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# Population Variation in the Mitochondrial-DNA of Two Marine Organisms: The Hard Shell Clam *Mercenaria spp.* and the Killifish, *Fundulus Heteroclitus*

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by

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## ABSTRACT

# POPULATION VARIATION IN THE MITOCHONDRIAL DNA OF TWO MARINE ORGANISMS: THE HARD SHELL CLAM, *MERCENARIA SPP.* AND THE KILLIFISH, *FUNDULUS HETEROCLITUS*

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Populations of two marine species were examined via restriction enzyme digestion of mitochondrial DNA (mtDNA) to determine levels and patterns of genetic variation . This technique is extremely sensitive to regional differentiation and gene flow between closely related organisms.

The first section of this dissertation focuses on the population genetics and systematics of the hard shell clam *Mercenaria*, an important aquaculture species. Fifteen clam populations were sampled along the east and Gulf coasts of the United States and assayed for mitochondrial genotype to determine the extent of geographic differentiation and thus evaluate the suitability of current hard shell clam breeding practices. Evidence based on mtDNA variation indicated that significant genetic differences do not exist between the more northerly populations of *M. mercenaria* sampled from New Jersey to North Carolina. These populations were characterized by

high probabilities of gene identity, I = 0.882 ± 0.060, and low percent nucleotide sequence divergence of their mtDNA, δ = 0.003 ± 0.002. The similarity was due to high levels of gene flow among these populations. The average effective migration rate between the northern populations was estimated to be $N_em$ = 3.6. Thus, stocks derived exclusively from northern populations should not be employed in "site-specific" breeding programs which employ crossing of clam stocks of varied geographic origin. All other populations sampled were significantly divergent as judged by $G_h$ tests for heterogeneity of mtDNA haplotype frequency and were characterized by intermediate levels of gene flow, genetic distance and sequence divergence consistent with subdivided populations of a species. *M. mercenaria* clams sampled from the Oregon Inlet, NC area exhibited mtDNA genotypes indicative of hybridization with *M. campechiensis*.

A phylogenetic analysis was conducted on the clam taxa *M. mercenaria, M. campechiensis* and *M. mercenaria texana* based on the information derived from mtDNA variation. This analysis detected similar degrees of divergence between all three taxa; δ = 0.053 ± 0.015 for *M. mercenaria* vs. *M. campechiensis*, δ = 0.044 ± 0.027 for *M. mercenaria* vs. *M. mercenaria texana*, and δ = 0.020 between *M. campechiensis* and *M. mercenaria texana*. The implication of this analysis is that considerable genetic divergence has occurred between these taxa which validates the use of "trait-specific" breeding approaches involving hybridization of the closely related species *M. mercenaria* and *M. campechiensis*. This analysis also indicated that the *texana* group may be of multiple maternal origin and in all probability deserves species distinction separate from *M. mercenaria*.

The results of the clam study are also of some note in that clam mtDNA is unique as compared to the majority of higher animals studied to date. It was found that clam populations demonstrate polymorphism in mtDNA size; the mtDNA molecule

ranged from 16.5 to 19.0 kilobases in length. In addition, individual clams were often found to be heteroplasmic; i.e., more than one type of mtDNA molecule was found in many individuals. Size heteroplasmy in clam populations ranged from 0 to 89 %. Many specimens were also heteroplasmic with respect to the nucleotide sequence of their mtDNAs; a condition called restriction site heteroplasmy. This condition occurred in 12 % of the individuals assayed in this study.

The second section of this study focuses on the short term evolutionary dynamics of the killifish, *Fundulus heteroclitus*, as indicated by mtDNA variation between partially isolated subpopulations. Considerable debate exists as to the primary forces maintaining observed clines in morphology and gene frequency in this species. Some hypotheses propose that limited gene flow is a primary factor while others implicate strong selection. An analysis of mtDNA variation in 480 individuals from five killifish subpopulations sampled on a very fine scale provided for the calculation of a realistic estimate of gene flow.

Effective migration rate, synonymous with gene flow, was calculated by two methods from mtDNA haplotype frequencies in the five subpopulations. Using the elementary relationship with $F_{st}$, $N_em$ was estimated to be 24.09. The corresponding value from a private alleles analysis was $N_em = 18.47$. These estimates indicate a very large potential for gene flow in *F. heteroclitus*; a finding not at all compatible with the hypothesis that restricted gene flow is an explanation for the maintenance of the clines. Therefore, substantial selection pressures must be invoked to account for the present-day clinal distributions of *F. heteroclitus*.

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# PREFACE

Advances in molecular biology and biochemistry have often had far reaching impacts in biological disciplines not immediately related to these fields. For example, the introduction of electrophoretic separation of allelic isozymes in the early 1960s resulted in an explosion of research and information concerning genetic variation in natural populations (Lewontin 1974). Thus, among the great accomplishments of the 1970s in population genetics was the accumulation of data related to longstanding disputes over the extent and meaning of alternative alleles at many genetic loci. Today, molecular biology has even been incorporated into such disciplines as Oceanography where it is employed in aquaculture, fisheries management, modeling, biogeography and taxonomy.

This dissertation focuses upon two major topics: the integration of molecular tools with applied aquacultural research and short term evolutionary dynamics. The first topic, discussed in Chapter 1 is devoted to hard clams, *Mercenaria spp.*, and to what one may learn about genetic differentiation between geographically disjunct but closely related populations. The second topic, covered in Chapter 2, focuses upon genetic differentiation among partially isolated populations of the fish *Fundulus heteroclitus*. The goal of Chapter 2 is to test hypotheses concerning genetic adaptation and the homogenizing effects of gene flow.

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# CHAPTER 1

Population variation in the mitochondrial DNA of the hard shell clam, *Mercenaria spp.*

# INTRODUCTION

Over the past 20 years, a great deal of research has focused on genetics of the hard shell clam, *Mercenaria spp.* During the last decade patterns of genetic variability in cultured clams have been widely studied via controlled matings and the electrophoretic detection of protein isozymes (see McHugh *et al.* 1982). However, a review of this research reveals considerable gaps in the knowledge of hard shell clam genetics. For example, while geographically distant populations of clams are assumed to be genetically distinct, there is no evidence to support this assumption (*cf.* Adamkewicz 1984a, 1984b, 1987; Dillon and Manzi 1987; Humphrey 1981).

The objective of this chapter is to elaborate upon the population genetics and systematics of the hard shell clam. It begins with a description of the current state of knowledge of clam biology as it relates to techniques for aquaculture. This is followed by a review of methods previously used to examine and quantify the genetic resources of *Mercenaria spp.* Finally, these methods are contrasted with a relatively new approach to the analysis of genetic variation in closely related organisms. This new approach analyzes populations for a different type of genetic variation--that exhibited by mitochondrial DNA.

## I. Clam biology and basic culture techniques

The hard shell clam inhabits marine and estuarine waters and is generally found in soft substrates such as sand or mud, where salinities range from 21-31 ppt, and where temperatures range from 18-30°C. *M. mercenaria* and *M. campechiensis* are most

commonly found in shallow intertidal areas with the exception that in the northern part of its range, *M. campechiensis* is restricted to deeper waters (roughly between low tide and 15 m). The lamellibranch gills of the hard shell clam serve as the primary organ for collection of food. Food particles such as small unicellular algae are retained on the gills and transported to the mouth by the combined ac n of mucous, cilia and cirri (Hyman 1967, Winter 1978). Assimilation efficiency in clams is approximately 75% (Tenore and Dunstan 1973) and clams exhibit relatively high values of growth efficiency as well (Tenore *et al.* 1973). These factors combined with man's ability to control the clam's life cycle are desireable attributes and contribute to successful clam aquaculture.

Hard shell clams are dioecious. Occasionally, protandry occurs and some sexually mature individuals undergo a permanent change in sex to female (Loosanoff 1939, Kennish 1980, Morton 1967). In the spring, and to a lesser extent again in the fall, water temperature and other cues (Nelson and Haskins 1949) trigger synchronous spawning. In the clam hatchery, broodstock are conditioned to spawn (Loosanoff and Davis 1950,1951). Mature individuals are stored in cool seawater (12°C) before the gonads have developed fully. Then at any time during the year they are removed and subjected to a gradual increase in water temperature up to 20°C accompanied by feeding. The elevated temperature and feeding are maintained for two to four weeks during which time the broodstock reach spawning condition (Manzi 1985). A typical female clam releases more than 20 million eggs per spawn and may spawn as often as once per week during spawning season (Davis and Chanley 1956, Ansell 1967). Typically, 100-200 individuals are mass spawned in a small trough over a period of several days with periodic replacement of spent animals with fresh, ripe stock.

At the time of spawning, eggs and sperm are shed into the water column where external fertilization occurs. Depending on water temperature, zygotes develop into free-swimming trochophores followed by straight-hinge veliger larvae (*ca.* 100 μm in length)

in one to two days (Manzi 1985). The duration of this pelagic veliger stage is three to four weeks in the wild (Carriker 1961) and under optimum food and temperature conditions is as little as one week in the hatchery (Manzi 1985).

On attaining a length of *ca.* 200 μm, the veliger larvae begin to metamorphose as pediveligers (having both a foot and a velum) and become epifaunal (Castagna and Kraeuter 1981). Very shortly thereafter, they complete metamorphosis and "set," i.e. they adopt a permanent sessile existence. These post-set juvenile clams continue to grow rapidly for the next two to four weeks (Manzi 1985). From a size of *ca.* 1 mm to 15 mm (anterior-posterior length) post-set clams are often called "seed." The minimum size at which clam seed are placed directly into field nurseries (the natural environment) is 10 mm (Castagna and Kraeuter 1977). Smaller seed cannot withstand pressures of predation unless predator exclusion measures such as those employed by Castagna and Kraeuter (1981) are undertaken. Alternative onshore nursery designs such as raceways (Hadley and Manzi 1984) and upflow systems (Bayes 1981, Manzi and Whetstone 1981) can accomodate even the smallest seed sizes.

Finally, seed larger than 10 mm are transfered to a grow-out site. Currently, the most cost effective method of grow-out entails the deployment of fiberglass trays filled with crushed stone and containing small seed planted at a density of at least $1000/m^2$ (M. Peirson, pers. comm.). In Virginia, the grow-out phase lasts 2-3 yr, in South Carolina 2 yr, and in Florida less than 2 yr, yielding marketable clams of 50 mm.

The techniques for clam culture as outlined above follow those developed by V. Loosanoff and colleagues in the 1950s for oysters and are detailed in several recent publications (*cf.*, Castagna and Kraeuter 1981, Claus 1981, Manzi 1985). The successes in clam culture research range from predator exclusion (Castagna and Kraeuter 1977; Kraeuter and Castagna 1980) and nutrition (Davis and Guillard 1958, Ukeles 1971) to the engineering and technical aspects of culture container design (Bayes 1981,

Lucas and Gerard 1981, Rodhouse *et al.* 1981). However, despite more than 30 years of research, cultured clam production in the U.S. remains latent. As outlined by Manzi (1985) the remaining constraints to clam culture can be categorized as environmental, imposed regulations, and lack of knowledge. The environmental type of constraint can usually be avoided by judicious choice of the site (location) of a clam culture venture. Regulations imposed by the government await review at the local, state and federal levels. Limitations in current knowledge indicate the need for further research into, among others, an area of critical importance: genetics (Bardach *et al.* 1972, Gall and Busach 1986, Manzi 1985, Newkirk 1983, Wilkins 1981).

## II. Studies of genetic variation in *Mercenaria*

### A. Loss of genetic variation in cultured populations:

It is well known that hatchery stocks of cultivated fish have less genetic variability than natural populations (Allendorf and Phelps 1980, Cross and King 1983, Ryman and Stahl 1980, also see Wilkins and Gosling 1983, Gall and Busack 1986). Stahl (1983), for example, determined through isozyme studies that his hatchery stocks of Atlantic salmon were at least 20% less heterozygous than corresponding natural populations.

The maintenance of sufficient levels of genetic variability in hatchery stocks in order to avoid inbreeding depression has also been a major concern in clam culture. For clams, loss of genetic variability in hatchery populations has been both demonstrated (Grassle 1976, Gosling 1981) and discounted (Dillon and Manzi 1987). In the recent study of Dillon and Manzi (1987) both wild and hatchery stocks were compared from Massachusetts and Virginia. They found no loss of heterozygosity (i.e. no inbreeding effect) in the hatchery populations although there was an indication of genetic drift.

Frequencies of some rare alleles had changed during the 4-5 generations during which the wild and hatchery populations were separated. This finding is not surprising when one considers the methods used to spawn clams.

B. Controlled mating and heterozygosity:

The suitability of certain clam strains as breeding stock has typically been researched via controlled matings and by electrophoretic detection of isozyme variants. The former approach requires extensive space and resources in order to conduct long-term breeding experiments. The latter approach, protein electrophoresis, is rapid and inexpensive deriving indirect information about the populations from gene products (proteins). Recent interest has centered on heterozygote deficiencies in natural populations and on the heterozygote advantage; that is--the observation that in natural populations, faster growing individuals are more heterozygous than slower growing members (Adamkewicz 1984a, 1984b; Gaffney and Scott 1984 and references therein). Clam culturists though, have found that a heterozygote advantage is not evident in the hatchery (Adamkewicz 1984b, Gaffney and Scott 1984). This observation holds true for other cultured bivalves as well (Beaumont *et al.* 1983, Foltz and Chatry 1986, Singh and Zouros 1978). In fact, a recent genetic analysis of native and inbred oyster populations (*Crassostrea virginica*) indicates that the faster growing hatchery animals are essentially monomorphic at 60% of the loci investigated (Paynter *et al.* 1988). Paynter's evidence gives support to the theory recently proposed by Adamkewicz (1984b) that selection fixes alleles which confer rapid growth in *M. mercenaria.*

The results from isozyme studies of *Mercenaria* to date can be summarized as follows:

1) a heterozygote advantage is often detected in natural populations of *Mercenaria* which does not occur in the hatchery (Adamkewicz 1984b, Manzi, pers. comm.),

2) although there is evidence of genetic drift in hatchery produced populations of clams, these populations do not exhibit inbreeding effects such as decreased heterozygosity (Dillon and Manzi 1987),

3) most studies report a deficiency of heterozygotes in natural populations of clams, indicating strong selection through differential viability of genotypes (Adamkewicz 1984b, Humphrey 1981, Mitton and Grant 1984, Singh and Green, 1984), and

4) no study has demonstrated regional differentiation among populations of *M. mercenaria* (Adamkewicz 1984a,b; Adamkewicz 1987; Dillon and Manzi 1987; Humphrey 1981; and others).

For many years, east coast clam culturists have included in their husbandry schemes the breeding of *M. mercenaria* stocks of varied geographic origin. This application of artificial selection for improved productivity was based on the empirically unsubstantiated belief that regional genetic differences existed between different clam stocks. Therefore, many recent studies of the hard shell clam have concentrated on the genetic characteristics of geographically diverse populations. However, no study of allozymes has given evidence of regional differentiation. Rather, electrophoretic isozyme analyses suggest that very little divergence has occurred between wild *M. mercenaria* populations along the east coast of the U.S. (Adamkewicz 1984a,b; Dillon and Manzi 1987; Humphrey 1981). Despite the lack of evidence supporting regional differentiation, independent clam culturists in several east coast locations continue to breed *M. mercenaria* stocks of varied geographic origin in an effort to produce a superior clam.

The genetic characteristics of populations from which cultured clam stocks have been derived have also been investigated. For most cultured species other than molluscs, geographically separated natural populations are known to exhibit pronounced genetic heterogeneity (Ryman 1983, Stahl 1983). For example, Ryman (1983) found both macro- and micro-geographic differences within the salmon. However, each study of isozyme variants in populations of *M. mercenaria* along the east coast has failed to demonstrate detectable divergence. Yet culturists continue to observe differences between clams from the northern and southern states. For example, Adamkewicz (1987) investigated the extent of genetic adaptation to local conditions in clam populations from Massachusetts, Virginia and South Carolina. Her selective breeding study showed a significant effect of parental origin on growth--clams with a greater southern genetic component grew best in all three locations.

III. Overview of *Mercenaria spp.* systematics

The systematics and morphology of the hard shell clam genus *Mercenaria* are outlined in Appendix A. Two species are recognized, the northern species, *M. mercenaria* (including the subspecies *M. mercenaria texana*) and the southern species, *M. campechiensis*. The two species are sympatric over a wide range of the Atlantic coast of the United States, *M. mercenaria* ranging from the Gulf of St. Lawrence to Florida and *M. campechiensis* from Virginia to Florida, Texas, Cuba and Mexico (Porter and Chestnut 1962; Menzel and Menzel 1965; Menzel 1968, 1970, 1971; Cummins 1966; Saila and Pratt 1975; Anderson *et al.* 1978). The most important phenotypic distinctions between the two, as far as the aquaculturist is concerned, are that 1) the northern species has excellent keeping qualities (up to two weeks on ice) while the southern species gapes and dehydrates after only several hours out of water (Menzel 1971) and 2) the growth rate of the southern clam exceeds that of *M. mercenaria* in their southern range by a

factor of approximately two (Menzel 1971). In the past, hybridization between the species has been widely used in clam culture to increase yields per unit time (Chestnut *et al.* 1957; Haven and Andrews 1957; Menzel 1963, 1964, 1966, 1971; Menzel *et al.* 1965, 1976). Such hybrids typically acquire the "good" qualities of each parent and are fertile (Haven and Andrews, 1957; Menzel, 1966, 1977); i.e., the fast growth of *M. campechiensis* and good keeping qualities of *M. mercenaria.*

Early studies found little or no morphological or physiological difference between the northern and southern clams (Loosanoff 1959, Loosanoff *et al.* 1966, Greenberg 1966, Hillman 1968, Menzel 1968, Merrill and Tubiash 1970, Tiffany 1972, Hinegardner 1974). However, Hopkins (1934) noted significant differences in oxygen consumption, Manwell (1963) found differences in hemoglobin content of their muscles, and Menzel (1963, 1964, and 1966) described the previously stated differences in growth and keeping quality.

It has been suggested that a significant degree of hybridization between *M. mercenaria* and *M. campechiensis* occurs in zones of sympatry such as the Indian River (Ft. Pierce) area of Florida (Menzel *et al.* 1965, Menzel 1968, Pesch 1974, Anderson *et al.* 1974, Dillon and Manzi, submitted). In addition, the variant *M. mercenaria texana*, found in the Gulf of Mexico, has been proposed by Menzel (1970) to be a natural hybrid. This conclusion was based on similarities between *M. mercenaria texana* and laboratory reared hybrids of *M. mercenaria* and *M. campechiensis.* Given the breeding practices described above it is important that culturists be able to distinguish among species from the field.

More recent studies have explored the possibility that both forms may in fact not be separate species. Pesch (1974) detected low levels of allelic protein variation between *M. campechiensis* and *M. mercenaria* and suggested that the two species have not yet achieved reproductive isolation based on the following:

1. the nuclear gene pools share many common alleles,

2. nuclear chromosomes are homologous,

3. successful hybridization in the lab and in nature, and

4. intergrades (naturally occurring hybrids) are numerous in zones of sympatry.

Humphrey (1981) studied allozyme variation and shell dimensions in the genus *Mercenaria* and found distinct differences between *M. mercenaria* and *M. campechiensis* at 4 enzyme loci. However, shell morphology was not an accurate delimiting characteristic between the two species. This is not surprising since shell morphology also depends on environmental characteristics; in particular, physical parameters such as temperature and salinity, nutrient conditions (e.g., dietary calcium) and edaphic conditions (substrate composition). Dillon and Manzi (submitted) discuss the environmental plasticity of clam shell morphology and derived a principal components analysis which employs several shell measurements to identify the two species with *ca.* 90% confidence.

These results notwithstanding, there continues to exist controversy over the species status of the two species primarily due to the following facts. First, the morphological characteristics used to distinguish the two species are continuous, contributing to variability and inappropriateness of shell morphology as a diagnostic character. Second, interspecific mating is thought to be widespread in known zones of sympatry. For example, populations near the Ft. Pierce, FL area have been estimated by Dillon and Manzi (submitted) to be comprised of as much as 87.5% hybrid individuals. Third, $F_1$ and $F_2$ cohorts and offspring of backcrosses are fertile in hybridization experiments (Menzel 1977). Lastly, the variant *M. mercenaria texana* has been proposed to be a natural hybrid (Menzel 1970). Given the overlap in distribution, a life history conducive to extensive gene flow and the genetic information detailed above for *M. mercenaria* and *M. campechiensis,* it is likely that contact zones between the two species

are numerous along the east coast and Florida. These zones have been shown to be important indicators of selection, gene flow and other processes associated with speciation (see Chapter 2). Therefore, several such zones were sampled in this study along the east coast and Florida as well as two populations of the Texas subspecies *M. mercenaria texana.*

IV. Analysis of mitochondrial DNA variation as a possible approach to resolving issues of genetic variation in hard shell clam populations

A. Genetic variation in the DNA of mitochondria:

Since protein and breeding studies have not detected a heritable genetic component for the growth and survival differences observed in clam stocks of varied geographic origin, there is a need to find some reliable measure of genetic variation. Fortunately, a tool now exists which has been shown to be extremely sensitive to regional differentiation and gene flow. The tool is restriction enzyme analysis of the mitochondrial genome. Information derived from mitochondrial DNA (mtDNA) is of a more direct nature than that derived from isozyme analysis and is not influenced by environmental factors as can be shell morphology. There are many other characteristics which contribute to the value and utility of mtDNA analyses in examining population structure. The following are listed by Avise *et al.* (1979b, 1984) and by Chapman *et al.* (1982):

1. patterns of mtDNA inheritance (essentially haploid) are independent of nuclear gene dynamics,
2. mitochondrial restriction genotypes (haplotypes) are unique and maternally inherited,

3. mitochondrial restriction genotypes are transmitted intact; since mitochondrial genomes of higher animals are not known to undergo recombination, the only cause of sequence change is mutation,
4. fixed mutations result in a new and recognizeable genotype, and
5. mtDNA mutates rapidly as compared to nuclear DNA; therefore, the rate of appearance of new mtDNA haplotypes is rapid.

With these points in mind, restriction enzyme analysis of clam mtDNA was used to investigate whether there is a genetic component to the geographic variation observed in clams. In order to better understand the significance of the results to be presented in both Chapters 1 and 2, the following outline of mtDNA characteristics is presented.

B. Overview of mtDNA analysis:

The mtDNA of metazoan animals is a closed circular molecule and is generally uniform in size, shape and gene arrangement. In addition, the genetic codes of mtDNAs are extremely variable--differing not only from nuclear DNA (nDNA), chloroplast DNA and that of unicellular organisms, but even from each other (Wallace 1982). According to the review by Wallace, mtDNAs of multicellular organisms are circles approximately 5 μm in length. This length corresponds to 16-17 kb (kilobase pairs). Wallace also notes that gene organization is usually conserved in multicellular animals including fruit flies, mice, birds, rats, frogs, cows and humans. Finally, mtDNA exhibits "economy of gene organization" (Borst and Grivell 1981). Unlike nDNA, there are few or no non-coding bases between gene sequences and transcriptional regulation is primitive due to the lack of leader, trailing, and stop regions. Each of these characteristics contributes to the economical nature of mtDNA.

MtDNA is cytoplasmically inherited; i.e., the genetic material within an individual's mitochondria is ultimately maternally inherited from the mitochondria

contained within the egg cytoplasm (Lansman *et al.* 1981, 1983a, 1983b; Powell and Zuniga 1983). In natural populations, offspring have not been shown to receive any paternal mitochondria and "paternal leakage" of mtDNA from generation to generation has been detected in only one instance (Satta *et al.* 1988). Paternal transmission of mtDNA is assumed to occur only at extremely low levels if at all (see Avise *et al.* 1987, Avise and Vrijenhoek 1987, Moritz *et al.* 1987, for reviews). MtDNA is therefore considered a very useful molecule for tracing maternal lineages.

Most species studied to date are homoplasmic with respect to mitochondrial genotype; i.e., every cell in an individual has mtDNA molecules identical to every other cell in that individual. However, as the number of studies of mtDNA variation increases, examples of heteroplasmy (differences among the mtDNA molecules within an individual) have become more frequent (Bentzen *et al.* 1988; Bermingham *et al.* 1986; Chapman 1987, 1989; Densmore *et al.* 1985; Hale and Singh 1986; Harrison *et al.* 1987; Moritz and Brown 1986; Moritz *et al* 1987; Mulligan and Chapman, in press; Sederoff 1984; Snyder *et al.*,1987). Organisms can be heteroplasmic in two ways. The most frequently observed form of heteroplasmy is due to variation in size of the mtDNA molecule. It is important to distinguish between size variation *from one individual to another* in a population which is termed "size polymorphism" and such variation *within an individual* which is termed "heteroplasmy." The second type of heteroplasmy occurs when a single individual possesses mtDNAs that differ in number or location of restriction sites.

Through the DNA thermostability analyses and restriction endonuclease studies of W. Brown and co-workers in the 1970s it was determined that mtDNA evolves (mutates) at a rapid rate relative to the rate of nuclear DNA mutation. Pairwise comparisons of nDNA of related species showed little nuclear sequence divergence while mtDNA sequences had diverged extensively (Brown *et al.* 1979). In addition, the same

group estimated the nuclear sequence difference between the nDNA of two monkey species to be 2% while their mtDNA exhibited 21% sequence divergence. Such a rapid change in mtDNA nucleotide sequence leads to heterogeneity between populations and species while differences often remain undetectable morphologically and in nDNA sequences.

One of the most widely used techniques for studying mtDNA sequence divergence is the qualification and quantification of fragments generated via restriction endonuclease digestion. These enzymes are isolated and purified from a variety of micro-organisms such as *Escherichia coli, Haemophilus influenzae,* and *Staphylococcus aureus* and recognize specific tetra-, penta- and hexanucleotide sequences in the double-stranded mtDNA molecule. They cleave both strands at a particular location in or near the recognized sequence of nucleotides. For example, the recognition and cleavage sites of the restriction enzyme *Hind* III could be depicted as follows:

5' A*AGCT T 3'

3' T TCGA*A 5'

where AAGCTT is the recognition sequence and "*" indicates the cleavage site. The name of each endonuclease (e.g., *Hind* III) has 2-3 parts, in accordance with the nomenclature system proposed by Smith and Nathans (1973). The first three letters are italicized with the first letter representing the genus and the next two letters designating the species of the micro-organism from which the enzyme is isolated. The fourth letter, if present indicates the strain in the producing organism and the Roman numeral indicates the restriction system. Thus, the name *Hind* III represents a type III restriction endonuclease isolated and purified from *H. influenzae*, strain Rd.

Restriction enzyme studies are of great utility because they allow quantification of genome heterozygosity free from many of the drawbacks associated with isozyme studies. For example, one reason that isozyme data (protein data) are less sensitive to

regional differentiation is that data are derived solely from the products of coding sequences while abundant heterozygosity occurs in non-coding sequences as well (Cooper and Schmidtke 1984). Restriction enzyme analysis of mtDNA, on the other hand, allows the detection of restriction sites which arise from single base pair (bp) changes or from additions, deletions, and rearrangements of nucleotide sequence anywhere in the mtDNA molecule. Such changes occur in both coding and non-coding sequences of DNA, albeit at unequal frequencies (Aquadro *et al.* 1984). Populations of organisms having a low percent difference in nucleotide sequence have many restriction sites in common and have similar restriction fragment patterns. Conversely, populations which have undergone more complete speciation will have fewer restriction sites in common because recognition sites will have been altered by one or more of the following:

a. removal or creation of new sites via nucleotide substitution,

b. movement of site positions via nucleotide sequence rearrangement (resulting in changed fragment length), and

c. biochemical modification of a site (e.g., methylation of DNA).

Data amassed to date indicate that most mtDNA genotypes are selectively neutral characters (Avise 1986, Birky *et al.* 1983,Brown 1983, Takahata 1983, Takahata and Slatkin 1983), i.e. they have no known selective value nor do they result in altered phenotypes. For example, restriction site changes which result from fixed mutations in silent positions of coding regions of the mitochondrial genome do not usually change amino acid sequence and thus have no adaptive significance since they have no measureable phenotypic effect. Most mutations in the D-loop have no known phenotypic effects as well. However, the assumption of selective neutrality of all restriction fragment patterns is the subject of considerable debate (*cf.* Aquadro *et al.* 1984, Avise *et al.* 1987, Clark 1985, Moritz *et al.* 1987). Without recombination, mutations in mtDNA

which lead to disfunction will be linked to any neutral mutations on the same molecule. If the disfunction leads to an altered probability of reproductive success then the entire mitochondrial genotype, including both selective and neutral variants, experiences selection.

