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MITOCHONDRIAL RESTRICTION ENZYME SCREENING AND PHYLOGENETIC RELATEDNESS IN THE HARD SHELL CLAM GENUS MERCENARIA. PART I. METHODOLOGY DEVELOPMENT

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Executive Summary

Development of successful breeding programs for the hard shell clam, Mercenaria, has been hindered by the length of time between the actual breeding event and acquisition of results. Additional complications have involved the need to determine whether observed differences in breeding stock from different locations are heritable. The development of new technologies has eliminated many of these problems and examination of mitochondrial nucleotide diversity is expected to facilitate the selection of breeding stock by distinguishing between Mercenaria stocks of different geographic origin.

Appropriate methodologies for extraction of clam mitochondrial DNA (mtDNA) for subsequent digestion by restriction enzymes have been developed at the O.D.U. Center for Biotechnology. In addition, procedures for restriction enzyme digestion have been refined and the appropriate enzymes for clam mtDNA analysis have been determined. The enzymes BamHI, ClaI, EcoRI, EcoV, and HindIII provide for unique DNA fragment patterns and were selected from the 19 endonucleases tested for further analysis of clam mtDNA. These restriction enzymes yield informative restriction fragment patterns which are easily resolved with an inexpensive and uncomplicated apparatus. Preliminary data indicate that differences may exist between clam populations; however, these data are not yet extensive enough to

indicate whether the polymorphisms are significant or whether mtDNA restriction fragment screening will allow distinction of geographic origin of Mercenaria.

This report outlines procedures which we have found to be most appropriate for the extraction and digestion of hard shell clam mtDNA. Although the solid phase procedures described in the original proposal yield mtDNA, several other forms of DNA are also co-extracted as are contaminating molecules (e.g., mucopolysaccharides) which prevent digestion of the mtDNA by restriction enzymes. We still consider solid phase extraction a viable approach and are attempting to resolve these problems by implementing procedural changes. In the interim we are utilizing mtDNA extracted via the described adaptation of the aqueous extraction procedure outlined by Stine (1986). The aqueous extraction has the advantage of yielding digestible mtDNA while its disadvantages include greatly reduced extraction efficiency and an increase in the amount of time required to complete the procedure as compared to solid phase extraction. Aqueous extraction of clam mtDNA has permitted us to satisfy our initial objectives: 1) extraction and determination of the qualitative characteristics of Mercenaria mtDNA, and 2) selection of appropriate and informative restriction enzymes for use in Mercenaria geographic origin studies. It is noteworthy that we have also successfully isolated and digested mtDNA from blue crabs (Callinectes sapidus) and from crayfish (Procambarus clarkii), data not shown, intimating the broad utility of the procedure for other invertebrate species.

Introduction

Hard shell clams, Mercenaria spp., are bivalves of significant commercial value in the Atlantic and Gulf coast states where they are both fished and cultured. Wild and cultured clam populations have been the subject of considerable research since the 1950's. Recent studies have concentrated on genetic and morphological characteristics in order to promote management of wild populations (c.f., Adamkewicz, 1987; Adamkewicz et al., 1984a and 1984b; Humphrey, 1981; Humphrey et al., 1982; Menzel, 1966 and 1971; Pesch, 1974).

Management and genetic improvement of cultured clams, as with other cultured aquatic organisms, lags far behind that produced by domestication of terrestrial organisms. Much current research into the genetics of cultured clams is directed toward stock improvement, elucidation of population heterogeneity, dynamics of reproduction, and domestication for use in aquaculture. Independent clam culturists in several east coast locations have, over the last few years, bred Mercenaria mercenaria stocks of varied geographic origin in an effort to produce a superior clam. This application of artificial selection for improved productivity is based on the as yet unsubstantiated belief that heritable regional differences exist between Mercenaria stocks of different geographic origin.

Two widely used techniques in these studies have been controlled matings and electrophoretic detection of allozyme variants. The former requires extensive devotion of space and resources in order to conduct long-term breeding studies. It is

important to emphasize that controlled mating experiments with clams require up to two years for the researcher to ascertain phenoypes (and thus genotypes) of the progeny. Thus, it is of paramount inportance that a simple and reliable assay be developed to determine the genotype of the breeding stock. An error in selection of breeding stock could require two years to become evident during which time the aquaculturist will have committed considerable resources. Such experiments have been conducted; notably by Adamkewicz (1987), Adamkewicz et al. (1984a and b) and by J. Manzi and coworkers at the Waddell Mariculture Facility in South Carolina (Manzi et al., 1986). Several of these stock assessment studies also incorporated electrophoretic techniques (Humphrey, 1981 and Humphrey et al., 1982). In general, it has been hypothesized that more heterozygous individuals exhibit increased metabolic efficiency, although electrophoretic data have failed to substantiate such an effect on aquaculture populations (c.f., Adamkewicz, 1987; Adamkewicz et al., 1984b; and Manzi et al., 1986). Similar results have been obtained for Mytilus edulis (Koehn et al., 1984).

