Chapter 4

Mitochondrial DNA from *Lumbriculus variegatus*: Isolation and Restriction Digest Analysis

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Contents

Introduction

This lab was designed to introduce students in upper division courses (Genetics, Cell Biology, Molecular Biology) to two concepts: the isolation and properties of mitochondrial DNA and restriction mapping using a real biological sample, with all of its ambiguity. It assumes students have a conceptual familiarity with DNA, restriction enzymes, and gel electrophoresis, as well as technical experience with micropipetting and determining sizes of DNA restriction fragments from gel results. Typically, completing the exercise requires three 3-hour lab periods, although it can condensed by isolating mitochondria and pouring agarose gels in advance. Preparation of solutions can make set up time extensive (4-6 hours), but many of these solutions are also used in plasmid miniprep procedures, so they may be available from labs that do minipreps.

Materials

For Entire Class of 24

A. *Lumbriculus variegatus* culture and handling
- 5 gallon plastic aquaria
- Air pumps
- Aquarium tubing
- Air stones
- Sinking goldfish pellets
- Brine shrimp nets
- Brita™ water filtering system

B. For lab exercise
- Refrigerated centrifuge capable of 12,000 x g, 4° C (Sorvall superspeed or similar)
- Centrifuge rotor for 40 mL centrifuge tubes (Sorvall SS-34 or similar)
- -20° C freezer
- 37° C water bath
- 70° C water bath
- Gel electrophoresis power supplies
- UV gel photodocumentation system (transilluminator, Polaroid camera)

Per Group of 6 Students

A. Equipment
- Horizontal gel electrophoresis apparatus (electrophoresis chamber, casting tray, comb)
- 40 mL glass homogenizer
- Balance

B. Materials
- 100 mL Mitochondrial Isolation Buffer
- 10 mL Mitochondrial Lysis Buffer
- 10 mL 2% SDS/0.4 N NaOH
- 10 mL KOAc/acetic acid
- 10 mL 1:1 phenol/chloroform
- 10 mL 100% Ethanol
- 10 mL 70% Ethanol
- 10 mL TE buffer
- 800 mL 1x TBE (volume depends upon gel electrophoresis chamber)
- 0.7% agarose/1X TBE gel (if poured ahead of time by instructor)
- 100 mL 1 ug/mL ethidium bromide
- DNA size markers (1 kilobase ladder or similar)

Per Team of 3 Students

A. Equipment
- P-20 micropipettor
- P-200 micropipettor
- P-1000 micropipettor
- Microcentrifuge tube rack
- 100 mL graduated cylinder
- Plastic funnel

B. Materials
- Cheesecloth
- Micropipettor tips for P-20/P-200 and P-1000
- 1.5 mL microcentrifuge tubes
- 0.5 mL microcentrifuge tubes
- BamHI restriction enzyme
- HindIII restriction enzyme
- 10X restriction enzyme buffer (Buffer H or Multi-Core)
- 1 mg/mL BSA
- 200 µg/mL RNase

Notes to Instructors

***Lumbriculus variegatus* (California blackworms)**

As described earlier in the handout, this worm was chosen for this lab because of convenience. These worms homogenize without too much trouble and give good yields of mitochondria by differential centrifugation. One source for large amounts of *Lumbriculus* is Aquatic Foods of Fresno, CA [phone (559) 291-0623, fax (559) 291-0601, web www.aquaticfoods.com). *Lumbriculus variegatus* is also available in small amounts from Carolina Biological. You might also find these worms in local pet stores, since many people use this worm as a feeder for aquarium fish.

The care and maintenance of these worms has been thoroughly described by Drewes (1996). Although spring water is preferred, the worms tolerate water filtered by a Brita™ system. Since Aquatic Foods sends worms in large amounts (3 pounds or more) large scale maintenance can be achieved for at least 1 semester by putting 200-300 grams of worms in 1 gallon of water in a 5 or 10 gallon aquarium. Regular feeding (every other day) is with sinking goldfish pellets, and water should be changed when murky (1-2 times per week). Even with feeding and water changing, cultures will sometimes crash and die off, so multiple cultures are recommended.

