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Mitochondrial DNA Variation in Striped Bass, Morone saxatilis, from the Rappahannock River, Virginia

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MITOCHONDRIAL DNA VARIATION IN STRIPED BASS, *MORONE SAXATILIS*, FROM THE RAPPAHANNOCK RIVER, VIRGINIA

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ABSTRACT

Restriction endonuclease analysis of mitochondrial DNA was used to examine genetic variation of striped bass, Morone saxatilis, within the Rappahannock River, Virginia. Ovarian tissue from twenty-three gravid females was collected in the spring of 1986. Mitochondrial DNA was isolated and digested with 4 restriction enzymes: Hind III, Eco RI, Eco RV, and Bcl I. Five size polymorphisms ranging from 17.5-17.8 kilobases were identified and designated as genotypes A, B, C, D/E, and F. The D/E genotype is heteroplasmic and contains 2 different size molecules, 17.65/17.75 kilobases within the mitochondria.

These data were compared with published and unpublished data to determine if Rappahannock striped bass are distinct from those in regions of the Upper Chesapeake Bay, and whether genotypic frequencies within the Rappahannock River remain constant year after year. Comparisons of genotypic frequencies of striped bass from the Rappahannock River and the Potomac River, Choptank River, and Worton Point in 1984 and 1986 suggest that Rappahannock M. saxatilis are genetically distinct from those in the Potomac River and may be distinct from those in the Choptank River and Worton Point.

Comparison of genotypic frequencies found in Rappahannock striped bass in 1984, 1986, and 1987 produced controversial results which may or may not indicate that the distribution of genotypes remained fairly constant. Based on reported molecular weights alone, a sudden shift in genotypic frequencies is apparent in 1987. Such a sudden change in the frequency distribution is difficult to explain in light of past tagging studies which support homing in female striped bass. However, after a direct comparison of samples representing the data sets involved, no differences in migration distances were observed. This supports the conclusion that frequencies remained generally constant between 1984, 1986, and 1987, and that female striped bass do return to the natal river to spawn. Although these data are preliminary and should not be used for management purposes, they provide a basis for additional studies already under way to identify stocks within the Chesapeake Bay.

MITOCHONDRIAL DNA VARIATION IN STRIPED BASS, *MORONE SAXATILIS*, FROM THE RAPPAHANNOCK RIVER, VIRGINIA

INTRODUCTION

Fishery management is defined as the application of scientific knowledge to the problems of providing a sustained optimum yield of fishery products for commerical and recreational use (Everhart and Youngs 1981). The contemporary objective of fisheries management according to the Fisheries Conservation and Management Act of 1976 (16 USC 1802, PL94-265), or FCMA, is to ensure the long-term biological and economic success of the fisheries. Prior to making policy decisions concerning the fishery, the present condition of the fish stock(s) should be assessed as should the possible results of the actions being considered (Gulland 1983).

Stock assessment is concerned with the collection and analysis of data on the identification, distribution, abundance, recruitment, mortality, and hence, the status of fishery stocks. The first step in these analyses is "to determine to what extent the fish population and the fishery based on it can be treated as a unit system" (Gulland 1976). Defining the 'unit system' or stock is sometimes complex, particularly when this unit stock as defined at one point in time may change due to environmental or human influence.

The FCMA defines a stock as "a species, subspecies or geographical grouping, or other category of fish capable of management as a unit." A

stock is also defined as that portion of a fish population which is considered actually or potentially exploitable (Ricker 1975), or as a unit which can be independently exploited or managed and contains as few reproductively isolated units as possible (Royce 1972). Cushing (1968) takes a strictly biological approach in defining the ideal unit stock as having a single spawning ground to which the adults return year after year. A stock, therefore, can be defined as both a biological and/or management unit. For the purpose of this thesis, the broader management interpretation provided by the FCMA of 1976 will be used. The biological or genetic definition of a stock may or may not coincide with the ultimate interpretation of a manageable unit. Attempting to manage a fishery as a single unit, however, when it consists of two or more stocks may prove ineffective and unnecessarily expensive.

Historically, fishery management has focused largely on the management of total abundance and available harvest. Ecology and population dynamics or stock assessment have dominated fisheries research, and scant attention has been given to the genetic make-up of the exploited populations. Consequently, very little is known and/or understood concerning the genetics of the various species (Allendorf, Ryman, and Utter 1987). Under such limitations, short-term efforts to restore the economic success of a fishery may prove temporarily advantageous, but the long-term survival of the species is not so easily ensured. Harvest or restoration of exploitable fish without regard to differential reproduction and survival due to different genotypes may alter the genetic composition of the stock. This may ultimately result in the economic extinction of the fishery or in a worse case, the biologic

extinction of the species. Therefore, the genetic structure of an exploited stock or species should be determined before implementing management strategies (Allendorf et al. 1987). As early as 1937, Merriman (1941) realized the importance of thorough scientific research in management: "Regulations intended for the conservation of the striped bass should be based on facts. If they are based on inadequate knowledge...they will be guess-work and in all probability futile."

Allendorf et al. (1987), cite several reasons that genetic data have been so rarely applied to fisheries management:

1. Marine resources, as opposed to other major food sources, are harvested from wild stocks with nebulous mobile boundaries.
2. Taxonomists, who usually do not make the subtle distinctions between individuals and their boundaries, have dominated fishery management in matters of systematics, and geneticists have been hesitant to become involved in the development of management plans.
3. The results from genetic studies sometimes contradict those from previous ecological studies or long-standing assumptions and conceptions concerning stock separation or mixing.

Lack of available or affordable technology may also have been a factor in the past. Presently, however, the technology is available and reasonably economical, and the genetic data base for several important commercial and recreational species is rapidly growing. Genetics are

becoming critical in stock identification and assessment, particularly when the stock and the fishery concerned are depressed.

The striped bass, Morone saxatilis, also known as striper, rock, or rockfish, has long been an important commercial and recreational species (Merriman 1941; Fay, Neves, and Pardue 1983) from North Carolina to Canada (Strand, Norton, and Adriance 1980). Earliest records for striped bass landings date back to 1887 when, according to reports at that time, the species was quite abundant (Koo 1970). The stock then steadily declined until 1934 when catches for the entire Atlantic coast totalled only 1.1 million pounds. The stock soon rebounded and followed an upward trend through 1970 (Koo 1970). Although the dominant year class of 1970 produced huge landings in 1973, subsequent Atlantic coast catch records reveal a gradual decline, with periodic upswings, in the harvest of striped bass (Boreman and Austin 1985).

This decline may be partitioned into the effects of overfishing, environmental stresses, natural fluctuations, or some synergistic combination of these factors. Management regulations imposed by the cooperating states of the Atlantic States Marine Fisheries Commission (ASMFC 1981) in 1982 account for most of the decline subsequent to that year. Current ESBS (Emergency Striped Bass Study) research is addressing these problems and attempting to determine the underlying cause of the decline. The genetic implications of such reductions are important in fisheries management, and the identification of the stock(s) is the first step in their determination.

Although this first step, identification of the stock(s), has been attempted for the striped bass (c.f. Vladykov and Wallace 1952; Raney

1957; Morgan, Koo, and Krantz 1973; Otto 1975), it may be that previous criteria were not adequately stringent to delineate reproducing units of stocks. Due to the depressed state of the stocks and the fishery, management efforts have increased dramatically over the last few years, particularly after 1982. A fishery management plan (FMP), which ideally should be in effect before a fishery is threatened, was belatedly developed in 1981 from historic data on population structure. Subsequent closer analyses suggest that some of the long-term assumptions upon which these management decisions are made, may be invalid, for example, age at maturity and growth rates (Berlinsky, O'Brien, and Specker 1988), and the concept of a single Chesapeake Bay stock (Chapman 1987). The long-term effectiveness of the FMP is not yet determined, but the number of fish does seem to be increasing. This may be due to a natural recovery or to the directed efforts to protect the large 1982 year class which has now entered the fishery, or to a combination of the two. Interstate management efforts have recently been hampered by the lack of stock identification and assessment.

This study examines the genetic structure of striped bass within the Rappahannock River over a four year period and compares it with fish of the upper Chesapeake Bay. Prior to stock identification, the appropriateness of a particular technique should be determined. One objective of this study is to examine the usefulness of mitochondrial DNA analysis in detecting variation and possible genetic markers within the Rappahannock River. The two questions to be answered by these and comparative data are:

1. Are the striped bass in the Rappahannock River genetically distinct from striped bass in the Upper Chesapeake Bay?
2. Do the genotypic frequencies observed in striped bass vary from year to year within a particular river?

The answers to these questions are critical if mtDNA is to be used in identifying stocks for long-term stock assessment and monitoring as required by the 1988 Interstate Fisheries Management Plan (ASMFC 1988). The spatial and temporal existence of a geographically or genetically distinct stock in the Rappahannock River and other Chesapeake Bay tributaries would suggest that the present approach to managing the lower Chesapeake Bay as a unit is not appropriate.

LITERATURE REVIEW

Four major stocks of striped bass have been identified on the Atlantic coast: a Hudson River stock, a Chesapeake Bay stock (Raney and deSylva 1953; Raney, Woolcott, and Mehring 1954; Raney 1957; Lewis 1957; Lund 1957), a Roanoke River-Albermarle Sound stock (Vladykov and Wallace 1952, Raney and Woolcott 1955), and a South Atlantic stock (Raney et al. 1954, Raney and Woolcott 1955, Lund 1957). The Chesapeake Bay stock contributes the largest percentage to the coastal migratory population, up to 90% depending on year class strength (Berggren and Lieberman 1977).

Morphometrics, Meristics, and Tagging

Many attempts have been made to delineate stocks within the Chesapeake Bay and its tributaries. Initially, morphometric (Lund 1957), meristic (Vladykov and Wallace 1952, Lewis 1957, Raney 1957, Murawski 1958), and tagging (Massman and Pacheco 1961, Nichols and Miller 1967) studies identified at least four stocks within the Bay: the Upper Bay, the James River (Massman and Pacheco 1961), the Potomac River (Vladykov and Wallace 1952, Nichols and Miller 1967), and a York-Rappahannock complex (Lewis 1957, Raney 1957, Murawski 1958). Some of this previous research indicates that other identifiable stocks may exist in the Rappahannock, York (Lund 1957, Massman and Pacheco 1961), and Pamunkey rivers (Raney and deSylva 1953).

Questions arose concerning environmental influences on the plastic morphometric and meristic characteristics during development and their role in defining fish stocks (Vladykov 1934, Cushing 1975). Increasing evidence suggests that three factors - temperature, space, and salinity - play important roles in the development of morphometric and meristic characters. In general, higher than average temperatures, lower salinity, or a crowded living space are each associated with a low number of segments and related characters. The extent of their influence, however, is not clearly understood (Vladykov 1934), and Cushing (1975) states that attempts to define fish stocks using morphometrics and meristics are useful only when genetic differences not affected by the environment cannot be detected.

Protein Analyses

With the development of electrophoresis and improved electrophoretic techniques, genetic variation, as expressed by variation in protein structure, within a population can be determined with relative ease (Allendorf and Utter 1979). Each gene locus has different alleles which may specify particular enzymes or proteins that differ in their net electrical charge. Electrophoresis allows indirect observation of genetic population structure by direct observation of these enzymes, the final product of gene activity. In gel electrophoresis, tissue extracts such as soluble proteins and enzymes are placed on or are embedded in a suitable gel and subjected to an electrical field. A particular protein will move through the gel towards the negative or

positive electrode. The mobility of each protein depends on the potential gradient applied between the electrodes, the net charge of the protein, and the size and shape of the protein.

