Improved Methods for Isolation of Fish mtDNA by Ultracentrifugation and Visualization of Restriction Fragments Using Fluorochrome Dye: Results From Gulf of Mexico Clupeids

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The isolation of mitochondrial DNA (mtDNA) for studies of restriction-site polymorphisms among fish populations is achieved by either of two general methods. The methods are those of Lansman et al. (1981) which uses ultracentrifugation in cesium-chloride/ethidium-bromide gradients to separate mtDNA from nuclear DNA, and Chapman and Powers (1984) which uses low-speed centrifugations and organic extractions to precipitate mtDNA. Although the method of Chapman and Powers (1984) results in mtDNA of lesser purity, it apparently requires fewer hours to isolate mtDNA from a greater number of specimens than the method of Lansman et al. (1981), and avoids the high cost of purchasing and servicing a typical ultracentrifuge.

The technique chosen for visualization of restriction fragments depends upon the amount of mtDNA obtainable from the specimens of interest and its purity. Neither of the commonly reported techniques, such as end-labeling and ethidium-bromide staining, is specific for mtDNA. Since end-labeling is about 1000× more sensitive, a more highly purified preparation is required than for ethidium bromide. Radio-labeled hybridization probes are highly specific for mtDNA as well as very sensitive, but require many steps for preparation (Maniatis et al. 1982) and must be periodically remade. Biotinylation may provide longer-lived hybridization probes (Graves et al. 1990) but making them also requires several steps.

Here we introduce a new combination of isolation and visualization methods for mtDNA studies which incorporate attributes of some of the above techniques and which may aid investigators developing plans to begin mtDNA studies. Our approach was developed for potential use in a population study of Spanish sardine *Sardinella aurita*, as well as of other clupeids of the western Atlantic, while mindful of the need to process statistical sample sets. Incorporated in this presentation of methodology is a brief account of our initial findings on Gulf clupeids.

Materials and methods

Sample collection and preparation

Sardinella aurita and *Opisthonema oglinum* were collected aboard commercial purse seine vessels in the nearshore regions of central-west and north Florida between April and August 1989. Male and female gonads from these samples were excised fresh or semifresh and immediately stored in 10 mL of cold MSB-Ca^{++} (Table 1) for up to 7 days at 4°C. The tissue was blotted dry, weighed, minced, and then homogenized in 2 to 3 volumes of cold MSB-Ca^{++} by 5 strokes of a chilled 15-mL Dounce homogenizer. The homogenates were transferred to chilled 50-mL polyethylene centrifuge tubes after addition of disodium EDTA (0.2 M, pH 7.5) to a final concentration of 10 mM.

The homogenate was centrifuged at 800 × g for 10 minutes at 4°C after which the supernatant was decanted and respun. These two spins remove nuclei and cellular debris. Mitochondria were then pelleted by centrifugation at 20,000 × g for 20 minutes at 4°C. The pellet was washed with 10–20 mL of cold MSB-EDTA (Table 1) and repelleted by centrifugation for 15 minutes at 20,000 × g.

The pellets were suspended in 2 mL of cold STE (Table 1) and the mitochondrial membranes dissolved by adding 0.10 mL of 25% SDS, vortexing briefly and incubating at 37°C for 5–8 minutes. The entire lysate was then transferred to 3.0-mL Beckman polyallomer or polycarbonate centrifuge tubes containing enough cesium chloride (Sigma #C-3032) to produce a concentration of 1.1 g CsCl per mL of lysate as in Lansman et al. (1981). To this was added 0.133 mL of 0.025 M ethidium bromide. The solution was then mixed by inverting several times, and the refractive index of each tube adjusted to 1.389 at 20°C with CsCl or STE. Solution density at this R_f causes the DNA to float lower in the tube than at the R_f given by Lansman et al. (1981).

Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

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Table 1
Solutions used in isolation and purification of mtDNA.