Isolation of Mitochondria and Mitochondrial DNA

There are 2 keys for this to be successful: keep things cold and keep things gentle. As many steps as possible should occur on ice or at 4^{o} C. A refrigerated centrifuge and microcentrifuge (or centrifuges in a cold room) is highly recommended. All centrifuge steps can occur in a microcentrifuge if a large refrigerated centrifuge is unavailable, although during mitochondrial isolation, the large, amorphous pellet after the first spin (low speed) may take up most of the microcentrifuge tube. Combining 2 or 3 supernatants into 1 new microcentrifuge tube for the second (high speed) spin will result in enough mitochondria in each pellet to continue.

Once the mitochondria have been disrupted by SDS, the mtDNA is quite fragile. So all the mixing steps during the isolation of mtDNA should be by gentle inversion, not vortexing. Although phenol/chloroform solutions are toxic and present disposal issues (Bloom *et al.* 1996), the organic extraction is crucial to the success of mtDNA isolation, so it shouldn't be skipped. Alternatives to phenol/chloroform extraction are currently being pursued, but I have had no success with commercial miniprep kits.

Restriction Digests

These 2 enzymes were chosen because they're relatively inexpensive, fairly reliable, and also cut pUC18 plasmid in the multiple cloning site, allowing for the possibility of creating mtDNA libraries in pUC18. The wide variety of available restriction enzymes means this exercise could be renewed each semester or year with a different set of enzymes, resulting in an increasingly detailed restriction map. RNase is included in the digests to degrade mtRNA that can hinder the detection of small mtDNA fragments on the gel.

Agarose Gel Electrophoresis

Any of the wide variety of horizontal minigel setups should work, although a gel 15 cm long is recommended because of increased resolution of larger mtDNA fragments. For background on agarose gel electrophoresis, consult Bloom, *et al.*, (1996) or many other lab manuals in genetics, biochemistry, cell biology, or molecular biology. If desired, students may pour their own gels; if so, allow 30-45 minutes for this procedure. Methylene blue gel staining, while safer and more convenient than ethidium bromide, is not sensitive enough to detect all of the mtDNA fragments. Use and disposal of ethidium bromide solutions is described in Bloom *et al.* (1996) as well as many other lab manuals; because of its toxicity, gel staining and photography is often best done by instructors rather than students. Gel staining is accomplished by covering the gel after electrophoresis in a 1 ug/mL ethidium bromide solution for 15 minutes, followed by visualization on an ultraviolet light box and Polaroid photography.

Restriction Mapping

Recently, we've been able to clone into plasmid pUC18 restriction fragments from single and double digests of mtDNA involving Bam HI, *Hind III* and many other restriction enzymes, and in some cases the cloned fragments are quite small (< 700 base pairs). Since we've never seen these small fragments on gels, its becoming clear to us that in order to make an accurate

restriction map of *Lumbriculus variegatus* mtDNA, we will need to combine results from agarose gels of restriction digests and from cloning and library construction.

Timing

This is obviously a lab exercise that requires multiple lab periods. Shown below are 2 suggested strategies for how to break this exercise up. It is recommended that isolated mitochondria be used immediately for mtDNA isolation; storing mitochondria in the freezer before mtDNA isolation generally gives poor results.

Strategy 1	Lab #1	Isolation of mitochondria (done in advance by instructor)
		Isolation of mtDNA (1 - 1.5 hours)
		Set up restriction digests (30 minutes)
		After 45 minutes - 1 hour, instructor places
		Restriction digests in freezer
	Lab #2	Inactivation of restriction digests (15 min)
		Loading of gels (30 minutes)
		Spectrophotometry (30 minutes)
		After 2-3 hours of electrophoresis
		Instructor stains and photographs gels
	Lab #3	Analysis of gel results
Strategy 2	Lab #1	Isolation of mitochondria (1 hour)
		Isolation of mtDNA (1-1.5 hours)
		Store in freezer
	Lab #2	Set up restriction digests (30 minutes)
		During digestion (45 minutes - 1 hour)
		Spectrophotometry and/or pour gels
		Loading of gels (30 minutes)
		After 2-3 hours of electrophoresis
		Instructor stains and photographs gels
	Lab #3	Analysis of gel results

