menhaden (*B. patronus* Goode 1879), distributed along the Gulf of Mexico coast from Florida Bay to the Gulf of Campeche, Mexico (Whitehead 1985) (Figure 1). The small-scaled group includes 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, Mexico (Whitehead 1985) (Figure 1). Anderson (2007) used mitochondrial DNA sequence and nuclear microsatellite data to validate that large-scaled and small-scaled menhadens comprise two distinct evolutionary lineages.

Identification of Atlantic menhaden can be particularly difficult. They are sympatric over part of their range with yellowfin menhaden. But, 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). Atlantic menhaden are also morphologically similar to gulf menhaden, although their ranges are not believed to overlap (Bigelow *et al.* 1963). 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).

Of the North American *Brevoortia*, Atlantic menhaden undertake the longest coastal migrations and have the most temporally and geographically protracted spawning season (Whitehead 1985). 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). The migration distance 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 (Checkley *et al.* 1988).

Due in part to their spatially and temporally protracted spawning season, 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 Atlantic coast estuaries within this range serve as nursery grounds for larval and juvenile Atlantic menhaden. Larvae feed within six days of fertilization and enter the estuaries and metamorphose after 30 to 90 days (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"

While cultural historian H. Bruce Franklin's (2007) naming of Atlantic menhaden as "the most important fish in the sea" is a gross hyperbole, Atlantic menhaden do have 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 generate $236 million in annual revenue (Southwick Associates and Loftus 2006). Additionally, Atlantic menhaden 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 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 and nearby waters. 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), although the actual removals from the Bay have decreased by 28% over the same period (ASMFC 2005). The 2006 total landings continue the downward trend at 13% less than the previous five year average (ASMFC 2007). Nonetheless, Atlantic menhaden account for 6% of all U.S. commercial fishery landings (by weight), making the fishery the fifth largest (by weight) in the country (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 far surpasses the value of the traditional reduction 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 type, Atlantic menhaden may regulate water quality. As filter-feeders, Atlantic menhaden remove particulates (plankton and detritus) from the water column, which may enhance water clarity and mitigate problems associated with eutrophication (Kemp *et al.* 2005). Durbin and Durbin (1975) suggested

that large schools of adult Atlantic menhaden can significantly impact local phytoplankton and zooplankton concentrations. This impact, however, may not be eutrophication mitigation. Lynch *et al.* (in review) evaluated the age-specific ingestion rates of Atlantic menhaden and found that age-1+ menhaden consumed zooplankton and exhibited no ingestion of phytoplankton. These findings suggest that older menhaden may, in fact, enhance phytoplankton production (and by proxy eutrophication) by relaxing grazing pressure.

Atlantic Menhaden Stock Structure Analyses

Though the extensive seasonal migrations of Atlantic menhaden might suggest that the species comprises 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 independently to fishing pressure (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 as few as one and as many as three different stocks, principally on the basis of meristics and morphometrics (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 the result of phenotypic plasticity due to environmental factors (i.e., water temperature at spawning time) than heritable characters. Epperly (1989) found allele frequency differences at the transferrin locus 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 of Atlantic menhaden, 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 that the Atlantic menhaden resource be

considered a single 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 regards Atlantic menhaden as one coastwide stock, although the transferrin 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 describe genetic stocks in clupeiforms. Allozymes are polymorphic proteins, the alleles of which can be separated primarily by charge using gel electrophoresis. Allozyme analysis identified three stocks of northern anchovy (*Engraulis mordax*) and differentiated Pacific herring (*Clupea pallasi*) from the Bering Sea and the eastern North Pacific (Vrooman *et al.* 1981, Grant and Utter 1984). Allozyme analysis described panmictic populations of Atlantic herring (*C. harengus*), cape anchovy (*E. capensis*), and bay anchovy (*Anchoa mitchilli*) (Grant 1984, Jørstad *et al.* 1991, Grant 1985, Morgan *et al.* 1995, respectively). The discriminatory power of allozymes, however, is limited because the evolutionary rate of the amino acid sequences of most proteins is very low (many changes at the DNA level

do not result in different amino acids) and not all amino acid substitutions result in charge changes.

Analysis of DNA, which investigates changes at the nucleotide level, provides a higher level of genetic resolution than analysis of allozymes. While proteins are usually under intense selective pressure, many regions of DNA are neutral and can mutate with little functional consequence. Originally, restriction fragment length polymorphism (RFLP) analyses were used to study DNA variation. In RFLP analysis, restriction enzymes are used to cut the DNA strand at specific restriction sites, generating fragments that are separated electrophoretically. Individuals with different RFLP patterns have different genetic sequences. RFLP studies can be conducted on the entire mitochondrial genome or an amplified mitochondrial gene region. 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 entire 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 investigation of population structure in clupeids because of their rapid evolutionary rates. Microsatellites, also known as variable number tandem repeats, are nuclear loci consisting of short

sequence repeats (usually 2-5 nucleotides) which are generally assumed to be selectively neutral. Alleles with different repeat numbers are created 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 the template DNA strand. As a result, copies of the repeat are either added to or deleted from the new strand of DNA. The fast rate of mutation seen in microsatellites can lead to homoplasy, so they are best suited for intraspecific studies.

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 Sea 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 also 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 microsatellite primers show interspecific 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, high resolution perspective that complements nuclear microsatellite loci for analysis of population structure. The mitochondrial genome, with its lack of recombination, maternal inheritance, and high evolutionary rates, 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 to be the most conserved in fishes (Meyer 1993). COI codes for the final electron acceptor protein in the electron transport chain of cellular respiration and is under intense selective pressure. In fact, COI is emerging as the standard genetic region to sequence in the Barcode of Life project as a species-specific diagnostic tool for many taxa (Ratnasingham and Hebert 2007). Yu *et al.* (2005), surveying 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 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 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 large-scaled sequences to species (Atlantic and gulf menhaden) using this gene region. García *et al.* (2008), similarly, found evidence of the existence of only one species of menhaden, Brazilian menhaden (*Brevoortia aurea*), and no support for a second purported species, Argentine menhaden (*B. pectinata*), in the southwestern Atlantic using cytochrome *b* sequences.

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 genes do not evolve at a rate fast enough to make them useful for intraspecific comparisons, non-coding nuclear gene regions, such as internal transcribed spacer 1 (ITS-1), have fewer selective constraints and a faster evolutionary rate which can be used to investigate population structure (Avise *et al.* 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, Brendtro *et al.* (2008) 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.

PROJECT OBJECTIVES

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 and around Chesapeake Bay, it is important to understand the genetic stock structure of the species. If Atlantic menhaden exhibit significant, genetically-based stock structure, there will be spatial partitioning of unique genetic variation, and regional fishing pressure could lead to the loss of unique genetic variation. Depletion of unique genetic variation may reduce a stock's ability to respond to shifting environmental pressures because a portion of the genetic differentiation among fish populations is considered adaptive (Higgins and Lynch 2001, Ryman 1981).

In this study, analysis of sequence data from the mitochondrial COI gene region and allele frequencies of seven nuclear microsatellite loci were used 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. Young-of-the-year (YOY) and yearling (age-1) fish were sampled 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). Gulf menhaden, finescale menhaden, and yellowfin menhaden were sampled as outgroups. The following null hypotheses were 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 in 2006 and 2007).

$H_{0,3}$: 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.

*$H_{0,5}$: There is no genetic difference among large-scaled menhaden (*B. tyrannus *and* B. patronus*) from five geographic regions along the U.S. Atlantic coast and Gulf of Mexico.*

MATERIALS AND METHODS

Sample Collection

YOY and yearling Atlantic menhaden were sampled from throughout the species's range in 2006 and 2007. YOY gulf menhaden were sampled from the Gulf of Mexico in 2006 and 2007 as an outgroup. Collections were grouped into five broad geographic regions: New England (Massachusetts), mid-Atlantic (New Jersey), Chesapeake Bay (Virginia), U.S. south Atlantic (South Carolina), and Gulf of Mexico (gulf, yellowfin, and finescale menhaden) (Figure 1). All menhaden were identified on the basis of morphological characters and capture location by local experts (New England: Gary Nelson, Massachusetts Division of Marine Fisheries; mid-Atlantic: Heather Corbett, New Jersey Division of Fish and 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; gulf menhaden: William Dailey, Texas A&M University; yellowfin and finescale menhaden: Joel Anderson, Texas Parks and Wildlife Department) (Appendix 1). Voucher specimens were retained from all U.S. Atlantic coast regions in 2007 to corroborate field identifications. 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 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 if sample sizes are not sufficiently large. In these cases, it can be difficult to get precise estimates of genetic relationships because of an incomplete representation of the total genetic variation. 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 evaluated at the nuclear internal transcribed spacer 1 (ITS-1) and 36 nuclear microsatellite loci were screened for variation within Atlantic menhaden. From the outcome of these preliminary surveys, COI and seven microsatellites were selected to characterize all samples.

Extraction and Amplification

Total genomic DNA was extracted from each tissue sample using a Qiagen DNeasy® Tissue Kit (Qiagen, Valencia, C.A.) following the manufacturer's protocol. The mitochondrial control region, COI, ITS-1, and eight microsatellite loci (Asa2, Asa4, Asa16, Brown *et al.* 2000; Aa16, Faria *et al.* 2004; AsaB020, AsaC334, AsaD055, Julian and Bartron 2007; SarBH04, Pereyra *et al.* 2004), were amplified using the polymerase chain reaction (PCR) (Table 1, Appendices 2, 3).

Ten μL sequencing reactions were amplified by PCR. Five μL of each PCR amplification product were 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 for sequencing 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, ITS-1 PCR products were cloned to separate the two alleles before sequencing. One μL of the nuclear product was cloned into a plasmid vector using the TOPO-TA plasmid cloning system (Invitrogen Corporation, Carlsbad, C.A.) 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 TOP10F' *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 amplified in a 10μL reaction using M13 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 product 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 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, Madison, W.I.) prior to sequencing. PCR products were prepared for sequencing using the ABI PRISM® Big Dye^{TM} Terminator v 3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, C.A.) at a 1:8 dilution of the manufacturer's protocol and subsequently sequenced on an 80cm capillary ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems). Samples were sequenced in the forward and reverse direction and 20% of samples were re-analyzed from DNA extraction through sequencing.

The chromatographic curves for each 80cm capillary 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., Ann Arbor, M.I.), and aligned using the ClustalW algorithm (Thompson *et al.* 1994) for multiple alignments in MacVector 9.0.1 (MacVector Inc., Cary, N.C.).

Microsatellite Analysis

Microsatellite loci were PCR amplified with locus-specific fluorescent labels. Following amplification, 1μL of PCR product for each locus was combined with PCR products from three other unique locus/fluorescent label combinations (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 sequencing on a 36cm capillary ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol.

The chromatic peaks for each microsatellite sequence were scored using GeneMarker AFLP/Genotyping Software v 1.60 (SoftGenetics, State College, P.A.). 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 samples were re-analyzed from DNA extraction through allele scoring.

Descriptive Statistics

Once aligned, the mitochondrial sequences were characterized in Arlequin 3.11 (Excoffier *et al.* 2005) to determine the following: number of haplotypes (N_h); number of polymorphic sites (*S*), variable base pair (bp) locations within a sequence set; number of transitions (Ts), point mutations from either a purine to a purine (A↔G) or a pyrimidine to a pyrimidine (C↔T); number of transversions (Tv), point mutations from a purine to a pyrimidine (A or G→C or T) or a pyrimidine to a purine (C or T→A or G); and number of indels (insertions to or deletions from a sequence). The amount of sequence variation for each mitochondrial gene region within each collection was estimated in Arlequin 3.11

(Excoffier *et al.* 2005) using the following diversity indices: haplotype diversity (h, the probability that two randomly chosen haplotypes [gene sequences] 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). Net nucleotide sequence divergence (δ, number of net nucleotide substitutions per site between populations) was calculated in MEGA 4.1 (Tamura *et al.* 2007) using the Kimura two-parameter model (Kimura 1981). For the microsatellite data, Genepop 3.4 (Raymond and Rousset 1995) was used to determine observed heterozygosity (H_O) and expected heterozygosity (H_E); Arlequin 3.11 (Excoffier *et al.* 2005) was used to determine allelic diversity (A; number of alleles per locus), and Microsatellite Analyzer (MSA) (Dieringer *et al.* 2003) was used to determine the allele size range (a.s.r.). The amount of microsatellite variation within each collection was estimated in FSTAT 2.9.3.2 (Goudet 1995) using the following indices: allelic richness (R_s; number of alleles per locus, corrected for sample size) and gene diversity (D; heterozygosity). To determine if the distribution of microsatellite genotypes conformed to the expectations of Hardy-Weinberg equilibrium for each locus at each collection location, three separate tests (probability, heterozygote deficiency, and homozygote deficiency) following the methods of Guo and Thompson (1992) are available in Genepop 3.4 (Raymond and Rousset 1995) and can be adjusted for multiple testing using sequential Bonferroni correction (Rice 1989). All three tests share the same null hypothesis (random distribution of alleles) and differ in their alternative hypotheses. The probability test is a two-tailed test with the p-value corresponding to the sum of random allelic counts with the same or lower probability. If the null hypothesis

for the probability test was rejected, more powerful one-tailed heterozygote excess and deficiency tests (in which the *p*-value considers only one end of the distribution) were performed.

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.). To investigate the possibility of mutational saturation, graphs of nucleotide substitution vs. genetic distance (overall and by third codon position) were constructed for mitochondrial control region and COI sequences in DAMBE (Xia and Xie 2001).

Phylogenetic trees are used describe the genetic relationships among species. A maximum parsimony tree was constructed for all mitochondrial COI sequences and nodal support was assessed with bootstrapping resampling (Felsenstein 1985) of 100 pseudoreplicates, 10 random addition sequences, with TBR branch swapping algorithm. The most appropriate nucleotide substitution model for the region was determined from a series of 56 likelihood ratio tests executed in ModelTest 3.7 (Posada and Crandall 1998). The accumulation of nucleotide substitutions 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 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 selected model was used in PAUP* 4.0 (Swofford 2000) to produce maximum likelihood and Bayesian analysis trees (Mr. Bayes; Huelsenbeck and Ronquist 2001). Support of the maximum likelihood internal branches was tested using bootstrap resampling (Felsenstein 1985) of 10 pseudoreplicates, 10 random addition sequences, with TBR branch swapping algorithm.

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 (no longer extant) between sampled taxa. With a network, mutations generally occur from the most common, interior haplotypes (still extant). Rarer haplotypes, as a result, are more likely to be related to these common haplotypes than to each other. Median-joining networks for COI and control region haplotypes were drawn in Fluxus 4.2.0.1 (Bandelt *et al.* 1999) to investigate population-level relationships.

Genetic relationships among collection locations based on microsatellite loci data were represented spatially in Genetix 4.04 (Belkhir *et al.* 2000) and estimated by constructing neighbor-joining trees using modified Cavalli-Sforza chord distances (D_A; Nei *et al.* 1983) and Nei's standard genetic distances (D_{ST}; Nei 1972) calculated 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 (Takezaki and Nei 1996).

Population Structure

A hierarchical analysis of molecular variance (AMOVA) was used to test for population structure using mitochondrial COI sequencing data and nuclear microsatellite loci data. The AMOVA partitions genetic variance into covariant components at predefined levels (individual, sub-region, and region). The fixation indices, Φ_{ST} for haplotypes and F_{ST} or R_{ST} for microsatellites, 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} (Weir and Cockerham 1984), based on the distribution of number of different alleles (not considering relationships among alleles), and R_{ST} (Slatkin 1995), based on the distribution and relationship of alleles (sum of squared allele size differences). The R_{ST} algorithm is generally considered only appropriate as a measure for microsatellite data when sample sizes are equal or larger than 50 (Ruzzante 1998). 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 (eg., 2007 YOY menhaden in Chesapeake Bay), between years within an age class at a location (eg., 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), among Atlantic coast regions (eg., New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic), among large-scaled regions (eg., New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, Gulf of Mexico), and between putative species (eg., Atlantic and gulf menhaden). Estimates of population pairwise Φ_{ST} and F_{ST}/R_{ST} were calculated in Arlequin 3.11 (Excoffier *et al.* 2005) as a

measure of genetic distance between individual groupings and adjusted for multiple tests using sequential Bonferroni correction (Rice 1989).

RESULTS

Evaluation of Mitochondrial Gene Region Variability

To determine which mitochondrial gene region would be appropriate to evaluate Atlantic menhaden population structure, a 535bp fragment of the mitochondrial control region and a 459bp fragment of cytochrome *c* oxidase subunit I (COI) were sequenced for 28 individuals in a preliminary study. 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 0.997, 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 0.960, and a mean nucleotide sequence diversity (π) of 0.0267. The haplotype diversity estimate for COI was high, but not as elevated as the control region estimate. Both gene regions revealed similar patterns of intraspecific relationships (Figure 2).

Mutational saturation curves lend some support to the hypothesis that the control region may be at risk of homoplasy. The rate of transitions appears to plateau and the rate of transversions approaches that of transitions for the control region data but the evidence is less substantial for COI (Figure 3). Based on this qualitative preliminary analysis, COI was chosen as the mitochondrial marker for this study and all samples were surveyed for this gene region.

Cytochrome c *Oxidase Subunit 1*

The COI fragment was sequenced for 339 individuals (Atlantic menhaden, n = 289, gulf menhaden, n = 50). The fragment contained 99 polymorphic sites overall (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 3). Haplotype diversity (h) estimates for the Atlantic menhaden sampling locations ranged from 0.932 to 0.956, with an overall (pooled) estimate of 0.941. Mean nucleotide sequence diversity (π) estimates for Atlantic menhaden sampling locations ranged from 0.0258 to 0.0295, with an overall (pooled) estimate of 0.0274. The mean number of pairwise differences (k) ranged from 11.8 to 13.5, with an overall (pooled) estimate of 12.6.

The median-joining network for the 109 COI Atlantic menhaden haplotypes showed two extensive star-shaped clusters (clades) separated by 17 nucleotide changes with one minor exterior grouping of three anomalous samples separated by 24 nucleotides (Figure 4). A contingency table of Atlantic coast sampling locations indicated that the two primary clades were equally represented along the U.S. Atlantic coast ($x^2 = 0.478$; $x^2_{0.05,3} = 7.815$; $p>0.05$; Table 4).

For the combined large-scaled menhadens, haplotype diversity (h) estimates ranged from 0.879 to 0.956, with an overall (pooled) estimate of 0.940. Mean nucleotide sequence diversity (π) estimates for Atlantic and gulf menhaden sampling locations ranged from 0.0071 to 0.0295, with an overall (pooled) estimate of 0.0258. The mean

number of pairwise differences (k) ranged from 3.24 to 13.5, with an overall (pooled) estimate of 11.8.

The median-joining network for all 124 Atlantic and gulf COI haplotypes showed the same star-shaped phylogeny (Figure 5). Clade I, the "ubiquitous large-scaled" clade, was composed of all gulf menhaden samples and 64% of Atlantic menhaden samples. Clade II, the "Atlantic-only" clade, comprised 35% of Atlantic menhaden samples. Clade III, the anomalous samples, comprised the remaining 1% of Atlantic menhaden.

Adding samples from the small-scaled menhadens, the COI fragment was sequenced for a total of 389 individuals (Atlantic menhaden, $n = 289$, gulf menhaden, $n = 50$, yellowfin menhaden, $n = 25$, finescale menhaden, $n = 25$). The fragment contained 105 polymorphic sites, 107 transitions, 7 transversions, and produced 145 haplotypes (Table 3). Haplotype diversity (h) estimates for the sampling locations across all four species ranged from 0.879 to 0.956, with an overall (pooled) estimate of 0.952. The overall (pooled) estimate of mean nucleotide sequence diversity (π) estimates across all species was 0.033. The overall (pooled) estimate of mean number of pairwise differences (k) was 15.2.

The median-joining network for all 145 COI haplotypes showed three extensive star-shaped clusters (clades) separated by 15 and 22 nucleotide changes, respectively (Figure 6). Clade I, the "ubiquitous large-scaled" clade was composed of all gulf menhaden samples, 64% of the Atlantic menhaden samples, and 12% of the yellowfin menhaden samples. Clade II, the "Atlantic-only" clade, was composed of 35% of the Atlantic menhaden samples, 16% of the yellowfin menhaden samples, and 16% of the finescale menhaden samples. Clade III, the "small-scaled" clade, was composed of 1%

of the Atlantic menhaden samples (the anomalous samples), 72% of the yellowfin menhaden samples, and 84% of the finescale menhaden samples. The three anomalous Atlantic menhaden were further sequenced for the mitochondrial control region to test for possible misidentification. The control region sequences for these individuals were included in a network with North American *Brevoortia* control region sequences from Anderson (2007). The median-joining network of 214 haplotypes showed the same three extensive star-shaped clusters as COI in this study: the "ubiquitous large-scaled" clade, the "Atlantic-only" clade, and the "small-scaled" clade (Figure 7). However, using control region, the anomalous sequences clustered with the large-scaled clades.