MSB-Ca^{++}	0.21	M	Mannitol
	0.07	M	Sucrose
	0.05	M	Tris-HCl, pH 7.5
	3.0	mM	$CaCl_2$
MSB-EDTA	0.21	M	Mannitol
	0.07	M	Sucrose
	0.05	M	Tris-HCl, pH 7.5
	0.01	M	EDTA, pH 7.5
TE	10	mM	Tris-HCl, pH 8.0
STE	10	mM	Tris-HCl, pH 8.0
	100	mM	NaCl
	1	mM	EDTA, pH 8.0
20 × SSC	3.70	M	NaCl
	0.37	M	Na citrate, adjust pH to 7.0 with 10 N NaOH, sterilize by autoclaving
10 × TAE	0.40	M	Tris-Base
	1.15%	v/v	glacial acetic acid
	0.025	M	EDTA, pH 8.0

Density-gradient ultracentrifugation

The cornerstone of our isolation method is low-volume ultracentrifugation using the Beckman TL-100 Tabletop Ultracentrifuge. The short rotor radius and high available g-forces of this product serve to significantly reduce spin times over current practices. The main requirement is simply to "downsize" existing protocols to be compatible with 3-mL centrifuge tubes. Isolation of mtDNA using the TL-100 appears to be less labor-intensive as well as faster in producing highly purified mtDNA than existing techniques.

Ultracentrifugation was accomplished at approximately 200,000 × g (70,000 rpm) overnight (15–17 hours) at 4°C in a fixed-angle rotor (Beckman TLA-100.3) with six positions. Deceleration was set at 5. Following ultracentrifugation the mtDNA bands, which are visible in fluorescent light 0.5–0.7 mm below the nuclear DNA bands (Fig. 1), were withdrawn using an 18-gauge stainless-steel hypodermic needle inserted through the top of the tube to a position slightly below the mtDNA band. Withdrawn sample volume was then adjusted to 0.5 mL with STE and extracted several times with NaCl-saturated n-butanol at room temperature to remove the ethidium bromide (Lansman et al. 1981).

Following butanol extraction, the mtDNA was ethanol-precipitated using essentially the procedure of Maniatis et al. (1982). Sample molar concentrations were adjusted by dilution with 0.33 mL of cold, sterile, distilled H_2O. Exactly two volumes of cold ethanol were added to the solution which is briefly vortexed.

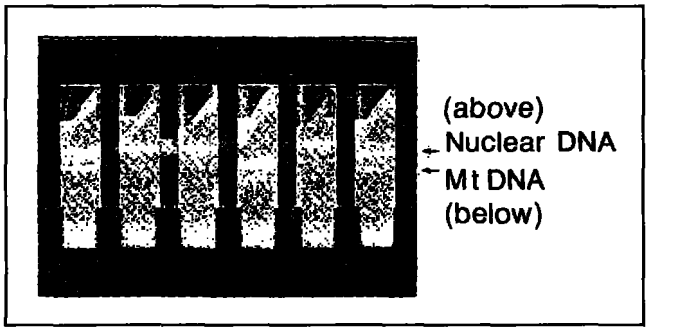

Figure 1
Appearance of nuclear and mitochondrial DNA bands in UV light after 17 hours of ultracentrifugation in CsCl/ethidium-bromide gradients at 200,000 × g and 4°C: upper bands are nuclear DNA, lower bands mtDNA. Material at the top of the tubes is the "skin" which forms during ultracentrifugation. Starting tissue was 1–2.5 g of ripe ovary from Spanish sardine *Sardinella aurita*.

The sample is stored overnight at −20°C after which mtDNA is pelleted by centrifugation in the fixed-angle rotor in the Beckman TL-100 at 30,000 × g for 30 minutes at 2°C. After complete drying by evaporation (no odor of ethanol remaining) the mtDNA pellet is dissolved in 50 μL of cold TE (Table 1) and stored indefinitely at −20°C.

