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Develop a fishery stock identification laboratory and identify coastal and estuarine stocks that are dependent on the Chesapeake Bay and its tributaries

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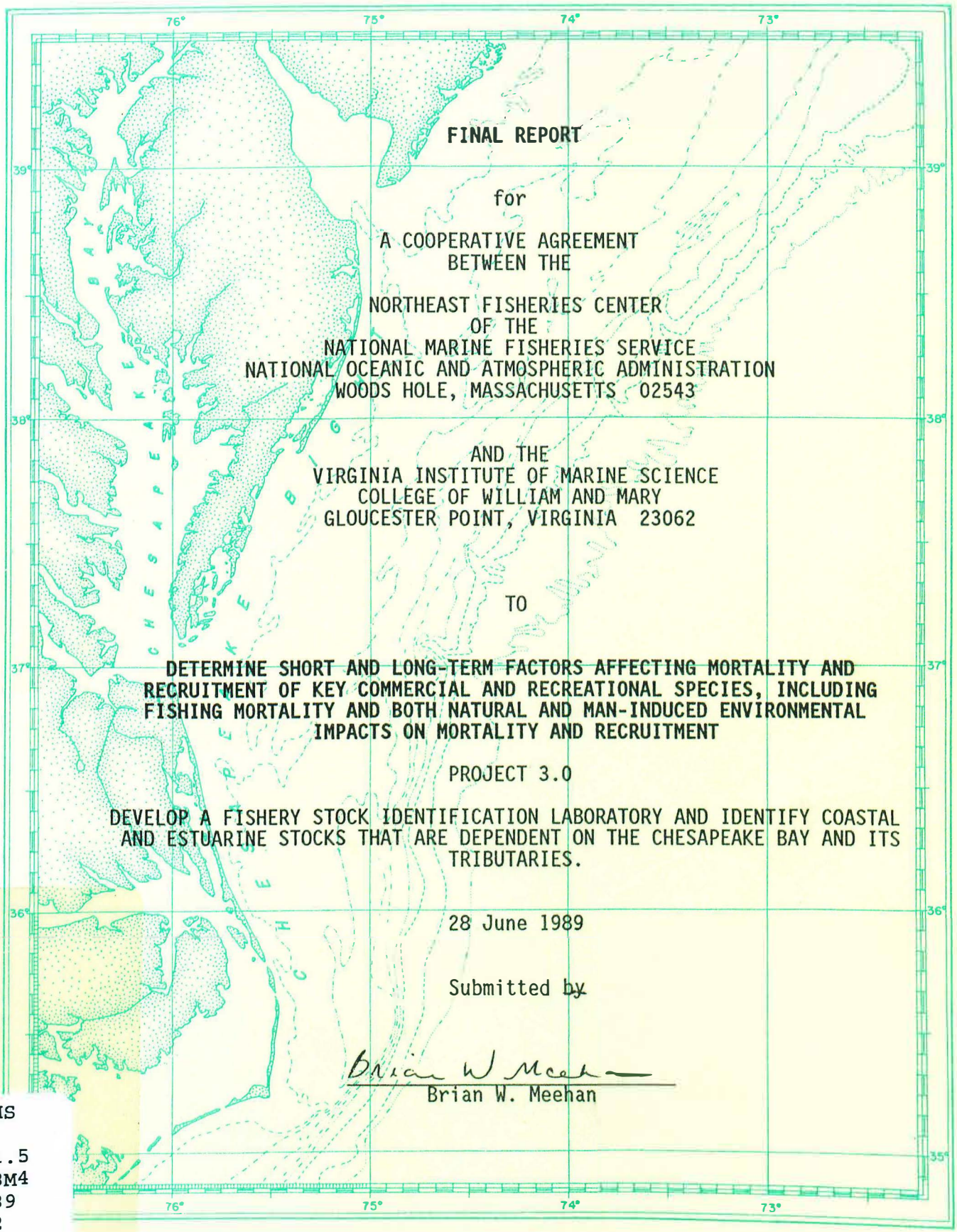

FINAL REPORT

for

A COOPERATIVE AGREEMENT
BETWEEN THE

NORTHEAST FISHERIES CENTER
OF THE
NATIONAL MARINE FISHERIES SERVICE
NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
WOODS HOLE, MASSACHUSETTS 02543

AND THE
VIRGINIA INSTITUTE OF MARINE SCIENCE
COLLEGE OF WILLIAM AND MARY
GLOUCESTER POINT, VIRGINIA 23062

TO

DETERMINE SHORT AND LONG-TERM FACTORS AFFECTING MORTALITY AND RECRUITMENT OF KEY COMMERCIAL AND RECREATIONAL SPECIES, INCLUDING FISHING MORTALITY AND BOTH NATURAL AND MAN-INDUCED ENVIRONMENTAL IMPACTS ON MORTALITY AND RECRUITMENT

PROJECT 3.0

DEVELOP A FISHERY STOCK IDENTIFICATION LABORATORY AND IDENTIFY COASTAL AND ESTUARINE STOCKS THAT ARE DEPENDENT ON THE CHESAPEAKE BAY AND ITS TRIBUTARIES.

28 June 1989

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Introduction

Stock identification and, subsequently, knowledge of stock movements and mixing are essential prerequisite for assessment and management programs of both commercial and recreational fisheries (Ryman and Utter, 1987; Kumph et al., 1987). This has been recognized by the Chesapeake Bay Stock Assessment Committee (CBSAC) and with funds provided by CBSAC.I (1985-1986) a laboratory devoted to biochemical/genetic analyses for stock identification has been established at the Virginia Institute of Marine Science (VIMS). This laboratory is specifically designed for conducting analyses of biomolecules for the identification of fisheries stocks. Primary equipment includes six 500 volt power supplies capable of handling twelve starch gels for isoenzyme analysis, two 3,000 volt power supplies each designated for an isoelectric focusing unit for quantitative and qualitative analysis of soluble proteins, and one 500 volt power supply capable of handling five submerged gel units for DNA analyses. Additional support equipment includes two high-speed refrigerated centrifuges, two microcentrifuges, two -20^{o}C and a -80^{o}C freezer, a low-shear continuous homogenizer, a U.V.-Vis. scanning densitometer with microprocessor and a micro-computer for data handling and word processing. Some unique advantages of this lab are; the ability to use an array of biochemical and genetic markers, capabilities of handling large numbers of samples with minimal cost and effort, and most importantly, the stability required for long term studies and monitoring.

Funding received from the CBSAC as part of CBSAC.II (1986-1987) was used for; 1, research and development of techniques for the processing of

large numbers of diverse samples efficiently, and 2, the initiation of pilot studies of three key commercial species. The three pilot studies serve as demonstrations of the broad range of applications of molecular genetic approaches to fisheries problems. Biochemical techniques that were adopted by this laboratory were originally developed for clinical or evolution studies that dealt with small sample sizes of limited diversity in which sample procurement and processing were not confounded by time constraints or peculiarities of the target organism's life history strategy. Also, few laboratories were designed to integrate a complete array of biochemical genetic techniques. The VIMS fisheries genetics laboratory has overcome many of these obstacles and is now capable of processing a diverse array of species in a relatively short time. The ability to secure tissues from large numbers of fish in an expedient fashion is a requirement dictated by existing fisheries sampling strategies in which fish are captured in large lots. Specimen collections are coordinated with a number of ongoing fisheries projects at VIMS, and it is not uncommon to have large numbers of specimens pass through the system on a single day.

When a species is targeted for study, a preliminary assay can be conducted of an array of biochemical genetic/markers. The most effective genetic markers for an intensive stock identification project are selected in accordance with the results from the preliminary assay. This initial survey of the complete array of available markers is a necessary step for identifying fisheries stocks in an efficient and cost effective manner. Unlike previous attempts to resolve fisheries stocks using biochemical markers this laboratory is not restricted to a single technique, often selected without a priori information, thus saving both time and money. For example, if a preliminary survey reveals that no polymorphisms are detected

using isoenzymes, often the case with marine fishes, this technique is eliminated from continued studies and emphasis can be shifted to analysis of mtDNA restriction fragments and/or isoelectric focusing of soluble tissue proteins. In essence, funding agencies are relieved from the pitfalls of committing all their funds to a single technique which may or may not be the most appropriate for addressing the objectives at hand.

The three pilot studies initiated with funds from CBSAC-II are: 1, Analysis of Biochemical/Genetic Markers for Delineating Natural Stocks of Striped Bass; 2, A Genetic Evaluation of Blue Crab Populations in Delaware Bay, Chesapeake Bay and North Carolina; and 3, Genetic Structure of Oyster Populations Within the Chesapeake Bay. The basic objective shared by each of these studies was to evaluate biochemical genetic markers that might serve to discriminate stocks of each species. Each of these studies have been very successful and are discussed in detail in the following three sections. A masters thesis supported by CBSAC I and II is also included as part of this final report.

Pilot Study 1: Analysis of biochemical/genetic markers for delineating natural stocks of striped bass. Mitochondrial DNA restriction morph patterns of Chesapeake Bay striped bass, Morone saxatilis.

Abstract

The striped bass, Morone saxatilis, is a key recreational and commercial fish within the Chesapeake Bay and other Atlantic coast estuarine systems. In response to dramatic declines in abundance during the past ten years, extensive management and monitoring programs of this fishery have been instituted. To determine if the Chesapeake Bay contains one or more striped bass spawning stocks the mtDNA restriction morph patterns of 143 individuals collected from the lower Chesapeake Bay were analyzed. All detectable mtDNA variation was limited to total molecule size differences, no restriction site changes were observed. Apparent shifts in the frequency of restriction morphs occurred from year to year and fish from different rivers throughout the Bay were similar within years. These data possibly indicate that distinct river specific spawning stocks of striped bass probably do not occur within the Chesapeake Bay.

Introduction

The striped bass, Morone saxatilis, has long been a vital and productive fishery along Atlantic coastal waters and inland estuaries (Merriman, 1941). Decade long declines in the commercial landings of this fish have brought about extensive monitoring programs, as well as,

regulations that limit landings (Boreman and Austin, 1985). A number of attempts have been made to supplement natural populations within major estuarine systems along the Atlantic coast. The effectiveness of these regulatory and stocking programs is uncertain. Fisheries management programs, including all stocking efforts, should have as a foundation as accurate an assessment of the fish stock as possible (Ryman and Utter 1987). This includes, but is not limited to, knowledge of the geographic range and genetic integrity of the stock. Whether the striped bass within major Atlantic coast estuarine systems constitute discrete spawning stocks or if they represent a larger coast-wide stock is instrumental to the development of effective management practices and, subsequently, the revitalization of this key commercial and recreational fish.

Over the years, many attempts have been made to delineate striped bass stocks including tagging-recapture studies, meristics, elemental composition of otoliths, and others (Setzler et al. 1980, Waldman et al. 1988). More recently, a number of investigators have relied in electrophoretic resolution of serum proteins, allozymes and eye lens proteins for comparisons among striped bass stocks (Morgan et al., 1975; Sidell et al., 1978; Sidell et al., 1980 and Fabrizio, 1987). These studies have indicated that there is some genetic structuring of striped bass along the Atlantic coast. Fabrizio (1987) was able to reliably identify Hudson River and Chesapeake Bay striped bass stocks within a Rhode Island mixed stock fishery. Fabrizio used isoelectric focusing of eye lens proteins coupled with traditional morphometric techniques. Eye lens proteins are quantitative, phenotypic characters relatively far removed from the genotype and they are subject to both environmental and ontogenic influences.

Most recently Chapman (1989, 1987a) and Wirgin et al. (1989) analyzed mtDNA restriction morph patterns of striped bass from along the Atlantic coast, including the Chesapeake Bay. The analysis of mtDNA restriction fragment patterns is a relatively recent technique in population genetics studies that can provide an accurate assessment of the genetic structure and integrity of populations (Avise, 1986). It appears that because mtDNA is simpler and has fewer associated regulating mechanisms that counter and correct for mutation events it may evolve much more rapidly than nuclear DNA (Brown et al., 1979). Therefore, mtDNA is more likely to reveal differences among recently segregated populations than nuclear DNA. Also, restriction fragment analysis of mtDNA reveals base pair or sequence changes directly; whereas, nuclear DNA composition is usually inferred from phenotypic expressions (allozymes and proteins) of the DNA.