Such results are partially due to environmental and physiological effects on the enzymes being studied as well as effects of other endogenous enzymes upon the former. Thus, current procedures involving protein analysis attempt to assess the genotype of a specific organism at a point in metabolism far removed from the nucleic acid sequence coding for the protein. Isozyme analysis can be complicated by post-transcriptional and post-translational events involving additional genes necessary

for the expression of the gene coding for the isozymes being studied. These modifications may occur in response to changes in any number of internal or external parameters. It is, therefore, not unreasonable to expect that an assay based directly on nucleotide sequence of a gene would be a more reliable estimate of the genotype of a population. Mitochondrial genes are often chosen over nuclear genes for sequence analysis because of the relative ease with which they may be obtained. In addition, mitochondrial DNA (mtDNA) analysis is quite a sensitive indicator of regional differentiation over short time periods. Thus, we have chosen restriction enzyme analysis of mtDNA to answer the question of whether there are heritable regional differences between Mercenaria stocks of different geographic origin. Such an anlysis conducted prior to controlled mating experiments could save time, labor, expense, and a great deal of valuable hatchery space and facilities.

Mitochondria are autonomous organelles. In animals, mtDNA occurs as a covalently closed circular molecule of approximately 17,000 base pairs (17 kb) in length. There are an average of about 10,000 mtDNA molecules per cell and mtDNA is strictly maternally inherited (paternal mitochondria being excluded from the oocyte around the time of fertilization (Chapman et al., 1982 and Lansman et al., 1983)). Purified mtDNA may be digested with bacterially derived enzymes called restriction endonucleases and the resulting DNA fragments electrophoresed through an agarose gel. Restriction patterns thus obtained are unique and may be used as the basis for estimating maternal phylogeny. Endonucleases may be used on mtDNA samples from large numbers of

individuals and the fragment patterns for each enzyme compared. When comparing one individual with another, an endonuclease will no longer recognize a restriction site in which a mutation has introduced a change in nucleotide sequence. In a population with such a sequence change, two bands of the original restriction pattern for that enzyme will be replaced by one band with a molecular size of approximately the sum of the two missing bands. The reverse is true when a mutation creates a new restriction site in the mitochondrial genome of a population. An excellent review of restriction fragment analysis theory and the use of mtDNA in phylogenetic comparisons can be found in Stine (1983). Evidence from a large number of laboratories suggests that restriction fragment analysis of mtDNA is a useful taxonomic tool at or below the level of congenerics.

Analyses of mtDNA restriction fragment patterns have proven to be a useful tool in detecting phylogenetic relationships among other closely related organisms (Adams et al., 1982; Aquadro et al., 1983; Avise et al., 1984; Brown et al., 1979; Cann et al. 1984; Chapman et al., 1984; and Ferris et al., 1981). This type of analysis is quite sensitive to regional differentiation over short periods and should be an ideal method for screening cultured and wild clam populations during selection of breeding stock. According to Avise et al. (1979) there are five characteristics of mtDNA restriction analyses which contribute to their value and utility. They are as follows:

a. restriction genotypes are unique,

b. restriction genotypes are transmitted intact; since

mitochondrial genomes do not undergo recombination, the only cause of sequence change is mutation,

c. fixed mutations result in new and recognizable genotypes,

d. mtDNA mutates at a rate approximately 10-fold greater than nuclear DNA; therefore, the rate of appearance of new genotypes is rapid, and

e. restriction enzyme assays are simple, rapid, and reproducible from generation to generation.

On the basis of these characteristics it is proposed that restriction enzyme analyses have the potential to assess mitochondrial diversity within and among populations of cultured and wild clams.

Researchers, however, have had a great deal of difficulty isolating mtDNA from molluscs (Stine, 1986; Avise, pers. comm.). In some instances where mtDNA is obtained, it is contaminated to such an extent as to be rendered undigestible by restriction enzymes. Over the past two years, our ongoing research has resulted in development of a practical approach to the isolation of mtDNA in populations of mammalian cells (Brick-Miller, 1987). This approach, termed "solid phase" mtDNA extraction, has the following advantages:

a. it requires as little as 200,000 cells (which can be obtained via needle biopsy) and need not result in the death of the animal,

b. it can be performed on large numbers of animals and is not restricted to a particular organism,

c. it is reproducible and analyses can easily be repeated at a later date, and

d. it is convenient (all reagents and materials can be readily purchased and rapid extraction of a tissue sample can be performed in 3-4 hours).

Solid phase mtDNA extraction has been adapted for use with clams with mtDNA yields several fold greater than the yields from aqueous extraction procedures. In addition, we have extracted clam mtDNA by the procedures of Bogenhagen et al., 1974; Avise et al., 1984; Chapman et al., 1984; Gonzalez-Villaseñor et al., 1986; Kessler et al., 1984; Palva et al., 1983 and Powel et al., 1985. In every instance we have encountered problems with obtaining intact digestible mtDNA and have thus adapted Stine's aqueous extraction procedure (Stine, 1986) while continuing to optimize solid phase extraction procedures. Molluscan mtDNA obtained via this aqueous extraction method yields digestible mtDNA from individual Mercenaria clams.

Objectives

Traditional molecular biology techniques have been increasingly utilized in applied areas of research in an effort to bring their greater powers of resolution and increased sensitivities to the solution of problems at the organismic level. Antibodies, isozymes, restriction enzymes and nucleotide sequencing are commonly used to genetically distinguish between populations of closely related organisms. In view of the recent advances made in our laboratory, restriction enzyme analysis is the method we have chosen to apply to the questions posed by aquaculture geneticists. We are completing development of procedures for both aqueous and solid phase separation of nuclear and mitochondrial DNA from small amounts of tissue. We have demonstrated that small covalently closed circular DNA embedded in an agarose block can be digested by several restriction enzymes and that clam mtDNA yields unique fragment patterns following digestion with restriction enzymes.

