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Mitochondrial Restriction Enzyme Screening and Phylogenetic Relatedness in the Hard Shell Clam Genus Mercenaria. *Part II. Population Variation*

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MITOCHONDRIAL RESTRICTION ENZYME SCREENING AND PHYLOGENETIC RELATEDNESS IN THE HARD SHELL CLAM GENUS MERCENERIA. PART II: POPULATION VARIATION

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Executive Summary

This report focuses on the population genetics and systematics of the hard shell clam *Mercenaria*, an important aquaculture species, as determined by restriction enzyme digestion of mitochondrial DNA (mtDNA). 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, $I = 0.882 \pm 0.060$, and low percent nucleotide sequence divergence of their mtDNA, $\delta = 0.003 \pm 0.002$. 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; $\delta =$

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.

Introduction

Over the past 20 years, a great deal of research has focused on genetics of the hard shell clams, *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 report is to elaborate upon the population genetics and systematics of the hard shell clam as determined by 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. Previous 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 and DiMichele 1989). 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.

II. 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. 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.*

III. 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, 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., *Hin*d 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 *Hin*d 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 quantitation 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 1983). In other words, mtDNA analysis could potentially provide a more sensitive estimate of genetic variation within and between geographically disjunct populations of clams.

IV. 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.

Objectives

Traditional molecular biological tools have been increasingly utilized in areas of applied research. Antibodies, isozymes, restriction enzymes and nucleotide sequencing are now commonly employed to genetically distinguish between populations of closely related organisms. The techniques outlined in Part I of this study (Brown and Wolfinbarger 1987) were used to accomplish the objectives of Part II.

In completion of Part II we have determined the degree of genetic differentiation within and between geographically disjunct but closely related populations of the hard shell clam genus *Mercenaria.* Mitochondrial restriction fragment patterns have been analyzed for 11 samples of *M. mercenaria* obtained from 9 geographically distant locales considered to be native clam populations ranging from Martha's Vinyard, MA to Indian River, FL. 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.

In addition, mitochondrial DNA (mtDNA) variation has been examined in two samples each of *M. campechiensis* and *M. mercenaria texana* for comparison with *M. mercenaria.* The data on mtDNA variation presented in this report indicate that considerable genetic divergence has occurred between the sibling species *M. mercenaria* and *M. campechiensis* and that mtDNA restriction enzyme analysis will be a useful tool in the identification of base populations for use in trait-specific breeding approaches. Our information also shows that similar degrees of genetic divergence have occurred between

M. mercenaria texana and the former two taxa. This supports the contention that *texana* deserves species distinction separate from *M. mercenaria.*

The information generated by both phases of this study is intended to contribute to current efforts in aquacultural genetics. Aquaculturists may employ the techniques outlined in Part I (Brown and Wolfinbarger 1987) and the data outlined herein to evaluate stock histories and the suitability of current breeding practices for clams and other cultured marine bivalves.

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 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.

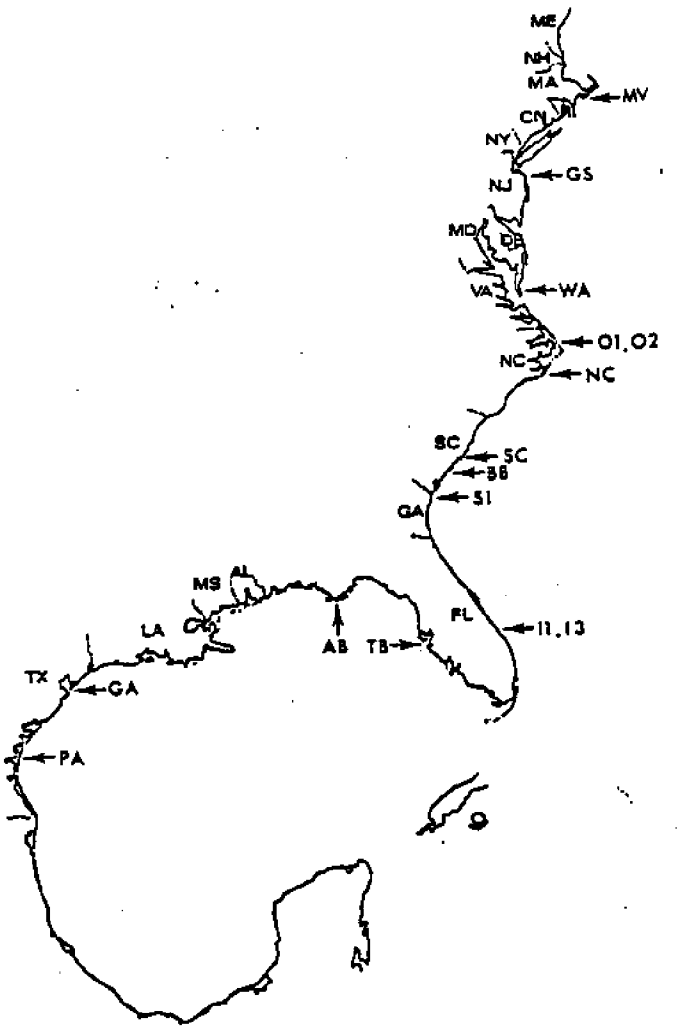


Figure 1. Sampling locales of *Mercenaria spp.* populations along the east and Gulf coasts of the United States. The location where clam populations were sampled is indicated by an arrow followed by the abbreviation given to that sample. The abbreviations are MV: Martha's Vineyard, MA, GS: Great Sound, 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*. The TB and AB populations are *M. campechiensis*. The GA and PA populations are *M. mercenaria texana*.

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, *Hin*d III, *Pst* I and *Pvu* II as listed in Appendix C. However, data from *Hin*d 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 type of analysis provided for 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, δ, 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, δ 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.

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

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).

