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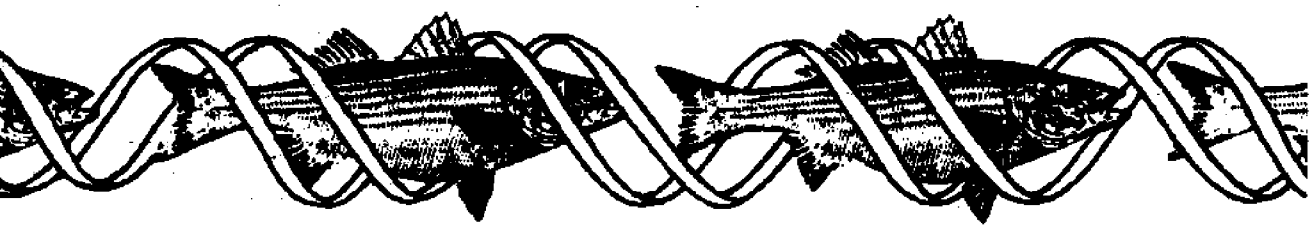
Robert W. Chapman
Dennis A. Powers



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Robert W. Chapman
The Chesapeake Bay Institute
The Johns Hopkins University
Shady Side, Maryland 20764

and

Dennis A. Powers
Biology Department
and The McCollum-Pratt Institute
The Johns Hopkins University
Baltimore, Maryland 21218

Copies of this publication are available from

Maryland Sea Grant College
University of Maryland
1222 H.J. Patterson Hall
College Park, MD 20742

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PREFACE

This report outlines a method for rapid and inexpensive isolation of mitochondrial DNA from fish. Working with seven fish species and two hybrids, Robert Chapman and Dennis Powers used mature oocytes and liver tissues to obtain digestible DNA. The electrophoretic patterns are comparable in quality to the highly purified mtDNA used in other laboratories. The advantages of this new method are its simplicity and speed. To cite one instance, twenty-four samples were simultaneously taken in less than six hours, some 20 times faster than current laboratory methods for mtDNA isolation. The procedure is applicable to many species and should be useful for processing large numbers of samples on a routine basis in modestly equipped laboratories.

INTRODUCTION

In the past two decades, techniques derived from molecular biology have made many contributions to our understanding of natural populations. The most extensively employed technique, gel electrophoresis of enzymes and other proteins, has become well integrated into the study of population genetics and related fields. Major factors in the broad application of electrophoresis are its speed, simplicity, and its more or less direct relation to the dynamics of genes in populations.

Most recently, mitochondrial DNA (mtDNA) analyses via restriction endonuclease digestions have been employed to gain further insight into population dynamics (cf. Aquadro and Greenberg 1983; Avise et al. 1979a, b; Avise and Lansman 1983; Brown 1980, 1983; Brown et al. 1979; Ferris et al. 1983; Lansman et al. 1981, 1983; Hauswirth and Laipis, 1982a, b; to name only a few). The number of studies has grown substantially in the past few years, while the number of laboratories taking advantage of this powerful technology has not kept pace. In part, the restriction on growth results from the technical approach that many investigators use. A number of published procedures employ extensive, tedious methods for isolating mtDNA and/or expensive, short lived radionuclides (Lansman et al. 1981). Using ultracentrifugation to obtain highly purified mtDNA has placed limits on sample sizes that can be processed even in a well equipped laboratory. Lansman et al. (1981) published a protocol which eliminated this step, but samples isolated by this protocol contain large quantities of cytoplasmic nucleic acids (CNAs). The presence of CNAs creates several problems in digesting mtDNA which may be circumvented by digesting small amounts of sample in large volumes of buffer. The quantities of mtDNA in these digestions are so small that digestion fragments can be visualized only by autoradiographic techniques (Lansman et al. 1981).

This report summarizes a rapid technique for isolating mtDNA from fishes. The protocol permits many individuals to be processed simultaneously, needs only limited quantities of tissue, and is comparatively inexpensive. This procedure

should facilitate the use of mtDNA analysis in other laboratories.

METHODS AND MATERIALS

Tissue Selection. Fishes provide a number of tissues which may be used for isolating mtDNA. Critical considerations in order of importance are the ratio of nuclear to mitochondrial DNA, tissue volume, ease of homogenization and glycogen content. The ratio of nuclear to mitochondrial DNA is important because small quantities of nuclear DNA contamination will reduce and ultimately eliminate resolution of mtDNA fragments. The more easily tissue is homogenized, the less likely one is to erupt mitochondrial membranes. Homogenates rich in glycogen present several problems during centrifugation.

