Systematics of the North American menhadens: molecular evolutionary reconstructions in the genus *Brevoortia* (Clupeiformes: Clupeidae)

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Abstract—Evolutionary associations among the four North American species of menhadens (*Brevoortia* spp.) have not been thoroughly investigated. In the present study, classifications separating the four species into small-scaled and large-scaled groups were evaluated by using DNA data, and genetic associations within these groups were explored. Specifically, data from the nuclear genome (microsatellites) and the mitochondrial genome (mtDNA sequences) were used to elicit patterns of recent and historical evolutionary associations. Nuclear DNA data indicated limited contemporary gene flow among the species, and also indicated higher relatedness within the small-scaled and large-scaled menhadens than between these groups. Mitochondrial DNA sequences of the large-scaled menhadens indicated the presence of two ancestral lineages, one of which contained members of both species. This result may indicate genetic divergence (reproductive isolation) followed by secondary contact (hybridization) between these species. In contrast, a single ancestral lineage indicated incomplete genetic divergence between the small-scaled menhaden. These results are discussed in the context of the biology and demographics of each species.

Manuscript submitted 28 July 2006 to the Scientific Editor's Office.

Manuscript approved for publication 12 January 2007 by the Scientific Editor.

Fish. Bull. 205:368–378 (2007).

The North American species of menhaden (*Brevoortia* spp. Gill, 1861) support large commercial fisheries on the eastern Atlantic and Gulf of Mexico (hereafter, Gulf) coasts. Historically, this industry represented as much as 40% of all commercial landings in the United States (Vaughan and Merriner, 1991), and the Gulf menhaden (*B. patronus* Goode, 1878) supported, by weight, one of the largest single fisheries in the United States. In addition, menhaden are one of the most important participants in estuarine and nearshore food webs along the Atlantic coast (Gottlieb, 1998) and support various recreational fisheries in the Gulf and Atlantic (Kroger and Guthrie, 1972; Scharf and Schlicht, 2000; Bethea et al., 2004).

There are four species of menhaden present in North American waters, three of which are found in the Gulf of Mexico (Fig. 1). These species are further classified into two general groups, the small-scaled and large-scaled menhadens. These groups are named according to the relative size of scales adjacent to the lateral line but also reflect contrasts in other morphological characteristics (Dahlberg, 1970), population demographics (Christmas and Gunter, 1960), and migratory behavior (Gunter, 1945; Simmons, 1957; Tolan and Newstead, 2004). The Gulf menhaden (*B. patronus*) and Atlantic menhaden (*B. tyrannus* Latrobe, 1802) are members of the large-scaled group. *Brevoortia patronus* occurs wholly in the Gulf of Mexico, and dominates the menhaden fishery in the Gulf, with other menhaden species representing less than 1% of the annual catch (Ahrenholz, 1981). *Brevoortia tyrannus* is a semimigratory species found in large schooling populations and is targeted by a second reduction fishery. This species is found only on the Atlantic coast, and does not inhabit the Gulf (Ahrenholz, 1991). The small-scaled menhadens, yellowfin menhaden (*B. smithi* Hildebrand, 1941) and finescale menhaden, (*B. gunteri* Hildebrand, 1948), are present in smaller population sizes and have slightly overlapping distributions in the Gulf. *Brevoortia gunteri* has a distribution range restricted entirely to the northern and western Gulf coastal region. In contrast, *B. smithi* exists mainly in the eastern Gulf, although it ranges north on the Atlantic coast to Cape Lookout, North Carolina (Reintjes, 1959; Ahrenholz, 1991). *Brevoortia smithi* overlaps the eastern range of *B. patronus*, and also overlaps the southern range *B. tyrannus*. Considerable hybridization is thought to occur between *B. patronus* and *B. smithi* along the west coast of Florida, and between *B. tyrannus* and *B. smithi* along the east coast of Florida (Turner, 1969; Hettler, 1984).

Although the evolutionary relationships among the four North American menhaden species have not been explicitly examined, early investigations in species morphology indicated that *B. tyrannus* and *B. patronus* were Atlantic and Gulf complements of one another, and that *B. gunteri* and *B.*

smithi were western and eastern Gulf cognates (Christmas and Gunter, 1960). Dahlberg (1970) conducted an extensive morphological investigation of the Atlantic and Gulf menhadens, including compilation of older data as well as 14 novel physical character assessments that provided morphological support for the notion of small-scaled and large-scaled groups. Subsequent morphological assessments of menhaden at various life stages support this generally accepted taxonomic arrangement (Hettler, 1984; Ahrenholz, 1991; Tolan and Newstead, 2004). Avise et al. (1989) and Bowen and Avise (1990) examined mitochondrial DNA (mtDNA) fingerprints of the large-scaled menhaden complex (*B. patronus+tyrannus*), and two divergent lineages of large-scaled menhadens were identified, one of which was shared between the two species, and the other occurring only in *B. tyrannus*. Avise (1992) suggested that this type of pattern is what would be expected from historical divergence, followed by secondary contact between the species. Anderson and McDonald (2007) used microsatellites to characterize populations of western Gulf menhadens (*B. patronus* and *B. gunteri*) and found significant genetic differences between these species. However, the latter work was limited in its phylogenetic scope because 1) it included only two of the four species of *Brevoortia* found in North America, and 2) it did not include genomic or mtDNA sequence data, which are typically more reliable than microsatellites in discerning relationships among species.