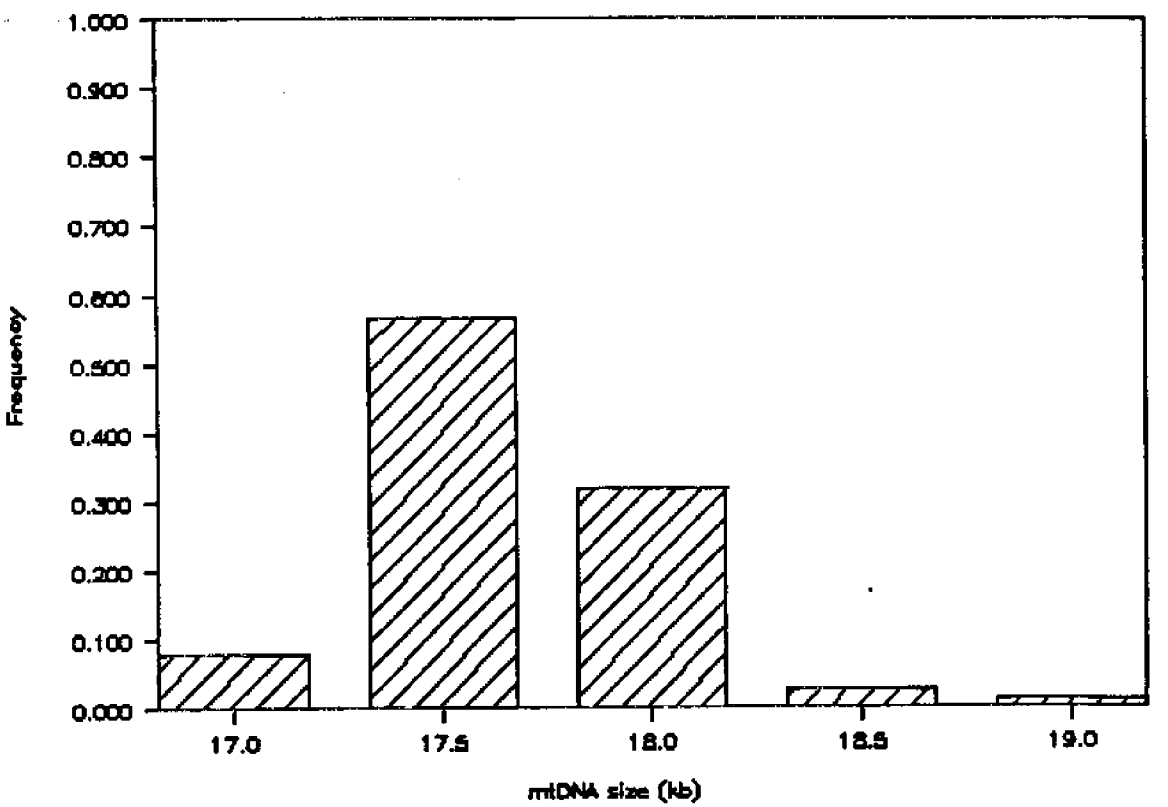
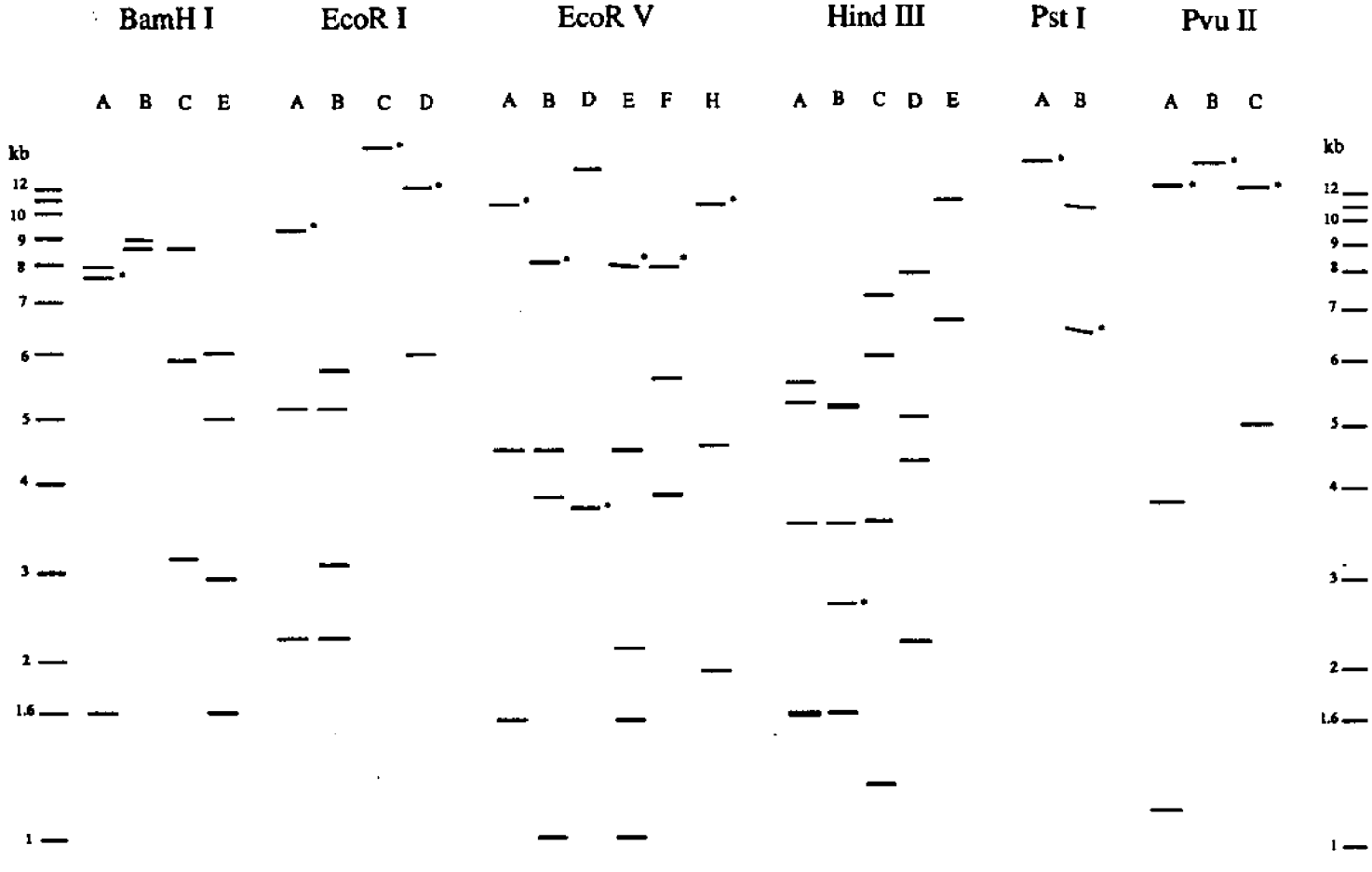


Figure 3. Graphic illustration of the restriction fragment patterns observed for the hard shell clam *Mercenaria spp.* Molecular weights of fragments for each pattern sum to *ca.* 17.5 kilobases (kb). Fragments of known molecular weight (shown along the left and right margins) are employed as a standard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



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 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

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

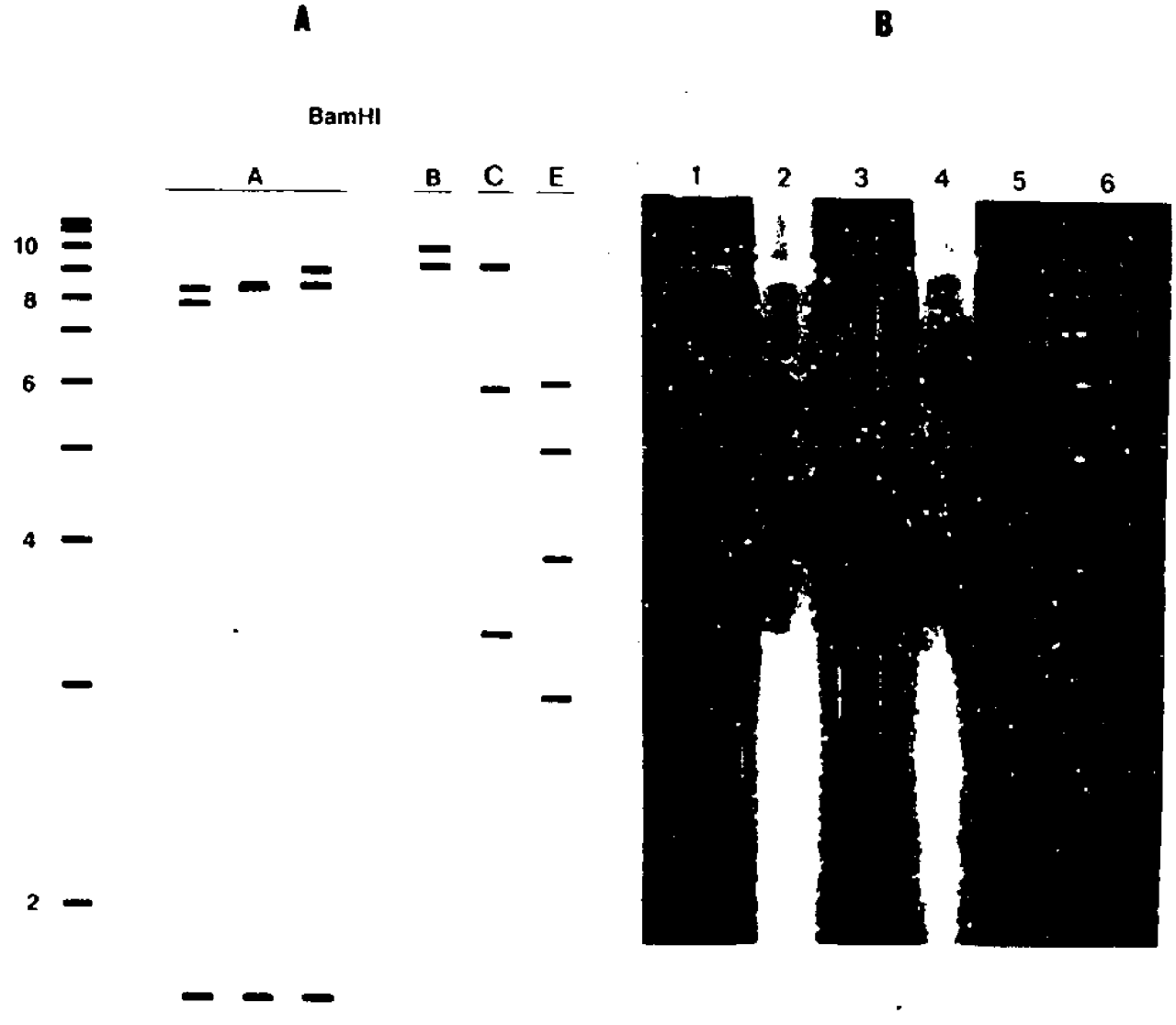
Pst I

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 genotypes of *Bam*H I. All 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 with the first "A" mtDNA genotype which totals 18.0 kb.


A
B
BamHI
A
B
C
E
10
8
6
4
2
1
2
3
4
5
6

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 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).