Restriction analysis of mtDNA as described here is a uniquely valuable method for studying phylogenetic relationships at the molecular level. In addition, mtDNA analysis, although prone to certain other biases (*cf.*, Chapman 1982), is not normally subject to many of the limitations of nDNA studies (i.e., recombination, inversion, and transposition of nucleotide sequences; Avise *et al.* 1979b). Another fundamental difference between the two genetic systems is that restriction analysis of mtDNA allows for detection of polymorphisms in both coding and non-coding sequences of DNA. Finally, due to its maternal inheritance and molecular dynamics, the effective number of genes is lower and the rate of gene fixation by drift is higher for mitochondrial vs. nuclear gene systems (Birky *et al.* 1983, Chapman *et al.* 1982, Takahata and Slatkin 1984). In other words, mtDNA analysis could potentially provide a more sensitive estimate of genetic variation within and between geographically disjunct populations of clams.

## V. Summary

The available evidence indicates that analysis of mitochondrial DNA by restriction enzymes can refine and enhance the information currently available for hard shell clams. In the past, analysis of mtDNA has proven to be quite sensitive to regional differentiation over short time periods. This is partially due to the fact that mtDNA mutates rapidly relative to nDNA such that the appearance of new mtDNA genotypes is rapid compared to nuclear genotypes. Indications are that mtDNA analysis can resolve genetic differences between clam populations, if they exist, where isozyme analysis could not.

Therefore, hard shell clam populations were assayed for mitochondrial genotype in order to determine the extent of geographic differentiation and thus evaluate the suitability of current hard shell clam breeding practices.

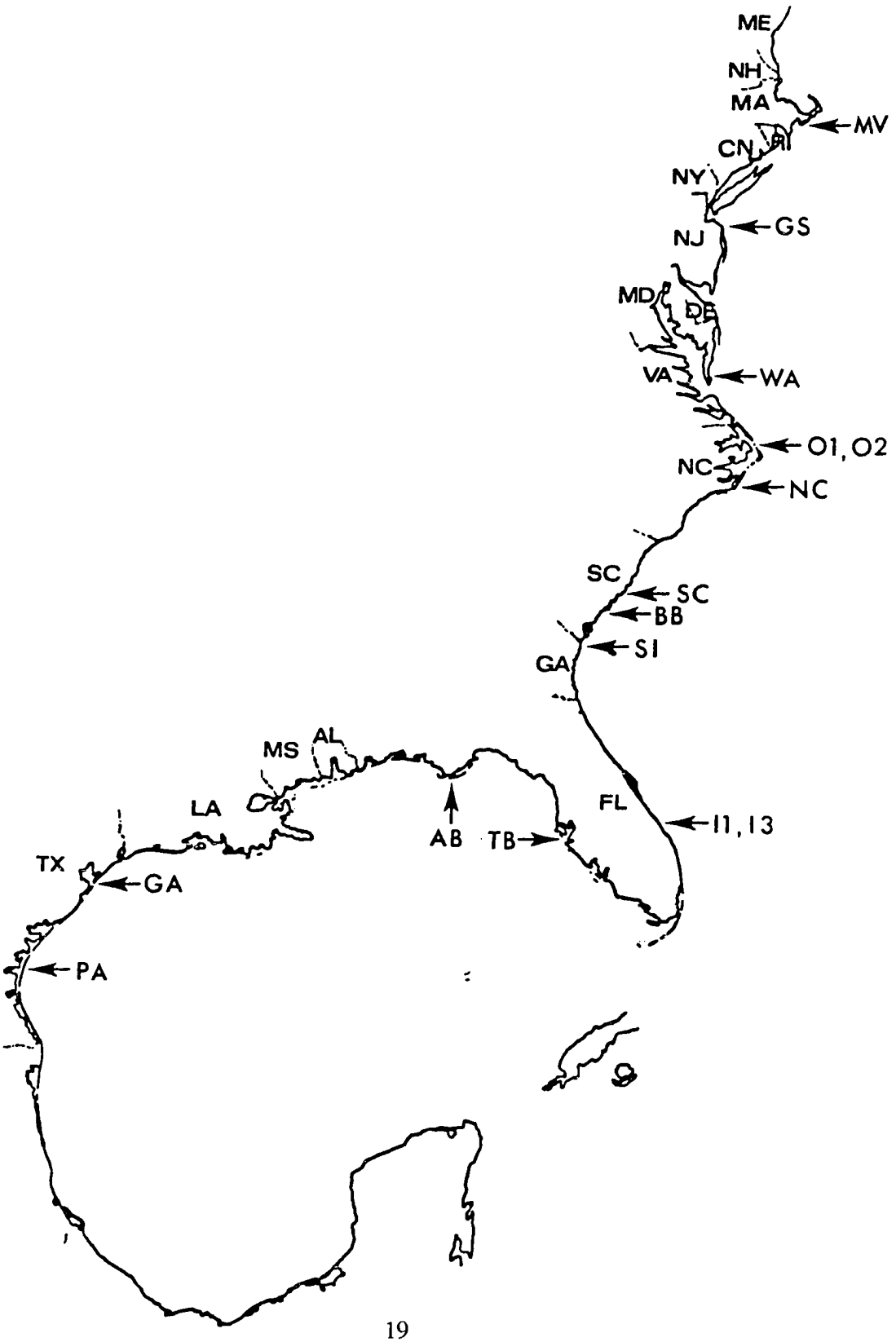
# METHODS

## I. Collection of Specimens

Fifteen hard shell clam samples were taken from 13 geographically distant locales and were considered to be natural clam populations. These locations, shown in Figure 1, are: Martha's Vinyard, MA (MV); Great Sound, NJ (GS); Hog Island--Wachapreague, VA (WA); Oregon Inlet, NC (two samples, O1 and O2); Beaufort, NC (NC); Folly River, SC (SC); Bull's Bay, SC (BB); Skidaway Island--Wassaw Sound, GA (SI); Indian River--Ft. Pierce, FL (two samples, I1 and I3); Tampa Bay, FL (TB); Appalachicola Bay, FL (AB); Galveston, TX (GA); and Port Aransas, TX (PA). The 11 east coast populations constitute specimens of *M. mercenaria* or, in some cases, hybrids between *M. mercenaria* and *M. campechiensis*. Duplicate samples (O2 and I3) were due to resampling from Oregon Inlet, NC and Indian River, FL to examine the potential occurrence of hybrid clam populations in those areas. The Tampa Bay, FL (TB) and Appalachicola Bay, FL (AB) populations are known to consist entirely of *M. campechiensis* (Humphrey 1981). Clams sampled from Galveston and Port Aransas, TX (GA and PA, respectively) were identified as *M. mercenaria texana* by a local expert based on sample location and on shell morphology.

All populations were analyzed by the G-test described below. However, due to incomplete data for several populations, only the following nine populations were included in the remaining analyses: GS, WA, NC, O1, O2, I1, I3, AB and PA. Attempts were made to collect at least 25 individuals from each population. This sample size was considered appropriate because individuals from the same sampling locale were

Figure 1. Sampling locales of *Mercenaria spp.* populations along the east and Gulf coasts of the United States. The location where a clam population was sampled is indicated by an arrow followed by the abbreviation given to that sample. The abbreviations are MV: Martha's Vineyard, MA; GS: Great Sound, NJ; WA: Wachapreague, VA at Hog Island; O1: first sample from Oregon Inlet, NC; O2: second sample from Oregon Inlet, NC; NC: Beaufort, NC; SC: Folly River, SC; BB: Bull's Bay, SC; SI: Skidaway Island, GA at Wassaw Sound; I1: Indian River, FL near Ft. Pierce; I3: Indian River, FL near Ft. Pierce; TB: Tampa Bay, FL; AB: Appalachicola Bay, FL; GA: Galveston, TX; and PA: Port Aransas, TX. The seven east coast populations are *M. mercenaria* or in the case of Oregon Inlet and Indian River may be hybrids between *M. mercenaria* and *M. campechiensis*. The TB and AB populations are *M. campechiensis*. The GA and PA populations are *M. mercenaria texana*.



expected to exhibit homogeneity of mtDNA genotype due to its maternal inheritance. Clams were either carried or shipped live to the laboratory where they were sacrificed and the hepatopancreas (*ca.* 1 g wet weight) was excised for immediate extraction and purification of mtDNA.

II. Extraction and purification of mtDNA

Recently, investigators have found that for many invertebrates, and molluscs in particular, mucopolysaccharides copurify with mtDNA. If these contaminants are not removed prior to lysis of the mitochondria then the mtDNA obtained will be encapsulated by a refractory sheath which prevents restriction enzyme digestion. This problem was first noted in the gastropod *Cepaea nemoralis* and resolved by Stine (1986) using a derivative of the rapid mtDNA isolation procedure set forth by Chapman and Powers (1984). Stine's technique was modified as outlined below for use on clams and yielded sufficient quantities of pure mtDNA to do at least six restriction enzyme digests per specimen.

The ingredients of all buffers and solutions referred to in this text are listed in Appendix B. Clam hepatopancreas was diced and homogenized in five volumes of cold STEK buffer containing 140 µg/ml ethidium bromide. Homogenization is a critical step because over-homogenation leads to broken mitochondria and loss of mtDNA. When care was taken not to overhomogenize the tissue, either of the following two techniques yielded intact mitochondria. If using a Dounce-type homogener, two strokes of the B pestel were performed followed by one stroke with the A pestel. This is the homogenization procedure recommended by Stine (1986). When using a Tekmar UltraTurak motor-driven homogenizer the tissue was homogenized at 40% power for 10 sec.

The isolation techniques which follow have been previously outlined by Brown and Wolfinbarger (1987) but are restated here for clarity. The homogenate was underlain by five volumes of cold STEP buffer and centrifuged for 40 min at 4°C and 13000 x g. The resulting mucopolysaccharide interphase and supernatant were aspirated off and discarded taking care not to disturb the loose mitochondrial layer atop the tissue pellet. The mitochondria/tissue pellet was resuspended in fresh STEK, underlain with STEP and centrifugation repeated. The supernatant was discarded as before and the sucrose gradient repeated until mucopolysaccharide at the interphase was nearly undetectable (usually three passes). After the final sucrose gradient spin, the resulting tissue pellet was resuspended in 300 µl cold STEK followed by the addition of 300 µl of 5% Nonidet P-40 in STEK (final NP-40 concentration 2.5%) to lyse all except nuclear membranes. Unlysed material and debris were pelleted by spinning the lysate for 10 min at 13000 x g and 4°C. The supernatant was then poured into a microfuge tube and the pellet discarded. This supernatant was extracted twice with phenol to denature and remove proteins as follows.

Approximately 300 µl of water-saturated redistilled phenol were added to the lysate supernatant. This was vortexed briefly to mix and spun for 10 min at 4°C in a microfuge to separate the aqueous and organic phases. The upper aqueous phase, containing mtDNA and some additional protein, was removed to a new tube leaving behind the white protein layer and the colored phenol phase below. Again 300 µl phenol were added to the aqueous phase. The sample was vortexed and spun as before and the upper aqueous phase retained again. Next, 300 µl of chloroform:isoamyl alcohol (24:1) were added and the tube was vortexed briefly to mix. This was spun for five minutes at 4°C to separate aqueous and organic phases. The top aqueous phase was transferred to a new microfuge tube and two volumes of 95% ethanol were added. The tube was capped, gently mixed, and stored either at -80°C for 20 min or at -20°C for two hours to

precipitate the mtDNA. The solution was spun in a microfuge to pellet the DNA after which the ethanol was poured off. The tube itself was then inverted and DNA allowed to dry completely at 37°C. MtDNA was then rehydrated in 100 µl of sterile distilled water and stored at -20°C until digestion.

III. Characterization of mtDNA

The mtDNA of each individual clam was characterized by digesting it with the restriction enzymes *Bam*H I, *Eco*R I, *Eco*R V, *Hind* III, *Pst* I and *Pvu* II as listed in Appendix C. However, data from *Hind* III are incomplete for several of the populations (see Appendix D) and were therefore included only in the estimate of clam mtDNA molecular weight. The digestions were performed in microtiter plates with conical-bottomed wells. Each digestion contained 16.5 µl mtDNA sample, 2 µl 10 X core buffer (provided by the manufacturer), 0.5 µl of 4 mM spermine and 1 µl (10 Units) of restriction enzyme. Reaction mixtures were incubated at the recommended temperature (usually 37°C) for 3-4 hr. Reactions were terminated by the addition of 2 µl of STOP buffer to each digest. This final mixture was loaded into a 0.9% Ultra Pure Agarose (Bio-Rad, Inc.) gel in TEB along side a molecular weight standard and electrophoresed overnight at 35 V to separate the mtDNA fragments generated by the restriction enzyme digestion.

The mtDNA fragment patterns for each restriction enzyme for each individual were visualized as described by Chapman and Powers (1984) and Chapman and Brown (1989). Gels were stained in a solution of 0.5 µg/ml ethidium bromide (a toxic mutagenic compound) in TEB for 10 min then destained in deionized water for 10 min. The gels were then placed on a custom UV light transilluminator (Chapman and Powers 1984) which is focused on wavelengths that cause maximum fluorescence of the ethidium/DNA complexes (i.e., the mtDNA bands in the stained gel). The gels were

then photographed with Polaroid Type 55 film and a Kodak 23A filter at f-stop 4.8 for 15-40 min depending on the intensity of fluorescence. This method of UV visualization detects as little as 60 pg DNA per band (Chapman and Brown, 1989) thereby circumventing the need for using radioactivity to visualize restriction fragment patterns in this species.

From the photographs (or their negatives), fragment patterns were recorded for each enzyme and the molecular weights of unique fragments were estimated as compared to the molecular weight standard. MtDNA restriction fragment patterns were designated by upper case alphabetic symbols. The most common pattern for each enzyme was assigned the letter "A" and subsequent patterns were assigned the letters B, C, D, *etc.* in the order in which they were detected.

Individuals were assigned haplotypes (composite genotypes) which consisted of a list of the letters designating the fragment patterns produced by digestion with each enzyme from Appendix D. The order of enzymes in the haplotype is the same as in the Appendix: *Bam*H I, *Eco*R I, *Eco*R V, *Pst* I and *Pvu* II. For example, the haplotype CAAAA is a composite consisting of the "C" restriction fragment pattern for *Bam*H I and the "A" pattern for the other four enzymes. Individuals heteroplasmic with respect to mtDNA size were assigned a normal haplotype but were also designated as "size heteroplasmic." Individuals heteroplasmic with respect to restriction site have haplotypes which include all of the fragment patterns observed. For example, the six individuals with haplotype BA/DAAA exhibited fragment pattern "B" for *Bam*H I, both patterns "A" and "D" for *Eco*R I, and the common fragment pattern "A" for the remaining enzymes. Individual clams were also classified according to the predominant size (molecular weight) of their mtDNA.

IV. Statistical analysis of mtDNA haplotypes

The basic calculations were performed on a personal computer with 640K memory. Additional support was required to run J. Felsenstein's Bootstrap Analysis (an 80386 personal computer) and the canonical discriminant analysis (SAS on an IBM 4381 mainframe).

Genetic relationships were first examined by the calculation of a G-test statistic. This statistic, denoted $G_h$, was employed to detect heterogeneity of haplotype frequencies among populations. $G_h$ is distributed approximately as Chi-square and has several advantages as outlined by Sokal and Rolf (1969). Among these are ease of calculation and the property of additivity such that the overall G-test can be successively decomposed between populations until no further heterogeneity is detected. In addition, since this test could be conducted enzyme-by-enzyme, information was obtained for all populations--even those for which data were incomplete for some enzymes. There were no *a priori* "expected frequencies" in the $G_h$ analysis. Rather, the null hypothesis for this test, homogeneity of haplotype frequencies among samples, was intrinsic to the data.

A preliminary $G_h$ analysis was conducted on the distribution of restriction fragment patterns in all 15 samples. Next, $G_h$ tests were conducted on the distribution of haplotypes in the nine samples with complete five-enzyme haplotypes. The overall $G_h$ test was begun in both analyses by treating all sampled populations as a single panmictic assemblage. Testing for heterogeneity of haplotype frequency was continued on successively smaller sets of populations until haplotype frequencies within sets were deemed homogenous as determined by comparing $G_h$ to the critical value of Chi-square with degrees freedom equal to "(rows-1)(columns-1)." The formula used to calculate $G_h$, following Sokal and Rohlf (1969), was:

$G_h$ = 2 [ (sum of $f \ln f$ for each restriction pattern (or haplotype) in each population)

- (sum of $f \ln f$ of both column and row totals)

+ ($n \ln n$ of the total number of individuals included in the comparison) ]

where $f$ = the number of individuals with a particular restriction fragment pattern (or haplotype) and $n$ = the total number of individuals in a sample.

Variation in mtDNA was also examined within and between populations by a canonical discriminant analysis performed using the SAS CANDISC procedure. Variables for this analysis consisted of the presence or absence of heteroplasmy and each restriction fragment pattern, along with the predominant mtDNA size of each individual. This analysis provided a visual presentation of the trends in variation by the construction of 95 % confidence elipses for each population on axes representing discriminant functions. These discriminant functions were related to the subset of variables which best revealed differences between populations. The discriminant axes were labeled only with names of variables which explained a large portion of the variance and covariance and which were shown to be statistically significant ($\alpha = 0.01$) by a multivariate analysis of variance. The order of variable names on an axis represents the relative strength of the variables in accounting for variance between sites, while the arrows indicate the direction of trends.

Pairwise genetic distances were calculated between each population by the method of Takahata and Palumbi (1985). This method of genetic distance calculation takes into account the degree of similarity between restriction fragment patterns resulting from shared fragments. In essence, Takahata and Palumbi's method requires calculation of two identity probabilities from the presence and absence of restriction fragments. An identity probability is the probability that an allele sampled twice in one population, or once in each of two populations, is identical by descent; that is, is derived from a

common ancestor. In this study an "allele" was defined as a restriction enzyme recognition sequence on the mtDNA which produced a particular restriction fragment. The probability of gene identity within each partially isolated population, I, is a measure of genetic diversity which was calculated by Takahata and Palumbi's (1985) equation (17) for each population while J, the probability of gene identity between partially isolated populations, is a measure of interpopulation differentiation and was calculated pairwise using their equation (19). At equilibrium these quantities are intimately dependent upon effective population size ($N_e$), the mutation rate, and the rate of migration between the populations. The theoretical ramifications of J are that a decrease in migration rate is reflected as a decrease in J. The actual estimate of genetic distance, D, then followed by the elementary equation

$$D = -\ln (J/I)$$

These distance values were subjected to UPGMA cluster analysis (Sneath and Sokal 1973).

Values for percent sequence divergence, $\delta$, were also calculated pairwise between all populations. Since it was known *a priori* that the assumption of negligible intrapopulation variation was violated, Nei and Li's (1979) equation (25) was employed which corrects the metric by subtracting intrapopulation variation from total interpopulation variation. Simply put, $\delta$ quantifies the degree of genetic divergence between the DNA of two populations in terms of the proportion of shared restriction sites.

The relative degree of gene flow between the seven east coast *M. mercenaria* populations was investigated by calculating the effective migration rate, $N_em$ (see Chapter 2 of this dissertation for extensive discussion on the methods of calculation and use of effective migration rate as an estimate of gene flow). Effective migration rate was derived from the elementary relationship

$$N_em = (F_{st}^{-1} - 1) / 2$$

as outlined by Takahata and Maruyama (1981) and by Birky *et al.* (1983). An overall estimate of $N_em$ was calculated for the entire east coast *M. mercenaria* sample and individual $N_em$ values were derived pairwise between each population.

V. Phylogenetic analysis using mtDNA haplotypes

Systematic implications of the data were examined by "bootstrapping" with the BOOT program of the PHYLIP package (Joseph Felsenstein, Version 3.1, 1988) and by analysis with the PAUP program (Illinois Natural History Survey, Version 2.4.1, 1980). Both programs were input with the presence/absence of fragments for each of the mtDNA haplotypes. Bootstrapping runs were lengthy (*ca.* 14 hr run time on an 80386 personal computer) even though the program was altered by lowering the constant "maxtrees" to 10 to reduce memory requirements.

# RESULTS

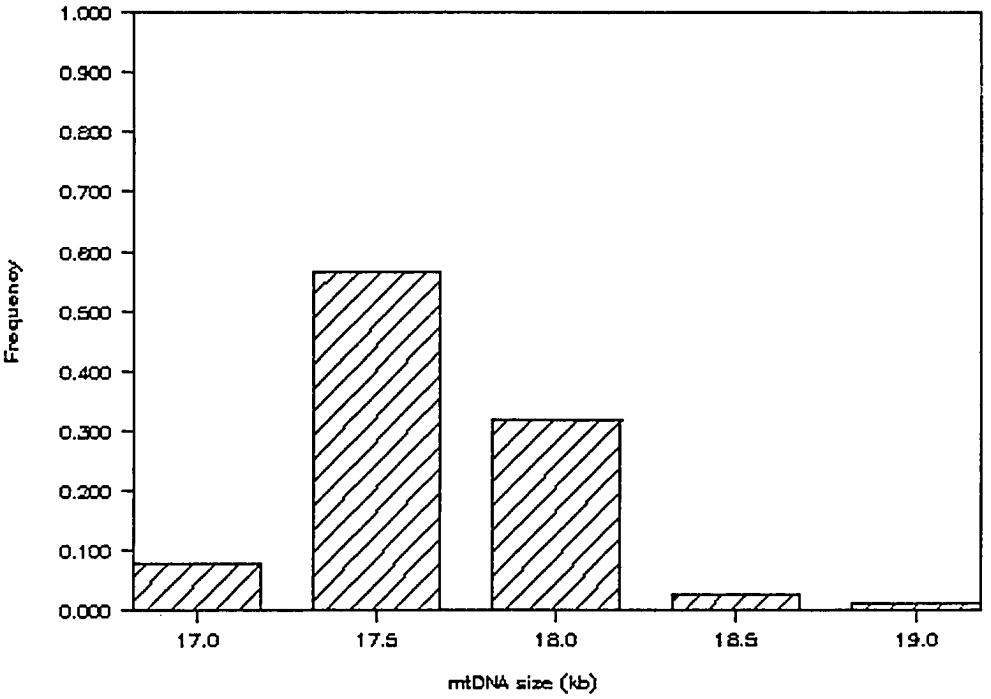
*Mercenaria spp.* populations were sampled along the U.S. east and Gulf coasts during 1986-1988 at the locations shown in Figure 1. A total of 317 individuals in 15 samples from 13 geographically distant locales were analyzed.

## I. Characteristics of hard shell clam mtDNA

More than 1900 restriction enzyme digests were performed in the characterization of clam mtDNA and its patterns of variation. These raw data are appended to the end of this manuscript as Appendix D. Although six restriction enzymes were employed as shown in Appendix C, data from *Hin*d III are incomplete for many individuals (see Appendix D) and are not included in the statistical analyses with the exception of clam molecular weight determination. An example of the incompleteness of *Hin*d III data is the population sampled from Martha's Vineyard (MV) shown in Appendix D. Instances where data for mtDNA restriction genotype were missing are represented by a "--."

The average length of the clam mtDNA molecule was estimated to be 17,480 ± 530 base pairs (17.5 kb) by summing all fragments in a pattern and averaging these totals for all patterns of all enzymes used (inclusive of *Hin*d III). This size was the most frequently encountered but was not the only size of mtDNA molecule detected. Many individuals exhibited molecules of size 17.0 kb, 18.0 kb and 18.5 kb. On rare occasion, individuals were examined with mtDNA as small as 16.5 kb and as large as 19.0 kb. The frequencies of each mtDNA size class observed in this study are shown in Figure 2. Some individuals exhibited more than one mtDNA size, i.e. were size heteroplasmic.

Figure 2. Frequencies of different mtDNA sizes detected in the *Mercenaria spp.* populations sampled along the U. S. east and Gulf coasts. MtDNA size is given in kilobases (kb).



A total of 24 different restriction fragment patterns were observed in this study as shown diagramatically in Figure 3. Some fragments (marked by an "*" in Figure 3) demonstrated variation in length as will be discussed below. The lengths of variable fragments in each pattern are standardized for the average total mtDNA size of 17.5 kb as presented in Table 1. For example, an individual with cut sites for the enzyme *Bam*H I which resulted in fragment pattern "A" as shown in Figure 3 would have bands on the gel located at 8.2, 7.7 and 1.6 kb (Table 1) if its mtDNA were 17.5 kb in length. However, an individual with a total mtDNA length of 18.0 kb would exhibit band lengths of 8.2, 8.2 and 1.6 kb when its mtDNA was digested by the enzyme *Bam*H I. Both individuals would have the same cut sites but the second individual has a larger mtDNA molecule. Figure 4 illustrates one example of size polymorphism in a population of hard shell clams.

When comparing two restriction enzyme digestion profiles, the disappearance of a large fragment accompanied by the appearance of two smaller fragments is assumed to occur via a mutational event which has resulted in a single nucleotide change in the mtDNA sequence recognized by that enzyme. In many cases, the clam digestion profiles can be related to one another by single restriction site changes. For example, *Bam*H I genotypes B and C in Figure 3 are related as follows. Individuals with genotype C have one additional restriction site in the 9.0 kb fragment which results in cleavage of this fragment into pieces 5.8 and 3.2 kb in length (refer to Table 1 for fragment lengths). On the other hand, neither the B, C nor E genotypes can be related by a simple sequence of site losses or gains to *Bam*H I genotype A. Likewise, genotypes A and B for the enzyme *Eco*R I shown in Figure 3 are closely related with two homologous fragments and a site gain in the size-variable 9.5 kb fragment of A which results in the appearance of two smaller fragments, one 5.7 kb and the other size-variable of *ca.* 3.1 kb in the B genotype. Genotypes C and D of *Eco*R I though, cannot be related to A or B by a simple

Figure 3. Graphic illustration of restriction fragment patterns observed for the hard shell clam *Mercenaria spp.* Molecular weights of fragments for each pattern sum to *ca.* 17.5 kilobases (kb). Fragments of known molecular weight (shown along the left and right margins) are employed as the standard for estimating the molecular weight of each unique clam mtDNA fragment. Thick bands indicate two fragments which have roughly the same molecular weight and thus demonstrate coincident electrophoretic mobility. An "*" indicates a fragment found to vary in size.