To assess the evolutionary relationships among all 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. Both maximum likelihood and Bayesian trees, using the HKY+I+Γ model, produced geographically unresolved structures (Figure 8, 9). Likewise, a maximum parsimony representation of the COI haplotypes produced no discernable structure among the species (Figure 10).

Internal Transcribed Spacer 1

The two genetically divergent clades of Atlantic menhaden 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 and subsequent mixing. 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 the phylogeographic structure inferred from analysis of COI sequences. Sixty clones from 12 individuals, representative of both mitochondrial clades, were sequenced. Of the 60 sequences, there were 45 haplotypes resulting in a haplotype diversity (h) of 0.979, nucleotide sequence diversity (π) of 0.0183, and a mean pairwise difference (k) of 10.2 nucleotides (Table 2). The median-joining network of the cloned nuclear ITS-1 sequences differed considerably from the distinct two clade mitochondrial structure and revealed no discernable structure among the putative species (Figure 11).

Microsatellites

Eight microsatellite loci, Aa16, Asa2, Asa4, Asa16, AsaB020, AsaD055, AsaC334, SarBH04, were amplified for the entire dataset (Atlantic menhaden, n = 289, gulf menhaden, n = 50, yellowfin menhaden, n = 25, finescale menhaden, n = 25). For the eight loci, sample allelic diversity (A) ranged from 1 to 21 alleles; the allelic richness (R_s) ranged from 1.00 to 15.1; and the gene diversity (D, heterozygosity) ranged from 0.000 to 0.938 (Table 5). Allele size ranges were similar for *B. tyrannus* and *B. patronus* (Figure 12). The genotypic distribution of all loci, except Asa16, conformed to the

expectations of Hardy-Weinberg equilibrium. The locus Asa16, produced a statistically significant result in the probability test and subsequently the heterozygote deficiency test, suggesting the presence of a null allele (a mutation in a primer binding site that results in non-amplification of an allele) (Table 6). The Microchecker analysis which examined the potential for scoring errors, large allele dropouts, and null alleles also indicated a heterozygote deficiency for Asa16. This locus was not included in any of the population structure analyses.

The genetic distance between sampling regions was estimated using two different methods. Nei's standard genetic distances (D_{ST}; Nei 1972) ranged from -0.0066 between the U.S. south Atlantic and New England to 0.844 between *B. smithi* and *B. patronus* (Table 7). Modified Cavalli-Sforza chord distances (D_A; Nei *et al.* 1983) ranged from 0.152 between the U.S. south Atlantic and Chesapeake Bay to 0.714 between *B. smithi* and *B. patronus* (Table 7). The neighbor-joining trees based on Nei's standard genetic distance (D_{ST}; Nei 1972) and modified Cavalli-Sforza chord distance (D_A; Nei *et al.* 1983) of microsatellite data separate the four North American menhaden species but do not show evidence of population structure within Atlantic menhaden (Figure 13, 14, 15).

Analysis of Molecular Variance

The AMOVAs of COI haplotype data and microsatellite genotype data were performed to evaluate the temporal and spatial partitioning of genetic variation within Atlantic menhaden. The mitochondrial (Φ_{ST}) AMOVA between early and late recruiting 2007 YOY in Chesapeake Bay attributed -0.62% ($p = 0.69$) of the variance to differences in recruitment time (Table 8). The microsatellite (F_{ST} and R_{ST}) AMOVAs attributed

-0.41% (p = 0.743) and 1.27% (p = 0.246) of the variance, respectively, to differences in recruitment early or late in the season (Tables 9, 10).

The mitochondrial (Φ_{ST}) AMOVA between 2006 YOY and 2007 yearling menhaden collected in Chesapeake Bay attributed -2.95% (p = 1.00) of the variance in the 2006 year class to sampling in successive years (Table 8). The microsatellite (R_{ST}) AMOVA attributed -0.828% (p = 0.730) of the variance to sampling in successive years (Table 10). The microsatellite (F_{ST}) AMOVA, on the other hand, produced significant results, attributing 1.80% (p = 0.0176) of the variance to differences within a cohort in Chesapeake Bay in successive years (Table 9).

The mitochondrial (Φ_{ST}) AMOVA between YOY and yearling menhaden collected in Chesapeake Bay in 2006 and also between YOY and yearling menhaden collected in Chesapeake Bay in 2007 attributed -0.77% (p = 0.96) and -0.66% (p = 0.97) of the variance, respectively, to differences between cohorts (Table 8). The microsatellite (F_{ST} and R_{ST}) AMOVAs attributed 1.06% (p = 0.0929), and -0.923% (p = 0.678) of the variance, respectively, between YOY and yearling menhaden in Chesapeake Bay in 2006. Likewise, the (F_{ST} and R_{ST}) AMOVAs attributed 0.0052% (p = 0.160), and 0.389% (p = 0.580) of the variance, respectively, between YOY and yearling menhaden collected in Chesapeake Bay in 2007 (Tables 9, 10).

The mitochondrial (Φ_{ST}) AMOVA among samples of YOY and yearling (combined) menhaden from four geographic regions along the U.S. Atlantic coast (New England, mid-Atlantic, Chesapeake Bay, and U.S. south Atlantic) attributed -0.87% (p = 1.00) of the variance to sampling location (Table 8). The R_{ST} AMOVA attributed

-0.0665% ($p = 0.634$) of variation due to sampling location (Table 10). The microsatellite F_{ST} AMOVA, however, produced significant results, attributing 0.575% ($p = 0.000$) of variation to sampling location (Table 9). An examination of alternate groupings (northern and southern sampling locations) with the F_{ST} AMOVA also produced significant results (0.00535%, $p = 0.00098$). No (F_{ST} and R_{ST}) pairwise comparisons, however, revealed statistically significant variation between any two sampling regions of Atlantic menhaden after sequential Bonferroni correction (Tables 11, 12).

The mitochondrial (Φ_{ST}) AMOVA among samples of YOY and yearling (combined) large-scaled menhaden from five geographic regions along the U.S. Atlantic coast and Gulf of Mexico (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and Gulf of Mexico) revealed significant partitioning of genetic variation, attributing 6.01% ($p = 0.000$) of the variance to sampling location (Table 8). The microsatellite (F_{ST} and R_{ST}) AMOVAs, likewise, revealed significant partitioning of genetic variation, attributing 4.68% ($p = 0.000$) and 16.89% ($p = 0.000$) of variation, respectively, to sampling location (Tables 9, 10). Additionally, (Φ_{ST}, F_{ST}, and R_{ST}) pairwise comparisons revealed statistically significant variation between the gulf menhaden sampling region and all other sampled regions (Tables 11, 12, 13).

DISCUSSION

Molecular Markers

The mitochondrial and nuclear markers employed in stock structure analyses need to be variable enough to detect spatial and temporal partitioning of genetic variation, if it exists (Hellberg *et al.* 2002). This study utilized the mitochondrial COI gene region and seven nuclear microsatellite loci, all of which revealed high levels of variation.

Previous analyses of COI sequences have demonstrated that it is typically one of the most conserved mitochondrial gene regions, exhibiting low levels of intraspecific variation, and it is often used for phylogenetic analyses (Saccone *et al.* 1999; Meyer 1993). However, in Atlantic menhaden, COI exhibited a high level of intraspecific variation. The COI variation noted in this study ($h = 0.941$) is the highest documented intraspecific COI haplotype diversity in vertebrates, indicating its potential utility for population level analysis in this species (high literature values include $h = 0.598$: giant otter, Garcia *et al.* 2007; $h = 0.547$: green and golden bell frog, Burnes *et al.* 2006). The within-species genetic diversity for Atlantic menhaden ($\pi = 2.74\%$) is an order of magnitude higher than the average within-species divergence reported for Australian marine fishes ($\pi = 0.39\%$, Ward *et al.* 2005), South China Sea fishes ($\pi = 0.319\%$, Zhang *et al.* 2007), Canadian freshwater fishes ($\pi = 0.302\%$, Hubert *et al.* 2008) (all values calculated using the Kimura-two-parameter model of nucleotide substitution). Additionally, all of the nucleotide substitutions within the entire dataset were synonymous, resulting in identical amino acid sequences that presumably maintain identical protein function.

Nuclear microsatellite loci typically exhibit a fast rate of mutation and high levels of polymorphism and are ideally suited for analyses of stock structure (O'Ryan *et al.*

1998, Hellberg *et al.* 2002). All seven microsatellite loci surveyed in this study revealed considerable within-sample variation (A = 5-21, H_{exp} = 0.435-0.923). Literature values of within-sample variation in other clupeids range from A = 1-56, H_{exp} = 0.066-0.98 (Table 14).

Species Identification

Atlantic, gulf, yellowfin, and finescale menhadens are currently recognized as four separate species. However, with the broad overlap of meristic and morphometric characters between Atlantic and gulf menhaden, and the ability of yellowfin menhaden to hybridize with both Atlantic and gulf menhaden, species identifications are problematic. Proper species identification is a critical first step in the analysis of intraspecific stock structure.

The two small-scaled menhadens, yellowfin and finescale, can be identified on the basis of morphological and molecular characters (Dahlberg 1970, Anderson 2007, this study). Yellowfin menhaden are distinguishable from finescale menhanden in having a higher number of vertebrae and scutes and a smaller head; both small-scaled menhadens are distinguishable from the large-scaled menhadens by the absence of a frontal groove, rounded scale pectinations, and higher scale counts (Dahlberg 1970). The two small-scaled menhadens can also be distinguished from each other and the large-scaled menhadens with genetic characters. Anderson (2007) separated yellowfin, finescale menhaden, and the large-scale menhadens using sequencing data from the mitochondrial control region and four microsatellite loci. Though no fixed differences have been established between the North American menhadens, significant allele

frequency differences have been detected at some loci. Yellowfin menhaden in this study (n = 25) were fixed for allele 130 at locus AsaB020 while this allele occurred at a frequency of 69% in finescale menhaden, 9% in gulf menhaden, and 1% in Atlantic menhaden. Similarly, finescale menhaden (n = 25) were fixed for allele 216 at locus Asa2 while this allele occurred at a frequency of 6% in yellowfin menhaden, 7% in gulf menhaden, and 10% in Atlantic menhaden.

Hybridization between yellowfin menhaden and the two putative large-scale menhadens has been reported to occur along the Atlantic and Gulf coasts of Florida (Turner 1969, Dahlberg 1970, Anderson and Karel 2007). Putative yellowfin/large-scale hybrids, identified on the basis of intermediate morphological and meristical characters, were found in southwestern and southeastern Florida (Turner 1969, Dahlberg 1970). The predominance of males suggested a postzygotic barrier to hybridization that would inhibit the establishment of a stable hybrid population (Dahlberg 1970).

Microsatellite loci and mitochondrial control region sequencing have been used to study the directionality of hybridization and potential for introgression in menhaden. Though frequency differences do not allow unambiguous identification of hybrids, Anderson and Karel (2007) identified putative yellowfin/gulf hybrids by major frequency differences at locus Asa2 (88% frequency of allele 195 in yellowfin menhaden; <5% frequency in large-scaled menhaden) and Asa 9 (90% frequency of allele 242 in large-scaled menhaden; absent in yellowfin menhaden). However, all potential hybrids grouped with yellowfin menhaden based on mitochondrial control region sequences. As the mitochondrial genome is maternally inherited, this result indicated that yellowfin menhaden was the maternal lineage in every case (Anderson and Karel 2007). Based on

an analysis of microsatellite alleles in yellowfin menhaden not identified as F_1 hybrids, Anderson and Karel (2007) concluded that there was a low rate of introgression between yellowfin menhaden and the large-scaled menhadens. This result supports the Dahlberg (1970) hypothesis of a postzygotic inhibition of a hybrid population. However, Anderson and Karel's (2007) samples sizes (n = 10 – 20) per location (New Jersey, Virginia, Maine, and North Carolina) may be insufficient to accurately estimate rates of introgression.

Although hybridization of yellowfin and large-scaled menhadens has been reported, there has been no documented case of hybridization between Atlantic and gulf menhaden. This is primarily due to the fact that there is no definitive diagnostic character to distinguish between these two putative species. The taxonomic key established by Dahlberg (1970) is currently still in use and provides location (i.e. Atlantic Ocean, Gulf of Mexico) as the primary distinguishing feature between the two species. All other key characters (i.e., gill filaments, vertebrae, predorsal scales, and ventral scutes) overlap between Atlantic and gulf menhaden (Dahlberg 1970). Species range for gulf menhaden has also been disputed. As early as Reintjes (1969) and as recently as Anderson and Karel (2007), gulf menhaden have been postulated to occur in southeast Florida based on morphological and mitochondrial data.

Even molecular characters have not resolved the large-scaled menhadens. In all three mitochondrial studies (mitochondrial genome RFLP, Avise *et al.* 1989; control region, Anderson 2007; COI, this study), Atlantic and gulf menhaden formed two well defined clades, but not by putative species. As previously mentioned, COI is a conserved gene region, but typically exhibits consistent (fixed) nucleotide differences between

species. It is, consequently, unusual for species to remain unresolved using COI as a marker.

COI is currently the standard gene region used for species identification in the Barcode of Life Datasystem. Not only do species tend to differ by one or more fixed nucleotide differences in this region, interspecific differences are substantially greater than intraspecific differences (Ratnasingham and Herbert 2007). In a survey of 207 fishes, all species had different COI sequences and differences between closely related species were 25 times higher (on average) than differences within species (Ward *et al.* 2005). In the present study, however, the nucleotide sequence diversity for large-scaled menhadens combined ($\pi = 0.0258$) is only 5 times higher than the within-clade diversity ($\pi = 0.0081, 0.0036$). In the case of the menhadens, the high variability of COI reduces its utility for barcoding but not for analyses of intraspecific population structure.

Based on the preliminary analysis, COI was chosen for this study rather than the mitochondrial control region. In both this study and Anderson (2007), the control region sequences appeared to exhibit mutational saturation. Similar levels of saturation were detected by García *et al.* (2008) among *cytochrome b* sequences in Brazilian menhaden (*B. aurea*).

Mutational saturation is demonstrated by plots of the number of transitions (by codon position if protein coding) and transversions (by codon position if protein coding) with genetic distance. Specifically, if the rate of transitions plateaus with increasing genetic distance and the rate of transversions approaches that of transitions, mutational saturation is indicated. In such cases, it is likely that the rate of transitions has not decreased with time, but that the limited number of character states (purine to purine or

pyrimidine to pyrimidine) results in "multiple hits" or homoplasy (for example, a series of transitions from A → G → A would appear as no change at all). The fact that transitions of the control region plateaued with increasing genetic distance and the rate of transversions approached them suggests that any Atlantic menhaden analyses based on control region nucleotide substitutions could be confounded by homoplasy. For population studies, genetic drift is a more important driving force, but mutation still comes into play.

Although in the initial assessment the COI sequences may have been less suspect of mutational saturation, both COI and control region exhibited high levels of variation. Thus, the same concerns noted for the control region may also apply for COI. Mutation saturation plots are a qualitative measure of homoplasy and not statistically based.

Though it was chosen, COI did not turn out to be an ideal molecular marker for this study as it could not resolve the small-scaled and large-scaled menhaden lineages. Three Atlantic menhaden grouped with the small-scaled menhadens in the COI phylogeny. This result could be from misidentification of individuals in the field, hybridization and introgression, or incomplete lineage sorting using COI as a marker. When sequenced for the control region, the three anomalous individuals clustered with the "ubiquitous large-scaled" clade. This result does not support misidentification or hybridization and introgression for these samples because the control region and COI relationships would have the same origin (e.g., the mitochondrial genome has no recombination). Since the evolutionary rate of the control region exceeds that of COI, this outcome suggests incomplete lineage sorting, an inability of COI to resolve the two major menhaden lineages.

Lineage sorting is the process of fixation of gene lineages between taxa. Incomplete lineage sorting occurs when ancestral polymorphism is retained in multiple taxa, resulting in an inability to distinguish between the groups with a specific gene region. Incomplete lineage sorting is a function of the molecular marker utilized, the taxonomic scale of analysis, and has been found in other lineages including cichlids in Lake Tanganyika (short interspersed element insertion data, Takahashi *et al.* 2001), schizorathicine fishes of Lake Rara, Nepal (proline tRNA and control region sequences, Dimmick and Edds 2002), and crotaphytid lizards (ND2 and Cyt*b* sequences, McGuire *et al.* 2007). These conclusions indicate that the control region may have been a more appropriate mitochondrial gene region to analyze for Atlantic menhaden population structure than COI.

While COI sequences may not completely resolve the four species of North American menhaden, the population structure revealed in this and previous mitochondrial surveys of large-scaled menhadens suggests the possibility of historical isolation between the two large-scaled clades. To obtain a nuclear perspective on the genetic differences between Atlantic and Gulf of Mexico large-scaled menhadens, ITS-1 was sequenced for a subset of samples, representing both mitochondrial clades, and seven microsatellite loci were analyzed for all samples. If the nuclear relationships were similar to the mitochondrial relationships, the result would indicate that the structure resulted from two independent evolutionary lineages (separate species), without contemporary gene flow. If the nuclear relationships were not consistent with the mitochondrial relationships, the result would suggest historical isolation and subsequent unidirectional gene flow.

Neither the ITS-1 nor microsatellite relationships were similar to the two-clade structure revealed by analyses of mitochondrial gene regions. The ITS-1 phylogeny produced no discernable structure, a high level of haplotype diversity ($h = 0.972$), and a low level of nucleotide sequence diversity ($\pi = 0.0122$) among the large-scaled menhaden clones. Intra-individual variation in ITS-1 phylogenetic studies is widely known to exist (Harris and Crandall 2000). In this study with 5 clones per individual, individuals had up to five different alleles. It is also important to note that all *B. gunteri* clones grouped as a species; all but one *B. smithi* sequence fell outside of the large-scaled group; and with that one *B. smithi* sample, all of the large-scaled individuals fell within one large polytomy. Despite the high intra-individual variation and haplotype diversity, ITS-1 does not provide any usable information to compare with the mitochondrial relationships.

The microsatellite results of this study, like Anderson (2007), did partition the North American menhadens into four groups. However, both studies indicated that the large-scaled menhadens are very closely related. Anderson (2007) surveyed variation at four microsatellite loci, estimating an F_{ST} of 0.110 between Atlantic and gulf menhaden. This study screened seven microsatellite loci, estimating an F_{ST} of 0.104 between Atlantic (all sampling regions grouped together) and gulf menhaden. Considering each Atlantic and Gulf sampling location independently, this study estimated F_{ST} values of 0.079-0.122 ($p = 0.000$). These F_{ST} values are more typical of differences between populations rather than species. For comparison, F_{ST} values between genetically distinct stocks based on microsatellites range from 0.002 to 0.226 (Table 15). Conversely, F_{ST} values between North American menhadens include: 0.488 between Atlantic and yellowfin menhaden, 0.412 between Atlantic and finescale menhaden, 0.411 between gulf and yellowfin

menhaden, 0.378 between gulf and finescale menhaden, and 0.355 between yellowfin and finescale menhaden (Anderson 2007).

Avise *et al.* (1989) grouped Atlantic and gulf menhaden into a *Brevoortia tyrannus/patronus* complex, noting "the species are very closely related and are of questionable taxonomic status." Bowen and Avise (1990) compared the population structure of the large-scaled menhadens, black sea bass, and sturgeon, three coastal taxa present in the Atlantic and Gulf of Mexico. The black sea bass exhibited reciprocal monophyly of the Atlantic and Gulf haplotypes while both the menhaden and sturgeon shared haplotypes between the Atlantic and Gulf of Mexico. Currently, the Atlantic and Gulf congeners have subspecies status for black sea bass and sturgeon, but not for the menhadens; the menhaden taxonomy conflicts with the genetic results.

While more morphological and genetic analyses of large scale menhadens should be undertaken, especially around Florida, the genetic data suggest that synonymizing Atlantic and gulf menhaden may be warranted. This situation parallels another within the genus *Brevoortia*. Historically, the existence of two menhaden species in the southwest Atlantic, Brazilian menhaden (*B. aurea* Spix & Agassiz 1829) and Argentine menhaden (*B. pectinata* Jenyns 1842) have been purported (Whitehead 1985). But, recent genetic data has confirmed that these two putative species, in fact, comprise only one species, *B. aurea* (García *et al.* 2008).