The main objectives of Part I of this study were to develop a rapid mtDNA extraction technique and restriction assay for estimating the mitochondrial diversity of aquaculture stocks used in Mercenaria culture and to determine which of the many restriction enzymes available generate informative patterns following digestion of Mercenaria mtDNA.

Due to the absence of sequence data for clam mtDNA, a large number of restriction enzymes must be examined in order to obtain maximal reliability of fragment patterns for each clam population. Once optimized, the techniques will be used in Part

II of the study to determine the extent of mtDNA divergence within and between chosen geographically separated populations of the genus Mercenaria. The information generated by the entire study is intended to contribute to current efforts in aquaculture genetics. The aim is to adapt state-of-the-art techniques for use in aquaculture. Aquaculturists may then use the data in evaluating stock histories and the suitability of current breeding practices.

Methods

In order to study geographic variation in the hard shell clam genus Mercenaria, restriction enzyme analyses have been performed on its mtDNA. Two mtDNA extraction procedures are being evaluated as they relate to this restriction enzyme analysis.

Solid phase extraction of mtDNA:

The solid phase procedure developed by Brick-Miller (1987) provides for the rapid isolation of mtDNA from small numbers of mammalian cells and has been adapted for clams as follows. Approximately 0.5 g of clam hepatopancreas is excised and minced. The tissue is homogenized on ice in 5 volumes of TEK buffer using 2 strokes with pestel B of a 15 ml Dounce homogenizer. An equal volume of molten ($50^{o}C$) 1.5% Ultra Pure agarose (Bio-Rad) in TAE buffer is quickly combined with 0.5 ml of the homogenate and transfered to a 1 cm^2 casting chamber. The cube is allowed to cool and is then cut into 18 small rectangular blocks (see Figure 1). Blocks containing clam tissue are lysed for 45 minutes at room temperature with a solution of 2% Nonidet P-40 in TEK buffer containing 0.2 mg/ml proteinase-K. The liberated mtDNA may then be electrophoresed into a 1 % agarose slab gel in TAE buffer at 10 V/cm for 2-3 hours. If the mtDNA is of sufficient purity it can subsequently be cut out of the separatory gel and digested in the block of agarose with restriction endonucleases by the procedure of Boehm et al. (1984).

Aqueous mtDNA extraction:

Because we have detected non-mtDNA in the banded DNA isolated

by solid phase extraction, we have also initiated an aqueous mtDNA extraction technique which has been adapted from Stine (1986) as follows. Clam tissue is excised, minced, diluted with 5 volumes of HOMOG and homogenized as described under solid phase procedures. The homogenate is transferred to an ultracentrifuge tube and an equal volume of STEP buffer is layered beneath it. Samples are centrifuged at 13,300 Xg for 50 minutes at 4°C in a swinging bucket rotor. The interface, top and bottom supernatants are carefully removed in that order and discarded. The tissue pellet is resuspended in HOMOG buffer and treated as in the previous step. The sucrose step gradient centrifugations remove mucopolysaccharides from the tissue. The interface and supernatants are removed and discarded. The tissue pellet is resuspended in 1.4 ml of ice cold 2 % Nonidet P-40 in TEK and divided equally into two 1.5 ml Eppendorf microcentrifuge tubes. Nonidet is a detergent which solubilizes all, except nuclear, membranes (Chapman et al., 1984). The tubes are held on ice for 15 minutes after which they are centrifuged (Eppendorf microfuge) at 12,000 r.p.m. and 4°C for 5 minutes. The supernatant from each tube is retrieved and transferred to a tube containing 0.8 ml of water-saturated redistilled phenol. After shaking to completely mix the aqueous and organic phases, the tubes are incubated at 37°C for 10 minutes, then held on ice for 10 minutes prior to centrifugation (Eppendorf microfuge) at 4° C for 10 minutes. The aqueous top layer is transferred to a third tube containing 0.8 ml chloroform:isoamyl alcohol (24:1) to extract any residual phenol. After gentle mixing and holding at room temperature for 5 minutes, the tubes are centrifuged (Eppendorf

microfuge) for 5 minutes. The upper aqueous phase is transferred to a final Eppendorf tube containing 150 ul ice cold 0.1 M spermine. The tube is then filled to 1.5 ml with ice cold sterile deionized water, mixed, and held on ice for 15 minutes. After centrifugation at 4°C for 10 minutes, small tan pellets may be observed. The supernatant is carefully pipetted off and the tube and pellet are washed with RINSE solution and centrifuged at 12,000 r.p.m. and 4°C for 5 minutes. The rinse solution is carefully removed and the tubes are inverted and dried under vacuum until the pellets are clear. Samples are then capped and stored in this condition at 4°C until being redissolved and used for restriction enzyme digestion.

Restriction enzyme digestion of mtDNA:

Dried pellets of mtDNA from individual clams are redissolved in RE buffer to a final concentration of approximately 0.25 ug mtDNA per ul buffer. Approximately 1 ug of mtDNA is used per digestion. In a sterile Eppendorf tube, 4 ul of mtDNA solution are combined with 12.5 ul of sterile deionized water and 2 ul of 10X core buffer (supplied by BRL or made according to BRL specifications and recommended for that particular restriction endonuclease). Then 0.5 ul of 4 mM spermidine is added followed by 1 ul (10 units) of restriction endonuclease. The contents are mixed and incubated at the appropriate temperature (usually 37°C) for 4-6 hours. The reaction is stopped by addition of 2 ul of STOP and samples are refrigerated until electrophoresis.