The analysis of mtDNA restriction fragment patterns is widely accepted, and recent studies of fish populations includes: skipjack tuna (Graves et al., 1984), sunfishes (Avise et al., 1984), some salmonids (Berg and Ferris, 1984; Wilson et al., 1984; Birt et al., 1986), the common mummichug (Gonzalez-Villansenor et al., 1986), striped bass (Chapman, 1987) and others (Avise, 1985). These studies strongly indicate that mtDNA of fishes contain as much or more genetic variation than nuclear DNA, and that restriction fragment analysis of mtDNA is useful in resolving closely related populations. We have examined mtDNA restriction morph patterns of 143 striped bass collected over a three year period from the lower Chesapeake Bay and compared this data with that collected by others (Chapman, 1987; Wirgin et al., 1989 and Wirgin et al., 1989) from within the Chesapeake Bay.

Methods

Striped bass were collected from the James and Rappahannock Rivers located in the lower Chesapeake Bay during the spawning seasons of 1986, 1987 and 1988. Various tissues (egg mass, heart or liver as appropriate) were used to obtain purified mtDNA using modifications of the procedures presented by Chapman and Powers (1984). The purified mtDNA was digested with the restriction endonuclease Eco RI and the size of all subsequent fragments visualized by ethidium bromide staining on a 1.0% agarose gel was determined against a 1 kilobase ladder. The restriction enzyme and 1 kilobase ladder were obtained commercially (BRL) and digests were conducted according to the manufacturers recommendations.

Variation within the mtDNA molecule of Chesapeake Bay striped bass is primarily restricted to 100 base pair changes in the molecule size as revealed by digestion with a number of different restriction enzymes (Chapman 1987). The restriction enzyme Eco RI was used for this study because the variable, diagnostic fragment ranges from 1.7 to 2.0 kilobases and these fragments can be accurately and reliably resolved by electrophoresis on a 1.0 % agarose gel. Following the nomenclature established by Chapman (1987a) the smallest mtDNA molecule, that which corresponds to the 1.7 kilobase fragment produced by an Eco RI digest, was designated "A" and larger molecules labeled "B", "C" and "F" in 100 base pair increments. Chapman (1987a) utilized the notation D/E to designate a heteroplasmic individual with two intermediate size molecules. For this study all heteroplasmic individuals were placed in an "other" category. The distribution of restriction morphs was compared within rivers and years

among males, females and hybrids and among rivers and years using a log-likelihood analysis of frequencies (G-test; Sokal and Rolf, 1981).

Results

The distribution of restriction morphs among the fish examined in this study is given in ,able 1. A comparison among males, females and hybrids using a G-test of independence indicated that there was not a significant difference in the distribution of restriction morphs across these types in 1987 or 1988 (G-test, 0.5>P>0.25 and 0.1>P>0.05, respectively). Therefore, these types were grouped for subsequent analyses. The numbers of individuals within each cell for all comparisons is fairly low and for all comparisons all cells with expected frequencies less than 5 were pooled. Within these limitations the results of these and subsequent G-tests should be regarded as general indicators of similarity among samples and not for providing fine resolution analysis among samples.

There was an apparent shift in the predominant restriction morphs from year to year. All five restriction morphs were present in the 1986 collection and restriction morph "C" was a relatively rare restriction morph as compared to restriction morph "B". In contrast, restriction morph "A" was exceptionally rare in the 1987 collection and restriction morph "C" was predominant, especially in the Rappahannock River. In 1988 restriction morphs "A" and "B" occurred in almost equal numbers; whereas, restriction morph "C" occurred in relatively low numbers overall. Striped bass analyzed by Chapman (1987) in 1984 and 1986 had similar type shifts in occurrence of restriction morphs between years.

Table 2 gives results of the G-test for restriction morph distributions within years among rivers and within rivers between years. All comparisons within rivers between years were highly significant except for the 1984 and 1986 comparison from the Choptank River sample. The Choptank River sample contains only 26 fish collected over two years and the robustness of the G-test may be affected by this small sample size. Examination of the data from Chapman (1987) revealed that restriction morph "C" increased and "B" decreased in occurrence from 1984 to 1986 in the Choptank River, coinciding with samples collected from all other locations.

In 1986 collections from the upper bay were not significantly different from one another, nor from the Rappahannock River collection in the central/lower Bay. Collections from the following year (1987) from the Rappahannock and James River, located in the lower Bay, were also not different from one another. The 1984 collection from the upper Bay was the only statistically significant within-year comparison of restriction morph distributions among rivers. As mentioned above, the 1984 collections contained exceptionally low numbers of individuals and the validity of the G-test is questionable, after pooling of cells with low expected frequencies it is still impossible to obtain more than one column of cells with expected frequencies greater than 5. If the Choptank River collection is excluded from this analysis, the 1984 Potomac River and Whorton Point collections are not significantly different from one another. The trends portrayed by these comparisons indicates that little mtDNA variation occurs among rivers each year; but, that significant mtDNA variation occurs from year to year throughout the Bay.

Discussion

Over the years there have been many attempts to determine if striped bass home to specific tributaries within the Chesapeake Bay. While some of the earlier studies of meristic and morphometric characters indicated that river specific stocks may occur, later studies of allozyme systems and serum proteins were inconclusive (Setzler et al., 1980, Sidell et al., 1980, Morgan et al., 1975, Grove et al., 1976). According to Sidell et al. (1980) the striped bass is among the least genetically variable species of teleost fish, and the mtDNA genome of striped bass seems to follow this trend. The variability within the striped bass mtDNA genome is primarily restricted to size polymorphisms and is low relative to other organisms with the more common restriction site changes or base pair substitutions. For example, in an examination of restriction fragment patterns of 100 blue fish there were more than 20 composite restriction morphs produced by digestion with only nine restriction enzymes (Table 3). The only base pair substitutions identified in striped bass have occurred in an exceptionally small percentage of individuals examined from various locations (Weisberg et al., 1987; Wirgin et al., 1989).

The most common source of mtDNA restriction morph variation is, in general, the result of nucleotide substitutions, and changes as a result of addition and deletion events usually occur with less frequency within limited regions of the molecule (Brown, 1983). It is uncertain why this trend is not applicable to the striped bass mtDNA genome; in fact, the opposite seems to occur. Possible explanations for this are that the mtDNA size variants are molecular artifacts and the lack of apparent substitution

events and subsequent site changes may be a manifestation of contemporary population dynamics and fisheries pressure.

Typically the vast majority of individuals within a reproductively intact population contain like mtDNA molecules, and mtDNA variants are restricted to relatively few individuals. Table 3 contains composite restriction morphs of 100 bluefish analyzed with nine restriction enzymes; 40% of the population contains a single composite restriction morph and the remaining mtDNA types occur at relatively low frequency (see also Avise et al. 1987 and Avise et al. 1979). The life history of striped bass is not a typical, it does not participate in unusual reproductive behavior such as brooding or parthenogenises. Therefore it is expected that transmission, evolution and maintenance of mtDNA genomes within striped bass populations should be as for other species and there is no presumed molecular basis for the lack of mtDNA variants.

The apparent lack of restriction site changes of striped bass mtDNA may be a result of extreme population fluctuations during recent years. The striped bass has been subjected to intense fishing pressure and the numbers of individuals within some spawning stocks have been low enough to threaten local extinction (Goodyear et al., 1985). In 1987 the size of the Rappahannock River striped bass stock during the spring spawning run was the largest on record; yet 62% of the this population was represented by 3-4 year old females, fish still too young to participate in spawning activity (Loesch and Kriete, 1987). This severe and almost regular bottlenecking of the population (mtDNA) genome of striped bass during the past ten years may be responsible for the low level of mtDNA variants.

The usual result of a bottlenecking event or severe population reduction is loss of genetic variability and the extent of genetic

information that is lost is proportional to the effective population size. The mtDNA genome is much more greatly affected by these type events than nuclear DNA because only female mtDNA is inherited reducing the effective population size by one half. In addition typically only one type mtDNA is carried by an individual female but the same individual is probably heterozygous at several nuclear gene loci. Under these conditions, a population that undergoes one or more severe bottlenecks could lose all of its mtDNA variability over a very short period of time (see Wilson et al. 1985 for a review). This would effectively create a population with a highly homogeneous mtDNA genome, relative to nuclear DNA.

If striped bass do move to specific tributaries in the Chesapeake Bay to spawn, then this behavior would have been established long before any affects of population reductions caused by fishing practices were realized, at best up to 10,000 years ago. If the mtDNA genome of striped bass stocks at this time was similar in composition to other contemporary organisms an array of mtDNA types should have been available for classical founder events to take place during the post-glacial sea level rise and the formation of these tributaries. These founder events in conjunction with population expansions within the newly formed tributaries would provide an excellent opportunity for the formation of tributary specific mtDNA genomes (DeSalle and Templeton 1988). These supposed recently founded populations would contain mtDNA genomes distinguishable by differences in the predominant mtDNA types. A population founded by individuals that carried mtDNA types in low frequency in the parent population would now contain these types in relatively high frequency within the the newly founded population. Subsequently, as bottlenecking tends to eliminate or reduce the occurrence of rarer genotypes, the high frequency mtDNA types diagnostic of these

tributary specific stocks would be maintained and most likely increase in frequency as a result of population declines. Therefore, the lack of unique restriction morphs, or homogeniety of the mtDNA genome, within striped bass collected from tributaries throughout the Chesapeake Bay strongly indicates that tributary specific mtDNA genomes were never established.

The rare restriction morphs reported by Weisberg et al. (1987) for striped bass within the Delaware Bay may be ancestral remnants and/or indications of macrogeographic structuring of coastal stocks. As many as four stocks of striped bass have been suggested along the Atlantic coast. The year to year mtDNA size variation may be a reflection of small (inadequate) sample sizes and these may represent highly variable tandem repeat regions that are unstable from generation to generation (Densmore et al. 1985, Moritz et al. 1987). Confirmation of these hypotheses is dependent on a large scale investigation of mtDNA restriction morphs of striped from along its entire Atlantic coast range.

In conclusion, the analysis presented here combined with that reported by Chapman (1989, 1987), Wirgin et al. (1989) and Furman (1989) is indicative of trends in the composition of Chesapeake Bay striped bass stocks critical to the development and execution of fisheries management strategies for this species. Available data on mtDNA restriction morphs, a sensitive indicator of population structuring, do not indicate that the Chesapeake Bay is composed of multiple spawning stocks of striped bass and management practices should be conducted accordingly. These findings also strongly support the need for a comprehensive population genetics study of striped bass with large sample sizes (>150 individuals) of all presumed stocks from throughout the Chesapeake Bay and other coa tal systems.

Table 1. Distribution of restriction morphs produced by Eco RI digests of mtDNA isolated from from Striped bass collected from the lower Chesapeake Bay in 1986,87,88.

River	Coll. Year	Type	Restriction morph N	A	B	C	F	O
Rappahannock	86	F	23	7	10	4	1	1
Rappahannock	87	F	46	0	16	24	3	3
James	87	F	9	1	5	0	2	1
James	87	M	16	0	7	6	2	1
James	87	H	1	0	1	0	0	0
James	88	F	24	7	9	6	1	1
James	88	M	6	4	2	0	0	0
James	88	H	18	10	7	1	0	0

Table 2. G-test of independence of the distribution of striped bass mtDNA Eco RI restriction morphs among Chesapeake Bay tribuaries as presented in Table # and data obtained from Chapman, 1987. Cells with expected frequencies less five were pooled when appropriate.

Comparison		Value	Value	d.f.	Prob.
This study:					
Rappahannock R.	(86 vs. 87)	7.80	19.96	3	P<<0.005
James R.	(87 vs. 88)	7.80	17.81	3	P<<0.005
Rappahannock R. (87) vs. James R.	(87)	7.80	5.97	3	0.25 P>0.1
Chapman, 1987:					
Potomac R., Choptank R. and Whorton Pt.	(84)	3.84	8.57	1	P<<0.005
Potomac R., Choptank R. and Whorton Pt.	(86)	9.49	4.23	4	0.25>P>0.1
Potomac R.	(84 vs. 86)	5.99	15.85	2	P<<0.005
Choptank R.	(84 vs 86)	5.99	2.6	2	0.50>P>0.25
Whorton Pt.	(84 vs 86)	5.99	13.22	2	P<<0.005
Rappahannock R., Potomac R. Choptank R., Whorton Pt.	(86)	12.6	12.06	6	0.10>P>0.05

Table 3. Composite restriction morphs of bluefish mtDNA produced by digestion with nine restriction enzymes. Restriction enzymes were Ava I, Hind III, Pvu II, Dra I, Eco RV, Sst I, Pst I, Sst II, and Nci I; respectively. All fish were collected from the lower Chesapeake during the spring and early summer of 1988.