The genetic information available from general protein and isozyme analysis far exceeds that obtainable from morphometric and meristic studies (Allendorf and Utter 1979). In the first electrophoretic study of M. saxatilis, Morgan, Koo, and Krantz (1973) examined serum proteins in juvenile and spawning individuals from the Potomac, Patuxent, Nanticoke, Choptank, and Elk rivers to determine if stocks existed in the upper Chesapeake Bay. They selected five proteins not related to age, sex, or time of collection, and determined that the Elk River striped bass were very distinct from all four locations. The Choptank and Nanticoke river striped bass were also distinct but to a lesser degree. The individuals in the Potomac and Patuxent rivers were indistinguishable from one another.

Otto (1975) collected striped bass from the Hudson River and the York, James, Rappahannock, and Potomac rivers of the Chesapeake Bay. He examined 28 enzyme loci, but found only three that were polymorphic (α-glycerophosphate dehydrogenase or α-GPDH, isocitrate dehydrogenase or IDH, and liver esterase). These proved adequate to discriminate between the Hudson river and Chesapeake Bay fish, but inadequate to discriminate river populations within the Bay. A likely problem associated with these data, however, is the lack of spawning adults in the collection (Sidell et al. 1978). All of the Chesapeake Bay striped bass were 1-2 years old while the Hudson River samples were young-of-year.

Grove et al. (1976) completed a similar study in 1974 and 1975 in which 8-15 morphometric and meristic characters and two polymorphic

liver enzyme systems (of 52 examined), α-GPDH and IDH, were used as stock discriminators. Striped bass were collected from the Roanoke, Hudson, Rappahannock, Potomac, Choptank, and Elk rivers. Overlap of morphometric and meristic character sets and lack of discriminating power in the liver enzymes resulted in the failure to distinguish subpopulations within the Chesapeake Bay. Striped bass from the Hudson and Roanoke Rivers, however, proved to be distinct from one another as well as from the Chesapeake Bay tributaries (Grove et al. 1976).

A subsequent study by Sidell et al. (1978, 1980) combined serum protein analysis as detailed by Morgan et al. (1973) and analysis of the polymorphic enzymes described by Otto (1975) and Grove et al. (1976). Spawning striped bass were collected from the Potomac, Choptank, Sassafras, Bohemia, Elk, and Rappahannock rivers as well as from the Chesapeake and Delaware Canal (C & D Canal). Some juveniles were collected in the C & D Canal and Bohemia River following the spawning season. Twelve of the 26 protein bands observed proved to be useful as stock discriminators. The serum enzymes α-GPDH and IDH, previously shown to be polymorphic in liver tissue of striped bass (Grove et al. 1976) were examined, although IDH was excluded from the final analysis due to inconsistent resolution. No significant differences were found among striped bass from the Chesapeake Bay tributaries which is in agreement with the results of Otto (1975) and Grove et al. (1976) . The same lack of heterogeneity was found even after grouping the fish into Mid- and Upper-Bay samples. These results support the conclusions of the previous morphometric and meristic studies (Vladykov and Wallace 1952, Lewis 1957, Raney 1957) in which the Upper Bay striped bass are classified as a homogenous stock within the Chesapeake Bay.

The dissimilarity between the results of Morgan et al. (1973) and Sidell et al. (1978, 1980) may be due to differences in sample preservation (Sidell et al. 1978, 1980). Blood samples collected during Morgan's study were centrifuged on the day of collection and then frozen at -15°C, while those taken during Sidell's study were held on ice for less than 2 hours before being centrifuged and stored in liquid nitrogen. Although Morgan's handling techniques are generally acceptable in such studies, proteins break down easily causing changes in their electrophoretic mobility. Other differences in handling and storage of samples and their subsequent analysis may be partially responsible for the differing conclusions of Morgan et al. (1973) and Sidell et al. (1978, 1980).

A more recent look (Rogier, Ney, and Turner 1985) at enzyme variation in landlocked striped bass of the Kerr Reservoir in North Carolina produced unique results. Spawning striped bass were collected in 1979 and 1980 from the Dan and Roanoke tributaries of the Kerr Reservoir. All sample tissues were stored on ice, centrifuged, and frozen on dry ice before storage at -90°C. Although 56 loci (31 enzyme systems) were initially surveyed, only 3 were polymorphic (creatine kinase 1, CK-1; inorganic pyrophosphatase 1, Ipp-1; and inosine triphosphatase, Itp). Based on this preliminary survey, the percentage of polymorphic loci, 5%, and the average heterozygosity estimate, 1.6%, are very low compared to other fish species examined (Nevo 1978, Kirpichnikov 1981). In the final results, allele frequencies of the 3 polymorphic loci were significantly different between the rivers in 1979 but not in 1980.

According to Lewontin (1974), only 33% of amino acid substitutions are detectable by electrophoresis. For many species such as striped

bass which is characterized by low heterozygosity and heterogeneity, electrophoresis of proteins may not adequately reveal the genetic variation present. The study of Rogier et al. (1985) also indicates that sampling should occur over a number of years to determine the year-to-year variation in gene frequencies. Although Rogier et al. (1985) did not collect their samples in the Chesapeake Bay, the problems encountered in their study of the Kerr Reservoir striped bass can very easily occur in the Bay as well. It is this type of interannual variation that can confound management strategies and has resulted in recommendations for annual river-by-river genetic (stock) monitoring in the rewrite of the 1988 Interstate FMP for striped bass (ASMFC 1988).

Isoelectric focusing is a type of electrophoresis which separates tissue proteins on the basis of their isoelectric points, the pH at which the protein is electrically neutral. Fabrizio (1987) used this technique to separate eye lens proteins of striped bass. She accurately distinguished fish from the Hudson River and Chesapeake Bay, which contributed to the Rhode Island trap net fishery. Prevous studies have shown that eye lens proteins are particularly suited for electrophoretic analysis of intraspecific differences (see Smith 1965, 1966, Smith and Goldstein 1967, Eckroat and Wright 1969, Peterson and Smith 1969, Bloemendal 1977, Fabrizio 1983), however the results may vary with the age, and thus the weight and length of the fish. Nutrition, exposure to toxins, and other factors may also affect eye lenses and their proteins (Hargis, Roberts, and Zwerner 1984; Hargis and Zwerner 1988).

Mitochondrial DNA Analysis

A technique only recently developed for identification of intraspecific differences in fish is restriction endonuclease analysis of mitochondrial DNA (mtDNA) (Avise, Lansman, and Shade 1979a; Brown et al. 1981; Berg and Ferris 1984; Chapman and Powers 1984). MtDNA is a double-stranded, circular molecule of approximately 17,000 base pairs, or 17 kilobases (kb) in striped bass. Restriction endonucleases (enzymes) recognize 4, 5, or 6 base pair sequences in the molecule and cleave the mtDNA at specific sites within these sequences. The resulting fragments are then separated by molecular weight through submerged gel electrophoresis and observed by staining or autoradiographic techniques. The number of restriction fragments equals the number of restriction (recognition) sites in the molecule. A single base pair substitution may cause the gain or loss of a restriction site.

MtDNA has many properties that make it a suitable and practical source of material for genetic studies. MtDNA is small, unlike nuclear DNA, and easily isolated in a sufficiently purified form for analysis by several methods (Avise et al. 1979a; Brown 1981; Chapman and Powers 1984). Nuclear DNA ia at least 25,000 times larger than mtDNA and contains introns and numerous repetitive sequences that make characterization of the genome difficult (Brown 1981, 1985). The mitochondrial genome of three species: mouse (Mus musculus)(Bibb et al. 1981), cow, and human (Anderson et al. 1981, 1982) has been completely sequenced. The relative simplicity of the mtDNA genome allows direct genotype analysis and comparison between populations or closely related species (Berg and Ferris 1984).

MtDNA is inherited maternally through the egg cytoplasm (Avise et al. 1979a; Giles et al. 1980) thus eliminating the complexities of recombination in meiosis (Avise et al. 1979a, Brown 1985). Phenotypes are transmitted intact and all sequence changes arise only by mutation (Avise et al. 1979a). Unlike nuclear DNA, mutations fixed in an individual result in a new phenotype that can be unambigously linked to its progenitor (Avise et al. 1979a).

Evolution of mtDNA is 5 to 10 times that of single-copy nuclear DNA possibly due to the lack of a repair function in mtDNA replication, a high rate of mutation fixation, or as a result of low functional constraints on the gene products (Brown, George, and Wilson 1979). Whatever the reason, rapid evolution of mtDNA allows for detection of relationships between recently diverged populations or species (Brown et al. 1979). This should help to confirm migration patterns, homing tendencies, and degree of mixing of stocks which is vital to stock assessment and management.

Restriction analysis of mtDNA offers several additional advantages over the standard protein analysis. All mtDNA within an individual is the same regardless of the tissue from which it was extracted (Avise et al. 1979b, Upholt and Dawid 1977). In contrast to the lack of heterogeneity encountered in the serum protein and isozyme analyses, mtDNA sequence heterogeneity is high among individuals of a species and individuals within a local breeding population (Avise et al. 1979a, Brown et al. 1982, Chapman and Powers in press).

MtDNA analysis, unlike protein analysis, focuses on the primary DNA sequence. Therefore, post translational modification through environmental influences such as temperature, space, and salinity do not alter

the data base. A single-base substitution within a mtDNA recognition sequence can be detected by examining the restriction pattern resulting from cleavage by restriction enzymes. As stated previously, protein electrophoresis can only detect 33% of possible amino acid substitutions (Lewontin 1974). Recent improvements in the technique and a substantial reduction in the time and money required for a complete restriction enzyme analysis have resulted in a more convenient and practical technique than in the past (Brown et al. 1981, Chapman and Powers 1984). MtDNA analysis is no longer so prohibitively expensive as to preclude its use over isozyme, eye lens protein, or general protein analysis, especially when one considers the wealth of additional information that becomes accessible. According to Graves and Dizon (1986), endonuclease analysis of mtDNA is presently the most powerful and practical tool available for studying the genetics underlying population structure.

Examination of striped bass mtDNA within the Chesapeake Bay was initially conducted by Chapman (1987) who evaluated the genotypic frequencies of 1982 year class males collected in 1984 and 1986 from the Potomac River, the Choptank River, and Worton Point near the mouth of the Sassafras River (Figure 1). He also collected 2 year old males in 1984 from the Rappahannock River (Chapman and Powers, in press; Figure 1) and compared their genotypic frequencies to those found in the Upper Bay in 1984. Within that year, significant differences existed between the Rappahannock sample and the pooled Upper Bay sample. The genotypic frequencies of the fish collected from the 3 locations in the Upper Bay, however, shifted between 1984 and 1986 possibly due to migration of fish from other rivers. The next step was to examine Rappahannock fish collected in 1986 (Figure 1) to determine whether the

differences observed in 1984 were still present. The results of Chapman's study provided not only a comparative data base for future studies but also established the technical and analytical foundation on which this study was based.