Agarose gel electrophoresis:

Digested samples are electrophoresed through a horizontal

1.0% Ultra Pure (Bio-Rad) agarose gel in TAE buffer overnight at 3.5 V/cm. The gel is then stained for 15 minutes in a solution of 0.5 ug/ml Ethidium Bromide (EtBr) in TAE and rinsed for 15 minutes in deionized water.

For viewing, the gel is placed directly on a UV Transilluminator and photographed for 1 second at f8 with Polaroid Type 57 film in a Polaroid MP-4 land camera. To increase band intensity, a Kodak Wratten gelatin filter 23 A is fitted immediately over the lens.

Electron Microscopy:

Clams were obtained from Cherrystone Aquafarms in September 1985 and February 1987. Tissues were examined via transmission electron microscopy. Small pieces of hepatopancreas, gill and mantle tissue were minced with a new razor blade and initially fixed in FIX 1 for 24 hours at 4°C. Tissue pieces were rinsed after primary fixation with 0.1 M phosphate buffer (pH 7.4) at 4°C then incubated in FIX 2 for 2 hours at 4°C. Again, samples were rinsed with 0.1 M phosphate buffer and dehydrated by six sequential 10 minute incubations at room temperature in EtOH (30%, 50%, 70%, 90%, 100% and 100%). Next, 100% acetone was used as an intermediate solvent in two 10 minute washes at room temperature. Infiltration was initiated by incubating the tissue samples in 50:50 (v:v) Epon resin:acetone for 16 hours at room temperature followed by 100% Epon resin at room temperature overnight. Lastly, samples were embedded in Epon and held for 48 hours at 67°C. Sections were cut on a diamond microtome and examined with Transmission Electron Microscopy.

Results and Discussion

Extraction of mtDNA:

The physiology and biochemistry of clam tissue is radically different from mammalian and other vertebrate tissues. Clams, like other molluscs, produce copius quantities of mucous which interfere with both the extraction and digestion of mtDNA. As such, the tissue used for extraction must be carefully chosen; mantle and gut tissues are particularly to be avoided as they contain excessive mucous and nucleases, respectively. In addition, other clam tissues contain high concentrations of nucleases which must be inactivated by relatively high concentrations of EDTA. The mtDNA can also be further protected from nuclease digestion by the addition of EtBr to the homogenization buffer. It is important to recall that EtBr, also used to stain DNA within agarose gels, is a powerful mutagen and gloves should be worn whenever handling solutions containing EtBr.

Solid phase extraction techniques are extremely gentle and sensitive. For example, Kedersha et al. (1986) have identified two novel and previously undetected organelle structures via a subcellular fractionation procedure similar in principle to the solid phase mtDNA extraction procedure herein described. Thus, it is not surprising that our data indicate more than one form of DNA present along with mtDNA in the wide solid phase band illustrated in lane 4 of Figure 2. The presence of additional bands was not noticeable under original electrophoretic conditions, however, the additional molecules complicate the "in

block" restriction enzyme digestion procedure. We are reevaluating the conditions utilized in the solid phase procedure in an effort to eliminate the extraneous DNA molecules. Chloroplast and bacterial plasmid studies in our lab have determined that the wide band often resolves into multiple bands, some of which may be small polydisperse extrachromosomal circular DNA (spcDNA) which have recently been described by Yamagishi (1986). Research continues with the objective of identifying which of these newly discovered bands is actually the mtDNA. We are modifying homogenization steps, washing the homogenate, and attempting to increase resolution and separation of multiple bands by decreasing voltage, changing ionic strength of buffers, and altering the separation gel. Lastly, the bands will be removed and purified via the Geneclean (BIO 101, Inc.) process as per the supplied protocol prior to treatment with restriction enzymes.

The quantity and quality of nucleic acid from solid phase and aqueous extraction procedures was measured spectrophotometrically. As can be seen from Table 1, the solid phase extraction yields an order of magnitude more nucleic acid material per gram of wet tissue than aqueous mtDNA extraction. In our laboratory, the aqueous procedure routinely yields 1-3 ug mtDNA per gram of wet tissue. Purity of the mtDNA solutions was estimated using the ratio of absorbance of DNA at 260 nm versus absorbance of protein at 280 nm. Absorbance is proportional to the amount of DNA (or protein) in a solution. It is notable from Table 2 that with increasing quantities of tissue in the solid

phase agarose block there occurs not only a decrease in purity of the DNA solution as indicated by the decreasing optical density ratio but also a drastic decrease in the amount of DNA extracted. Thus, DNA cannot be extracted in larger quantities simply by increasing the amount of tissue embedded in a block. Additional optical density data (not shown) indicate appreciable contamination of solid phase extracted mtDNA by polysaccharides as well as proteins. Comparable data for aqueous extracted mtDNA are not available at this time. We are evaluating the possible use of digestive enzymes in reducing the levels of contaminants in solid phase mtDNA preparations. Almost certainly related to high levels of polysaccharide contamination is the differential receptiveness of solid and aqueous extracted mtDNA to restriction enzyme digestion shown in Table 2.