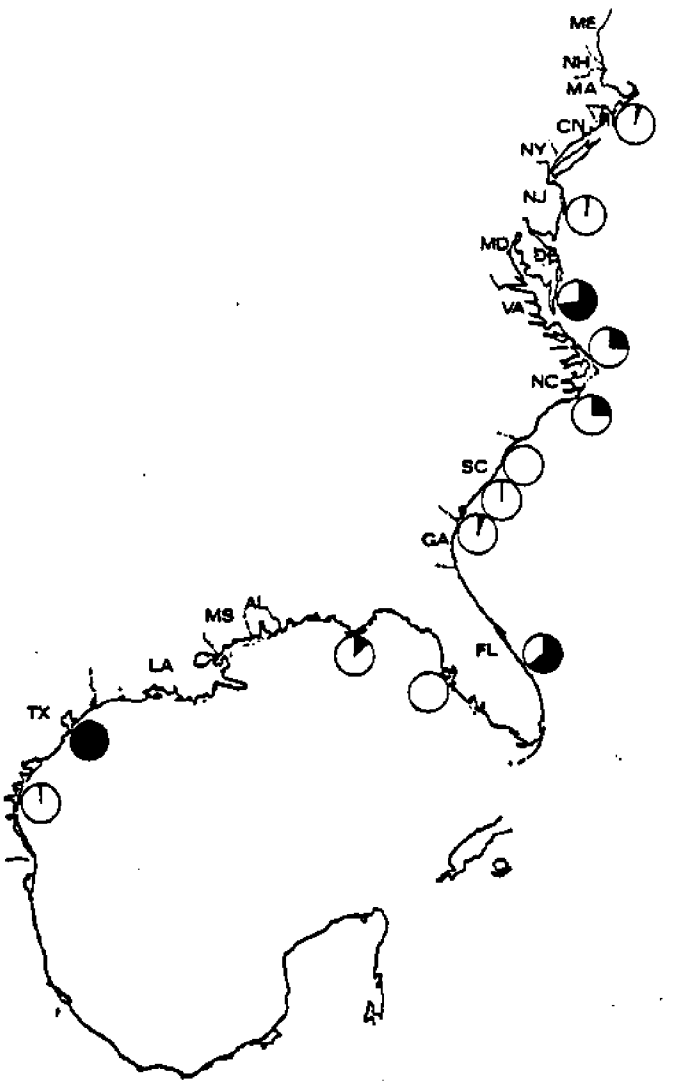


Figure 5. Frequencies of mtDNA heteroplasmy in *Mercenaria spp.* populations sampled along the east and Gulf coasts of the United States. Shaded areas indicate the percent of individuals sampled which were heteroplasmic for either size or restriction site. White areas indicate the percent of individuals 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.

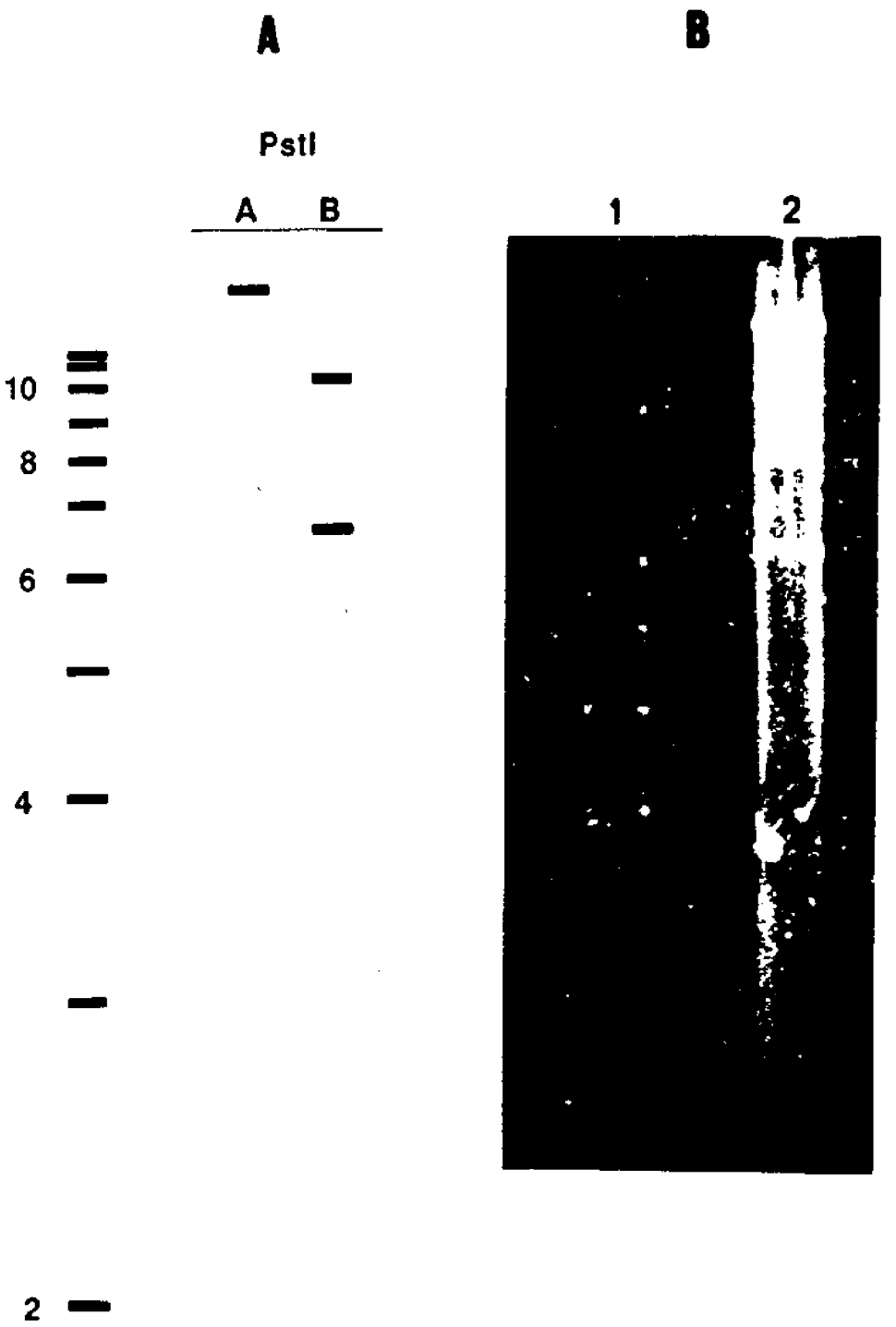

A
B
PstI
A
B
1
2
10
8
6
4
2

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.

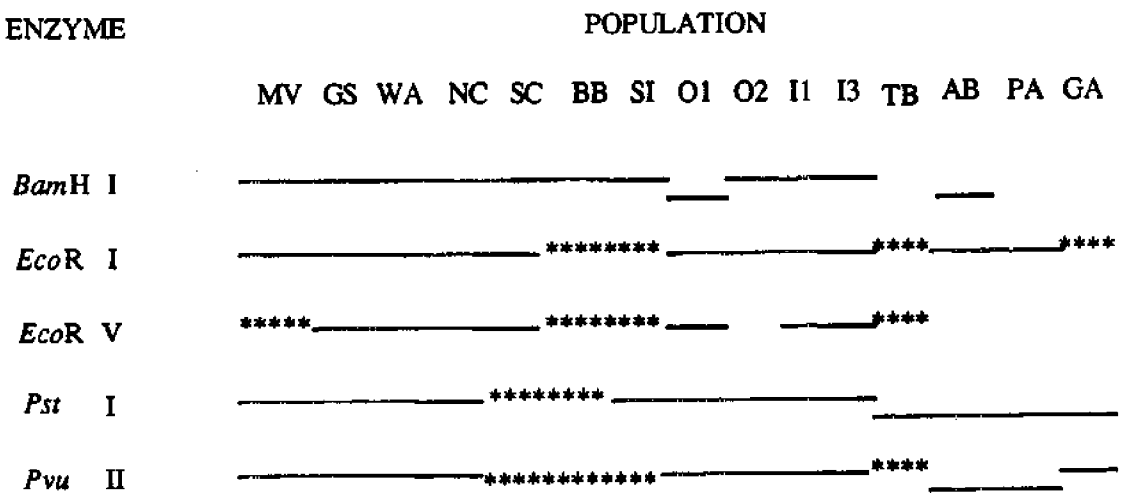


Table 2. Haplotype, mtDNA molecular weight and heteroplasmy for nine natural clam populations. Haplotypes are presented for those individuals sampled from the following populations (GS, WA, NC, O1, O2, I1, I3, AB and PA as shown in Figure 1) which had complete data for the five restriction enzymes *Bam*H 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.