Restriction morph	Individ.	Freq.
AAAAAAAAA	42	39.6
ABAAAAAAA	9	8.5
AAAACAAAA	8	7.5
AAAADAAAA	7	6.6
BAAAAAAAA	6	5.7
AAAABAAAA	6	5.7
ABAABAAAA	5	4.7
AAAAAAAAC	3	2.8
AAAAAAAAD	3	2.8
BAAACAAAA	3	2.8
CAAAAAAAA	2	1.9
CAAAAAACA	2	1.9
AABABAAAA	2	1.9
BBAAAAAAA	1	0.9
AABAAAAAA	1	0.9
AABACAAAA	1	0.9
AACAAAAAA	1	0.9
AACACAAAA	1	0.9
ABADAAAAA	1	0.9
BAAACAAAA	1	0.9
BAADAAAAA	1	0.9
BADAAAAAA	1	0.9
	106	100.5

Pilot study 2: Isolation and Restriction Fragment Analysis of Mitochondrial DNA From The Decapod Crustacean Callinectes sapidus.

Abstract

The blue crab, Callinectes sapidus, provides important commercial and recreational fisheries along coastal states of the Atlantic ocean and Gulf of Mexico. Along this range population epicenters are associated with the large estuarine systems of Delaware Bay, Chesapeake Bay, Albemarle and Pamlico Sounds, and the Gulf of Mexico. There is an immediate requirement for development of management strategies that will assure the stability of each of these major blue crab stocks and to do so a better understanding of their stock structure and integrity is essential. We have initiated an examination of the mtDNA restriction fragments of blue crabs to determine the extent of macrogeographic structuring of populations within Atlantic coastal waters and the Gulf of Mexico. Though isolation and restriction fragment analysis of mtDNA has become routine for an array of organisms this is not the case for many marine crustaceans and molluscs, including blue crabs. Here we present an isolation protocol that resolves this problem, and provide an initial characterization of blue crab mtDNA as determined with restriction enzymes.

Introduction

The blue crab, Callinectes sapidus, provides important commercial and recreational fisheries along coastal states of the Atlantic ocean and Gulf of Mexico. Before the 1960's the reported catches in the Chesapeake Bay varied widely. In 1960 a record catch was recorded for the dredge and pot fisheries, but since then harvest yields have declined (Stagg, 1986). There is an immediate need for development of management strategies that will assure stock stability, and to do so a better understanding of stock structure and integrity is required. Unfortunately, all of the necessary data to assess blue crab stocks is not presently available (Stagg, 1986).

Blue crabs occur in inland bays and estuaries within Atlantic coastal waters from Massachusetts to Florida and throughout the Gulf of Mexico. Along this range population epicenters are associated with the large estuarine systems of Delaware Bay, Chesapeake Bay, Albemarle and Pamlico Sounds, and the Gulf of Mexico. The life history strategy of blue crabs allows them to take advantage of adjacent high salinity coastal waters as larvae, and inland estuarine systems with abundant food and refuge habitats as adults. It is generally considered that the larvae spawned by crabs that have migrated from the Delaware Bay, Chesapeake Bay, and Albemarle and Pamilco Sounds to adjacent coastal waters are probably returned to these same systems as post-larvae and juvenile crabs. Exchange of individuals among these major estuarine systems or population epicenters is probably limited to a relatively small number of adult blue crabs wandering adjacent coastal waters. Blue crabs within the Gulf of Mexico are maintained as a separate stock because of prevailing currents within the Gulf and apparent lack of favorable habitat along the Atlantic coastal waters of Florida.

Hence, the Delaware Bay, Chesapeake Bay, Albemarle and Pamilco Sounds, and the Gulf of Mexico might each maintain a semi-isolated stock of blue crabs and each of these should be identified for effective execution of management plans.

Molecular genetic techniques have been useful for the identification and resolution of a number of fisheries stocks including numerous finfish species, and some molluscs and crustaceans (Kumpf et al., 1985). There have been a small number of studies of blue crab proteins (Mangum et al., 1987; Dendinger, 1980), but only a fraction of these have addressed the population dynamics of this species. Cole (1983) examined allozyme frequencies of blue crabs collected from north and south of Cape Hatteras. Cole (1978) examined allozymes of blue crabs collected from within the Chesapeake Bay and the immediately adjacent Chincoteague Bay. In both of these studies results were inconclusive because of small sample sizes (less than 50 individuals from any population) and a surprisingly low level of detected polymorphisms. However, these authors did report that slight differences occurred between the populations examined, that widely separated populations may be genetically distinguishable, and more extensive analyses may be warranted.

A recently developed molecular genetic technique, mitochondrial DNA (mtDNA) restriction fragment analysis, has been used for identification of a number of fisheries stocks and continued applications seem very promising (Komm et al., 1982; Avise, 1985). Mitochondrial DNA is thought to be under less stringent controls and limitations than nuclear DNA and may evolve more rapidly. Subsequently, it is more likely than nuclear DNA to reveal differences among populations separated for relatively short periods of time (Brown, 1983). In the absence of extreme bottlenecks, founder events, or strong selective pressures it is unlikely that nuclear gene divergence among

blue crab populations within the major estuarine systems will have occurred at a detectable level. However, new (mutated) mtDNA molecules are incorporated into populations relatively rapidly (Takahata and Slatkin, 1984), making it possible that blue crab subpopulations or stocks may be distinguishable by rare mtDNA genotypes.

We have conducted an initial examination of the mtDNA restriction fragments of blue crabs to determine if macrogeographic structuring of populations might be detectable within Atlantic coastal waters and the Gulf of Mexico. Though isolation and restriction fragment analysis of mtDNA has become routine for an array of organisms this is not the case for many crustaceans and molluscs, including blue crabs. We find (and others) that polysacharride and/or protein contaminants co-isolate with the mtDNA and prevent restriction enzymes from cleaving the molecule. This apparently occurs whether or not the mtDNA is purified by banding in a cesium chloride gradient and has been noted for other crustaceans, as well as, some molluscs (personal communication, various investigators). Here we present an isolation protocol that resolves this problem, and provide an initial characterization of blue crab mtDNA as determined with restriction enzymes.

Methods

Blue crabs from Tampa Bay, Florida; Albemarle Sound, North Carolina and the York River, Virginia were analyzed. The tissue that consistently provided the best yield of mitochondria and, hence, mtDNA was the hepatopancreas. Unextruded egg masses and muscle tissue did not provide as much mtDNA and required additional steps. If blue crabs were held in poor condition or for a great length of time the hepatopancreas atrophied and the

mtDNA yield was much lower; therefore, all isolations should be performed with hepatopancreas from fresh, live crabs. The only unique item used during the isolation is a low-shear continuous (LSC) tissue grinder (Yamato, Inc.) which ruptures tissue cells and leaves organelles intact. Otherwise, the protocol below is an adaptation from an array of standard laboratory protocols used by a number of investigators.

Mitochondrial isolation: Three to five grams of hepatopancreas is homogenized with the LSC grinder in cold (4^{o}C) 0.3M sucrose/TEK buffer (0.05M Tris, 0.01M EDTA, 0.2M KCl, 0.3M sucrose, pH 7.8) and collected in a 12ml centrifuge tube. Cellular debris are removed from this homogenate by centrifugation twice at 1,000g for 10 minutes. The supernatant, containing mitochondria, is placed over 3ml of 1.1M sucrose/TEK and centrifuged at 23,000g for 60 minutes. This produces a loose mitochondrial pellet at the bottom of the centrifuge tube and mucus at the sucrose density interface. The supernatant is gently poured off and the mitochondrial pellet is resuspended in approximately 7ml of 0.3M sucrose/TEK. Any remaining debris are removed from this mitochondrial suspension by centrifugation at 1,000g for ten minutes. The supernatant is then placed over 1.1M sucrose/TEK, and centrifuged at 23,000g for 60 minutes. After this final centrifugation, the supernatant is poured off and the tube with the mitochondrial pellet is placed upside down in a rack and allowed to drain.

mtDNA isolation: The mitochondrial pellet is resuspended in 400ul of TEK by vortex mixing and transferred to a 1.5ml polypropolene microcentrifuge tube. Five microliters of proteinase K (1 unit/ul) is added to the mitochondrial suspension, mixed and incubated at 37^{o}C for 15 minutes. This mitochondrial suspension is made to 1.5% non-Idet P-40, a non-ionic

detergent that solubilizes both inner and outer mitochondrial membranes but leaves a high proportion of nuclei intact. Following incubation of this suspension, 100ul of 5M NaCl is added and thoroughly mixed. To this, 70ul (10% in 0.7M NaCl) of hexadecytrimethylammonium bromide (CTAB) is added, thoroughly mixed, and allowed to incubate at 55^{o}C for 15 minutes. The CTAB combines with and precipitates proteins and exopolysaccharides that may be complexed with the mtDNA and the NaCl prevents CTAB-nucleic acid precipitation by forming ionic interactions with the nucleic acids (Ausubel et al., 1987). Proteins and polysacharrides are removed from this solution by extraction once with Chloroform:isoamyl alcohol, once with phenol and once again with chloroform:isoamyl alcohol following standard protocols (Maniatis et al., 1982, Schleif and Wensink, 1981). The purified nucleic acids are then precipitated by adding twice the volume of 95% ethanol, pelleted by centrifugation at 12,000g for 15 minutes and air dried at 37^{o}C. The dried mtDNA pellet is resuspended in sterile distilled water and stored at -20^{o}C until analysis with restriction enzymes.

Results

We have digested blue crab mtDNA that was isolated using the above protocol with a number of restriction enzymes including Eco RI, Sst I, Msp I, Nci I, Hind III, and Ava I. The enzymes that were most polymorphic were Sst I and Msp I as illustrated in figure 1. Using estimates of the size of restriction fragments produced by these enzymes the size of the blue crab mtDNA molecule is approximately 16.1 kilobases. The two restriction morphs produced by Sst I are illustrated in figure 2. The common restriction morph

"A" contains five fragments with approximate sizes of 5.4, 4.5, 3.8, 1.6 and 1.3 kilobases. The restriction morph "B" contains only four restriction fragments indicating the loss of the restriction site between fragments 4.5 and 1.6 kilobases yielding a 5.6 kilobase fragment. Of forty-four blue crabs examined from Virginia and North Carolina, restriction morph "A" was in 42 individuals and the restriction morph "B" was in two individuals. All 15 of the blue crabs examined from Florida contained the "A" restriction morph.

The restriction enzyme Msp I produces six different restriction morphs as illustrated in figure 3. The restriction morph "A" contains three fragments 6.1, 5.5, and 4.5 kilobases. The restriction morph "B1" has an additional restriction site indicated by the 0.9 and 3.7 kilobase fragments, "B2" has an additional restriction site splitting the 6.1 kilobase of "A" into 3.2 and 2.9 kilobase fragments, and "B3" has additional restriction site in the same region that splits the 6.1 kilobase fragment of "A" into 3.4 and 2.7 kilobase fragments. The restriction morph "C" contains an additional site cutting the 3.7 kilobase fragment of "B" into 1.5 and 2.2 kilobase fragments. The restriction morph "D" contains the most fragments with an additional site located on the 5.5 kilobase fragment of "C" yielding 1.5 and 3.9 kilobase fragments. Of the 45 blue crabs examined from Virginia and North Carolina three individuals contained restriction morph "B1", two individuals contained restriction morphs "C" and "D", and the remaining individuals contain restriction morph "A". Of the 15 blue crabs examined from Florida, 13 contain the common restriction morph "A". Two additional restriction morphs, "B2" and "B3", not observed in individuals from Virginia or North Carolina were were seen in individuals from Florida.

Discussion

The estimated size of the blue crab mtDNA molecule is within the range of other multicellular animals (15.7-19.5 kilobases). There have been very few published studies of mtDNA restriction fragment analysis of marine invertebrates and no other studies of any of the species of blue crabs. This can probably be attributed to the inhibition of restriction enzyme activity as indicated above. Of the few studies of marine invertebrates that have been conducted McLean et al. (1982) and Komm et al. (1982) characterized the mtDNA of the spiny lobster, Panulirus argus, with a number of restriction enzymes and electron microscopy. Their studies suggest that the mtDNA genome size of Panulirus argus is on the order of 16.2 kilobases. Saunders et al. (1986) estimated that the mtDNA genome size of the horseshoe crab, Limulus polyphemus, is in the range of 14.5 and 16.0. A number of studies of Mytilus edulis and Mytilus galloprovincialis have indicated that the mtDNA genome size of both these bivalves is in the order of 17.4 kilobases (Skibinski, 1985; Edwards and Skibinski, 1987). Snyder et al. (1987) reported an atypical mtDNA genome size of the deep-sea scallop Placopecten magellanicus of from 32.1 to 39.3 kilobases. This estimate is more than twice then what might be expected and is considered very unusual.