Student Outline

Introduction

In eukaryotic cells, most of the DNA is packaged in long, linear chromosomes found in the nucleus. Sexual reproduction, through meiosis and fertilization, results in nuclear DNA and its associated genes being transmitted from two parents to progeny organisms in predictable ways, most famously shown by the characteristic patterns and ratios discovered by Gregor Mendel. Outside of the nucleus, small amounts of DNA are found both in chloroplasts and mitochondria. In contrast to nuclear genes, the DNA of chloroplasts and mitochondria usually shows a uniparental (often maternal) inheritance pattern (Birky, 1995). Although maternal inheritance has often been explained as a consequence of unequal gamete size (and therefore

unequal cytoplasmic and organelle contribution), the prevalence of uniparental inheritance even in isogamous organisms indicates that other, more active mechanisms might be involved in inheritance of chloroplast and mitochondrial genes (Sears & VanWinkle-Swift 1994, Shitara *et al.*, 1998).

Mitochondrial DNA (mtDNA) has been extensively characterized from a wide variety of eukaryotes. Relatively small, it ranges in size from 16 kilobase pairs (kbase pairs) in many animals (DeSalle, *et al.* 1993) to 50-80 kbase pairs in fungi to over 100 kbase pairs in plants (Scheffler, 1999). Although the size of mtDNA varies between types of eukaryotes, the set of encoded proteins and RNAs shows fewer differences. In general, mtDNA encodes ribosomal RNAs, transfer RNAs, and a small set of polypeptides, mainly subunits of electron transport complexes I, III, and IV and ATP Synthase (Scheffler, 1999). Since these polypeptides are but a fraction of the total needed for function, mitochondria import hundreds of nuclearly-encoded polypeptides from their site of synthesis in the cytoplasm. The nature of mitochondrial protein import and the coordination of gene expression between mitochondria and the nucleus remains an area of active research (Neupert, 1997).

Traditional methods of mitochondrial DNA isolation involve ultracentrifugation in cesium chloride gradients (DeSalle, *et al.* 1993), which requires an investment of time and money not always available in undergraduate teaching laboratories. Since animal mtDNA is small and circular, it resembles many types of bacterial plasmid DNA. Today, we will take advantage of this similarity to isolate mtDNA using a modified plasmid isolation procedure (Pelonquin, *et al.* 1993, Tamura & Aotsuka, 1988). Our source of mitochondria is *Lumbriculus variegatus*, an aquatic member of Phylum Annelida (segmented worms) and relative of the earthworm. This organism was chosen for 3 reasons:

1) ease of maintaining live cultures in the lab (Drewes, 1996);
2) ease of homogenization to release intact mitochondria;
3) lack of research done on mitochondria and mtDNA of this organism

Isolation of Mitochondria

A. Weigh out approximately 5 g of California blackworms (*Lumbriculus variegatus*).

B. Combine ~5 g of blackworms with 25 mL of Mitochondrial Isolation Buffer (MIB) in 40 mL glass homogenizer on ice.

C. Keeping on ice, homogenize worms in MIB using 10 strokes with pestle A (loose) followed by 10 strokes with pestle B (tight).

D. To remove unhomogenized debris, filter homogenate through 4 layers of cheesecloth into a 40 mL centrifuge tube on ice.

E. Before centrifuging, balance (by visual inspection) your centrifuge tube with another group's tube. Discard homogenate if necessary to balance.

F. Centrifuge at 1,000 x g (3,000 rpm in SS-34 rotor), 4^{o} C, 5 minutes.

G. Transfer resulting supernatant to new 40 mL centrifuge tube on ice. Balance your centrifuge tube with another group's tube. Discard pellet in old tube (contains unbroken cells and nuclei).