Phylogeography

Phylogenetic analyses based on mitochondrial genome RFLP as well as control region and COI sequence data revealed the presence of two highly divergent lineages of

large-scaled menhadens (Bowen and Avise 1990, Anderson 2007, this study). Bowen and Avise (1990) noted the presence of one clade (α; "ubiquitous large-scaled" in this study) in menhaden collected along the U.S. Atlantic coast and Gulf of Mexico and the other clade (β; "Atlantic-only" in this study) in menhaden only collected from the Atlantic Ocean. Based on mitochondrial COI sequences of this study, the net nucleotide sequence divergence (δ) between the two clades was 3.70%. This value is somewhat smaller than the 5% sequence divergence noted between the two clades by Bowen and Avise (1990) based on RFLP analysis of the entire mitochondrial genome. The slight difference between the divergences estimated in the two studies is consistent with the fact that the mitochondrial genome contains both coding and non-coding gene regions and that COI is a coding region.

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. During a period of glaciation, sea level was lower and waters were cooler, making the Florida peninsula a more formidable barrier to gene flow. This barrier produced a phylogeny with an Atlantic clade and a Gulf clade. During a period of warming, sea level was higher, allowing for unidirectional gene flow from the Gulf of Mexico to the Atlantic via the Gulf Stream. Cooling subsequently limited mixing around Florida, with the Gulf signature remaining in the Atlantic population.

Anderson (2007) postulated that the distribution of these two clades in menhaden collected along the U.S. Atlantic coast supported very recent gene flow from the Gulf of

Mexico to the Atlantic because four of the eight "Atlantic-only" haplotypes in the study were collected from the northernmost sampling location. However, the postulated geographic cline in the distribution of the "Atlantic-only" clade was only qualitatively addressed and also must be moderated by the small sample size ($n = 37$) of Atlantic menhaden in that study. In the present study, a more extensive sampling regime for Atlantic menhaden ($n = 289$) refutes Anderson's (2007) hypothesis of recent gene flow based on a clinal distribution of the two clades in Atlantic menhaden. A chi-square analysis of Atlantic coast sampling locations indicated that the two mitochondrial clades were not heterogeneously distributed among Atlantic coast collection locations. In other words, there is no geographic cline in mitochondrial sequences along the Atlantic coast.

While the mitochondrial data suggest unidirectional gene flow from the Gulf of Mexico to the Atlantic coast in the recent geological past, analysis of nuclear microsatellites indicates a barrier to gene flow at the present time. This study surveyed 7 microsatellite loci and found an F_{ST} of 0.104 ($p = 0.000$) between Atlantic and gulf menhaden, a value that agrees well with Anderson's (2007) F_{ST} of 0.110 based on the analysis of 4 loci.

Population Structure

Stock structure analyses of Atlantic menhaden along the U.S. Atlantic coast have suggested as few as one and as many as three different stocks based on spawning time, spawning location, and migration tracks (June 1958, 1965; Sutherland 1963; June and Nicholson 1964; Nicholson 1972, 1978; Dryfoos *et al.* 1973; Epperly 1989). This study analyzed the distribution of allelic variation of rapidly evolving molecular characters to

evaluate population structure of Atlantic menhaden. The resulting AMOVAs did not attribute any significant portion of molecular variance to variation between the following group comparisons: YOY menhaden recruiting to 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).

While 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%, $p = 0.0000$). Grouping the U.S. Atlantic coast sampling regions also produces a slight, but significant, partioning between the northern and southern sampling locations ($F_{ST} = 0.00535$, $p = 0.00098$). This significant result from the F_{ST} AMOVA between the four U.S. Atlantic coast geographic regions may be a result of a latitudinal gradient in genetic variation or, conversely, a biologically insignificant event. Given enough genetic comparisons, statistically significant differences can be expected since some aberrations from complete panmixia do occur (Waples 1998).

The differences between the F_{ST} and R_{ST} AMOVA results can be accounted for by the different distance algorithms employed in the analyses. The F_{ST} AMOVA is based on the distribution of number of different alleles (not considering relationships among alleles) and uses the infinite allele model of mutation. As a result, F_{ST} may be more appropriate in situations of recent divergence when genetic drift is the predominant factor (Ruzzante 1998). The R_{ST} AMOVA is based on the distribution and relationship of alleles (sum of squared allele size differences) and uses the stepwise mutation model. The R_{ST} algorithm may be more appropriate in situations where mutation is a key factor (Ruzzante 1998). Additionally, the R_{ST} method is considered applicable for microsatellite data only when sample sizes are either equal or larger than 50 (Ruzzante 1998). Because mutation is an important feature in this dataset and because the sample sizes exceed 50, R_{ST} is a more reliable microsatellite method for comparing putative species. Conversely, for within species comparisons, F_{ST} is a more typical method.

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 sequential Bonferroni correction. These findings support the hypothesis that the significant results from the F_{ST} AMOVAs were biologically insignificant. The collective results indicate that there was no significant partitioning of genetic variation between the sampling regions of Atlantic menhaden, and the null hypothesis of a single U.S. Atlantic stock can not be rejected.

Finding no 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 (Whitehead 1985). They also undergo an ontogenetic shift in migration where larger fish migrate furthest north (Dryfoos *et al.* 1973, Quinlan *et al.* 1999). Atlantic menhaden are batch spawners, spawning multiple times during a year, and have the most temporally and geographically protracted spawning season of any North American *Brevoortia* (Whitehead 1985). This means that the area(s) where a fish spawns in one year could be a different from the area(s) that it spawns in the following year. Additionally, eggs are spawned in coastal, pelagic waters. 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, Quinlan *et al.* 1999). These characteristics appear to keep Atlantic menhaden – and their gene pool – well mixed.

Genetic analyses of other clupeids have failed to find population stucture. For Atlantic herring (*Clupea harengus*), Grant (1984) postulated that small scale migrations accounted for the lack of genetic divergence between adjacent areas (40 allozyme loci, 6 locations, $\approx$ 50 samples/location). Volk *et al.* (2007) found evidence of substantial gene flow between spawning locations of twaite shad (*Alosa fallax*) with no evidence of localized genetic drift due to habitat changes (12 microsatellite loci, 5 locations, 50 samples/location; F_{ST} = -0.0004 - 0.0089, $\alpha = 0.05$, non-significant after Bonferroni correction in all but one case). Gonzalez and Zardoya (2007) discovered minimal genetic differentiation among European sardines, hypothesizing it to be a result of weak isolation by distance (8 microsatellite loci, 9 locations, 50 samples/location; $R_{ST} = 0.001 - 0.083$, of 36 pairwise comparisons, only nine comparisons revealed significant values after

correction for multiple tests, $p < 0.0014$).

In contrast, some clupeid species exhibit significant stock structure, often attributed to the presence of geographic barriers or temporal reproductive isolation. Allozyme analysis revealed significant genetic differentiation between eastern North Pacific and Bering Sea Pacific herring (*C. pallasi*) because of adults homing to previous spawning locations (spawning site fidelity) and larval retention mechanisms (40 allozyme loci, 6 locations, ≈ 50 samples/location; Grant and Utter 1984). Similarly, O'Connell *et al.* (1998) used microsatellite analysis to confirm genetic isolation of the Bering Sea and Gulf of Alaska stocks of Pacific herring separated by the Alaska Peninsula (5 microsatellite loci, 7 locations, ≈ 50 samples/location; $R_{ST} = 0.11 - 0.16$, $p < 0.001$). And, Sugaya *et al.* (2008) exhibited significant genetic difference between Honshu and Hokkaido Island samples of Pacific herring due to natal homing (5 microsatellite loci, 10 locations 200/location$_{avg}$; $F_{ST} = 0.046\text{-}0.173$, $p < 0.05$, after sequential Bonferroni correction). Shaw *et al.* (1999) also found significant genetic structuring between Icelandic summer-spawners, Norwegian spring-spawners, and Norwegian fjord stocks of Atlantic herring (*C. harengus*), attributing differences to temporal isolation of spawning (4 microsatellite loci, 5 locations, ≈ 50 samples/location; $F_{ST} = 0.01 - 0.04$, $p < 0.001$; $R_{ST} = 0.08 - 0.28$, $p < 0.001$).

Implications for Management

Atlantic menhaden have had a rocky history of management in Virginia, the only state that still maintains a reduction fishery facility for the species. Atlantic menahden are the only species in Virginia state marine waters to be managed by the Virginia

Legislature, rather than the Virginia Marine Resource Commission. In 2005, the Atlantic States Marine Fisheries Commission (ASMFC), the regional managing authority, proposed a precautionary cap of 105,000 tons on the reduction fishery to assess the status of Atlantic menhaden in Chesapeake Bay relative to coastal systems (ASMFC 2005). By mandate, all ASMFC states were required to comply with the cap. However, during the 2006 legislative session, members of the Virginia General Assembly withdrew or killed four separate bills seeking to adopt the ASMFC cap. If Virginia was still non-compliant by July 2006, a commercial moratorium could have been established by federal law. However, Virginia Governor Tim Kaine instituted a 109,000 ton cap by gubernatorial proclamation July 31, 2006 which was accepted by the ASMFC (ASMFC 2006).

With concerns over the potential of localized depletion, the landings cap provided a precautionary management measure, allowing time to evaluate the connectivity and genetic relatedness of Atlantic menhaden in Chesapeake Bay and along the U.S. Atlantic coast. This is especially important because there has been a reported loss of unique genetic variation in other species such as Pacific cod, leopard darter, Japanese flounder, and American shad due to fishing pressure, habitat degradation, and hatchery stocking (Grant and Stahl 1988, Echelle *et al.* 1999, Sekino *et al.* 2003, and Brown *et al.* 2000, respectively).

Currently, Atlantic menhaden are managed as one coastwide stock. With high genetic variability and homogeneous distribution of the genetic variation, the results of this study indicate that the current management practices for Atlantic menhaden are sufficient for maintaining the genetic composition of the species. This finding is consistent with results of previous tagging studies for Atlantic menhaden (Dryfoos *et al.*

1973, Nicholson 1978). The genetic connectivity between New England, mid-Atlantic, Chesapeake Bay, and U.S. south Atlantic samples suggests that loss of unique genetic variation due to intensified fishing pressure in Chesapeake Bay is not likely. However, it is important to realize that gene flow of only a few migrants per generation, even for populations with millions of individuals such as the Atlantic menhaden, is enough to negate the effects of genetic drift (Mills and Allendorf 1996, Hartl and Clark 2007).

While the results of this study suggest that menhaden would eventually return to an area depleted by fishing effort without any significant loss of genetic variation, genetic analysis cannot estimate the time course of movements into the depleted area. Menhaden could repopulate a depleted area on a timescale of weeks, months, seasons, years, or decades. Short-term replenishment to areas affected by localized fishing pressure is better estimated with non-genetic techniques, such as mark-recapture or analysis of otolith microchemistry.

As the fifth largest U.S. commercial fishery (by weight) and a principal forage fish, Atlantic menhaden is an important species, economically and ecologically. Proper management requires the most current understanding of Atlantic menhaden stock structure. This study evaluated the potential for local depletion of genetic variation due to the recent consolidation of the reduction fishery to within and around Chesapeake Bay. While the taxonomic status of Atlantic and gulf menhaden remains questionable, the results from seven microsatellite loci suggest that Atlantic menhaden have complete spatial and temporal genetic connectivity. Consequently, the management of Atlantic menhaden as a single coastwide stock is appropriate at the present time.

TABLES

Table 1. Microsatellite loci tested in the present study for amplification in *Brevoortia* specimens. Loci listed in black successfully amplified variation; loci listed in gray did not.

Locus	GenBank Accession no.	Repeat motif [reported size]	Fluorescent Label	Primer sequence (5'-3')	Annealing temp	Reference	Comment
Aa14	AY617109	$(GT)_8$ [128-140]	PET	F: GAG AAG AGG GCA TTC G R: ATT TAG TGT GTG CCC AGC	61°C	*Faria et al. 2004*	no allele found
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 °C	Faria *et al.* 2004	
Aa20	AY617111	$(GT)_{16}$ [116-146]	NED	F: GGT GTA ATG CCC GTC CG R: CAG TGT GCA GAC CAG CC	48.6°C	Faria *et al.* 2004	Fixed
Af6	AY617112	$(CA)_{12}AT(CT)_8(CA)_{10}AA(CA)_8$ [159-171]	PET	F: AGG AGA TGT TTA TCC TGC C R: CAC AGA GGC ATA AAT GTG G	48.6°C	Faria *et al.* 2004	Fixed
Af11	AY617113	$(CA)_5CT(CA)_1$ [126-139]	VIC	F: CGA GTA CAA TCA AAA GCC R: AGC TTC CTC AGA CTG G	49.1 °C	Faria *et al.* 2004	did not amplify well
Af13	AY617114	$(CA)_{17}$ [170-178]	6-FAM	F: AGG ATA CAT AGT CTC CC R: CAA GTT GGA GTG TCA CG	59 °C	Faria *et al.* 2004	no allele found
Af15	AY617115	$(CA)_{11}$ [173-208]	NED	F: CCC ATT CAC TCT TTT TCT C R: GAG AGG AGT TGA GTA TGG	53 °C	Faria *et al.* 2004	no allele found

Table 1 cont. Microsatellite loci tested in the present study for amplification in *Brevoortia* specimens. Loci listed in black successfully amplified variation; loci listed in gray did not.

Locus	GenBank Accession no.	Repeat motif [reported size]	Fluorescent Label	Primer sequence (5'-3')	Annealing temp	Reference	Comment
Af20	AY617116	$(CA)_{11}$ [173-208]	VIC	F: AAT GGA CAT ATC TGC TGG R: ATG GAG GGC CAT ATT TCG	52.6 °C	Faria *et al* 2004	Fixed
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°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 °C	Brown *et al.* 2000	
Asa6	AF039659	$(AAC)_{12}$ [83-110]	PET	F: ACC TTC TGT TCT GTT TCA CCT G R: TTC ACT GTA ATG CAA TGT AAT GTT	48-62°C grad	Brown *et al.* 2000	no allele found
Asa8	AF039660	$(CTTG)_8$ [108-168]		F: TCC ATT CCA TTA CGT AGA GCA CT R: CCG GCA GGG CAC AGA AC		Brown *et al.* 2000	Fixed
Asa9	AF039661	$(TTTC)_7$ [156-266]	PET	F: GGG AAT AAG GGA TGT AGC CAA GAT R: AGG AGA AGG AAA GGG GAG TGA GAG	48.6°C	Brown *et al.* 2000	Fixed
Asa16	AF049462	$(GTT)_3(CCT)(GTT)_{12}$ [116-131]	VIC	F: CCG TCG CCG ATG TAG TTG CAG TGG R: ACG GGA GAA AAG AGT ATG TGT CCT TGA G	48.6 °C	Brown *et al.* 2000	Suspect null allele

Table 1 cont. Microsatellite loci tested in the present study for amplification in *Brevoortia* specimens. Loci listed in black successfully amplified variation; loci listed in gray did not.

Locus	GenBank Accession no.	Repeat motif [reported size]	Fluorescent Label	Primer sequence (5'-3')	Annealing temp	Reference	Comment
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°C	Julian & Bartron 2007	
AsaC010	EF014991	$(GTAT)_{16}$ [261-321]	PET	F: AAT AAT GTT GTG CTG GAT TGT G R: TTT ATT GTT ATT GTG ATG GAG GG	50.4 °C	Julian & Bartron 2007	Fixed
AsaC051	EF014992	$(GAAT)_7(GTAT)_{13}$ [248-316]	VIC	F: GTA AGT CGC TTT GGA CTA CCA G R: TCT AAA TGC CCA GGT AAA GAT G	48-62°C grad	Julian & Bartron 2007	no allele found
AsaC249	EF014994	$(CATA)_8(TTCT)_{13}$ [243-367]	6-FAM	F: TTA TTA CAA CGG TGA ATT GAG TG R: TAA GTG CAT GTT GTG TGT GAT G	62°C	Julian & Bartron 2007	Fixed
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 °C	Julian & Bartron 2007	
AsaD021	EF014996	$(CTAT)_{15}$ [249-301]	NED	F: TTA TTA CAA CGG TGA ATT GAG TG R: TAA GTG CAT GTT GTG TGT GAT G	62°C	Julian & Bartron 2007	Fixed
AsaD029	EF014997	$(CTAT)_{20}$ [182-254]	VIC	F: ATT ATG CAC AGG AAT CTG GAA G R: TGT GCT TAC AAA AGT GAC ATG G	48.6°C	Julian & Bartron 2007	Fixed

Table 1 cont. Microsatellite loci tested in the present study for amplification in *Brevoortia* specimens. Loci listed in black successfully amplified variation; loci listed in gray did not.

Locus	GenBank Accession no.	Repeat motif [reported size]	Fluorescent Label	Primer sequence (5'-3')	Annealing temp	Reference	Comment
AsaD030	EF014998	$(CTAT)_{23}$ [102-182]		F: CCA CAG CAT CAT CTC TTT ACT G R: ACC TTG AAT TTC TCC TTG GG		Julian & Bartron 2007	Amplifies wrong region?
AsaD031	EF014999	$(CTAT)_{14}$ [180-240]		F: TTC CTG ATA TTT CTT GTG AGG G R: ATT TCT GTG GAA ACC TTT TGG	48-62°C grad	Julian & Bartron 2007	no allele found
AsaD042	EF015000	$(CTAT)_{12}$ [162-214]	6-FAM	F: ACT GGT CAA TTG TAA GAC ACC C R: CAA GAT GAC CAA GGG TTA AGA C	48-62°C grad	Julian & Bartron 2007	no allele found
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°C	Julian & Bartron 2007	
AsaD278	EF015002	$(CTAT)_{17}$ [190-210]	NED	F: CCC TTA GTG AAT GTT GAG TGT G R: CTT AGG CCA CAT AGG TGA GTA TC	48-62°C grad	Julian & Bartron 2007	no allele found
Sar1A11	AY636120	$(GATA)_2GCTA$ [257-313]		F: GAG CTG GAA ATC TGG TGA TAT TTA G R: CCT GTT CAC AAG TTA GAG CAT TC	48-62°C grad	Pereyra *et al. 2004*	no allele found
Sar1D06	AY636123	$(TG)_{18}$ [120-158]	PET	F: CGG CTA TTC TTA GAC TAG GTG R: CCC CAT CAG CAA TGA ATA AG	48.6°C	Pereyra *et al.* 2004	didn't amplify well

Table 1 cont. Microsatellite loci tested in the present study for amplification in *Brevoortia* specimens. Loci listed in black successfully amplified variation; loci listed in gray did not.