As indicated above, if discrete blue crab stocks occur they would probably be of relatively recent origin and none of these stocks would be completely isolated. However, mtDNA restriction fragment analysis has revealed geographic structuring of semi-isolated populations of other organisms (Avise and Lansman 1983). Saunders et al. (1985) was able to identify distinct northern and southern populations of Limulus polyphemus along a continuous distribution in Atlantic coastal waters and the Gulf of

Mexico. Saunders et al. (1985) suggest that their data follows a stochastic model of genetic divergence for species with a limited gene flow along a continuous distribution. If this stochastic model of genetic divergence is of general application, then blue crab stocks or subpopulations may also be distinguishable by the appearance of recent restriction morphs. Both the number of individuals examined and the number of restriction enzymes used for analysis and presented in this paper are too low to draw conclusions regarding macrogeographic structuring of populations. However, it appears that the level of polymorphisms, expressed as different restriction morphs, is high as compared to allozyme studies (Cole 1978, Cole 1982). Continued studies that will provide the requisite data for determining the structure and integrity of blue crab stocks along the Atlantic coast and the Gulf of Mexico are recommended.

Acknowledgments

Blue crabs from North Carolina were provided by J. Hawkins and from Florida by P. Steele and T. Bert.

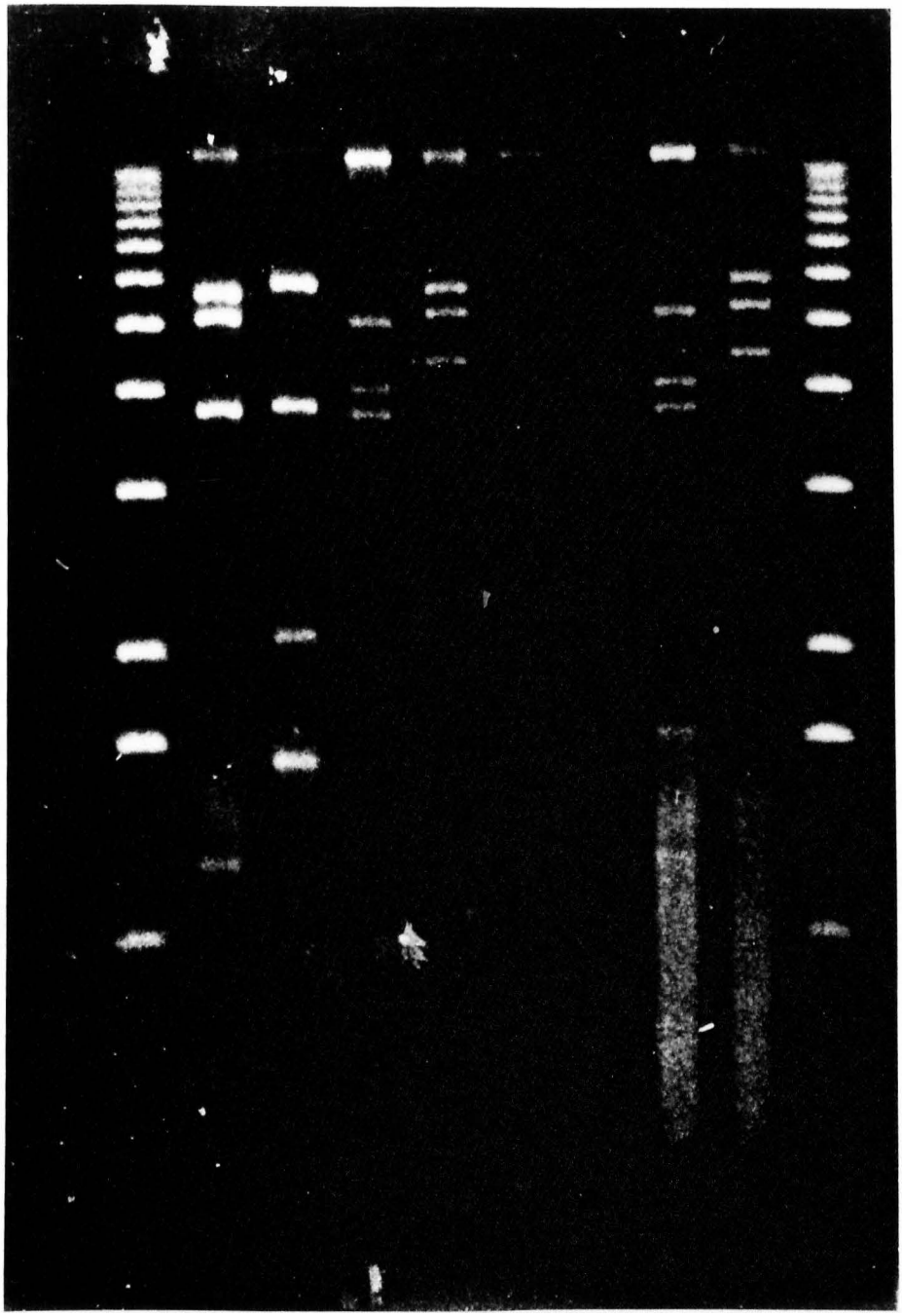
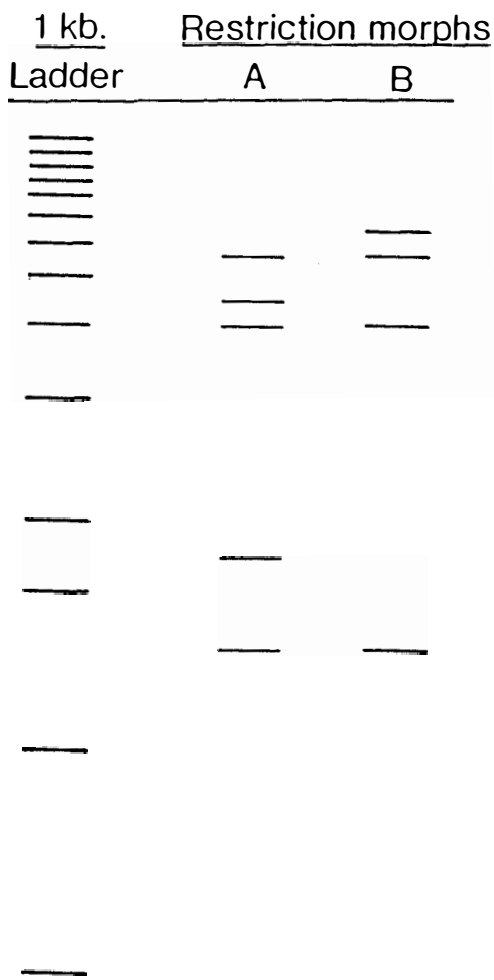


Figure 1. Photograph of blue crab mtDNA restriction fragments produced by cleavage of mtDNA isolated using the presented protocol and digested with the restriction enzymes Sst I (lanes 2, 4, 6 and 8) and Msp I (lanes 3, 5, 7 and 9). Lanes 1 and 10 contain a 1 kilobase ladder as a reference.

Sst I



	Va.	N.C.	Fla.
A	23	19	15
B	2	1	—
	25	20	15

Figure 2. Restriction morphs produced by digestion of Virginia (Va), North Carolina (NC) and Florida (Fla) blue crab mtDNA with the enzyme Sst I. Closed circle diagrams indicate the relative position of restriction fragments on the mtDNA molecule.

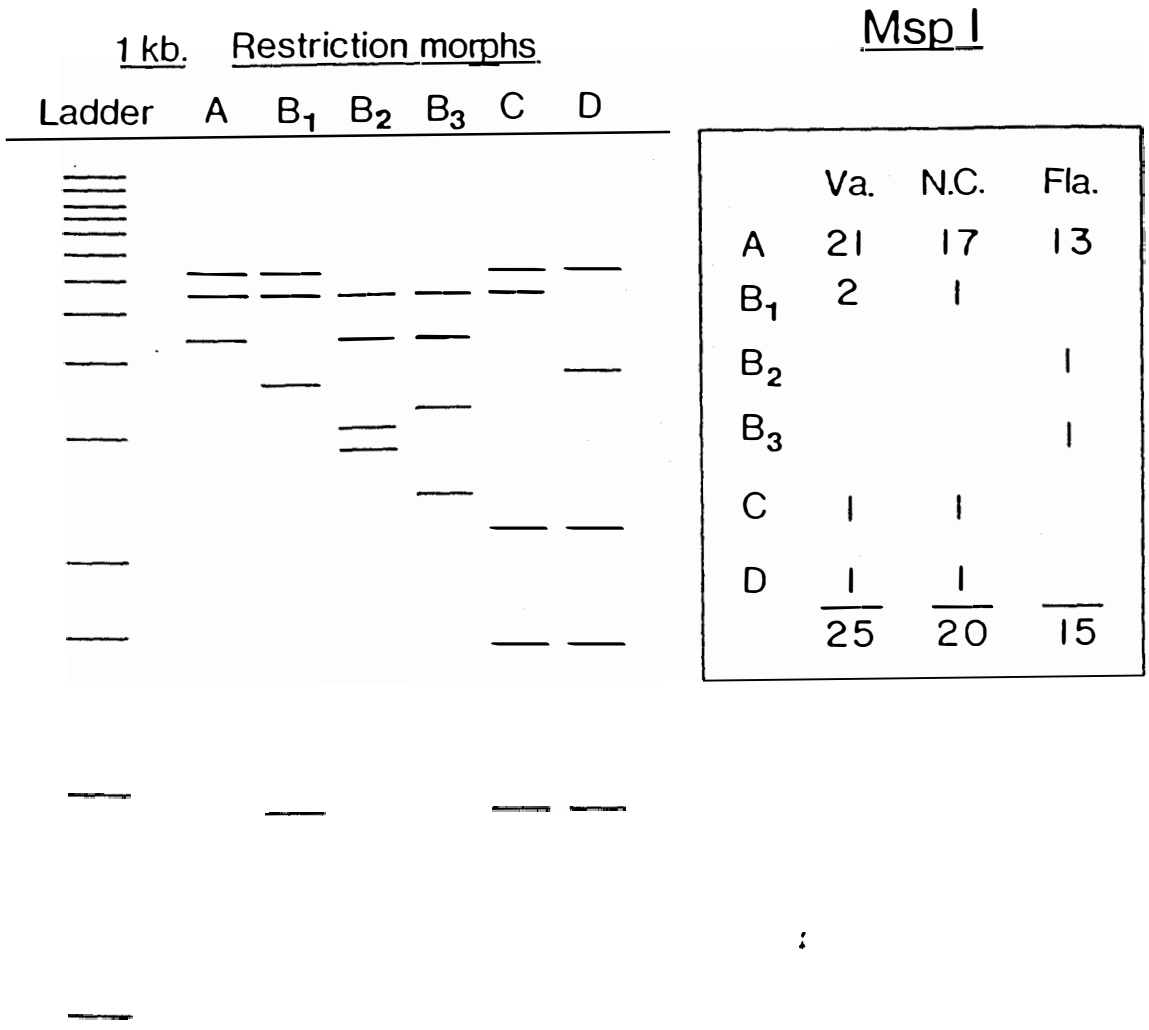


Figure 3. Restriction morphs produced by digestion of Virginia (Va) North Carolina (NC) and Florida (Fla) blue crab mtDNA with the enzyme Msp I. Closed circle diagrams indicate the relative position of restriction fragments on the mtDNA molecule.

Study 3. Genetic structure of oyster rocks within the Chesapeake Bay.

Abstract

The genetic structure of Chesapeake Bay Crassostrea virginica populations were examined at nine enzyme loci using standard starch gel electrophoresis. One of the populations examined is routinely exposed to natural invasions by the parasite Haplosporidium nelsoni (MSX) and adults are considered resistant to chronic infections. Other populations are distributed throughout the Bay encompassing a broad range of environmental and habitat conditions. Variations in allele frequencies among the populations were identified for a number of the loci examined. The data strongly suggests that selection pressures closely associated with environmental regimes and oyster pathogens indirectly influence the locale genome of oyster populations throughout the Bay. The manifestation of these selection pressures is establishment of discrete genomes for oyster populations within the Bay.