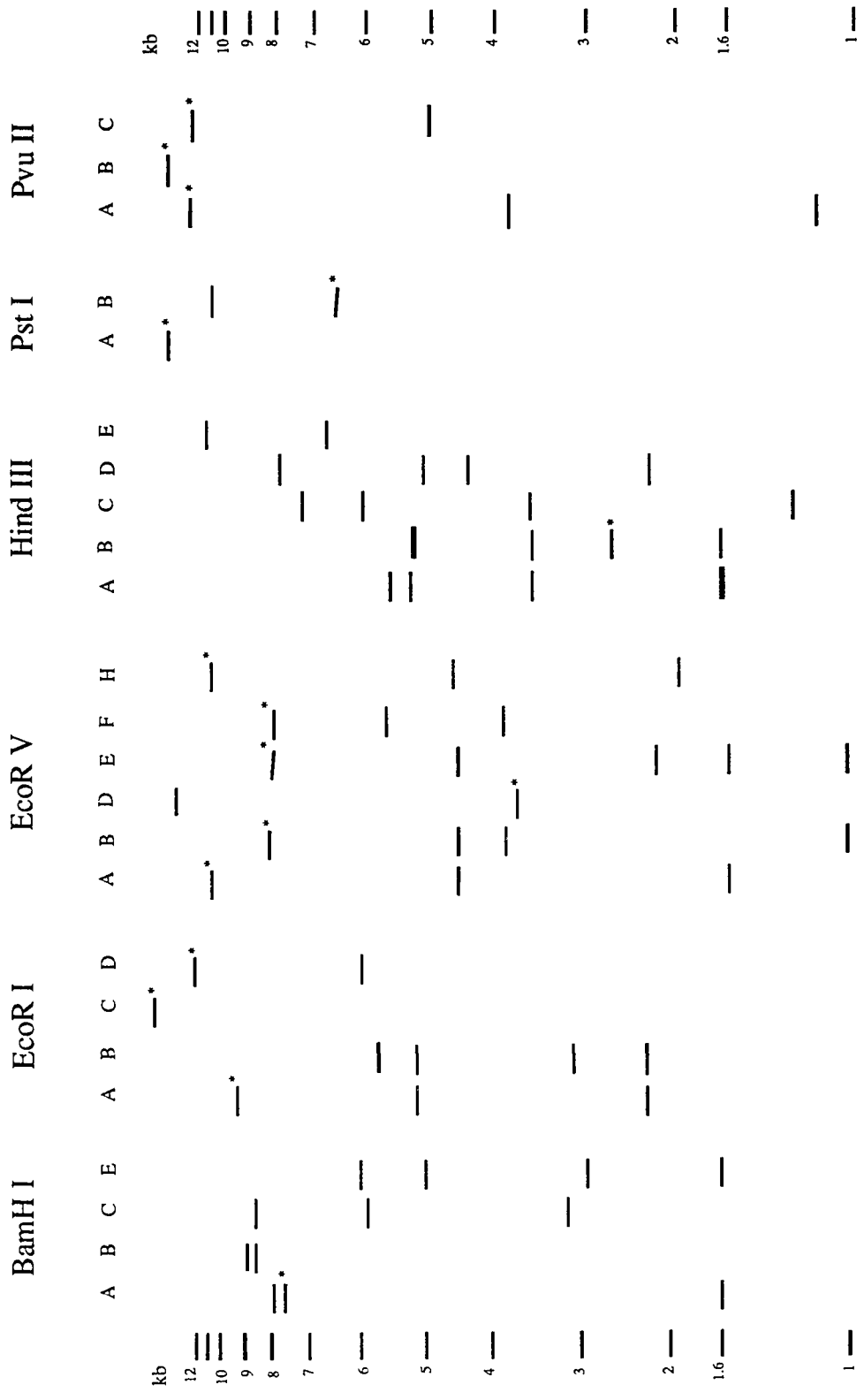


Table 1. Molecular weight estimates for *Mercenaria spp.* mtDNA restriction fragments as depicted in Figure 3. Size-variable fragments are marked with an "*." Molecular weights are in kilobases (kb) and generally sum to *ca.* 17.5 kb, the predominant molecular weight observed for clam mtDNA. Molecular weights of fragments marked with "*" was observed to vary in increments of 0.5 kb as described in text.

BamH I

| A | B | C | E |
|---|---|---|---|
| 8.2 | 9.0 | 8.5 | 6.0 |
| 7.7* | 8.5 | 5.9 | 5.0 |
| 1.6 | | 3.2 | 2.9 |
| | | | 1.6 |
| 17.5 | 17.5 | 17.6 | 15.5 |

EcoR I

| A | B | C | D |
|---|---|---|---|
| 9.5* | 5.7 | 17.5* | 12.0* |
| 5.2 | 5.2 | | 6.0 |
| 2.3 | 3.1 | | |
| | 2.3 | | |
| 17.0 | 16.3 | 17.5 | 18.0 |

EcoR V

| A | B | D | E | F | H |
|---|---|---|---|---|---|
| 10.7* | 8.2* | 14.0 | 8.2* | 8.2* | 10.7* |
| 4.5 | 4.5 | 3.7* | 4.5 | 5.6 | 4.5 |
| 1.6 | 3.9 | | 2.2 | 3.9 | 1.9 |
| | 1.1 | | 1.6 | | |
| | | | 1.1 | | |
| 16.8 | 17.7 | 17.7 | 17.6 | 17.7 | 17.1 |

Hind III

| A | B | C | D | E |
|---|---|---|---|---|
| 5.5 | 5.2 | 7.2 | 7.8 | 11.0 |
| 5.2 | 5.1 | 6.0 | 5.0 | 6.5 |
| 3.5 | 3.5 | 3.5 | 4.3 | |
| 1.6 | 2.6* | 1.3 | 2.2 | |
| 1.6 | 1.6 | | | |
| 17.4 | 18.0 | 18.0 | 19.3 | 17.5 |

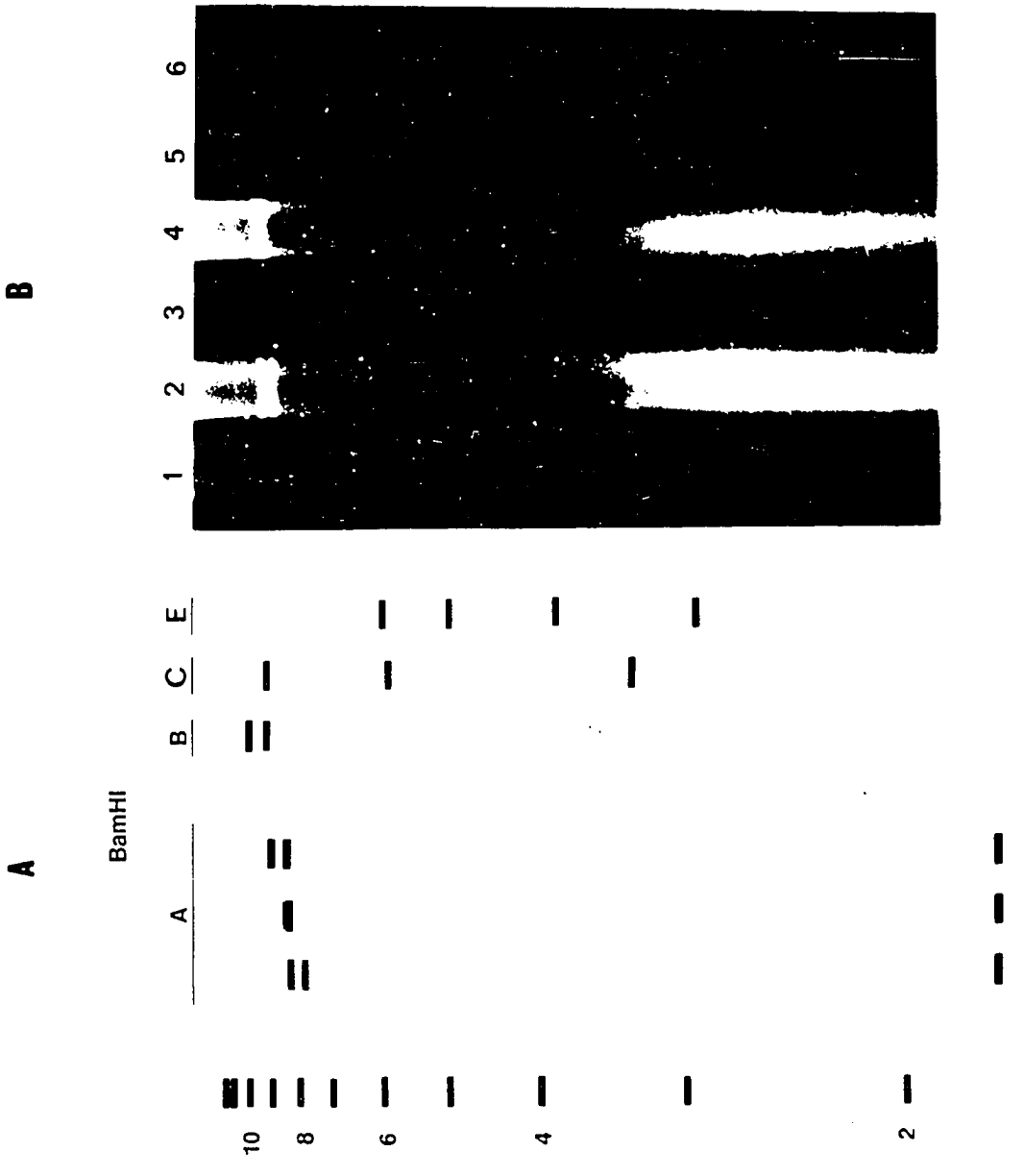
Pst I

| A | B |
|---|---|
| 17.5* | 10.5 |
| | 6.5* |
| 17.5 | 17.0 |

Pvu II

| A | B | C |
|---|---|---|
| 12.5* | 17.5* | 12.5* |
| 3.8 | | 5.0 |
| 1.2 | | |
| 17.5 | 17.5 | 17.5 |

Figure 4. Size polymorphism between mtDNAs of four individuals in a population of *M. mercenaria* clams when their mtDNA was digested with the restriction enzyme *Bam*H I. A. Diagram showing all restriction genotypes for *Bam*H I. The three patterns under genotype "A" have the same restriction cut sites but result from length differences in the 7.7 kb fragment (refer to Figure 3 and Table 1). A molecular weight standard is shown along the left margin of the diagram in which bands of 2, 4, 6, 8, and 10 kb are marked. This standard corresponds to the molecular weight standard shown in lane 6 of the gel photograph. B. Gel photograph showing individuals in lanes 1, 3, and 4 which have the second "A" mtDNA genotype totaling 17.5 kb and an individual in lane 2 with the first "A" mtDNA genotype which totals 18.0 kb.



process of inferring restriction site losses and gains. When this exercise is carried out for the fragment patterns of each enzyme in turn, the final analysis is that the clam populations sampled in this study exhibit many highly divergent patterns.

In the initial stages of this study many replicate isolations, extractions and digestions were conducted because of restriction fragment patterns resulting from heteroplasmy. After extensive repetition of isolations from the same animal and digests from duplicate extractions it was concluded that heteroplasmy (most often size but frequently site as well) is a common occurrence in hard shell clams. The distribution of heteroplasmy is shown in Figure 5. For each restriction enzyme employed in this study, size-variable fragments such as those depicted in Figure 4 were detected both within populations (size polymorphism) and within individuals (size heteroplasmy). In addition, for the enzymes *Bam*H I, *Eco*R I, *Eco*R V, *Pst* I and *Pvu* II combinations of two and occasionally three different restriction fragment patterns within an individual resulted in site heteroplasmic genotypes such as that illustrated in Figure 6.

II. Geographic distribution of mtDNA patterns in native east coast populations of *M. mercenaria*

Two sets of $G_h$ tests were conducted. The first was based solely on the distribution of restriction fragment patterns (not haplotypes) within populations as listed in Appendix D. As such, all sampled populations could be tested for heterogeneity of restriction fragment pattern frequencies. Preliminary results shown in Figure 7 indicate that the major trend in mtDNA variation along the east coast is toward homogeneity. It is noteable that the first sample from Oregon Inlet, NC (O1) is not significantly different from the *M. campechiensis* from Appalachicola Bay.

A second set of $G_h$ tests (results shown in Table 4) was based on the distribution of haplotypes among the nine populations with more complete data as listed in Table 2

Figure 5. Frequencies of mtDNA heteroplasmy in *Mercenaria spp.* populations sampled along the east and Gulf coasts of the United States. Shaded areas indicate the percent of individuals sampled which were heteroplasmic for either mtDNA size or restriction site. White areas indicate the percent of individuals which were not detectably heteroplasmic.

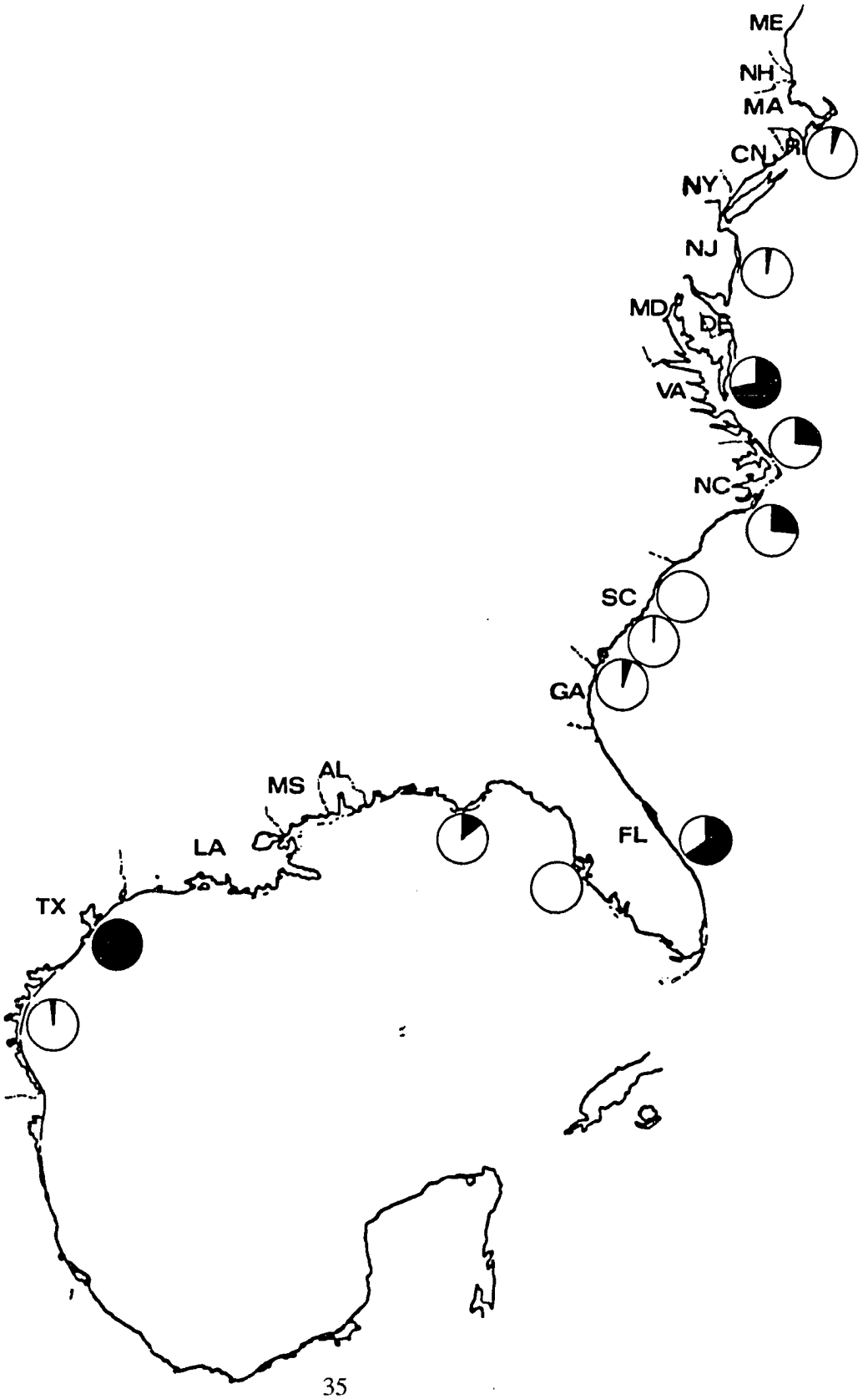


Figure 6. Restriction site heteroplasmy in an individual *M. campechiensis* clam. A. Diagram of restriction fragment patterns "A" and "B" for the enzyme *Pst* I. A molecular weight standard is shown along the left with fragments of 2, 4, 6, 8, and 10 kb marked. B. Gel photograph of an individual *M. campechiensis* for which both patterns "A" and "B" were observed. The presence of both patterns indicates that the individual possesses two types of mtDNA molecules with different nucleotide sequences.

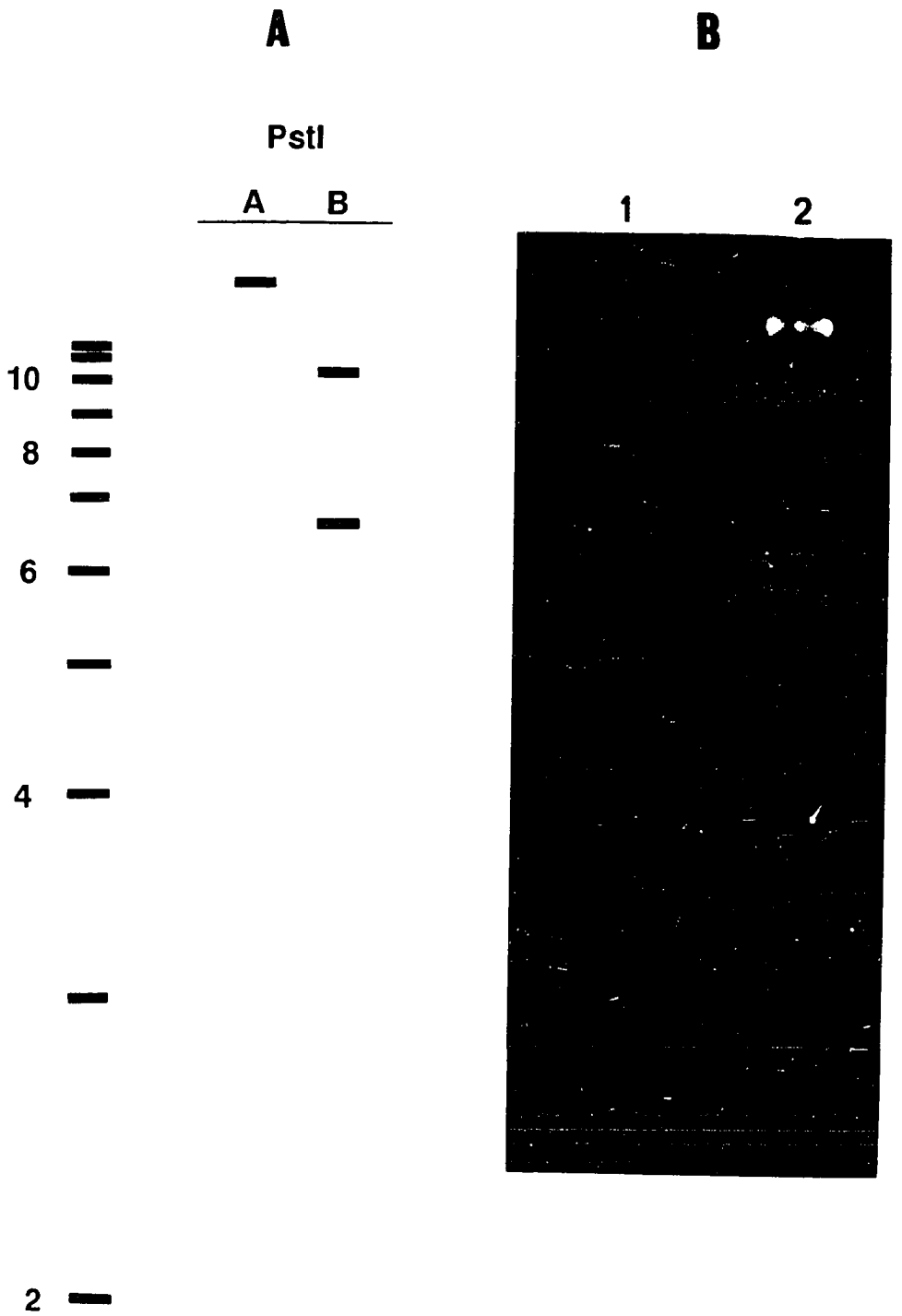


Figure 7. Results of preliminary $G_h$ tests conducted enzyme-by-enzyme for each of the 15 samples. Population designations are as in Figure 1. A solid line (——) indicates that populations were not significantly different based on restriction fragment patterns for that enzyme ($\alpha = 0.05$). Asterisks (*****) indicate populations for which data were not available for comparison with that particular restriction enzyme. Absence of a line for an enzyme comparison indicates that population was significantly different ($\alpha = 0.05$) from all other populations.

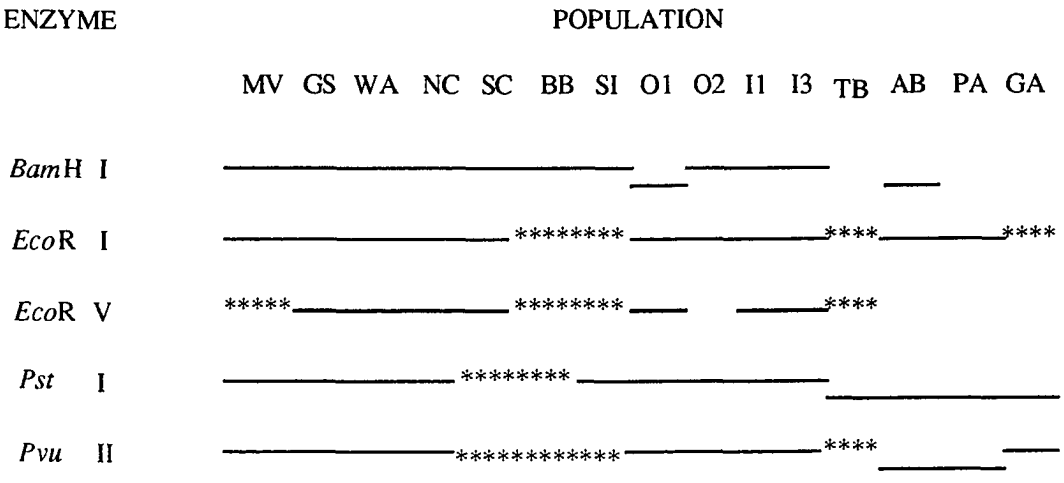


Table 2. Haplotype, mtDNA molecular weight and heteroplasmy for nine natural clam populations. Haplotypes are presented for those individuals sampled from the following populations (GS, WA, NC, O1, O2, I1, I3, AB and PA as shown in Figure 1) which had complete data for the five restriction enzymes *Bam*H I, *Eco*R I, *Eco*R V, *Pst* I, and *Pvu* II, in that order. Predominant molecular weight is the weight in kb of the most common sized mtDNA in the individual. The heteroplasmic condition is indicated as present "1" or absent "0" for both size of the individual's mtDNA molecule and restriction site as indicated by digestion with restriction enzymes.

| Individual | Population | Haplotype | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|
| 1 | GS | AAAAA | 18.0 | 0 | 0 |
| 2 | GS | AAAAA | 17.5 | 0 | 0 |
| 3 | GS | AAAAA | 17.5 | 0 | 0 |
| 4 | GS | AAAAA | 18.0 | 0 | 0 |
| 5 | GS | AAAAA | 18.0 | 0 | 0 |
| 6 | GS | AAAAA | 18.0 | 0 | 0 |
| 7 | GS | AAAAA | 17.5 | 0 | 0 |
| 9 | GS | AAAAA | 18.0 | 0 | 0 |
| 12 | GS | AAAAA | 18.0 | 0 | 0 |
| 13 | GS | AAAAA | 18.0 | 0 | 0 |
| 14 | GS | AAAAA | 18.0 | 0 | 0 |
| 15 | GS | AAAAA | 18.0 | 0 | 0 |
| 22 | GS | AAAAA | 18.0 | 0 | 0 |
| 23 | GS | AAAAA | 18.0 | 0 | 0 |
| 1 | WA | AAAAA | 18.0 | 0 | 0 |
| 2 | WA | AAAAA | 18.0 | 1 | 0 |
| 3 | WA | AAAAA | 17.5 | 1 | 0 |
| 4 | WA | AAAAA | 17.5 | 0 | 0 |
| 5 | WA | AAAAA | 17.5 | 1 | 0 |
| 6 | WA | AAAAA | 17.5 | 1 | 0 |
| 7 | WA | AAAAA | 17.5 | 1 | 0 |
| 8 | WA | AAAAA | 17.5 | 1 | 0 |
| 9 | WA | AA/DAAA | 18.0 | 1 | 1 |
| 10 | WA | AAAAA | 18.0 | 1 | 0 |
| 11 | WA | AAAAA | 17.5 | 1 | 0 |
| 12 | WA | AAAAA | 18.0 | 1 | 0 |
| 13 | WA | AAAAA | 17.5 | 1 | 0 |
| 15 | WA | AA/DAAA | 17.0 | 1 | 1 |
| 16 | WA | AA/DAAA | 17.0 | 1 | 1 |
| 17 | WA | AA/DAAA | 17.5 | 1 | 1 |
| 18 | WA | AA/DAAA | 17.5 | 1 | 1 |
| 19 | WA | AA/DAAA | 17.5 | 1 | 1 |
| 4 | NC | AAAAA | 18.0 | 0 | 0 |
| 5 | NC | AADAA | 18.0 | 0 | 0 |
| 6 | NC | AAAAA | 18.0 | 1 | 0 |
| 7 | NC | AAAAA | 18.0 | 0 | 0 |
| 9 | NC | AAAAA | 17.5 | 1 | 0 |
| 10 | NC | AAAAA | 17.0 | 0 | 0 |
| 11 | NC | AAAAA | 18.0 | 1 | 0 |
| 12 | NC | AAAAA | 18.0 | 1 | 0 |
| 13 | NC | AAAAA | 17.5 | 0 | 0 |
| 14 | NC | AAAAA | 17.5 | 0 | 0 |
| 15 | NC | AAAAA | 17.5 | 0 | 0 |
| 16 | NC | AAAAA | 17.0 | 0 | 0 |
| 17 | NC | AAAAA | 17.5 | 0 | 0 |

Table 2 continued

| Individual | Population | Haplotype | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|
| 3 | O1 | AAAAA | 17.5 | 1 | 0 |
| 4 | O1 | AAAAA | 17.5 | 0 | 0 |
| 5 | O1 | AAAAA | 17.5 | 0 | 0 |
| 6 | O1 | BCAAA | 17.5 | 1 | 0 |
| 10 | O1 | AAAAA | 18.0 | 0 | 0 |
| 13 | O1 | BA/DAAA | 17.5 | 1 | 1 |
| 14 | O1 | BAABA | 17.5 | 1 | 0 |
| 15 | O1 | BA/DAAA | 17.5 | 0 | 0 |
| 16 | O1 | BA/DAAA | 18.0 | 0 | 0 |
| 17 | O1 | AAAAA | 18.0 | 0 | 0 |
| 20 | O1 | BA/DAAA | 18.0 | 0 | 0 |
| 21 | O1 | BA/DAAA | 18.0 | 0 | 1 |
| 22 | O1 | BA/DAAA | 17.5 | 0 | 0 |
| 1 | O2 | AAHAA | 18.0 | 0 | 0 |
| 2 | O2 | AAHAA | 17.5 | 0 | 0 |
| 3 | O2 | AAHAA | 18.0 | 0 | 0 |
| 4 | O2 | AAHAA | 17.5 | 0 | 0 |
| 5 | O2 | AAAAC | 17.5 | 0 | 0 |
| 6 | O2 | AAHAB | 17.5 | 0 | 0 |
| 7 | O2 | AAAAA | 18.0 | 0 | 0 |
| 8 | O2 | AAHAA | 17.0 | 0 | 0 |
| 9 | O2 | AAHAA | 17.5 | 0 | 0 |
| 10 | O2 | AAHAA | 17.5 | 0 | 0 |
| 11 | O2 | AAHAA | 18.0 | 0 | 0 |
| 9 | I1 | AAAAB | 17.5 | 0 | 0 |
| 10 | I1 | AAAAB | 17.5 | 0 | 0 |
| 25 | I1 | AAAAA | 17.5 | 0 | 0 |
| 26 | I1 | AAAAA | 17.5 | 1 | 0 |
| 27 | I1 | AAAAA | 17.5 | 1 | 0 |
| 28 | I1 | AAAAA | 18.0 | 1 | 0 |
| 29 | I1 | AAAAA | 17.5 | 1 | 0 |
| 30 | I1 | AAAAA | 17.5 | 1 | 0 |
| 31 | I1 | AAAAA | 18.5 | 1 | 0 |
| 32 | I1 | AAAAA | 18.0 | 1 | 0 |
| 25 | I3 | AAAAA | 17.5 | 0 | 0 |
| 26 | I3 | AAAAA | 18.5 | 0 | 0 |
| 27 | I3 | AAAAA | 18.5 | 0 | 0 |
| 28 | I3 | AAAAA | 18.0 | 0 | 0 |
| 29 | I3 | AAAAA | 18.0 | 0 | 0 |
| 30 | I3 | AABBA | 17.0 | 0 | 0 |
| 31 | I3 | AAAAA | 18.0 | 0 | 0 |
| 32 | I3 | AAAAA | 17.0 | 0 | 0 |
| 33 | I3 | AAAAA | 17.5 | 0 | 0 |
| 34 | I3 | AAAAA | 18.5 | 0 | 0 |
| 35 | I3 | AAAAA | 17.5 | 0 | 0 |