Characterization of Mercenaria mtDNA:

MtDNA isolated from M. mercenaria is within the size range, as specified by Brown (1983), of 15.7 to 19.5 kilobases (kb). We have estimated clam mtDNA size to be 16.6 $\pm$ 0.8kb as compared to the relative movement of supercoiled DNA standards (BRL). Also, restriction patterns obtained by digesting the mtDNA with various endonucleases consist of bands, the total of whose approximate sizes is in the range of 17-19 kb. It is worth noting that in a restriction fragment pattern, small fragments (less than 0.5 kb) are usually not resolved on the gel. Also, movement of a DNA band through a gel is not only dependent upon the molecular size of the band but on the total amount of DNA contained in the band. Thus, for these and other reasons, especially for very large molecules, molecular size may be easily overestimated.

Seasonal variation in clam mtDNA:

Prototype studies of solid phase mtDNA extraction from clam tissue were carried out during the Summer and Fall of 1986 when very large and apparently discrete bands of mtDNA were routinely detected. Subsequent attempts at solid phase isolation of mtDNA from clams did not always yield identical results. Most clams yielded a large discrete mtDNA band while only a long smear of randomly sized (possibly nuclear) material was extracted from specimens obtained during the months of December and January. Two brief experiments and electron microscopic evaluation were performed in an attempt to determine the basis of this variation.

Temperature:

Ten cherrystone clams were obtained from Cherrystone Aquafarms near Cape Charles, Virginia, where the ambient water temperature was 1°C. All ten were slowly acclimated to 4°C, followed by slow acclimation of five clams to 23°C. All clams were then held at their respective temperatures (five clams at 4°C and five clams at 23°C) for three weeks after which time mtDNA was extracted from each.

Feeding:

Ten cherrystone clams were obtained from Cherrystone Aquafarms. All were slowly acclimated to 23°C. Five were not fed during the duration of the experiment while the other five clams were fed Tahitian Isochrysis galbana. Clams were fed by transfer, every other day, to an aerated container filled with an algal suspension of 10^5 algal cells/ml where they were allowed to filter until the water was cleared.

Afterwards, they were returned to the aquarium with the five unfed clams. The ten clams were treated as such for two weeks prior to mtDNA extraction.

Temperature effect experiments were inconclusive in that all specimens gave rise to smears with only faint mtDNA bands sometimes being visible. Feeding studies, though, indicated that feeding does remove the smearing effect as illustrated in Figure 3. It is suggested that during periods of environmental stress, clam digestive tissue may be "primed" for the onset of feeding with a multitude of enzymes. If so, digestion of DNA by endogenous nucleases could be minimized by inclusion of EtBr in the homogenization buffer.

A preliminary electron microscopic examination of clam hepatopancreas from September 1985 (secondary algal bloom) and February 1987 indicate that the quantity of mitochondria per cell may be greater in the September sampling (see Figure 4). However, the condition of the tissue as a whole appears to be much better in the February sample. Fixation and staining of the tissue samples were conducted by two different technicians and we intend to repeat the experiment this year in order to confirm our initial observations.

Choice of restriction enzymes:

At this time, 19 restriction endonucleases have been tested on Mercenaria mtDNA. Each enzyme tested is listed in Table 3 along with the number of bands detected following digestion of the clam mtDNA with that enzyme. A majority of the enzymes do not cleave clam mtDNA due to absence of recognition sites or perhaps due to residual mucopolysaccharide contamination. Of

those which do cleave mtDNA, BamHI, ClaI, EcoRI, EcoRV, and HindIII will be used in subsequent analyses of clam nucleotide sequence diversity. These are the enzymes which yield an informative restriction fragment pattern using the agarose gel system described here.

Conclusion

To obtain the baseline restriction fragment data for the phylogenetic relatedness portion of this study and in order to meet projected deadlines, an additional technique has been modified for extraction of mtDNA from the hard shell clam. The method was adapted from that of Stine (1986) which protects the DNA from endogenous nucleases and which removes mucopolysaccharides from the tissue preparation. It is mtDNA extracted by this method which has been used for all restriction enzyme digests reported here. The procedure takes approximately one hour longer than that described by Chapman et al. (1984) due to the two sucrose step gradients designed to remove mucous. We still consider the solid phase extraction technique to be the optimal approach for isolation of mtDNA due to its ease and simplicity and are currently reevaluating and revising the process.

Now that appropriate techniques have been developed, emphasis will shift to determination of mtDNA polymorphisms in specific clam populations including two populations previously studied by Adamkewicz (1987). The numbers and kinds of DNA fragments generated from individual clams by the specific restriction enzymes will be determined as well as the overlap (if any) of "polymorphs" between clam populations. We have indications of two such polymorphs as illustrated in Figure 5 with patterns for BamHI and EcoRI in two different populations.