Introduction

Unlike studies 1 and 2, the present study emphasizes the use of isoenzymes for genetically describing oyster populations or stocks. Isoenzymes are appropriate for this study because of the combined affect of the life history strategy of oysters and the selection regimes involved in establishing and maintaining discrete genomes. Unlike blue crabs and

striped bass, post-larval oysters are sedentary throughout their life and short-term selection pressures immediately post-settlement play a primary role in determining the genome of local populations. The gene pool of oyster populations may also be influenced by movement or transfer of oysters by an aggressive state repleation program. Motile species (eg. blue crab and striped bass) can actively move about in response to environmental conditions, inter and intraspecies interactions, or combinations thereof; all potentially acting as selection pressures on the population genome. Therefore, biochemical or genetic characteristics discriminating specific stocks of motile organisms are a manifestation of isolation and divergence over the long-term often associated with major physical and/or behavioral barriers. The population genome of sedentary invertebrates (eg. oysters) is principly determined by locale selection pressures within very short time frames, usually immediately post-settlement.

Since the advent of protein electrophoresis it has become possible to evaluate the genetic structure of natural populations (Powell, 1975; Selander, 1976). Using this technique, evidence has been presented that the genetic structure of bivalve populations can vary on both macrogeographic and microgeographic scales and that genomic variations are often associated with environmental and habitat differences. Koehn et al. (1973, 1976) pioneered this research with their work on the population genetics of Midiolus demissus and Mytilus edulis. Koehn (1983) clearly demonstrated the genetic fidelity of Mytilus edulis to local environmental conditions. During three successive years Mytilus edulis larvae, containing the Lap ^{94} (leucine aminopeptidase) allele, were transported by currents from oceanic waters into Long Island Sound. The immigration of larvae into the sound was

indicated by elevated frequencies of the Lap^{94} allele in the young set genome (immigrants) relative to the resident adults. Each year the immigrants were naturally culled from the populations by selective mortalities and the resident adult populations remained relatively unchanged. Selection may be occuring at the Lap locus or this locus may be coincidently marking oceanic larvae that happen to be disadvantaged in estuarine environments. Hilbish (1982) described a possible mechanism by which salinity may select for different alleles at the Lap locus. Under low salinity conditions, the Lap^{94} allele is probably selected against because its enzyme results in an excessive loss of nitrogenous wastes (see also Pierce, 1982).

Specific genetic studies of C. virginica have addressed geographic variations, environmental/habitat affects, affects on growth rates and affects on physiological condition. These studies indicate that the genetic structure of C. virginica populations is responsive and sensitive to environmental/habitat parameters; and, the genetic structure can affect survivorship, fitness, growth and physiological condition of individuals. Buroker (1983a) studied macrogeographic variations among C. virginica populations along the Atlantic coast and the Gulf of Mexico. He found that estimates of genetic similarity ranged from 96.2% to 99.7%, values expected for conspecifics. Buroker (1983a) also found that macrogeographic clines occured at the Lap locus along the Atlantic coast and at the Lap and Pgi (phosphoglucose isomerase) loci in the Gulf of Mexico. According to Buroker (1983a) individual alleles at the Lap and Pgi loci are favored over others at specific locations by environmental parameters, causing differential mortality and creating genetically discrete sympatric populations.

Genetic affects on growth and physiological condition of C. virginica were first suggested by Singh and Zouros (1978) and Zouros et al. (1980). They reported that the body weight of C. virginica was positively correlated with individual heterozygosity. The more heterozygous loci possessed by an individual the greater the weight and growth rate, as compared to more homozygous individuals of the same cohort. Assuming that growth rate and condition are related to the conversion efficiency of consumed energy to somatic tissue, Koehn and Shumway (1982) investigated the relationship to heterozygosity to energy available for growth. They demonstrated that the metabolic energy demand of C. virginica exposed to high temperature and low salinity (stressed conditions) was over twice as great for multiple locus homozygotes then for heterozygous individuals. They also reported that the relationship was additive, metabolic efficiency and tolerance to stress increased almost steadily with the addition of heterozygous loci. Koehn and Shumway (1982) suggested that heterozygous individuals are more metabolically fit and better able to tolerate environmental extremes. Foltz (1983) found that regardless of environmental conditions heterozygous individuals grow faster and are more physiologically fit than homozygous individuals. Singh and Zouros (1981) predicted that by increasing the heterozygosity of a C.virginica population to Hardy-Weinberg equilibrium a four fold increase in the mean weight of the population would result.

Three recent studies have been conducted on the genetic structure of C. virginica within the Chesapeake Bay. Rose (1984) investigated adjacent oyster rocks with only slight environmental disparity among them. To the other extreme, Buroker (1983b) examined C. virginica subpopulations throughout the length of the Bay encompassing broad reaches of C. virginica's geographic range. Though the populations examined by Buroker

(1983b) were geographically widespread within the Bay they were all from similar habitats, defined by salinity. The salinity range of all populations examined by Buroker (1983b) was between 9 and 15 0/00. Within the Chesapeake Bay oysters maintain viable and productive populations in salinities ranging from 2.0 to 30.0 0\00%. For the present investigation oyster populations were selected for study that represent the full extent of their environmental and geographic range within the Chesapeake Bay. Five of the populations examined encompass the full salinity extremes of two estuaries (the James and Rappahannock Rivers) one occurs in a relatively high, stable salinity area (Mobjack Bay) and one is located in moderate salinity in the northern reach of the Bay (Tred Avon River).

Methods

Adult oysters have been examined from the James River, Rappahannock River and the Mobjack Bay in the lower York River, Virginia. Both adult and spat oysters have been examined from the Tred Avon River, Maryland. The oysters examined from the James River were from Horsehead, Wreck Shoal and Nansemond Ridge. The oysters examined from the Rappahannock River were collected from Bowlers Rock and Corrotoman inlet. The oyster population within Mobjack Bay is subject to natural invasions by MSX, salinities range from 19-23 0\00.

Oysters were held in shallow outdoor tanks in flowing York River water until processed. For enzyme analyses a portion of the adductor muscle and digestive diverticular were dissected from each oyster, combined with an equal volume of distilled water and homogenized in an ice bath with a glass grinder fitted to a Wheaton stirrer. The homogenates were centrifuged at

1,000 x g for ten minutes to remove cellular debris and the supernatant was absorbed onto filter paper wicks and electrophoresed in 11% starch gels.

Genotypes were determined at nine enzyme loci which encode for the following six enzyme systems: leucine aminopeptidase (Lap-1 and Lap-2), phosphoglucose isomerase (Pgi), phosphoglucomutase (Pgm), alanopine dehydrogenase (Adh), strombine dehydrogenase (SDH), and an aminopeptidase (Ap-1 and Ap-2). The enzyme systems Pgi, Ap-1, Ap-2, Sdh and Adh were resolved using a LiOH discontinous buffer system and Pgm with a tris-maleate buffer system (Selander et al. 1969). Leucine aminopeptidase was resolved using the tris-citric acid buffer system of Rodhouse and Gaffney (1984). For each locus the fastest migrating allele, the most anodal, was designated "A" and slower alleles "B", "C", "D"..., respectively. For maintenance of continuity in scoring gels a portion of tissue from a previously typed oyster was run on each gel. This reference tissue was stored at -20 C until used. The data were compiled and initial analyses were conducted using the software package Biosys-1 (Swofford and Selander 1981). Mean heterozygosity was compared among the populations using a t-test and a G-test was used to determine if the occurrence of alleles was independent of population location (Sokal and Rohlf 1981).

Results

The allele frequency data at all loci for all populations examined is given in Table 1 and summaries of heterozygosity values in Table 2. These data can be most easily compared using Nei's genetic identity values given in Table 3 and Figure 1 in a clustering diagram. Nei's genetic identity is an expresssion of the average level of genetic similarity among populations

extrapolated over all loci. Figure 1 clearly illustrates that populations from within any river system are more closely related to one another than to those in other river systems. In addition, the Mobjack Bay population is distinctly different than all other populations examined.

Though slight genetic differences occurred among populations within and among river systems data analysis is primarily focused on comparisons with presumed MSX resistent stock. The population of oysters located in Mobjack Bay are considered MSX resistent, while those in the upper James River, Wreck shoal, have long been considered non-resistent to MSX. The theory has been that populations that thrive despite exposure to MSX must be resistent and those not exposed to the pathogen lack any opportunity to develop resistence. It should be noted, the potential for resisting chronic infections of MSX is present in all oysters, but, some are better at it then others. Faced with MSX infestation oysters less able to resist chronic infections succumb, and are selected against. This process has a winnowing affect on the population genome creating a genetically discrete stock of "resistent" oysters.

Differences in the genetic structure between the two populations were primarily manifest in the occurrence of alleles and not by overall heterozygosity levels. The mean heterozygosity of individuals at the Mobjack Bay (MJB) population was 0.464 and at the Wreck Shoal (WSH) population 0.471 (Table 2). The results of a t-test indicate that the mean heterozygosities are not significantly different between the two populations ($t=0.147$, $d.f.=12$, $P>0.1$). In comparison to other studies of bivalve molluscs including Crassostrea virginica these heterozygosity values are higher than average but not uncommon (see review by Berger 1983). The mean heterozygosity of oysters from ten populations within the Chesapeake Bay,

calculated from data presented by Buroker (1983), on the same loci examined in this study is 0.440 and the range is 0.404 -> 0.499.

The allele frequencies at five of the nine loci examined are different between the two populations (Table 4). The greatest differences occur at the Lap-2, Pgm-1 and Ap-1 loci; lesser differences occur at the Lap-1 and Adh loci (Table 1). At the Lap-2 locus alleles "C", "D" and "E" occur in decreasing frequencies within both populations; however, allele "C" occurs at a much higher frequency within the MJB population. At the AP-1 locus, alleles "A", "B" and "C" occur at equal frequencies in the MJB population, whereas these same alleles occur at varying frequencies within the WSH population. The locus exhibiting the greatest differences in allele frequencies between the two populations is Pgm-1. At this locus within the Wreck Shoal population allele "B" occurs at greatest frequency and within the MJB population allele "C" occurs at greatest frequency. The proportion of individuals with the "B" allele in the WSH population is 86%, in comparison, 90% of the individuals in the MJB population contain the "C" allele at this locus. The results of the G-test (Table 4) indicate these differences in the occurrence of alleles at the Lap-2, Pgm-1 and Ap-1 loci are associated with the population locations.

Discussion

It is very unlikely that the enzyme loci examined in this investigation are directly responsible for a mechanism used in resisting MSX infections in oysters. Rather, these loci, or other closely linked loci, may influence the metabolic fitness of individual oysters and provide the energetic advantage necessary for combatting chronic infections. Previous studies

have indicated that the genetic structure of bivalves is responsive to environmental regimes and habitat conditions (above). As a result of this process the gene structure of bivalve populations is winnowed by the selective mortalities of less fit individuals, eliminating less favorable genotypes. This process was observed in previous studies of Crassostrea virginica within the the Gulf of Mexico and the Chesapeake Bay (Buroker 1983a, 1983b; Rose 1984). Buroker (1983a) identified allele frequency clines along the Mississippi River delta and the Gulf of Mexico that he attributed to environmental characteristics.

During the late 1950's the haplosporidan parasite, Haplosporidium nelsoni, entered the Chesapeake Bay and rapidly infested Crassostrea virginica (Andrews 1984). Each year since then MSX infections have been responsible for high levels of oyster mortalities, though primary areas of infestation are confined to waters with salinities greater than 20 0/00 Haskin and Ford 1982). The oyster populations within high salinity waters that were able to survive the initial infestation and continue to thrive are thought to possess some form of enhanced resistance over those that succumbed to the disease (Andrews and Frierman 1974). Both laboratory and field experiments have shown that oysters from resistant populations that are exposed to the pathogen have lower levels of infection and mortalities than oysters from apparently non-resistant populations (Andrews 1968). When the progeny of these resistant oysters were reared over a number of successive generations they exhibited a similar level of tolerance to MSX (Andrews and Frierman 1974; Haskin and Ford 1979). These observations have indicated that the apparent ability to resist chronic infections of MSX may be an inherited characteristic.

The general response of molluscs to an infection or invasion is hemocytic phagocytosis and/or encapsulation of the foreign substance (Cheng and Rifkin 1970; Bayne 1982). Crassostrea virginica responds to systemic infections of MSX by producing hemocyte aggregations around the parasite, known as hemocytosis (Ford 1986). Oysters that exhibit resistance to MSX have a higher level of response to the infection, expressed as hemocyte activity, then do oysters that have not demonstrated resistance. This indicates that oysters able to resist MSX infections either, 1) maintain a greater population of hemocytes and await infection, 2) respond to invasion by initiating rapid proliferation of hemocytes, or 3) contain a more efficient population of hemocytes (see Fisher and Newell 1986). Whether any combination of these three mechanisms or some additional mechanisms are employed there must be an associated energetic cost for this very active cellular response. In addition to the energetic requirements of the oysters' defense mechanism, energy expenditures are required for the replacement of metabolic substances consumed by MSX or lost from damaged cells, as well as repair of damaged tissue (Mengebier and Wood 1969; Feng and Canzonier 1970; Douglas and Haskin 1976).