H. Centrifuge 12,000 x g (10,000 rpm in SS-34 rotor), 4^{o} C, 10 minutes.

I. Discard resulting supernatant. Resuspend light-brown portion of pellet (mitochondria) in 1.0 mL of Mitochondrial Lysis Buffer (MLB) by pipetting up and down. Underlying black portion of pellet does not resuspend.

J. Proceed to isolation of mitochondrial DNA.

Isolation of Mitochondrial DNA

The general plan for isolating DNA from these mitochondria involves 3 steps:

1. Disrupting the mitochondrial inner and outer membranes with detergent (sodium dodecyl sulfate, SDS)
2. Removing proteins by selective precipitation (high salt) and extraction (phenol/chloroform)
3. Precipitation of DNA (ethanol)

A. Aliquot 200 µL of your mitochondrial suspension into each of three 1.5 mL microcentrifuge tubes. Keep these tubes on ice whenever possible.

B. Add 100 µL of 2% SDS/0.4 M NaOH to each microcentrifuge tube. Mix contents of tubes by inverting each tube 10 times. The mitochondrial suspension should change from an opaque brown to a more transparent amber color. This indicates that the mitochondrial membranes have been solubilized by the SDS. Incubate tubes on ice for 5 minutes.

C. Add 150 µL of KOAc/acetic acid solution to each microcentrifuge tube. Mix contents of tubes by inverting each tube 10 times. The contents should become cloudy as proteins and lipids precipitate due to the high ionic strength (5 M acetate, 3 M sodium) of the KOAc solution. Incubate tubes on ice for 5 minutes.

D. Centrifuge tubes in microcentrifuge at 14,000 rpm for 5 minutes at 4^{o} C (cold room).

E. Transfer the resulting supernatants into 3 new 1.5 mL microcentrifuge tubes.

F. Add 500 µL of phenol/chloroform (TE-saturated) to each tube. Mix contents by inverting each tube 20 times, to ensure thorough mixing of the aqueous and organic (phenol/chloroform) solutions.

G. Centrifuge tubes in microcentrifuge at 14,000 rpm for 5 minutes at 4^{o} C. This will result in 2 layers in the microcentrifuge tubes: bottom layer - phenol/chloroform top layer - aqueous, contains mtDNA.

H. Using a p1000 micropipettor, carefully pull the top layer out of each tube and transfer into 3 new 1.5 mL microcentrifuge tubes.

I. Add 1000 µL (1.0 mL) of ice-cold 100% ethanol to each tube. Mix by inverting each tube 10 times. Incubate on ice for 5 minutes.

J. Centrifuge tubes in microcentrifuge at 14,000 rpm for 10 minutes at 4^{o} C. After centrifuging, each tube should contain a small white pellet of mitochondrial DNA.

K. Remove and discard the ethanol supernatant from each tube. Add 500 µL of 70% ethanol to each tube. Mix by inverting each tube 10 times. Make sure the mtDNA pellet comes free of the wall of the microcentrifuge tube. This will wash away most of the remaining salt and EDTA.

L. Centrifuge tubes in microcentrifuge at 14,000 rpm for 2 minutes at 4^{o} C. Remove and discard 70% ethanol supernatant.

M. Let mtDNA pellets air dry 10-15 minutes, or until transparent. Resuspend all 3 pellets in a total volume of 20 µL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA.) This is most easily done by resuspending one pellet in 20 µL of TE, transferring that 20 µL into the next tube, resuspending the second pellet in the same 20 µL, then transferring that 20 µL into the third tube and resuspending the third pellet, which results in all three pellets suspended in the same 20 µL of TE buffer.

Restriction Digest

Analysis of DNA molecules is often hampered by their large size. One way of generating specific smaller DNA molecules from larger ones is through the use of restriction enzymes, which are sequence specific DNA endonucleases. The 2 restriction enzymes we will use to generate fragments of mitochondrial DNA are:

Bam HI - from *Bacillus amyloliquefaciens* site - 5' G▼G A T C C 3'

Hind III - from *Hemophilus influenzae* site - 5' A▼A G C T T 3'

Each group will set up **one** of the following restriction digests, in a 0.5 mL microcentrifuge tube, as assigned by the instructor.