In the present study, the taxonomic relationships among the four North American menhaden species are examined by using both nuclear satellite DNA (microsatellites) and mitochondrial DNA sequences (mtDNA). Microsatellites are short runs of repetitive sequence (usually a two to four base motif repeated multiple times) that tend to be highly variable in vertebrates, are codominant (both parental copies of the marker can be scored for progeny), and are selectively neutral. Because of these properties, they are used extensively for examinations of population structure, paternity, and kinship in vertebrates (Wright and Bentzen, 1994; Jarne and Lagoda, 1996). Five DNA microsatellites were scored in populations of menhaden comprising the range of the four North American species. In addition, mtDNA sequencing was conducted on a short segment of the mtDNA control region for a subset of menhaden representing the four species. Mitochondrial DNA assays are beneficial because the mitochondrial genome of fishes is presumably not affected by recombination, and direct maternal lineages can be examined in contrast to the potentially admixed genotypes of nuclear DNA. In addition, genome-wide genetic studies (i.e., those that include both nuclear and mitochondrial data) are favored over single-locus or single-genome treatments because different genetic loci may yield conflicting results (Hare and Avise, 1998). These data revealed distinctive patterns which, when examined in the context of the biogeographic setting of each species, broaden the existing information with which the evolutionary history of North American menhadens may be elucidated.

Materials and methods

Sample collection and DNA isolation

Fin clips from individual menhaden were collected from bay systems covering the extent of the North American range of *Brevoortia* (Fig. 1) from 2002 through 2004 (see Brown et al., 1996 for a discussion of the implications and assumptions of pooling multiple year genetic data in clupeids). Adult fish samples from Texas were collected with a 184 m gill net partitioned into four sections ranging in mesh sizes from 76 mm to 152 mm, and all juveniles and young adult menhaden were collected in bag-seine hauls. Menhaden from locations outside of Texas were obtained through appropriate state and federal agencies (see "Acknowledgments" section). Fin tissue was excised from larger fish and placed in 70% ethanol; for smaller fish, whole individuals were preserved in ethanol. DNA was extracted from 200 mg of each fin clip or whole fish by using a Puregene® miniprep kit (Gentra Systems, Minneapolis, MN) and following the manufacturer's instructions.

Microsatellite data set

Genomic DNA from 20 sampling locales (Fig. 1) was used as a template to amplify microsatellite loci by touchdown polymerase chain reaction (PCR) with fluorescent end-labeled forward primers. Primer pairs were designed around microsatellite regions initially isolated from another clupeid, American shad (*Alosa sapidissima* Wilson, 1811); these primers have subsequently been evaluated for examinations of the genus *Brevoortia* (Anderson and McDonald, 2007). PCR products were electrophoresed with a Beckman-Coulter CEQ™ 8000 automated capillary system, with a 400 base pair (bp) standard (Beckman Coulter, Inc., Fullerton, CA), according to the manufacturer's suggested protocol. Microsatellite allele sizes were estimated with Beckman-Coulter fragment analysis software (Beckman Coulter, Inc., Fullerton, CA), by using allelic bins that were based on the analysis of 1276 DNA samples from the four North American menhaden species collected over four years (data not shown).

The freeware program FSTAT vers. 2.9.3.2 (Goudet, 1995) was used to calculate observed (H_o) and expected (H_e) heterozygosity and allelic diversity (k_a), and to identify microsatellite loci which deviated significantly from Hardy-Weinberg equilibrium expectations (HWE). Deviation from HWE was tested by calculating the inbreeding coefficient (F_{is}) in the overall data set, and within each individual sampling locale. Statistical significance was assessed in a randomization procedure with 100 iterations. For this analysis, 13 populations of *B. patronus* (n=20) ranging from southern Texas to southern Florida were used. Individuals were then pooled into species and sampling locales (n=20 in each sampling locale, in locales where two species were captured, 20 of each species were used). The genetic divergence (F_{st}, Weir and Cockerham, 1984) among the four

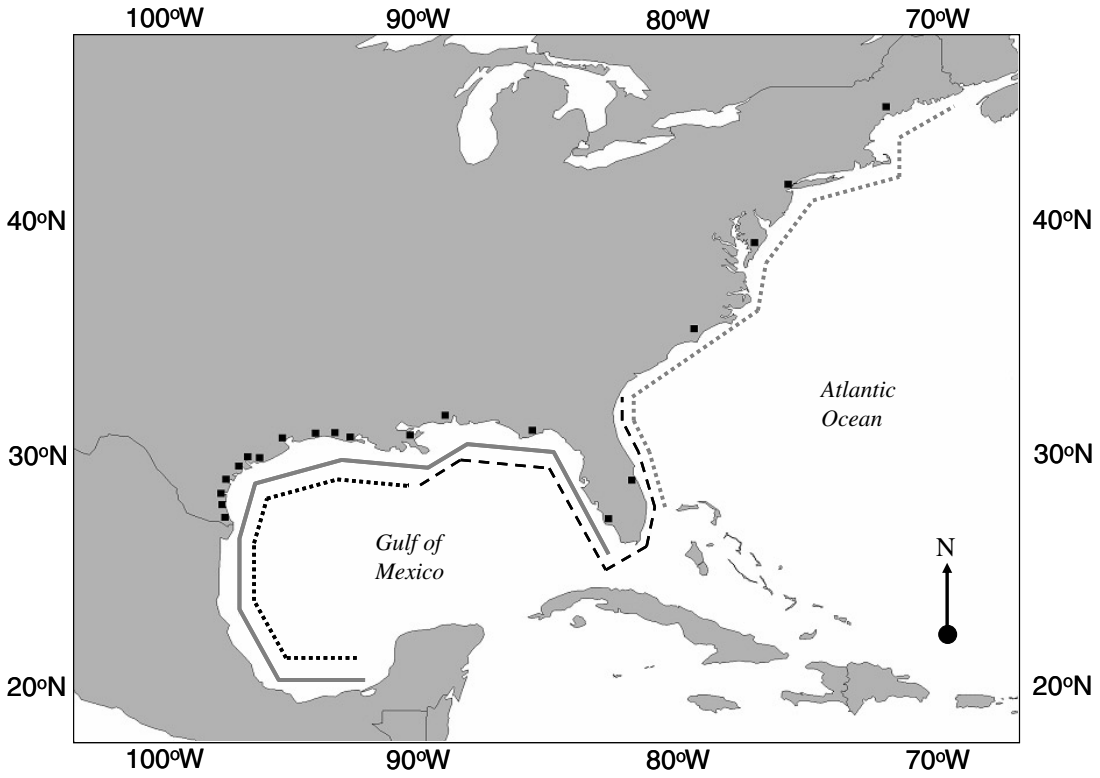

Figure 1

Geographic range of the four menhaden species: Gulf menhaden (*Brevoortia patronus*)—smooth gray line, Gulf; Atlantic menhaden (*B. tyrannus*)—dotted gray line, Atlantic; fine-scale menhaden (*B. gunteri*)—dotted black line, western Gulf; and yellowfin menhaden (*B. smithi*)—dashed black line, eastern Gulf). Sample sites are indicated by black boxes.