Fluorometric quantification of mtDNA

Yield quantification followed Paul and Myers (1982). Standard curves were generated from fluorescence of known DNA quantities in a 1.5×10^{-4} M solution of Hoechst 33258 (CalBiochem Cat. #SR5A03-0388). Fluorescence was measured using an Aminco J4-7439 Fluoro-Colorimeter (GE F4T4/BL UV lamp and an R-136 phototube) at photomultiplier settings of 1, 3, and 10. mtDNA concentrations were calculated using the geometric mean regression of the standard curve. Yields were determined as μg mtDNA per g of starting tissue.

Restriction endonuclease digestion

Immediately prior to digestion with restriction enzymes, samples were treated with RNAse-A to a concentration of 10 μg/mL and incubated at room temperature for 1 hour. Samples were then heated to 70°C for 5 minutes and cooled on ice before addition of the restriction enzymes. In this initial study only two six-base-pair restriction endonucleases were used: *Eco*-RI and *Hin*d-III. Digestions were carried out according to the reaction conditions specified by the manufacturers. Reaction mixtures contained 0.2–1.0 μg of mtDNA in a final volume of 20 μL adjusted with

sterile, distilled water. Using a Hamilton syringe, 2.0 μL of the digestion buffer was added to the tube and mixed by vortexing, then at least two units of the restriction enzyme were added and also mixed by vortexing. The reaction mixtures were allowed to incubate at 37°C for at least 2–3 hours, but sometimes as long as overnight. The reaction was stopped by the addition of 0.4 μL of 0.5 M EDTA (pH 7.5).

mtDNA fragment visualization using Hoechst 33258

Restriction fragments were separated on horizontal (submerged) agarose slab gels with the Hoechst 33258 fluorochrome incorporated as described in DeFlaun and Paul (1986). The fluorochrome is a 1:30 dilution of 1.5×10^{-4} M solution of Hoechst 33258 with 1 × SSC (i.e., 1:20 dilution of 20 × SSC, Table 1). The gel is created by boiling a 1.1% solution of agarose in distilled H_2O containing 10% 10 × TAE (Table 1) buffer until clear. The suspension is allowed to cool to the touch before adding a 1.1% volume of the Hoechst 33258 dilution. After casting, the gel is allowed to set for at least 30 minutes in an opaque box to prevent photo-deactivation of the fluorochrome.

For running, the gel is immersed in a tank buffer of 500 mL distilled H_2O, 56 mL of 10 × TAE (Table 1), and 5.6 mL of the Hoechst 33258 dilution. Samples containing 10 μL of digested mtDNA from the reaction mixture, 1 μL of 10 × TAE, and 2 μL of tracking dye (Bromophenol blue) are then micropipetted into the wells. Outside lanes contained *Hin*d-III digested λ-DNA standards.

Gels are electrophoresed at 50 volts for approximately 3 hours, or until the tracking dye covers three-quarters of the length of the gel. The gel apparatus remains isolated from light during running. Following the run, gels were placed on a UV transilluminator and photographed with a Polaroid MP-4 Land-camera using Polaroid Type 667 black-and-white film.

Measurement and analysis of mtDNA fragment patterns

Photographs of the gels were processed on a computer-automated optical pattern-recognition system developed by Biosonics Inc., Seattle, Washington. Fragment sizes were calculated by the global form of the reciprocal method as described by Elder and Southern (1987).

Results

Ovarian tissue from *Sardinella aurita* and *Opisthonema oglinum* consistently produced visible mtDNA bands in the CsCl/ethidium-bromide gradient by ultracentrifugation in fewer than 17 hours (Fig. 1), but the testicular tissue did not. If carefully extracted, the mtDNA fraction was sufficiently void of contamination by RNA and nuclear DNA. With the Beckman TL-100 it is undoubtedly possible to reduce the time required for ultracentrifugation even further, since higher speeds of up to 100,000 rpm are available using polycarbonate tubes.