Locus	GenBank Accession no.	Repeat motif [reported size]	Fluorescent Label	Primer sequence (5'-3')	Annealing temp	Reference	Comment
Sar1D01	AY636121	$(CA)_{20}GG(CA)_3$ [182-256]		F: GCT CTG GTC GGA GGC TCT ATC R: GGT GTT CAC GTG GGC TGG TA	48-62°C grad	Pereyra *et al.* 2004	no allele found
Sar1H11	AY636122	$(TG)_{11}TA(TG)_6$ [150-200]		F: CAC GGC ACG TTA CGT TTC AG R: CCA GCG TGT CAT GAA ATG ATG	48-62°C grad	Pereyra *et al.* 2004	no allele found
SarBA07	AY636114	$(GA)_{12}$ [68-136]	NED	F: CTC CTC ACT CAG CCG CTA AGG A R: GGG TAA CAT TTC GGC AAG TGC T	48.6°C	Pereyra *et al.* 2004	Fixed
SarBA08	AY636115	$(CA)_{26}$ [201-301]	NED	F: GTG ATA CTC TCT GCC TTG GA R: GCA CTT TGT CCT AGT AAA TAG C	48-62°C grad	Pereyra *et al.* 2004	no allele found
SarBC05	AY636116	$(TC)_5TT(TC)_4$ [120-182]	PET	F: GAA CGC AGA CAT AAA AGG GTC R: GGG TAT GTG GTG ATT ATC GTT C	48-62°C grad	Pereyra *et al.* 2004	no allele found
SarBD09	AY636117	$(CA)_6(GA)_8$ [269-303]		F: GGT CAT CTG CTT CAA CAA CAC R: GCA GCC TGT CTG AAA CTC TG	48-62°C grad	Pereyra *et al.* 2004	no allele found
SarBG09	AY636118	$(GA)_6GT(GA)_{26}$ [149-163]		F: GGT GGA AAG AAC ACT GGT CA R: GGT TCA CTA TGC AGG CTA TG	48-62°C grad	Pereyra *et al.* 2004	no allele found
SarBH04	AY636119	$(GT)_9$ [181-271]		F: CGA GTT TGT CCC ACA CCT GGA G R: CTC CAA GCA CCG AGA GCA TC		Pereyra *et al.* 2004	

Table 2. 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 of 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	19.0 ± 8.67
COI	28	20	44	41	8	0	0.960 ± 0.0241	0.0267 ± 0.0141	9.44 ± 4.46
ITS-1	60	45	81	54	23	14	0.979 ± 0.0110	0.0183 ± 0.0094	10.2 ± 4.73
ITS-1 (large-scale only)	50	38	59	36	18	11	0.972 ± 0.0154	0.0122 ± 0.0065	6.80 ± 3.26

Table 3. Sequence statistics of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on cytochorme *c* oxidase subunit I (COI) sequence data by region, clade, 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	0.940 ± 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	118	50	62	63	3	0.932 ± 0.0144	0.0267 ± 0.0134	12.2 ± 5.57
U.S. south Atlantic	69	44	66	67	2	0.954 ± 0.0166	0.0295 ± 0.0149	13.5 ± 6.16
Atlantic menhaden only	289	109	97	99	6	0.941 ± 0.0083	0.0274 ± 0.0137	12.6 ± 5.68
gulf menhaden	50	25	34	33	5	0.879 ± 0.0419	0.0071 ± 0.0041	3.23 ± 1.70
large-scaled menhaden combined	339	124	99	101	7	0.940 ± 0.0079	0.0258 ± 0.0129	11.8 ± 5.36
"Atlantic only" clade	236	98	76	76	7	0.925 ± 0.0136	0.0081 ± 0.0045	3.71 ± 1.88
"ubiquitous large-scaled" clade	100	23	22	22	1	0.732 ± 0.0456	0.0036 ± 0.0024	1.65 ± 0.98
finescale menhaden	25	16	43	42	1	0.940 ± 0.0310	0.0220 ± 0.0116	10.0 ± 4.77
yellowfin menhaden	25	17	53	53	3	0.923 ± 0.0449	0.0335 ± 0.0173	15.4 ± 7.10
small-scaled menhaden combined	50	27	57	58	3	0.936 ± 0.0231	0.0261 ± 0.0133	12.0 ± 5.50
Overall	389	145	105	107	7	0.952 ± 0.0062	0.0331 ± 0.0164	15.2 ± 6.80

Table 4. Contingency table testing the independence of geographic representation between the two cytochrome *c* oxidase subunit I (COI) sequence clades of large-scaled menhadens (*Brevoortia tyrannus* and *B. patronus*) for all Atlantic coast regions (New England, mid-Atlantic, Chesapeake Bay, and U.S. south Atlantic). The "Atlantic only" clade consists only of Atlantic menhaden; the "ubiquitous large-scaled" clade consists of Atlantic and gulf menhaden. $x^2 = \sum_i \sum_j \frac{(f_{ij} - \hat{f}_{ij})^2}{\hat{f}_{ij}}$, where f_{ij} = observed counts (bolded), $\hat{f}_{ij}$ = expected counts (italicized), and degrees of freedom (v) = (i rows -1)(j columns -1). For this data set, $x^2_{0.05,3} = 7.815$.

All Atlantic coast regions: $\mathbf{x^2 = 0.478} < 7.815 \rightarrow$ **NS**					
Clade	**New England**	**mid-Atlantic**	**Chesapeake Bay**	**U.S. south Atlantic**	Total
"Atlantic only"	**19** *17.5*	**17** *18.2*	**39** *41.0*	**25** *23.5*	**100** 0.35 (% of total)
"ubiquitous large-scaled"	**31** *32.0*	**35** *33.3*	**78** *74.9*	**42** *42.9*	**186** 0.64 (% of total)
Total	50	52	117	67	

Table 5. Population statistics of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on eight microsatellite loci by region and overall samples: number of individuals (*n*), allele size range (a.s.r.), allelic diversity (*A*), allelic richness (R_s), and gene diversity (*D*).

Locus	Repeat motif	GenBank accession		New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic	gulf menhaden	finescale menhaden	yellowfin menhaden
Aa16	$(CA)_4$ AA $(CA)_3$ AA $(CA)_8$	AY617110	*n*	47	51	117	53	49	24	24
			a.s.r.	139-155	139-155	139-155	139-157	135-161	135-155	141-157
			A	7	5	7	7	9	6	4
			R_s	4.98	4.07	4.40	4.70	4.69	4.54	2.91
			D	0.733	0.626	0.630	0.712	0.447	0.507	0.323
Asa2	$(TTC)_{13}$	AF039657	*n*	44	50	114	68	49	25	24
			a.s.r.	210-237	213-234	210-237	216-237	210-237	216	213-216
			A	6	8	9	7	10	1	2
			R_s	4.15	5.00	4.91	5.01	5.15	1.00	1.85
			D	0.600	0.660	0.651	0.657	0.641	0.000	0.120
Asa4	$(ACC)_3$ $(AAC)_{12}$ $(AGC)_6$	AF039658	*n*	44	47	102	53	44	25	24
			a.s.r.	168-204	162-222	156-198	165-201	165-198	165-216	159-177
			A	9	12	14	12	10	7	5
			R_s	5.46	7.52	6.71	6.59	6.19	6.33	3.90
			D	0.722	0.840	0.821	0.782	0.780	0.799	0.645
Asa16	$(GTT)_3$ CCT $(GTT)_{12}$	AF049462	*n*	45	50	111	69	45	25	24
			a.s.r.	247-292	223-286	223-304	223-289	223-298	244-277	244-268
			A	16	17	21	18	15	8	5
			R_s	13.2	14.4	15.1	12.8	12.8	7.88	5.00
			D	0.901	0.912	0.918	0.901	0.904	0.738	0.605

Table 5 cont. Population statistics of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on eight microsatellite loci by region and overall samples: number of individuals (*n*), allele size range (a.s.r.), allelic diversity (*A*), allelic richness (R_s), and gene diversity (*D*).

Locus	Repeat motif	GenBank accession		New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic	gulf menhaden	finescale menhaden	yellowfin menhaden
AsaB020	(GAT)15	EF014990	n	47	49	116	66	49	24	24
			a.s.r.	142-184	130-193	130-196	130-187	130-196	127-139	130
			A	13	19	20	19	19	4	1
			Rs	9.44	10.9	10.0	10.0	12.4	3.17	1.00
			D	0.897	0.902	0.887	0.888	0.938	0.473	0.000
AsaD055	(CTAT)10	EF015001	n	44	36	113	63	45	25	24
			a.s.r.	254-314	254-326	238-334	238-326	258-330	242-290	238-294
			A	17	14	18	17	20	13	11
			Rs	10.3	10.4	10.7	11.3	11.4	10.2	8.76
			D	0.911	0.917	0.912	0.923	0.917	0.910	0.885
AsaC334	(GTAT)17	EF014995	n	49	51	114	63	49	11	17
			a.s.r.	118-146	122-138	122-146	118-138	118-154	134-158	134-158
			A	7	5	7	6	9	7	6
			Rs	4.77	3.20	4.33	3.68	7.65	7.00	5.16
			D	0.594	0.436	0.636	0.570	0.857	0.809	0.597
SarBH04	(GT)9	AY636119	n	40	49	111	56	44	21	24
			a.s.r.	179-221	179-221	179-221	179-221	179-221	169-215	189-221
			A	12	12	13	12	12	10	7
			Rs	6.27	6.43	5.89	7.22	5.57	8.37	4.26
			D	0.721	0.760	0.735	0.793	0.451	0.851	0.306

Table 6. Hardy-Weinberg equilibrium test of association at the locus level for Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) using the methods of Guo and Thompson (1992) based on eight microsatellite loci by region and overall samples: number of samples (no. samp.),* number of genotypes (no. geno.), observed heterozygosity (H_O), and expected heterozygosity (H_E). Significance values are based on 10,000,000 Markov chain randomizations. The two-tailed probability test (p_{prob}) was performed for all samples. The one-tailed heterozygote deficiency test ($p_{heterodef}$) and one-tailed heterozygote excess test ($p_{heteroexc}$) were performed if the probability test had significant results. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 6 = 0.0083$). *samples <50 do not produce reliable test results.

Locus		New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic	gulf menhaden	finescale menhaden	yellowfin menhaden
Aa16	no. samp.	47	51	117	53	49	24	24
	no. geno.	13	10	14	14	11	7	4
	H_O	0.723	0.588	0.590	0.887	0.449	0.500	0.375
	H_E	0.734	0.625	0.630	0.913	0.447	0.507	0.325
	p_{prob}	0.122 ± 0.0012	0.571 ± 0.0010	0.606 ± 0.0020	0.242 ± 0.0017	0.693 ± 0.0041	0.313 ± 0.0020	1.00 ± 0.0000
Asa2	no. samp.	44	50	114	68	49	25	24
	no. geno.	10	15	18	15	14	1	2
	H_O	0.614	0.540	0.579	0.662	0.653		0.143
	H_E	0.600	0.658	0.651	0.657	0.661		0.117
	p_{prob}	0.524 ± 0.0017	0.251 ± 0.0021	0.0219 ± 0.0008	0.504 ± 0.0019	0.432 ± 0.0039		1.00 ± 0.0000
Asa4	no. samp.	44	47	102	53	44	24	24
	no. geno.	16	23	30	20	18	13	6
	H_O	0.75	0.809	0.824	0.698	0.681	0.583	0.500
	H_E	0.723	0.839	0.823	0.801	0.791	0.794	0.641
	p_{prob}	0.819 ± 0.0018	0.372 ± 0.0033	0.0227 ± 0.0012	0.0126 ± 0.0007	0.0560 ± 0.0007	**0.0012 ± 0.0001**	**0.0046 ± 0.0001**
	$p_{heterodef}$						**0.0002 ± 0.0000**	0.199 ± 0.0012
	$p_{heteroexc}$						1.00 ± 0.0000	0.803 ± 0.0011
Asa16	no. samp.	45	50	111	69	45	25	24
	no. geno.	32	36	65	40	31	12	7
	H_O	0.660	0.808	0.729	0.797	0.700	0.880	0.640
	H_E	0.809	0.877	0.856	0.900	0.812	0.741	0.582
	p_{prob}	0.0053 ± 0.0005	0.0473 ± 0.0018	**0.0000 ± 0.0000**	0.0587 ± 0.0022	0.0272 ± 0.0010	0.844 ± 0.0014	0.544 ± 0.0012
	$p_{heterodef}$			**0.0000 ± 0.0000**				
	$p_{heteroexc}$			1.00 ± 0.0000				

Table 6 cont. Hardy-Weinberg equilibrium test of association at the locus level for Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) using the methods of Guo and Thompson (1992) based on eight microsatellite loci by region and overall samples: number of samples (no. samp.),* number of genotypes (no. geno.), observed heterozygosity (H_O), and expected heterozygosity (H_E). Significance values are based on 10,000,000 Markov chain randomizations. The two-tailed probability test (p_{prob}) was performed for all samples. The one-tailed heterozygote deficiency test ($p_{heterodef}$) and one-tailed heterozygote excess test ($p_{heteroexc}$) were performed if the probability test had significant results. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 6 = 0.0083$). *samples <50 do not produce reliable test results.

Locus		New England	mid-Atlantic	Chesapeake Bay	U.S. south Atlantic	gulf menhaden	finescale menhaden	yellowfin menhaden
AsaB020	no. samp.	47	49	116	66	49	24	23
	no. geno.	29	33	60	41	38	5	1
	H_O	0.787	0.898	0.905	0.833	0.439	0.458	
	H_E	0.894	0.902	0.887	0.888	0.458	0.473	
	p_{prob}	0.0153 ± 0.0006	0.0663 ± 0.0027	0.444 ± 0.0061	0.416 ± 0.0060	0.0293 ± 0.0014	0.0592 ± 0.0004	
AsaC334	no. samp.	49	51	114	63	49	11	17
	no. geno.	12	6	14	10	25	8	7
	H_O	0.531	0.431	0.588	0.556	0.878	0.909	0.529
	H_E	0.593	0.435	0.636	0.570	0.857	0.814	0.595
	p_{prob}	0.417 ± 0.0028	0.734 ± 0.0012	0.428 ± 0.0012	0.588 ± 0.0018	0.494 ± 0.0017	0.775 ± 0.0012	0.142 ± 0.0013
AsaD055	no. samp.	44	36	113	63	45	25	24
	no. geno.	34	26	60	48	34	19	20
	H_O	0.841	0.917	0.894	0.905	0.867	0.960	0.833
	H_E	0.910	0.916	0.916	0.924	0.916	0.912	0.883
	p_{prob}	0.386 ± 0.0036	0.106 ± 0.0019	0.029 ± 0.0012	0.633 ± 0.0037	0.0182 ± 0.0016	0.496 ± 0.0032	0.705 ± 0.0022
SarBH04	no. samp.	40	49	111	56	44	21	24
	no. geno.	17	20	28	23	15	13	7
	H_O	0.675	0.694	0.631	0.661	0.409	0.381	0.333
	H_E	0.720	0.759	0.739	0.791	0.450	0.816	0.307
	p_{prob}	0.492 ± 0.0048	0.368 ± 0.0042	0.382 ± 0.0050	0.0486 ± 0.0014	0.352 ± 0.0077	**0.0000 ± 0.0000**	1.00 ± 0.0000
	$p_{heterodef}$						**0.0000 ± 0.0000**	
	$p_{heteroexc}$						1.00 ± 0.0000	

Table 7. Modifed Cavalli-Sforza chord distances (D_A; Nei *et al.* 1983) (above diagonal) and Nei's standard genetic distances (D_{ST}; Nei 1972) (below diagonal) for Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04).

	New England	**mid-Atlantic**	**Chesapeake Bay**	**U.S. south Atlantic**	**gulf menhaden**	**finescale menhaden**	**yellowfin menhaden**
New England		0.227	0.184	0.178	0.363	0.626	0.684
mid-Atlantic	0.0263		0.181	0.169	0.378	0.618	0.670
Chesapeake Bay	0.0285	0.0136		0.152	0.352	0.600	0.661
U.S. south Atlantic	-0.00656	0.0156	0.0123		0.344	0.615	0.666
gulf menhaden	0.335	0.408	0.423	0.332		0.658	0.714
finescale menhaden	0.668	0.614	0.614	0.632	0.774		0.427
yellowfin menhaden	0.745	0.702	0.702	0.718	0.844	0.324	

Table 8. Hierarchical variance partitioning and 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 grouped by recruitment 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-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and gulf menhaden), and by putative species (Atlantic and gulf menhaden). The distance method used was pairwise differences with 10,000 permutations. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	Variance	**% total**	**Φ Statistics**	***p***
Grouped by recruitment time				
between recruitment times	-0.0389	-0.621	$\Phi_{ST} = -0.00621$	0.692
within a recruitment time	6.31	100.621		
Grouped by age class (in successive years)				
between years	-0.174	-2.95	$\Phi_{ST} = -0.0295$	1.00
within year	6.06	102.95		
Grouped by age class (within 2006)				
between age classes	-0.0437	-0.773	$\Phi_{ST} = -0.00773$	0.963
within an age class	5.70	100.773		
Grouped by age class (within 2007)				
between age classes	-0.0430	-0.659	$\Phi_{ST} = -0.00659$	0.969
within an age class	6.57	100.659		
Grouped by region (Atlantic coast only)				
Among regions	-0.0547	-0.87	$\Phi_{ST} = -0.00873$	1.00
within a region	6.32	100.87		
Grouped by region (Atlantic and Gulf combined)				
Among regions	0.360	6.01	$\Phi_{ST} = 0.0601$	**0.00000**
within a region	5.63	93.99		

Table 8 cont. Hierarchical variance partitioning and 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 grouped by recruitment 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-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and gulf menhaden), and by putative species (Atlantic and gulf menhaden). The distance method used was pairwise differences with 10,000 permutations. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	Variance	**% total**	**Φ Statistics**	***p***
Grouped by putative species (Atlantic and Gulf menhaden)				
between species	1.24	18.2	$\Phi_{ST} = 0.175$	**0.00000**
among regions within a species	-0.0447	-0.655	$\Phi_{SC} = -0.00800$	1.00
within a region	5.63	82.5		

Table 9. Hierarchical variance partitioning and analysis of molecular variance (AMOVA) among Atlantic menhaden (*Brevoortia tyrannus*) and gulf menhaden (*B. patronus*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) grouped by recruitment 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 latitude for the Atlantic coast samples (north = New England and mid-Atlantic, south = Chesapeake Bay and U.S. south Atlantic), by region for large scale-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and gulf menhaden), and by putative species (Atlantic and gulf menhaden). The distance method used was number of different alleles (F_{ST}) with 10,000 permutations. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	Variance	**% total**	***F* Statistics**	***p***
Grouped by recruitment time				
between recruitment times	-0.0109	-0.41	F_{ST}=-0.00413	0.743
within a recruitment time	2.65	100.41		
Grouped by age class (in successive years)				
between years	0.0483	1.80	F_{ST}=0.0180	**0.0176**
within year	2.64	98.20		
Grouped by age class (within 2006)				
between age classes	0.0271	1.06	F_{ST}=0.0106	0.0929
within an age class	2.52	98.94		
Grouped by age class (within 2007)				
between age classes	0.0140	0.52	F_{ST}=0.00520	0.160
within an age class	2.67	99.48		
Grouped by region (Atlantic coast only)				
among regions	0.0152	0.575	F_{ST}= 0.00575	**0.00000**
within a region	2.62	99.42		
Grouped by latitude (north, south: Atlantic coast only)				
between north and south	-0.00294	-0.112	F_{ST}= 0.00535	**0.00098**
within the latitudinal grouping	0.0171	0.646	F_{SC}= 0.00646	**0.00000**
within a region	2.62	99.47		

Table 9 cont. Hierarchical variance partitioning and analysis of molecular variance (AMOVA) among Atlantic menhaden (*Brevoortia tyrannus*) and gulf menhaden (*B. patronus*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) grouped by recruitment 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 latitude for the Atlantic coast samples (north = New England and mid-Atlantic, south = Chesapeake Bay and U.S. south Atlantic), by region for large scale-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and gulf menhaden), and by putative species (Atlantic and gulf menhaden). The distance method used was number of different alleles (F_{ST}) with 10,000 permutations. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	Variance	**% total**	***F* Statistics**	***p***
Grouped by region (large-scaled menhaden combined)				
among regions	0.128	4.68	F_{ST}= 0.0468	**0.00000**
within a region	2.61	95.32		
Grouped by putative species (Atlantic and Gulf menhaden)				
between species	0.344	11.50	F_{ST}= 0.120	**0.00000**
among regions within a species	0.0153	0.517	F_{SC}= -0.00584	**0.00000**
within a region	2.61	88.0		

Table 10. Hierarchical variance partitioning and analysis of molecular variance (AMOVA) among Atlantic menhaden (*Brevoortia tyrannus*) and gulf menhaden (*B. patronus*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) grouped by recruitment 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-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and gulf menhaden), and by putative species (Atlantic and gulf menhaden). The distance method used was number of different alleles (R_{ST}) with 10,000 permutations. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	Variance	**% total**	***F* Statistics**	***p***
Grouped by recruitment time				
between recruitment times	6.98	1.27	F_{ST}=0.0127	0.246
Within a recruitment time	541.3	98.73		
Grouped by age class (in successive years)				
between years	-5.31	-0.828	F_{ST}=-0.00828	0.730
Within year	647.3	100.83		
Grouped by age class (within 2006)				
between age classes	-5.17	-0.923	F_{ST}=-0.00923	0.678
Within an age class	565.3	100.923		
Grouped by age class (within 2007)				
between age classes	-2.25	-0.389	F_{ST}= -0.00389	0.580
Within an age class	581.9	100.389		
Grouped by region (Atlantic coast only)				
among regions	-0.382	-0.0665	F_{ST}= -0.00066	0.634
within a region	574.1	100.0665		
Grouped by region (large-scaled menhaden combined)				
among regions	13.29	16.89	F_{ST}= 0.169	**0.00000**
within a region	65.37	83.11		