species, and among all populations, was determined by using FSTAT software. A neighbor-joining (N-J) dendrogram was constructed with genetic divergence as a distance metric, and populations were revealed as terminal nodes by using the freeware program MEGA, vers. 2.1 (Kumar et al., 2001).

With a small subset of the data (to accommodate differences in sample size among species), we used a Bayesian procedure, as implemented in the freeware program Structure 2.1 (Pritchard et al., 2000), to examine historical admixture among species. Admixture may be defined as any measure of combination and introgression of genotypes (through hybridization) between two distinctive genetic sources, such as definitive populations, subspecies, or species. Structure 2.1 uses a Markovian chain Monte Carlo method (MCMC) to assign individuals to population clusters such that satisfaction of Hardy-Weinberg and linkage equilibrium expectations are maximized within populations. Because of the mechanics of the MCMC method, each iteration in a simulation is necessarily connected to the previous iteration, and therefore initial iterations are subject to the stochastic nature of random sampling. In order to account for this, data from simulations should not be collected until summary statistics begin to converge (to "burn-in"). A burn-in length of 10,000 repetitions was chosen by examination of summary statistics for convergence in preliminary runs. A run length of 100,000 repetitions was chosen after several preliminary trials, after which parameter values were evaluated for consistency. For all analyses, the admixture model was used, and allele frequencies were assumed to be independent among species. Although this procedure allows estimation of the number of populations present (K), while simultaneously assigning individuals, we constrained the population number to $K = 4$, to reflect the prior assumption that only four species were present. Individuals were then assigned profiles that included the proportion of their genotype contributed by each species cluster. These profiles were then aligned by species in order to visually examine the influence of genetic admixture upon species groups.

Multilocus expected heterozygosity, estimated from microsatellite allele frequencies, was used to generate relative estimates of the value $N_e\mu$ for each of the four species, where N_e is the effective population size and μ represents the overall mutation rate, assuming a stepwise mutation model (Valdes et al., 1993). The estimator θ_F (Xu and Fu, 2004) was used to approximate $N_e\mu$ and was calculated as follows:

$$\theta_F = 0.5\ (F^{-2} - 1),$$

where F = multilocus mean homozygosity ($1 - H_e$).

Although estimates of N_e from small genetic samples are notoriously inaccurate and may do a poor job of reflecting overall census size, relative estimates of N_e have been used to test evolutionary hypotheses (Nielsen, 1997), and theoretically may be used to examine relative differences in multiple populations at the same loci. The assumptions of the present study are that the mutation rate (μ) is not significantly different among the four examined species, and that genetic variability is related to population size. For this analysis, four loci were used because of the highly significant deviation from equilibrium frequencies that was observed at locus AF39660 (see "Results" section).

Sequencing data set

Sequencing templates were generated with PCR by using primers specific to an 840-bp fragment of the mtDNA control region. The heavy strand primer (HN20) was a previously described universal primer (Bernatchez and Danzmann, 1993) (5′-CGGGGTTTGACATGAATAT), whereas the light strand primer (940r) was a novel sequence primer designed specifically for menhaden (3′-TGTAATACTAGTGCGCAGATGGTAC). Each sample was amplified twice, and the amplicons were combined before purification to assure a high quality, concentrated product. PCR products were purified by using QIAquick® PCR purification kits (Qiagen Inc., Valencia, CA). The resulting purified fragments were used in two sequencing reactions in a nested design. The novel heavy strand primer (520f) was internal (5′-GGAACCAAATGCCAGGAATAGT), whereas the light strand primer was the same used in PCR. This nested design resulted in fragments which overlapped one another for 360 bp and which resided within the original 840-bp PCR fragment. Sequences were electrophoresed and analyzed on a Beckman-Coulter CEQ™ 8000 capillary sequencer by using default module (LFR-1) parameters (Beckman Coulter, Inc., Fullerton, CA). Raw sequences were trimmed and edited, and forward and reverse sequences were conjoined by using the software package Sequencher™ vers. 4.2 (Gene Codes Corp., Ann Arbor, MI). Whole sequences were then aligned by using Clustal X freeware (Jeanmougin et al., 1998). Sequences were submitted to GenBank by means of Sequin freeware (National Center for Biotechnology Information, Bethesda, MD) as batch submissions (accession numbers EF119342-EF119454).

Sequence data were obtained from 113 individuals from the four species (*B. tyrannus,* n=37; *B. smithi,* n=32; *B. patronus,* n=30; *B. gunteri,* n=14), and samples were selected to represent the full geographic range of each species (sampling locations in Maine, New Jersey, Virginia, North Carolina, Atlantic and Gulf Florida, and Texas). Sequence statistics, including nucleotide diversity (π) and haplotype diversity (h), were estimated for each species, and the genetic similarity among populations (measured using D_a, Nei, 1987) was calculated by using the freeware program DnaSP 4.0 (Rozas et al., 2003). The variances of sequence diversity estimates were used to construct 95% confidence intervals around each mean. The averaged frequency of each base and the estimated ratio of transitions to transversions (ts/tv) were calculated with the freeware program DAMBE (Data Analysis in Molecular Biology and Evolution, Xia and Xie, 2001). Because of the extreme level of variation found in menhaden mtDNA, both in this study and in a previous study (Bowen and Avise, 1990), we employed a mutation saturation test as instituted in DAMBE. With this analysis, we compared the genetic distance between sequences to the number of transitions and transversions occurring between them. When all pairwise comparisons of sequences were plotted in this manner, mutation saturation was indicated by a plateau at which transversions approach or outnumber transitions at higher genetic distances. The genetic distance employed for this analysis (*K2P*) is described in Kimura (1980).