MATERIALS AND METHODS

Field Collection

Thirty-five gravid striped bass were collected from Naylor's Point, Blanfield Point, and Carter's Wharf in the Rappahannock River, Virginia during the spring spawning run of 1986 (Figure 1). The sampled fish represent the 1977 to 1985 year classes with approximately 56% representing the 1982 year class. Once collected, the striped bass were transported on ice to the Virginia Institute of Marine Science (VIMS), and within 24 hours all fish were measured, weighed, and sampled for tissues. Stage of sexual maturity was also recorded and scales were removed for subsequent age determination. Fresh ovarian tissue was excised from the fish and placed immediately on ice. Within 30 minutes, all tissues were transferred to a -20^{o}C freezer. Several months later, all samples were moved to a -72^{o}C freezer (So-Low Chilling Machine^{R}) until they were required for further processing.

Mitochondrial DNA Isolation

The laboratory procedure employed in this study was a modification of Chapman and Powers technique (1984) which substantially reduces the time and effort previously required to isolate mtDNA by traditional methods. Many of the time-consuming steps have been eliminated and replaced by more expedient procedures.

The resulting mtDNA is not as pure as with traditional methods, it is more than adequate for the needs of this study.

Three to 5 grams of frozen ovarian tissue were thawed and homogenized in 5 volumes of cold TEK buffer (50mM Tris, 10mM EDTA, 1.5% KCl, pH 7.5) to lyse the cells. A Yamato^{R} low-shear continuous tissue homogenizing system was used to ensure that the cells were not excessively homogenized causing destruction of the mtDNA and possible nuclear DNA contamination.

The homogenate was transferred to 15 ml polycarbonate centrifuge tubes and centrifuged at 1000 x g for 10 minutes. Three layers typically resulted: a bottom layer containing cell membranes, a middle layer of mitochondria, glycogen and proteins in an aqueous solution, and an upper layer of fat. The mitochondrial layer was drawn off, avoiding the transfer of fat, to another centrifuge tube and TEK buffer was added to a final volume of 10-13 ml. Centrifugation at 1000 x g was repeated and the mitochondria were then transferred to a high speed centrifuge tube. The supernatant was spun for 60 minutes at 18,000 x g which resulted in the formation of a dense, clear glycogen pellet overlain by a loose mitochondrial pellet. The aqueous phase containing cellular debris was discarded. The pellet was resuspended in TEK and centrifuged for 30 minutes at 18,000 x g. This second high speed spin further purified the mitochondria. The aqueous phase was again discarded leaving a dense pellet containing glycogen and mitochondria.

To lyse the mitochondria, I added 0.5 ml of 5% Non-idet-P-40 (NP-40 in TEK) to each sample. NP-40 is a non-ionic detergent capable of lysing the mitochondrial membrane. The pellet was resuspended by vortexing and transferred to a sterile 1.5 ml microcentrifuge tube. The

samples were left at room temperature for 10-15 minutes to allow the NP-40 to completely lyse the mitochondria. A test for complete lysis is the clearing of the solution shortly after mixing.

The lysed mitochondria were then centrifuged at 12,000 x g for 15 minutes. The supernatant containing mtDNA was transferred to another microtube leaving the pellet containing the broken membranes behind. NP-40 is incapable of lysing nuclear membranes so any nuclear DNA material present at this time is pelleted with the mitochondrial membranes. Three hundred microliters of redistilled (Chapman and Powers 1984) and buffered phenol (Maniatis, Fritsch, and Sambrook 1982) was added to each sample and thoroughly mixed. The mixture was then centrifuged at 12,000 x g for 15 minutes.

Following centrifugation, 3 distinct layers typically resulted: a bottom layer of phenol, a middle layer of precipitated proteins, and an upper aqueous phase containing nucleic acids. This upper layer was transferred to a clean microcentrifuge tube and the phenol extraction repeated to further purify the mtDNA. The upper aqueous layer was again drawn off to another microcentrifuge tube and 0.2 ml of a 24:1 chloroform:iso-amyl alcohol solution was added and mixed thoroughly to remove traces of phenol.

The mtDNA-chloroform:iso-amyl alcohol mixture was centrifuged as before at 12,000 x g for 15 minutes. Two clear, but immiscible layers resulted. The upper layer was transferred to a clean microcentrifuge tube and 2 volumes of cold 95% ethanol was added to precipitate the mtDNA. MtDNA is soluble is water but not in ethanol. The samples were then placed in the freezer at -20^{o}C where they were held for at least 2 hours.

The precipitated mtDNA was pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C. The ethanol was decanted and the samples were dried at 37°C. The dried pellets were rehydrated with 100 microliters (µl) sterile water and immediately digested with restriction enzymes or stored at -20°C until needed.

Mitochondrial DNA Digestion

The mitochondrial DNA was initially digested (cut) with 16 restriction enzymes: Hind-III, Eco-RI, Eco-RV, Bcl-I, Bgl-I, Nci-I, Sma-I, Sst-I, Sst-II, Xba-I, Bam-HI, Pst-I, Sal-I, Cla-I, Pvu-II, and Ava-I (Bethesda Research Laboratories). For comparative purposes, I chose Hind III, Eco RI, and Bcl I which had been used successfully in an earlier study of striped bass mtDNA in the Upper Chesapeake Bay (Chapman 1987). These enzymes produce small molecular weight fragments $\leq$ 3.6 kb that occur in a portion of the gel in which small size differences of approximately 100 bp are easily detected (Chapman, personal communication). Although the smallest fragment produced by Eco RV is 4.7 kb, detection of size variations was still possible. All samples were first isolated, digested, electrophoresed, and stained for analysis.

Selected samples (see Results) were then reanalyzed using end-labelling with ATP(^{35}S) which enhances visualization of digestion fragments. The method used here was a modification of that described by Maniatis et al. (1982) (See Appendix B). The Klenow fragment, cold phosphate dGTP, dCTP, dTTP (if required), and ^{35}SdATP were added to the digestion reaction and the samples were incubated at 37°C for 3 hours.

After incubation, the labelled mtDNA was precipitated by adding two volumes of cold 95% ethanol to each sample. The soluble unincorporated label remained in the ethanol. The resulting solution was well-mixed and centrifuged at 12,000 x g for 15 min at $4^{o}C$. The ethanol was removed by pipet, and the sample was then dried at $37^{o}C$ and rehydrated in 10 µl TEB (89mM Tris, 2.5mM EDTA, 74mM Boric Acid, pH 8.3) and 2 ul STOP solution (0.02% bromophenol blue, 0.50% SDS, 20.0% glycerol). After a 3-5 second centrifugation to assure complete mixing, the samples were immediately loaded onto a gel or held at $-20^{o}C$ until needed. For a discussion on the problems which may be encountered using this technique, see Appendix A.

Electrophoretic Separation

Agarose gels (agarose in TEB) were prepared during sample digestion to allow the gel adequate time (> 1 hr.) to harden. Agarose concentrations were adjusted from 0.8-1.0% to magnify the differences between variable bands. An increase in the concentration of the gel slowed the separation of the fragments during electrophoresis, but ultimately allowed for tighter, sharper bands. A lower gel concentration allowed for more rapid separation of the bands when detection of minor size differences was not necessary. Molecular weight determinations were possible with the addition of a standard 1 kb ladder (BRL) which can be radiolabelled or stained with ethidium bromide (EtBr)(Maniatis *et al*. 1982). For each standard, 2 µl of a 1:90 dilution of the ladder, as shipped, was added.

Gels were run overnight (12-16 hrs.) at 25V and $\leq$40 milliamps. Once the run was complete, the gels were removed from the gel unit and

tray and stained in EtBr for approximately 30 minutes. The gels were removed from the EtBr, rinsed briefly with TEB, and photographed over a 165 x 165 mm UV transmitting filter (Hoya Optical, U-340) which was illuminated from below by 3 20-watt fluorescent bulbs (Westinghouse, FS-series, sunlamps). The UV filter allowed only light of approximately 310 nm to penetrate to the gel which was placed directly on the filter. The photographic system consisted of a Polaroid MP-4 camera equipped with a Kodak 23A orange filter. The filter further enhances the contrast between the fluorescent bands and the background by absorbing shortwave radiation and transmitting the longer red-orange wavelengths. Polaroid Type-55 film was exposed for 10-15 minutes at f4.5. Exposure time depended on the intensity of the stain. The film was developed for 1 minute and the negative was placed in 18% sodium sulfite for 5 minutes, washed with water overnight, coated with photoflo and air-dried.

The gels were then transferred back to destain (TEB) for several minutes before placing them in 10% Acetic Acid/10% methanol solution for 5-15 minutes. This acidifies the gels and thus precipitates and immobilizes the mtDNA in the gel matrix. The reaction was complete when the marker dye turned pale yellow. The gels were dried face-down onto Whatman 3MM filter paper for 1-2 hours at 60^{o}C until completely flat. In a darkroom, the dried filter paper was taped, gel-side up, to a piece of cardboard, and Kodak XAR-5 X-ray film was secured with tape over the gel. Excess cardboard flaps were folded over the film and gel and clamped securely so that the film lay flat on the gel. This "set-up" was then wrapped entirely in aluminum foil to ensure that no light

penetrated to the film. The wrapped gel and film were held in a light-tight drawer for 1-14 days depending on the incorporation of the label by the mtDNA.

The x-rays were developed in the following manner:

1. GBX x-ray developer- no more than 4 minutes
2. Kodak Stop (1% Acetic acid) - 2 minutes
3. Commercial Fix - 5 minutes
4. Wash in water for 10-15 minutes
5. Dry at room temperature

The final result is an x-ray that exactly, and more clearly, represents the photographic negative of the EtBr-stained gel.

Data Interpretation

Each gel was examined for differences in the restriction patterns among individual samples. Migration distances were estimated by ruler to the nearest tenth of a millimeter from the gel, photographic negative, and/or x-ray for each restriction fragment (represented as bands on the gel) of each individual sample. Pattern differences, i.e. gain or loss of fragments or different size fragments, were noted for each enzyme. Molecular weight determinations for each fragment were based on the migration distances of the molecular weight standard. A best fit regression function using Lotus 1-2-3 was formulated for each gel and its standard. The molecular weight for the sample restriction fragments on that gel were computed from this function. Total molecule weight was determined by addition of the various fragments.

Each sample produced a fragment pattern, as determined by the number and molecular weight of the restriction fragments produced by digestion with an enzyme. For each enzyme, the pattern was classified as a particular genotype labelled A, B, C, etc. In this and earlier experiments, 'A' represents the smaller molecules while B, C, etc. represent larger molecules. The frequency of occurrence of each genotype was recorded and compared to those found by Chapman (1987), Chapman and Powers (in press), and Meehan and Banford (unpublished) to determine if differences existed between geographic locations within the Chesapeake Bay and between sampling years within the Rappahannock.

The G-statistic was used to test for Goodness-of-Fit (Sokal and Rohlf 1981) to an expected genotypic frequency distribution generated from the total observed frequencies. As G-values were neither summed or partitioned, the William's Correction was incorporated to lessen the Type I error, thus producing a more conservative test. Although the G-test is usually accepted as the stronger test in most cases (Conahan 1970, Sokal and Rohlf 1981), these results were compared to those obtained using the more conservative Chi-square Goodness-of-Fit test. Larntz (1978) states that at a significance level of 5%, and expected frequencies between 1.5 and 4, G rejects the null hypothesis too often and was not a close approximation to the Chi-square distribution when the observed frequencies were 0 or 1. Larntz' study, however, did not consider the William's Correction. With the exception of a few cases, the results obtained with the X^2 statistic differed only in the level of significance from those generated with the G-statistic. In the exceptional cases, the result was considered non-significant in agreement

with the Chi-square test, as the X^2 value generated by the G-test was usually of marginal significance.