Table 2

Individual	Population	Haplotype	Predominant MW	Heteroplasmy Size	Heteroplasmy 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	Heteroplasmy 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	Heteroplasmy 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	17.5	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.	< 0.00

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

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 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.

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.

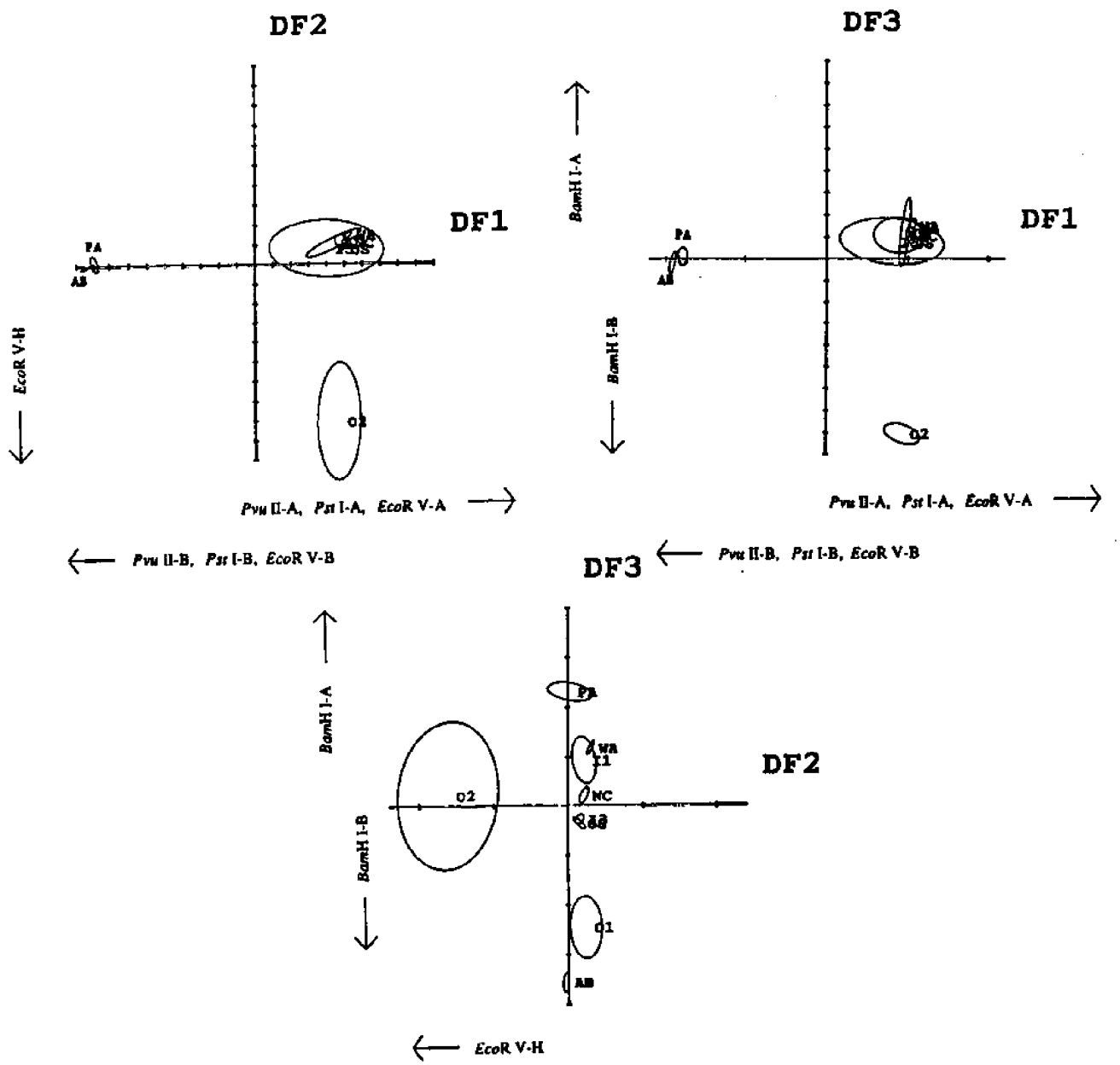


Table 5. Gene identity probabilities for each of nine natural clam populations. Population designations are as in Figure 1. The probability of gene identity is calculated as described in the text. Values along the diagonal are within population gene identity, I, while values in the upper triangle represent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

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								

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.

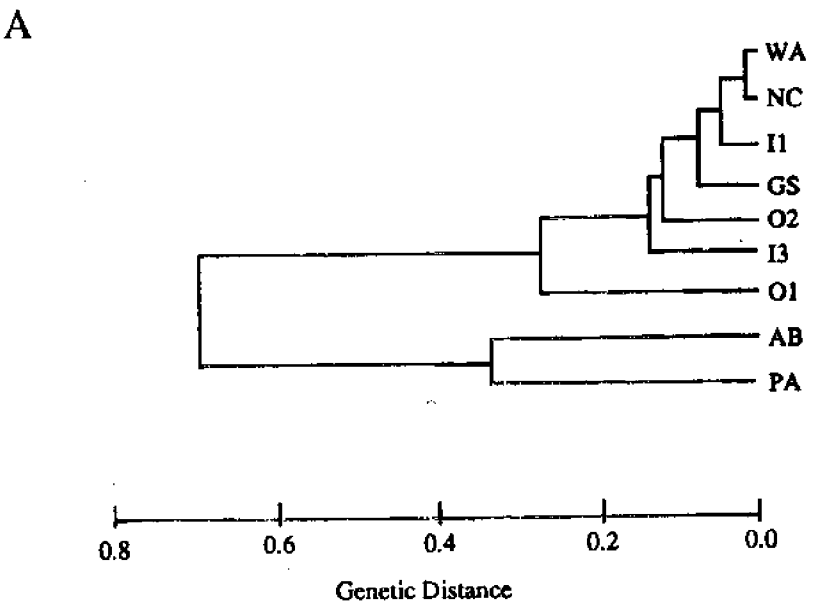
The overall F_{st} for east coast clam populations was calculated from the data in Table 3 to be $F_{st} = 0.411$ which yields an intermediate estimate for effective migration rate of $N_em = 0.717$. Pairwise estimates of effective migration rate between subpopulations are shown in Table 8. These values 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 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 *texana*.

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

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, δ, from Table 7.



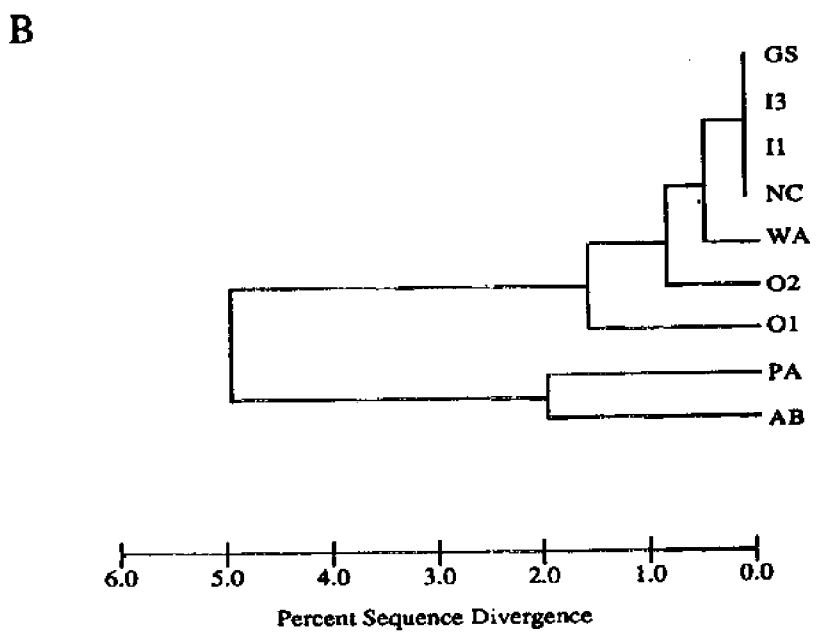


Table 8. Pairwise estimates of effective migration rate, N_em, between the seven east coast populations of *M. mercenaria*. Population designations are as in Figure 1. N_em is calculated from F_{st} as described in the text.

	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						

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, $p < 0.005$).