Considering all of these criteria, mature oocytes are the best source of mtDNA. The ratio of mtDNA to nuclear DNA is very large, perhaps as high as 3000:1 w/w (Benbow pers. comm.). In spawning season, the ovaries may outweigh all other tissues except muscle; however, in most fishes this tissue is available only during a limited spawning season. Liver tissue is the next best source of mtDNA. While not nearly as rich in mtDNA as oocytes, it is richer than other tissues except the gills and outweighs gill tissue by a large factor. Liver tissue often has a large glycogen content and numerous low molecular weight RNAs, which complicate mtDNA isolation. In addition to its other virtues, ovarian tissue can be held at 4°C for 2 weeks prior to homogenization while liver can be held only 12-96 hr in this condition.

Some investigators have recommended muscle as a source of mtDNA. We cannot recommend this tissue because it is difficult to homogenize and one must strike a delicate balance between cell disruption and mitochondria destruction. The relatively low abundance of mitochondria in muscle demands large amounts of tissue (approx. 100 gm). The dilution factors (1:9) necessary to separate muscle fibrils from mitochondria require centrifugation of large volumes (on the order of 1 L), which limits the number of samples that

can be processed simultaneously and requires somewhat specialized equipment.

Mitochondrial Isolation. We routinely homogenize 1-5 gm of liver or mature oocytes in 5 volumes of TEK buffer (TEK; 50 mM Tris, 10 mM EDTA, 1.5% KCl, pH 7.5) with a single stroke of a motor driven glass teflon homogenizer. The tissue can be overground by additional strokes leading to nuclear DNA contamination and loss of mitochondria. The homogenate is then transferred to a 50 ml polypropylene disposable centrifuge tube. A 15% sucrose-TEK solution is then underlayered by passing a long stem pasteur pipette through the homogenate and allowing gravity feed to form a sharp miniscus. The homogenate is then centrifuged (1000 x g) for 10 min. at 4°C. If care is exercised in layering, the sharp boundary will remain intact. The supernatant is drawn off into another 50 ml disposable centrifuge tube and underlain by a second 15% sucrose-TEK solution. Centrifugation at 1000 x g is repeated. If mature oocytes are used, the second low speed spin may be ommitted. The supernatant from the second low speed spin is drawn off and mitochondria pelleted by centrifugation at 18,000 x g for 1 hr at 4°C. If the tissue contains large quantities of glycogen, this spin will produce a loose mitochondrial pellet overlying a dense clear pellet. The supernatant is drawn off and discarded. The loose mitochondrial pellet is then drawn off leaving the clear pellet behind. The mitochondrial pellet is resuspended in TEK to 10 ml and centrifuged at 18,000 x g for 30 min. At the end of this spin many of our samples do not form a clear pellet. If a clear pellet does form, it is advisable to wash and pellet the mitochondria a third time. Mature oocytes do not need this third wash.

Mitochondrial DNA Isolation. The mitochondrial pellet is resuspended in 0.9 ml TEK per 2.5 gm starting tissue and placed in a sterile microcentrifuge tube. The suspension is then made to 1% non-idet by addition of a 10% non-idet-TEK solution. The suspension will clear almost immediately; however it is advisable to allow 5-10 min. for complete lysis. The suspension is then centrifuged at 12,000 x g for 10 min. to remove debris. Since non-idet does not attack nuclear membranes, any intact nuclei carried over from the

low speed spins will pellet here. The supernatant is drawn off and mixed with an equal volume of water saturated with redistilled phenol to precipitate proteins. The sample is shaken thoroughly, allowed to stand 5 min. and spun at 12,000 x g for 10 min. A clear phase may form at the bottom of the tube and be overlain by precipitated proteins and phenol. A white flocculant protein precipitate will separate the phenol phase from the upper aqueous phase which contains nucleic acids. The aqueous phase is drawn off and reextracted with phenol. Following a second 12,000 x g centrifugation, the aqueous phase should be clear (not opaque) and little or no protein should be evident at the phenol-aqueous phase boundary. If the aqueous phase is not clear, the phenol extraction step should be repeated.

The transparent aqueous phase is drawn off and extracted with a 24:1 chloroform:iso-amyl alcohol solution to remove traces of phenol. The upper aqueous phase is drawn off and mixed with 2 volumes of cold (4°C) 95% ethanol. The sample is placed at -20°C for 2 hr to ensure complete precipitation of nucleic acids. Nucleic acids are pelleted by centrifugation at 12,000 x g for 10 min. at 4°C. The ethanol is decanted and the pellet allowed to dry at 37°C. Drying is complete when the white precipitate turns translucent. The sample is then rehydrated in 100 µl sterile water per gm starting tissue and should be either digested immediately or frozen at -20°C. The sample can also be held in liquid phase at -20°C by adding an equal volume of anhydrous glycerol.