Table 10 cont. Hierarchical variance partitioning and analysis of molecular variance (AMOVA) among Atlantic menhaden (*Brevoortia tyrannus*) and gulf menhaden (*B. patronus*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) grouped by recruitment 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-scale samples (New England, mid-Atlantic, Chesapeake Bay, U.S. south Atlantic, and gulf menhaden), and by putative species (Atlantic and gulf menhaden). The distance method used was number of different alleles (R_{ST}) with 10,000 permutations. Bolded *p*-values indicate significance ($p<0.05$) after Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	Variance	**% total**	***F* Statistics**	***p***
Grouped by putative species (Atlantic and gulf menhaden)				
between species	41.28	38.75	F_{ST} = 0.386	**0.00000**
among regions within a species	-0.120	-0.113	F_{SC} = -0.00185	0.727
within a region	65.37	61.36		

Table 11. Estimates of pairwise F_{ST} (below diagonal) and respective p-values (above diagonal) between regions of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) after 10,000 permutations. Bolded p-values indicate significance ($p<0.05$) after sequential Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	New England	**mid-Atlantic**	**Chesapeake Bay**	**U.S. south Atlantic**	**all Atlantic**	**gulf menhaden**	**finescale menhaden**	**yellowfin menhaden**
New England		0.252	0.180	0.973		**0.000000**	**0.000000**	**0.000000**
mid-Atlantic	0.00293		0.748	0.0811		**0.000000**	**0.000000**	**0.000000**
Chesapeake Bay	0.00233	-0.00211		0.0270		**0.000000**	**0.000000**	**0.000000**
U.S. south Atlantic	-0.00631	0.00679	0.00505			**0.000000**	**0.000000**	**0.000000**
all Atlantic						**0.000000**	**0.000000**	**0.000000**
gulf menhaden	0.0786	0.117	0.122	0.0818	0.104		**0.000000**	**0.000000**
finescale menhaden	0.371	0.327	0.320	0.333	0.309	0.436		**0.000000**
yellowfin menhaden	0.447	0.410	0.374	0.402	0.360	0.512	0.584	

Table 12. Estimates of pairwise R_{ST} (below diagonal) and respective p-values (above diagonal) between regions of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) after 10,000 permutations. Bolded p-values indicate significance ($p<0.05$) after sequential Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	New England	**mid-Atlantic**	**Chesapeake Bay**	**U.S. south Atlantic**	**all Atlantic**	**gulf menhaden**	**finescale menhaden**	**yellowfin menhaden**
New England		0.288	0.981	0.972		**0.00000**	**0.00000**	**0.00000**
mid-Atlantic	-0.00002		0.496	0.243		**0.00901**	**0.00000**	**0.00000**
Chesapeake Bay	-0.0112	-0.00214		0.883		**0.00000**	**0.00000**	**0.00000**
U.S. south Atlantic	-0.0116	0.00111	-0.00559			**0.00089**	**0.00000**	**0.00000**
all Atlantic						**0.00000**	**0.00000**	**0.00000**
gulf menhaden	0.0908	0.101	0.110	0.0862	0.118		**0.00000**	**0.00000**
finescale menhaden	0.717	0.628	0.621	0.662	0.637	0.734		**0.00000**
yellowfin menhaden	0.764	0.683	0.673	0.711	0.686	0.769	0.182	

Table 13. Estimates of pairwise Φ_{ST} (below diagonal) and respective p-values (above diagonal) between regions of Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*) based on cytochorme *c* oxidase subunit I (COI) sequence data after 10,000 permutations. Bolded p-values indicate significance ($p<0.05$) after sequential Bonferroni correction (initial $\alpha = 0.05 / 2 = 0.025$).

	New England	**mid-Atlantic**	**Chesapeake Bay**	**U.S. south Atlantic**	**all Atlantic**	**gulf menhaden**	**finescale menhaden**	**yellowfin menhaden**
New England		0.854	0.787	0.705		**0.00000**	**0.00000**	**0.00000**
mid-Atlantic	-0.0142		0.962	0.722		**0.0000**	**0.00000**	**0.00000**
Chesapeake Bay	-0.0100	-0.0112		0.440		**0.0000**	**0.00000**	**0.00000**
U.S. south Atlantic	-0.0108	-0.00991	-0.00299			**0.0000**	**0.00000**	**0.00000**
all Atlantic						**0.0000**	**0.00000**	**0.00000**
gulf menhaden	0.244	0.213	0.192	0.236	0.178		**0.00000**	**0.00000**
finescale menhaden	0.595	0.563	0.580	0.545	0.564	0.804		0.298
yellowfin menhaden	0.482	0.450	0.485	0.438	0.478	0.705	0.00347	

Table 14. Literature values of within-sample microsatellite variation: number of loci (# loci), allelic diversity (A; alleles per locus) and expected heterozygosity (H_E).

Species	# loci	A	H_E	Reference
allis shad (*Alosa alosa*)	8	3-9	0.241-0.805	Faria *et al.* 2004
American shad (*A. sapidissima*)	6	7-29	0.73-0.92	Brown *et al.* 2000
Atlantic herring (*Clupea harengus*)	9	15.2-17.7	0.818-0.843	Mariani *et al.* 2005
	9	2-21	0.269-0.932	Jørgensen *et al.* 2005
	12	12-56	0.66-0.98	McPherson *et al.* 2001
	12	9-56	0.73-0.95	Miller *et al.* 2001
	4	13-41	0.88-.95	Shaw *et al.* 1999
Atlantic menhaden (*B. tyrannus*)	4	3-20	0.67	Anderson 2007
finescale menhaden (*B. gunteri*)	4	1-11	0.41	Anderson 2007
	5	3-13	0.066-0.823	Anderson and McDonald 2007
gulf menhaden (*B. patronus*)	4	4-19	0.67	Anderson 2007
	5	8-21	0.126-0.923	Anderson and McDonald 2007
Pacific herring (*C. pallasi*)	5	8-44	0.815-0.945	Sugaya *et al.* 2008
	14	5-49	0.20-0.96	Olsen *et al.* 2002
	5	12-26	0.737-0.942	O'Connell *et al.* 1998
twaite shad (*A. fallax*)	9	4-12	0.151-0.811	Volk *et al.* 2007
	8	3-7	0.234-0.692	Faria *et al.* 2004
yellowfin menhaden (*B. smithi*)	4	2-8	0.37	Anderson 2007

Table 15. Statistically significant literature values of F_{ST} between genetically distinct stocks (after sequential Bonferroni correction).

Species	# Loci	F_{ST}	Reference
Arctic charr (*Salvelinus alpines*)	5	0.031 - 0.055, $p < 0.05$	Westgaard *et al.* 2004
Atlantic herring (*Clupea harengus*)	4	0.008 - 0.043, $p < 0.001$	Shaw *et al.* 1999
coastal cutthroat trout (*Oncorhynchus clarki clarki*)	6	0.064-0.226, $p < 0.05$	Wenburg *et al.* 1998
cod (*Gadus morhua*)	6	0.001-0.109, $p < 0.05$	O'Leary *et al.* 2007
common carp (*Cyprinus carpio*)	4	0.05, $p < 0.001$	Lehoczky *et al.* 2005
Japanese flounder (*Paralichthys olivaceus*)	11	0.012-0.077, $p < 0.005$	Sekino and Hara 2001
Pacific herring (*C. pallasii*)	5	0.046-0.173, $p < 0.05$	Sugaya *et al.* 2008
Patagonian toothfish (*Dissostichus eleginoides*)	8	0.017-0.059, $p < 0.05$	Smith and McVeagh 2000
redfin culter (*Culter erythropterus*)	5	0.062, $p < 0.001$	Wang *et al.* 2006

FIGURES

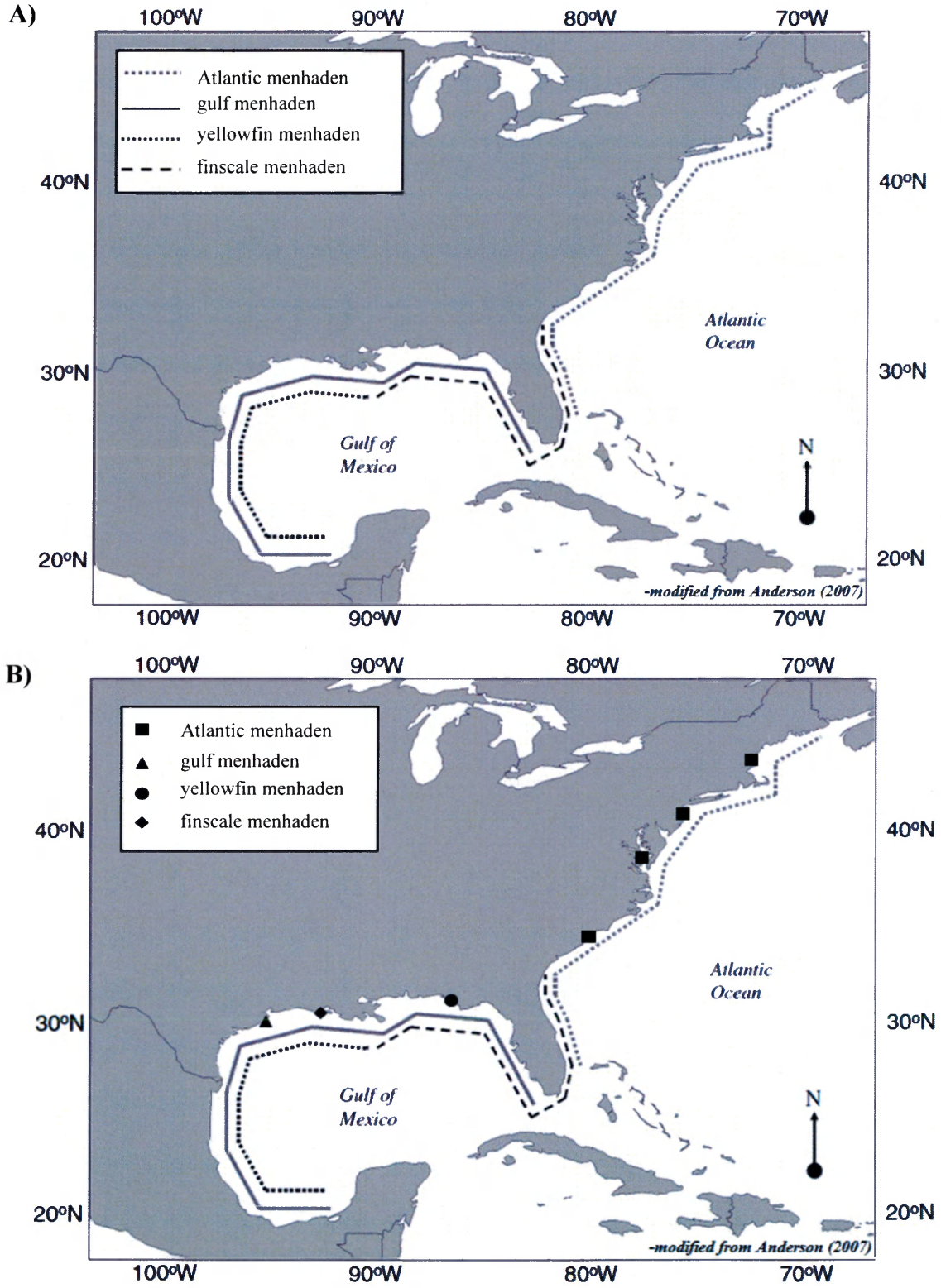

Figure 1. **A)** Map of ranges of the North American menhaden species: Atlantic menhaden (*Brevoortia tyrannus*), gulf menhaden (*B. patronus*), yellowfin menhaden (*B. smithi*), and finescale menhaden (*B. gunteri*). **B)** Map of sampling locations in the Atlantic Ocean and the Gulf of Mexico.

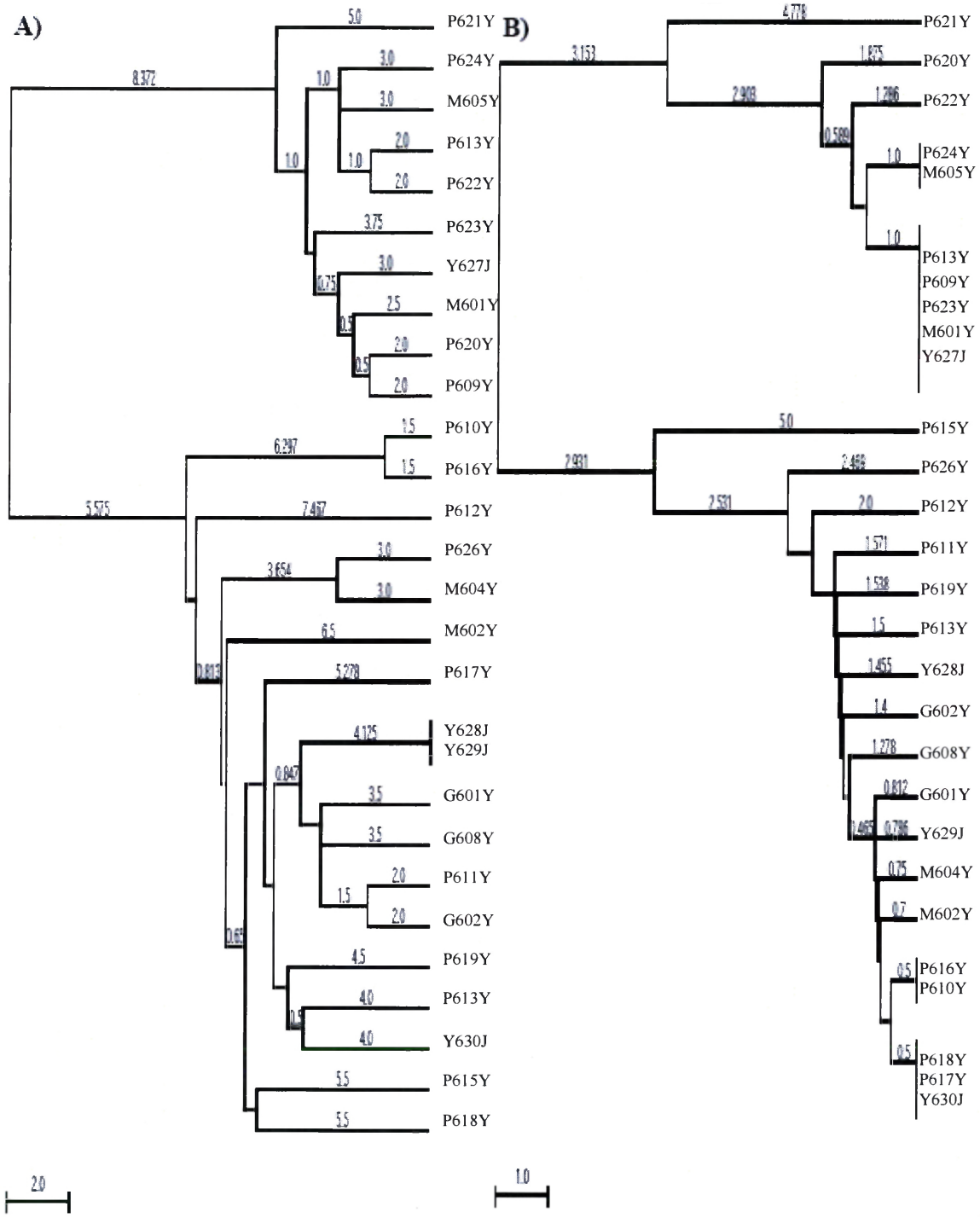

Figure 2. Unweighted pair group method with arithmetic mean (UPGMA) trees of a preliminary survey of Atlantic menhaden (*Brevoortia tyrannus*, *n*=25) and gulf menhaden (*B. patronus*, *n*=3) **A)** mitochondrial control region sequences and **B)** cytochrome *c* oxidase subunit I sequences. The samples are coded by region (M, P, Y=Chesapeake Bay, G=Gulf of Mexico), age (J=yearling, Y=YOY) and year collected (6=2006, 7=2007). Divergence of clades is given as an absolute number of base pair differences.

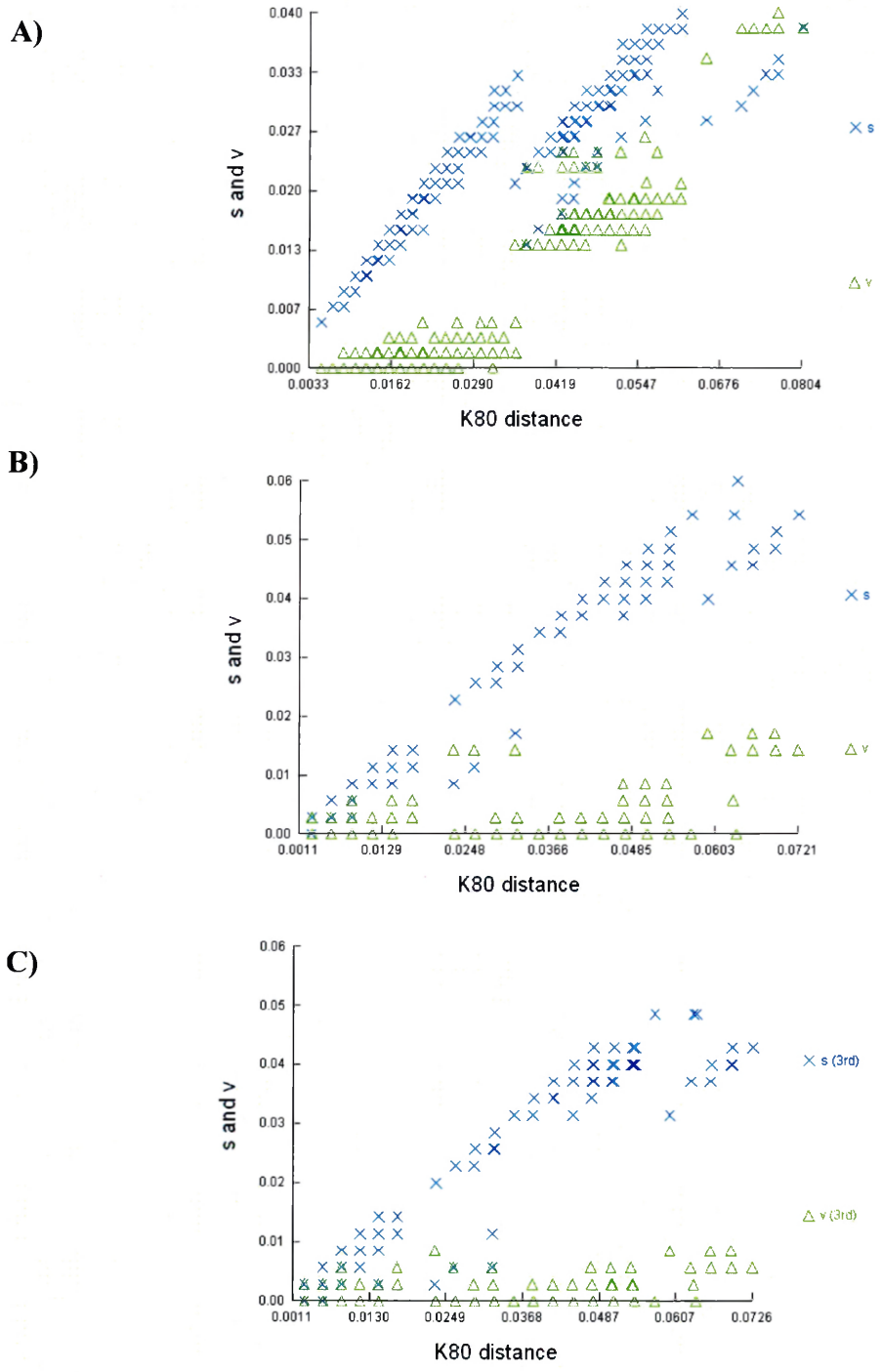

Figure 3. Nucleotide substitution patterns observed in pairwise comparisons of an initial survey of *Brevoortia* mitochondrial **A)** control region sequences overall, **B)** cytochrome *c* oxidase subunit I (COI) sequences overall, and **C)** COI third codon positions. The x-axis is the Kimura (1980) distances between sequences; the y-axis represents transitions (blue Xs) and transversions (green triangles).