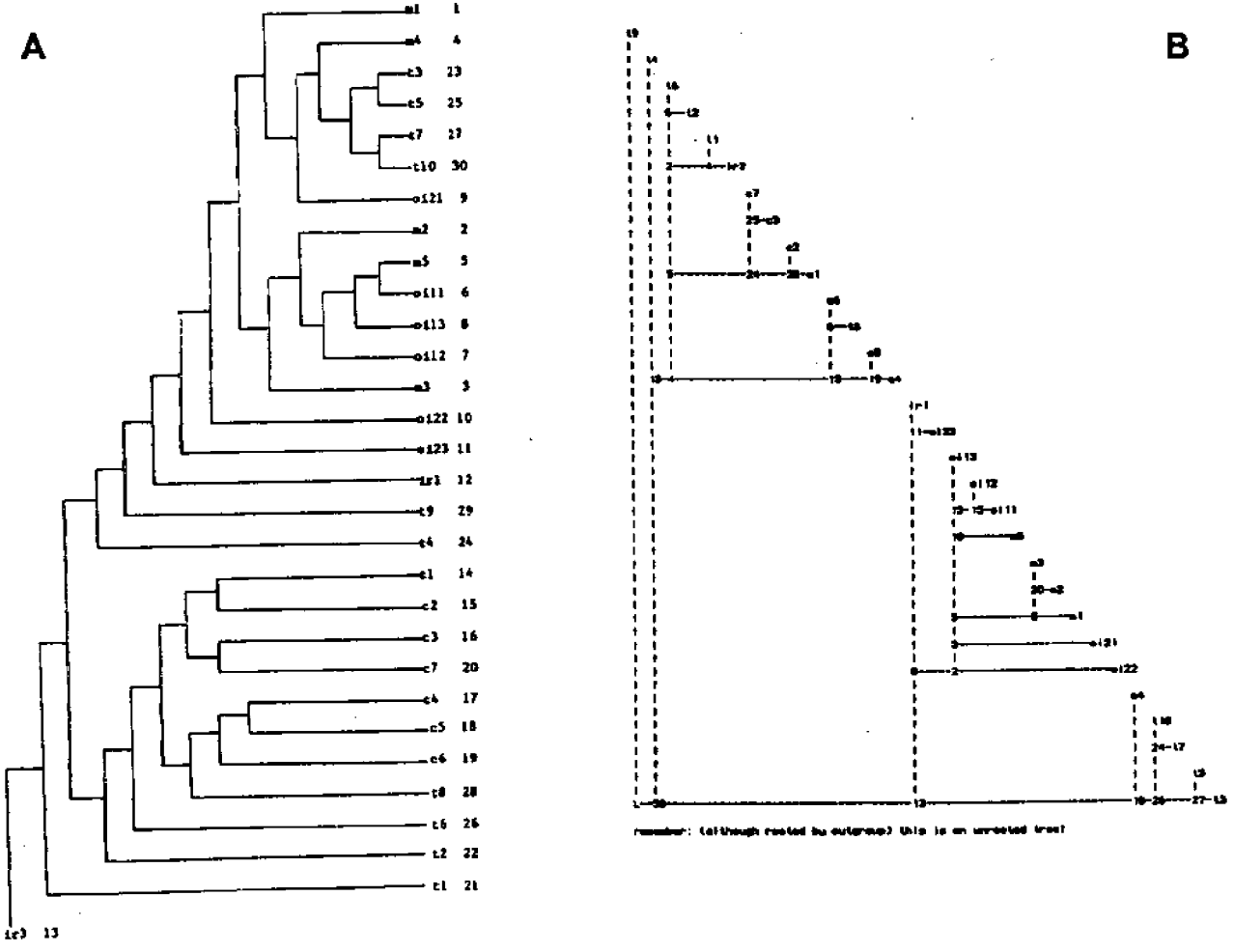
Considering genetic distance and percent sequence divergence, variation on the whole among the three taxa *mercenaria*, *campechiensis* and *texana* occurs at the level of species. Comparisons from Table 6 between the taxa *mercenaria* vs. *campechiensis* (average $D = 0.527$) and *mercenaria* vs. *texana* ($D = 0.868$) 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 are shown in Appendix E) 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 δ, employed information on shared fragments (which were assumed to indicate homologous sequences of mtDNA). Estimates of D and δ 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 δ over D due to the fact that δ 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 δ 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

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.

equation from Slatkin's (1985) private alleles technique for estimating N_em 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 δ 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 (δ = 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 ± 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, δ values between the taxa *mercenaria* and *texana* are not significantly different from those between *mercenaria* and *campechiensis* (δ = 0.044 ± 0.027 and δ = 0.053 ± 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 δ, 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

Conclusion

Hard shell clam aquaculture is becomming a productive Virginia industry. Impediments to progress in the domestication of clams and other cultured invertebrates include the inapplicability of traditional livestock breeding strategies, the lack of stock identification techniques, and uncertainty of the value of different native stocks to selective breeding programs. Current trends in aquaculture research include the development of rapid and reliable techniques for assaying genetic variability in cultured stocks. We have employed restriction enzyme digestion of hard shell clam mtDNA to ultimately determine the appropriateness of current breeding practices.

This method of analysis was chosen based on the generally accepted view that the rate of evolution of mtDNA is greater than that of nuclear DNA--thus, the rate of appearance of new mitochondrial genotypes is fairly rapid. In addition, the mode of inheritance of mtDNA is maternal; fixed mutations in the molecule are transmitted intact; and recombination does not occur. In view of these features it was deemed that with reasonably large sample sizes, restriction enzyme analysis of mtDNA would be the best indicator of population level phenomena in clams.

During 1987, Phase I of the project, laboratory procedures and experimental protocol were developed and optimized for extraction of hard shell clam mtDNA and its subsequent digestion with restriction enzymes. A battery of over 20 restriction enzymes were then tested to determine which were informative with regard to restriction site variation. The results of Phase I are presented by Brown and Wolfinbarger (1987).

In Phase II, 1988, efforts were focused on assaying the mtDNAs of *M. mercenaria* clams from nine populations along the U.S. Atlantic coast, two Gulf coast populations of *M. campechiensis*, and two populations of *M. mercenaria texana* from Texas. Geographic and phylogenetic relationships were evaluated based on variation in mtDNA genotypes. 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 exhibited 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 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.

The most noteable findings of this study for clam breeders are 1) populations of hard shell clams along the southeastern coast of the United States are genetically distinct, 2) northeast populations of hard shell clams are genetically homogenous but may, in some instances, be distinguished by diagnostic mtDNA haplotypes, 3) mtDNA analysis upholds the separate taxonomic status of *M. mercenaria* and *M. campechiensis*. These findings should markedly affect decisions on clam husbandry practices and when considered will facilitate the formation of sound clam breeding programs in Virginia.

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Literature Cited

Abbott, R. 1954. American seashells. D. Van Nostrand Co., Inc., Princeton, NJ. 543 pp.

Adamkewicz, L. 1987. Geographical effects on growth rate in the hard clam *Mercenaria mercenaria*. Ann. Meeting of the National Shellfish. Assoc. 9-13 August 1987. Halifax, Nova Scotia. (abstract).

Adamkewicz, L., S. Taub and J. Wall. 1984b. Genetics of the clam *Mercenaria mercenaria*. II. Size and genotype. Malacologia 25: 525-533.

Adamkewicz, L., S. Taub, and J. Wall. 1984a. Genetics of the clam *Mercenaria mercenaria*. I. Mendelian inheritance of allozyme variation. Biochem. Genet. 22: 215-219.

Allendorf, F. W., and S. R. Phelps. 1980. Loss of genetic variation in hatchery stock of cutthroat trout. Trans. Amer. Fish. Soc. 109: 537-543.

Anderson, W., W. Keith, F. Mills, M. Bailey, and J. Steinmeyer. 1978. A survey of South Carolina's hard clam resources. SC Wildlife and Marine Resources Dept., Mar. Res. Center, Tech. Rept. No. 32. 32pp.

Aquadro, C., N. Kaplan, and K. J. Risko. 1984. An analysis of the dynamics of mitochondrial DNA sequence evolution. Mol. Biol. Evol. 1: 423-434.

Avise, J. C. 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. Phil. Trans. R. Soc. Lond. B. 312: 325-342.

Avise, J. C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb, and N. C. Saunders. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18: 489-522.

Avise, J. C., and R. A. Lansman. 1983. Polymorphisms of mitochondrial DNA in populations of higher animals. Pages 147-164 *in* M. Nei and R. Koehn, eds. Evolution of genes and proteins. Sinauer, Sunderland, MA.

Avise, J. C., R. A. Lansman, and R. Shade. 1979b. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. I. Population structure and evolution in the genus *Peromyscus*. Genetics 92: 279-295.