Newell (1985) described some of the physiological consequences of MSX infections upon oysters. His overall findings were that infected oysters had decreased clearance rates and no change in oxygen consumption rates as compared to non-infected oysters. It is expected that metabolism, and hence oxygen consumption rates, would decrease with decreased feeding activity. Since this was not the case in the experiments conducted by Newell (1985), metabolic reserves are probably being consumed and energy diverted from digestive processes to other functions. Considering the circumstances, this energy may be diverted to hemocyte production and activity or some other

defense mechanism. Eventually, metabolic reserves become depleted, the infected oyster cannot energeticly afford to combat the infection and it succumbs. However, if an individual oyster possesses an efficient metabolic system it may be able to meet the energy requirements for combatting the parasite.

Koehn and Shumway (1982), Rodhouse and Gaffney (1984) and Rodhouse et al. (1986) have shown that there is a relationship between metabolic efficiency and the genetic structure of C. virginica. These studies have indicated that genetically advantageous oysters have more energy available for growth and are better able to resist stress. The results of the present investigation indicate that this same relationship might allow some oysters to resist chronic level infections of MSX. The enzyme loci examined in the above investigations were included in this study; therefore, differences expressed at these loci between the two populations may be indicative of differences in the metabolic efficiency between the two populations. According to Newell (1985) oysters identified as resistant are still infected with the parasite but the infection is localized in the gills, indicating that spread of the parasite is being actively resisted by the oyster.

The results of this investigation support a long standing assumption that there is probably a genetic basis for resistance to chronic infections of MSX in the American oyster, Crassostrea virginica. Since shortly after infestation of the MSX pathogen so-called MSX resistant stocks of oysters have been maintained and selectively bred in hatcheries. The successive progeny of these "resistant" oysters demonstrated equal or increased resistance to the pathogen (Haskin and Ford 1979); therefore, it has been assumed that there must be a genetic basis for this phenomenon. Despite a

relatively large amount of research concerning environmental selection and genetic effects on fitness characteristics in marine bivalves, including oysters, this is the first investigation indicating a correlation between the genetic structure of oyster populations and resistance to the pathogen MSX. Additional genetic studies should be conducted under controlled experimental conditions and on presumed resistant stocks maintained in hatcheries.

In addition, it appears that isoenzymes may function as stock discriminators for oysters within the Chesapeake Bay. However, care must be exercised in making comparisons among presumed stocks. As mentioned above, discrete oyster stocks are probably established by the affect of short-term selection pressures at a particular location. These selection pressures are not restricted to pathogens (MSX, Dermo) but may include environmental, as well as, habitat factors (eg. salinity, temperature regime). Geographic distances between oyster populations may not be a significant factor in establishing oyster stocks.

Because of the recent declines in oyster populations management efforts are being steered toward revitalizing natural stocks. Two techniques that have been receiving attention are the management of brood stocks and the hatchery production of seed oysters. Brood stocks, potentially capable of supplying spat to a number of subpopulations, can be nurtured and protected from over exploitation by fishing. Sucess of these revitalization efforts may be dependent on recognizing the genetic requirements, population genome, of managed populations.

Table 1. Allele frequencies at eight enzyme loci for eight populations within the Chesapeake Bay. Population and locus abbreviations are as indicated in the text, N=number of individuals examined. (James River populations are NAN=Nansemond Ridge, HHD=Horsehead, WSH=Wreck Shoal; Tred Avon River populations are TRB=Adults, TRC=Spat; Rappahannock River populations are RBR=Bowlers Rock, RCN=Corrotoman Inlet and MJB=Mobjack Bay population.)

	POPULATION							
LOCUS	NAN	HHD	WSH	TRB	TRC	RBR	RCN	MJB
Lap-1								
(N)	221	223	157	116	49	132	102	102
A	0.095	0.117	0.108	0.082	0.143	0.091	0.083	0.123
B	0.729	0.626	0.726	0.741	0.622	0.697	0.686	0.696
C	0.172	0.235	0.159	0.168	0.235	0.212	0.225	0.176
D	0.005	0.022	0.003	0.009	0.000	0.000	0.005	0.005
E	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000
Lap-2								
(N)	130	124	133	113	48	121	78	56
A	0.008	0.012	0.000	0.053	0.094	0.012	0.006	0.000
B	0.092	0.036	0.034	0.212	0.135	0.087	0.109	0.009
C	0.392	0.585	0.429	0.385	0.542	0.550	0.519	0.625
D	0.277	0.262	0.297	0.279	0.188	0.240	0.250	0.268
E	0.212	0.101	0.218	0.066	0.031	0.099	0.115	0.098
F	0.015	0.004	0.023	0.004	0.010	0.012	0.000	0.000
G	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgi								
(N)	221	149	157	116	49	143	103	118
A	0.027	0.037	0.041	0.047	0.020	0.045	0.044	0.047
B	0.624	0.688	0.650	0.720	0.694	0.678	0.636	0.712
C	0.335	0.275	0.303	0.220	0.276	0.266	0.311	0.233
D	0.014	0.000	0.006	0.009	0.010	0.010	0.010	0.008
E	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000
Pgm-1								
(N)	130	94	156	69	19	135	58	103
A	0.150	0.154	0.157	0.123	0.079	0.204	0.353	0.063
B	0.681	0.734	0.750	0.754	0.789	0.685	0.543	0.209
C	0.142	0.101	0.087	0.109	0.132	0.104	0.103	0.689
D	0.027	0.000	0.006	0.014	0.000	0.007	0.000	0.039
E	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.000

Table 1 (cont.).

	POPULATION							
LOCUS	NAN	HHD	WSH	TRB	TRC	RBR	RCN	MJB
Pgm-3								
(N)	221	176	157	101	48	144	115	118
A	0.025	0.026	0.035	0.020	0.052	0.024	0.083	0.051
B	0.215	0.276	0.188	0.178	0.125	0.240	0.222	0.237
C	0.620	0.625	0.650	0.520	0.521	0.646	0.570	0.597
D	0.111	0.057	0.099	0.228	0.250	0.083	0.113	0.076
E	0.027	0.017	0.025	0.054	0.052	0.007	0.013	0.038
F	0.002	0.000	0.003	0.000	0.000	0.000	0.000	0.000
Adh								
(N)	147	157	157	103	46	144	101	117
A	0.034	0.010	0.006	0.000	0.000	0.000	0.000	0.410
B	0.459	0.455	0.347	0.359	0.337	0.247	0.356	0.359
C	0.337	0.341	0.439	0.364	0.478	0.455	0.426	0.231
D	0.170	0.194	0.204	0.248	0.152	0.257	0.218	0.000
E	0.000	0.000	0.000	0.029	0.033	0.042	0.000	0.000
F	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000
Sdh								
(N)	145	158	157	104	44	143	99	118
A	0.062	0.089	0.092	0.034	0.011	0.115	0.096	0.106
B	0.338	0.475	0.471	0.385	0.466	0.423	0.505	0.466
C	0.372	0.351	0.350	0.413	0.375	0.388	0.308	0.394
D	0.197	0.085	0.086	0.144	0.136	0.066	0.091	0.034
E	0.031	0.000	0.000	0.024	0.011	0.007	0.000	0.000
Ap-1								
(N)	99	144	156	115	47	138	98	100
A	0.303	0.267	0.375	0.322	0.351	0.380	0.408	0.325
B	0.348	0.319	0.199	0.200	0.255	0.330	0.235	0.325
C	0.343	0.403	0.426	0.478	0.394	0.290	0.357	0.325
D	0.005	0.003	0.000	0.000	0.000	0.000	0.000	0.025
E	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.000

Table 2a. Heterozygosity Values for NAN Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 221	Lap-2 130	Pgi 221	Pgm-1 130	Pgm-3 221	Adh 147	Sdh 145	Ap-1 99
H	0.431	0.716	0.497	0.493	0.556	0.646	0.704	0.669
H(UNB)	0.432	0.719	0.498	0.495	0.557	0.648	0.706	0.672
H(D.C.)	0.389	0.569	0.457	0.338	0.439	0.639	0.586	0.535

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.589 (S.E. 0.038)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.591 (S.E. 0.039)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.494 (S.E. 0.037)
MEAN NUMBER OF ALLELES PER LOCUS = 4.75 (S.E. 0.41)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2b. Heterozygosity Values for HHD Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 223	Lap-2 124	Pgi 149	Pgm-1 94	Pgm-3 176	Adh 157	Sdh 158	Ap-1 144
H	0.539	0.578	0.450	0.427	0.529	0.639	0.636	0.664
H(UNB)	0.540	0.580	0.451	0.429	0.531	0.641	0.638	0.666
H(D.C.)	0.457	0.460	0.416	0.255	0.489	0.541	0.532	0.403

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.558 (S.E. 0.031)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.560 (S.E. 0.031)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.444 (S.E. 0.032)
MEAN NUMBER OF ALLELES PER LOCUS = 4.38 (S.E. 0.32)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2c. Heterozygosity Values for WSH Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 157	Lap-2 133	Pgi 157	Pgm-1 156	Pgm-3 157	Adh 157	Sdh 157	Ap-1 156
H	0.436	0.679	0.485	0.405	0.531	0.645	0.639	0.638
H(UNB)	0.437	0.681	0.486	0.407	0.533	0.647	0.641	0.640
H(D.C.)	0.382	0.579	0.459	0.282	0.497	0.637	0.510	0.519

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.557 (S.E. 0.038)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.559 (S.E. 0.038)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.483 (S.E. 0.039)
MEAN NUMBER OF ALLELES PER LOCUS = 4.50 (S.E. 0.33)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2d. Heterozygosity Values for TRB Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 116	Lap-2 113	Pgi 116	Pgm-1 69	Pgm-3 101	Adh 103	Sdh 104	Ap-1 115
H	0.415	0.722	0.431	0.405	0.643	0.676	0.659	0.628
H(UNB)	0.417	0.725	0.433	0.408	0.646	0.680	0.662	0.630
H(D.C.)	0.379	0.522	0.466	0.319	0.495	0.660	0.490	0.504

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.572 (S.E. 0.047)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.575 (S.E. 0.047)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.479 (S.E. 0.036)
MEAN NUMBER OF ALLELES PER LOCUS = 4.50 (S.E. 0.33)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2e. Heterozygosity Values for TRC Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 49	Lap-2 48	Pgi 49	Pgm-1 19	Pgm-3 48	Adh 46	Sdh 44	Ap-1 47
H	0.537	0.643	0.442	0.353	0.645	0.634	0.623	0.657
H(UNB)	0.543	0.650	0.447	0.363	0.652	0.640	0.631	0.664
H(D.C.)	0.592	0.521	0.388	0.316	0.542	0.652	0.477	0.447

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.567 (S.E. 0.040)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.574 (S.E. 0.040)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.492 (S.E. 0.039)
MEAN NUMBER OF ALLELES PER LOCUS = 4.13 (S.E. 0.40)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2f. Heterozygosity Values for RBR Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 132	Lap-2 121	Pgi 143	Pgm-1 135	Pgm-3 144	Adh 144	Sdh 143	Ap-1 138
H	0.461	0.623	0.467	0.478	0.518	0.665	0.653	0.663
H(UNB)	0.463	0.625	0.469	0.480	0.520	0.667	0.655	0.665
H(D.C.)	0.439	0.537	0.462	0.304	0.438	0.597	0.524	0.543

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.566 (S.E. 0.033)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.568 (S.E. 0.033)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.481 (S.E. 0.032)
MEAN NUMBER OF ALLELES PER LOCUS = 4.25 (S.E. 0.37)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2g. Heterozygosity Values for RCN Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 102	Lap-2 78	Pgi 103	Pgm-1 58	Pgm-3 115	Adh 101	Sdh 99	Ap-1 98
H	0.471	0.643	0.497	0.569	0.607	0.644	0.633	0.651
H(UNB)	0.474	0.647	0.500	0.574	0.609	0.647	0.636	0.654
H(D.C.)	0.500	0.551	0.379	0.293	0.417	0.634	0.505	0.449

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.589 (S.E. 0.025)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.593 (S.E. 0.025)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.466 (S.E. 0.037)
MEAN NUMBER OF ALLELES PER LOCUS = 3.88 (S.E. 0.30)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 2h. Heterozygosity Values for MJB Population

	LOCUS AND SAMPLE SIZE							
ALLELE	Lap-1 102	Lap-2 56	Pgi 118	Pgm-1 103	Pgm-3 118	Adh 117	Sdh 118	Ap-1 100
H	0.469	0.528	0.437	0.476	0.577	0.650	0.615	0.683
H(UNB)	0.472	0.533	0.439	0.478	0.579	0.652	0.618	0.686
H(D.C.)	0.402	0.500	0.492	0.301	0.466	0.675	0.441	0.520

MEAN HETEROZYGOSITY PER LOCUS (BIASED ESTIMATE) = 0.554 (S.E. 0.032)
MEAN HETEROZYGOSITY PER LOCUS (UNBIASED ESTIMATE) = 0.557 (S.E. 0.032)
MEAN HETEROZYGOSITY PER LOCUS (DIRECT-COUNT ESTIMATE) = 0.475 (S.E. 0.038)
MEAN NUMBER OF ALLELES PER LOCUS = 4.00 (S.E. 0.19)
PERCENTAGE OF LOCI POLYMORPHIC (0.95 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (0.99 CRITERION) =100.00
PERCENTAGE OF LOCI POLYMORPHIC (NO CRITERION) =100.00

Table 3. Nei's (1978) unbiased genetic identity (above diagonal) and unbiased genetic distance (below diagonal) values.