RESULTS

Of the fish sampled, 23 produced acceptable results. Many of the tissue samples were depleted prior to end-labelling in attempts to obtain interpretable data. Some individual material which was very limited in quantity was held until digestion and end-labelling techniques could be perfected. The -70^{o}C freezer in which the samples were held was inoperable for 5 days due to an electrical storm. All remaining samples, including some that had not been analyzed, thawed completely and no mtDNA was recoverable after that time.

Although 10 enzymes cleave the mtDNA consistently (Hind III, Eco RI, Eco RV, Bcl I, Bgl I, Ava I, Nci I, Sst I, Sst II, Pvu II), 4 enzymes were most useful in revealing differences among striped bass individuals: Hind III, Eco RI, Eco RV, Bcl I. Table 1 lists the specific sequence recognized by each of these enzymes and the respective cleavage sites.

The genotypic frequencies obtained with Hind III, Eco RI, Eco RV, and Bcl I are presented in Table 2. There are 5 genotypes represented with total molecular weights ranging from 17.5-17.8 kb. No gain or loss of restriction sites caused by substitution, deletion, or addition of nucleotides was observed. Variation in the total molecular weight was reflected in the approximately 100 bp change in the size of the variable fragment. The restriction patterns produced by each enzyme are presented in Figures 2 and 3.

The B genotype, represented primarily by 1982 year class females, comprises 44% of the fish sampled. The heteroplasmic D/E genotype is unusual in revealing 2 different size molecules within the same individual.

In a comparison of the genotypic frequencies observed in samples taken in 1986 from the Rappahannock River and the pooled frequencies found in Upper Bay samples (Chapman 1987), significant differences were found which were similar to those seen in the same comparison in 1984 (Chapman 1987 and Chapman and Powers, in press)(Tables 3A and 3B). An examination of the data from each of the 3 locations in the Upper Bay yields a somewhat different conclusion when compared to the 1984 (Chapman and Powers, in press) and 1986 Rappahannock data (Table 4). In 1984 the genotypic frequency distribution in the Choptank was not significantly different from that in the Rappahannock, while the Potomac distribution was by far the most distinct. The same comparison between the 1986 Rappahannock sample and Chapman's (1987) Upper Bay samples produced similar results for the Potomac River striped bass showing that they were genetically distinct from the Rappahannock fish. The Choptank fish were significantly different, while mtDNA samples from Worton Point, a true Upper Bay location, did not differ significantly in genotypic frequencies from those in the Rappahannock fish. In both 1984 and 1986, M. saxatilis in the Potomac River, the sampling site closest to the Rappahannock, displayed the least genetic similarity to the Rappahannock fish.

In order to determine whether yearly variation in genotypic frequencies occurred within a sampling site, specifically the Rappahannock River, data from 1984 (Chapman and Powers, in press), 1986, and 1987

(Meehan and Banford, unpublished) were analyzed for deviations from expected frequencies. Comparison of the 1986 collection of females presented in this study to the 1984 collection of 2 year old males (Chapman and Powers, in press) revealed no significant differences between the corresponding genotypic frequencies (Table 5). Although their 1984 sample did not produce any representatives of the C, D/E, and F genotypes which account for 26% of the 1986 sample, the predominance of the B genotype, 17.6 kb, was found in both collections.

Further comparison of these 1984 and 1986 samples the 35 females collected in the spring of 1987 (Meehan and Banford, unpublished; (Table 6), revealed startling differences, including the presence of a new heteroplasmic genotype, C/F (17.7/17.8) (Tables 5 and 6, Figure 4). Unlike the 1984 and 1986 data, the C genotype (17.7 kb) is predominant comprising 54% of the total sample, and the A genotype is completely absent.

Because this analysis compared data generated in two different laboratories (Chapman 1987, at the Chesapeake Bay Institute and Meehan and Banford unpublished, at VIMS), I was concerned whether this rapid frequency shift was real or merely an artifact of slightly different techniques in either data generation or interpretation. Although genetically possible, such a sudden change in frequencies is difficult to explain. To determine whether the shift was real, samples from the 1987 collection were electrophoresed on the same gel with tissues provided by Chapman. A discussion of this analysis and my conclusions are found on pages 37-41.

DISCUSSION

Geographic Variation

The primary objective of this study was to compare striped bass mtDNA genotypes from the Rappahannock with other populations in the Chesapeake Bay. The hope was to find a clear genetic marker, such as a unique genotype, that would specifically identify these fish as originating in the Rappahannock River. While no such specific marker was found, a comparison of genotypic frequencies indicated that *M. saxatilis* in the Rappahannock River are distinct from those in regions of the Upper Chesapeake Bay.

Evidence provided by a comparison of genotypic frequencies found in the Upper Bay striped bass in 1984 and 1986 (Chapman 1987) to those found in the Rappahannock in 1984 (Chapman and Powers, in press) and 1986 indicate that distinct differences exist between fish from these two regions of the Chesapeake Bay (Table 3). The major contributors to the degree of heterogeneity seen in this and other comparisons are shifts in the frequency distributions of common genotypes within a river. The rare genotypes, such as D/E and F, are interesting in an evolutionary sense but may not be diagnostic of stock differences. For example, the A genotype, although not predominant, occurs at a relatively high frequency in the 1984 and 1986 Rappahannock samples,

while only 5 of 109 fish sampled in the Upper Bay displayed the A genotype in 1984 and 1986 combined (Table 3). Closer examination of the frequencies found within the Potomac, Choptank, and Worton Point as they compare with the Rappahannock (Table 4), it appears that factors other than geographic distances may be at work in establishing genotypic frequencies. The spawning grounds of the Rappahannock and Potomac River are approximately 120 river miles apart, but M. saxatilis are known to travel great distances within the Bay. Striped bass are also not as bound by salinity as is the closely related white perch, Morone americana (Bowen 1987), or by other known geographic barriers that would prevent mixing of these spawning groups during the remainder of the year.

Past tagging (Vladykov and Wallace 1938, 1952; Nichols and Miller 1967), morphometric and meristic studies (Vladykov and Wallace 1952, Lund 1957) support the existence of a relatively static Potomac River stock distinct from the Lower Chesapeake Bay tributaries. When the Potomac was not found to be morphometrically or meristically unique as to be classified as a separate stock from the Maryland portions of the Bay, the Potomac was grouped with other Upper Bay samples and tested against lower Bay tributaries for significant heterogeneity (Lewis 1952, Raney 1952, Murawski 1958). In each case, the Upper Bay sample proved significantly different from the Rappahannock, James and York Rivers. Subsequent biochemical assays did not support the existence of sub-populations or stocks in the lower Chesapeake Bay. With the exception of one serum protein analysis which indicated the presence of Upper Bay stocks (Morgan, Koo, and Krantz 1973), no other evidence for the existence of river stocks was found (Otto 1975, Grove et al. 1976, Sidell et

al. 1978, 1980), probably due to the lack of heterogeneity typical of striped bass enzyme systems.

It has been suggested that some level of differentiation exists among the Chesapeake Bay tributaries based on salinity. Morgan et al. (1973) proposed a geologic origin for river stocks. During the Wisconsin glaciation almost 35,000 years ago, sea level dropped 200-300 feet below the present level causing the salt wedge in the Chesapeake Bay to retreat onto the continental shelf. The Susquehanna became a huge river into which all the Chesapeake Bay tributaries drained. As sea level began to rise 10,000-12,000 years ago, the salt wedge,and consequently the fresh and brackish waters of the striped bass spawning grounds, moved up the Susquehanna. Before the salt wedge reached the mouth of the James River, striped bass could spawn throughout the area. On reaching the James River, however, the salt wedge split creating one salt wedge in the James and one in the Susquehanna. This provided two hydrographically restricted areas for the striped bass to spawn. According to Morgan et al. (1973), this process continued for each tributary as the salt wedge moved with rising sea level up the Susquehanna.

Morgan et al. (1973) also proposed that the time scale involved in such a process supported the evidence for clearly defined stocks in each of the James, York, and Rappahannock rivers and a lack of distinct stocks within the Upper Bay. Clearly, the salt wedge and spawning grounds in the James River were well-established before those of the York and Rappahannock rivers and long before those of the Upper Bay. Lewis (1957), Lund (1957), Raney (1957), and Murawski (1958) classify

the James as the most well-defined stock followed by the York-Rappahannock system and the Upper Bay.

The theory proposed by Morgan et al. (1973) may provide an adequate explanation for the differences observed in the genotypic frequencies of striped bass from the Rappahannock and Potomac rivers. As mentioned earlier, however, salinity defines only the spawning grounds and not general tolerances throughout the range of the species. This theory would only hold true if during the development of the spawning grounds 10,000-12,000 years ago, spawning striped bass developed certain responses to specific environmental cues which resulted in subsequent generations to return to a specific natal river. This is a possibility, of course, as female M. saxatilis are thought to exhibit homing. Another factor to consider is differential migration. Massman and Pacheco (1961) suggested that York and Rappahannock fish may migrate northward in the Bay, while Mansueti (1961) concluded from tagging studies that very few striped bass migrated from Maryland waters into Virginia tributaries.

Chapman's (1987) Upper Bay data indicates that migration of male M. saxatilis between rivers may indeed exist. Genotypic frequencies of fish taken from each of the 3 sampled locations, Potomac River, Choptank River, and Worton Point, reveal an increase between 1984 and 1986 in the number of fish exhibiting the C genotype, from 15% to 49%. Mutation alone cannot possibly account for this rapid shift in overall frequencies. Migration from other areas of the Upper or Lower Bay must therefore be responsible. It is impossible to determine the river(s) of

origin based on available data, but the genotypic frequencies characteristic of the Rappahannock River during that time indicate that the Rappahannock is not the source of the C genotype.

As noted by Kriete, Merriner, and Austin (1978), the extent of striped bass migration is in part dependent on year class size. In years of below average or average abundance, fish tend to remain in the natal river throughout their second year before entering the migratory population. However, in years of higher than average abundance, a larger percentage of fish ≤ 2 years old join the migratory population. The studies presented here primarily involve the 1982 and 1983 year classes which were smaller than average (Colvocoresses 1984). Now that the stock(s) is(are) rebuilding, migratory habits may be changing in response to a larger population size. Mixing of fish from various regions of the Chesapeake Bay may be more extensive.

Whether geologic history, distinct migratory habits, density-dependent stock fluctuations, or other unknown factors play a significant role in establishing genotypic frequencies is unclear from the available data. Extensive sampling is necessary to obtain a more comprehensive picture of genetic differences among striped bass. The presence of genetically distinct stocks within the rivers of the Chesapeake Bay would indicate the presence of biological differences as well. In either case, the future of the Chesapeake striped bass fishery may be more vulnerable to overfishing than is presently suggested. The elimination of a genotype or genotypes in a particular river may result in the elimination of a population and the fishery it supports. The genetic composition and varying biological requirements of river stocks, if present, should be considered in the management of the species.