Both transitions and transversions were included in phylogenetic analyses. Before evolutionary reconstruction of haplotypes, the freeware program Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the model of sequence evolution which had the highest likelihood. Briefly, 56 nested models of evolution were tested against raw sequence data with the program PAUP 4.0b1 (Phylogenetic Analysis Using Parsimony, available from Sinauer Associates, Inc., Sunderland, MA). Modeltest was then used to perform hierarchical likelihood ratio testing (hLRT) in order to identify the model with the highest likelihood. Concurrently, the Akaike information criterion (AIC) weights of the four most likely models were also examined during model selection. The appropriate model was subsequently used in a maximum-likelihood (ML) procedure in PAUP 4.0b1, with 10 replicates, to reconstruct an unrooted haplotype phylogeny. Because of the enormous computational load associated with maximum likelihood of highly variable loci, a concurrent N-J tree was constructed by using the 2-parameter distance of Kimura (1980); this tree was bootstrapped (1000 replicates over nucleotides, Felsenstein, 1985) to evaluate the significance of major interior nodes that correlated with nodes on the maximum-likelihood tree.

Results

Microsatellite data set

Microsatellite samples were distributed from southern Texas to the Gulf of Maine, the northern extent of *B. tyrannus*. *Brevoortia tyrannus* was sampled in three locations (all Atlantic coast), *B. smithi* in two locations (one Atlantic, one eastern Gulf), *B. patronus* in 14 locations (all Gulf coast), and *B. gunteri* in three locations (all western Gulf coast). Of the five microsatellite markers used in this study, all had observed levels of heterozygosity that were lower than anticipated under Hardy-Weinberg expectations when tested by using samples

Table 1

Short tandem repeat locus statistics (listed by National Center for Biotechnology Information accession number) averaged over 13 populations of Gulf menhaden (*Brevoortia patronus*, n=260). Statistics include the DNA base motif (each allele is distinguished by "x" number of repetitions of the motif), observed (H_o) and expected (H_e) heterozygosity, number of alleles (k_a), and deviation from Hardy-Weinberg expected genotype frequencies (measured by deviation of F_{is} from 0).

Locus	Primer sequence	Motif	H_o	H_e	k_a (overall)	F_{is} (overall)
AF049462	*F: GGAGGCACAGGTGTGGTATT*					
	R: TTTGGAGGGAGAGAAACGTC	$[GTT]_x$	0.84	0.93	31	0.09
AF039658	*F: TAATAAACCCCGTTGGGACA*					
	R: GCTGATGTTCCTCCATCTCC	$[CAA]_x$	0.78	0.87	23	0.10
AF039657	*F: GCCATTACTCCAAGTTGCTTTT*					
	R: CGTGGCACAACATAGTCATCA	$[CTT]_x$	0.70	0.73	20	0.05
AF039661	*F: TGCTTTAATCCGGAATGGAC*					
	R: GGGGAGTGAGAGAACGAGTG	$[CTTT]_x$	0.15	0.20	10	0.24
AF039660	*F: GGAGCTCAGCACATCTCTCC*					
	R: CTGACATGGCCCAGTAGGTT	$[GTTT]_x$	0.26	0.58	13	0.55*

* indicates that F_{is} is significantly different from zero in a majority of *B. patronus* populations sampled after correction for multiple tests, determined by a randomization procedure with 1000 iterations.

of *B. patronus* individuals from the Gulf of Mexico (n=260, Table 1). One locus (AF39661) had a high overall value of F_{is}, but was not significantly different than zero based on randomization tests. However, a second locus (AF39660) had lower than expected heterozygosity at a significant level in a majority of the populations assayed. Whether the explanation for decreased levels of heterozygosity is biological or simply sampling error is unclear; however analyses excluding this marker had little effect on tree topology or other results. Multilocus estimates of genetic divergence (F_{st}) were lowest within the predefined menhaden groups; that is, the level of divergence seen in the comparisons between *B. patronus+tyrannus* and *B. smithi+gunteri* was lower than any other pairwise comparisons among species (Table 2). Similarly, the N-J tree constructed from microsatellite data was deeply bifurcated (Fig. 2) and the two major groupings on this tree corresponded to populations of small-scaled and large-scaled menhaden, exclusively. Four major clusters on this tree corresponded to the populations of each of the four assayed species. The greatest level of within-species divergence was observed between the Atlantic and Gulf populations of *B. smithi*. The divergence among these populations was an order of magnitude higher than the mean divergence from comparisons among populations of the same species, and was 5.5 standard deviations removed from the mean of all F_{st} comparisons combined (F_{st}^{smithi}=0.0523, $F_{st}^{overall}$=0.0054, σ^2=0.0094).

Table 2

Genetic divergence among Atlantic menhaden (*Brevoortia tyrannus*), Gulf menhaden (*B. patronus*), yellowfin menhaden (*B. smithi*), and finescale menhaden (*B. gunteri*), as measured by using multilocus microsatellite data (F_{st}, above the diagonal) and mtDNA sequence similarity among populations (D_a, below the diagonal).

	B. tyrannus	*B. patronus*	*B. smithi*	*B. gunteri*
B. tyrannus	0	0.110	0.448	0.412
B. patronus	0.005	0	0.411	0.378
B. smithi	0.077	0.093	0	0.355
B. gunteri	0.071	0.088	0.004	0

Bayesian analysis of population structure resulted in four well-defined clusters corresponding to the four assayed species (Fig. 3). However, no single species contained exclusively one cluster; each species had individuals with composite genotypes including influence from other clusters. In particular, admixture among the two large-scaled species was indicated by individuals of each species in which a majority of the genotype was contributed by the complimentary species. A smaller level of admixture was indicated in the small-scaled species. However, one interesting result was a single individual *B. gunteri* which had 0.723 of its genotype contributed by the *B. patronus* cluster. This is significant in that, although the possibility of hybridization in the western Gulf has not been ruled out (Anderson and McDonald, 2007), a verified F1 *B. patronus+gunteri* hybrid has not been documented in the Gulf.