*	POPULATION	1	2	3	4	5	6	7	8
1	NAN.	*****	0.986	0.989	0.982	0.977	0.981	0.977	0.879
2	HHD.	0.014	*****	0.988	0.976	0.986	0.989	0.984	0.880
3	WSH	0.011	0.013	*****	0.985	0.985	0.989	0.987	0.861
4	TRB.	0.018	0.025	0.015	*****	0.994	0.976	0.972	0.849
5	TRC	0.024	0.014	0.015	0.006	*****	0.986	0.978	0.862
6	RBR	0.019	0.012	0.011	0.024	0.014	*****	0.991	0.874
7	RCN.	0.023	0.017	0.013	0.029	0.023	0.009	*****	0.881
8	MJB	0.129	0.128	0.150	0.163	0.149	0.134	0.127	*****

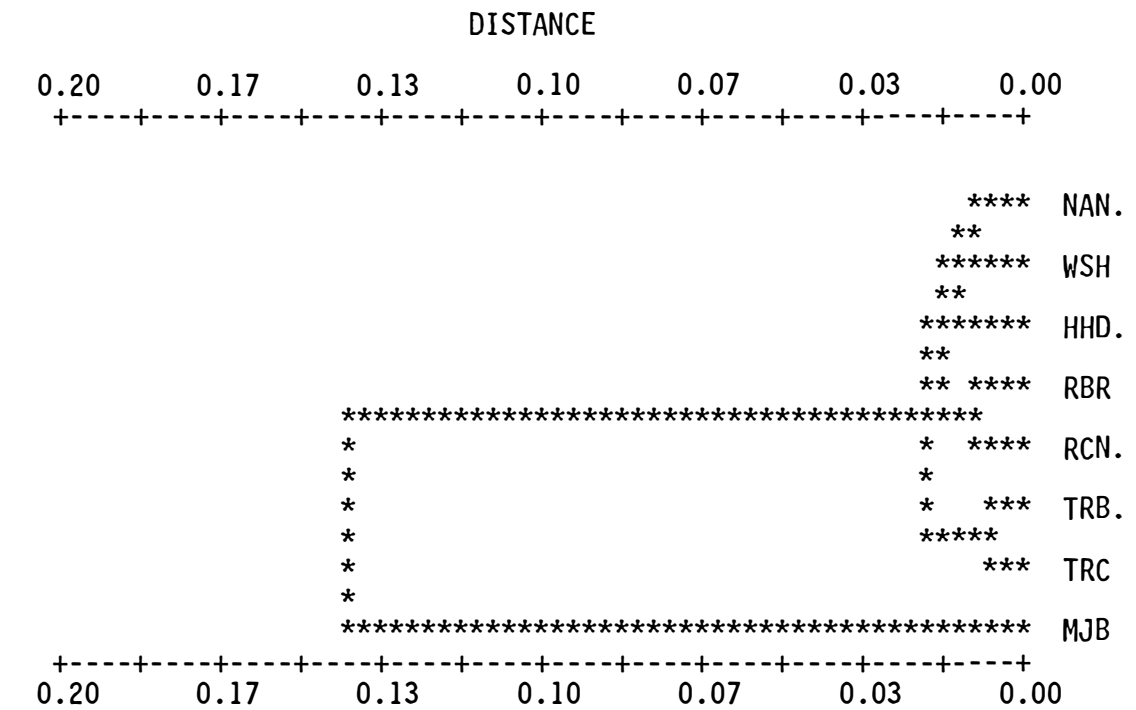


Fig. 1 Cluster diagram using unweighed pair group method (Swofford and Selander, 1981) of genetic identity values from Table 3.

Table 4. G test of independence of occurrence of alleles for each locus between locations.

locus	d.f.	G-test G-value	Significance
Lap-1	2	5.99	P<0.1
Lap-2	3	26.9	P<0.005
Pgi		2	3.23 n.s.
Pgm-1	3	229	P<0.001
Pgm-3	4	4.33	n.s.
Adh		2	5.4 P<0.1
Sdh		3	5.34 n.s.
Ap-1		3	20.4 P<0.005
Ap-2		2	3.13 n.s.

G total=298.3, d.f.=24, significance P<0.001

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

A Thesis

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Carol Furman

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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

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.

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°C. The ethanol was removed by pipet, and the sample was then dried at 37°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°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 ≤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 $\leq$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.

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

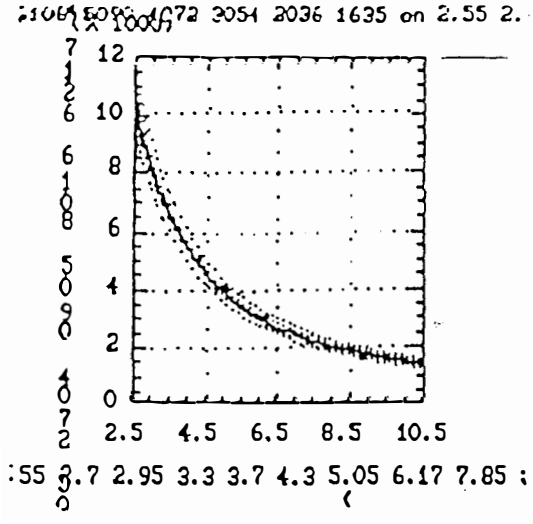
APPENDIX C

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.

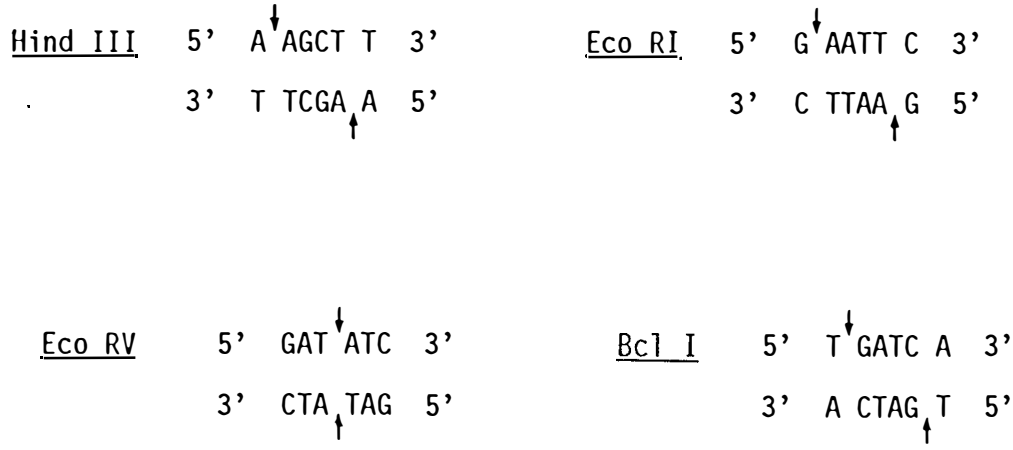


Table 2. Observed genotypic frequencies in the Rappahannock River in 1986.

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

Location	GENOTYPE 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

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

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.