Table 2 continued

| Individual | Population | Haplotype | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|
| 1 | AB | ACBBB | 17.5 | 0 | 0 |
| 2 | AB | A/BC/A/DB | 17.5 | 1 | 1 |
| 3 | AB | AC/A/DBB] | 18.0 | 1 | 1 |
| 4 | AB | A/BCBB/A] | 18.0 | 1 | 1 |
| 15 | AB | BABBB | 17.5 | 0 | 0 |
| 16 | AB | BABBB/A | 18.0 | 0 | 1 |
| 17 | AB | BABBB/AE | 17.5 | 0 | 1 |
| 18 | AB | BABBB | 17.5 | 0 | 0 |
| 19 | AB | BABBB | 18.0 | 0 | 0 |
| 20 | AB | BABBB | 17.5 | 0 | 0 |
| 21 | AB | BABBB | 18.0 | 0 | 0 |
| 23 | AB | BABBB | 17.5 | 0 | 0 |
| 26 | AB | BABBB | 17.5 | 0 | 0 |
| 30 | AB | BABBB | 17.5 | 0 | 0 |
| 31 | AB | BABBB | 18.0 | 0 | 0 |
| 32 | AB | BABBB | 17.5 | 0 | 0 |
| 33 | AB | BABBB | 18.0 | 0 | 0 |
| 38 | AB | BABBB | 17.0 | 0 | 0 |
| 1 | PA | AABBB | 17.0 | 0 | 0 |
| 2 | PA | AABBB | 17.5 | 0 | 0 |
| 3 | PA | AAFBB | 17.5 | 0 | 0 |
| 4 | PA | AABBB | 17.5 | 0 | 0 |
| 5 | PA | AABBB | 17.5 | 0 | 0 |
| 6 | PA | AABBB | 18.0 | 0 | 0 |
| 7 | PA | AABBB | 17.5 | 0 | 0 |
| 8 | PA | EADAC | 17.0 | 0 | 0 |
| 10 | PA | AABAB | 17.5 | 0 | 0 |
| 12 | PA | AABAB | 17.5 | 0 | 0 |
| 13 | PA | AABAB | 18.0 | 0 | 0 |
| 14 | PA | ECDAC | 17.5 | 0 | 0 |
| 15 | PA | AABAB | 18.0 | 0 | 0 |
| 16 | PA | AAB/DBB | 18.0 | 0 | 1 |
| 17 | PA | AAFBB | 18.0 | 0 | 0 |
| 18 | PA | EADBB | 17.5 | 0 | 0 |
| 19 | PA | AABBB | f7.5 | 0 | 0 |
| 20 | PA | AABBB | 17.5 | 0 | 0 |
| 21 | PA | AABAB | 17.5 | 0 | 0 |
| 22 | PA | AAEAB | 17.5 | 0 | 0 |
| 23 | PA | AABBB | 17.5 | 0 | 0 |
| 24 | PA | AABBB | 17.5 | 0 | 0 |
| 25 | PA | AAFBB | 18.0 | 0 | 0 |
| 26 | PA | EADAC | 18.0 | 0 | 0 |
| 28 | PA | AAFBB | 17.5 | 0 | 0 |
| 29 | PA | EADBC | 17.0 | 0 | 0 |
| 30 | PA | BABAB | 17.5 | 0 | 0 |
| 31 | PA | AABBB | 17.5 | 0 | 0 |
| 32 | PA | AABAB | 17.5 | 0 | 0 |

Table 3. Distribution of mtDNA haplotypes among nine natural clam populations (data compiled from Table 2). The code in column one is used in subsequent phylogenetic analyses to refer to clam mtDNA haplotypes. Population designations are as in Figure 1. Values in the body of the table are the numbers of individuals with each haplotype in each population. Each individual has only one haplotype.

| | | | POPULATION | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Code | Haplotype | | GS | WA | NC | O1 | O2 | I1 | I3 | AB | PA | TOTAl |
| m1 | 1 | AAAAA | 14 | 12 | 13 | 5 | 1 | 8 | 10 | | | 63 |
| ir1 | 2 | AAAAB | | | | | | 2 | | | | 2 |
| oi22 | 3 | AAAAC | | | | | 1 | | | | | 1 |
| t4 | 4 | AABAB | | | | | | | | | 6 | 6 |
| ir3 | 5 | AABBA | | | | | | | 1 | | | 1 |
| t1 | 6 | AABBB | | | | | | | | | 11 | 11 |
| t6 | 7 | AAB/DBB | | | | | | | | | 1 | 1 |
| m4 | 8 | AADAA | | | 1 | | | | | | | 1 |
| t9 | 9 | AAEAB | | | | | | | | | 1 | 1 |
| t2 | 10 | AAFBB | | | | | | | | | 4 | 4 |
| oi21 | 11 | AAHAA | | | | | 8 | | | | | 8 |
| oi23 | 12 | AAHAB | | | | | 1 | | | | | 1 |
| m3 | 13 | AA/DAAA | | 6 | | | | | | | | 6 |
| c7 | 14 | ACBBB | | | | | | | | 1 | | 1 |
| c2 | 15 | AC/A/DBBB | | | | | | | | 1 | | 1 |
| c3 | 17 | A/BCBB/AB | | | | | | | | 1 | | 1 |
| c1 | 18 | A/BC/A/DBBB | | | | | | | | 1 | | 1 |
| oi13 | 19 | BAABA | | | | 1 | | | | | | 1 |
| t8 | 20 | BABAB | | | | | | | | | 1 | 1 |
| c6 | 21 | BABBB | | | | | | | | 12 | | 12 |
| c4 | 22 | BABBB/A | | | | | | | | 1 | | 1 |
| c5 | 23 | BABB/AB | | | | | | | | 1 | | 1 |
| oi12 | 24 | BA/DAAA | | | | 6 | | | | | | 6 |
| oi11 | 25 | BCAAA | | | | 1 | | | | | | 1 |
| t3 | 27 | EADAC | | | | | | | | | 2 | 2 |
| t7 | 28 | EADBB | | | | | | | | | 1 | 1 |
| t10 | 29 | EADBC | | | | | | | | | 1 | 1 |
| t5 | 30 | ECDAC | | | | | | | | | 1 | 1 |
| | n | | 14 | 18 | 14 | 13 | 11 | 10 | 11 | 18 | 29 | 138 |

Table 4. Results of $G_h$ analysis for heterogeneity of haplotype frequency among nine natural clam populations. Population designations are as in Figure 1. $G_h$ values are listed for each successive comparison along with the appropriate degrees freedom and the probability of that $G_h$ value. $G_h$ is distributed as Chi-square.

GS, NC, O1, O2, I1, I3, AB, PA:

| | |
|---|---|
| $G_h$ = | 364.1 |
| df = | 216 |
| prob. | < 0.005 |

GS, WA, NC, O1, O2, I1, I3:

| | |
|---|---|
| $G_h$ = | 124.5 |
| df = | 60 |
| prob. | < 0.005 |

GS, WA, NC, O2, I1, I3:

| | |
|---|---|
| $G_h$ = | 69.24 |
| df = | 15 |
| prob. | < 0.005 |

GS, WA, NC, I1, I3:

| | |
|---|---|
| $G_h$ = | 24.57 |
| df = | 8 |
| prob. | < 0.005 |

GS, NC, I1, I3:

| | |
|---|---|
| $G_h$ = | 6.534 |
| df = | 3 |
| prob. | 0.05<p<0.1 |

AB, PA:

| | |
|---|---|
| $G_h$ = | 62.56 |
| df = | 16 |
| prob. | < 0.005 |

O1, AB:

| | |
|---|---|
| $G_h$ = | 42.17 |
| df = | 10 |
| prob. | < 0.005 |

O1, O2, I1, I3, AB:

| | |
|---|---|
| $G_h$ = | 142.6 |
| df = | 60 |
| prob. | < 0.005 |

O1, O2, AB:

| | |
|---|---|
| $G_h$ = | 85.06 |
| df = | 26 |
| prob. | < 0.005 |

I1, I3, AB:

| | |
|---|---|
| $G_h$ = | 58.17 |
| df = | 18 |
| prob. | < 0.005 |

I1, O2:

| | |
|---|---|
| $G_h$ = | 27.7 |
| df = | 6 |
| prob. | < 0.005 |

I1, I3:

| | |
|---|---|
| $G_h$ = | 4.334 |
| df = | 2 |
| prob. | 0.1<p<0.5 |

and summarized in Table 3. These populations were GS, WA, NC, O1, O2, I1, I3, AB, and PA. In this second analysis, discrimination of populations is possible. The only group of populations not significantly different by $G_h$ was GS, NC, I1, I3 ($G_h$ = 6.5 with 3 degrees freedom).

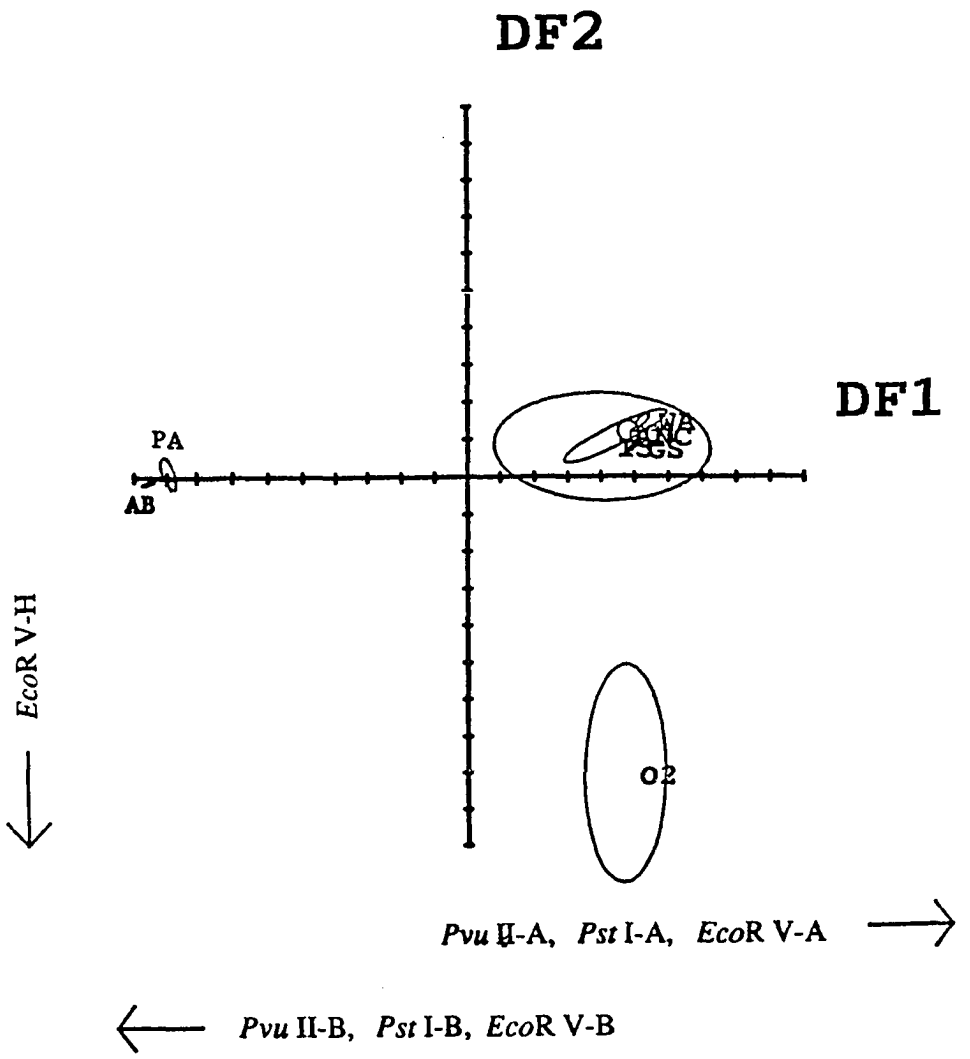
Canonical discriminant analysis was employed not as an exploratory tool but for descriptive purposes. For the nine populations with complete data, GS, WA, NC, O1, O2, I1, I3, AB and PA, the multivariate test for differences between populations indicated no significant difference ($\alpha$ = 0.01) between any of the east coast *M. mercenaria* populations except the second Oregon Inlet sample (O2). This relationship is illustrated by the confidence elipses for discriminant functions 1-3 shown in Figure 8.

Table 5 contains identity probabilities for the same nine populations. The diagonal element is within population gene identity, I, while the upper triangle values are gene identities between populations, J. A cursory examination of these data indicates that northern east coast populations of *M. mercenaria* exhibit low degrees of heterogeneity (GS and WA have I values of 1.00 and 0.88, respectively) while all other populations exhibit moderate to high levels of differentiation (I values range from 0.85 to 0.36). Concurrently, only for the interpopulation comparisons of GS vs. WA, NC and I1 does there appear to be a very high degree of interpopulation similarity while the remaining comparisons indicate at least moderate (e.g., J = 0.70) to large (e.g., J = 0.23) levels of genetic differentiation. This observation agrees with $G_h$ tests by haplotype indicating no significant difference between GS, NC, I1 and I3 and with significant differences between haplotype frequencies of all other populations.

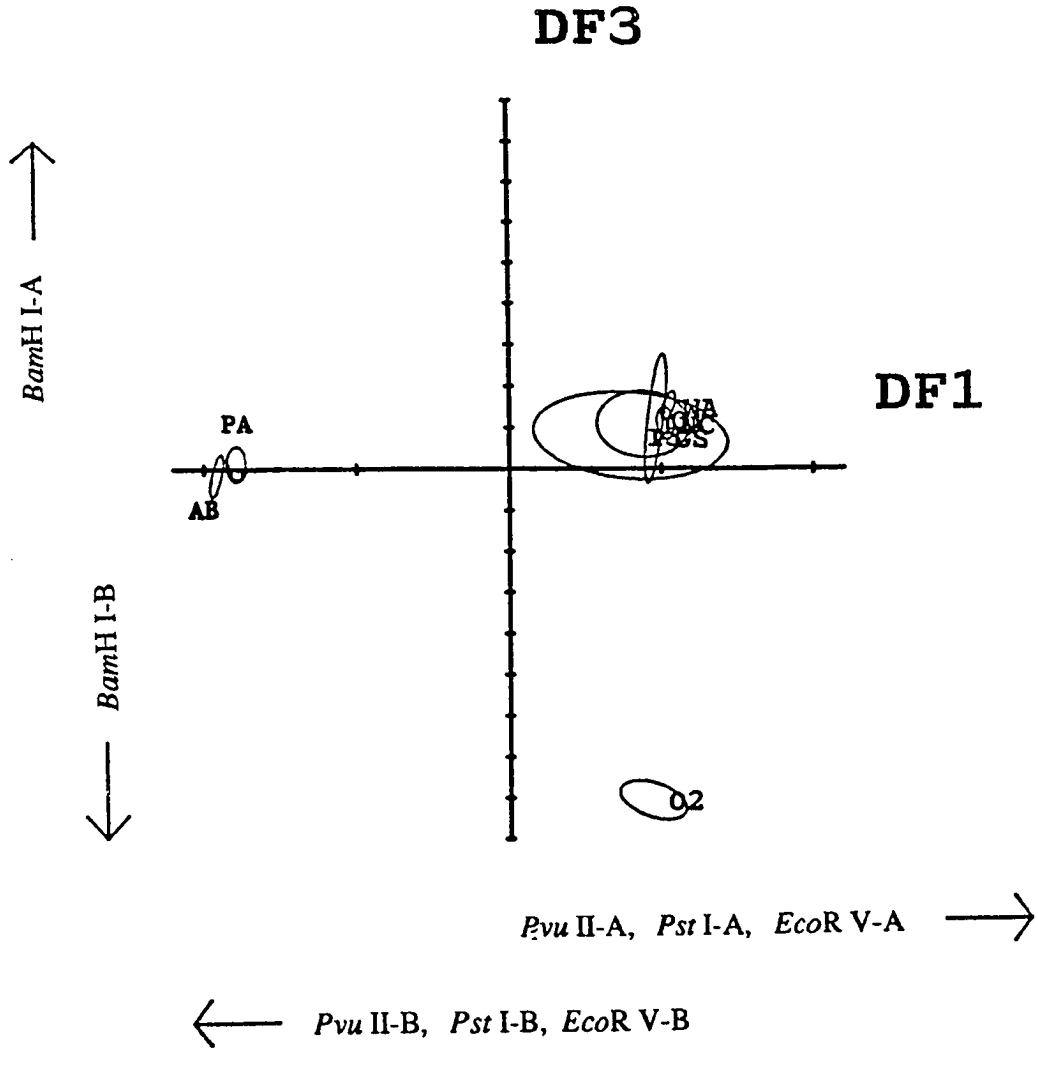
Pairwise genetic distance estimates, D, are given in Table 6 for these nine samples. Excluding comparisons with Oregon Inlet populations, D values between *M. mercenaria* populations (ranging from 0.008-0.307) are consistent with typical genetic distances associated with large subdivided populations within a species. The

Figure 8. 95% confidence elipses for discriminant functions 1, 2, and 3 from the canonical discriminant analysis run on variables for the nine populations: GS, WA, NC, O1, O2, I1, I3, AB and PA. The discriminant axes are labeled only with the names of variables which best revealed differences between populations and which were shown to be statistically significant ($\alpha = 0.01$) by multivariate analysis of variance. The order of names for an axis indicates the relative strength of those variables in accounting for variation between populations while the arrows indicate the direction of trends. Variable names are a combination of the name of the restriction enzyme followed by the letter designating a particular fragment pattern from Figure 3. A. Plot of discriminant function 1 vs. discriminant function 2. B. Plot of discriminant function 1 vs. discriminant function 3. C. Plot of discriminant function 2 vs. discriminant function 3.

A.



B.



C.

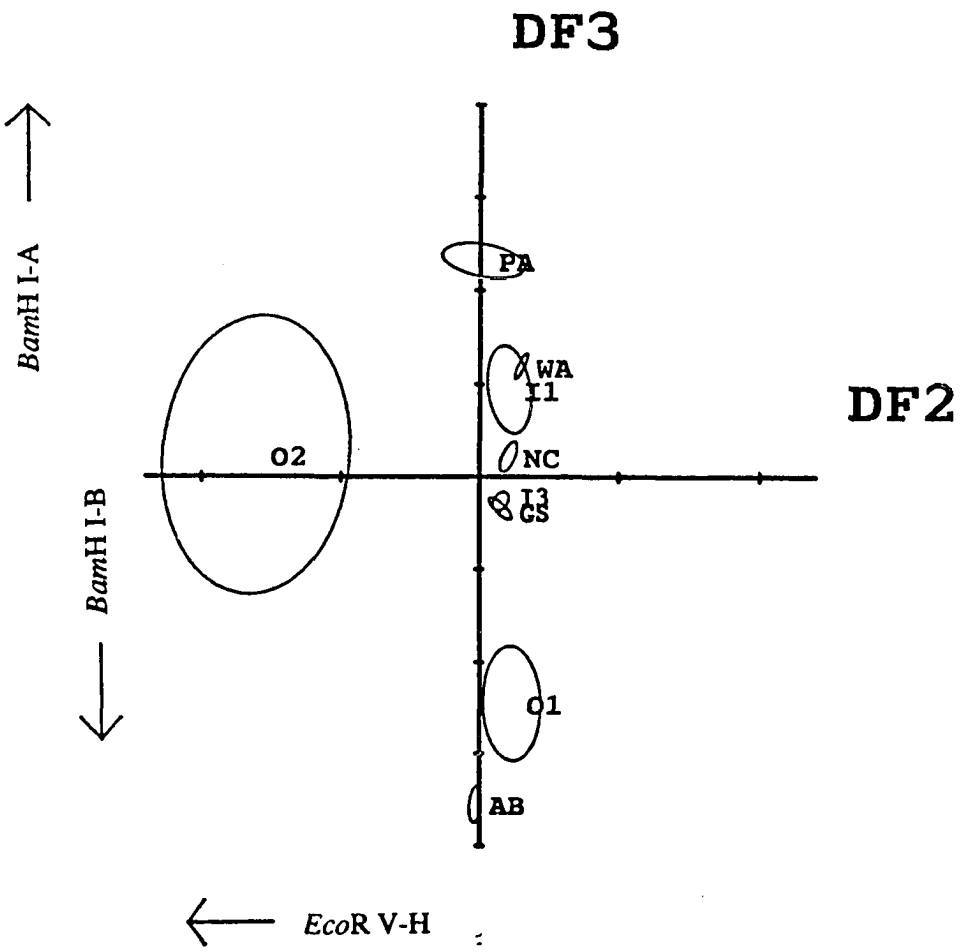


Table 5. Gene identity probabilities for each of nine natural clam populations. Population designations are as in Figure 1. The probability of gene identity is calculated as described in the text. Values along the diagonal are within population gene identity, I, while values in the upper triangle represent between population gene identities, J.

| | GS | WA | NC | O1 | O2 | I1 | I3 | AB | PA |
|---|---|---|---|---|---|---|---|---|---|
| GS | 1.000 | 0.867 | 0.852 | 0.542 | 0.733 | 0.886 | 0.707 | 0.351 | 0.240 |
| WA | | 0.880 | 0.852 | 0.558 | 0.733 | 0.827 | 0.707 | 0.354 | 0.240 |
| NC | | | 0.838 | 0.532 | 0.723 | 0.812 | 0.696 | 0.346 | 0.239 |
| O1 | | | | 0.544 | 0.479 | 0.512 | 0.530 | 0.281 | 0.232 |
| O2 | | | | | 0.738 | 0.702 | 0.640 | 0.374 | 0.237 |
| I1 | | | | | | 0.849 | 0.674 | 0.356 | 0.238 |
| I3 | | | | | | | 0.692 | 0.349 | 0.253 |
| AB | | | | | | | | 0.380 | 0.268 |
| PA | | | | | | | | | 0.360 |

Table 6. Pairwise genetic distance estimates, D, between nine natural clam populations. Population designations are as in Figure 1. Calculation of D is discussed in the text.

| | WA | NC | O1 | O2 | I1 | I3 | AB | PA |
|---|---|---|---|---|---|---|---|---|
| GS | 0.081 | 0.075 | 0.353 | 0.170 | 0.043 | 0.179 | 0.676 | 1.041 |
| WA | | 0.008 | 0.244 | 0.098 | 0.045 | 0.106 | 0.576 | 0.948 |
| NC | | | 0.262 | 0.086 | 0.038 | 0.095 | 0.566 | 0.920 |
| O1 | | | | 0.291 | 0.307 | 0.154 | 0.497 | 0.667 |
| O2 | | | | | 0.122 | 0.110 | 0.401 | 0.840 |
| I1 | | | | | | 0.134 | 0.546 | 0.932 |
| I3 | | | | | | | 0.428 | 0.730 |
| AB | | | | | | | | 0.323 |
| PA | | | | | | | | |

notable exceptions again include intraspecies comparisons with O1 (average D = 0.268 is high in comparison to other intraspecies D values for *M. mercenaria* which averaged 0.093).

Pairwise percent sequence divergence estimates, δ, for the same nine populations are shown in Table 7. Consistent with distance measures, δ values estimated between *M. mercenaria* populations are low ranging from 0.001 to 0.025.

Genetic distances and percent sequence divergence estimates between populations were clustered via UPGMA. Figure 9a shows the phenogram from genetic distance which indicates close similarity between the east coast *M. mercenaria* populations. Note that the last group to join in the *M. mercenaria* cluster is population O1 from Oregon Inlet, NC. Figure 9b shows the phenogram derived from percent sequence divergence. Phenetic clustering based on δ is concurrent with the genetic distance analysis except with regard to the order of inclusion of populations into the *M. mercenaria* cluster.

The overall $F_{st}$ for east coast clam populations was calculated from the data in Table 3 to be $F_{st} = 0.411$ which yields an intermediate estimate for effective migration rate of $N_em = 0.717$. Pairwise estimates of effective migration rate between subpopulations are shown in Table 8. These values range from $N_em = 0.08$ between O2 and GS to $N_em = 20.39$ between I1 and I3 indicating that levels of gene flow vary from negligible to extensive throughout *M. mercenaria*'s range.

III. Phylogenetic analysis of mtDNA haplotypes between taxa

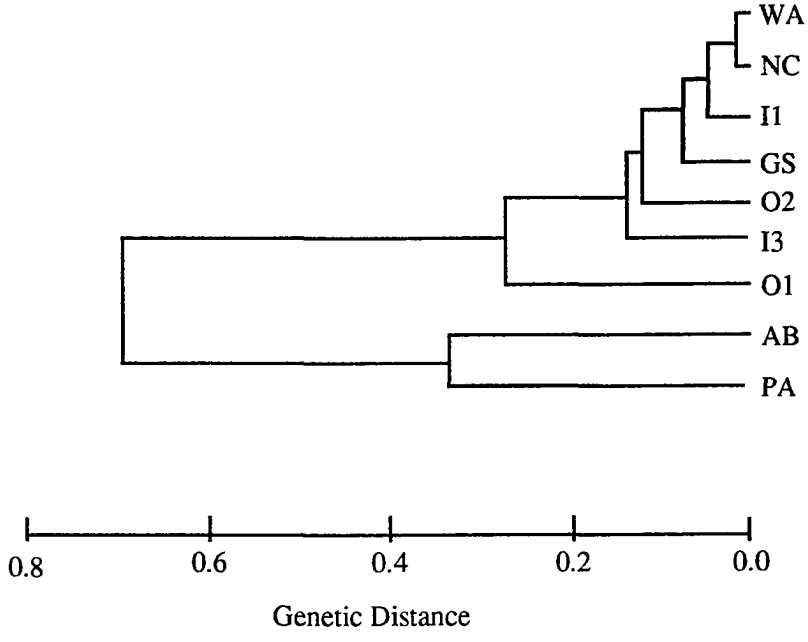
*M. mercenaria texana* sampled from the Galveston location was highly polymorphic. Many specimens from this location exhibited unique restriction fragment patterns which shared no fragments at all with either *mercenaria* or *campechiensis*. In addition, site heteroplasmy was noted in most specimens which made it impossible to

Table 7. Percent nucleotide sequence divergence, δ, between nine natural clam populations. Population designations are as in Figure 1. Calculation of δ is discussed in the text.

| | WA | NC | O1 | O2 | I1 | I3 | AB | PA |
|---|---|---|---|---|---|---|---|---|
| GS | 0.004 | 0.001 | 0.016 | 0.025 | 0.001 | 0.001 | 0.062 | 0.031 |
| WA | | 0.004 | 0.014 | 0.007 | 0.005 | 0.005 | 0.054 | 0.027 |
| NC | | | 0.015 | 0.005 | 0.002 | 0.002 | 0.058 | 0.028 |
| O1 | | | | 0.022 | 0.017 | 0.017 | 0.020 | 0.078 |
| O2 | | | | | 0.005 | 0.005 | 0.059 | 0.029 |
| I1 | | | | | | 0.002 | 0.061 | 0.029 |
| I3 | | | | | | | 0.058 | 0.090 |
| AB | | | | | | | | 0.020 |
| PA | | | | | | | | |

Figure 9. UPGMA phenograms summarizing the relationships between nine natural clam populations including *M. mercenaria, M. campechiensis,* and *M. mercenaria texana.* Population designations are as in Figure 1. A. Phenogram based on genetic distance, D, from Table 6. B. Phenogram based on percent nucleotide sequence divergence, $\delta$, from Table 7.