The only apparent drawback of the fixed-angle rotor over the swinging-bucket rotor is that in the fixed-angle rotor the cross-sectional area of the fluid in the tube is greatest while under centrifugation, but becomes less when the rotor is at rest and least when the tube is upright. Thus, the scab-like material that forms on top of the gradient during centrifugation protrudes down into the gradient when the tube is upright (Fig. 1). However, if the refractive index is adjusted carefully, both DNA bands will form below the protrusion of this scab-like material. Since this material does not crumble after centrifugation at 4°C, it can be removed or pushed aside to withdraw the mtDNA.

The mean yield of mtDNA from fresh *S. aurita* gonads stored in MSB-Ca^{++} for 2 days at 4°C was 1.90 ± 0.60 μg mtDNA per g of tissue. The mean yield from fresh *S. aurita* gonads stored in MSB-Ca^{++} for 7 days at 4°C was 1.18 ± 0.45 μg mtDNA per g of tissue. The semifresh *O. oglinum* processed after 5 days of storage yielded 1.01 ± 0.66 μg mtDNA per g of ovarian tissue. Isolation of mtDNA from material quick-frozen on liquid nitrogen was also achieved, but yields were reduced by as much as 56% from that of the semifresh samples.

Various concentrations of mtDNA were electrophoresed on Hoechst 33258/agarose gels. The minimum concentration required to produce clear, resolvable restriction fragments (at the 500-base pair (bp) level) was 11.8 ng/μL in 20-μL wells. Fragment mobilities from separate digestions with the two restriction endonucleases indicated the mtDNA genome size for *S. aurita* to be 16,263–16,353 bp, and that of *O. oglinum* to be 15,186–15,683 bp.

Discussion

The techniques presented here have successfully addressed some of the potential problems associated with performing a large population study using mtDNA. Purification of sufficient quantities of mtDNA to perform a detailed restriction-site analysis has been