Estimates of genetic variability including H_e, k_a, and θ_F were higher in large-scaled species of menhaden than in small-scaled species (Table 3). Multilocus expected heterozygosity ranged from 0.672 in *B. patronus* and

Table 3

Genetic diversity estimates over four nuclear loci (AF49462, AF39658, AF39657, and AF39661) and the mtDNA control region locus, for Atlantic menhaden (*Brevoortia tyrannus*), Gulf menhaden (*B. patronus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*). The diversity estimates include allelic diversity (*k,* alleles/locus) at each microsatellite locus and overall (k_a), multilocus expected heterozygosity (H_e), relative effective population size estimated from multi-locus heterozygosity (θ_F), the 95% confidence range of mtDNA nucleotide diversity (π), and the 95% confidence range of mtDNA haplotype diversity (h). Sample sizes were equal (n=39) in all comparisons unless otherwise noted.

Species	*AF49462* (*k*)	*AF39658* (*k*)	*AF39657* (*k*)	*AF39661* (*k*)	k_a	H_e	θ_F	π	h
Brevoortia tyrannus	20	14	15	3	13.0	0.67	4.15	0.064–0.086 (n=37)	0.99–1.00 (n=37)
Brevoortia patronus	19**	16**	9**	4**	12.0	0.67	4.15	0.046–0.054 (n=30)	0.99–1.00 (n=30)
Brevoortia gunteri	11	11	3	1	6.5	0.41	0.90	0.038–0.055 (n=14)	0.95–1.00 (n=14)
Brevoortia smithi	8	4	2	2	4.0	0.37	0.76	0.030–0.037 (n=32)	0.92–1.00 (n=32)

** Allelic counts for *B. patronus* are different from those presented in Table 1 because fewer individuals were used to account for smaller sample sizes in the three remaining species.

B. tyrannus, to 0.413 in *B. gunteri* and 0.370 in *B. smithi*. This disparity is due to near fixation of alleles in both small-scaled species at a single locus which is highly polymorphic in both large-scaled species (AF39657), but also reflects decreased variability at all marker loci. As a result, the estimates of θ_F in large-scaled species were four to five times larger than those for small-scaled species.

Sequencing data set

The mtDNA sequencing data set indicates an enormous amount of genetic variation in the control region of menhaden (Table 3), although this variation was not evenly distributed across the entire alignment. In particular, regions of sequence conservation in the data set were directly adjacent to highly degenerate regions more typical of the mitochondrial control region of clupeids (Grant et al., 1998). Over a total dataset of 360 bp, 149 sites were polymorphic, and pairwise sequence comparisons resulted in an average of 28.7 nucleotide differences among haplotypes. Of 113 individuals assayed, 106 haplotypes were identified in the four species. Despite this high level of sequence divergence, only three single-base deletions were detected in the multiple alignment of 113 individuals (deletions were treated as missing data). The control region of menhaden is A-T rich (contains greater than 50% adenine and thymine bases), with averaged base frequencies of A = 0.269, T = 0.487, C (cytosine) = 0.156, and G (guanine) = 0.167, and with no evidence for heterogeneity of base frequencies among taxa (χ^2=59.40, P≈1.00).

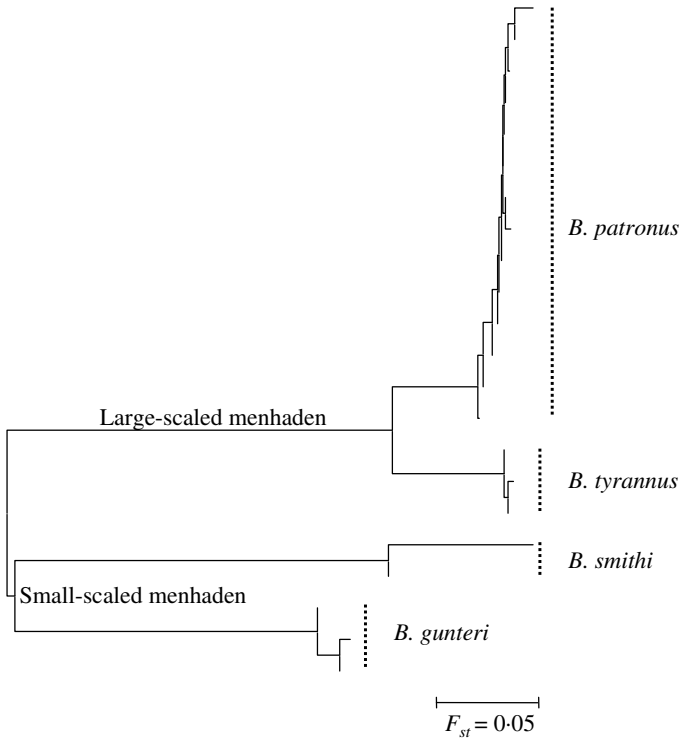

Figure 2

Neighbor-joining dendrogram of populations (n=20, in each population) of Gulf menhaden (*Brevoortia patronus*), Atlantic menhaden (*B. tyrannus*), finescale menhaden (*B. gunteri*), and yellowfin menhaden (*B. smithi*). The distance metric used for neighbor-joining was F_{st} (Weir and Cockerham, 1984), which is the estimated proportion of genetic variance that is due to between-population effects.

The estimated ts/tv ratio was 2.1:1. As was the case in comparisons using microsatellites, the mtDNA distance among species (D_a) was lowest in comparisons between species within small-scaled and large-scaled groups (Table 2).