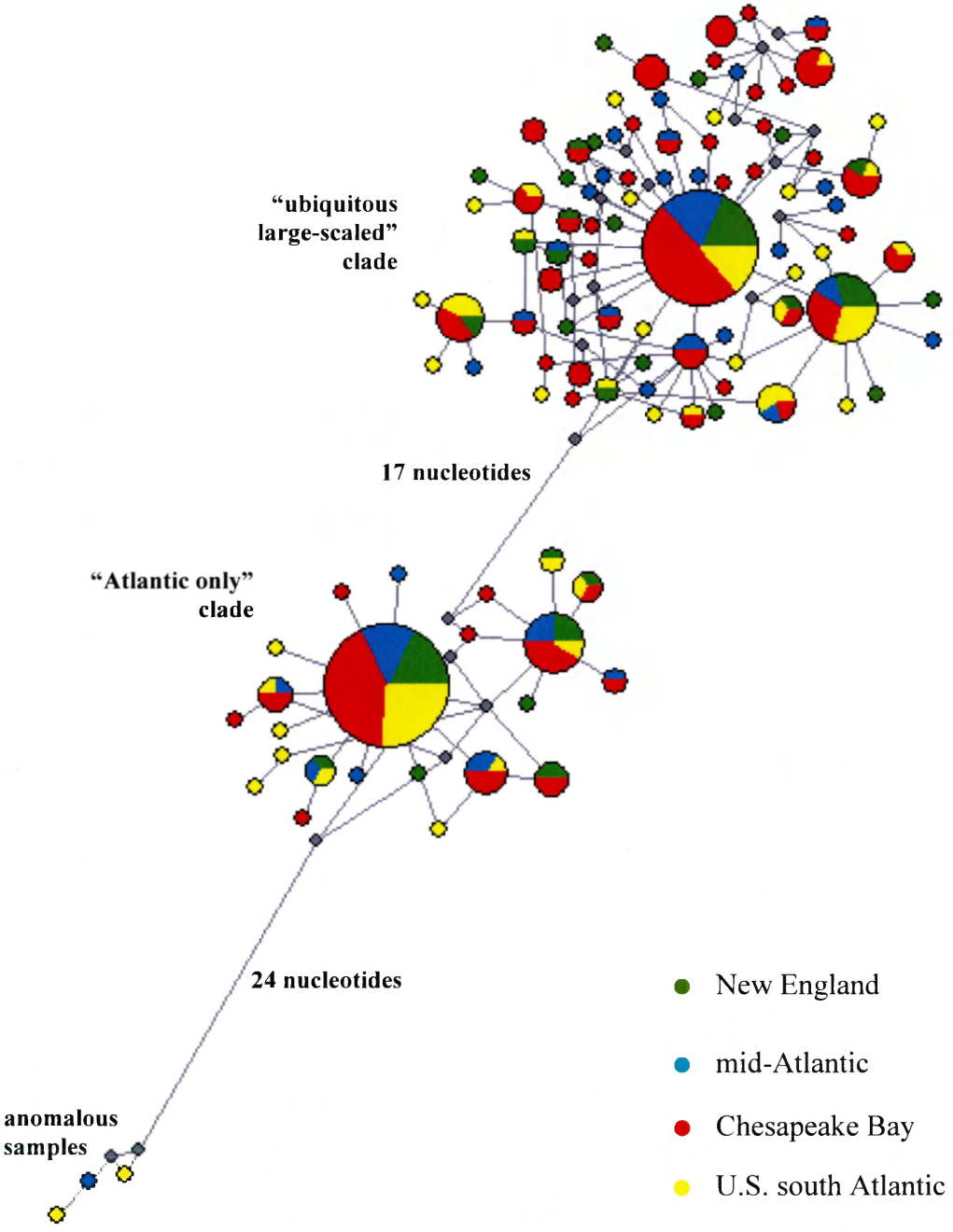

Figure 4. Median-joining network of 109 Atlantic menhaden (*Brevoortia tyrannus*, *n*=289) cytochrome *c* oxidase subunit I (COI) haplotypes. The observed haplotypes are sized according to frequency by regional proportions: New England (*n*=50; green), mid-Atlantic (*n*=53; aqua), Chesapeake Bay (*n*=117; red), U.S. south Atlantic, (*n*=69; yellow). The hypothesized intermediate haplotypes are displayed in gray.

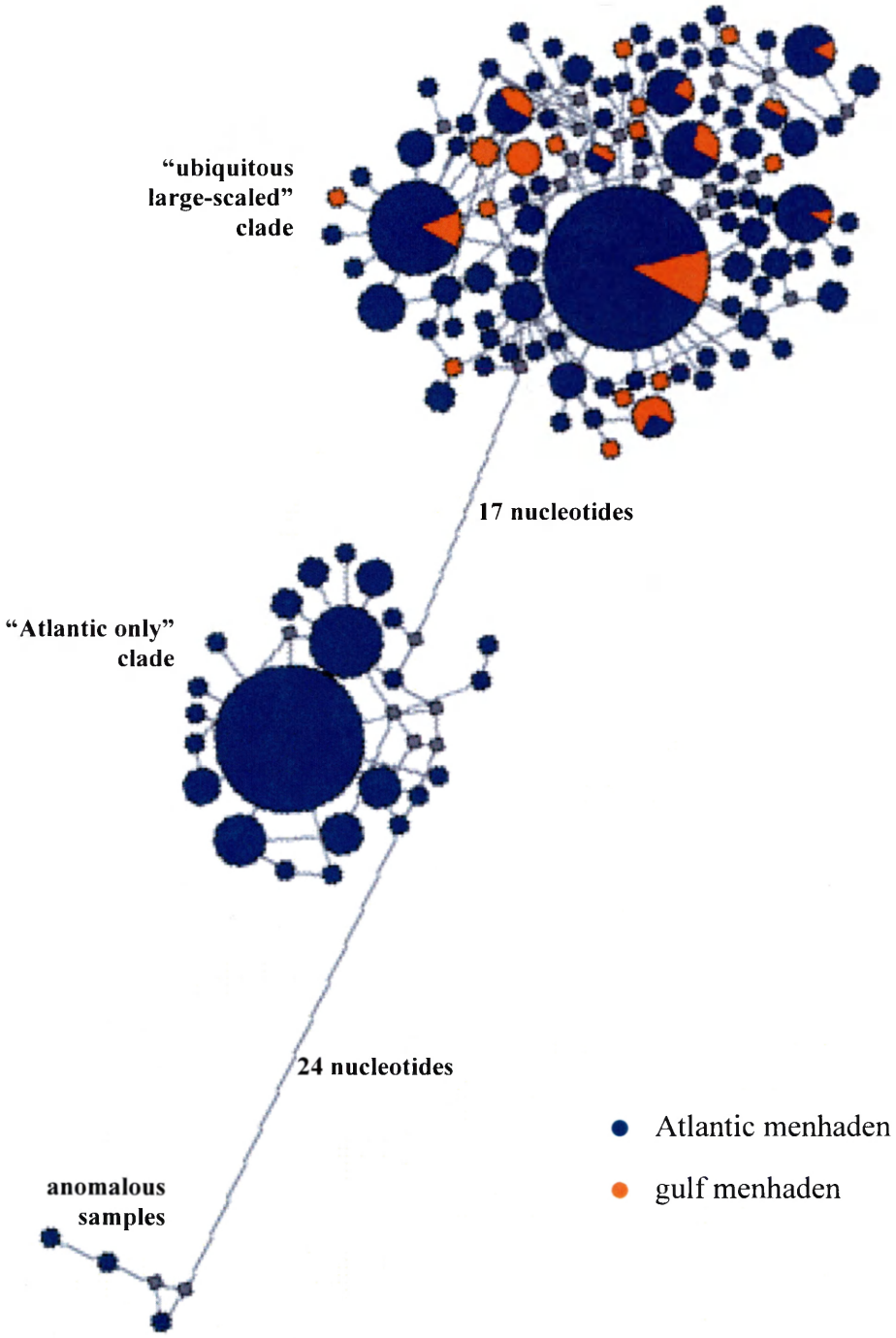

Figure 5. Median-joining network of 124 Atlantic menhaden (*Brevoortia tyrannus*, *n*=289), gulf menhaden (*B. patronus*, *n*=50) cytochrome *c* oxidase subunit I (COI) haplotypes. The observed haplotypes are sized according to frequency by species proportion: Atlantic menhaden (navy), gulf menhaden (orange). The hypothesized intermediate haplotypes are displayed in gray.

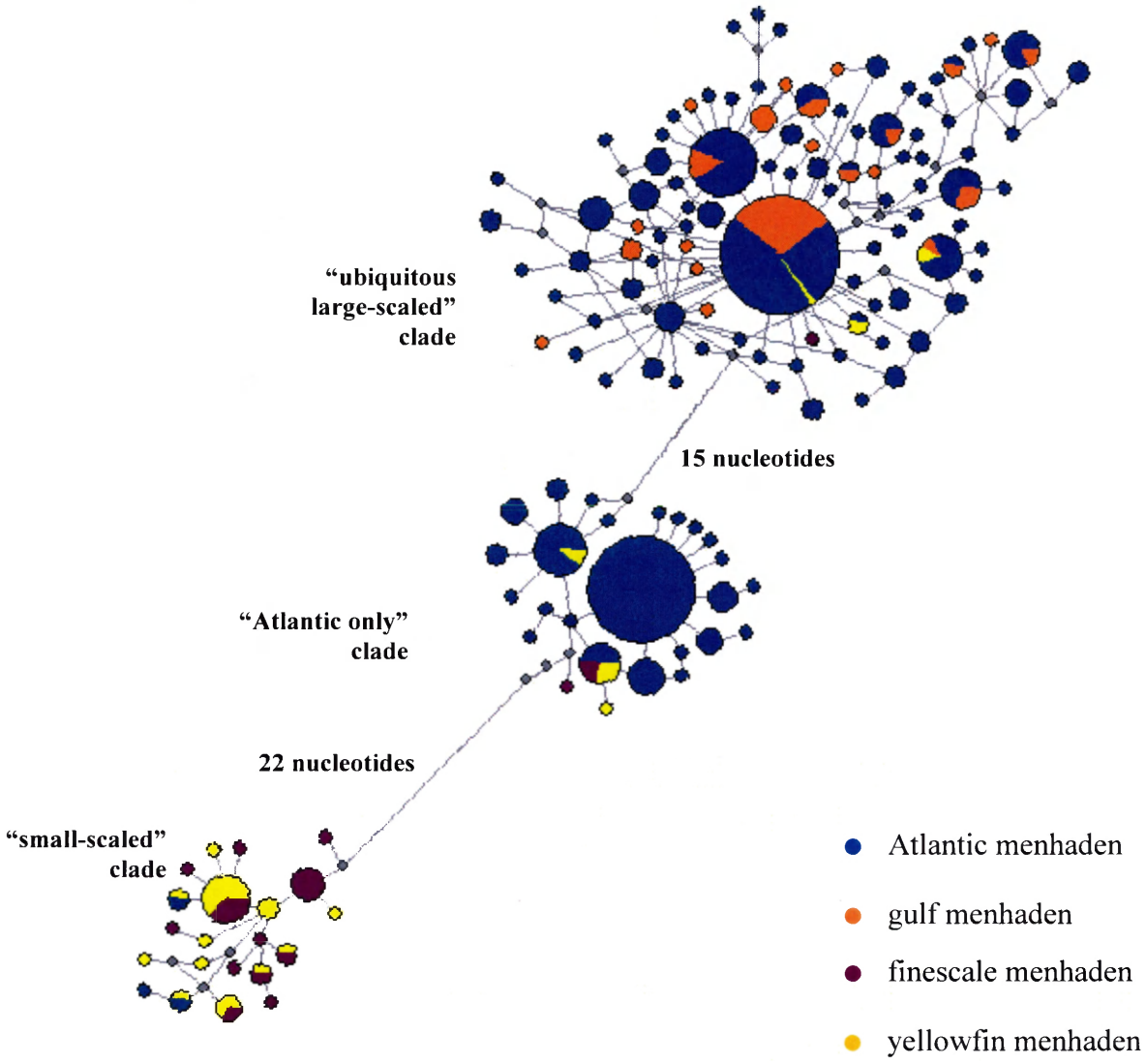

Figure 6. Median-joining network of 145 Atlantic menhaden (*Brevoortia tyrannus*, *n*=289), gulf menhaden (*B. patronus*, *n*=50), yellowfin menhaden (*B. smithi*, *n*=25), and finescale menhaden (*B. gunteri*, *n*=25) cytochrome *c* oxidase subunit I (COI) haplotypes. The observed haplotypes are sized according to frequency by species proportion: *Brevoortia tyrannus* (navy); *B. patronus* (orange); *B. gunteri* (purple); *B. smithi* (yellow). The hypothesized intermediate haplotypes are displayed in gray.

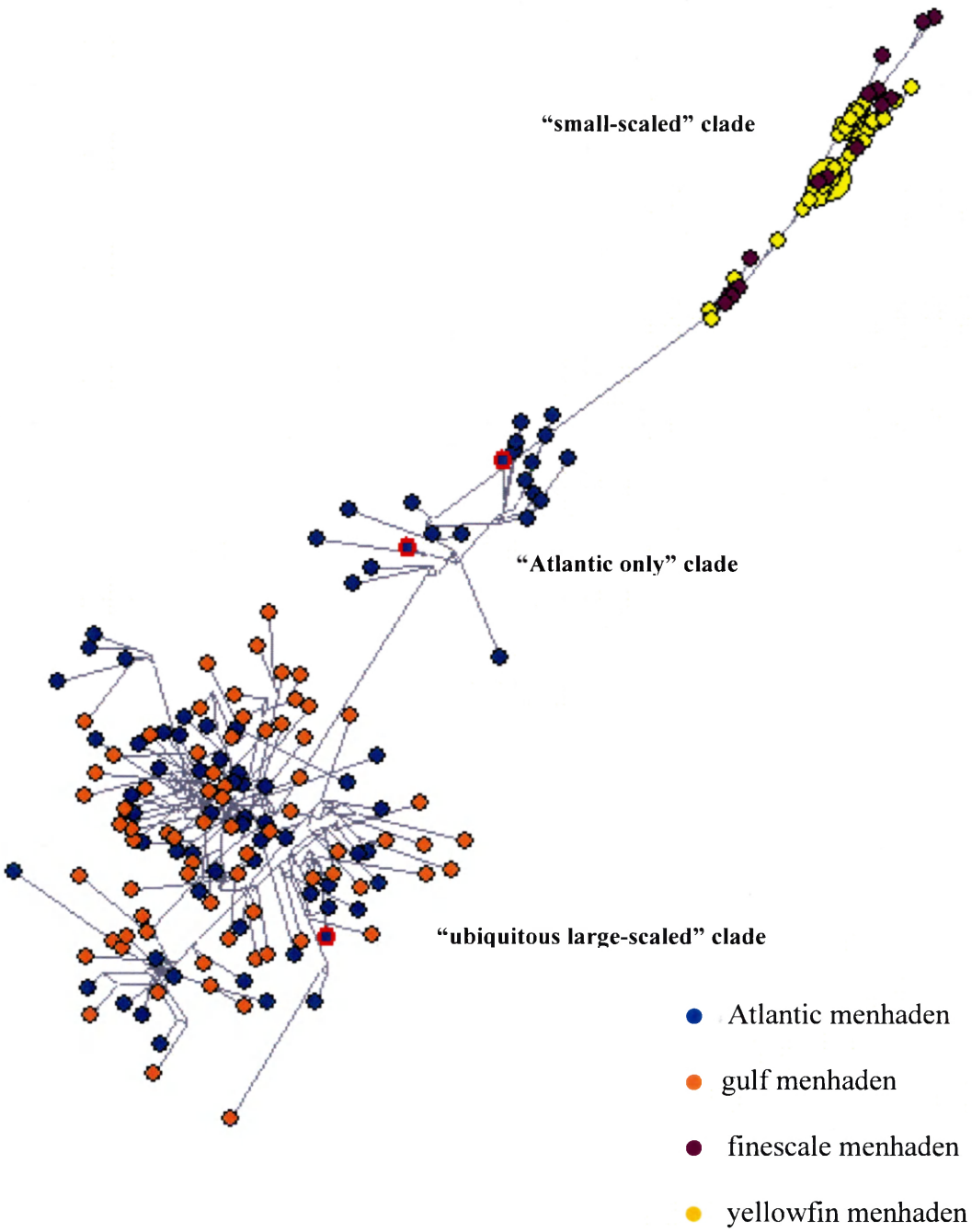

Figure 7. Median-joining network of 214 Atlantic menhaden (*Brevoortia tyrannus*, *n*=76), gulf menhaden (*B. patronus*, *n*=73), yellowfin menhaden (*B. smithi*, *n*=57), and finescale menhaden (*B. gunteri*, *n*=15) control region haplotypes from Anderson (2007) and three anomalous samples from this study (outlined in red). The observed haplotypes are sized according to frequency by species proportion: *Brevoortia tyrannus* (navy); *B. patronus* (orange); *B. gunteri* (purple); *B. smithi* (yellow).

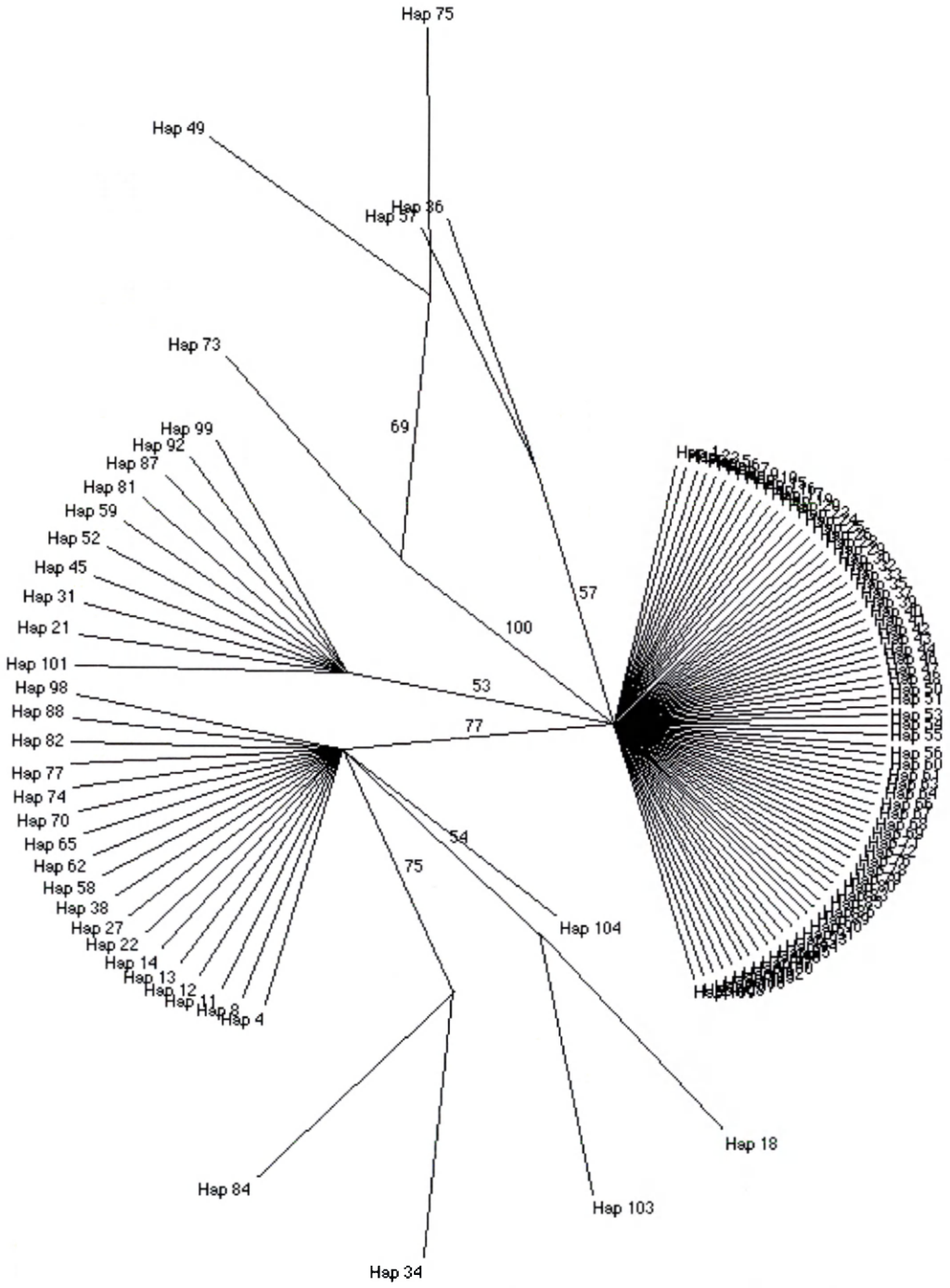

Figure 8. Unrooted maximum likelihood tree of 145 Atlantic menhaden (*Brevoortia tyrannus*, *n*=289), gulf menhaden (*B. patronus*, *n*=50), yellowfin menhaden (*B. smithi*, *n*=25), and finescale menhaden (*B. gunteri*, *n*=25) cytochrome *c* oxidase subunit I (COI) haplotypes using maximum likelihood distances calculated by the HKY + I + Γ model (Hasegawa *et al*. 1985). For haplotype codes by region and sample, see Appendix 3. Nodal support was assessed with bootstrapping (10 pseudoreplicates, 10 random addition) and the numbers below each data bipartion indicate bootstrap support (if >50%).