Avise, J. C., J. E. Neigel, and J. Arnold. 1984. Demographic influences on mitochondrial DNA lineage survivorship in animal populations. J. Mol. Evol. 20: 99-105.

Avise, J. C., J. E. Neigel, and J. Arnold. 1984. Demographic influences of mitochondrial DNA lineage survivorship in animal populations. J. Mol. Evol. 20: 99-105.

Avise, J. C., and R. C. Vrijenhoek. 1987. Mode of inheritance and variation of mitochondrial DNA in hybridogenetic fishes of the genus *Poeciliopsis*. Mol. Biol. Evol. 4: 514-525.

Barnes, R. S. K. 1984. A synoptic classification of living organisms. Sinauer, Sunderland, MA. 273 pp.

Beaumont, A. R., C. M. Beveridge, and M. D. Budd. 1983. Selection and heterozygosity within single families of the mussel *Mytilus edulis* (L.). Mar. Biol. Lett. 4: 151-161.

Bentzen, P., W. C. Leggett, and G. G. Brown. 1988. Length and restriction site heteroplasmy in the mitochondrial DNA of american shad (*Alosa sapidissima*). Genetics 118: 509-518.

Bermingham, E., T. Lamb, and J. C. Avise. 1986. Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates. J. Heredity 77: 249-252.

Birky, C. W., T. Maruyama, and P. Fuerst. 1983. An approach to population and evolutionary genetic theory for genes in mitochondrial and chlorophasts, and some results. Genetics 103: 513-527.

Borst, P., and L. Grivell. 1981. Small is beautiful--portrait of a mitochondrial genome. Nature 290: 443-444.

Bowen, B. W. 1987. Population structure of the white perch, *Morone americana*, in lower Chesapeake Bay as inferred from mitochondrial DNA restriction analysis. Unpub. Master's thesis. College of William and Mary in Virginia. 33pp.

Brown, B. L. and Wolfinbarger, L. 1987. Mitochondrial restriction enzyme screening and phylogenetic relatedness in the hard shell clam genus *Mercenaria*. Part I. Methodology development. Virginia Sea Grant Report No. VSG-87-02. 33pp.

Brown, W. M. 1983. Evolution of animal mitochondrial DNA. Pages 62-88 *in* M. Nei and R. Koehn, eds. Evolution of Genes and Proteins. Sinauer Assoc. Inc., Sunderland, MA.

Brown, W. M., M. George, and A. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. 71: 4617-4621.

Brummett, A. R., and J. N. Dumont. 1981. Initial stages of sperm penetration into the egg of *Fundulus heteroclitus*. J. Exp. Zool. 210: 417-434.

Buroker, N. E. 1984. Gene flow in mainland and insular populations of *Crassostrea* (Mollusca). Biol. Bull. 166: 550-557.

Chapman, R. W. 1987. Changes in the population structure of male striped bass, *Morone saxatilis*, spawning in three areas of the Chesapeake Bay from 1984 to 1986. Fish. Bull. 85: 167-170.

Chapman, R. W. 1989. Spatial and temporal variation of mitochondrial DNA haplotype frequencies in the striped bass 1982 year class. Copeia (in press).

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

Chapman, R. W., and D. A. Powers. 1984. A method for rapid isolation of mitochondrial DNA from fishes. Maryland Sea Grant Tech. Rept. UM-SG-TS-84-05. 11pp.

Chapman, R. W., J. Stephens, R. A. Lansman, and J. C. Avise. 1982. Models of mitochondrial DNA transmission genetics and evolution in higher eucaryotes. Genet. Res., Camb. 40: 41-57.

Chestnut, A., W. Fahy, and H. Porter. 1957. Growth of young *Venus mercenaria, Venus campechiensis* and their hybrids. Proc. Nat. Shellfish. Assoc. 47: 50-56.

Clark, A. G. 1985. Natural selection with nuclear and cytoplasmic transmission. II. Tests with Drosophila from diverse populations. Genetics 111: 97-112.

Clark, A. G. 1988. Deterministic theory of heteroplasmy. Evolution. 42: 621-626.

Cooper, D. and Schmidtke, J. 1984. DNA restriction fragment length polymorphisms and heterozygosity in the human genome. Hum. Genet. 66: 1-16.

Cross, T. F., and J. King. 1983. Genetic effects of hatchery rearing in Atlantic salmon. Aquaculture 33: 33-40.

Crow, J. F., and M. Kimura. 1970. An Introduction to Population Genetics Theory. Harper and Row, New York. 591 pp.

Cummins, R. 1966. Hard clam explorations off the southeastern United States. Comm. Fish. Rev. 28: 33-42.

Dall, W. H. 1909. Synopsis of the family Veneridae and of the North American recent species. Proc. U.S. Natl. Museum. 26: 335-412.

Densmore, L. D., J. W. Wright, and W. M. Brown. 1985. Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenetic and bisexual lizards (Genus *Cnemidophorus*). Genetics 110: 689-707.

Dillon, R. T. and J. J. Manzi. 1987. Hard clam *Mercenaria mercenaria*, broodstocks: genetic drift and loss of rare alleles without reduction in heterozygosity. Aquaculture 60: 99-105.

Dillon, R. T., and J. J. Manzi. (submitted). Genetics and shell morphology in a hybrid zone between the hard clams, *Mercenaria mercenaria* and *M. campechiensis*.

Foltz, D. W., and M. Chatry. 1986. Genetic heterozygosity and growth rate in Louisiana oysters (*Crassostrea virginica*). Aquaculture 57: 261-269.

Gaffney, P. M., and T. M. Scott. 1984. Genetic heterozygosity and production traits in natural and hatchery poplations of bivalves. Aquaculture 42: 289-302.

Gall, G. A. E., and C. A. Busach, eds. 1986. Genetics in Aquaculture II. Elsevier, New York. 386 pp.

Gosling, E. M. 1981. Genetic variability in hatchery produced Pacific oysters (*Crassostrea gigas* Thunberg). Aquaculture 26: 273-287.

Grassle, J. F. 1978. The genetic component of variable growth and survival in seed quahogs. Page 6 *in* Marine Resource Devleopment and Management. A Rept. on the WHOI Sea Grant Program for July 1975 - July 1976. NOAA Sea Grant 04-6-158-44016.

Greenberg, M. J. 1966 Species specific effects of acetylcholine on bivalve rectums. Science 154: 1015-1017.

Hale, L. R., and R. Singh. 1986. Extensive variation and heteroplasmy in size of mitochondrial DNA among geographic populations of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 83: 8813-8817.

Harrison, R. G., D. M. Rand, and W. C. Wheeler. 1985. Mitochondrial DNA size variation within individual crickets. Science 228: 1446-1448.

Harrison, R. G., D. M. Rand, and W. C. Wheeler. 1987. Mitochondrial DNA variation in field crickets across a narrow hybrid zone. Mol. Biol. Evol. 4: 144-158.

Haven, D., and J. Andrews. 1957. Survival and growth of *Venus mercenaria, Venus campechiensis* and their hybrids in suspended trays and on natural bottoms. Proc. Nat. Shellfish. Assoc. 47: 43-49.

Hillman, R. 1968. Histochemical studies of mucous in the mantle of two species of venerid clams. Pages 564-567 *in* Proc. Symp. Mollusca. Part II. (Mar. Biol. Assn. India).

Hinegardner, R. 1974. Cellular DNA content of the mollusca. Comp. Biochem. Physiol. 47A: 447-460.

Holmes, R. S., and G. S. Whitt. 1970. Developmental genetics of the esterase isozymes of *Fundulus heteroclitus*. Biochem. Genet. 4: 471-480.

Hopkins, H. 1934. Catalase and oxidative processes in animal tissues as possible factors in adaptation. Biol. Bull. 67: 115-125.

Humphrey, C. M. 1981. Ecological genetics of the hard clams (*Mercenaria mercenaria* Linne and *Mercenaria campechiensis* Gmelin): Electrophoretic estimates of enzyme variation and the use of shell morphology as a species indicator. Unpub. Ph. D. thesis. University of Georgia. 93 pp.

Kume, M., and K. Dan. 1968. Invertebrate Embryology. [Trans. from Japanese]. NOLIT Publishing House, Belgrade, Yugoslavia. 605pp.

Lansman, R. A., J. C. Avise, and C. Aquadro. 1983a. Extensive genetic variation in mitochondrial DNAs among geographic populations of the deer mouse, *Peromyscus maniculatus*. Evolution 37: 1-16.

Lansman, R. A., J. C. Avise, and M. Huettel. 1983b. Critical experimental test of the possibiltiy of "paternal leakage" of mitochondrial DNA. Proc. Natl. Acad. Sci. 80: 1969-1971.