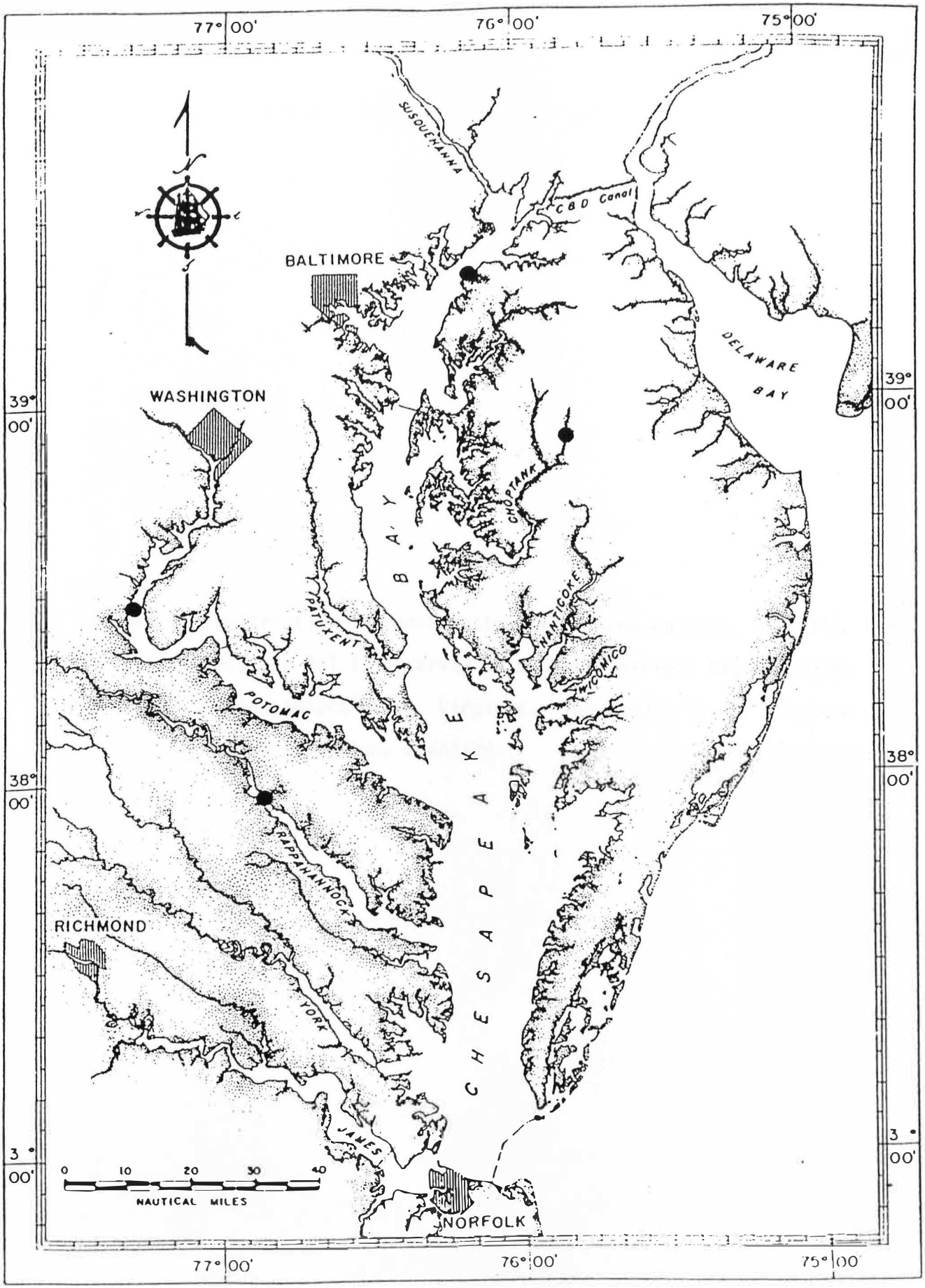
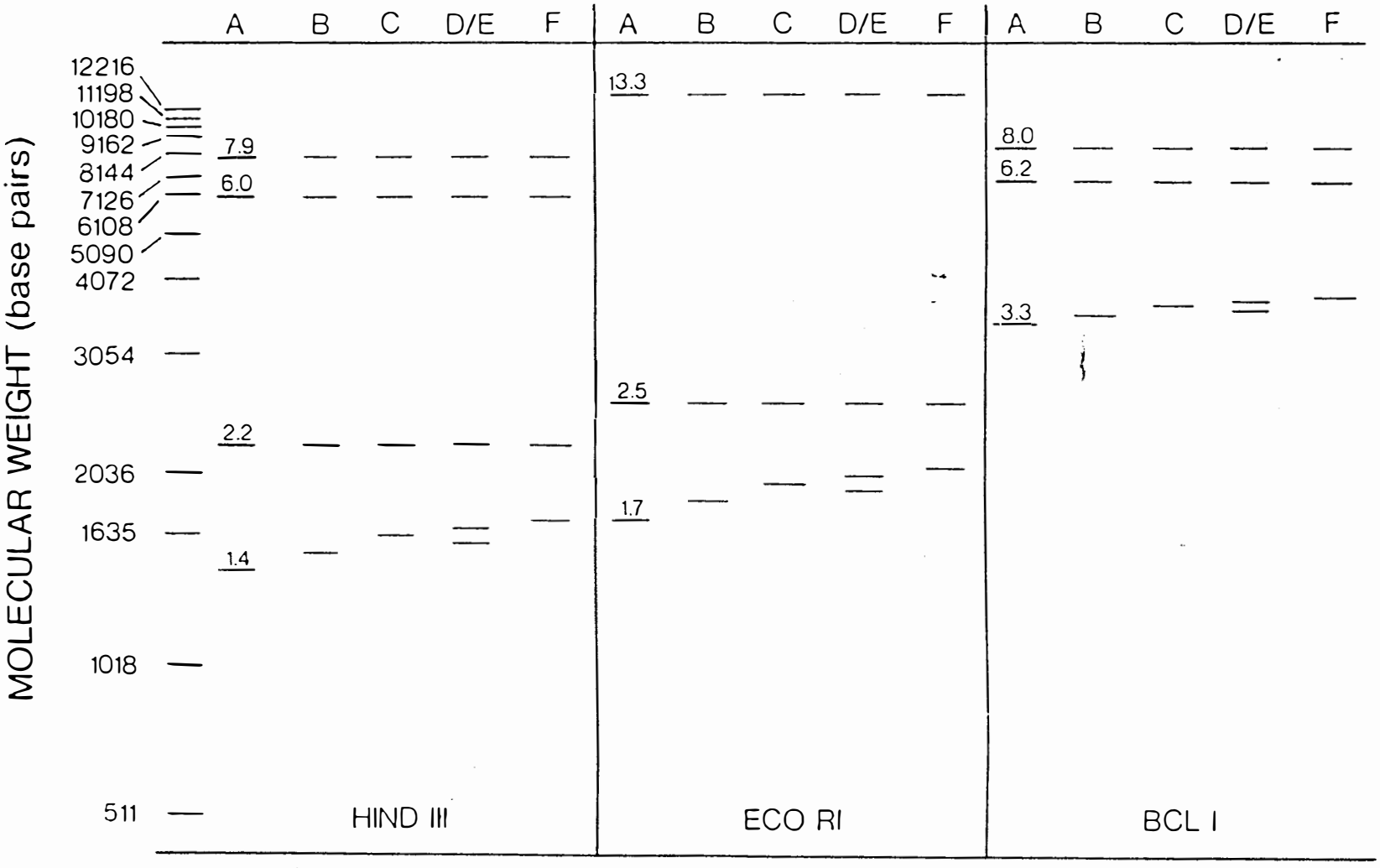
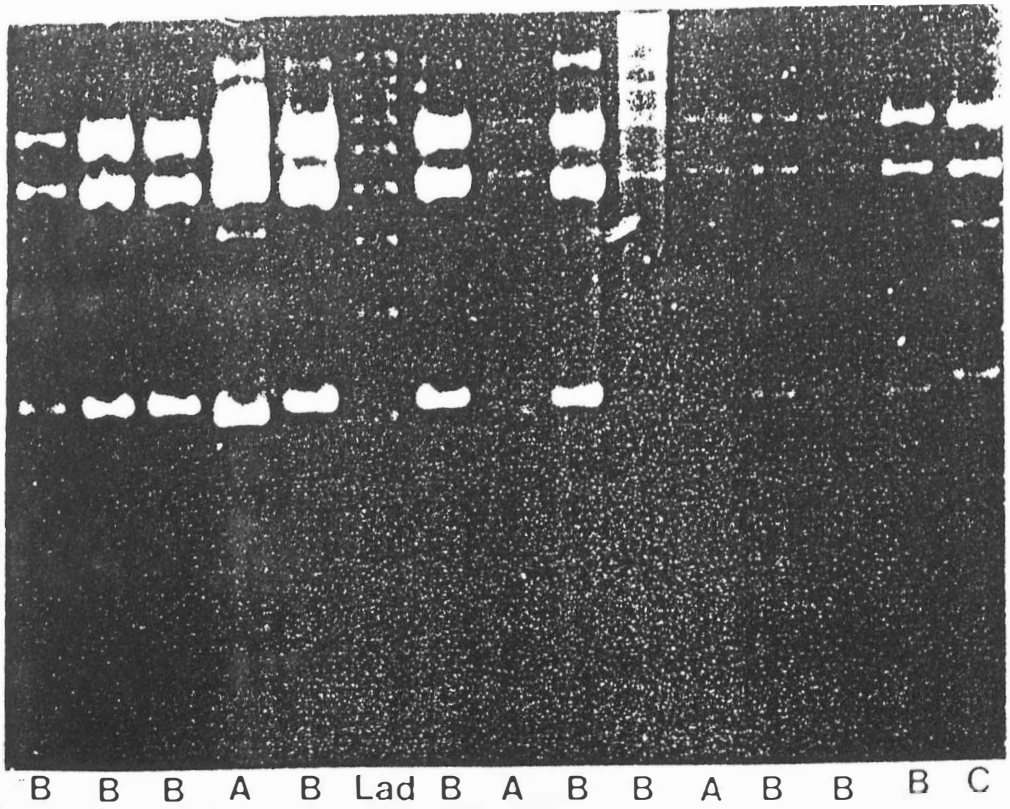

77° 00'
76° 00'
75° 00'
SUSQUEHANNA
C & D Canal
BALTIMORE
DELAWARE BAY
WASHINGTON
39° 00'
CHOPTANK
PATUXENT
NANTICOKE
WICOMICO
POTOMAC
38° 00'
RAPPAHANNOCK
RICHMOND
YORK
JAMES
CHESAPEAKE BAY
37° 00'
0 10 20 30 40
NAUTICAL MILES
NORFOLK

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

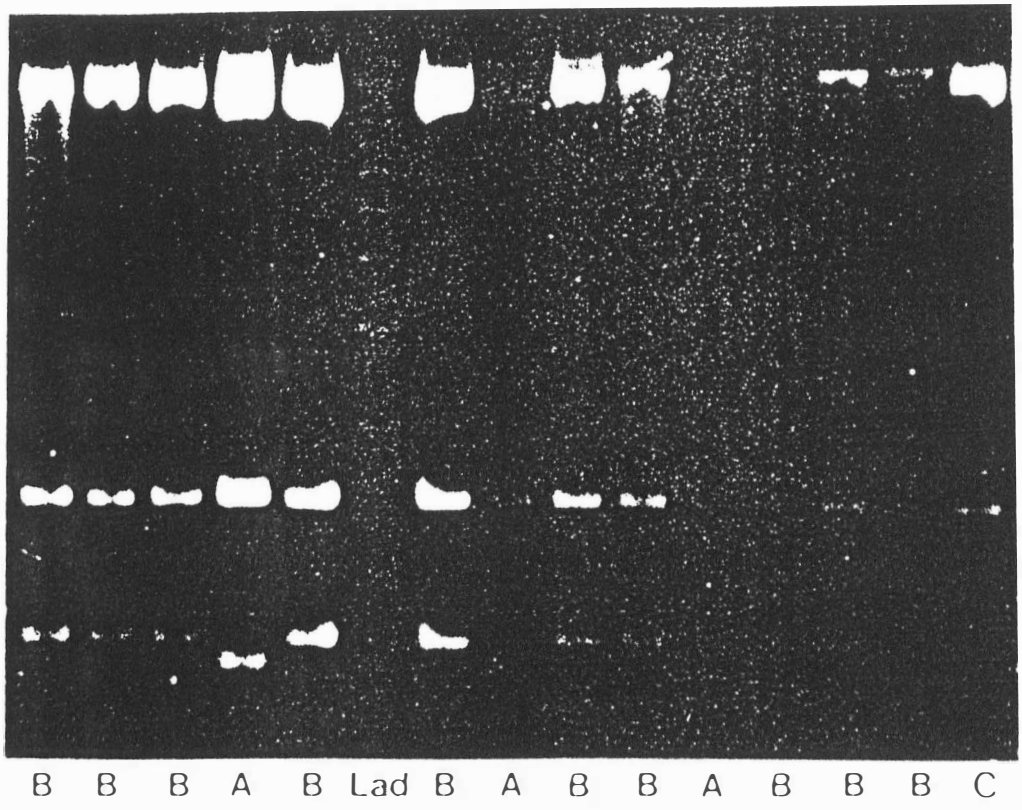
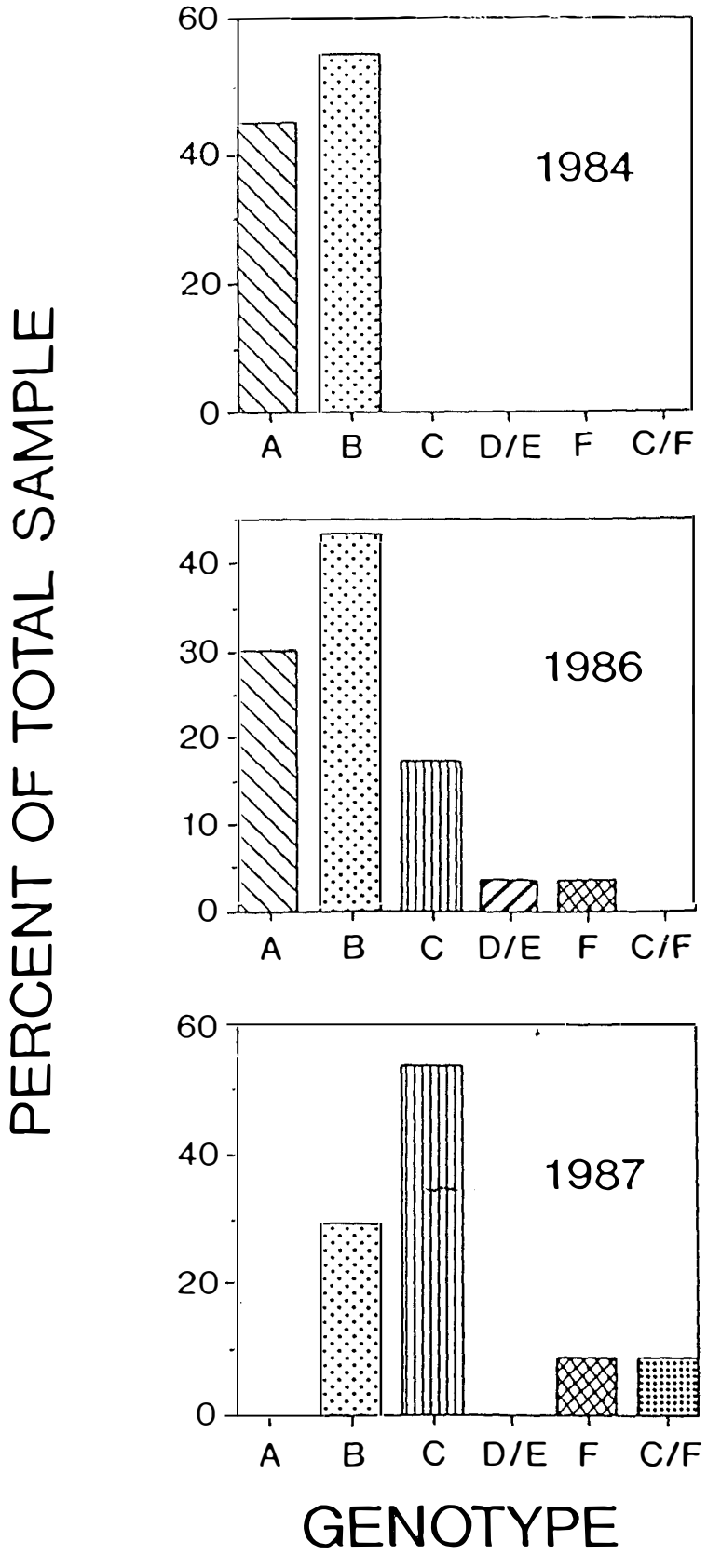

B B B A B Lad B A B B A B B B C

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.