Mitochondrial DNA Digestion. The procedure outlined generates enough mtDNA for about 5 digestions per gram liver tissue and about 20 digestions per gram mature oocytes. We routinely take 18 µl of sample and digest it in 2 µl of 10x digestion buffer containing 5-10 units of enzyme. Reactions are allowed 4 hr at 37°C to go to completion. The digested sample may be run immediately or stored for months at -20°C.

Selection of Restriction Enzymes. We recommend that only enzymes with recognition sites of five or six nucleic acid sequences be used (e.g., Hind-III, Xba-I, Bgl-I, etc.). These enzymes produce on the average three or four mtDNA

fragments with molecular weights usually exceeding 500 base pairs (b.p.). Restriction enzymes recognizing four base sequences produce many more cuts and many fragments of low molecular weight (approx. <500 b.p.). The isolation procedure outlined above yields numerous low molecular weight (<200 b.p.) RNAs, which interfere with identification of low molecular weight mtDNA fragments.

Identification of Restriction Fragments. We routinely separate mtDNA fragments on 1.1% to 0.8% agarose gels following the methods of Maniatis et al. (1982). Normally we allow the tracking dye (bromophenol blue) to fully elute from gel before the run is terminated. This ensures that most low molecular weight nucleic acids will elute from the gel and not interfere with photography. At the end of a run, gels are removed and placed in a solution of electrophoresis buffer to which ethidium bromide (0.5 µg/ml) has been added. The gel is allowed to incubate up to 1 hr and is then destained for 30 min. in electrophoresis buffer.

While some investigators (Maniatis et al. 1982) have recommended that ethidum bromide be added to the electrophoresis bath during the run--the separation of mtDNA fragments can then be monitored by direct visualization under UV light--we do not recommend this procedure. Ethidium bromide is a powerful carcinogen. Its addition to the electrophoresis bath can lead eventually to contamination of the laboratory, to say nothing of the electrophoresis units.

Photographic Techniques. We have assembled a photographic system which illuminates the gel with UV light tightly focused around 310 nm and permits identification of as little as 1 ng DNA per fragment. The system consists of three 20 watt fluorescent bulbs (Westinghouse, FS-series, sunlamps), a 165 mm x 165mm UV transmitting, visible absorbing filter (Hoya Optical, U-340), a Kodak 23A orange filter, and a Polaroid MP-4 land camera. The sunlamps emit a series of sharp spectral lines between 250 and 360 nm and a broad spectrum above 400nm. The U-340 filter absorbs 99+% of the spectrum below 260 nm or above 390nm and transmits more than 70% of the spectrum between 310 and 350 nm (Hoya

Optical specifications). The Kodak 23A filter absorbs virtually all wavelengths above 500nm.

We normally place gels directly on the U-340 filter and expose Polaroid Type-55 film for 4 min. at f4.5. (A 35 mm camera and panatomic X film may be substituted for the Polaroid MP-4). The film is then removed and allowed to develop for 1 min. The negative is immersed in 22% sodium sulfite for 5 min., washed with dH_2O for 5 min., coated with photoflo and dried. We have exposed film up to 30 min. without appreciable loss of resolution (blurring of high molecular weight bands) or background exposure, by inserting a 1 cm crown glass plate between the gel and Kodak 23A filter. During long exposures, it is also important to limit light leakage from the transilluminator. Although the 23A filter is quite effective at quenching illuminations below 500nm and the film is not particularly sensitive to short wavelengths, the glass plate helps greatly during prolonged exposures.

Further Purification Methods. Supercoiled mtDNA can be separated from linear mtDNA, nuclear DNA and low molecular weight RNAs by a variety of methods. We have employed two techniques with success: low melt agarose isolation and column chromatography. The low melt agarose isolation (Vogelstein and Gillespie 1979) is superior in speed and quantitative yield, but slightly more expensive.

We prepare mini-sub gels (BRL, Inc.) using an 0.8% LMP Agarose (BRL, Inc.) in TBE buffer. Undigested samples are loaded onto this gel and electrophoresis conducted for 2 hr (50 ma) at 4°C. The gels are stained as outlined above and supercoiled mtDNA bands are cut from the gel. Supercoiled mtDNA is isolated by melting the agarose at 37°C in a microtube, extracting twice with phenol, followed by an alcohol precipitation.