Lansman, R.A., R. Shade, J. Shapiro, and J. C. Avise. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Mol. Evol. 17: 214-226.

Linnaeus, C. 1758. Systema Naturae. Vol. I. Tenth ed. [Photographic facsimile made in 1956]. Jarrold and Sons Ltd., Norwich.

Loosanoff, V. 1959. The size and shape of metamorphosing larvae of *Venus* (*Mercenaria*) *mercenaria* grown at different temperatures. Biol. Bull. 117: 308-318.

Loosanoff, V. L., and H. C. Davis. 1950. Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. Biol. Bull. 98: 60-65.

Loosanoff, V., H. Davis, and P. Chanley. 1966. Dimensions and shapes of larvae of some marine bivalve molluscs. Malacologia 4: 351-435.

Manwell, C. 1963. The chemistry and biology of hemoglobin in some marine clams. I. Distribution of the pigment and properties of the oxygen equilibrium. Comp. Biochem. Physiol. 8: 209-218.

Manzi, J. J. 1985. Clam Aquaculture. Pages 275-310 *in*: Crustacean and mollusk aquaculture in the United States. J. V. Huner and E. E. Brown, eds. AVI Publishing Co., Inc., Westport, CN.

McHugh, J. L., M. W. Summer, P. J. Flagg, D. W. Lipton, and W. J. Behrens. 1982. Annotated bibliography of the hard clam (*Mercenaria mercenaria*). NOAA Tech. Rep. NMFS SSRF-756, U.S. Dept. of Commerce, Washington, DC. 848 pp.

Menzel, W. 1963. Seasonal growth of the northern quahog, *Mercenaria mercenaria*, and the southern quahog, *Mercenaria campechiensis* in Alligator Harbor, Florida. Proc. Nat. Shellfish. Assoc. 52: 37-46.

Menzel, W. 1964. Observations of quahog clams and their hybrids. Am. Zool. 4: 292.

Menzel, W. 1966. Studies of the F_1 and F_2 hybrids of the northern and southern quahog clams. Proc. Nat. Shellfish. Assoc. 57: 2 (abstract).

Menzel, W. 1968. Chromosome number in nine families of marine pelecypod molluscs. Nautilus. 82: 45-58.

Menzel, W. 1970. The species and the distribution of quahog clams (*Mercenaria*). Proc. Nat. Shellfish. Assoc. 60: 8 (abstract).

Menzel, W. 1971. Selection and hybridization in oysters and clams. Mollusc Chaser, Vol. 9. South Florida Shell Club, Inc. Miami, FL. 2 pp.

Menzel, W., and M. Menzel. 1965. Chromosomes of two species of quahog clams and their hybrids. Biol. Bull. 129: 181-188.

Menzel, W., E. Cake, M. Haines, R. Martin, and L. Olsen. 1976. Clam mariculture in northwest Florida: observations on selection and hybridization. Proc. Nat. Shellfish. Assoc. 66: 103 (abstract).

Merrill, A., and Tubiash, H. 1970. Molluscan resources of the Atlantic and Gulf coasts of the United States. Pages 925-948 *in* Proc. Symp. Mollusca. Part III. (Mar. Biol. Assn. India).

Mitton, J. B., and M. C. Grant. 1984. Associations among protein heterozygosity, growth rate and developmental homeostasis. Ann. Rev. Ecol. Syst. 15: 479-499.

Monnerot, M., J.-C. Mounolou, and M. Solignac. 1984. Intra-individual length heterogeneity of *Rana esculenta* mitochondrial DNA. Biol. Cell 52: 213-218.

Moritz, C., and W. M. Brown. 1986. Tandem duplication of D-loop and ribosomal RNA sequences in lizard mitochondrial DNA. Science 233: 1425-1427.

Moritz, C., and W. M. Brown. 1987. Tandem duplications in animal mitochondrial DNAs: Variation in incidence and gene content among lizards. Proc. Natl. Acad. Sci. 84: 7183-7187.

Moritz, C., T. E. Dowling, and W. M. Brown. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18: 269-292.

Mulligan, T. J., and R. W. Chapman. 1989. Mitochondrial DNA analysis of Chesapeake Bay white perch, *Morone americana*. Copeia (in press).

Nei, M., and W. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. 76: 5269-5273.

Neigel, J. E., and J. C. Avise. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. Pages 515-534 *in* E. Nevo and S. Karlin, eds. Evolutionary Processes and Theory. Academic Press, New York.

Olivo, P. D., M. J. Van de Walle, P. J. Laipis, and W. W Hauswirth. 1983. Nucleotide sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA D-loop. Nature 306: 400-402.

Paynter, K. T., and L. DiMichele. 1989. Growth of tray cultured oysters in the Chesapeake Bay. Aquacultue (in press).

Pesch, G. 1974. Protein polymorphisms in the hard clams *Mercenaria mercenaria* and *Mercenaria campechiensis*. Biol. Bull. 146: 393-403.

Porter, H., and A. Chestnut. 1962. The offshore clam fishery of North Carolina. Proc. Nat. Shellfish. Assoc. 51: 67-73.

Powell, J., and Zuniga, M. 1983. A simplified procedure for studying mtDNA polymorphisms. Biochem. Genetics 21: 1051-1055.

Rand, D. M. 1987. Population biology of mitochondrial DNa in the crickets, *Gryllus pennsylvanicus* and *Gryllus firmus*. Unpub. Ph. D. thesis. Yale University. 169 pp.

Rand, D. M., and R. G. Harrison. 1986. Mitochondrial DNA transmission genetics in crickets. Genetics. 114: 955-970.

Ryman, N. 1983. Patterns of distribution of biochemical genetic variation in salmonids: Differences between species. Aquaculture 33: 1-21.

Ryman, N., and G. Stahl. 1980. Genetic changes in hatchery stocks of brown trout (*Salmo trutta*). Can. J. Fish. and Aquat. Sci. 38: 1562-1575.

Saila, S., and S. Pratt. 1975. Mid-Atlantic Bight Fisheries. Pages 6-1 to 6-125 *in* Coastal and Offshore Environmental Inventory, Cape Hatteras to Nantuckett Shoals, Mar. Exp. Sta., Grad. School of Oceanography, Univ. of Rhode Island. Mar. Publ. Ser. 2.

Satta, Y., N. Toyohara, C. Ohtaka, Y. Tatsara, T. K. Wantanbe, E. T. Matsuura, S. I. Chiguse, and N. Takahata. 1988. Dubious maternal inheritance of mitochondrial DNA in *Drosophila simulans* and evolution of *Drosophila mauritiana*. Genet. Res., Camb. 52: 1-6.

Saunders, N. C., L. G. Kessler, and J. C. Avise. 1986. Genetic variation and geographic differentiation in mitochondrial DNA of the horseshoe crab, *Limulus polyphemus*. Genetics 112: 613-627.

Scheltema, R. S. 1971. The dispersal of the larvae of shoal water benthic invertebrate species over long distances by ocean currents. Pages 7-28 *in* D. J. Crisp, ed. European Marine Biology Symposium. Vol. 4. Cambridge University Press, New York.

Sederoff, R. R. 1984. Structural variation in mitochondrial DNA. Adv. Genet. 22: 1-108.

Singh, S. M., and E. Zouros. 1978. Genetic variation associated with growth rate in the american oyster (*Crassostrea virginica*). Evolution 32: 342-353.

Slatkin, M. 1985b. Rare alleles as indicators of gene flow. Evolution 39: 53-65.

Smith, H., and D. Nathans. 1973. A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. J. Mol. Biol. 81: 419-423.

Sneath, P., and R. Sokal. 1973. Numerical taxonomy. W. H. Freeman and Co., San Francisco. 573 pp.

Snyder, M., A. R. Fraser, J. LaRoche, K. E. Gartner-Kepkay, and E. Zouros. 1987. Atypical mitochondrial DNA from the deep-sea scallop *Placopecten magellanicus*. Proc. Natl. Acad. Sci. 84: 7595-7599.

Sokal, R. R., and F. J. Rohlf. 1981. Biometry. W. H. Freeman and Co., San Francisco. 859 pp.

Solignac, M., J. Genermont, M. Monnerot, and J.-C. Mounolou. 1987. Drosophila mitochondrial genetics: evolution of heteroplasmy through germ line cell divisions. Genetics 117: 687-696.

Solignac, M., J. Genermont, M. Monnerot, and J.-C. Mounolou. 1984. Genetics of mitochondrial in Drosophila: inheritance in heteroplasmic strains of *D. mauritiana*. Mol. Gen. Genet. 197: 183-188.