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Year-to-Year Variation

When it occurs yearly variation in the M. saxatilis stock composition of a particular river should be reflected by corresponding shifts in the genotypic frequency distribution. This is, of course, assuming the genotypic frequencies are not homogenous throughout the Chesapeake Bay and its tributaries. In the Maryland waters of the Chesapeake Bay, year-to-year frequency variation does exist (Chapman 1987) and may be explained by different migratory habits (Chapman 1987; Adamkewicz, Chapman, and Powers 1987).

In the comparison of 1982 year class males taken in the spring of 1984 and 1986 from the Potomac River, Choptank River, and Worton Point, Chapman found significant differences between the three areas in 1984 but not in 1986 (Table 7). As males generally do not migrate out of the natal tributary until after their second year (Massman and Pacheco 1961, Mansueti and Hollis 1963), Chapman suggested that the 1984 sample of 2 year old males represented the matriarchal genotypic frequencies which were apparently distinct among the three locations. He postulated that during their third year, the males migrated out of their natal rivers and mixed with males from the Upper and Lower Bay, and that the 1986 sample represented males which reentered the rivers indiscriminantly and without regard to their natal river. Therefore, the mtDNA sequences found in 1986 include those originating elsewhere in the Bay, possibly the Lower Bay. The shift in restriction fragment patterns is most apparent in Chapman's (1987) comparison of genotypes in 1984 and 1986 within each sampling site (Table 8).

Yearly variation within the Rappahannock River was examined by comparing genotypic frequencies in 1984 (Chapman and Powers, in press),

1986, and 1987 (Meehan and Banford, unpublished; Table 5). Although the 1984 sample is composed entirely of 1982 year class males and the 1986 and 1987 samples are all females primarily of the 1982 and 1983 year class, respectively, the comparison is valid as 2 year old males represent the matriarchal genotypes within the natal river. The general distribution of the common mitochondrial genotypes, A, B, C, etc., revealed a major shift towards higher frequencies among the larger molecules. One explanation for this shift in genotypic frequencies may be interannual changes in year class distribution. The B genotype was predominant in both the 1984 and 1986 samples which are composed mostly (100% and 56%, respectively) of 1982 year class fish, while the C genotype was most common in the 1987 collection which was largely (71%) 1983 year class females (Table 6). Due to the small sample size of the 1984 and 1986 samples, the sudden increase in the frequency of the C genotype is probably not an accurate reflection of the actual rate of increase, but the differences are clear.

Of course, it is possible that one or more Rappahannock samples do not accurately represent the frequency of mtDNA sequences in M. saxatilis during that sampling year or that Chapman (1987) and Meehan and Banford (unpublished) used slightly different techniques to score the data. Assuming that the genotypic frequencies found in 1984, 1986, and 1987 are representative of true frequencies within the stocks involved, one must also assume that year-to-year genetic variation does exist within the Rappahannock River as it did in the enzymatic study of striped bass in the Kerr Reservoir, North Carolina in 1979 and 1980 (Rogier et al. 1985).

Although minor shifts in frequencies are expected to occur over time due to mutation, selection, or random genetic drift, sudden distributional changes are not. Variable spawning success or fishing pressure may cause major shifts in genotypic frequencies within a single year, but there is no indication that either was a factor in the Rappahannock between 1982 and 1987.

The increased frequency of the C genotype in 1987 in this case must be due to migration from other river systems as mutations would not have accumulated to a detectable level within one year. This finding does not then support the theory that female striped bass home as suggested by previous tagging studies (Mansueti 1961, Massman and Pacheco 1961, Nichols and Miller 1967). It is possible that the increase in the number of the C-type restriction pattern arose from a small proportion of breeding females in the early 1980's when the stocks were at an all time low and that the less common C/F genotype was sufficiently rare as to remain undetected in the previous samples. However, the complete absence of the A genotype is difficult to explain. One or more of the Rappahannock samples may be extremely localized and unique, or other unknown factors may play a very important role in determining genotypic frequencies.

An important consideration when comparing data generated by different laboratories is whether sample handling, data generation, statistical treatment, and interpretation were consistent. The technique used to generate mtDNA restriction fragments by me and Meehan and Banford were derived directly from that developed by Chapman and Powers (1984). Therefore, no variability was caused by sample processing and data generation. Scoring of the gels, i.e. labelling a restriction

fragment pattern as genotype A, B, C, etc., however, was initially a problem. Slight variations (~0.5mm) in the measurement of migration distances can result in different genotypic designations.

In an effort to determine if gels were scored consistently between the two laboratories, samples provided by Chapman and Meehan were digested and electrophoresed side-by-side. According to the genotypic labels given to the samples each provided, Chapman's 'A' was identical to Meehan and Banford's 'B' as determined by migration distances. Therefore, for the same fragment pattern, molecular weights as determined by Meehan and Banford were slightly higher (~100 bp) than those reported by Chapman. My scoring of the gels was consistent with that of Chapman's which would explain the shift in reported genotypic frequencies toward the larger molecules in 1987.

Chapman and Meehan and Banford's approach to scoring the gels were basically the same, although different tools were used to measure migration distances. These distances were used to generate a standard regression curve which was then used to determine the molecular weight of the unknown sample DNA. Meehan and Banford measured migration distances of fragments directly from the gel. Chapman used a digitizer to measure migration distances from a photograph of the gel. An example of the regression analysis generated by Meehan and Banford is found in Appendix C.

The discrepancy in molecular weight determinations could be a result of error in the measurement of migration distances, rounding error, or inherent in the generation of the regression curve. In most mtDNA population studies, an error of 100 bp would not affect the interpretation of the final results since most species exhibit restriction

fragment length polymorphisms, or a gain or loss of restriction sites. Detection of a restriction site gain or loss in a sample is accomplished by simple comparison to a molecule which exhibits the original or unaltered restriction fragment pattern. The molecular weight of each fragment is not as important as the actual change in the pattern; the total molecule size usually remains the same. Striped bass are unusual in that the only variation seen thus far in the mtDNA is variation in the total molecule size, not in the gain or loss of restriction sites. Therefore, in interpreting the results it is important to determine, as accurately as possible, the total molecular weight and particularly that of the variable bands. An error of 50-100 bp may significantly affect the interpretation of the results.

In comparing striped bass data generated at different labs, side-by-side electrophoresis of samples previously scored by each lab should be emphasized in order to ensure consistency in molecular weight determinations. Because the actual weight of the striped bass mtDNA molecule can only be determined through sequencing, a long and tedious process, consistency between researchers in determining molecular weights is necessary to allow meaningful interpretation of the results.

Until the problem is resolved and based on the analyses here, I conclude that the general distribution of the common mitochondrial genotypes, A, B, C, etc. was the same in 1984, 1986, and 1987, and that the apparent shift in genotypic frequencies in 1987 (Meehan and Banford unpublished) was due to differences between laboratories in the scoring of the data. A stable genotypic distribution in the rivers would support the homing theory for female striped bass as suggested by tagging studies. If females did not return to the natal river to spawn and

indiscriminately mixed with females from other rivers, one would expect frequencies in all rivers to be the same. Again, this is not evident in the data presented here.

When fish from different geographical locations are to be compared for the presence of distinct mtDNA sequences indicating separate stocks, an understanding of the year-to-year variation in a particular site becomes most important. Ideally, genotypic frequencies representing striped bass from distinct geographic locations should only be compared within the same sampling year. Until differential migration between the sexes is confirmed or disproven, comparisons should also be limited to those between fish of the same sex. It is important to note that males $\leq$ 2 years old, however, reflect the matriarchal genotype and can be treated as such in comparisons. When sampling more than one year class, careful consideration should be given to the genetic contribution of each year class to the total sample. Migration patterns which vary with age could have a major impact on the genetic composition of striped bass within a river.

MtDNA analyses can clearly detect intraspecific variation that may or may not be apparent through morphometric, meristic, or biochemical analyses. Until the extent of mtDNA variation is determined and the implications of that variation is understood, it may be wise to use the information provided by mtDNA analyses in conjunction with that provided through other more standard techniques. As with past striped bass stock identification studies, dependency on one technique alone may not provide enough or even accurate data in order to allow population distinctions for well-founded management decisions.

Statistical Considerations

There are several precautions that need to be considered when analyzing these data, the most obvious of which are sample size and randomization. The validity of statistical analyses applied to a sample of 11-35 individuals is questionable in most population studies using morphometrics, meristics, and protein analyses. The majority of published mtDNA analyses, however, involve less than 18 individuals to represent a specific location. Many, if not most, of these same studies compare populations represented by 1 individual (see Lansman et al. 1983, Skibinski et al. 1985, Bermingham and Avise 1986, Saunders et al. 1986). Therefore, it is possible that the data presented here do not accurately represent the sampled population.

Associated with sample size is randomization of the collection in time and space. Due to regulations restricting the collection of striped bass during the spawning season and the constraints of the budget and available manpower, random collection of fish along the river was not possible. In each of the data sets examined here, striped bass were taken in pound nets at discrete locations within a small section of the rivers. Duplicate sampling over time and along a broader geographic range were not attempted for the reasons stated above.

Of the two problems mentioned here, randomization is of greater statistical importance. Assuming random sampling and a significance level of 5%, the possibility of committing a Type I error, rejecting a true hypothesis, is only 5% regardless of the size of the sample. The possibility of committing a Type II error, accepting a false hypothesis, is much greater. Plans for future sampling will hopefully be able to

avoid the problems associated with these data; however, this can only be accomplished with a more extensive and costly sampling scheme.

Another problem associated with data analyses concerns the scope of the study. Striped bass mtDNA may be equally heterogenous throughout the Chesapeake Bay such that no genetically distinct stocks are identified. Should this be true, mtDNA analyses may be more useful in identifying larger more geographically isolated stocks such as those in the Hudson River, Chesapeake Bay, and the Albemarle Sound. However, we are far from assessing and understanding total mtDNA variation within the Chesapeake Bay and its tributaries, and such determinations cannot be attempted until more extensive sampling is completed. The data and conclusions presented here provide a basis for additional studies, some of which are already in progress, but they are only preliminary in nature and are not an adequate foundation for management decisions.

SUMMARY AND CONCLUSIONS

The striped bass, Morone saxatilis, has long been an important commercial and recreational species within the Chesapeake Bay. Concern over the management of the species has increased over the last decade in response to the alarming decline in the harvest. Identifying the stocks to be managed is one of the first steps in developing an effective managment plan. Although stock identification within the Chesapeake Bay has been attempted using morphometric, meristic, tagging, and protein analyses, confusion concerning the existence of river stocks still exists.

Restriction endonuclease analysis of mitochondrial DNA is a tool that may help resolve the existence of discrete striped bass stocks within the Chesapeake Bay. The data and analyses presented here provide a preliminary assessment of mtDNA variation within the Rappahannock River, Virginia and between the Rappahannock River and 3 locations in the upper Chesapeake Bay. The results suggest several conclusions:

1. Restriction endonuclease analysis of mitochondrial DNA indicates the existence of genetic variation that may be diagnostic of river stocks within the Chesapeake Bay.

2. No clear genotypic marker was found, but genotypic frequencies should provide information adequate to identify separate river stocks if, and where, they exist.