A variety of column chromatography techniques can separate mtDNA from nuclear and low molecular weight DNAs. We refer the interested reader to any number of texts on these techniques. We would recommend techniques using sodium chloride rather than phosphate salts. We have

collected nucleic acids from fractions containing up to 1 M NaCl by alcohol precipitation. Phosphate salts must be dialyzed before nucleic acids are collected.

RESULTS

Digestable mtDNA can be obtained from intact organisms in as little as 4 hr using the technique outlined above. The time required depends markedly upon the number of samples being processed and the tissue employed. Liver requires more extensive processing than mature oocytes due to its glycogen content. We have processed as many as 24 samples simultaneously obtaining digestable mtDNA in less than 6 hr. In most cases, the volume of homogenate and the number of rotor positions required to centrifuge this volume at 18,000 x g is the rate limiting step.

We have isolated mtDNA from seven fish species (Lepomis macrochirus, Morone saxatilis, M. americana, Fundulus heteroclitus, Alosa sapidissima, A. pseudoharengus and A. aestivalis) and two hybrids (M. saxatilis x M. chrysops and M. chrysops x M. saxatilis). The restriction digests (Hind-III, Xba-I, Eco RI, Bgl-I, and Pvu-II) of M. saxatilis and (Xba-I and Hind III) L. macrochirus (Fig. 1) are identical with those obtained in laboratories which use highly purified mtDNA (J. Grossfield pers. comm.; Avise et al. in press).

The photographic techniques outlined above can detect about 1 ng DNA per band. This estimate is based on detection of the 564 fragment from Hind III digestions of 0.1 µg lambda DNA. Sensitivity is dependent upon thoroughly removing nuclear DNA and limiting mtDNA breakage.

DISCUSSION

The transmission of mtDNA molecules within cell lineages and among individuals is, at present, poorly understood. Theoretical treatments of plausible mechanisms have been advanced (Chapman et al. 1982; Birky et al. 1983), but empirical tests of modeling assumptions are not extensive. In part, the paucity of critical data is due to extensive and tedious methods used to isolate mtDNA. These

Figure 1. Restriction digests of fish mtDNAs. (A) Hind III digests of Morone saxatilis in lanes 1-7. Lane 8, Hind III digest of lambda DNA as a molecular weight standard. (B) Hind III digests of Lepomis macrochirus purpurescens (lanes 1,2) and Lepomis macrochirus macrochirus (lanes 3,4). (C) Morone americana mtDNA in lanes 2-7, lambda DNA standard in lane 1. Lane 2 Hind III, lane 3 Bgl-I, lane 4 EcoRI, lane 5 Sst-I, lane 6 undigested, lane 7 Nci-I.

methods severely restrict the number of individuals that can be surveyed and, thereby, limit the data base. Mitochondrial DNA analyses also hold great potential in elucidating dynamics of natural populations (cf. Lansman et al. 1981). Studies of this type require substantial sample sizes that can only be obtained through herculean efforts with existing methods of isolation (cf. Avise et al. 1983).

The rate limiting step in "classical" procedures is density gradient centrifugation for 30-40 hr followed by dialysis for another 24-48 hr. Since swinging bucket rotors have at most six positions, the ultra centrifuge-dialysis step consumes 2/3 day per sample. Given some preparation time before this step, it is not uncommon for the purification of twelve samples to require one week. While the ultracentrifugation dialysis techniques does yield mtDNA of high purity, it is slow, costly, requires a high level of technical expertise and expensive equipment. Moreover, published techniques refer to freshly excised tissue (usually liver), which often requires that live animals be transported to the lab.

The isolation procedure outlined above is a great improvement over existing methods. We have isolated mtDNA from 24 samples simultaneously in less than six hours. This is approximately a twenty-fold decrease in isolation time. Circumventing density gradient centrifugation also results in great savings of technician time and capital expense. While mtDNA isolated by our method does contain some low molecular weight nucleic acids (<200 bp.), these contaminants do not interfere with identification of restriction fragments.

Mitochondrial DNA fragments of Fundulus heteroclitus have been cloned from material isolated by the technique outlined here (Powers et al. in prep.). These fragments have been used in southern blots against F. heteroclitus and Morone saxatilis mtDNAs. DNA-DNA hybridization naturally improves resolution and sensitivity; however, it is unnecessary when large quantities of liver tissue are available or when mtDNA is isolated from eggs. We have yet to employ end-labling techniques to the mtDNA fragments

generated by our procedure. In view of the clarity of our gels, we anticipate no difficulty with this technique.

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