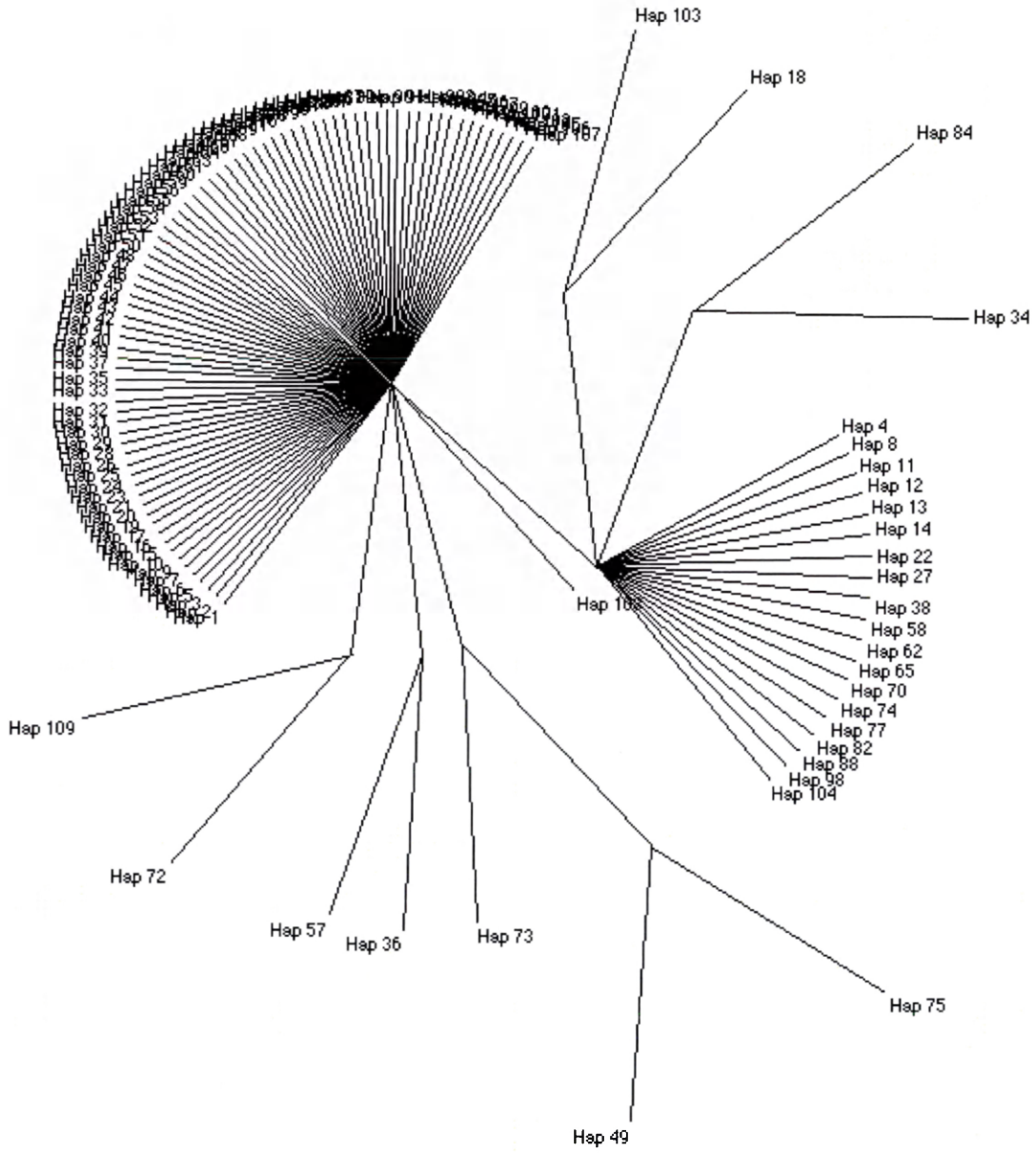

Figure 9. Unrooted Bayesian tree of 145 Atlantic menhaden (*Brevoortia tyrannus*, *n*=289), gulf menhaden (*B. patronus*, *n*=50), yellowfin menhaden (*B. smithi*, *n*=25), and finescale menhaden (*B. gunteri*, *n*=25) cytochrome *c* oxidase subunit I (COI) haplotypes using maximum likelihood distances calculated by the HKY + I + Γ model (Hasegawa *et al*. 1985). For haplotype codes by region and sample, see Appendix 3. The analysis used Markov Chain Monte Carlo simulations with 4,000,000 generations sampled every 1,000 generations.

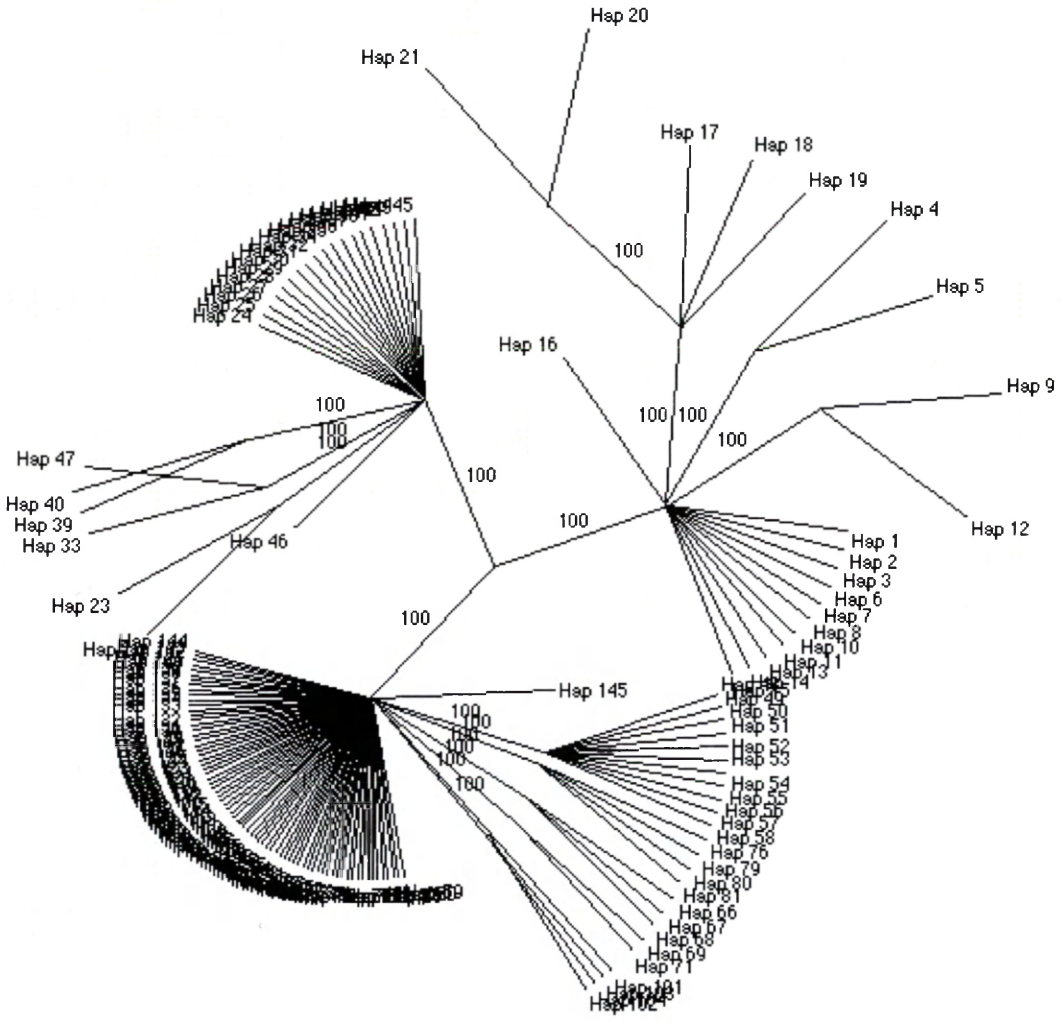

Figure 10. Unrooted maximum parsimony tree of 145 Atlantic menhaden (*Brevoortia tyrannus*, *n*=289), gulf menhaden (*B. patronus*, *n*=50), yellowfin menhaden (*B. smithi*, *n*=25), and finescale menhaden (*B. gunteri*, *n*=25) cytochrome *c* oxidase subunit I (COI) haplotypes. For haplotype codes by region and sample, see Appendix 3. Nodal support was assessed with bootstrapping (100 pseudoreplicates, 10 random addition) and the numbers below each data bipartion indicate bootstrap support (if >50%).

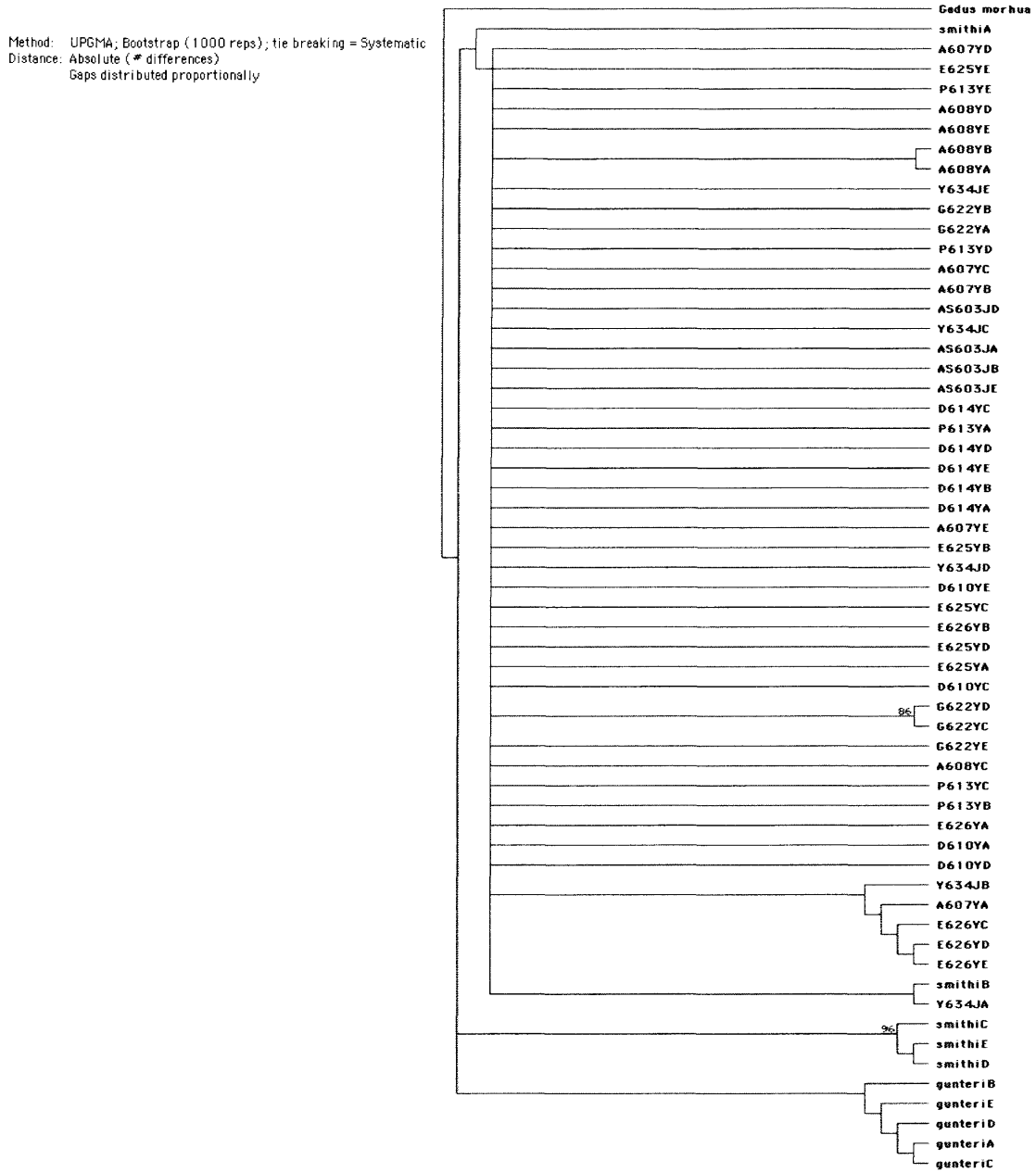

Figure 11. Unweighted pair group method with arithmetic mean (UPGMA) tree of an initial survey of Atlantic menhaden (*Brevoortia tyrannus*, *n*=9), gulf menhaden (*B. patronus*, *n*=1), yellowfin menhaden (*B. smithi*, *n*=1), and finescale menhaden (*B. gunteri*, *n*=1) internal transcribed spacer 1 sequences. The samples are coded by location (New England = A, mid Atlantic = D, Chesapeake Bay = M, P, Y, U.S. south Atlantic = AS, gulf menhaden = G, yellowfin menhaden = smithi, finescale menhaden = gunteri), age (J = yearling, Y = YOY), year collected (6=2006, 7=2007), and clone number (the final digit). Divergence of clades is given as absolute number of base pair differences. Nodal support was assessed with bootstrapping (1,000 pseudoreplicates, systematic tie breaking) and the numbers of below each data bipartion indicate bootstrap support (if >50%).

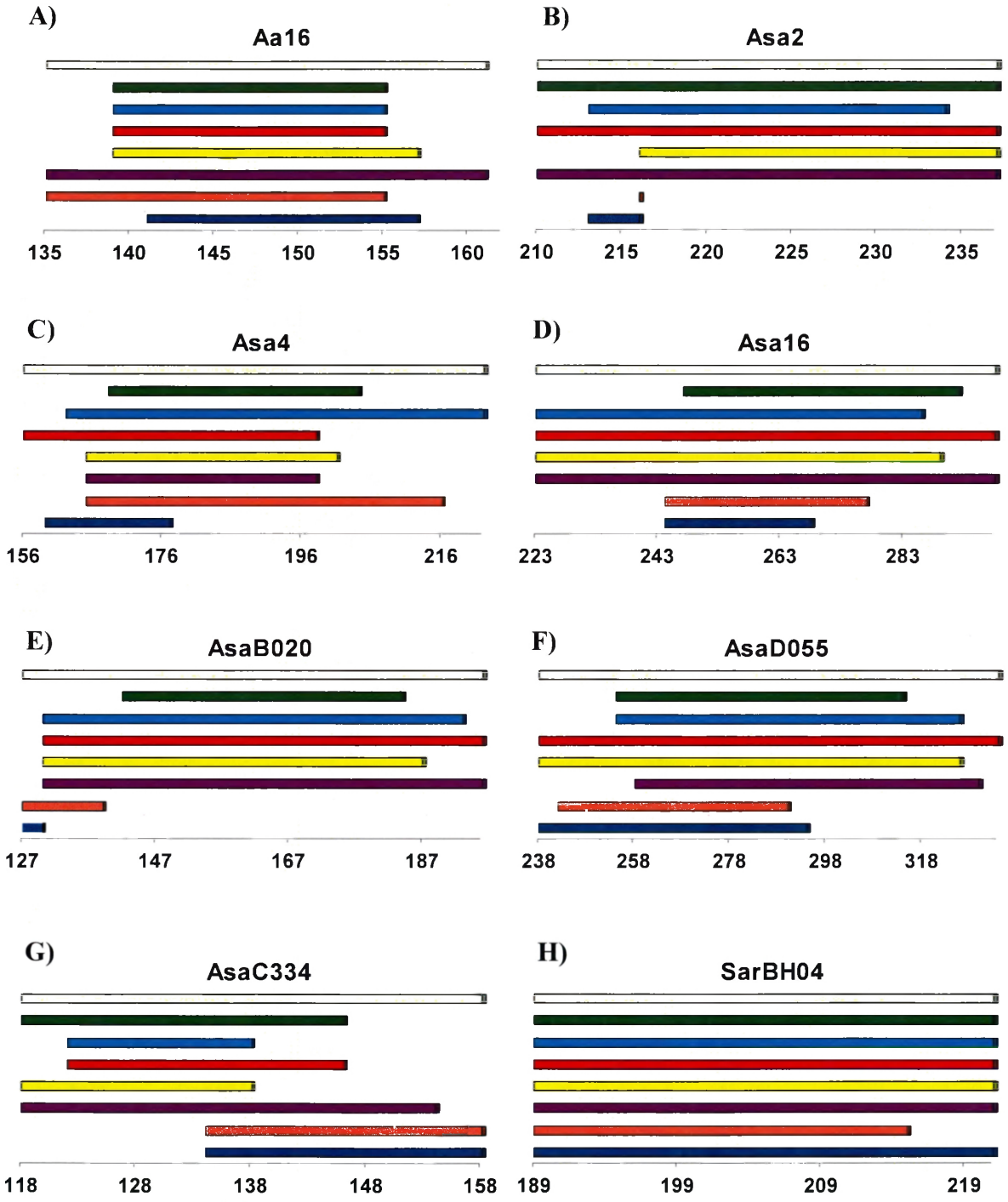

Figure 12. Allele ranges by region for the 8 microsatellite loci: **A)** Aa16, **B)** Asa2, **C)** Asa4, **D)** Asa16, **E)** AsaB020, **F)** AsaD055, **G)** AsaC334, **H)** SarBH04. Samples are coded by region in descending order: full allele range (white); *Brevoortia tyrannus*, New England (green), mid-Atlantic (aqua), Chesapeake Bay (red), U.S. south Atlantic (yellow); *B. patronus* (purple); *B. gunteri* (orange); *B. smithi* (navy).

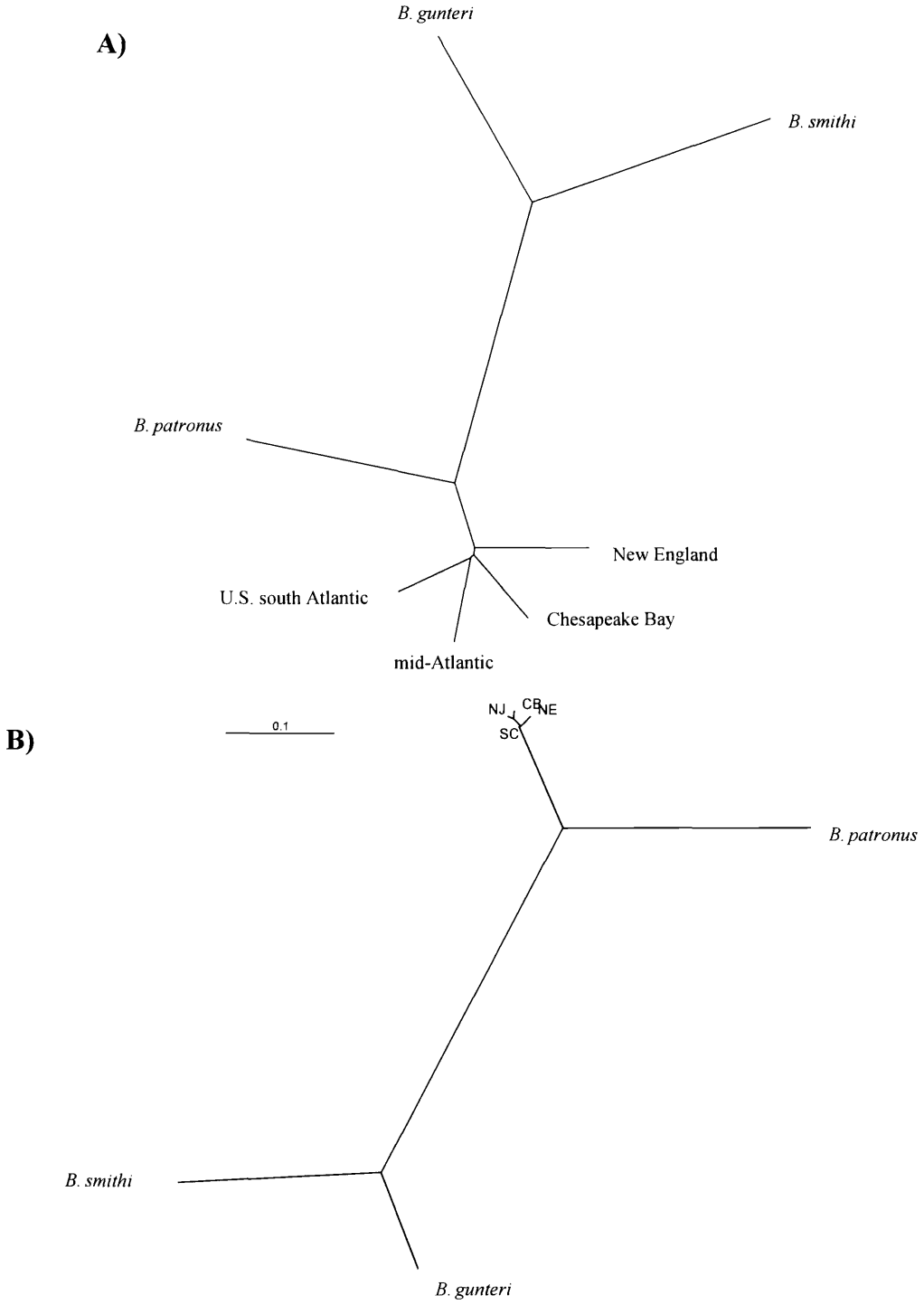

Figure 13. Unrooted neighbor-joining trees of Atlantic menhaden (*Brevoortia tyrannus*) sampling regions: New England (NE), mid-Atlantic (NJ), Chesapeake Bay (CB), and U.S. south Atlantic (SC); gulf menhaden (*B. patronus*); finescale menhaden (*B. gunteri*); and yellowfin menhaden (*B. smithi*) with 7 microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) using **A)** modified Cavalli-Sforza chord distance (D_A; Nei *et al.* 1983) and **B)** Nei's standard genetic distance (D_{ST}; Nei 1972).