3. The Rappahannock River striped bass are genetically distinct from those in the Potomac River and may be distinct from those in other locations in the Upper Chesapeake Bay. This supports previous tagging, morphometric, and meristic studies.

4. Factors other than geographic distance, such as geologic history, or distinct migratory habits, may be important in establishing the genetic differences between striped bass in the Rappahannock and Potomac rivers.

5. Significant variation in genotypic frequencies may or may not be present in Rappahannock striped bass between 1984, 1986, amd 1987. Discrepancies between designation of genotypic labels and molecular weight determinations must be resolved before it can be determined whether year-to-year variation exists.

6. More extensive and comprehensive sampling is necessary to fully resolve genetic variation within the Chesapeake Bay.

7. Should genetically distinct stocks be identified, present management strategies may not be appropriate to ensure long-term preservation of the species.

SUGGESTED FUTURE RESEARCH

In order to accurately resolve the mtDNA variation within the Chesapeake Bay striped bass population, current sampling should be continued and a more comprehensive sampling strategy must be implemented. The ideal sampling strategy should include the following:

1. Equal representation of males and females within the sample.

2. Equal representation of sampled year classes and recognition of the individual genetic contribution of each year class.

3. Random sampling throughout the river or sampling area, particularly during the spawning season.

4. Seasonal sampling to determine if shifts in genotypic frequencies occur within the year due to the anadromous habits of the species.

5. Increasing the sample size to 50-100 individuals to more accurately represent true genotypic frequencies.

6. Sampling should be repeated every two years for at least 10 years.

Although such an ideal sampling scheme is usually impractical, improvements in the present approach are necessary to obtain results which are trusted to reflect true mtDNA variation.

APPENDIX A

The isolation and digestion procedures for striped bass mtDNA are well-established (Chapman and Powers 1984), but several problems may be encountered while learning the technique.

Contamination is a constant threat. Bacterial contamination from the air, hands, or counter-top may cause complete degradation of the mtDNA or the restriction enzymes. This results in the presence of little or no DNA or whole, uncut DNA. Careful handling of the solutions and proper technique reduces the possibility of contamination.

Unbuffered phenol stored at room temperature degrades within one to two months of preparation and is no longer effective in removing proteins. This degradation results in loss of the mtDNA. Buffering the phenol according to Maniatis et al. (1982), division into small aliquots, and freezing during storage eliminates the problem.

Without the proper equipment, the amount of mtDNA in a sample is not easily determined. Although it is not necessary to know exact quantities, it is helpful to know relative quantities. Starting with an equal amount of material, two different ovarian samples may produce very different quantities of mtDNA depending on the stage or condition of the ovary. Pellet size is also not a good indicator as the presence of large quantities of RNA or other impurities sometimes produce a large pellet when little mtDNA was present. Over-dilution with sterile water renders mtDNA undetectable in EtBr-stained gels,

while overloading of DNA causes blurring of the bands and reduces the accuracy in determining the molecular weight of the fragments.

End-labelling, on the other hand, requires a minimal amount of DNA to be detected through autoradiography. For EtBr-stained gels a dilution of the samples to only 50-60 µl allows 4-5 digests, while end-labelling allows a dilution to 100 µl producing at least 13 digests with the same amount of starting tissue. Although a more tedious process, end-labelling is advantageous when the sample material is limited. Sample storage is also an important consideration. Although it is convenient to freeze samples, prolonged storage of striped bass ovarian tissue reduces the quantity of intact mtDNA for analyses.

Complete mixing of the DNA, enzyme, buffer, and label (if used) is essential to obtain complete digestion , and thus, accurate results. Partial digestions, if not recognized, can lead to erroneous conclusions. Partial digestion is easily recognized by the presence of whole, uncut mtDNA at the upper end of the gel. Over-digestion usually occurrs when excessive enzyme is used or the reaction is allowed to continue beyond 3 hours. The sample appears as a series of many bands in excess of the 'normal' restriction pattern. During over-digestion, fragments re-anneal (rejoin) creating new restriction sites and new fragments of varying molecular weights.

Recognition and interpretation of the results in the presence of partial or over-digestions is an important and necessary step in understanding the data. Misinterpretation is usually avoided by summing the molecular weights of the observed fragments. Partial or over-digestion typically produce total molecular weights inconsistent with the known mtDNA molecule size of vertebrates (16-20 kb).

APPENDIX B

End-labelling Reaction Mixture with Klenow Fragment (Chapman)

(quantities per sample)

Klenow	0.2 units ~ 0.04 µl
Reaction buffer	1.00 µl
Restriction enzyme	0.50 µl
Cold phosphate dGCT, dCTP, dTTP	1.00 µl (if required)
$(^{35}S)^*$ label	0.20 - 0.30 µCi
MtDNA solution	7.00 µl (of a 100 µl dilution)
Sterile water	to 20 µl total volume

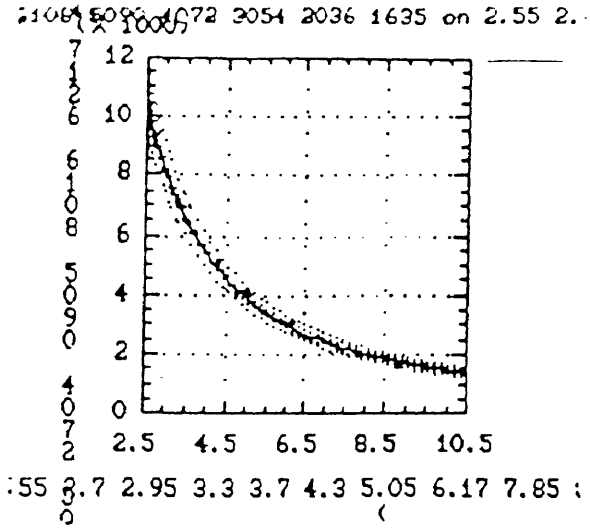
APPENDIX C

Variable Fragments

Distance	kb	Corrected kb
7.90	2.04	2.02
8.15	1.95	1.93

Migration distance (cm)	bp
2.25	12246
2.35	11198
2.55	10180
2.70	9162
2.95	8144
3.30	7126
3.70	6108
4.30	5090
5.05	4072
6.17	3054
7.85	2036
8.80	1635

7.85 cm on standard curve = 2.060 kb which is +0.024. Fragment sizes are therefore corrected by subtracting 0.02.



Regression Analysis - Multiplicative model: Y = aX^b

Dependent variable: 10180 9162 8144 7126 Independent variable: 2.55 2.7 2.95 3.3

Parameter	Estimate	Standard Error	T Value	Prob. Level
Intercept*	10.5789	0.0474784	222.816	2.22045E-16
Slope	-1.43075	0.0311665	-45.9066	5.60139E-11

* NOTE: The Intercept is equal to Log a.

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	Prob. Level
Model	3.5930	1	3.5930	2107.4125	.00000
Error	.0136395	8	.0017049		
Total (Corr.)	3.6066533	9			

Correlation Coefficient = -0.998107 R-squared = 99.62 percent.
Stnd. Error of Est. = 0.0412909

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Table 1. Four restriction enzymes and their recognition sequences. Arrows indicate restriction sites.

Hind III	5'	A↓AGCT T 3'
	3'	T TCGA↑A 5'
Eco RI	5'	G↓AATT C 3'
	3'	C TTAA↑G 5'
Eco RV	5'	GAT↓ATC 3'
	3'	CTA↑TAG 5'
Bcl I	5'	T↓GATC A 3'
	3'	A CTAG↑T 5'

Table 2. Observed genotypic frequencies in the Rappahannock River in 1986.

OBSERVED GENOTYPIC FREQUENCIES

GENOTYPE	# SAMPLES	TOTAL MOLECULAR WEIGHT (kb)*
A	7	17.5
B	10	17.6
C	4	17.7
D/E	1	17.65/17.75
F	1	17.8
Total fish	23	

GENOTYPIC FREQUENCIES BY YEAR CLASS

	GENOTYPE					
YEAR CLASS	A	B	C	D/E	F	TOTAL
77	1	1				2
80	1					1
81	2	1			1	4
82	3	6	3	1		13
83		1	1			2
undet'd		1				1
Total fish	7	10	4	1	1	23

* Molecular weights as determined by the methods of Robert W. Chapman of the Chesapeake Bay Institute, Shady Side, Maryland.

Table 3. Distribution of mtDNA genotypes and G tests for random distribution in the Upper Bay and Rappahannock River, 1984 and 1986. Expected values are in parentheses.

A. **1984**

	GENOTYPE						
Location	A	B	C	D/E	F	N	G
*Upper Bay	3 (8.0)	31 (28.0)	6 (4.0)			40	13.56** df=2
$Rappahannock	9 (4.0)	11 (14.0)	0 (2.0)			20	
Total	12	42	6			60	

B. **1986**

	GENOTYPE						
Location	A	B	C	D/E	F	N	G
*Upper Bay	2 (6.8)	21 (23.3)	34 (28.5)	4 (3.8)	8 (6.8)	69	16.68** df=4
Rappahannock	7 (2.3)	10 (7.8)	4 (9.5)	1 (1.3)	1 (2.3)	23	
Total	9	31	38	5	9	92	

*From Chapman (1987)
$From Chapman and Powers (in press)

Table 4. Comparison of genotypic frequencies in the Rappahannock River and three locations in the upper Chesapeake Bay in 1984 and 1986. Expected values are in parentheses.

1984

LOCATION	GENOTYPE A	B	C	D/E	F	N	G-VALUE
*Rappahannock	9	11	0			20	
	(5.0)	(13.3)	(1.7)				14.65**
$Potomac	0	13	3			16	df=2
	(4.0)	(10.7)	(1.3)				
*Rappahannock	9	11	0			20	
	(7.3)	(10.9)	(1.8)				5.99
$Choptank	3	7	3			13	df=2
	(4.7)	(7.1)	(1.2)				
*Rappahannock	9	11				20	
	(5.8)	(14.2)					9.18**
$Worton Point	0	11				11	df=1
	(3.2)	(7.8)					

1986

LOCATION	GENOTYPE A	B	C	D/E	F	N	G-VALUE
Rappahannock	7	10	4	1	1	23	
	(3.2)	(7.2)	(9.5)	(1.4)	(1.8)		18.36**
$Potomac	0	6	17	2	3	28	df=4
	(3.8)	(8.8)	(11.5)	(1.6)	(2.2)		
Rappahannock	7	10	4	1	1	23	
	(4.5)	(8.3)	(6.4)	(1.9)	(1.9)		10.54**
$Choptank	0	3	6	2	2	13	df=4
	(2.5)	(4.7)	(3.6)	(1.1)	(1.1)		
Rappahannock	7	10	4	1	1	23	
	(4.1)	(9.9)	(6.8)	(0.5)	(1.8)		7.15
$Worton Point	2	12	11	0	3	28	df=4
	(4.9)	(12.1)	(8.2)	(0.5)	(2.2)		

*From Chapman and Powers (in press)
$From Chapman (1987)

Table 5. Genotypic frequencies in the Rappahannock River in 1984, 1986, and 1987 and G-tests for goodness-of-fit. Expected values are in parentheses.