A




B

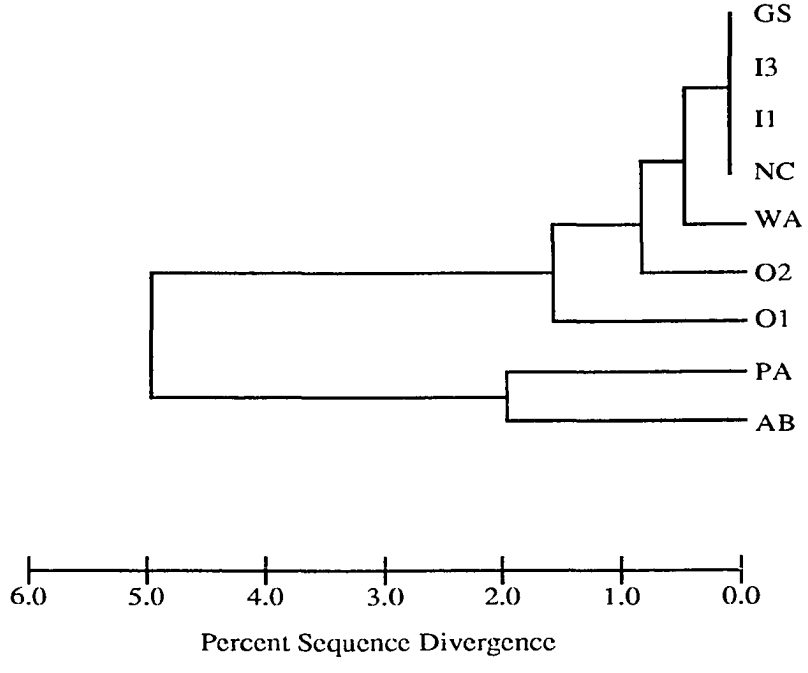


Table 8. Pairwise estimates of effective migration rate, $N_em$, between the seven east coast populations of *M. mercenaria*. Population designations are as in Figure 1. $N_em$ is calculated from $F_{st}$ as described in the text.

| | WA | NC | O1 | O2 | I1 | I3 |
|---|---|---|---|---|---|---|
| GS | 2.00 | 4.75 | 0.49 | 0.08 | 0.87 | 0.87 |
| WA | | 4.21 | 3.03 | 1.01 | 4.65 | 3.34 |
| NC | | | 1.02 | 0.19 | 0.64 | 0.83 |
| O1 | | | | 3.70 | 1.47 | 1.16 |
| O2 | | | | | 0.48 | 0.18 |
| I1 | | | | | | 20.39 |
| I3 | | | | | | |

define the new restriction fragment patterns for several of the enzymes employed in this study. Fortunately, the Port Aransas sample had a very low degree of heteroplasmy so that novel restriction fragment patterns were determined for most of the individuals sampled. Therefore, only data from the PA population has been included in discussions of relatedness between *mercenaria*, *campechiensis* and t*exana*.

A qualitative consideration of fragment patterns is fruitful prior to the quantitative analysis. It appears from a cursory examination of Table 2 that *texana* clams share ancestry with *campechiensis* as well as with *mercenaria*. For illustrative purposes, a reasonable simplification of the data is that a representative haplotype for *mercenaria* is AAAAA, for *campechiensis* is BABBB, and for *texana* is a combination of the two resulting in AABBB or AABAB. This relationship is important in that it recurrs in the statistical treatments described below as well as in the computer generated phylogenetic analyses.

Although many restriction fragment patterns were shared between the three taxa--*mercenaria*, *campechiensis* and *texana* (especially for *Eco*R I), there were no common haplotypes. However, since the populations being compared are closely related species, homologous fragments were considered to be shared apomorphic characters. This assumption was employed in the phylogenetic analyses where the haplotypes were interrelated based on fragment homologies.

In the preliminary $G_h$ analysis (Figure 7), *M. campechiensis* (population AB) was statistically indistinguishable from *M. mercenaria texana* (population PA) based on restriction fragment pattern frequencies for four out of five enzymes employed (data from Appendix D). The canonical discriminant analysis (refer to Figure 8) also indicated a high degree of similarity between *M. campechiensis* (AB) and *M. mercenaria texana* (PA). However, based on the more sensitive analysis of composite haplotypes shown in

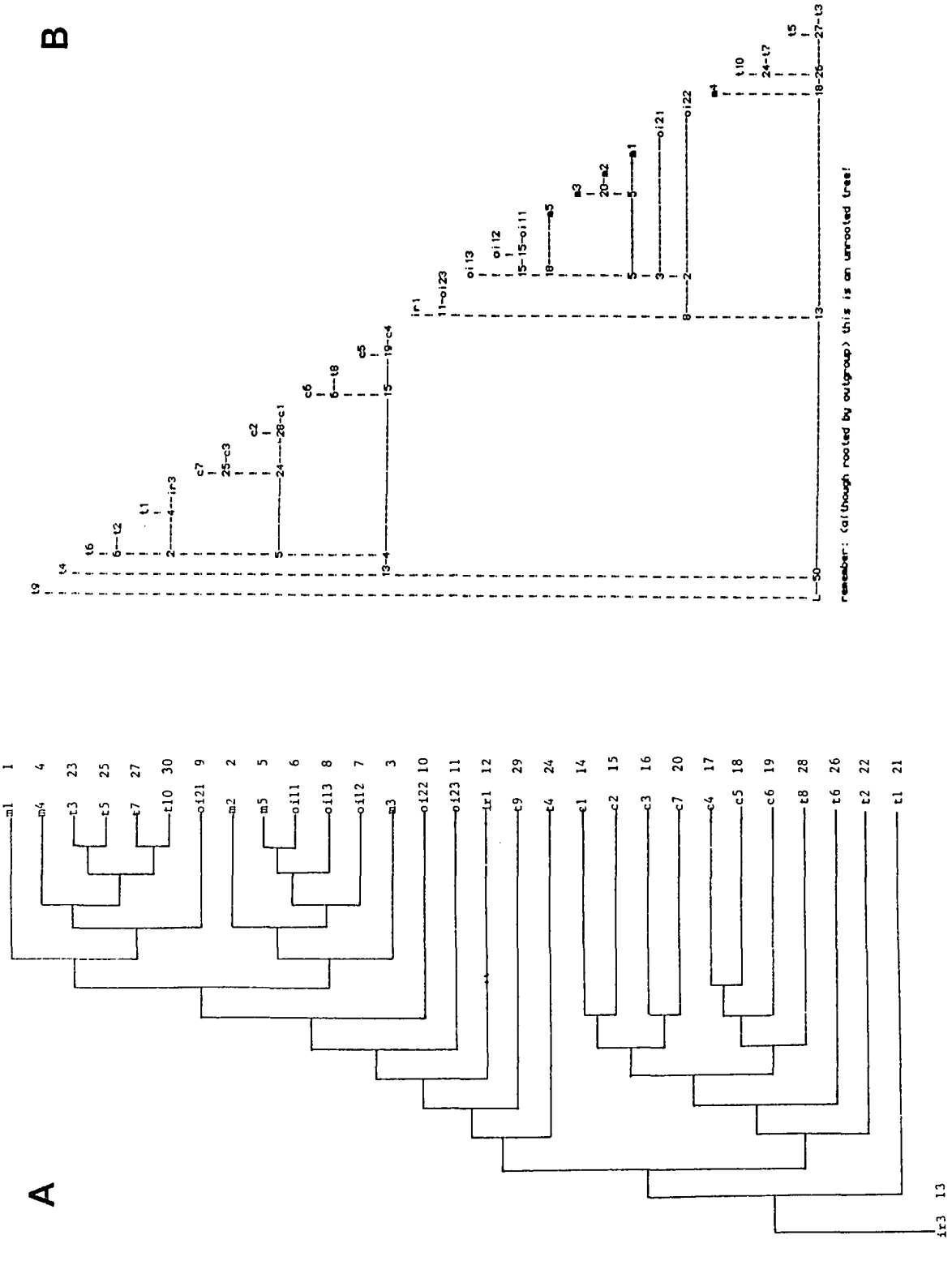
Table 4, AB and PA are significantly different ($G_h = 62.5$ with 16 degrees freedom, $p < 0.005$).

Considering genetic distance and percent sequence divergence, variation on the whole among the three taxa *mercenaria*, *campechiensis* and *texana* occurs at the level of species. Comparisons from Table 6 between the taxa *mercenaria* vs. *campechiensis* (average D = 0.527) and *mercenaria* vs. *texana* (D = 0.868) are consistent with interspecies comparisons. However, compared to the other interspecies distances estimated in the present study, the genetic distance between *campechiensis* and *texana* is relatively low (D = 0.323).

The δ values from Table 7 indicate a slightly different relationship. Comparisons between all three taxa yield quantitatively similar estimates of percent sequence divergence. Although not significant, δ for *mercenaria* vs. *campechiensis* is slightly higher than for *mercenaria* vs. *texana* (average $\delta = 0.044 \pm 0.027$) and for *campechiensis* vs. *texana* (where $\delta = 0.020$).

The D and δ values calculated between populations indicate similar degrees of mtDNA divergence between all three taxa. Therefore, the degrees of relatedness between haplotypes of these taxa (δ values between each haplotype) were investigated in order to determine whether the maternal ancestry of *texana* could be identified. Several different runs of BOOT and PAUP were conducted with virtually every possible haplotype (as well as several hypothetical haplotypes) tried as outgroups. Examples of phylogenetic outcomes from both BOOT and PAUP are shown in Figure 10. There was one general outcome of these phylogenetic analyses. They consistently resolved haplotypes into two primary groups: a *mercenaria* / *texana* complex and a *campechiensis* / *texana* complex. This result combined with a qualitative interpretation of the *texana* haplotypes listed in Table 2 indicates that the *texana* group may be of multiple maternal origin.

Figure 10. Phylogenetic clustering of *Mercenaria spp.* haplotypes by two computer programs, PAUP and BOOT. Data were input as a matrix of the presence/absence of each restriction fragment for all haplotypes in the taxa *mercenaria, campechiensis*, and *texana*. A. Phenogram from the PAUP package interrelating haplotypes. Both the code and the haplotype from Table 3 are shown at the terminal end of each branch in the tree. B. Phenogram from the BOOT program of the PHYLIP package. Only the code appears at the terminal end of each branch in the tree. Numbers at the forks indicate the numbers of times the group consisting of haplotypes above and to the right of that fork occurred among 50 bootstrap replicates.



# DISCUSSION

## I. Characteristics of hard shell clam mtDNA

The characteristics of clam mtDNA are unique as compared to the majority of higher animals studied to date. Few of the generalizations frequently claimed for mtDNA (*cf.* INTRODUCTION and reviews by Avise and Lansman 1983, Brown 1983) were found to hold true for *Mercenaria spp.* Unique attributes include the facts that 1) clam populations demonstrate polymorphism in mtDNA size, 2) this size polymorphism is often manifest as heteroplasmy in individual clams, and 3) individual clams are also encountered which are heteroplasmic with respect to the presence or absence of restriction sites. Such attributes are not unheard of in studies of mtDNA variation. Observations of extensive size polymorphism within populations and size heteroplasmy are becoming more frequent. Hale and Singh (1986) described size polymorphism and size and site heteroplasmy in natural populations of *Drosophila melanogaster.* Bermingham *et al.* (1986) described size polymorphism and size heteroplasmy in the fish *Amia calva* and two species of *Hyla* frogs. Chapman (1987) observed size polymorphism and size heteroplasmy in another fish, *Morone saxatilis* and Mulligan and Chapman (in press) have described extensive size heteroplasmy in the white perch, *M. americana.* There are also numerous other reports including those of size polymorphism in populations of crickets (Harrison *et al.* 1985) and lizards (Moritz and Brown 1986, 1987).

A. MtDNA size polymorphism in clam populations

In the present study, size polymorphism in clam populations occurred at a frequency of 42%; i.e., only 58% of the individuals assayed had the common size of mtDNA, 17.5 kb (refer to Figure 2). This observation is similar to that of Snyder *et al.* (1987) who reported a high frequency of size polymorphism in natural populations of the scallop, *Placopecten magellanicus*. This bivalve is unique in that its mtDNA is atypically large. In their study, only 50% of the animals assayed had the common mtDNA size (34 kb). The rest of the individuals possessed mtDNAs ranging in size from 32.1 to 39.3 kb. In most other studies where size polymorphism was detected, it has occurred at maximum frequencies of *ca.* 20%.

The utility of employing mtDNA size variants as indicators of genetic and evolutionary relationships is subject to debate (Moritz *et al.* 1987). Chapman (1987) for example, found that Chesapeake Bay striped bass populations were characterized by six mtDNA length polymorphisms rather than restriction site differences. His results were concurrent with previous stock delineations based on morphology and isozyme variation while providing for a greater separation among populations. Several breeding studies have been conducted on the transmission of mtDNA size variation (fruit flies, Solignac *et al.* 1984, 1987; and crickets, Harrison *et al.* 1985, Rand and Harrison 1986). The former studies indicated that mtDNA size variants are inherited without constraint and that the processes of genetic drift and sorting operate as previously described. The latter studies showed shifts in the frequencies of size variants from heteroplasmic mother to offspring which they attributed to selection for smaller mtDNAs. Based on their results, Harrison *et al.* (1987) emphasize that size variation is not a useful marker for studying population structure in crickets. It is likely that size variation in each of these examples occurs at different rates and by different processes of transmission and segregation.

In many cases mtDNA size variation is traced to tandem duplication of a particular segment located within the control region: the D-loop (Moritz *et al.* 1987). The repeated segments range in size from very small (10 bp in cattle, Olivo *et al.* 1983) to very large (as much as 8.0 kb in the lizard *Cnemidophorus cozumela cozumela*, Moritz and Brown 1987 and 8.5 kb in the newt *Triturus*, Wallis 1987). In clams, mtDNAs were found to differ by increments of *ca.* 500 bp with some molecules observed as small as 16.5 kb and some as large as 19.5 kb (Figure 2). However, the canonical discriminant analysis detected no correlation between geographic location and size polymorphism in clam populations $r^2 = 0.0919$. Data from the present study indicate that mtDNA size variation is not an effective discriminator of population structure in clams. Therefore, since the primary objective of this study was to determine geographic patterns in clam mtDNA variation, no attempt was made to elaborate upon the molecular dynamics giving rise to size polymorphism in *Mercenaria spp.*

B. MtDNA size heteroplasmy in individual clams

Heteroplasmy for mtDNA size has been documented in natural populations of several species as mentioned above. Frequencies of size heteroplasmy range from quite low (0.06% in the lizard *C. tesselatus*, Densmore *et al.* 1985) to as high as 100% (*M. americana*, Bowen 1987 and Mulligan and Chapman, in press; *Rana esculenta*, Monnerot *et al.* 1984). Size heteroplasmy in the clam populations censused by the present study occurred at an average frequency of 22% (ranging from 0 to 89%). However, like mtDNA size polymorphism in populations, size heteroplasmy in individuals is not an effective discriminator of population structure in clams. The canonical discriminant analysis indicated that the occurrence of size heteroplasmy was not correlated with sample location, $r^2 = 0.5338$.

Size heteroplasmic clams were given restriction genotypes for each enzyme based on the standard fragment patterns shown in Figure 3. It is noted that classifying individuals differing in size but not site as the same genotype as those which are not size heteroplasmic results in the loss of information, especially with regard to calculation of D and δ. However, at this time no reasonable method is available to account for size variation in such a polymorphic population. By referring to Figure 3 and Table 1 where the 12 size-variable fragments are marked by an "*", a quick calculation shows that if patterns due to different sized molecules had also been assigned unique designating names, there would have been at least 99 different restriction fragments for the five main mtDNA sizes and the six enzymes. As it is, 51 distinct fragments were identified.

There is no concensus of opinion as to the molecular dynamics leading to size heteroplasmy in mtDNA. Size differences within individuals could be due to accumulation of small-scale additions/deletions (Bermingham *et al.* 1986) or to large insertion events (Clarke 1988, Hale and Singh 1986, Wallis 1987), and/or to processes such as replication slippage (Moritz and Brown 1987, Moritz *et al.* 1987, Streisinger *et al.* 1966). What is clear from the current literature however, is that in organisms which demonstrate extensive size heteroplasmy, the condition arises by frequent mutation (Bentzen *et al.* 1988, Hale and Singh 1986, Rand 1987 Rand and Harrison 1986) and that larger-sized mtDNA variants may be at a selective disadvantage (Bentzen *et al.* 1988, Rand and Harrison 1986).

C. Restriction site heteroplasmy in hard shell clams

Reports of restriction site heteroplasmy are rare in the primary literature. Hale and Singh (1986) found one instance of site heteroplasmy in 92 isofemale lines of *D. melanogaster*. Satta *et al.* (1988) report restriction site heteroplasmy in *D. simulans*. Bentzen *et al.* (1988) found 4% of shad (*Alosa sapidissima*) to be site heteroplasmic. In

the clam populations studied here, 12% of the individuals were heteroplasmic with respect to restriction site. A typical example of the site heteroplasmy observed in clams is shown in Figure 6. The restriction site heteroplasmy was geographically widespread, occurring in four of the nine populations and like size polymorphism and size heteroplasmy was not correlated with latitude as determined by the canonical discriminant analysis ($r^2 = 0.1830$).

Although infrequently observed, site heteroplasmy can be accounted for by theoretical models of mtDNA transmission genetics (Birky *et al.* 1983, Chapman *et al.* 1982, Clark 1988, Takahata and Maruyama 1981). Under these models the condition may arise via recent mutation within a single female generation or by paternal transmission. In general, these theories predict that mtDNA variants arising by either mechanism are rapidly sorted out by stochastic sampling processes in most organisms, resulting in homoplasmic offspring. In order to account for the increasingly frequent observation of extensive heteroplasmy, additional models have been proposed by Clark (1988). Clark's models include the following factors: mutation, natural selection, mutation/selection, and paternal leakage.

Any or all of these factors could be employed to explain the empirical observation of site heteroplasmy in clams. For example, the mutation rate in clam mtDNA may be higher than the rate at which the new mtDNA variants are sorted out within the cell lines of individual clams. Of course, this is contrary to the observation in other organisms that heteroplasmy for size is much more common than for site. Alternatively, there may be a fitness advantage for some heteroplasmic clam genotypes. For example, heteroplasmic genotypes for *Eco*R I (A/D and C/A/D) were observed in 9% of the assayed individuals as calculated from Appendix D. A combination of rapid mutation rate for base substitution with fitness differences/efficiency of mtDNA replication associated with

mtDNA size may also account for the degree of heteroplasmy observed in this study. Such a mechanism was proposed by Hale and Singh (1986).

Another factor which could generate the levels of restriction site heteroplasmy observed in clams is the possibility of a paternal contribution to the zygotic mtDNA pool (paternal leakage). Satta *et al.* (1988) have observed heteroplasmy in *Drosophila* which is best explained by paternal transmission of mtDNA. In addition Chapman and Brown (submitted) give empirical support for paternal transmission in fishes. In the case of pelecypods it is known that both the sperm head and tail are taken into the egg at fertilization (Kume and Dan 1968), indicating that mitochondria from the sperm midpiece are likely to be incorporated into the clam zygote. Such an occurrence is also known in fishes (Brummett and DuMont 1979). The possibility of paternal transmission is reinforced by records that molluscan spermatozoa enter eggs at the vegetal pole and lie in position near that pole until both the first and second polar bodies have been extruded at the animal pole (Kume and Dan 1968). Finally, according to Kume and Dan (1968) egg and sperm pronuclei come together and unite near the center of the egg only after the maturation divisions of the egg have been completed. Thus, it is possible that paternal mtDNA may be contributed to the mitochondrial pool of the developing embryo and if the paternal complement is different from the maternal mtDNA then the offspring may be heteroplasmic.

Conventional mtDNA theories do not account for intermolecular recombination events. Although no mechanism is known for such a process, evidence has recently been obtained indicating that recombination does in fact occur between mtDNA molecules (C. Stine, pers. comm.). Therefore, this mechanism may also be proposed to account for the extensive site heteroplasmy observed in *Mercenaria.*

II. Geographic variation in *M. mercenaria* mtDNA

Although $G_h$ tests conducted enzyme-by-enzyme (Figure 7) indicated homogeneity of east coast *M. mercenaria* populations, the $G_h$ analyses by haplotype (Table 4) implied that only the following populations are relatively homogenous: GS, NC, I1 and I3. The exclusion of WA from this group is due to the frequent occurrence of the *Eco*R I genotype A/D which is also found in O1 and AB. The occurrence of this genotype may indicate the influence of offshore *M. campechiensis*. In addition, other populations also have unique diagnostic haplotypes which occur at high enough frequencies to be useful in population discrimination (e.g., AADAA in NC, Table 2). A major finding then of this study is that mtDNA analysis does provide for the discrimination between east coast *M. mercenaria* populations.

The remaining analytical treatments, estimation of D and $\delta$, employed information on shared fragments (which were assumed to indicate homologous sequences of mtDNA). Estimates of D and $\delta$ are better indicators of geographic structuring than $G_h$ because they account for more than qualitative differences in patterns--they account for similarities and differences between haplotypes. These analyses therefore provide quantitative estimates of the degree of genetic relatedness between the sampled clam populations. In addition, there is a benefit of $\delta$ over D due to the fact that $\delta$ is corrected for intrapopulation variation which is in some cases quite substantial (see I values in Table 5).

The estimates for D, pairwise genetic distance, are significantly greater than those of Humphrey (1981) whose data on allozyme variation along the same geographic range indicate D = 0.001 to 0.016. Thus it is clear that mtDNA is a better discriminator of genetic variation among east coast populations of *M. mercenaria*. The high degree of similarity between the northern populations (GS, WA and NC) indicated by the D and $\delta$ values in Tables 6 and 7 may be in part due to the homogenizing effects of gene flow

(both artificial and natural). Artificial gene flow could result from relaying or transplanting of clams between these areas followed by interbreeding with native clams. Although this cannot be discounted, every effort was made to sample clams from natural areas where the possibility of artificial gene flow was minimal. In contrast, the potential for natural gene flow in *M. mercenaria* could be appreciable due to extensive larval dispersal. Under reasonable environmental conditions, clam zygotes develop into straight-hinge veliger larvae in two days. The veliger stage remains planktonic, feeding on microalgae for up to four weeks (Loosanoff and Davis 1951). During this time larvae may be widely dispersed depending on the strength of water currents and the extent of tidal cycles in which they are entrained. Thus, the species can be dispersed as though it were motile simply because its life history involves a planktonic larval stage. Even so, it is known for many species that gene flow is often much less than would be expected given the ability of an organism to disperse (Endler 1977).

As stated earlier, the overall estimate of effective migration rate calculated from the total sample's mtDNA data is $N_em = 0.717$. This value indicates that on the whole, the extent of gene flow between local *M. mercenaria* populations is intermediate and comparable to that expected based on studies of gene flow in other marine pelecypods. Buroker (1984) made qualitative estimates of gene flow between contiguous mainland as well as insular populations of the oyster genus *Crassostrea*. Using his data and the equation from Slatkin's (1985) private alleles technique for estimating $N_em$ (see Chapter 2 of this dissertation) it can be seen that all populations he sampled, both contiguous ($N_em = 2.4$) and insular ($N_em = 8.8$), demonstrate high levels of gene flow. Slatkin (1985) also found extensive gene flow ($N_em = 42.0$) when analyzing nuclear allele data from *Mytilus edulis*. Such a high value for $N_em$ indicates panmixia, although no information is included as to the geographic extent of sampling of the mussel populations.

When estimates of $N_em$ calculated pairwise between *M. mercenaria* populations are considered (Table 8) two general conclusions can be made. First, the northern east coast populations GS, WA and NC appear to experience high levels of interpopulation gene flow ($N_em$ = 2.0 to 4.7). This corroborates the implications of the genetic distance and percent sequence divergence analyses. Second, duplicate samples (O1 and O2, I1 and I3) exhibit very high effective migration rates ($N_em$ = 3.7 and 20.4, respectively). This is expected if the assumption of random mating holds true for local nondisjunct populations of clams.

The accuracy of the $N_em$ values in Table 8 was investigated by comparison with $N_em$ calculated with data from a recent investigation of nuclear allele variation in natural east coast clam populations. Data were extracted from Dillon and Manzi (1987) which yielded an estimated $N_em$ value between Massachusetts and Virginia of 2.5 by the private alleles approach. This is quite similar to the northern east coast levels of gene flow calculated here on the basis of mtDNA.

One additional feature of east coast *M. mercenaria* populations is worthy of discussion. $G_h$ tests (Figure 7 and Table 4) and the canonical discriminant analysis by population (Figure 8) indicate a relationship between O1 and AB clams. Estimates of D between O1 and other *M. mercenaria* populations in Table 6 are very high and $\delta$ values in Table 7 for O1 are an order of magnitude greater than those for the other intraspecies comparisons. Closer inspection of the data indicates similarities in *Bam*H I and *Eco*R I genotypes between O1 (*mercenaria*) and AB (*campechiensis*) reflected by the low percent sequence divergence between O1 and AB ($\delta$ = 0.02, Table 7). This prompted a more thorough enquiry into the distribution of *M. campechiensis*. It was found that *M. campechiensis* once existed in commercial quantities off the North Carolina coast near Beaufort Inlet (Porter and Chestnut 1960) and off Oregon Inlet (H. Porter, pers. comm.). This would account for the observation of *campechiensis*-like genotypes in O1

(individuals 6,13-16,20-22, Table 2), in O2 (individuals 5 and 6, Table 2), in WA (individuals 9, 15-19, Table 2) and also in NC (individual 5, Table 2). There apparently exists some degree of natural hybridization between *M. mercenaria* and *M. campechiensis* along the east coast.

On the other hand, none of the analyses conducted in the present study indicated as great a presence of *M. campechiensis* in the Indian River area as was reported by Dillon and Manzi (submitted). The only indication of *M. campechiensis,* is the occurrence of *campechiensis*-like genotypes for *Eco*R V, *Pst* I and *Pvu* II in the haplotypes of three Indian River individuals. Recall that the $G_h$ tests by haplotype shown in Table 4 indicated that GS, NC, I1 and I3 were the only group of populations studied which are not signigicantly different ($0.05<p<0.10$). If previous reports are correct (87.5 % hybrids) then the bulk of hybrids at the Indian River, FL location must be of *M. mercenaria* maternal origin indicating unidirectional gene flow. Empirical studies to date support neither reproductive isolation in areas where the two species are sympatric nor a fitness effect of maternal ancestry in clams.

III. MtDNA variation between the taxa

$G_h$ tests conducted enzyme-by-enzyme (Figure 7) indicate that for four of five enzymes employed, *M. campechiensis* and *M. mercenaria texana* are not significantly different although the $G_h$ based on haplotypes (Table 4) does discriminate between the two taxa ($p < 0.005$). The similarity between the taxa *campechiensis* and *texana* is reiterated in the genetic distance analysis. The value of D calculated between *campechiensis* and *texana* of 0.323 in Table 6 is much lower than the average interspecies comparisons for *mercanaria* vs. *texana* ($D = 0.868 \pm 0.131$) and somewhat lower than the average comparison of *mercanaria* vs. *campechiensis* ($D = 0.527 \pm 0.094$). However, as stated earlier, D values are not corrected for intrapopulation

variation and may be misleading. Therefore, percent sequence divergence must be considered as well.

Unlike the interspecies comparisons for genetic distance, $\delta$ values between the taxa *mercenaria* and *texana* are not significantly different from those between *mercenaria* and *campechiensis* ($\delta = 0.044 \pm 0.027$ and $\delta = 0.053 \pm 0.015$, respectively from Table 7). However on the whole, the interspecies comparisons are an order of magnitude greater than intraspecies sequence divergences (excluding Oregon Inlet) leading to the conclusion that *mercenaria* are roughly as different from *texana* as they are from *campechiensis*. In all probability, *texana* deserves species distinction separate from *M. mercenaria*.

IV. Phylogenetic analysis of mtDNA haplotypes

Given the multiplicity of haplotypes exhibited by the PA clams, the extreme divergence detected in the GA sample, the limited potential for gene flow between *mercenaria*, *campechiensis*, and *texana* as indicated by known Gulf Stream circulation patterns, and the rather extensive genetic divergence between the three taxa indicated by estimates of D and $\delta$, there is little indication that *texana* are in fact natural hybrids as proposed by Menzel (1970). If *texana* were in fact hybrids, one would expect to find *mercenaria* or *campechiensis* haplotypes or both, instead of the divergent haplotypes observed here. Therefore, the systematic implications for *texana* were investigated further by determining relationships between haplotypes of the three taxa.

The phylogenetic analyses shown in Figure 10 indicate that the group of *texana* haplotypes observed in the PA sample is polyphyletic; i.e., the haplotypes are descended from two or more maternal lineages. Evidence for this comes from the BOOT (bootstrapped mixed parsimony) and PAUP analyses (shown in Figures 10a and 10b, respectively) which clustered some *texana* haplotypes with *mercenaria* and some with

*campechiensis*. In almost every instance the *texana* haplotypes t3, t5, t7 and t10 clustered with *mercenaria* haplotypes m1, m4 and oi21, while *texana* haplotypes t2, t6 and t8 clustered consistently with the 7 *campechiensis* haplotypes (codes referring to haplotypes are shown in Table 3). The pattern of clustering indicates at least two distinct maternal origins for the PA population of *texana*.