Digest	mtDNA	10X enzyme buffer	1 mg/mL BSA	200 ug/mL RNase	Enzyme
1	12 µL	2 µL buffer E	2 µL	2 µL	2 µL Bam HI
2	12 µL	2 µL buffer E	2 µL	2 µL	2 µL *Hind III*
3	10 µL	2 µL buffer E	2 µL	2 µL	2 µL Bam HI and 2 µL *Hind III*

Collect contents in bottom of microcentrifuge tubes by pulsing in microcentrifuge. Mix contents by pipetting up and down a few times. Incubate tubes in 37^{o} C water bath for 1 hour. After 1 hour, transfer tubes to 70^{o} C water bath for 10 minutes to inactivate restriction enzymes. Store restriction digests at -20^{o} C until next lab period.

Electrophoresis of mtDNA Fragments

Agarose gel electrophoresis will be performed as before (Bloom *et al.*, 1996) to separate mtDNA fragments from restriction digests and estimate their size.

A. Mix 10 µL of your restriction digest from last week with 2 µL loading dye.

B. Load all 12 µL of this digest-dye mixture into adjacent wells of a 0.7% agarose/TBE gel. DNA standards (1 kb ladder) will be loaded onto each gel by your instructor.

C. The gel will be run at 95 V for 2-3 hours, until the dyes in the loading buffer are well resolved.

D. Your instructor will stain the gel with ethidium bromide and photograph the gels under ultraviolet illumination to visualize the DNA fragments and standards. Next week you will be given a copy of the gel photograph.

Results

From the gel photograph, measure the distance traveled for the DNA standards and record these values in Table 1. Also measure the distance traveled for the fragments of mtDNA in your restriction digest and in the other two types of digests, so that you have data from a Bam HI single digest, a *Hind III* single digest, and from a Bam HI and *Hind III* double digest. Record these values in Table 2.

Analysis

A. On graph paper or through a spreadsheet program, plot $\log_{10}$(size) of the 1 kilobase ladder DNA standards vs. distance traveled during electrophoresis. Draw a straight line through these 13 points.

B. Use this graph as a standard curve to estimate the sizes of the DNA fragments in each of the three restriction digests. For each fragment, find the distance traveled on the x-axis of your graph, and draw a vertical line from this point to the data line from the DNA standards. From the intersection of your vertical line with the data line, draw a horizontal line over to the y-axis. Where the horizontal line intersects the y-axis will indicate the estimated $\log_{10}$(size) for that mtDNA fragment, which can then be converted to the size using your calculator's antilog (or 10^x) function. Round off these values to the nearest 100 base pairs. Record both the $\log_{10}$(size) and the size in base pairs in Table 2.

C. Estimation of total size of *Lumbriculus variegatus* mtDNA.

From your estimation of mtDNA fragment size in part B, estimate the total size of mtDNA by adding up the sizes of fragments in each digest and recording these values in Table 2. If the total sizes from each of the 3 digests are not closely matched, that may indicate multiple DNA fragments of similar size in some digests that were unresolved by electrophoresis. It may also indicate that some digests contain many small DNA fragments that are not visualized well by this system.

D. Restriction mapping of *Lumbriculus variegatus* mtDNA.

Draw a restriction map of *Lumbriculus variegatus* mtDNA, indicating total size, number of sites for each of your restriction enzymes, and their relative position and spacing. Keep in mind the following information (Rapoza, 1999).

1. Remember that the sizes of these DNA fragments are estimates. You may need to fiddle with your initial estimates to get everything to add up.

2. Compare the results of each single digest to the results of your double digest. Bands present in the single digest that are present in the double digest represent DNA fragments that don't contain a site for the other restriction enzyme. Bands present in a single digest that are not present in the double digest represent DNA fragments that do contain a site for the other restriction enzyme.