	GENOTYPE								
YEAR	A	B	C	D	C/D	D/E	F	N	G VALUE
*1984	9	11	0			0	0	20	
	(7.4)	(9.8)	(1.9)			(0.5)	(0.5)		6.50
1986	7	10	4			1	1	23	
	(8.6)	(11.2)	(2.1)			(0.5)	(0.5)		df=4
*1984	9	11	0	0	0			20	
	(3.3)	(7.6)	(6.9)	(1.1)	(1.1)				41.32**
$1987	0	10	19	3	3			35	
	(5.7)	(13.4)	(12.1)	(1.9)	(1.9)				df=4
1986	7	10	4	0	0	1	1	23	
	(2.8)	(7.9)	(9.1)	(1.2)	(1.2)	(0.4)	(0.4)		24.85**
$1987	0	10	19	3	3	0	0	35	
	(4.2)	(12.1)	(13.9)	(1.8)	(1.8)	(0.6)	(0.6)		df=6

*From Chapman (1987)
$From Meehan and Banford (unpublished)

Table 6. Genotypic frequencies in the Rappahannock River, 1987.

	GENOTYPE							
Year Class	A	B	C	D	C/D	D/E	F	
1980			1					1
1981				1				1
1982		2	3	1				6
1983		6	15	1	3			25
1984		2						2
Total		10	19	3	3			35

(Meehan and Banford, unpublished)

Table 7. Frequency of mtDNA genotypes and G-tests for random distribution between the Potomac River, Choptank River, and Worton Point in 1984 and 1986. Expected values are in parentheses.

1984

Location	GENOTYPE A	B	C	D/E	F	G value
Potomac River	0	13	3			
	(1.2)	(12.4)	(2.4)			
Choptank River	3	7	3			10.57**
	(1.0)	(10.1)	(2.0)			p<.05
Worton Point	0	11	0			
	(0.8)	(8.5)	(1.7)			

1986

Location	GENOTYPE A	B	C	D/E	F	G value
Potomac River	0	6	17	2	3	
	(0.8)	(8.5)	(13.8)	(1.6)	(3.2)	
Choptank River	0	3	6	2	2	10.34
	(0.4)	(4.0)	(6.4)	(0.8)	(1.5)	p>.10
Worton Point	2	12	11	0	3	
	(0.8)	(8.5)	(13.8	(1.6)	(3.2)	

Derived from Chapman (1987).

Table 8. Frequency of mtDNA genotypes and G tests for random distributions in the Potomac River, Choptank River, and Worton Point between 1984 and 1986. Expected values are in parentheses.

Location	Year	GENOTYPE A	B	C	D/E	F	G-value
Potomac River	1984		13	3	0	0	
			(6.9)	(7.2)	(0.7)	(1.1)	7.12**
	1986		6	17	2	3	p<.01
			(12.1)	(12.7)	(1.2)	(1.9)	
Choptank River	1984	3	7	3	0	0	
		(1.5)	(5.5)	(4.0)	(1.0)	(1.0)	2.85
	1986	0	3	6	2	2	0.1<p<.05
		(1.5)	(5.5)	(4.0)	(1.0)	(1.0)	
Worton Point	1984	0	11	0		0	
		(0.5)	(6.2)	(3.4)		(0.8)	10.51**
	1986	2	12	11		3	p<.01
		(1.4)	(15.8)	(8.6)		(2.2)	
Combined	1984	3	31	6	0	0	
		(1.8)	(18.7)	(14.7)	(1.5)	(3.9)	26.62**
	1986	2	21	34	4	8	p<.01
		(3.1)	(32.3)	(25.3)	(2.5)	(5/1)	

From Chapman (1987). G values generated after appropriate pooling of genotypic classes.

Figure 1. Location of sampling stations in the Rappahannock River, Potomac River, Choptank River, and at Worton Point.

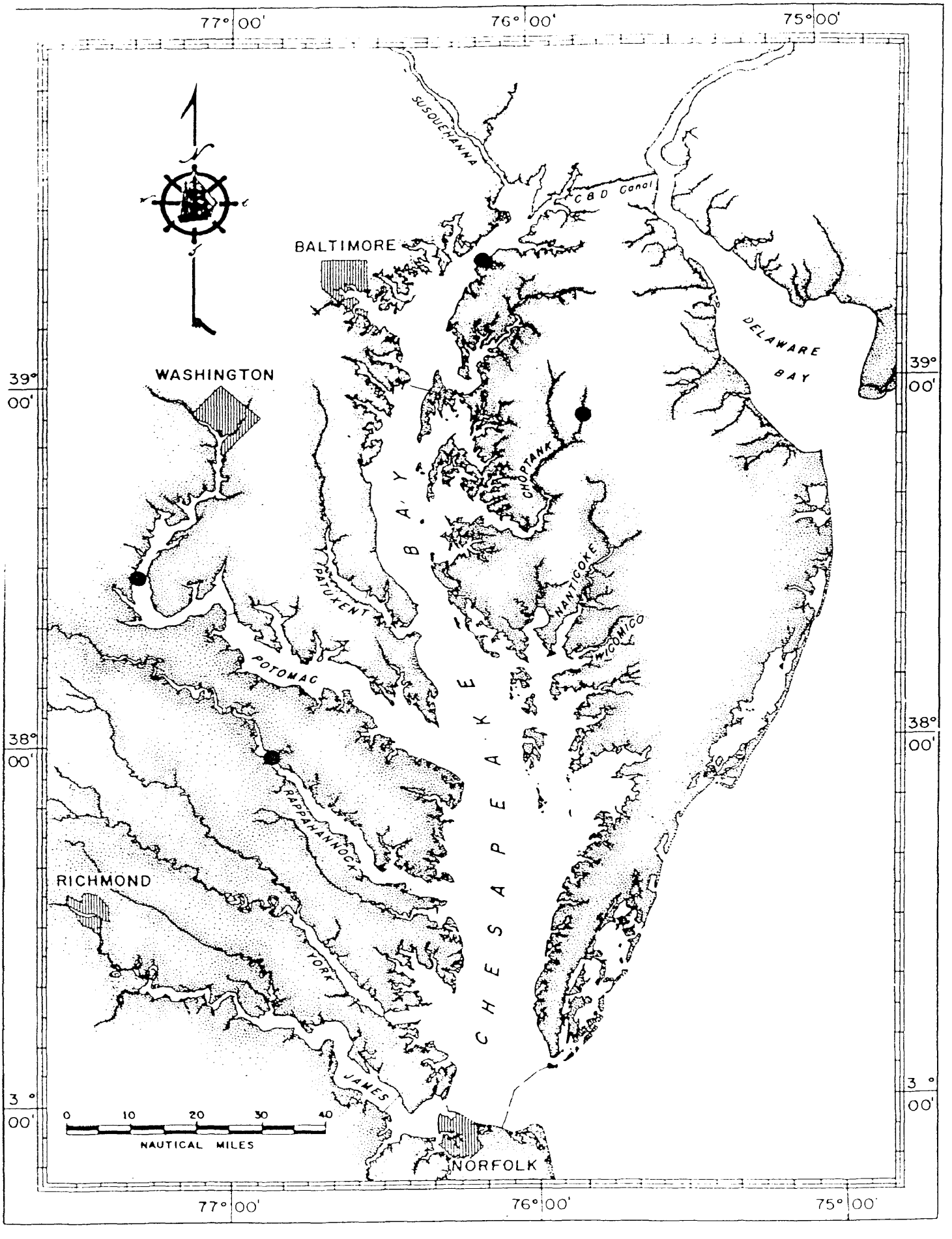
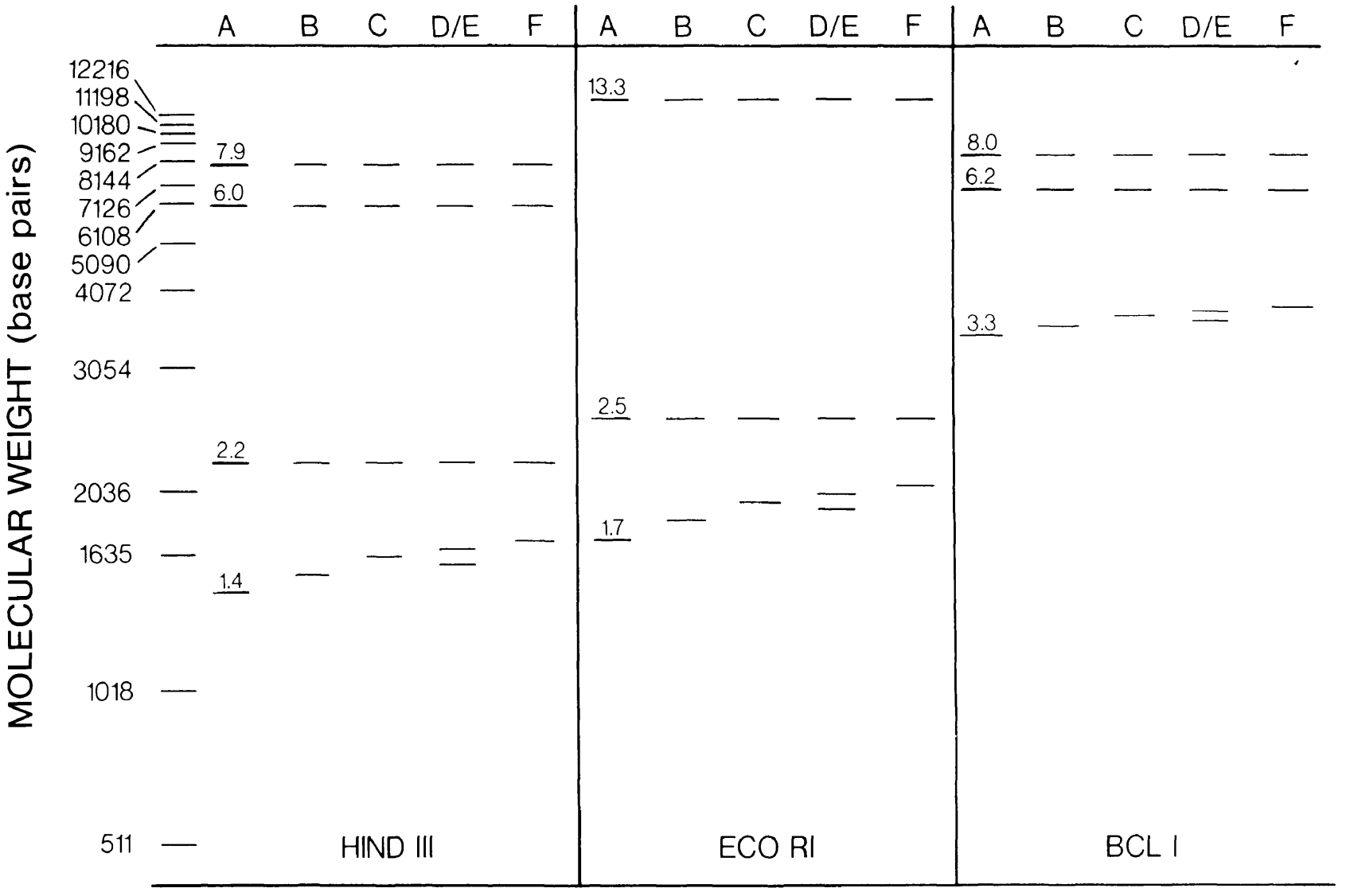
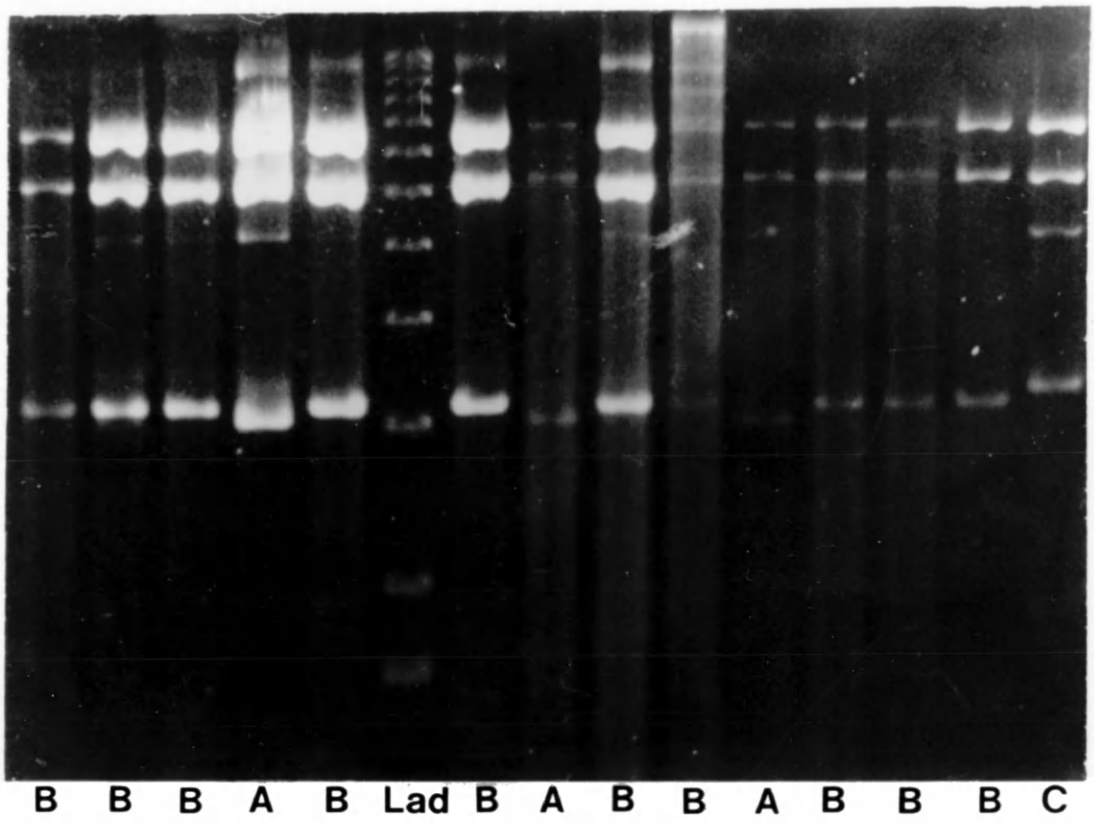