This haplotypic distribution can be accounted for by the theoretical genetic models of Avise *et al.* (1984) and Neigel and Avise (1986) which involve stochastic extinction ("phylogenetic sorting") of mtDNA lineages. In essence, assuming that the speciation event in *Mercenaria* was relatively recent, *texana* would be expected to be polyphyletic in maternal ancestry for *ca.* 2-4 $k$ generations (where $k$ = carrying capacity). In other words, the length of time since reproductive isolation of these species may not yet have been sufficient to allow sorting and random extinction of mtDNA lineages to result in monophyly. By making some conservative assumptions as to generation length and carrying capacity, application of these models indicates that the speciation event separating *texana* from the other taxa has occurred relatively recently.

# CONCLUSION

Where analysis of genetic variation in nuclear alleles has failed to discriminate between Atlantic coast populations of *M. mercenaria*, mtDNA analysis succedes. Although there appears to be a good deal of gene flow along this range, associated with pelagic larval dispersal, the more southerly populations sampled in this study exhibit significant regional differentiation among one another based on restriction fragment digestion profiles. The degree of mtDNA variation exhibited by clams is greater in magnitude than other coastal marine species studied to date, but the pattern of variation is somewhat consistent: genetic homogeneity of northern populations and regional differentiation of southern populations (*cf.* Saunders *et al.* 1986).

In a study of the horseshoe crab *Limulus polyphemus* by Saunders *et al.* (1986), two widely divergent haplotypic assemblages were detected in samples taken from New Hampshire to the Gulf of Mexico. The northern assemblage consisted of a few genotypes which were very closely related while the southern assemblage was much more diverse. The two haplotypes differed by at least nine mutational steps over a very short geographic distance in northern Florida. However, unlike the Saunders *et al.* (1986) study of *Limulus*, there is little evidence of a distinct north-south genetic break in the east coast clam samples. On the contrary, although the clams exhibit many highly distinct mtDNA variants, they are either widely dispersed (e.g., the *Hin*d III A and B genotypes for populations with this information listed in Appendix D) or they are restricted to single populations as private alleles (e.g., genotype H for *Eco*R V as in Appendix D). Rather, in the case of hard shell clams mtDNA analysis indicates a zone of

hybridization between the two sibling species *M. mercenaria* and *M. campechiensis* in the vicinity of Oregon Inlet, NC. MtDNA analysis also provides information on another estranged zone of hybridization, Indian River, FL. The populations sampled in this study from the Indian River area near Ft. Pierce, FL do not show evidence of extensive hybridization between the taxa *mercenaria* and *campechiensis* as previously predicted.

The appropriateness of two of the most widely used hard shell clam breeding strategies can be evaluated on the basis of the data presented here. Both practices stem from the assumption that significant genetic variation exists between the natural stocks from which founding parents are derived. These approaches to clam husbandry may be termed "site-specific" and "trait-specific." The former approach employs breeding of clams of varied geographic origin along the Atlantic coast in order to obtain a more productive stock. Evidence based on mtDNA variation indicates that sufficient genetic differences do not exist between the more northerly populations of *M. mercenaria* to warrant their use in such programs. Thus many of the growth differences observed in breeding studies involving exclusively northern *mercenaria* stocks may have been due solely to environmental variation or simply to management practices within the hatchery and growout phases of clam culture. However, based on the levels of genetic variability within and between east coast clam populations presented here, founding stocks may now be prudently chosen which demonstrate sufficient genetic variability to warrant their use as base populations in selective breeding programs.

The "trait-specific" approach to breeding involves hybridization of the closely related species *M. mercenaria* and *M. campechiensis*. Morphological similarities and possible large scale hybridization in natural zones of sympatry of the two species have perpetuated the controversy over their separate species status. Data on mtDNA variation presented here indicate that considerable genetic divergence has occurred between the

two and as such will be useful in identifying base populations for use in trait-specific breeding approaches as well.

The information of mtDNA variation collected here is also pertinent to another taxonomic unit within the genus *Mercenaria*. The estimates of genetic distance and percent sequence divergence were sizeable and similar for the three taxa: *M. mercenaria*, *M. campechiensis*, and *M. mercenaria texana* indicating similarity in the degree of genetic divergence which has occurred between them. This supports the contention that *texana* deserves species distinction separate from *M. mercenaria*. Phylogenetic analyses were performed based on the relatedness between haplotypes of the three taxa. These analyses showed evidence that *texana* has arisen relatively recently and that it is a polyphyletic complex, having multiple (minimally two) distinct maternal origins.

# CHAPTER 2

Population variation in the mitochondrial DNA of the killifish, *Fundulus heteroclitus*

# INTRODUCTION

In Chapter 1 of this dissertation, considerable attention was paid to the effects of *gene flow* upon current distributions of mtDNA haplotypes in natural populations. This is as it should be, because gene flow figures prominently in virtually all theories of evolutionary and population genetics. Gene flow is not, however, the only force molding genetic distributions in nature. Since the origin of population genetics theory during the turn of the century, it has been widely appreciated that *finite population size, natural selection and non-random mating* could also play important roles in gene frequency distribution. A great deal of study has been directed toward determination of the relative magnitude and interactions of each of these forces, both theoretically and empirically.

In the last portion of this dissertation, an illustration of the complexity of the interaction among these forces will be addressed using the killifish, *Fundulus heteroclitus*. Unlike hard shell clams, the killifish is considered to be a species of limited dispersal potential. Strong gene frequency clines have been observed in this species and considerable debate exists regarding the primary forces maintaining these clines. This chapter first reviews the knowledge regarding patterns of genetic variation in *F. heteroclitus*. Next, a description is provided of current hypotheses pertaining to the maintenance of killifish gene frequency clines and the adaptive importance of genetic variation. Finally, the issue of limited gene flow in this species is investigated by analysis of mtDNA variation within and between killifish populations sampled on a very fine scale.

I. Synopsis of *Fundulus heteroclitus* genetics, biochemistry and physiology

A. Ecology and life history

The killifish, *Fundulus heteroclitus*, is widely distributed along the North Atlantic coast from Labrador (Kendall 1909) to Mexico, including Delaware and Chesapeake Bays (Hildebrand and Schroeder 1928, Bigelow and Schroeder 1953). Its extensive range is not surprising considering it can withstand salinities in the range 0 - 120.3 ppt (Griffith 1974) as well as temperatures from -1.5 to 36.3°C (Garside and Chin-Yuen-Kee 1972). The habitat of *F. heteroclitus* is typified by shallow brackish coves, tidal creeks and salt marshes (Hildebrand and Schroeder, 1928; Bigelow and Schroeder, 1953). Although killifish populations are distributed over a wide and seemingly continuous geographic range, the actual distribution of their habitats is often clumped and disjunct. Where these shallows do occur, killifish are a key component of the estuarine food web--playing an important trophic role as both predator and prey (Kneib 1986).

Killifish mature sexually during their first year of life and have a maximum life expectancy of four years (Valiella 1977). It is probably safe to assume that individuals spawn only once based on an average life expectancy of one to two years. Newman (1907) observed pairwise mating in killifish after a brief period of courtship and Relyea (1967) noted that eggs were small, demersal and adherent. Killifish are known to exhibit egg stranding behavior (similar to that of the grunion) which presumably increases survival (Able and Castagna 1975, Taylor 1976). Although no studies have been conducted pertaining to larval and juvenile dispersal, Kneib (1986) and Taylor *et al.* (1979) observed that larvae remained in the shallow pools of the hatching zone (and even on the surface of the marsh during low tides) for up to two months before they began to move with the tidal flow as do adults.

Bigelow and Schroeder (1953) contend that the killifish is a stationary species. Conversely, Massmann (1954) observed that killifish often ascend purely freshwater streams for great distances (e.g., as many as 72 km above the brackish/freshwater line in James River, VA). Spawning migrations were observed in the Raritan River, NJ by Chichester (1920) to progress from the mouth of the river to the brackish/freshwater line. There the fish remained for the duration of the summer and moved back to deeper channel waters with the onset of cold weather. Lotrich (1975) conducted a tagging study in a tidal creek in Delaware. He estimated a very restricted summer home range of 36 m for adult *F. heteroclitus* with a possible annual dispersal of 2 km associated with overwintering in freshwater areas. However, several anomalous recaptures (as much as 375 m away from the site of original capture) indicate that the actual home range of *F. heteroclitus* may not be so limited as his observations suggest.

On the whole, the available information of *F. heteroclitus* ecology and life history suggests a very low potential for short range dispersal during the breeding season associated with only a limited migratory potential. This implies decreased gene flow and favors geographic differentiation in gene frequencies.

B. Zoogeography of *F. heteroclitus*

The zoogeography of *F. heteroclitus* has been well studied by Relyea (1967) who confirmed that two morphological races of *F. heteroclitus* inhabit the north Atlantic coast and Chesapeake Bay. In addition, he proposed a likely scenario for the recent evolutionary past of *F. heteroclitus* based on his data of observed morphological distributions. Reylea (1967) postulated that the most recent Pleistocene glaciation probably pushed *F. heteroclitus* southward to the vicinity of the Suwanee Straits and/or the Miocene Islands. Then during the Miocene, this "pre-*heteroclitus*" as he puts it

founded a fundulid stock on the Miocene Islands which differentiated into *F. grandis* now endemic in the Gulf of Mexico.

Relyea (1967) hypothesized that subsequent to the most recent glaciation, *F. heteroclitus* populations have remained continuous along the Atlantic coast and have undergone racial differentiation into northern and southern races in response to genotype/environment interactions. He notes that racial differences in *F. heteroclitus* are parallel to those demonstrated by *F. majalis* and other inshore marine fishes (e.g., *Menidia spp.*) which also exhibit faunal distinctions north and south of Cape Hatteras, NC. Reylea (1967) did not recognize significant effects of geological events on the racial differentiation in *F. heteroclitus* nor did he discriminate the two forms nomenclaturally.

C. Studies of geographic variation in *F. heteroclitus* morphological characters

Other researchers have also detected extensive geographic variation in morphological characters (*cf.* Brummett and Dumont 1981, Morin and Able 1983, Able and Felley 1986). In these studies, investigators examined egg morphology and found distinct differences between northern and southern populations. Morin and Able (1983) showed that several characters varied clinally both along the north Atlantic coast and within the Chesapeake and Delaware Bays. There existed a concordance such that morphologies of fish from the upper Bays corresponded to those from the north Atlantic while the morphologies from the southern Bay locations corresponded to morphologies from the south Atlantic. When the extent of difference between the environments of the upper Bays and the north Atlantic were noted they hypothesized that it was much more likely that the clines arose due to secondary intergradation of relict populations rather than due to a selective response to an environmental gradient.

D. Inquiry into *F. heteroclitus* genetics

The genetics of killifish have been studied in depth over the past twenty years. Via protein electrophoresis, at least 45 enzyme-coding loci have been examined. Studies of killifish genetic loci have revealed not only high degrees of allelic polymorphism (Cashon *et al.* 1981, DiMichele *et al.* 1986, Holmes and Whitt 1970, Mitton and Koehn 1975, Palumbi *et al.* 1980) but extensive zoogeographic variation as well (Able and Felley 1986, Morin and Able 1983, Place and Powers 1978, Powers 1972). This zoogeographic variation is often manifest as gene frequency clines (*sensu* Huxley 1942: a gradient of a measurable character [such as gene frequency change] along a geographic transect of the species' range). A review of available data indicates that two races or subspecies of *F. heteroclitus* are evolving and that their populations interact to form the clines. These clines have been shown to be stable and correlated with steep thermal and salinity gradients.

Patterns of *F. heteroclitus* genetic variation have been characterized by Powers *et al.* (1986). Of the 16 enzyme-coding loci which have been examined in killifish populations along the eastern Atlantic coast of North America, all but two exhibit clinal changes of one type or another in allele frequency. Four of these loci are distinctly clinal with northern killifish populations fixed for one allele while southern populations are fixed for the alternative allele. Of alleles at eight other loci showing directional changes with latitude, northern populations are fixed for one allele while killifish from other latitudes express from two to six alternative alleles. Finally, two loci are fixed for the same allele in populations at both the extreme northern and southern ends of the transect and are variable only in middle latitude populations. In general, the contact zone between northern and southern genotypes, or center of the cline, occurs at *ca.* latitude 41°N which corresponds to Vince Lombardi's Truckstop off of Interstate 95 in New York , NY.

1. Thermal shifts in *F. heteroclitus* clines

Electrophoretic protein patterns of Chesapeake Bay killifish populations suggest clines in gene frequency similar to north Atlantic east coast clines. Approximately 50% of the loci that have been studied in both north Atlantic and Chesapeake Bay populations vary in a similar manner such that the "northern" allele for an east coast cline is the predominant allele detected in upper Chesapeake Bay samples. In direct comparisons of east coast and Chesapeake Bay clines with thermal profiles, Powers *et al.* (1986) show that Chesapeake Bay gene frequency clines are displaced southward by distances of 160-320 km. They then note that Chesapeake Bay water temperatures (location approximately 37-39°N latitude) are cooler that east coast waters of the same latitude. Rather, Chesapeake Bay temperatures are roughly the same as east coast water temperatures 160-320 km north (approximately 39-41°N latitude). They take this concordance of clines as the first line of evidence supporting thermal selection in *F. heteroclitus*.

A second line of evidence taken to support thermal selection is related to the locations of clines for several *F. heteroclitus* loci. Analytical treatments indicate that if different selective pressures act on different loci then the location of their clines may be shifted (Mayr 1970 Endler 1977). Just such shifts covering a distance of 864 km are empirically documented by Powers *et al.* (1986) for 11 protein loci.

2. Fitnesses and *F. heteroclitus* clines

One approach to verifying the significance of selection as a driving force of *F. heteroclitus* clines is to identify the existence of fitness differences between allelic isozymes. As the first step in this approach, several laboratory studies have examined the biochemical properties of allelic isozymes themselves and detected functional kinetic differences. Such results are exemplified by the detection of different catalytic

efficiencies of allozymes by Place and Powers (1979, 1984) and by correlations of metabolic differences with genotype found by Powers *et al.* (1979, 1983).

The second step is the identification of physiological differences in *Fundulus* genotypes which are associated with environmental variation. Mitton and Koehn (1975) have studied the responses of allozymes to variable environmental conditions. Similar responses have been described in reports of developmental, hatching and physiological differences associated with genetic variation at particular loci associated with environmental consequences such as respiratory stress (DiMichele and Powers 1982a, DiMichele *et al.* 1986).

Finally, biochemical and physiological differences must be shown to be relevant in the natural situation. As evidence, swimming performance differences related to differential kinetics of Ldh-B allozymes have been recorded by DiMichele and Powers (1982b) and by Powers *et al.* (1983) while Paynter *et al.* (submitted) have implicated environmentally induced selection at the same locus. All of these studies were aimed at establishing the adaptive significance of electrophoretic protein variants. Identification of fitness differences was taken as evidence of a direct mechanistic relationship between biochemical differences of allozymes and variation in environmental components.

E. The question of primary *vs.* secondary intergradation

Spatial variation in killifish gene frequencies has been attributed to selection associated with either primary intergradation (contact between geographic forms which diverged while remaining in contact) or secondary intergradation (contact between geographic forms which at one time diverged in isolation) as defined by Endler (1977). Relyea (1967) advocated primary intergradation and maintained that the geological information is not sufficient to explain the observed patterns of morphological variation in *F. heteroclitus* races. Conversely, Morin and Able (1983) were confident that two

subspecies of *F. heteroclitus* arose during the Wisconsin glaciation followed by secondary intergradation. Powers *et al.* (1986) asserted simply that natural selection is one of the fundamental driving forces which establish and maintain the *F. heteroclitus* clines. As evidence they presented data on the shapes and locations of nuclear gene frequency clines in the historical context of the most recent Pleistocene glaciation event as well as data from the previous studies of the adaptive significance of allozymes. However, without additional information they could not assign responsibility for the initial establishment of the cline to either primary or secondary intergradation.

F. Hypotheses of intergradation in *F. heteroclitus*

Based on observed zoogeographic variation in morphological and nuclear gene frequency, two intergradation scenarios have been considered. In the case of the primary intergradation model, Powers *et al.* (1986) suggest that during the [Wisconsin] glaciation, populations may have remained continuous characterized by a highly polymorphic gene pool but a very slow migration rate. As the glacier retreated, new cooler habitats were uncovered which were invaded by various well-adapted individuals. Clines in these areas of range-extension could have arisen in response to thermal selection and must have been maintained by reduced gene flow (slow migration and dispersal). Under these conditions the new populations could have differentiated genetically due to purely stochastic processes and would tend to be more and more homozygous as the range was extended northward. This scenario is supported by the ecological observations of Relyea (1967), by observations of reduced genetic variability of northern *F. heteroclitus* populations Powers *et al.* (1986) and by the concordance of clines for several loci between Chesapeake and North Atlantic populations (Place and Powers 1978, Powers and Place 1978, Cashon *et al.* 1981, Morin and Able 1983).

In the case of secondary intergradation, glaciation must have resulted in the fragmentation of habitat and formation of numerous disjunct populations. Genetic divergence of *F. heteroclitus* populations occurred in allopatry due to chance and/or adaptive forces. Retreat of the glacier and subsequent flooding of the areas with warmer water provided the context for secondary contact and mixing of the groups. In these zones of mixing, clines were formed which depended on selection, competition, rates of migration, *etc.* Comparisons made by Powers *et al.* (1986) of clinal variation between populations of Chesapeake Bay and the North Atlantic show north-south "shifting effects" (*cf.* Endler 1977) which tend to support the possibility of secondary intergradation. In addition, the concordance of clines which they observed is just as consistent with a model of secondary intergradation as it is with primary intergradation. Morin and Able (1983) cite additional evidence for secondary intergradation in *F. heteroclitus*. However, in their opinion, temperature and salinity dissimilarities between the upper Bay and North Atlantic coastal environments argues against selection and for differentiation by isolation.

II. Models of primary and secondary intergradation

Endler (1977) and Mayr (1942) have defined the processes of primary and secondary intergradation as they relate to zones of contact between phenotypically divergent populations. Primary intergradation describes the consequences of contact between geographically distinct but contiguous populations. Initial divergence between the groups is due to the gradual response of the ancestral population to selective pressure associated with an environmental gradient. Despite continuous contact, selection can overcome gene flow resulting in gene frequency clines.

Secondary intergradation is the term used to refer to consequences of contact between formerly disjunct populations. Isolating mechanisms evolve originally when

discontinuities in the environment segregate portions of the ancestral population which are small enough to allow genetic drift and/or adaptation to predominate. When contact is resumed, the zone of interbreeding usually exhibits steep gradients.

Once established, clines are affected by a complex of forces which relate to gene flow and balance. These include but are not limited to: barriers to gene flow (both physical and genetic), population density, and the rate of dispersal. Evidence of Barton (1983) and Barton and Hewitt (1985) indicates that geographic/environmental barriers may be of much greater consequence in the establishment of clines than in their maintenance.

The extent of selective pressure is determined by the degree of environmental variation and by genotypes and gene frequencies themselves. If the selective pressure favoring alternative alleles in different locations is greater than the rate of gene flow between them, then genetic differentiation will occur. Put another way, migration will decay clines unless sufficiently strong selection acts. If dispersal is very short range then selection pressures do not have to be large to elicit changes in gene frequency.

The effective rate of gene flow ($N_e m$) from larger subpopulations is greater than from smaller ones (Barton and Hewitt 1985, Endler 1977). In the event that the gene complexes of the emmigrants are not well adapted for the environment to which they disperse, the result is less gene flow than would be estimated from a direct observation of dispersal (Mayr, 1970). In addition, if population density fluctuates, the rate of gene flow can vary extensively over time. This causes large variance in census size and family size and can result in decreased effective population sizes ($N_e$) (Avise *et al.* 1988, Crow and Kimura 1970, Fisher and Ford 1950, Gall 1987). There is substantial evidence that effective gene flow ($N_e m$) tends to be spatially restricted between natural populations as compared to dispersal of individuals which is in turn much less than migration (Bishop 1972, Crumpacker and Williams 1973, Slatkin 1985b). Marine

species with planktonic larvae are usually considered to be an exception to this generalization (Scheltema 1971).

III. Mitochondrial DNA studies support the model of secondary intergradation

Powers *et al.* (1986) conducted a preliminary analysis of mitochondrial DNA (mtDNA) haplotypes of northern (Maine) and southern (Georgia) *F. heteroclitus*. Since the two races were found to be distinguishable the investigators suggested that mtDNA analysis might be an appropriate method by which to resolve the primary *vs.* secondary intergradation issue.

Smith (1988) presented data on mtDNA haplotype frequencies along the north Atlantic coast and within the Bay. He verified the existence of the northern and southern races within Chesapeake Bay. In addition, within the Bay proper he found southern genotypes while northern mitochondrial complements were restricted to the headwaters of its tributaries and to the extreme upper Bay. The transition from southern to northern genotype was found to be abrupt in both the Bay and Atlantic coast samples. This information strongly favors theories involving secondary intergradation and virtually negates primary intergradation in this species.

In light of the strong evidence supporting secondary contact between northern and southern races of *F. heteroclitus* we are left with the question "Are the observed gene frequency clines maintained by strong selection or by limited gene flow?" If the ecological and life history observations implicating a limited potential for gene dispersal in *F. heteroclitus* are correct (Able and Castagna 1975, Bigelow and Schroeder 1953, Hildebrand and Schroeder 1928, Lotrich 1975, Relyea 1967,Richards and Castagna 1970), then selective pressures required to maintain the observed clines need not be very large. On the other hand, if gene flow is extensive as suggested by the observations of

Butner and Brattstrom (1960), Chichester (1920), and Massmann (1954), then strong selection must be invoked to account for maintenance of the clines.

In *F. heteroclitus* there is little concordance in observations of dispersal and population size and as stated by Powers *et al.* (1986) there exists a precise estimate of neither one. Therefore, in order to address hypotheses concerning the adaptive importance of genetic variation and the effects of gene flow, a realistic estimate of the effective rate of gene flow, $N_em$, is necessary. Hence this study in which the issue of limited gene flow was investigated via mtDNA analysis of five partially isolated populations of *F. heteroclitus*.

## MATERIALS AND METHODS

I. Field collections and laboratory analysis

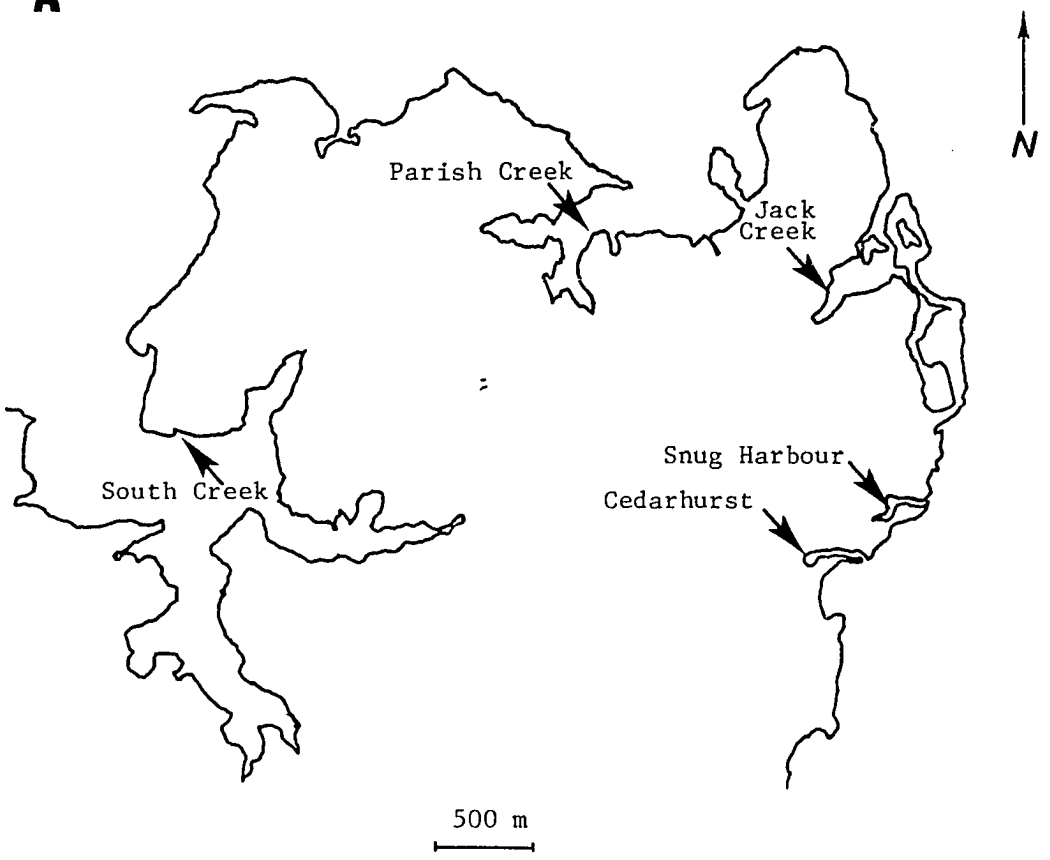
Adult specimens of *F. heteroclitus* were collected during late August and early September 1988 from five tidal creeks in Shady Side, MD. The sampling stations were located along a continuous shoreline at Cedarhurst, Snug Harbour, Jack Creek, Parish Creek and South Creek. These stations are shown in Figure 11. Approximately 100 individuals were sampled with minnow traps over a period of several days from each of the five locations for a total sample size of N = 480 individuals. The killifish were transported immediately to the laboratory at Chesapeake Bay Institute in Shady Side where they were sacrificed. When ripe females were obtained, eggs were used as the source for mtDNA extraction. Otherwise, livers were excised from both females and males as the tissue source for mtDNA.

The procedure for rapid extraction of mtDNA from individual *F. heteroclitus* was basically the same as outlined by Chapman and Powers (1984) with the following changes.

1. Tissue was homogenized in 0.25 M Sucrose TEK (Appendix B) using a Tekmar UltraTurak apparatus.
2. The sucrose step gradient centrifugation was omitted.
3. Mitochondrial DNA was precipitated by the addition of 0.6 volume of isopropanol, incubation at 4°C for 10 min, followed by 10 min microcentrifugation.

Figure 11. Location of samples and distances between sampling locales for *Fundulus heteroclitus* subpopulations taken along a continuous shoreline in Shady Side, MD. A. Map of Shady Side Penninsula in a Maryland area of upper Chesapeake Bay. Sites are indicated by an arrow. B. Shoreline distances in meters between sampling locations along Shady Side Penninsula.

**A**




**B**

| | Jack | Parish | Snug | South |
|---|---|---|---|---|
| Cedarhurst | 2712 | 4882 | 930 | 8370 |
| Jack Creek | | 2635 | 2325 | 5812 |
| Parish Creek | | | 4340 | 5115 |
| Snug Harbor | | | | 7905 |
| South Creek | | | | |

4. Mitochondrial DNA was resuspended in 50 µl sterile distilled water then frozen until digestion.

This procedure yielded sufficient mtDNA of high enough purity to perform 6 restriction enzyme digests per sample and detect the resulting fragment patterns by ultraviolet illumination alone.

In order to digest *F. heteroclitus* mtDNA, 8.5 µl aliquots of each purified sample were incubated in a total volume of 10 µl for 1 hr with the restriction endonuclease *Bcl* I, *Bgl* I, *Hin*d III, *Sma* I, *Sst* I, or *Xba* I according to the manufacturers' recommendations. Reactions were stopped by the addition of 1 µl STOP buffer (Appendix B) then the samples were electrophoresed through 0.8 % agarose gels in TBE buffer (Appendix B) overnight at 35-40 V. Gels were stained with ethidium bromide, viewed, and photographed as described in Chapter 1. From the photographs or their negatives, fragment patterns were recorded and molecular weights of unique fragments were determined.

Mitochondrial DNA restriction fragment patterns were assigned upper case alphabetic symbols according to the designations of Smith (1988). Each individual was then assigned a haplotype, or composite genotype, as outlined in Chapter 1 of this dissertation. The frequencies of these haplotypes in each of the five populations were the raw data employed in determining the genetic relationships between the populations as well as in the computation of $N_em$.