3. Bands present in the double digest that are not in either of the single digests should sum up in pairs or in threes to the sizes of a fragment in one or the other single digest.

4. It is possible to have restriction sites for different enzymes so close together that no fragment appears between them in the double digest. If you suspect this is the case, draw these sites as overlapping on your map.

5. Make sure to consider the possibility that 2 or more DNA fragments are so similar in size that our electrophoresis system cannot resolve them, and therefore these multiple

fragments appear as one band on the gel. This will probably be the simplest explanation for apparently "missing" DNA fragments in certain digests.

6. Remember there is no "right" answer for this restriction map. We're the first researchers to ever analyze *Lumbriculus variegatus* mtDNA, so this is uncharted territory and therefore our maps are a "rough draft" that will no doubt be revised by future researchers. In other words, make your best guess.

Acknowledgements

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Appendix A

Sources and Suppliers

Most of the supplies came from Fisher Scientific (1-800-766-7000, www.fishersci.com) since we have a state contract with them, but in most cases, these are standard supplies and reagents available from almost any scientific supply company.

Specific supplies

40 mL glass Dounce homogenizer (tissue grinder) from Kontes
Fisher catalog number - K885300-0040, $79.50 US each

1 kilobase (kb) DNA ladder molecular weight standard (from Promega)
Fisher catalog number - PR-G5711, $90.00 US/250 µL (enough for 50 gels)

Lumbriculus variegatus (California blackworms)
Aquatic Foods of Fresno, CA [phone (559) 291-0623, fax (559) 291-0601, www.aquaticfoods.com]. $64.00 US/3 pounds of worms

Appendix B

Solutions

Stock solutions

1.0 M Tris, pH 8.0 for 400 mL

48.4 g Tris (Tris[hydroxymethyl]methylamine, MW - 121.1 g/mol)
Dissolve in ~350 mL dH_2O, pH to 8.0 with HCl
Final volume to 400 mL, store at room temperature

4.0 N NaOH for 400 mL

64.0 g NaOH (sodium hydroxide, MW - 40.0 g/mol)
Dissolve in ~300 mL dH_2O
Final volume to 400 mL, store at room temperature

10% SDS for 400 mL

40 g SDS (sodium dodecyl sulfate, sodium lauryl sulfate, MW - 288.4 g/mol)
Dissolve in ~350 mL dH_2O
Final volume to 400 mL, store at room temperature

0.5 M Na_2EDTA, pH 8.0 for 400 mL

74.4 g EDTA (ethylenediamine tetraacetic acid, disodium salt, MW - 372.2 g/mol), dissolve in ~ 300 mL dH_2O, adjust pH to 8.0 with 4.0 N NaOH, final volume to 400 mL, store at room temperature

5.0 M KOAc for 400 mL

196.3 g KOAc (potassium acetate, MW - 98.14 g/mol)
Dissolve in ~ 250 mL dH_2O
Final volume to 400 mL, store at room temperature

5 mg/mL ethidium bromide stock solution

Dissolve 50 mg of ethidium bromide in 10 mL dH_2O
Handle carefully, wear rubber gloves, and avoid breathing dust
Store in refrigerator (4^oC) in foil-wrapped container

10 mg/mL RNase (DNase-free)

Dissolve 100 mg RNase in 10 mL TE buffer
Heat at 100^o C for 15 minutes to inactivate DNase
Cool slowly to room temperature
Store in 1.0 mL aliquots in freezer (-20^o C)

Solutions for isolation of mitochondria and mtDNA

Mitochondrial isolation buffer (MIB) - 0.25 M sucrose, 30 mM Tris-HCl, pH 8.0, 50 mM Na_2EDTA

For 400 mL

Dissolve 34.2 g sucrose in ~300 mL dH_2O
Add 12 mL of 1.0 M Tris-HCl, pH 8.0
Add 40 mL of 0.5 M Na_2EDTA
Final volume to 400 mL, store in refrigerator (4^o C)