Hierarchical likelihood ratio tests indicated that the K81uf+I+Γ model (Kimura, 1981) was the most appropriate model of sequence evolution at the control region locus. This model also had the second highest AIC weight, at 0.23. The K81uf+I+Γ model approximates the rate of heterogeneity across sites (a property which can simply be described as unequal probabilities of mutations among sites) by using a gamma distribution, after variant sites have been removed. Haplotype phylogenies generated with both the M-L method and the N-J cluster method indicated three divergent lineages of menhadens (Fig. 4), and these lineages were identical in haplotype membership between the two tree-building methods. The first lineage had 99% bootstrap support and included haplotypes contributed from both *B. patronus* and *B. tyrannus*. This main group of large-scaled haplotypes (n=59) was characterized primarily by transitions and had minimal (<7) transversions among haplotypes. A second, small group of divergent Atlantic menhaden occurred in the haplotype phylogeny, with 100% bootstrap support. This second group comprised the remaining eight large-scaled haplotypes and differed from the first group by 14+ transversions per haplotype, likely the result of mutation saturation. Plots of transitions and transversions indicated that this data set was saturated; the rate of transversions begins to rival that of transitions at approximately a Kimura distance of 0.10 (Fig. 5A). Although saturation is indicated in cases where divergent haplotype clades are compared, saturation is not indicated in comparisons involving within-clade comparisons (Fig. 5, B–D). Thus, the main effect of saturation on this data set was on longer (more divergent) branches. A third lineage on the maximum likelihood tree had 100% bootstrap support and consisted of haplotypes contributed by *B. gunteri* and *B. smithi* in paraphyly. Haplotypes in this clade indicated incomplete sorting of mtDNA haplotypes within the small-scaled menhadens. However, there was clear evidence for genetic divergence between this group and both large-scaled clades, and 100% bootstrap support for monophyly of the small-scaled haplotypes.

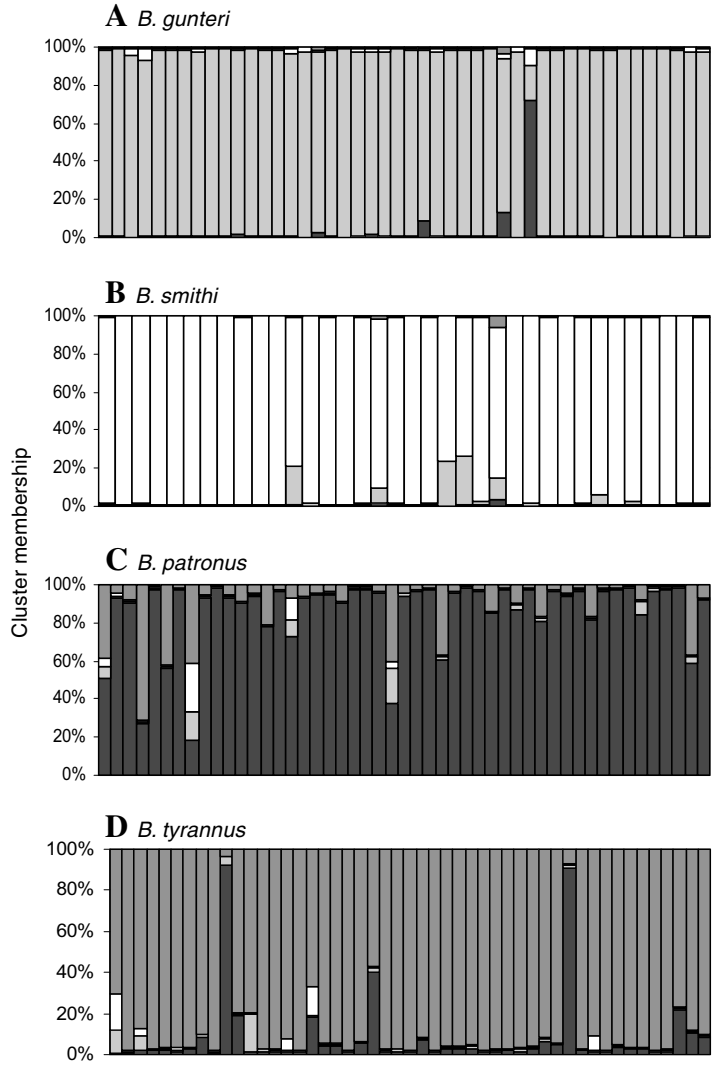

Figure 3

Bayesian assignment tests resulted in recovery of four putative source populations of genotypic data. Individuals were separated into their respective species groups (**A–D**), and genetic identity was assigned based upon the percentage of an individual's genotype contributed by each of the four source populations, as indicated on the *y*-axis. Genetic source populations are represented by light grey (finescale menhaden [*Brevoortia gunteri*], white (yellowfin menhaden, [*B. smithi*]), black (Gulf menhaden [*B. patronus*]), and dark grey (Atlantic menhaden [*B. tyrannus*]).

Discussion

The morphological differences among the four North American menhaden species have been examined previously at egg, larval, juvenile, and adult stages (Dahlberg, 1970 [and references therein]; Hettler, 1984; Ahrenholz, 1991; Tolan and Newstead, 2004), and previous classifications have the species divided between small-scaled and large-scaled groups (Dahlberg, 1970). The results presented here reinforce the conventional hypothesis that *B. smithi* and *B. gunteri* share recent common ancestry as do *B. patronus* and *B. tyrannus*. Both classes of genetic markers showed

significant genetic divergence between the two menhaden types, and this distinction is reinforced by demographic characteristics shared within these groups. Specifically, overall census sizes (Christmas and Gunter, 1960), species ranges (Christmas and Gunter, 1960), and migratory behavior (Gunter, 1945; Simmons, 1957; Tolan and Newstead, 2004) are markedly different between the small-scaled and large-scaled species pairs, yet similar between species within these pairs. The differences in the demographics of small-scaled and large-scaled menhaden are reflected in nuclear-based estimates of $N_e\mu$, which are four to five times larger in large-scaled species. Smaller population sizes, and relatively shorter overall coastal ranges, are reflected in the lower genetic variability of small-scaled menhaden compared to large-scaled menhaden. In addition, nutritional and trophic differences among these species in the western Gulf have previously been documented (Castillo-Rivera et al., 1996; Castillo-Rivera and Kobelkowsky, 2000) and may represent mechanisms for the divergence of population demographic parameters among these groups.