Stahl, G. 1983. Differences in the amount and distribution of genetic variation between natural populations and hatchery stocks of Atlantic salmon. Aquaculture 33: 23-32.

Stine, O. C. 1986. The origin of *Cepaea nemoralis* in Lexington, Virginia: mitochondrial DNA and climatic selection. Unpub Ph.D. thesis. Univ. of Virginia. 82 pp.

Streisinger, G., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye. 1966. Frameshift mutations and the genetic code. Cold Spring Harbor Symp. Quant. Biol. 31: 77-84.

Takahata, N. 1983. Population genetics of extranuclear genomes under the neutral mutation hypothesis. Genet. Res., Camb. 42: 235-255.

Takahata, N., and M. Slatkin. 1983. Evolutionary dynamics of extranuclear genes. Genet. Res., Camb. 42: 257-265.

Takahata, N., and S. Palumbi. 1985. Extranuclear differentiation and gene flow in the finite island model. Genetics 109: 441-457.

Takahata, N., and T. Maruyama. 1981. A mathematical model of extranuclear genes and the genetic variability maintained in a finite population. Genet. Res., Camb. 37: 291-302.

Tiffany, W. 1972. Aspects of excretory ultrafiltration in the bivalved molluscs. Comp. Biochem. Phys. 43A: 527-536.

Wallace, D. 1982. Structure and evolution of organelle genomes. Microbiol. Rev. 46: 208-240.

Wallis, G. P. 1987. Mitochondrial DNA insertion polymorphism and germline heteroplasmy in the *Triturus cristatus* species complex. Heredity 58: 229-238.

Wilkins, N. P., and E. M. Gosling, eds. 1983. Genetics in aquaculture. Elsevier Science Publishers, New York. 425 pp.

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, irrefular, 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 the hard shell clam *Mercenaria spp.*

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
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*. The sequence of nucleotides on the mtDNA molecule which is recognized by each of the enzymes is listed in the right hand column..

BamH I	G*GATCC
EcoR I	G*AATTC
EcoR V	GAT*ATC
Hind III	A*AGCTT
Pst I	CTGCA*G
Pvu II	CAG*CTG

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

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Heteroplasmy Size	Site
1	A	–	–	–	A	A	18		
2	A	–	–	–	A	A	18		
3	A	–	–	–	A	A	18		
4	–	–	–	–	A	A	17		
5	–	–	–	–	A	A	17		
7	–	–	–	–	A	A	19		
8	–	–	–	–	A	A	17		
9	–	–	–	–	A	A	17		
10	–	–	–	–	A	–	17.5		
11	–	–	–	–	A	A	19		
12	–	–	–	–	A	–	17		
13	–	–	–	–	A	A	17.5		
14	–	–	–	–	A	A	17.5		
15	–	–	–	–	–	A	17.5		
16	–	A/B	–	–	A	A	18	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	Site
1	A	A	A	–	A	A	18		
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		
5	A	A	A	B	A	A	18		
6	A	A	A	B	A	A	18		
7	A	A	A	B	A	A	17.5		
8	–	–	–	B	–	–	17.5		
9	A	A	A	B	A	A	18		
12	A	A	A	–	A	A	18		
13	A	A	A	–	A	A	18		
14	A	A	A	–	A	A	18		
15	A	A	A	A	A	A	18		
16	–	–	A	–	–	–	18		
22	A	A	A	–	A	A	18		
23	A	A	A	–	A	A	18		
24	–	A	A	–	–	–	17.5		

APPENDIX D

Location: WA

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Heteroplasmy Size	Heteroplasmy Site
1	A	A	A	A	A	A	18		
2	A	A	A	–	A	A	18	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	X	X
10	A	A	A	B	A	A	18	X	
11	A	A	A	B	A	A	17.5	X	
12	A	A	A	B	A	A	18	X	
13	A	A	A	B	A	A	17.5		
14	A	–	A	B	–	–	18		
15	A	A/D	A	B	A	A	17	X	X
16	A	A/D	A	B	A	A	17	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	Pvu II	Predominant MW	Heteroplasmy Size	Heteroplasmy Site
2	B	A	–	–	–	–	17		
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		
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		
17	A	A	A	–	A	A	18		
18	–	–	–	–	A	A	17.5		
19	A	–	A	–	–	–	17.5		
20	B	A/D	A	–	A	A	18		
21	B	A/D	A	–	A	A/?	18		X
22	B	A/D	A	–	A	A	17.5		

APPENDIX D

Location: O2

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Heteroplasmy Size	Heteroplasmy Site
1	A	A	H	A	A	A	18		
2	A	A	H	A	A	A	17.5		
3	A	A	H	A	A	A	18		
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		
8	A	A	H	A	A	A	17		
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		

Location: NC

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Heteroplasmy Size	Heteroplasmy Site
3	A	–	–	–	–	–	18		
4	A	A	A	–	A	A	18		
5	A	A	D	–	A	A	18		
6	A	A	A	–	A	A	18	X	
7	A	A	A	–	A	A	18		
8	–	–	–	–	A	–	18		
9	A	A	A	A	A	A	17.5	X	
10	A	A	A	A	A	A	17		
11	A	A	A	A	A	A	18	X	
12	A	A	A	B	A	A	18	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		
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	Heteroplasmy Site
6	–	–	A	A	–	–	18		
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	X	X

APPENDIX D

Location: BB

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

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		
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		

Location: I 1

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant 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	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	X	

Location: I3

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant 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		
29	A	A	A	A	–	A	18		
30	A	A	B	A	–	A	17		
31	A	A	A	A	–	A	18		
32	A	A	A	A	–	A	17		
33	A	A	A	A	–	A	17.5		
34	A	A	A	A	–	A	18.5		
35	A	A	A	A	–	A	17.5		

Location: TB (M. c.)

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Heteroplasmy Size	Site
1	A	–	–	–	–	–	17.5		
8	A	–	–	–	–	–	18		
9	A	–	–	–	–	–	17.5		
10	A	–	–	–	–	–	18		
11	A	–	–	–	A	–	17.5		
12	–	–	–	–	–	B	17.5		
14	–	–	–	–	A	–	17.5		
15	A	–	–	–	–	–	19		
24	A	–	–	–	–	–	18.5		

APPENDIX D

Location: AB (M. c.)

Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Heteroplasmy Size	Heteroplasmy 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	X	X
4	A/B	C	B	B	B/A	B	18	X	X
15	B	A	B	–	B	B	17.5		
16	B	A	B	–	B	B/A	18		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		
20	B	A	B	–	B	B	17.5		
21	B	A	B	–	B	B	18		
22	B	–	–	–	–	B	18		
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		
32	B	A	B	–	B	B	17.5		
33	B	A	B	–	B	B	18		
34	B	–	–	B	–	B	17		
35	B	–	–	A	–	B	17		
36	B	–	B	B	–	–	17.5		
37	B	–	B	B	–	–	17.5		
38	B	A	B	B	B	B	17		

APPENDIX D

	Location:	GA (M.m.t.)					Predominant	Heteroplasmy	
Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	MW	Size	Site
1	B	–	–	–	–	–	18		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		X
14	A	–	–	–	–	–	18		
15	A	–	B/A	–	–	–	18		X
17	B	–	A/B	–	–	–	18		X
19	A/B	–	B/A	E	–	–	17.5		X
20	A	–	B/A	–	–	–	18		X
21	A	–	E/A	–	–	–	18		X
22	A	–	B/A	–	–	–	18		X
23	D/A	–	A/D	–	–	–	18		X
24	A	–	A/D	–	–	–	18		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		X
31	A/B	–	B/A	–	–	–	17.5		X
32	A	–	B/A	–	–	–	17.5		X

APPENDIX D

	Location:	PA (M.m.t.)						Heteroplasmy	
Indiv.	BamH I	EcoR I	EcoR V	HindIII	Pst I	Pvu II	Predominant MW	Size	Site
1	A	A	B	–	B	B	17		
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		
7	A	A	B	–	B	B	17.5		
8	E	A	D	–	A	C	17		
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		
14	E	C	D	–	A	C	17.5		
15	A	A	B	–	A	B	18		
16	A	A	B/D	–	B	B	18		X
17	A	A	F	–	B	B	18		
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		
26	E	A	D	–	A	C	18		
27	–	–	–	–	–	B	17.5		
28	A	A	F	–	B	B	17.5		
29	E	A	D	–	B	C	17		
30	B	A	B	–	A	B	17.5		
31	A	A	B	–	B	B	17.5		
32	A	A	B	–	A	B	17.5		