Mitochondrial Lysis Buffer - 150 mM NaCl, 50 mM Na_2EDTA, 10 mM Tris-HCl, pH 8.0 for 400 mL
Dissolve 3.50 g NaCl (sodium chloride, MW - 58.44 g/mol) in ~300 mL dH_2O, add 40 mL of 0.5 M EDTA (stock solution),
Add 4.0 mL of 1.0 M Tris-HCl, pH 8.0 (stock solution)
Final volume up to 400 mL, store in refrigerator (4^o C)

2% SDS/0.4 N NaOH - for 100 mL

Combine 70 mL dH_2O
20 mL 10% SDS stock solution
and 10 mL 4.0 N NaOH
Make up fresh day of mtDNA isolation

KOAc/acetic acid - 5 M acetate, 3 M potassium

for 400 mL

240 mL 5.0 M KOAc (stock solution)
46 mL glacial acetic acid
114 mL dH_2O
Store in refrigerator (4^oC)

1:1 phenol/chloroform

Contrary to many published protocols, the most effective 1:1 phenol/chloroform solution for the isolation of mtDNA from *Lumbriculus variegatus* is not buffer-equilibrated. In fact, traditional buffer-equilibrated phenol/chloroform solutions phenol/chloroform solution used in this mtDNA isolation is prepared as follows:

A) Melt crystalline phenol (molecular biology grade) in 68^o water bath until fully liquefied.

B) Combine 100 mL liquefied phenol with 100 mL chloroform in brown glass bottle.

C) Add 20 mL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to phenol/chloroform. Mix by shaking.

D) Store at 4^oC (refrigerator) with minimal light exposure (wrapping bottle in foil helps). Good for 1-2 months if kept at 4^oC and in the dark.

TE - 10 mM Tris-HCl, pH 8.0, 1 mM EDTA
for 400 mL

4 mL 1.0 M Tris-HCl, pH 8.0 (stock solution)
0.8 mL 0.5 M EDTA (stock solution)
395 mL dH_2O
Store in refrigerator (4^oC)

Solutions for restriction digests

200 ug/mL RNase
Dissolve 20 µL 10 mg/mL RNase stock solution in 980 µL dH_2O
Make up fresh on day of restriction digests

1 mg/mL bovine serum albumin (BSA)
Dissolve 100 µL 10 mg/mL BSA (from restriction enzyme supplier) in 900 µL dH_2O
Make up fresh day of restriction digests

Solutions for electrophoresis

10X Tris/Borate/EDTA (TBE) electrophoresis buffer
for 1000 mL (1.0 L)
Dissolve in ~700 mL dH_2O

1 g NaOH
108 g Tris
55 g boric acid
7.4 g EDTA, disodium salt

Final volume to 1000 mL with dH_2O, store at room temperature

1X TBE electrophoresis buffer

Combine 9.0 L dH_2O and 1.0 L 10X TBE in carboy
Store at room temperature

Gel staining solution (1 ug ethidium bromide/mL)

Dissolve 100 µL of 5 mg/mL ethidium bromide stock solution
in 500 mL dH_2O
Store in foil-wrapped container in refrigerator (4^oC)

Appendix C
Student Data Sheets

Table 1 Agarose Gel Electrophoresis Results of 1 kilobase DNA ladder

size in base pairs	$\log_{10}$(size)	distance traveled
10,000	_____	_____
8,000	_____	_____
6,000	_____	_____
5,000	_____	_____
4,000	_____	_____
3,000	_____	_____
2,500	_____	_____
2,000	_____	_____
1,500	_____	_____
1,000	_____	_____
750	_____	_____
500	_____	_____
250	_____	_____

Table 2 Agarose Gel Electrophoresis Results of mtDNA restriction fragments

Bam HI digest

Distance traveled	$\log_{10}$(size)	size in base pairs
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Total size of mtDNA in base pairs = _____

Hind III digest

Distance traveled	$\log_{10}$(size)	size in base pairs
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Total size of mtDNA in base pairs = _____

Bam HI and *Hind III* double digest

Distance traveled	$\log_{10}$(size)	size in base pairs
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Total size of mtDNA in base pairs = _____