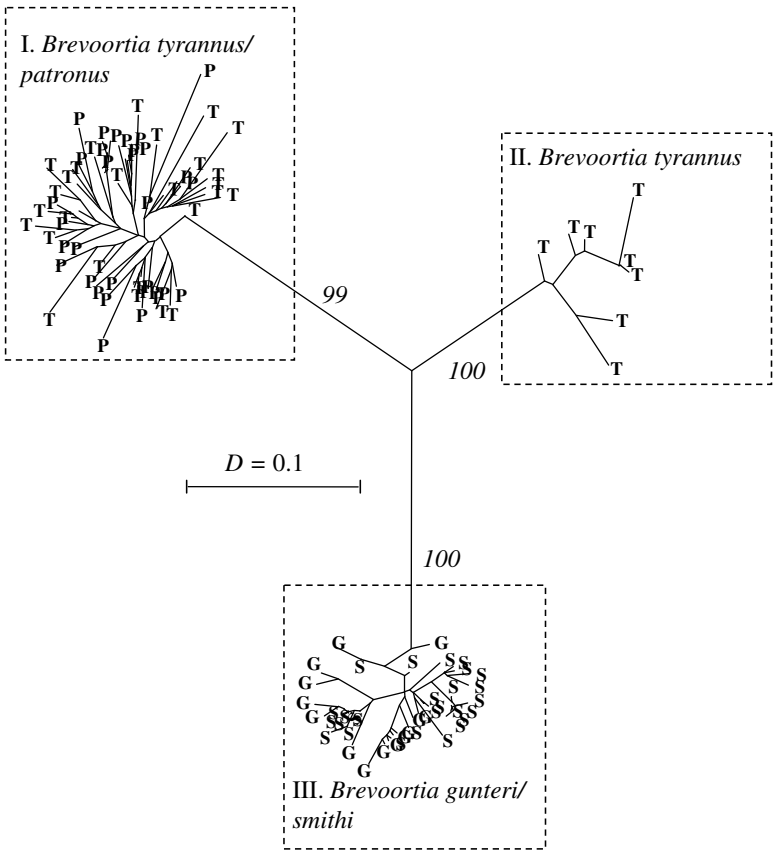

Figure 4

Maximum likelihood plot of unrooted menhaden mtDNA samples. The numbers at the base of each of three divergent clades (99, 100, and 100) are the bootstrap support values from a neighbor joining analysis. The genetic distance (*D*) between links in the phylogeny is a modified version of Kimura's distance (1981), which includes gamma-distributed rate variation after invariant sites are removed, as outlined in Modeltest software (Posada, 1998). The roman numerals correspond to clade numbers as discussed in the text, including clade I (mixture of haplotypes from Gulf menhaden [*Brevoortia patronus*] and Atlantic menhaden [*B. tyrannus*]), clade II (haplotypes from *B. tyrannus* only), and clade III (mixture of haplotypes from yellowfin menhaden, *B. smithi* and finescale menhaden, *B. gunteri*).

In both the present study and the study of Bowen and Avise (1990), high levels of genetic variation were detected in the mtDNA genome of menhaden. Indeed, because of the high mutation rate of the mtDNA region surveyed in this study, saturation was a likely source of bias in comparisons among divergent lineages. A second similarity between this study and that of Bowen and Avise (1990) is the presence of two divergent large-scaled haplotype groups, one that is confined to *B. tyrannus*, and a second which includes haplotypes from both species. Avise (1992) suggested that a likely explanation for this pattern is recent gene flow between these species, rather than incomplete lineage sorting. A Bayesian population assignment of nuclear genotypes seems to support the possibility of admixture between these species, with individuals from both species containing genetic signatures of the complementary group. Epperly (1989, [and references therein]), suggested the existence of two subpopulations of *B. tyrannus* in the Atlantic: one on the southern Atlantic coast (below 40°N) and a subpopulation north of Long Island, New York. Juveniles from these subpopulations differed in meristic and morphological characteristics, and also had different biochemical profiles (Epperly, 1989). Epperly (1989) suggested that the spawning times for these two subpopulations may be different, allowing for recruitment to occur in these areas at different times of the year. Variance in spawning times may be a mechanism for maintenance of divergence between haplotype clades for large-scale menhaden recovered in this study. However, it is likely that the variation in morphology and biochemistry seen in northern and southern forms of Atlantic menhaden is affected by the influence of recurrent gene flow between this species and its Gulf cognate. Avise (1992) demonstrated a broad-scale demographic break between Atlantic and Gulf forms of both terrestrial and marine vertebrates (including menhaden); however, in many species, haplotypes representative of Gulf forms were present in individuals collected in the southern Atlan-

tic. Locales farther north in the Atlantic lack the Gulf haplotypes; in this scenario it would be expected that Atlantic menhaden collected farther north would lack the Gulf menhaden influence. Indeed, in the divergent group of Atlantic menhaden examined in the present study, four of the eight haplotypes came from juveniles collected from the northernmost sampling locale (Maine). The remaining haplotypes came from a single adult from a northern locale (New Jersey), two adults from southern locales (North Carolina), and a southern locale juvenile (North Carolina).