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TABLE 1

Buffer Recipes[a]

Buffer	pH	Contents
FIX 1[b]	7.4	3% Glutaraldehyde 0.5M sucrose (Ultra Pure enzyme grade) 0.15M KCl 0.1% BSA in 0.05M Phosphate Buffer
FIX 2[b]	7.4	1% OsO_4 in 0.1M Phosphate Buffer
HOMOG	7.4	0.25M sucrose (Ultra Pure enzyme grade) 140 ug/ml EtBr (added after auto-claving) in TEK
RE	8.0	50mM Tris-HCl
RINSE	7.5	75% EtOH 0.3M NaOAc 0.01M $MgCl_2$
STEP	7.4	1.1M sucrose (Ultra Pure) in TEK
STOP	8.0	0.1% (w/v) Bromphenol Blue 1.0% (w/v) SDS 0.1M Na_2EDTA 50% (v/v) glycerol
TAE	8.0	0.4M Tris-base 0.02M Acetic acid 0.001M Na_2EDTA
TEK	7.4	50mM Tris-HCl 10mM Na_2EDTA 1.5% KCl

[a] All buffers except FIX 1 and FIX 2 are steam autoclaved for 30 minutes to sterilize.

[b] Do not autoclave this buffer.

TABLE 2

Optical Density of Solid Phase and Aqueous Extracted *Mercenaria* mtDNA

	OD260[a] / OD280	ug DNA obtained per g wet tissue	Digestion by restriction enzymes
Solid phase[b]:			
0.0025g tissue	1.7	165.5	N
0.01g tissue	1.5	28.2	N
0.06g tissue	1.3	2.5	N
0.1g tissue	0.8	0.5	N
Aqueous:			
Mercenaria			
live	1.7	2.0	Y
frozen	1.6	62.0	N
cNA[c]	1.6	144.0	N

a For an aqueous solution of pure DNA, OD260/OD280 = 1.8. An increase in protein contamination leads to a decrease in the value of the ratio.

b Amount of tissue embedded within a block of agarose.

c Total nucleic acid extraction.

TABLE 3

Restriction Endonuclease Activity on *M. mercenaria* mtDNA

Enzyme	Activity	# cut sites
Alu I	N	--
Ava I	N	--
Bam HI	Y	2/3
Bcl I	N	--
Bgl I	N	--
Bgl II	?	?
BstE II	?	?
Cla I	Y	2
Dra I	Y	>50
EcoR I	Y	3/4
EcoR V	Y	1
Hind III	Y	3/4
Pst I	N	--
Pvu II	N	--
Sal I	N	--
Sma I	N	--
Sst II	N	--
Xba I	N	--
Xho I	N	--