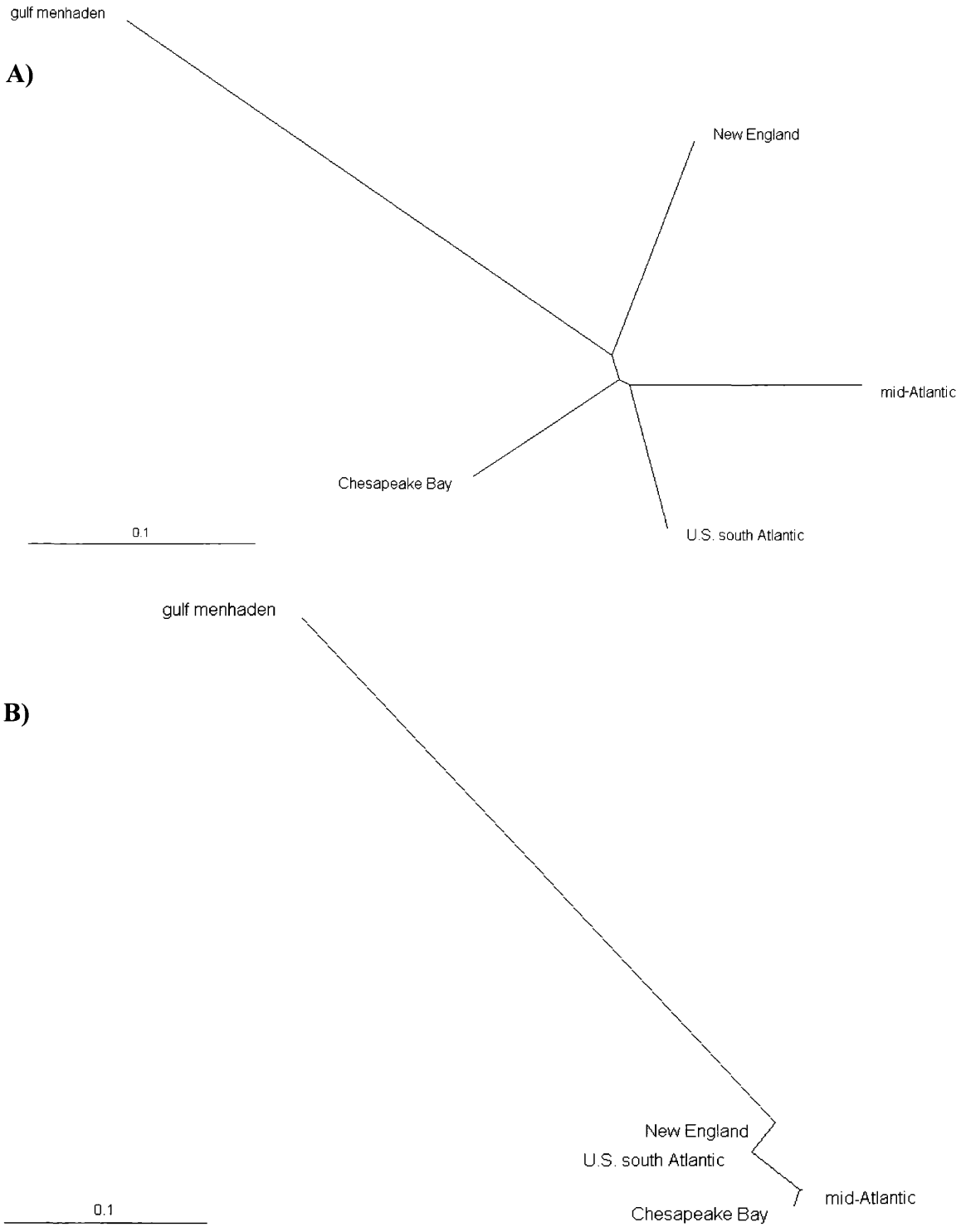

Figure 14. Unrooted neighbor-joining trees of Atlantic menhaden (*Brevoortia tyrannus*) sampling regions (New England, mid-Atlantic, Chesapeake Bay, and U.S. south Atlantic) and gulf menhaden (*B. patronus*) as an outgroup with 7 microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) using **A)** modified Cavalli-Sforza chord distance (D_A; *Nei et al.* 1983) and **B)** Nei's standard genetic distance (D_{ST}; Nei 1972).

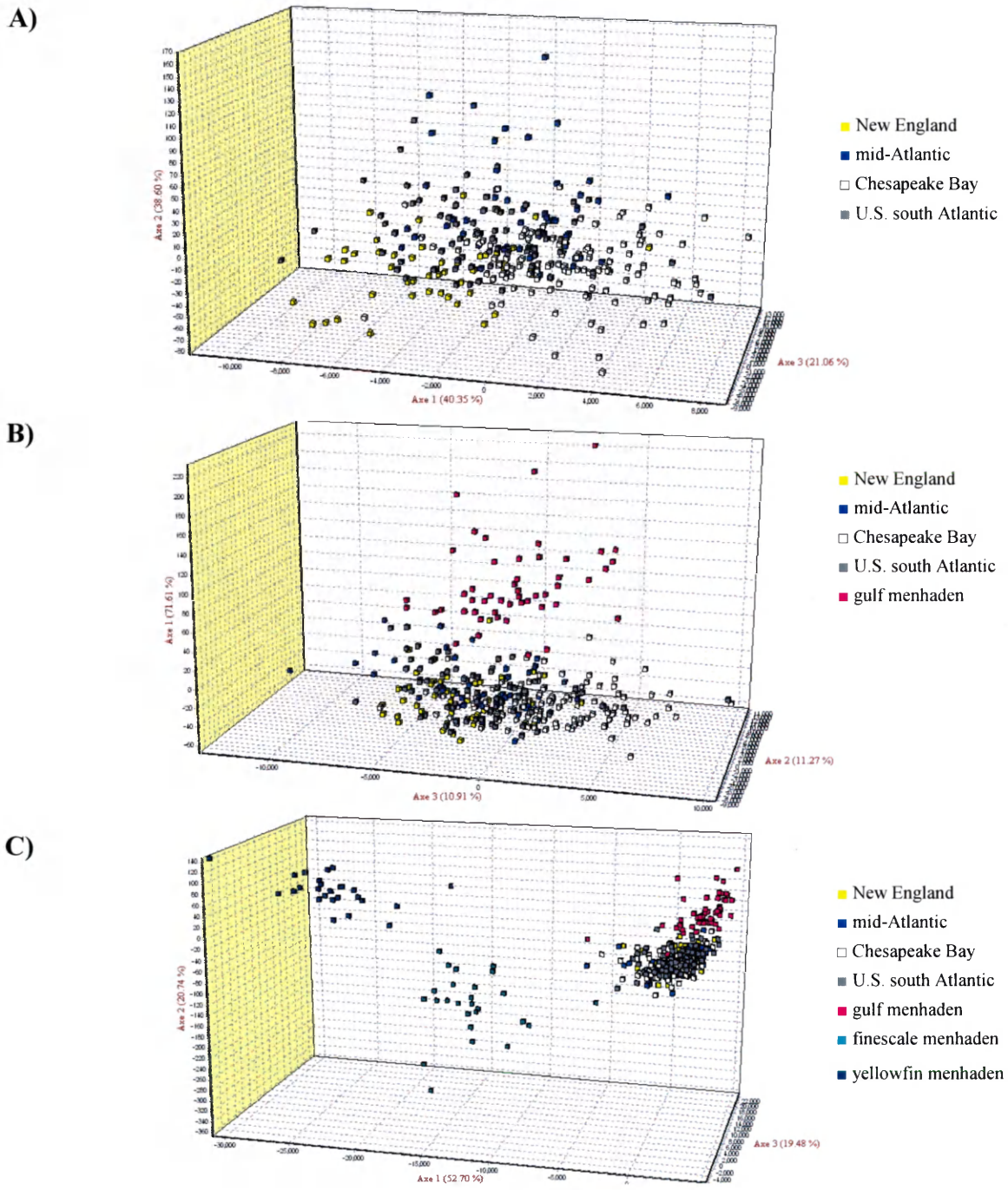

Figure 15. Spatial representation of genetic relationships based on 7 microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) between **A)** Atlantic menhanden, *Brevoortia tyrannus*: New England (yellow), mid-Atlantic (blue), Chesapeake Bay (white), U.S. south Atlantic (gray), **B)** large-scale menhaden, *B. tyrannus*: New England (yellow), mid-Atlantic (blue), Chesapeake Bay (white), U.S. south Atlantic (gray); *B. patronus* (magenta), **C)** North American *Brevoortia*, *B. tyrannus*: New England (yellow), mid-Atlantic (blue), Chesapeake Bay (white), U.S. south Atlantic (gray); *B. patronus* (magenta); *B. gunteri* (aqua); *B. smithi* (navy).

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APPENDICES

Appendix 1. Sample collection.

Please see attached electronic version.

Appendix 2. 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' (Brendtro *et al.* 2008)

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

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

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°C annealing for 1 minute
	72°C extension for 2 minutes
1 cycle	72°C extension for 5 minutes
Hold	4°C

Microsatellites

For each 5μL PCR reaction,

- 2.78μL sterile, filtered H_2O
- 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 °C annealing for 45 seconds*
	72°C extension for 45 seconds
1 cycle	72°C extension for 7 minutes
Hold	4°C

* Primers and annealing temperatures specific to each locus are listed in Table 1.

Appendix 3. **A)** Aligned mitochondrial control region sequence data, **B)** aligned mitochondrial cytochrome *c* oxidase subunit I (COI) sequence data, **C)** COI haplotypes, **D)** COI amino acid sequence data, **E)** aligned nuclear internal transcribed spacer 1 (ITS-1) sequence data, and **F)** allele scores for seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) collected from *Brevoortia* specimens.

Appendix 3. **A)** Aligned mitochondrial control region sequence data collected from 28 *Brevoortia* specimens.

Please see attached electronic version.

Appendix 3. **B)** Aligned mitochondrial cytochrome *c* oxidase subunit I (COI) sequence data collected from 389 *Brevoortia* specimens.

Please see attached electronic version.

Appendix 3. **C)** Cytochrome *c* oxidase subunit I (COI) haplotypes from 389 *Brevoortia* specimens coded by region and sample.

Haplotype Code:	***B. smithi***	***B. gunteri***	***B. patronus***	***B. tyrannus***
	X			
		X		
			X	
				X
			X	X
	X	X		
	X			X
		X		X
	X	X		X
	X		X	X

haplotypes	haplotypes	haplotypes	haplotypes	haplotypes
1	30	59	88	117
2	31	60	89	118
3	32	61	90	119
4	33	62	91	120
5	34	63	92	121
6	35	64	93	122
7	36	65	94	123
8	37	66	95	124
9	38	67	96	125
10	39	68	97	126
11	40	69	98	127
12	41	70	99	128
13	42	71	100	129
14	43	72	101	130
15	44	73	102	131
16	45	74	103	132
17	46	75	104	133
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19	48	77	106	135
20	49	78	107	136
21	50	79	108	137
22	51	80	109	138
23	52	81	110	139
24	53	82	111	140
25	54	83	112	141
26	55	84	113	142
27	56	85	114	143
28	57	86	115	144
29	58	87	116	145

haplotype	sample
1	SMI23
2	GUN25
3	GUN15
3	GUN19
3	GUN24
3	Gun3
3	Gun7
4	GUN11
5	Gun10
5	SMI14
6	GUN17
7	GUN14
8	Gun8
8	Smi2
9	Gun9
10	Gun5
11	GUN12
11	GUN13
11	GUN16
11	GUN21
11	Smi1
11	SMI15
11	SMI16
11	SMI18
11	SMI19
11	SMI24
11	SMI25
12	SMI21
13	GUN18
13	GUN23
14	GUN20
15	Smi10
16	E717Y
16	SMI20
17	GUN22
17	smi11
17	SMI22
18	SMI13
19	SMI12
20	D705Y
20	SMI17
21	E715Y
22	AS603J
23	E705Y
24	Gun4
25	A615Y
25	AS613J
26	Y729J
27	SH708Y

haplotype	sample
28	M764Y
29	D725Y
29	Y639J
30	A621Y
30	A622Y
30	CH737J
30	D603Y
30	D702Y
30	D703Y
30	Gun6
30	M605Y
30	SH718Y
30	Y707Y
30	Y711Y
30	Y719Y
30	Y745J
31	E721Y
31	GH701Y
31	M763Y
32	D723Y
33	P622Y
34	Smi8
35	D708Y
35	D712Y
35	E707Y
35	P620Y
35	Y720Y
35	Y725Y
36	Gun1
36	Gun2
36	P621Y
36	SH723Y
36	SH729Y
36	Smi5
36	Smi9
36	Y747J
37	AS608J
38	CH731J
39	Y721Y
40	AS612J
40	D701Y
40	Y716Y
40	Y738J
41	Y637J
42	Smi3
43	E624Y
44	E722Y
45	D626Y
46	A607Y

haplotype	sample
46	A612Y
46	A613Y
46	A616Y
46	AS602J
46	AS607J
46	CH726J
46	CH727J
46	CH728J
46	CH734J
46	CH735J
46	CH736J
46	CH740J
46	D611Y
46	D614Y
46	D624Y
46	D722Y
46	D724Y
46	D726Y
46	D727Y
46	E703Y
46	E704Y
46	E708Y
46	E712Y
46	GH703Y
46	M601Y
46	M751Y
46	M753Y
46	M754Y
46	M756Y
46	M759Y
46	P609Y
46	P613Y
46	P623Y
46	SH706Y
46	SH709Y
46	SH713Y
46	SH719Y
46	Y628J
46	Y642J
46	Y703Y
46	Y709Y
46	Y710Y
46	Y712Y
46	Y714Y
46	Y727J
46	Y732J
46	Y733J
46	Y773Y
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haplotype	sample
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47	D709Y
47	E706Y
48	D610Y
48	M760Y
49	A617Y
50	E720Y
51	D706Y
52	G720Y
53	Y640J
54	P625Y
55	G723Y
55	Y748J
56	D711Y
56	D718Y
56	E711Y
56	G713Y
56	M603Y
56	Y734J
57	Y636J
58	P619Y
58	Y629J
58	Y635J
59	Y713Y
60	P615Y
60	P626Y
61	G614Y
62	D616Y
63	D608Y
64	E701Y
65	E623Y
66	Y724Y
67	A620Y
68	D601Y
69	A609Y
70	G604Y
71	G703Y
71	M757Y
71	P616Y
71	Y627J
71	Y774Y
72	Y632J
73	AS604J
74	D721Y
75	D620Y
76	AS611J
77	D606Y
77	M604Y
78	E716Y

haplotype	sample
78	SH722Y
79	D612Y
80	E627J
81	AS614J
81	E628J
81	E714Y
81	G712Y
81	SH705Y
81	Smi4
81	Y641J
81	Y643J
81	Y737J
82	Y706Y
83	G718Y
84	M767Y
85	AS610J
85	G602Y
85	G704Y
85	G721Y
86	G710Y
87	M766Y
87	Y771Y
88	A611Y
89	G624Y
90	A608Y
90	Smi6
91	Y739J
92	D704Y
93	G605Y
94	E724Y
95	G601Y
96	Y728J
97	Y749J
98	SH710Y
99	A603Y
99	D604Y
100	Y722Y
101	E709Y
102	CH732J
102	G702Y
102	G709Y
102	SH724Y
102	Y638J
102	Y742J
102	Y750J
103	A604Y
104	P614Y
104	P624Y
105	G708Y

haplotype	sample
106	A610Y
106	Y634J
107	SH707Y
108	D619Y
109	E713Y
110	CH738J
111	CR620J
112	D602Y
112	D625Y
112	Y735J
112	Y743J
113	E719Y
113	M606Y
114	Y704Y
115	E625J
115	SH721Y
116	G608Y
116	G622Y
117	SH717Y
118	E702Y
119	E626J
120	D627Y
121	E710Y
121	G701Y
121	G716Y
121	M758Y
121	Y768Y
122	C618J
122	CH730J
122	D717Y
122	E622Y
122	Y726J
123	A602Y
123	A618Y
123	AS609J
123	AS616J
123	CH739J
123	D609Y
123	D623Y
123	D719Y
123	E718Y
123	E725Y
123	G618Y
123	G620Y
123	G714Y
123	GH702Y
123	M607Y
123	M765Y
123	SH712Y

haplotype	sample
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123	Y717Y
123	Y775Y
124	A619Y
124	AS606J
124	P611Y
125	G611Y
126	G617Y
126	G619Y
126	G621Y
127	G603Y
128	CH741J
129	SH711Y
130	C619J
131	C617J
131	M752Y
131	Y746J
132	D605Y
133	D613Y
133	Y630J
134	Y715Y
135	Y631J
136	G724Y
137	G610Y
138	D716Y
139	D607Y
140	AS615J
141	G707Y
141	SH728Y
142	A601Y
142	A605Y
142	A614Y
142	A625Y
142	AS601J
142	AS605J
142	CH729J
142	CH733J
142	CR621J
142	D615Y
142	D617Y
142	D621Y
142	D622Y
142	D707Y
142	D710Y
142	D714Y
142	D720Y
142	E723Y
142	G606Y
142	G607Y

haplotype	sample
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142	G711Y
142	G715Y
142	G717Y
142	G719Y
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142	G725Y
142	GH704Y
142	M608J
142	M755Y
142	M761Y
142	P610Y
142	P617Y
142	P618Y
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142	SH716Y
142	SH727Y
142	Smi7
142	Y633J
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142	Y708Y
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142	Y723Y
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142	Y731J
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142	Y740J
142	Y741J
142	Y744J
142	Y769Y
142	Y770Y
142	Y772Y
143	A623Y
143	M602Y
144	A606Y
145	D618Y
145	P612Y

Appendix 3. **D)** Aligned mitochondrial cytochrome *c* oxidase subunit I (COI) amino acid sequence data collected from 389 *Brevoortia* specimens.

Please see attached electronic version.

Appendix 3. **E)** Aligned nuclear internal transcribed spacer 1 (ITS-1) sequence data collected from 12 × 5 clones of *Brevoortia* specimens with outgroup *Gadus morhua*.

Please see attached electronic version.

Appendix 3. **F)** Allele scores for seven microsatellite loci (Aa16, Asa2, Asa4, AsaB020, AsaD055, AsaC334, SarBH04) collected from 389 *Brevoortia* specimens.

Please see attached electronic version.

VITA

ABIGAIL J. LYNCH

Born in Fairfax, Virginia on 30 June 1983. Graduated valdictorian from J.E.B. Stuart High School in 2001. Earned B.A. in English and B.S. in biology from the University of Virginia in 2005. Entered the Master of Science program at the School of Marine Science, Virginia Institute of Marine Science, College of William & Mary in 2005. Received the John A. Knauss Marine Policy Fellowship for 2008.