## II. Statistical analysis

### A. Quantification of genetic relationships between populations

Genetic relationships between populations were first quantified by the calculation of the G-test statistic for heterogeneity of haplotype frequencies among the five

subpopulations. The G-test statistic, denoted $G_h$,was calculated as described in Chapter 1. Next, pairwise genetic identities within and between each sampling location were determined by the methods of Takahata and Palumbi (1985) also described in Chapter 1. Genetic distance, D, was then calculated by the elementary equation

$$D = - \ln (J/I).$$

Finally, percent sequence divergence between each pair of subpopulations was calculated as described in Chapter 1 and according to Nei and Li (1979). For the *F. heteroclitus* data, Nei and Li's (1979) metric, $\delta$, was calculated with a PASCAL program written by R. Chapman and defined by a series of equations given by Nei and Li (1979).

Estimates of effective migration rate,$N_em$, were computed by two methods. The first was by calculating $F_{st}$ and the second was by Slatkin's private allele approach (Slatkin 1981). Both measures were calculated treating mtDNA haplotypes as "alleles" at a single locus.

B. Calculation of $N_em$ via $F_{st}$

Wright (1943) outlined the theoretical basis of $F_{st}$ which is defined as the correlation of two alleles chosen at random in a subpopulation relative to the whole population. The quantity is calculated simply by the formula

$$F_{st} = V_p/|\bar{p}(1-\bar{p})|$$

where $\bar{p}$ is the mean frequency of an allele among subpopulations and $V_p$ is the variance in that allele frequency among the subpopulations calculated as the differences between subpopulation means and the grand mean. This correlation coefficient can be related to several other quantities from population genetics theory depending on the assumptions made (e.g., selective neutrality of alleles or levels of mutation and migration). Wright (1943) also showed that if mating is assumed to be random within each subpopulation, then

$$F_{st} = 1/(4N_em+1)$$

for nuclear alleles in diploid organisms. Takahata and Maruyama (1981) and Birky *et al.* (1983) extended this to apply to mtDNA yielding the relationship

$$F_{st} = 1/(2N_em+1)$$

where the change is due to the fact that mtDNA is uniparentally (maternally) transmitted. When the latter equation is employed, $F_{st}$ can be used to estimate effective gene flow, $N_em$, from data on mtDNA variation.

C. Calculation of $N_em$ via private alleles

The analysis of private alleles proposed by Slatkin (1981) is just one of several additional methods available to study gene flow in subdivided populations. This analysis was chosen for the present study because it is highly dependent on migration and does not require the assumptions of selective neutrality and negligible mutation. The method involves calculations based on alleles restricted to only one of the subpopulations sampled. Unless there exists a very high level of dispersal, these "private alleles" are not likely to be carried to other subpopulations by migrants (Slatkin 1985b).

The quantity $N_em$ was calculated from *F. heteroclitus* mitochondrial haplotypes according to the method outlined by Slatkin (1985b) substituting the terms "mitochondrial haplotype" for "allele" and "subpopulation" for "deme". In this computation, average *rare* allele frequencies were assumed to be the average frequencies of haplotypes (alleles) found in at least one but not all subpopulations (demes). By Slatkin's (1981) terminology, i denotes the number of subpopulations in which a haplotype was found; p denotes the frequency of the haplotype in population i; and $\bar{p}$ denotes the mean frequency of a haplotype in all populations where it was detected. Those haplotypes which appeared in only one population (i = 1) were used to calculate

$\bar{p}(1)$ which is called the conditional average frequency of *private* alleles. Finally, this quantity was used to estimate $N_em$ by the equation

$$N_em = [e^{(\ln(\bar{p}(1))+2.44)/-0.505)}]/(N/25)$$

which is derived from Slatkin's (1985b) equation pertaining to the linear portion of his Figure 1 plot of $\bar{p}(1)$ *vs.* $N_em$. This value is also corrected for average sample size, N, as employed by Slatkin (1985b).

# RESULTS

*F. heteroclitus* subpopulations were sampled along a continuous shoreline at locations indicated by arrows in Figure 11a. These stations ranged from 930 meters to 8370 meters apart as indicated in Figure 11b. Several other inshore marine fishes occurred in the traps along with killifish including *F. majalis*, *Cyprinodon variegatus*, and *Anguilla rostrata.*

Virtually no difference was detected between the extractability of mtDNA from fresh eggs and liver. Both tissues yielded mtDNA in sufficient quantity and quality to perform at least six digests per individual. The mtDNA of all 480 individual *F. heteroclitus* was digested with each of the five restriction enzymes for a total of 2400 digestions. The average length of the mtDNA molecule was estimated to be 16,860 ± 184 base pairs (16.86 kb).

The fragment patterns observed for each of the restriction enzymes employed to digest mtDNA are shown diagramatically in Figure 12 and the molecular weights of the fragments are listed in Table 9. A total of 24 different restriction fragment patterns were observed in the study, encompassing 45 separate restriction sites on the mitochondrial genome. Based on the fragment patterns, 25 distinct haplotypes were identified. The distribution of these haplotypes among the five subpopulations is shown in Table 10. From this table it can be seen that there is a great variety of haplotypes which are fairly evenly distributed across the total population. Of the individuals surveyed, 83 % were genotype BCBBB. This is the haplotype identified by Smith *et al.* (1988) as purely southern. This haplotype was found in all subpopulations sampled and varied only

Figure 12. Graphic illustration of restriction fragment patterns observed for the the killifish, *F. heteroclitus*. Molecular weights of fragments for each pattern sum to *ca.* 16.8 kilobases (kb). Fragments of known molecular weight (shown along the left and right margins) are employed as a standard for estimating the molecular weight of each unique clam mtDNA fragment. Thick bands indicate two fragments which have roughly the same molecular weight and thus comigrate to the same location during electrophoresis.

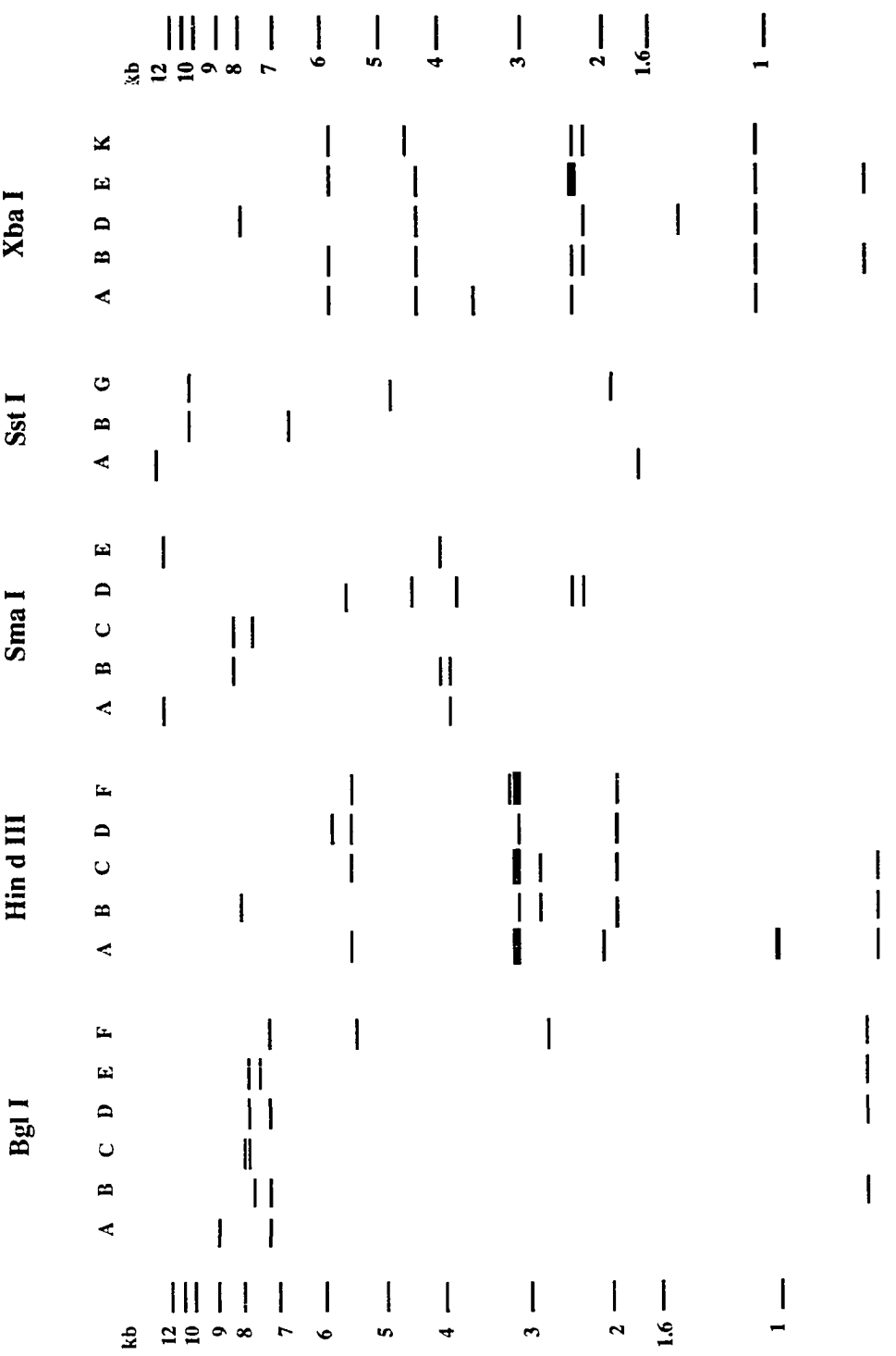


Table 9. Molecular weight estimates for *Fundulus heteroclitus* mtDNA restriction fragments as depicted in Figure 11. Molecular weights are given in kilobases (kb) and generally sum to *ca.* 16.8 kb. Sums much less than 16.8 kb indicate loss of small molecular weight fragments from gels during electrophoresis.

Bgl I

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| 9.0 | 8.4 | 8.4 | 8.0 | 8.0 | 7.4 |
| 7.4 | 7.4 | 8.0 | 7.4 | 7.6 | 5.4 |
| | 0.6 | | 0.6 | 0.6 | 2.7 |
| | | | | | 0.6 |
| 16.4 | 16.4 | 16.4 | 16.0 | 16.2 | 16.1 |

Hind III

| A | B | C | D | F |
|---|---|---|---|---|
| 5.4 | 8.0 | 5.4 | 5.6 | 5.4 |
| 3.0 | 3.0 | 3.0 | 5.4 | 3.2 |
| 3.0 | 2.7 | 3.0 | 3.0 | 3.0 |
| 2.0 | 1.8 | 2.7 | 1.8 | 3.0 |
| 0.9 | 0.5 | 1.8 | | 1.8 |
| 0.9 | | 0.5 | | |
| 0.5 | | | | |
| 15.7 | 16.0 | 16.4 | 15.8 | 16.4 |

Sma I

| A | B | C | D | E |
|---|---|---|---|---|
| 13.0 | 8.2 | 8.2 | 5.5 | 13.0 |
| 3.8 | 4.0 | 7.5 | 4.3 | 4.0 |
| | 3.8 | | 3.8 | |
| | | | 2.4 | |
| | | | 2.2 | |
| 16.8 | 16.0 | 15.7 | 18.2 | 17.0 |

Sst I

| A | B | G |
|---|---|---|
| 13.9 | 10.1 | 10.1 |
| 1.7 | 6.5 | 4.8 |
| | | 1.9 |
| 15.6 | 16.6 | 16.8 |

Xba I

| A | B | D | E | K |
|---|---|---|---|---|
| 5.9 | 5.9 | 8.0 | 5.9 | 5.9 |
| 4.3 | 4.3 | 4.3 | 4.3 | 4.5 |
| 3.5 | 2.3 | 2.1 | 2.3 | 2.3 |
| 2.3 | 2.1 | 1.5 | 2.3 | 2.1 |
| 1.1 | 1.1 | 1.1 | 1.1 | 1.1 |
| | 0.6 | | 0.6 | |
| 17.1 | 16.3 | 17.0 | 16.5 | 15.9 |

Table 10. Distribution of mtDNA haplotypes among five *F. heteroclitus* subpopulations sampled in Shady Side, MD. Values in the body of the table are the numbers of individuals with each haplotype in each subpopulation. Each individual has only one haplotype.

| | | POPULATION | | | | | |
|---|---|---|---|---|---|---|---|
| HAPLOTYPE | | Cedarhurst | Jack Creek | Parish Creek | Snug Harbour | South Creek | TOTALS |
| 1 | BCBBB | 80 | 74 | 82 | 78 | 84 | 398 |
| 2 | BCBBK | | | 1 | | 1 | 2 |
| 3 | BCBBA | | 1 | | | | 1 |
| 4 | BCBBE | | | 1 | 1 | | 2 |
| 5 | BCBBD | | 3 | 1 | 3 | 2 | 9 |
| 6 | BCBGB | | 1 | 2 | 1 | 2 | 6 |
| 7 | BCBAB | | 2 | 1 | 1 | | 4 |
| 8 | BCABB | 5 | 1 | | 1 | 1 | 8 |
| 9 | BCABA | | | 1 | | | 1 |
| 10 | BCAAB | | 1 | | | | 1 |
| 11 | BCDBB | | 1 | 1 | | | 2 |
| 12 | BCEBB | 1 | | | 1 | 1 | 3 |
| 13 | BCCBB | | | | 1 | | 1 |
| 14 | BABBB | 1 | | | 1 | | 2 |
| 15 | BAAAB | | | | 1 | | 1 |
| 16 | BAAAA | | | 1 | | | 1 |
| 17 | BBBBB | 1 | 7 | 2 | 2 | 4 | 16 |
| 18 | BDBBB | | | | 1 | | 1 |
| 19 | BEBBB | | | | | 1 | 1 |
| 20 | ACBBB | 1 | 6 | | | | 7 |
| 21 | CCBBB | | 2 | | 2 | 2 | 6 |
| 22 | DCBBB | 1 | | | | 2 | 3 |
| 23 | DABBB | | | | 1 | 1 | 2 |
| 24 | ECBBB | | | | | 1 | 1 |
| 25 | FCBBB | | 1 | | | | 1 |
| | n | 90 | 100 | 93 | 95 | 102 | 480 |

slightly in frequency among sampling locales, $G_h = 9.58$ (df = 4, $p \approx 0.05$). Forty-six percent of the remaining 24 haplotypes can be considered private alleles as they were restricted to occurrence in only one sampling locale. The overall distribution of mtDNA profiles is consistent with the observations of Smith *et al.* (1988) and indicates that *F. heteroclitus* endemic to the Shady Side, MD portion of Chesapeake Bay are of the southern race.

Mitochondrial haplotype frequencies from Table 10 were used to calculate an overall $G_h$. This G-statistic enquired as to the homogeneity of haplotype distribution among subpopulations. Based on the distribution of the 25 haplotypes in the five subpopulations, $G_h = 110.6$ (with 96 degrees freedom). This value is not significant ($0.5<p<0.1$) when compared to the corresponding critical value of Chi-square indicating homogeneity of haplotypes among the five subpopulations. That is to say, no significant geographic structuring was detected by $G_h$ among the five locales sampled.

Thus, on the basis of $G_h$ the five local groups of killifish would be considered components of one homogenous subpopulation. This conclusion, however, is not entirely supported by further analysis of haplotype distribution. Casual observation of Table 11 indicates that on the basis of gene identity probabilities within (I) and between (J) the sampling locales, there exists at least a moderate level of genetic differentiation (average I = 0.86 and average J = 0.75). When these identity probabilities are employed in calculation of pairwise genetic distance, D, between sampling locations, again a moderate level of differentiation is implied (Table 12). Genetic distances between pairs of subpopulations range from 0.09 to 0.19. In addition, the pairwise percent sequence divergence measures, $\delta$, shown in Table 13 also indicate moderate levels of geographic differentiation ($\delta$ ranges from 0.04 - 0.10; compare to Wilson 1985). In other words, the nucleotide sequences of mtDNA of average individuals in any two subpopulations is different by 4 % to 10 %. Powers *et al.* (1986) gave an estimate of $\delta = 0.035$ between

Table 11. Gene identity probabilities for the five local killifish subpopulations. The diagonal element of the table consists of I values, within population identity, while the upper portion values are between population identities, J. Calculations were performed as described in Chapter 1 using data presented in Table 10.

| | Cedar | Jack | Parish | Snug | South | |
|---|---|---|---|---|---|---|
| Cedarhurst | 0.915 | 0.746 | 0.752 | 0.752 | 0.752 | |
| Jack Creek | | 0.786 | 0.745 | 0.745 | 0.745 | Average J |
| Parish Creek | | | 0.911 | 0.750 | 0.751 | 0.7490 |
| Snug Harbour | | | | 0.851 | 0.751 | |
| South Creek | | | | | 0.851 | |

Table 12. Genetic distance, D, between each of the five local killifish subpopulations. Calculation of D was performed as described in Chapter 1 using the values from Table 11.

| | Jack | Parish | Snug | South | |
|---|---|---|---|---|---|
| Cedarhurst | 0.131 | 0.194 | 0.160 | 0.160 | Average |
| Jack Creek | | 0.130 | 0.094 | 0.094 | distance |
| Parish Creek | | | 0.160 | 0.160 | 0.1409 |
| Snug Harbour | | | | 0.125 | |
| South Creek | | | | | |

Table 13. Percent nucleotide sequence divergence, δ, between each of the five local killifis subpopulations. Calculation of δ was as discussed in Chapter 1 using the data presented Table 10.

| | Jack | Parish | Snug | South |
|---|---|---|---|---|
| Cedarhurst | 0.101 | 0.084 | 0.035 | 0.035 |
| Jack Creek | | 0.047 | 0.064 | 0.094 |
| Parish Creek | | | 0.048 | 0.079 |
| Snug Harbor | | | | 0.033 |
| South Creek | | | | |

the northern and southern killifish races. However, their value is not directly comparable with those presented here because their estimate included invariant enzymes while invariant enzymes were excluded from the present study.

The $F_{st}$ values listed in Table 14 constitute another measure of differentiation between subpopulations. These were computed for each haplotype but only values for the most common haplotype (BCBBB) are shown. $F_{st}$ values indicate that on the average, just less than 4 % of the variation in mitochondrial haplotype detected in this study can be attributed to variation between sampling locales.

Estimates of effective migration rate were computed as $N_em$ from the equation relating to $F_{st}$ and by the method using private alleles (see Materials and Methods and Table 15). Using the value $F_{st}$ = 0.0203 (calculated for the total sample), $N_em$ is estimated to be 24.09. The corresponding value from the private alleles analysis is $N_em$ = 18.47. As will be discussed, these estimates are very similar and their order of magnitude indicates a very large potential for gene flow in *F. heteroclitus*.

Table 14. $F_{st}$ and effective migration rate, $N_em$, estimates between each of the five local killifish subpopulations. Equations for $F_{st}$ and $N_em$ are given in the text and were calculated using the data presented in Table 10. A. $F_{st}$ comparisons. B. $N_em$ comparisons. C. $F_{st}$ and $N_em$ calculated for the total sample frequencies of the common haplotype, BCBBB.

A. $F_{st}$

| | Jack | Parish | Snug | South | |
|---|---|---|---|---|---|
| Cedarhurst | 0.0367 | 0.0406 | 0.0398 | 0.0395 | Average $F_{st}$ |
| Jack Creek | | 0.0361 | 0.0366 | 0.0370 | 0.0390 |
| Parish Creek | | | 0.0438 | 0.0417 | |
| Snug Harbour | | | | 0.0379 | |
| South Creek | | | | | |

B. $N_em$

| | Jack | Parish | Snug | South | |
|---|---|---|---|---|---|
| Cedarhurst | 13.13 | 11.82 | 12.06 | 12.17 | Average $N_em$ |
| Jack Creek | | 13.34 | 13.15 | 13.01 | 12.38 |
| Parish Creek | | | 10.92 | 11.50 | |
| Snug Harbour | | | | 12.68 | |
| South Creek | | | | | |

C. For haplotype BCBBB in the total population

| | |
|---|---|
| $F_{st}$ | 0.0203 |
| $N_em$ | 24.09 |

Table 15. Rare alleles in local *F. heteroclitus* subpopulations compiled from data in Table 10. The variable i is the occupancy number of a haplotype; i.e., it indicates the number of subpopulations in which a particular haplotype is present. The function $\bar{p}(i)$ denotes the mean frequency of haplotypes for the different occupancy numbers. The function $\bar{p}(1)$ then, is the mean frequency of haplotypes found in only one subpopulation; also called the frequency of "private alleles." The method by which $N_em$ is calculated from $\bar{p}(1)$ is discussed in the text.

| i | $\bar{p}(i)$ |
|---|---|
| 1 | 0.0101 |
| 2 | 0.0165 |
| 3 | 0.0180 |
| 4 | 0.0181 |
| 5 | 0.6958 |

Private Alleles:

$\bar{p}(1) =$ 0.0101

$N_em =$ 18.4674

# DISCUSSION

Previous studies of mtDNA variation in *F. heteroclitus* (Powers *et al.* 1986, Smith *et al.* 1988) have verified the existence of two races (or subspecies) by examining the large scale distribution of haplotype frequencies. The general implication of these studies is a scenario of secondary intergradation between two divergent populations which resulted in the establishment of gene frequency clines. The issue which remains of interest is what factors are acting to maintain the clines. Theoretical treatments have attributed the maintenance of clines mainly to a complex balance between selection and gene flow. However, we know that several other factors are implicit in the determination of selective pressure and rates of gene flow. These factors include barriers (both material and genetic), population density, frequency and distribution of subpopulations, and the rates of dispersal and migration. The complexity of the interaction is reinforced by the fact that the latter three factors also affect one another. This study, in contrast to previous investigations, was designed to examine intrapopulation variation in mtDNA so that one of the aforementioned factors, gene flow, could be investigated on a scale fine enough that complicating effects of the other factors would be minimal.

## I. Genetic relationships between subpopulations

Although no significant geographic structure was indicated by $G_h$, the five local groups of killifish exhibit at least a moderate degree of genetic divergence associated with a large variety of haplotypes. One reason for detection of such a large variety is the large sample size: *ca.* 100 individuals per subpopulation. The question arises though, as to the

cause of such a large number of local variants. At this point, introduction of an historical perspective is appropriate. The following account involves a good deal of speculation. However, I strive to adhere to the dictum of Darlington (1957), "Zoogeography, if it is to tell things about the past, should be consulted with forethought, common sense, an open mind, and a remembrance of human fallibility."

The geological record tells us that there were four ice ages during the Pleistocene, each of which had cataclysmic effects on floral and faunal distributions in North America. Animals (such as "*F.* pre-*heteroclitus*") which were basically northern species, experienced severe reductions in population size. They were pushed southward as the ice sheets grew, then northward again as the sheets withdrew. As the most recent ice receded, rainfall increased and sea level rose *ca.* 80-100 m (Curray 1969). Thus, about 10,000 years BP, the Atlantic rose and flooded the Susquehanna River Basin creating what we know now as Chesapeake Bay. Based on the information of Curray (1969), Chesapeake Bay has retained its current proportions for the last *ca.* 3000 yr.

According to the secondary intergradation model, the Bay was in all liklihood initially repopulated by a single founding stock of northern *F. heteroclitus* as they retreated along the glacier's boundary. Since the evidence of Relyea (1967) indicates that this species is basically limited by water temperature, this northern race may have predominated in the Bay only as long as the temperatures were cool. The subsequent warming (which amounted to 4-8$^{o}$C) allowed the invasion of southern *F. heteroclitus* into the Bay which effectively pushed northern members into the upper Bay and tributary headwaters. Most of the available data on morphology (Morin and Able 1983, Brummett and Dumont 1981) and gene frequencies (Powers and Place 1978, Cashon *et al.* 1981, Powers *et al.* 1986, Smith *et al.* 1988) support this proposed zoogeographic description with northern *F. heteroclitus* in the Bay being glacial relicts.

Based on the data of Smith *et al.* (1988) and on the short time since recolonization of the Bay, it seems reasonable to assume that the common genotype BCBBB, observed in 83 % of all individuals in the present study, probably predates the recolonization event. In addition, there are several private allele haplotypes which appear to be related to the northern haplotype and could thus predate the recolonization event. However, there is also a reasonable chance that the majority of the remaining haplotypes evolved *in situ*. This conclusion in based on several lines of evidence. First, most of the variant haplotypes recorded here are but a single site removed from the common haplotype. Second, Carson (1968), Hardin (1959) and Fisher and Ford (1950) have described a mechanism by which populations which undergo considerable fluctuation in $N_e$ can exhibit increased levels of variation. It could be, for example, that as temperatures warmed and southern *F. heteroclitus* invaded the Bay, that they experienced an explosive increase in numbers followed by increased dispersal into new habitats. A large increase in $N_e$ would carry with it an increase in the population's mutation rate. That killifish populations have experienced great expansion over recent evolutionary past is consistent with the evidence of Powers *et al.* (1986) and Morin and Able (1983). Third, some of these variant haplotypes may be associated with evolutionary "hot spots" on their mtDNA. It is known that certain locations on the mtDNA molecule (e.g., the D-loop) can experience repeated gains and losses of restriction sites (Moritz *et al.* 1987). Lansman *et al.* (1983) for example, observed hypervariable restriction sites which appeared and disappeared over the course of evolution in the deer mouse *Peromyscus maniculatus*. Fourth, there is now evidence of molecular recombination between mtDNAs (C. Stine, pers. comm.). This process would provide for the possibility of changes within a lineage as well as incorporation of a paternal component into an mtDNA lineage if paternal transmission were to occur at a low frequency. In depth investigations to resolve which, if any, of these processes occur in *F. heteroclitus* are beyond the scope

of this study. It suffices to say that the subpopulations sampled show evidence of partial isolation and patterns of genetic variability which can be accounted for by known mechanisms and which implicate at least a moderate potential for the transfer of genes.

II. Estimates of gene flow between subpopulations

The calculation of $N_em$ by two separate methods addresses the validity of the use of private alleles analysis on mtDNA data. Simulation studies by Barton and Slatkin (unpub data) as well as Slatkin (1987) both arrived at similar estimates of $N_em$ from both the $F_{st}$ and private alleles methods using allozyme data. Bowen (1987) estimated gene flow and migration in white perch populations by a private alleles analysis of mtDNA data. Based on the relatively more rapid rate of evolution of the mtDNA molecule, he considered estimates of $N_em$ to be biased conservatively, if at all. In Chapter 1 of this dissertation, data were extracted from previous studies of hard shell clam allozyme variation to calculate $N_em$ estimates for comparison to those calculated in that section. $N_em$ estimated from allozymes was virtually identical to those derived from the mtDNA data collected from natural clam populations over the same geographic range. In light of these studies, the similarity of *Fundulus* $N_em$ values as estimated by $F_{st}$ and by private alleles validates both the use of private alleles analysis for the estimation of $N_em$ from mtDNA data and the accuracy of the estimates themselves.

The proportion of private alleles detected in these killifish samples is very small ($\overline{p}(1) = 0.01$, Table 15). Thus, the overall effective migration rate estimated by private alleles analysis of *F. heteroclitus* mtDNA frequencies ($N_em = 18.47$, Table 15) indicates a very high degree of gene flow between the local subpopulations ($N_em > 10$ indicates panmixis, Slatkin 1985b). This result would not be anticipated based solely on killifish life history patterns.

The two most important population parameters affecting the estimate ($N_e$ and the potential for dispersal) are both extremely difficult to quantify. Effective population size, $N_e$, in *F. heteroclitus* has been estimated by several investigators to be *ca.* 25,000 based on calculations from the data of Valiela *et al.* (1987) and Lotrich (1975). But essentially, $N_e$ is determined by the less numerous sex, or in the case of mtDNA by the mean number of breeding females, $N_f$ (Birky *et al.* 1983, Avise *et al.* 1988). Thus, assuming a 1:1 sex ratio, $N_e$ for the present calculations may be on the order of 12,000 or smaller.

In addition, $N_e$ is not a static parameter. Effective population size is certainly influenced by historical zoogeography. For example, it has already been shown that killifish population sizes are likely to have fluctuated widely over the species' recent biological past. This would greatly affect $N_e$ as previously discussed. Several other factors tend to reduce $N_e$, including nonrandom family size distributions (Gall, 1987) and instability of local population structure such as that brought about by frequent extinctions and recolonizations (discussed by Slatkin 1987). Also, Endler (1977) showed that differences in $N_e$ between subpopulations would affect the amount of gene flow between them. Because all of these processes are likely to affect killifish populations, it is probable that $N_e$ is not nearly so large as 25,000. This being the case, the potential for dispersal in *F. heteroclitus* must be very great.