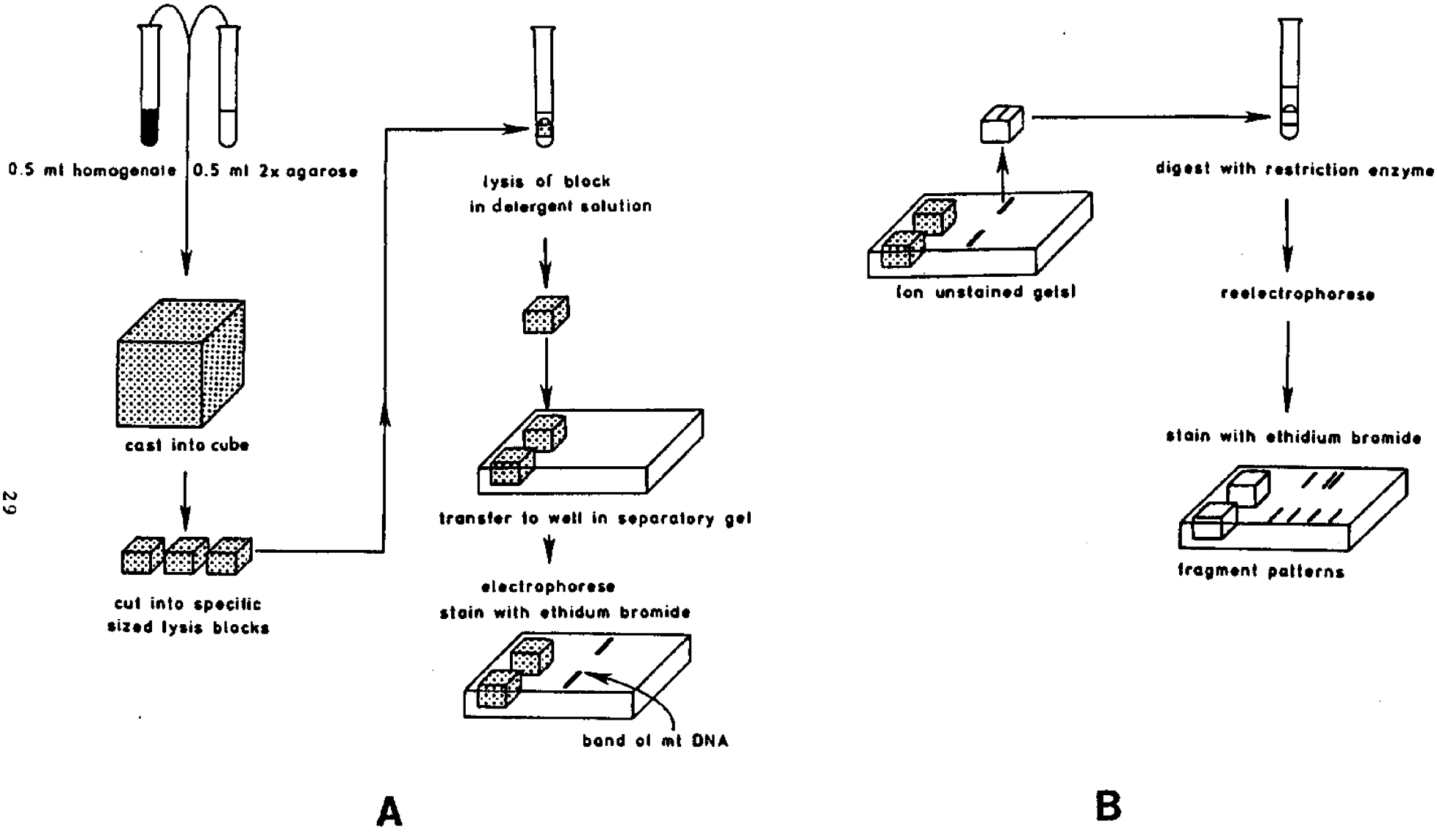

Figure 1. Solid phase mtDNA extraction and digestion procedures.

A. Solid phase mtDNA extraction, and
B. Solid phase mtDNA digestion.

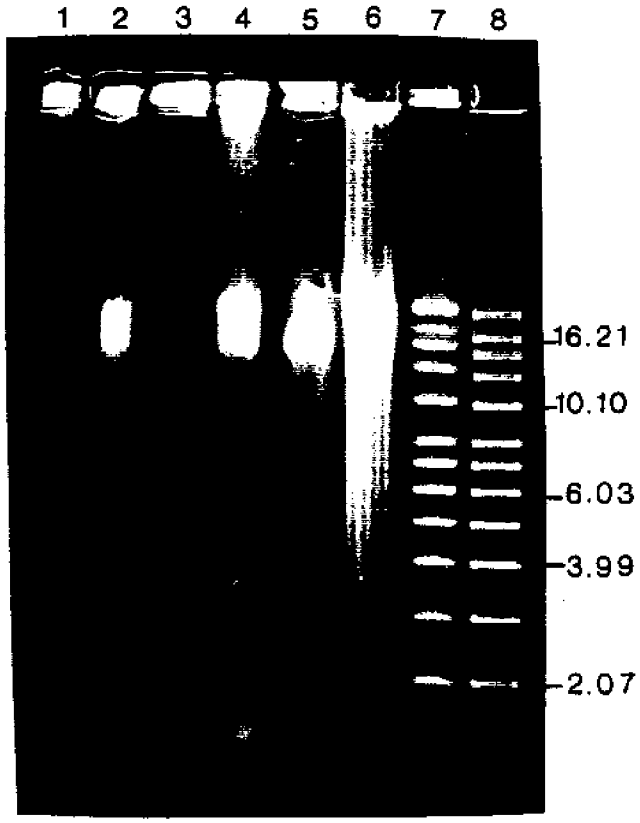

Figure 2. Composite 0.75% separatory agarose gel illustrating solid phase mtDNA extraction. Lane 1 is an untreated block of tissue; lane 2 is a tissue block treated with detergent; lane 3 is an untreated block of intact mitochondria; lane 4 is a block of mitochondria treated with detergent; lane 5 is aqueous extracted mtDNA embedded in an agarose block; lane 6 is aqueous extracted mtDNA pipetted directly into the well (lanes 5 and 6 are purposefully overloaded to approximate the amount of DNA in lanes 1-4); lane 7 contains supercoiled molecular weight standards (BRL) embedded in an agarose block; and lane 8 is supercoiled molecular weight standards pipetted directly into the well. Numbers shown represent molecular size (Kb) of the bands indicated in the supercoiled ladder.

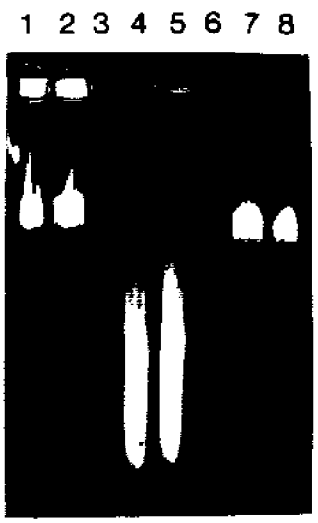

Figure 3. Seasonal variation in clam mtDNA. Lanes 1 and 2 contain tissue from clams obtained during the Fall (water temperature 24°C). Lanes 4 and 5 contain tissue from clams obtained during the Winter (water temperature 1°C). Lanes 7 and 8 contain tissue from clams acclimated to 23°C and fed every other day.