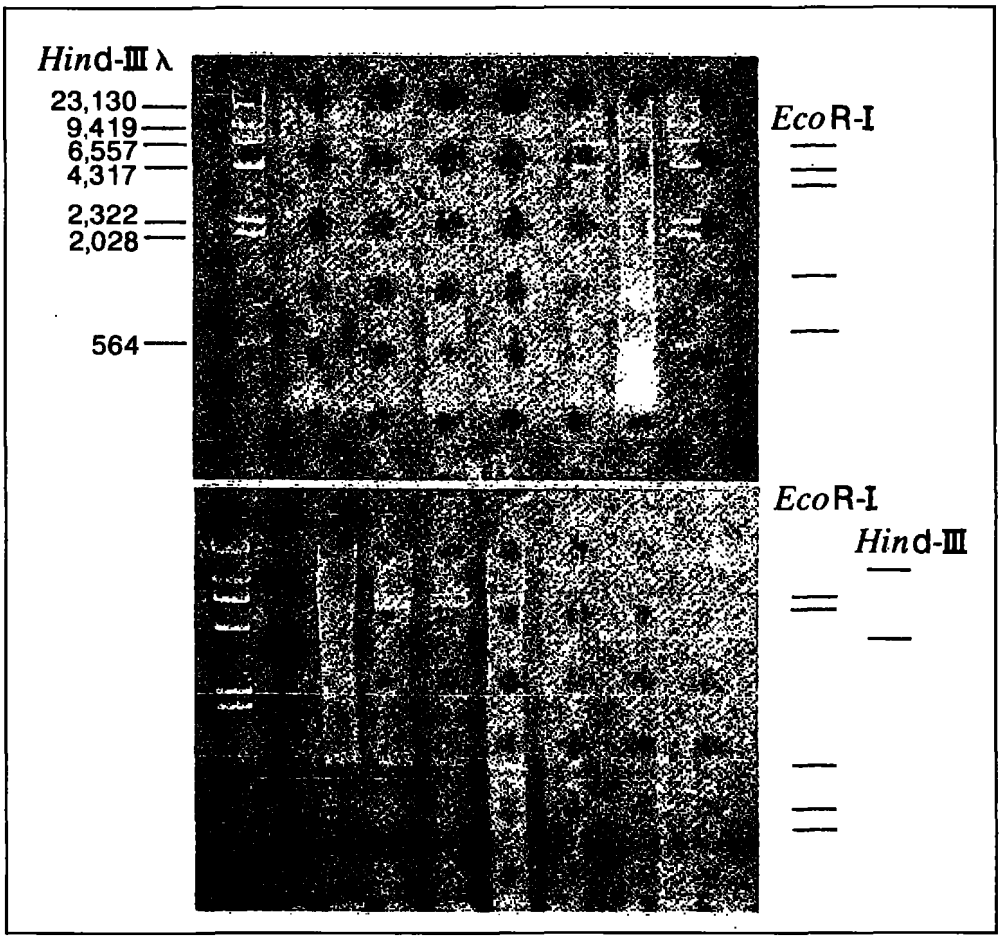

Figure 2
Top: Restriction digests with *Eco*-RI of mtDNA from Spanish sardine *Sardinella aurita* visualized on a routine Hoechst/agarose gel photographed in transilluminated UV light. Lane 1 is the *Hin*d-III digested λ-DNA standard. Bottom: *Eco*-RI (left) and *Hin*d-III digests of mtDNA of threadherring *Opisthonema oglinum*. All digests were carried out on ~0.3 μg of mtDNA for 2.5 hours, then electrophoresed for 3 hours at 50 volts. The *Hin*d-III digest of *S. aurita* mtDNA (not shown) produces five fragments compared with two for that of *O. oglinum*.

accomplished through low-volume density-gradient separation in a relatively inexpensive tabletop ultracentrifuge which is essentially portable. This simple overnight ultracentrifugation followed by extraction of the mtDNA and subsequent ethanol precipitation would allow mtDNA isolation from at least 30 individuals in a normal work-week by a single worker. Five grams of fresh, ripe, ovarian tissue should produce enough mtDNA per individual to be digested separately by 20 restriction endonucleases and still be resolved on Hoechst 33258/agarose gels.

Agarose gels incorporating the Hoechst 33258 fluorochrome (DeFlaun and Paul 1986) is a good substitute for staining of restriction fragments using ethidium bromide. Whereas Hoechst 33258 binds specifically and quantitatively to AT-rich mtDNA (Latt and Stetten 1976) ethidium bromide has mixed RNA-DNA specificity (Le Pecq and Paoletti 1966) reducing its resolving power and making visualization of small fragments (<500 bp) difficult. The suggestion of Lansman et al. (1981), that minimally 0.5 μg of mtDNA be used per digestion to insure visualization of fragments with ethidium-bromide staining, limits its resolving power to about 20 ng of DNA. In contrast, a zone of fragments containing only 7.0 ng of DNA (~1.8 μg per digestion) should be visible on Hoechst 33258/agarose gels, and these can be viewed immediately following electrophoresis on a UV transilluminator.

Our estimate of the sizes of the mtDNA genomes of *Sardinella aurita* and *Opisthonema oglinum* is close to those obtained for other clupeids (Kornfield and Bogdanowicz 1987, Avise et al. 1989). Ours are probably very good estimates since the pattern-recognition system used here greatly reduces measurement error of fragment mobility inherent in the use of stereomicroscopes and other manual methods. This survey of only a few individuals did not reveal any polymorphisms at *Eco*-RI or *Hin*d-III in either species, but the restriction fragments produced at both restriction sites are quite distinct between the species (Fig. 2). With new microextraction methods for mtDNA available (Graves et al. 1990), it should be possible to eventually separate or identify even the eggs and very young larvae of *S. aurita* and *O. oglinum* obtained in field samples. The ability to do so could be used to provide estimates of egg and larval mortality rates in the

earliest life-history stages of these and other important pelagic species.

Acknowledgments

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