Underestimation of the potential for dispersal in killifish may also account for the high level of gene flow calculated in this study. For example, it is possible that egg dispersal is much greater than hypothesized. However, a high degree of egg dispersal is unlikely since, as previously cited, killifish eggs are known to be demersal, adhesive and to develop under conditions of desiccation. Another possibility is that killifish larvae could frequently occur in deeper portions of tidal creeks where they may be consistently entrained in tidal currents. However, this explanation is also unlikely since young killifish are known to remain in shallow pools on the marsh surface (< 8 cm in depth) for

up to eight weeks after hatching (Taylor *et al.* 1979). Based on the empirical evidence to date it is more likely that adult killifish have greater dispersal tendencies than previously thought. Endler (1977) described a distance, $\bar{x}$, over which gene flow occurs. This distance is essentially the distance between parent and offspring. From direct observations of adult killifish movement (Lotrich 1975), a lower limit for $\bar{x}$ can be estimated to be 4-190 m. Based on the allozyme data of Powers and Place (1978), $\bar{x}$ ranges from 1000-2000 m. In the present study, the greatest distance between subpopulations was 8370 m between Cedarhurst and South Creek (Figure 11b). Gene flow between these two subpopulations was estimated to be $N_em = 12.17$ (Table 14) indicating panmixis. Thus, since effective population sizes may be small in killifish, adult migration rates must be considerable and the distance between parent and offspring may even exceed the 8000 m limit of sampling in this study.

Waples (1987) examined effective migration rate in several other marine shore fishes, many of which have life histories indicative of high dispersal. He ranked fishes according to their supposed capabilities of dispersal as indicated by their life history. At one end of Waples' scale, *Clinocottus analis* was classified as having a low dispersal capability (spawns intertidally, has low fecundity, and larvae are collected only near rocky shores--the adult habitat). At the other end of his scale was the species classified as having very high dispersal capability, *Medialuna californiensis*. This fish is cited as having an extended larval history, whose larvae are captured over a large range of distances and whose larvae are often associated with drifting kelp. Were *F. heteroclitus* to be classified according to Waples' scale, it would be classified as a species with low dispersal capability.

Waples (1987) examined variation in nuclear allele frequencies within samples of fish taken along the coasts of California and Baja. Interestingly, although his estimate of $N_em$ based on rare alleles concurred with predictions for the high dispersal species

($N_em$ = 26.1), the estimate of effective migration rate for the low dispersal species did not. *C. analis* gave $N_em$ = 6.7, indicating intermediate to high levels of gene flow. Thus, the results of both Waples (1987) study and the present one indicate that life history alone is not an adequate predictor of dispersal potential.

It is tempting to think of gene flow as equivalent to dispersal of individuals. However, gene flow entails successful establishment of alien genes--a process more accurately visualized as the distance between the sites of parent and offspring reproduction. As an illustration, observations of movement and dispersal (e.g., Hildebrand and Schroeder 1928, Lotrich 1975) have provided information which has been taken to indicate that the current potential for gene flow is quite small in *F. heteroclitus*. If this conclusion is correct, then the observed clines in gene frequency may be maintained by weak selection pressure. However, migration and gene flow as estimated in this study are quite high and not at all compatible with the hypothesis that restricted gene flow is an explanation for the maintenance of *F. heteroclitus* gene frequency clines. Therefore, since clines are thought to be maintained by a selection/gene flow balance and it is demonstrated here that gene flow is high, then substantial selection pressures must be invoked to account for the present-day gene frequency distributions in *F. heteroclitus*.

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# APPENDIX A

Classification and distinguishing morphological characteristics of taxa within the genus *Mercenaria*. The information listed here was pooled from four primary sources: Linnaeus (1758), Dall (1909), Abbott (1954) and Barnes (1984).

| | |
|---|---|
| Kingdom | Animalia |
| Phylum | Mollusca |
| Class | Bivalvia |
| Order | Venerida |
| Suborder | Heterodonta |
| Superfamily | Veneracea |
| Family | Veneridae |
| Subfamily | Venerinae |

Genus *Mercenaria* Schumacher 1817

Shell large, thick and trigonal with concentric lamellar sculpture; lunule large, heart-shaped and bounded by an incised line. Internal margins crenulate. Three cardinal teeth in each valve; two bifid cardinals in left valve, one bifid in right. Posterior dorsal margin of right valve grooved to receive the edge of the left valve.

species *mercenaria* Linne 1758

Shell 8-13 cm in length, ovate-trigonal, about 5/6 as high as long; heavy and quite thick. Moderately inflated. Numerous concentric growth lines which are prominant and distantly spaced near the umbo. Exterior center of valves smooth and/or glossy. Exterior color dirty-gray to white. Interior white, commonly with purple stainings. Entire lunule is 3/4 as wide as long (spade-shaped).

variety *notata* Say 1822

Brown zig-zag mottlings on valve exterior and no interior purple coloration.

variety *texana* Dall

Confined to northern Gulf of Mexico. Shell suborbicular and inflated. Exterior center of valves is smooth and/or glossy. However, also has large, irregular, coalescing, flat-topped concentric ribs.

species *campechiensis* Gmelin 1792

Shell 8-15 cm in length, much more obese than *mercenaria*; highly inflated beaks. Heavier shell lacks smooth central area on outside of valves and ends are blunt. Entire lunule is as wide as long (heart-shaped). Always white internally. Sometimes with purplish stain on escutcheon and brown mottlings on side. Young

frequently have external brown zig-zag lineation like the *notata* markings. Valve sculpture is dense, low, thin concentric lamellation. Young often have purplish flush in cavity of beak.

## APPENDIX B

Buffers and solutions used in the extraction and digestion of mtDNA from both the hard shell clam *Mercenaria spp.* and the killifish *Fundulus heteroclitus.*

| | | |
|---|---|---|
| Homogenization Buffer | 30 mM | Tris-HCl pH 7.4 |
| | 2.50% | KCl |
| | 0.3 M | Sucrose |
| | 30 mM | CaCl2 |
| | 140 ug/ml | Ethidium Bromide (MUTAGEN!) |
| TEB | 0.089 M | Tris |
| | 0.089 M | Boric Acid |
| | 0.002 M | EDTA |
| TEK | 50 mM | Tris-HCl pH 7.4 |
| | 10 mM | EDTA |
| | 1.50% | KCl |
| STEK | 0.25 M | Sucrose in TEK |
| STEP | 1.1 M | Sucrose in TEK |
| STOP | 0.89 M | Tris |
| | 0.89 M | Boric Acid |
| | 0.02 M | EDTA (tetrasodium) |
| | 0.25% | Bromophenol Blue |
| | 50% | Glycerol |
| | 1% | SDS |
| NP-40 Lysis Solution | 5% | Non-idet P-40 (Sigma) in TEK |

## APPENDIX C

Restriction endonucleases employed in the digestion of mtDNA of clams, *Mercenaria spp.* (A.) and of killifish, *Fundulus heteroclitus* (B.). The sequence of nucleotides on the mtDNA molecule which is recognized by each of the enzymes is listed in the right hand column. The site where the enzyme cleaves the mtDNA molecule is indicated by an asterisc (*).

### Chapter 1

| | |
|---|---|
| *Bam* H I | G*GATCC |
| *Eco*R I | G*AATTC |
| *Eco*R V | GAT*ATC |
| *Hin*d III | A*AGCTT |
| *Pst* I | CTGCA*G |
| *Pvu* II | CAG*CTG |

### Chapter 2

| | |
|---|---|
| *Bcl* I | T*GATCA |
| *Bgl* I | GCCNNNN*NGGC |
| *Hin*d III | A*AGCTT |
| *Sma* I | CCC*GGG |
| *Sst* I | GAGCT*C |
| *Xba* I | T*CTAGA |

# APPENDIX D

Clam mitochondrial genotypes, sizes and heteroplasmy for individuals sampled in all locales. Location abbreviations are as follows: MV: Martha's Vineyard, MA; GS: Great Sound, NJ; WA: Wachapreague, VA at Hog Island; O1: Oregon Inlet, NC (first sample); O2: Oregon Inlet, NC (second sample); NC: Beaufort, NC; SC: Folley River, SC; BB: Bull's Bay, SC; SI: Skidaway Island, SC; I1: Indian River, FL near Ft. Pierce (first sample); I3: Indian River, FL near Ft. Pierce (second sample); TB: Tampa Bay, FL; AB: Apalachicola Bay, FL; GA: Galveston, TX; PA: Port Aransas, TX. Missing restriction genotypes are denoted by "--" where no data was obtainable. The column "Predominant MW" gives the predominant molecular weight of that individual's mtDNA if heteroplasmic (and "X" also appears under "Size"), or the sole molecular weight of that individual's mtDNA if it was homoplasmic with respect to mtDNA size. In the column "Site" under "Heteroplasmy," individuals marked with an "X" demonstrated two or more different restriction fragment patterns for a single restriction enzyme.

Location: MV

| Individual | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Heteroplasmy Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | -- | -- | -- | A | A | 18.0 | | |
| 2 | A | -- | -- | -- | A | A | 18.0 | | |
| 3 | A | -- | -- | -- | A | A | 18.0 | | |
| 4 | -- | -- | -- | -- | A | A | 17.0 | | |
| 5 | -- | -- | -- | -- | A | A | 17.0 | | |
| 7 | -- | -- | -- | -- | A | A | 19.0 | | |
| 8 | -- | -- | -- | -- | A | A | 17.0 | | |
| 9 | -- | -- | -- | -- | A | A | 17.0 | | |
| 10 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 11 | -- | -- | -- | -- | A | A | 19.0 | | |
| 12 | -- | -- | -- | -- | A | -- | 17.0 | | |
| 13 | -- | -- | -- | -- | A | A | 17.5 | | |
| 14 | -- | -- | -- | -- | A | A | 17.5 | | |
| 15 | -- | -- | -- | -- | -- | A | 17.5 | | |
| 16 | -- | A/B | -- | -- | A | A | 18.0 | X | X |
| 17 | -- | A | -- | -- | -- | A | 17.5 | | |
| 18 | -- | -- | -- | -- | -- | A | 17.5 | | |
| 19 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 20 | -- | -- | -- | -- | A | A | 17.5 | | |
| 22 | -- | -- | -- | -- | A | -- | 17.5 | | |

Location: GS

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Heteroplasmy Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | A | A | -- | A | A | 18.0 | | |
| 2 | A | A | A | B | A | A | 17.5 | | |
| 3 | A | A | A | -- | A | A | 17.5 | | |
| 4 | A | A | A | B | A | A | 18.0 | | |
| 5 | A | A | A | B | A | A | 18.0 | | |
| 6 | A | A | A | B | A | A | 18.0 | | |
| 7 | A | A | A | B | A | A | 17.5 | | |
| 8 | -- | -- | -- | B | -- | -- | 17.5 | | |
| 9 | A | A | A | B | A | A | 18.0 | | |
| 12 | A | A | A | -- | A | A | 18.0 | | |
| 13 | A | A | A | -- | A | A | 18.0 | | |
| 14 | A | A | A | -- | A | A | 18.0 | | |
| 15 | A | A | A | A | A | A | 18.0 | | |
| 16 | -- | -- | A | -- | -- | -- | 18.0 | | |
| 22 | A | A | A | -- | A | A | 18.0 | | |
| 23 | A | A | A | -- | A | A | 18.0 | | |
| 24 | -- | A | A | -- | -- | -- | 17.5 | | |

Appendix D continued

Location: WA

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | A | A | A | A | A | 18.0 | | |
| 2 | A | A | A | -- | A | A | 18.0 | X | |
| 3 | A | A | A | -- | A | A | 17.5 | X | |
| 4 | A | A | A | -- | A | A | 17.5 | | |
| 5 | A | A | A | -- | A | A | 17.5 | X | |
| 6 | A | A | A | -- | A | A | 17.5 | X | |
| 7 | A | A | A | B | A | A | 17.5 | X | |
| 8 | A | A | A | B | A | A | 17.5 | X | |
| 9 | A | A/D | A | B | A | A | 18.0 | X | X |
| 10 | A | A | A | B | A | A | 18.0 | X | |
| 11 | A | A | A | B | A | A | 17.5 | X | |
| 12 | A | A | A | B | A | A | 18.0 | X | |
| 13 | A | A | A | B | A | A | 17.5 | | |
| 14 | A | -- | A | B | -- | -- | 18.0 | | |
| 15 | A | A/D | A | B | A | A | 17.0 | X | X |
| 16 | A | A/D | A | B | A | A | 17.0 | X | X |
| 17 | A | A/D | A | B | A | A | 17.5 | X | X |
| 18 | A | A/D | A | B | A | A | 17.5 | X | X |
| 19 | A | A/D | A | A | A | A | 17.5 | X | X |
| 20 | A | -- | A | -- | -- | -- | 17.5 | | |
| 21 | -- | -- | -- | -- | A | -- | 17.5 | | |

Location: O1

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 2 | B | A | -- | -- | -- | -- | 17.0 | | |
| 3 | A | A | A | -- | A | A | 17.5 | X | |
| 4 | A | A | A | -- | A | A | 17.5 | | |
| 5 | A | A | A | -- | A | A | 17.5 | | |
| 6 | B | C | A | -- | A | A | 17.5 | X | |
| 7 | -- | -- | -- | -- | A | A | 17.5 | | |
| 8 | B | -- | A | -- | -- | -- | 17.5 | | |
| 9 | -- | -- | -- | -- | A | A | 17.5 | | |
| 10 | A | A | A | -- | A | A | 18.0 | | |
| 11 | B | -- | -- | -- | A | A | 17.5 | | |
| 12 | A | -- | A/? | -- | -- | -- | 17.5 | | X |
| 13 | B | A/D | A/? | -- | A | A/? | 17.5 | X | X |
| 14 | B | A | A | -- | B | A | 17.5 | X | |
| 15 | B | A/D | A | -- | A | A | 17.5 | | |
| 16 | B | A/D | A | -- | A | A | 18.0 | | |
| 17 | A | A | A | -- | A | A | 18.0 | | |
| 18 | -- | -- | -- | -- | A | A | 17.5 | | |
| 19 | A | -- | A | -- | -- | -- | 17.5 | | |
| 20 | B | A/D | A | -- | A | A | 18.0 | | |
| 21 | B | A/D | A | -- | A | A/? | 18.0 | | X |
| 22 | B | A/D | A | -- | A | A | 17.5 | | |

Appendix D continued

Location: O2

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | A | H | A | A | A | 18.0 | | |
| 2 | A | A | H | A | A | A | 17.5 | | |
| 3 | A | A | H | A | A | A | 18.0 | | |
| 4 | A | A | H | A | A | A | 17.5 | | |
| 5 | A | A | A | A | A | C | 17.5 | | |
| 6 | A | A | H | -- | A | B | 17.5 | | |
| 7 | A | A | H | A | A | A | 18.0 | | |
| 8 | A | A | H | A | A | A | 17.0 | | |
| 9 | A | A | H | -- | A | A | 17.5 | | |
| 10 | A | A | H | A | A | A | 17.5 | | |
| 11 | A | A | H | A | A | A | 18.0 | | |

Location: NC

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 3 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 4 | A | A | A | -- | A | A | 18.0 | | |
| 5 | A | A | D | -- | A | A | 18.0 | | |
| 6 | A | A | A | -- | A | A | 18.0 | X | |
| 7 | A | A | A | -- | A | A | 18.0 | | |
| 8 | -- | -- | -- | -- | A | -- | 18.0 | | |
| 9 | A | A | A | A | A | A | 17.5 | X | |
| 10 | A | A | A | A | A | A | 17.0 | | |
| 11 | A | A | A | A | A | A | 18.0 | X | |
| 12 | A | A | A | B | A | A | 18.0 | X | |
| 13 | A | A | A | B | A | A | 17.5 | | |
| 14 | A | A | A | B | A | A | 17.5 | | |
| 15 | A | A | A | B | A | A | 17.5 | | |
| 16 | A | A | A | A | A | A | 17.0 | | |
| 17 | A | A | A | A | A | A | 17.5 | | |

Location: SC

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 6 | -- | -- | A | A | -- | -- | 18.0 | | |
| 8 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 9 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 11 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 12 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 13 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 14 | -- | A | -- | -- | -- | -- | 17.5 | | |
| 17 | -- | -- | A | -- | -- | -- | 17.5 | | |
| 18 | -- | -- | -- | C | -- | -- | 17.5 | X | |
| 19 | C | A/D | A | A | -- | -- | 18.0 | X | X |

Appendix D continued

Location: BB

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | -- | -- | -- | -- | -- | 17.0 | | |
| 2 | A | -- | -- | -- | -- | -- | 17.0 | | |
| 3 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 4 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 5 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 6 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 7 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 8 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 9 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 10 | A | -- | -- | -- | -- | -- | 18.5 | | |
| 11 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 12 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 15 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 17 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 18 | A | -- | -- | -- | -- | -- | 18.5 | | |
| 19 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 21 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 22 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 23 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 24 | A | -- | -- | -- | -- | -- | 18.0 | | |

Location: SI

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Pvu II | Predominant MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 2 | -- | -- | -- | -- | A | -- | 17.5 | X | |
| 3 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 4 | -- | -- | -- | -- | A | -- | 18.0 | | |
| 5 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 8 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 10 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 11 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 12 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 13 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 14 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 15 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 18 | A | -- | -- | -- | A | -- | 17.5 | | |
| 19 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 20 | A | -- | -- | -- | A | -- | 17.5 | | |
| 21 | A | -- | -- | -- | A | -- | 17.5 | | |
| 22 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 23 | A | -- | -- | -- | A | -- | 17.5 | | |

Appendix D continued

Location: I 1

| Indiv. | BamHI I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 9 | A | A | A | -- | A | B | 17.5 | | |
| 10 | A | A | A | -- | A | B | 17.5 | | |
| 12 | -- | A | -- | -- | -- | -- | 17.5 | | |
| 25 | A | A | A | B | A | A | 17.5 | | |
| 26 | A | A | A | B | A | A | 17.5 | X | |
| 27 | A | A | A | B | A | A | 17.5 | X | |
| 28 | A | A | A | B | A | A | 18.0 | X | |
| 29 | A | A | A | B | A | A | 17.5 | X | |
| 30 | A | A | A | B | A | A | 17.5 | X | |
| 31 | A | A | A | B | A | A | 18.5 | X | |
| 32 | A | A | A | B | A | A | 18.0 | X | |

Location: I3

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 25 | A | A | A | A | -- | A | 17.5 | | |
| 26 | A | A | A | A | -- | A | 18.5 | | |
| 27 | A | A | A | A | -- | A | 18.5 | | |
| 28 | A | A | A | A | -- | A | 18.0 | | |
| 29 | A | A | A | A | -- | A | 18.0 | | |
| 30 | A | A | A | A | -- | A | 17.0 | | |
| 31 | A | A | A | A | -- | A | 18.0 | | |
| 32 | A | A | A | A | -- | A | 17.0 | | |
| 33 | A | A | A | A | -- | A | 17.5 | | |
| 34 | A | A | A | A | -- | A | 18.5 | | |
| 35 | A | A | A | A | -- | A | 17.5 | | |

Appendix D continued

Location: TB (M. c.)

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 8 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 9 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 10 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 11 | A | -- | -- | -- | A | -- | 17.5 | | |
| 12 | -- | -- | -- | -- | -- | B | 17.5 | | |
| 14 | -- | -- | -- | -- | A | -- | 17.5 | | |
| 15 | A | -- | -- | -- | -- | -- | 19.0 | | |
| 24 | A | -- | -- | -- | -- | -- | 18.5 | | |

Location: AB (M. c.)

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | C | B | -- | B | B | 17.5 | | |
| 2 | A/B | C/A/D | B | B | B | B | 17.5 | X | X |
| 3 | A | C/A/D | B | B | B | B | 18.0 | X | X |
| 4 | A/B | C | B | B | B/A | B | 18.0 | X | X |
| 15 | B | A | B | -- | B | B | 17.5 | | |
| 16 | B | A | B | -- | B | B/A | 18.0 | | X |
| 17 | B | A | B | -- | B/A | B | 17.5 | | X |
| 18 | B | A | B | -- | B | B | 17.5 | | |
| 19 | B | A | B | -- | B | B | 18.0 | | |
| 20 | B | A | B | -- | B | B | 17.5 | | |
| 21 | B | A | B | -- | B | B | 18.0 | | |
| 22 | B | -- | -- | -- | -- | B | 18.0 | | |
| 23 | B | A | B | -- | B | B | 17.5 | | |
| 24 | B | -- | -- | -- | -- | -- | 17.5 | | |
| 25 | B | A | -- | -- | -- | -- | 17.5 | | |
| 26 | B | A | B | -- | B | B | 17.5 | | |
| 27 | B | -- | -- | -- | B | -- | 17.5 | | |
| 28 | B | -- | -- | -- | B | -- | 17.5 | | |
| 29 | B | -- | -- | -- | -- | -- | 17.5 | | |
| 30 | B | A | B | -- | B | B | 17.5 | | |
| 31 | B | A | B | -- | B | B | 18.0 | | |
| 32 | B | A | B | -- | B | B | 17.5 | | |
| 33 | B | A | B | -- | B | B | 18.0 | | |
| 34 | B | -- | -- | B | -- | B | 17.0 | | |
| 35 | B | -- | -- | A | -- | B | 17.0 | | |
| 36 | B | -- | B | B | -- | -- | 17.5 | | |
| 37 | B | -- | B | B | -- | -- | 17.5 | | |
| 38 | B | A | B | B | B | B | 17.0 | | |

Appendix D continued

Location: GA (M.m.t.)

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | MW | Heteroplasmy Size | Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | B | -- | -- | -- | -- | -- | 18.0 | | X |
| 2 | A | -- | B/A | -- | B | -- | 17.5 | | X |
| 3 | A | -- | -- | -- | -- | -- | 17.5 | | |
| 4 | A | -- | B/A | -- | B | -- | 17.5 | | X |
| 5 | A | -- | B/A | -- | -- | -- | 17.5 | | X |
| 12 | A | -- | B/A | -- | -- | -- | 17.5 | | X |
| 13 | A | -- | B/A | -- | -- | -- | 18.0 | | X |
| 14 | A | -- | -- | -- | -- | -- | 18.0 | | |
| 15 | A | -- | B/A | -- | -- | -- | 18.0 | | X |
| 17 | B | -- | A/B | -- | -- | -- | 18.0 | | X |
| 19 | A/B | -- | B/A | E | -- | -- | 17.5 | | X |
| 20 | A | -- | B/A | -- | -- | -- | 18.0 | | X |
| 21 | A | -- | E/A | -- | -- | -- | 18.0 | | X |
| 22 | A | -- | B/A | -- | -- | -- | 18.0 | | X |
| 23 | D/A | -- | A/D | -- | -- | -- | 18.0 | | X |
| 24 | A | -- | A/D | -- | -- | -- | 18.0 | | X |
| 25 | D/A | -- | A/D/B | D | -- | A/? | 19.5 | | X |
| 26 | D/A | -- | B/A | -- | -- | A/? | 17.5 | | X |
| 27 | A | -- | B/A | -- | -- | A/? | 17.5 | | X |
| 28 | A/B | -- | B/A | -- | -- | -- | 17.5 | | X |
| 29 | A/B | -- | B/A | E | -- | -- | 17.5 | | X |
| 30 | A/B | -- | B/A | -- | -- | -- | 18.0 | | X |
| 31 | A/B | -- | B/A | -- | -- | -- | 17.5 | | X |
| 32 | A | -- | B/A | -- | -- | -- | 17.5 | | X |

Appendix D continued

Location: PA (M.m.t.)

| Indiv. | BamH I | EcoR I | EcoR V | HindIII | Pst I | Predominant Pvu II | Predominant MW | Heteroplasmy Size | Heteroplasmy Site |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | A | B | -- | B | B | 17.0 | | |
| 2 | A | -- | B | -- | B | B | 17.5 | | |
| 3 | A | A | F | -- | B | B | 17.5 | | |
| 4 | -- | A | B | -- | B | B | 17.5 | | |
| 5 | A | -- | B | -- | B | B | 17.5 | | |
| 6 | A | A | B | -- | B | B | 18.0 | | |
| 7 | A | A | B | -- | B | B | 17.5 | | |
| 8 | E | A | D | -- | A | C | 17.0 | | |
| 9 | -- | A | -- | -- | -- | -- | 17.5 | | |
| 10 | A | -- | B | -- | A | B | 17.5 | | |
| 11 | -- | A | -- | -- | -- | B | 17.5 | | |
| 12 | A | A | B | -- | A | B | 17.5 | | |
| 13 | A | A | B | -- | A | B | 18.0 | | |
| 14 | E | C | D | -- | A | C | 17.5 | | |
| 15 | A | A | B | -- | A | B | 18.0 | | |
| 16 | A | A | B/D | -- | B | B | 18.0 | | X |
| 17 | A | A | F | -- | B | B | 18.0 | | |
| 18 | E | A | D | -- | B | B | 17.5 | | |
| 19 | A | A | B | -- | B | B | 17.5 | | |
| 20 | A | A | B | -- | B | B | 17.5 | | |
| 21 | A | A | B | -- | A | B | 17.5 | | |
| 22 | A | A | E | -- | A | B | 17.5 | | |
| 23 | A | A | B | -- | B | B | 17.5 | | |
| 24 | A | A | B | -- | B | B | 17.5 | | |
| 25 | A | A | F | -- | B | B | 18.0 | | |
| 26 | E | A | D | -- | A | C | 18.0 | | |
| 27 | -- | -- | -- | -- | -- | B | 17.5 | | |
| 28 | A | A | F | -- | B | B | 17.5 | | |
| 29 | E | A | D | -- | B | C | 17.0 | | |
| 30 | B | A | B | -- | A | B | 17.5 | | |
| 31 | A | A | B | -- | B | B | 17.5 | | |
| 32 | A | A | B | -- | A | B | 17.5 | | |

## AUTOBIOGRAPHICAL STATEMENT

Bonnie Lynn Brown was born in Birmingham, AL on 22 June 1960. She received a Bachelor of Science in Biology from the University of Alabama in Birmingham in 1981. She has held numerous instructional and research positions at both the University of Alabama in Birmingham and Old Dominion University. During her tenure at ODU she has held the following appointments as well as numerous technical and assistantship positions:

Assistant Research Scientist at the Chesapeake Bay Inst., Johns Hopkins University
Associate Investigator, Virginia Sea Grant Program, Old Dominion University
Director and Instructor, ODU Sea Camps, Old Dominion University
Instructor of Mathematics, Old Dominion University.
Instructor of Biology, Tidewater Community College, Suffolk, VA Campus.

She has published/co-authored the following:

Chapman, R. W., and B. L. Brown. (submitted). Mitochondrial DNA transmission in two fish species: evidence of paternal leakage.

Chapman, R. W., and B. L. Brown. 1989. Two methods to detect DNA fragments produced by restriction enzymes. Anal. Biochem. 177: 199-202.

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Brown, B. L. 1986. Aquaculture as a teaching tool in secondary eduaction. Mid-Atlantic Marine Education Association, Virginia Beach, VA. (abstract).

Butt, A., and B. L. Brown. 1986. Civilization and sea level change; past and present. Pages 24-28 *in* IEEE Oceans '86 Conference Proceedings. Washington, DC.

Brown, B. L., and A. Savitsky. 1985. Sea turtles of Chesapeake Bay. Technical Rept. to Virginia Dept. of Highways and Transportation, Richmond, VA.

Brown, B. L., and R. MacGregor. 1980. Effects of blinding and pinealectomy on gonadal activity in the catfish, *Ictalurus punctatus*. Alabama Academy of Science, Auburn. (abstract).

Academic Honors:

Outstanding Ph.D. Student in Oceanography, Old Dominion University, 1989
Mentor, Governor's Science & Technology Program, 1987
Sigma Xi Scientific research Society, 1986
Fellowship, Dept. of Oceanography, Old Dominion University, 1982-1984
National Dean's List 1977-1984
Outstanding Undergraduate in Biology, Univ. of Alabama in Birmingham, 1981
Fellowship, Univ. of Alabama in Birmingham, 1981
Phi Kappa Phi National Honor Society, 1981