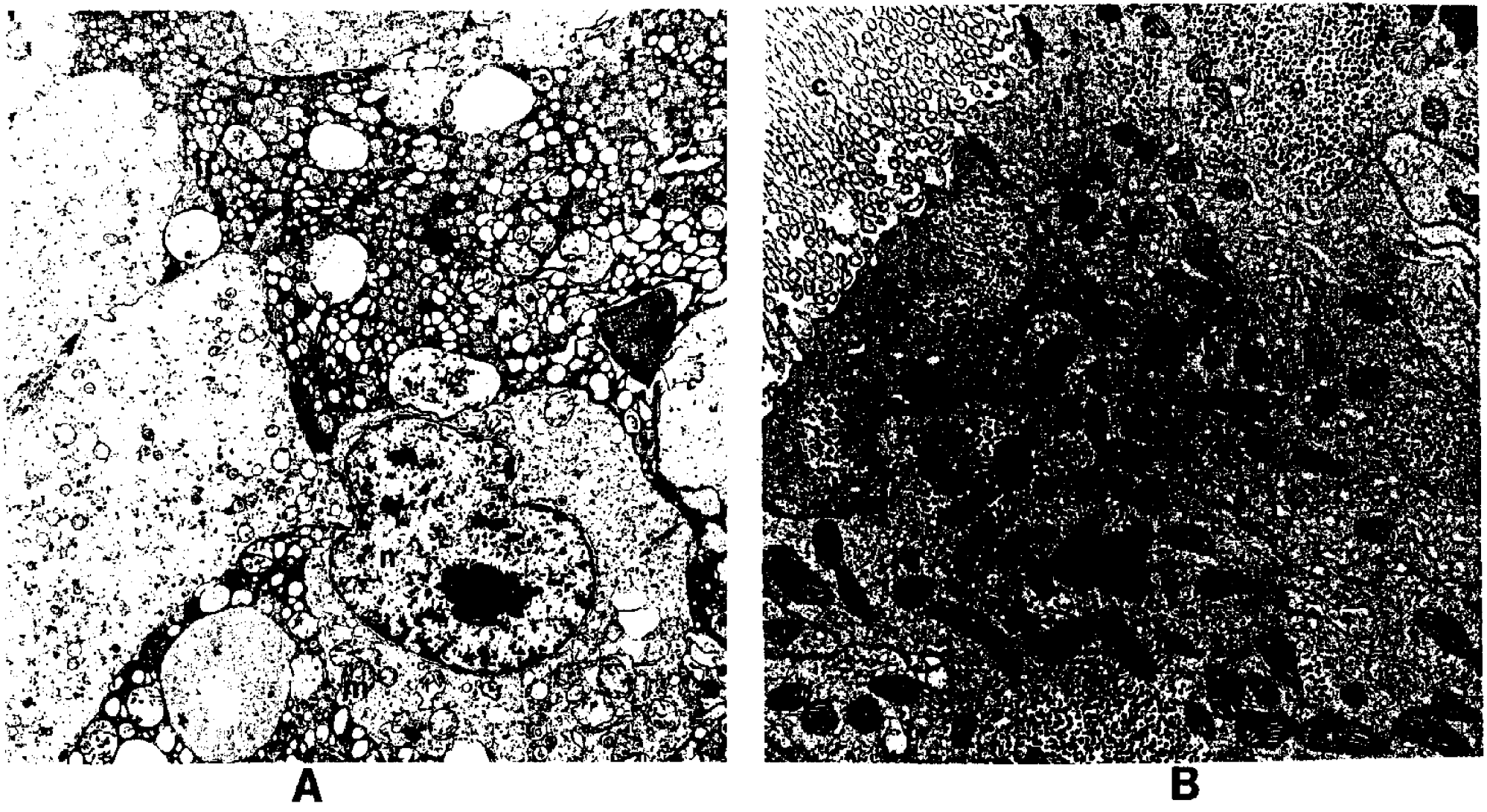

Figure 4. Electron micrographs of M. mercenaria hepatopancreas: A. September 1985 and B. February 1987. The September fixation is very poor as evidenced by bloated mitochondria and crenulated nuclei. Abbreviations: c, cilia; g, presumptive glycogen storage granules; m, mitochondrion; n, nucleus.

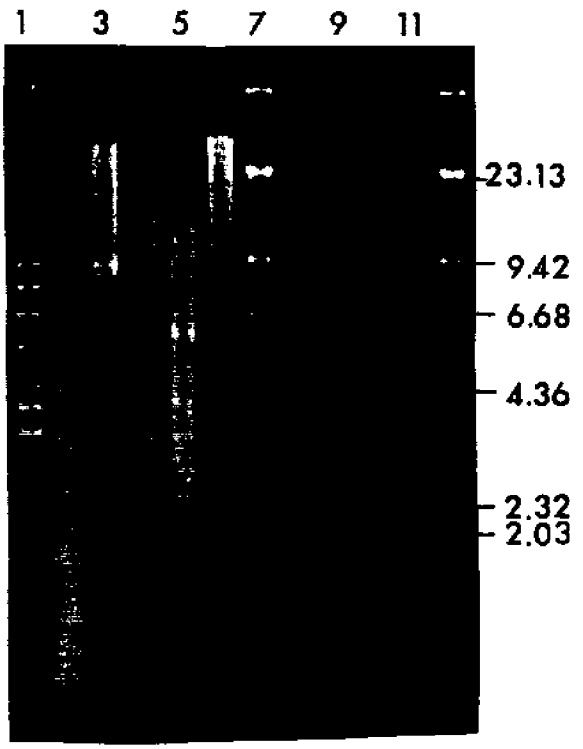

Figure 5. Restriction fragment patterns typical of two populations of M. mercenaria. Lane 1 contains supercoiled molecular weight markers; lane 2 contains undigested mtDNA; lanes 3-6 contain mtDNA from an individual of population 1 digested with BamHI, EcoRI, HindIII, and XhoI, respectively; lanes 7 and 12 contain lambda phage DNA cut with HindIII as linear molecular weight markers; lanes 8-11 contain mtDNA from an individual of population 2 digested with BamHI, EcoRI, HindIII, and XhoI, respectively. Numbers shown represent molecular sizes (Kb) of the lambda phage bands in lanes 7 and 12.