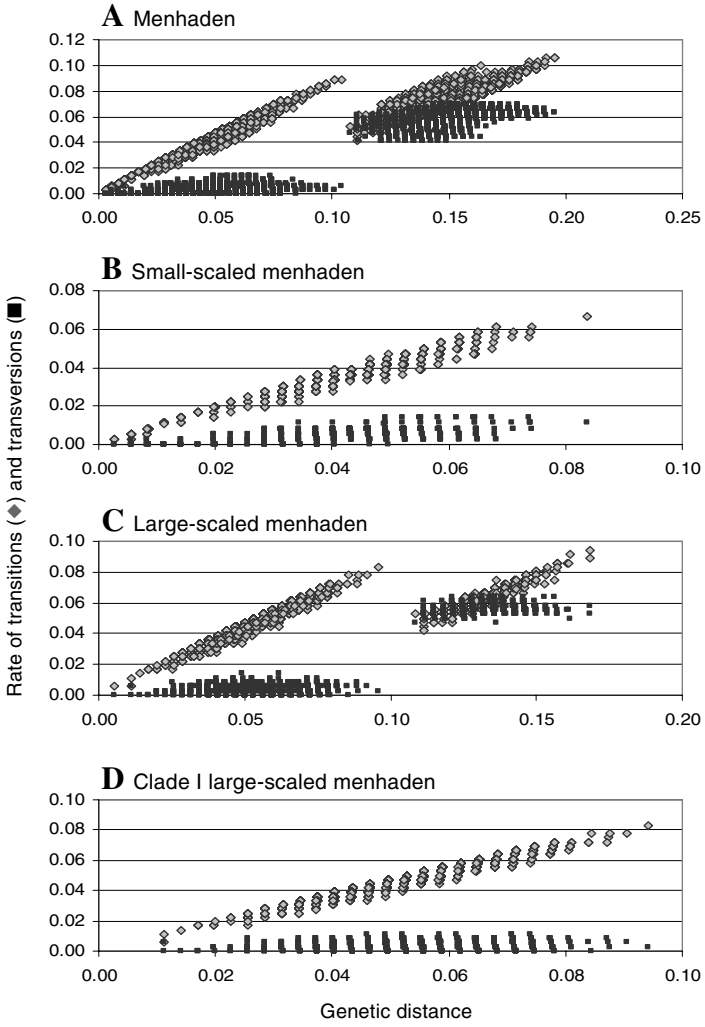

Figure 5

Saturation plots of menhaden mtDNA data. The *x*-axis represents Kimura (1980) distances between sequences, whereas the *y*-axis represents the rate of transitions (♦) and transversions (■). A saturation plot was constructed for (**A**) mtDNA samples from all four species, (**B**) samples from small-scaled menhaden only (yellowfin menhaden [*Brevoortia smithi*] and finescale menhaden [*B. gunteri*]), (**C**) samples from large-scaled menhaden only (Gulf menhaden [*B. patronus*] and Atlantic menhaden [*B. tyrannus*]), and (**D**) large-scaled menhaden from mtDNA clade I only.

Previous treatments have recognized two subpopulations of *B. smithi* in the Gulf and Atlantic, and Hildebrand (1963) characterized the eastern Gulf *B. smithi* as intermediate in form between *B. gunteri* and the Atlantic form of *B. smithi*. An interesting note in our data set is the large divergence estimate between two sampled populations of *B. smithi* obtained in the microsatellite analysis. This estimate of divergence between these populations (F_{st}^{smithi}=0.0523) is the largest of any comparison and is an order of magnitude greater than the mean comparison among populations of the same species (F_{st}^{mean}=0.0054). *Brevoortia smithi* is the only species of menhaden that has significantly large populations in both the Gulf and Atlantic, and populations of this species are rare towards the southern tip of Florida (Reintjes, 1959; Dahlberg, 1970). Thus, vicariance may play a role in divergence between these populations. It is clear that biological differences between Atlantic and Gulf forms of *B. smithi* extend into the genetic data presented here, resulting in long branches in the microsatellite-based topology. In addition, the smaller relative census sizes of small-scaled menhaden species has likely resulted in a faster and more distinctive pattern of divergence among populations of these species than what has occurred in large-scaled species. Further investigation is needed in order to determine if the genetic divergence between Gulf and Atlantic forms of *B. smithi* is genome-wide.

A final peculiarity of the data was a single individual *B. gunteri* which had a genotype that indicated influence from *B. patronus* (Fig. 3). Specifically, this individual had an estimated 72.3% of its genotype contributed by a cluster roughly representative of *B. patronus*, whereas all other individuals of this species had less than a 10% contribution from this cluster (averaged around 2.7%). Although high rates of hybridization between *B. smithi* and both large-scaled menhaden species have been documented in peninsular Florida (Turner, 1969; Hettler, 1984), hybrids between *B. gunteri* and *B. patronus* have not been documented (Anderson and McDonald, 2007). Visual inspection of the individual in question yielded no morphological evidence of hybridization. In addition, Bayesian assignment can be expected to perform better if more loci are sampled. Although five microsatellite loci were adequate to recover evidence for population structure in the present data set, individual assignments based on limited genetic loci will comprise a high level of uncertainty

(Corander et al., 2006). However, given the result here and a similar individual possible occurrence documented in Anderson and McDonald (2007), rare hybridization events between *B. gunteri* and *B. patronus* cannot be ruled out.

Acknowledgments

The following are to be acknowledged for their assistance in completing this work. B. Mobley, K. Anderson, and H. Hendrickson provided technical laboratory assistance. B. Bumguardner, B. Karel, M. Fisher, J. Mambretti, T. Stelly, D. McDonald, S. Grant, and four anonymous reviewers provided thoughtful comments on the manuscript. Texas samples were collected by numerous individuals during routine monitoring of Texas estuarine ecosystems by staff of Texas Parks and Wildlife Department, Coastal Fisheries Division. The following provided samples from outside of Texas: R. McMichael and the staff of the fisheries-independent monitoring program at the Florida Fish and Wildlife Conservation Commission, especially T. Tuckey, D. Adams, and C. Idelberger; J. W. Smith at the National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Beaufort Laboratory in Beaufort, NC; and B. Tarbox at the Southern Maine Community College. This research was funded in part by a Federal Aid in Sport Fish Restoration Grant (F-144-R).

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