77° 00'
76° 00'
75° 00'
SUSQUEHANNA
C & D Canal
BALTIMORE
DELAWARE BAY
WASHINGTON
39° 00'
CHOPTANK
B A Y
PATUXENT
NANTICOKE
WICOMICO
POTOMAC
38° 00'
RAPPAHANNOCK
C H E S A P E A K E
RICHMOND
YORK
JAMES
3 ° 00'
0 10 20 30 40
NAUTICAL MILES
NORFOLK

Figure 2. Restriction fragment patterns of three enzymes, Hind III, Eco RI, and Bcl I, observed in mtDNA of striped bass from the Rappahannock River, Virginia. The ladder is a 1 kilobase molecular weight standard.



RESTRICTION FRAGMENT PATTERNS FOR THREE INFORMATIVE ENZYMES.

Figure 3. Three restriction fragment patterns observed in striped bass from the Rappahannock River, Virginia.


B B B A B Lad B A B B A B B B C

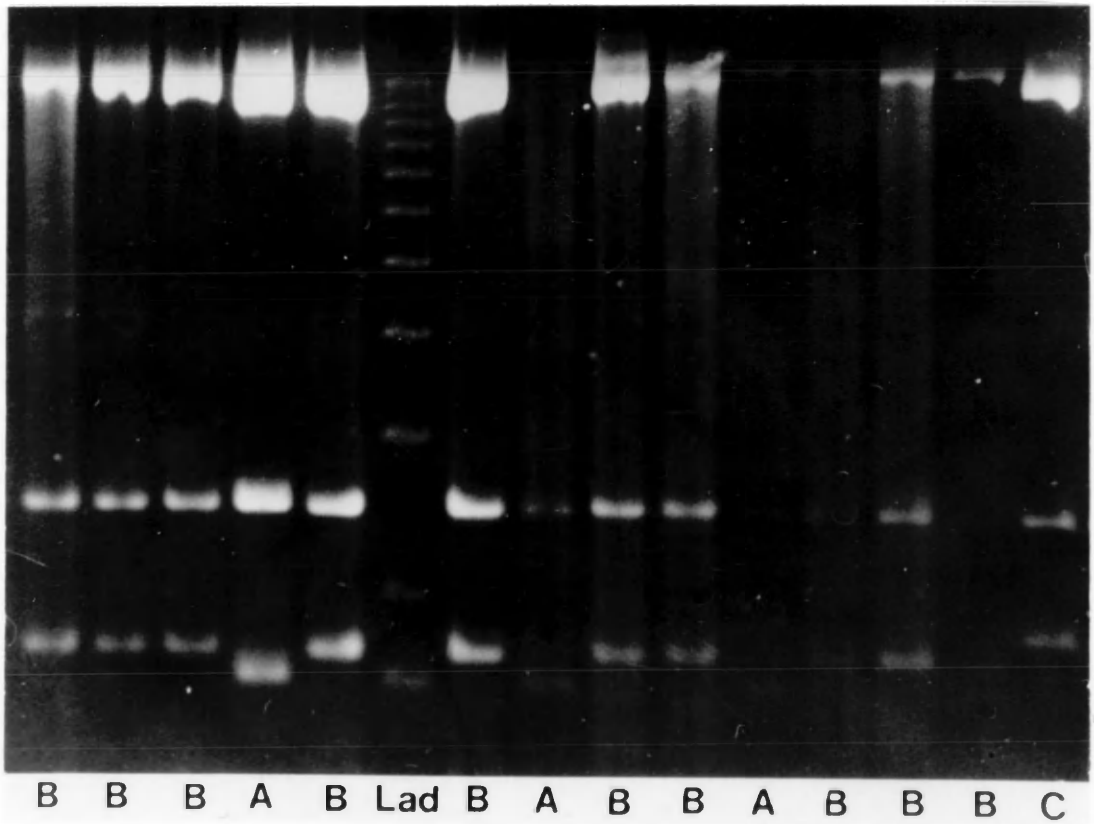
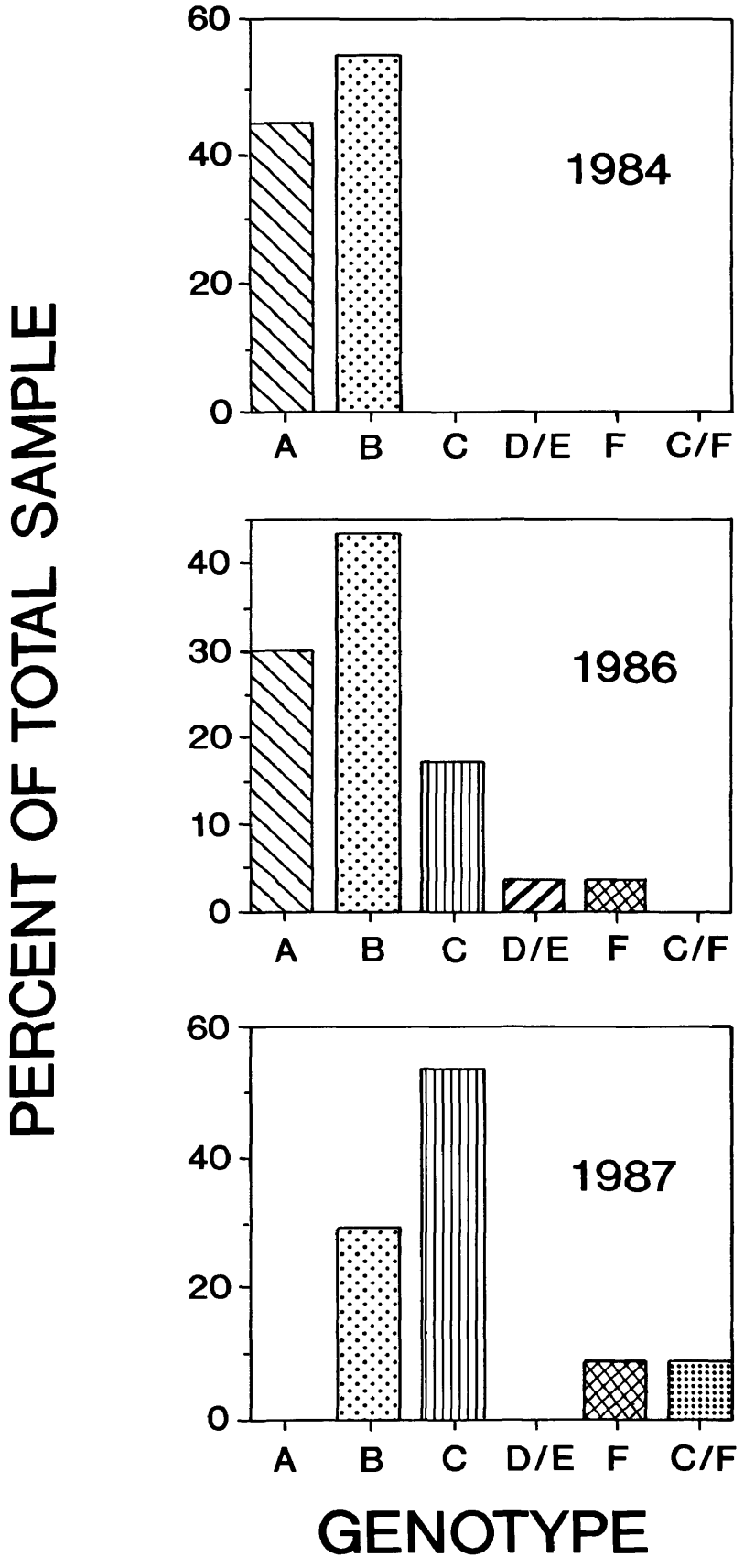

B B B A B Lad B A B B A B B B C

Figure 4. Percent occurrence of genotypes in Rappahannock River striped bass in 1984, 1986, and 1987.


PERCENT OF TOTAL SAMPLE
60
40
20
0
1984
A
B
C
D/E
F
C/F
40
30
20
10
0
1986
A
B
C
D/E
F
C/F
60
40
20
0
1987
A
B
C
D/E
F
C/F
GENOTYPE

VITA

CAROL FURMAN

Born in Greenville, South Carolina, 14 April 1960. Graduated from Christ Church Episcopal School in 1978. Received Bachelor of Arts degree with double major in Biology and Spanish from Wake Forest University, Winston-Salem, North Carolina, in 1982. Entered Master's program at the Virginia Institute of Marine Science, College of William and Mary in 1983. Completed M.A. degree in May 1989, and entered doctoral program in Genetics at Texas A & M University.