Chapter 3

Introduction to the Molecular Phylogeny of Insects

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Introduction

Purpose: This laboratory introduces DNA analysis as a tool for the study of evolution. We isolate DNA from insects and make PCR (polymerase chain reaction) products from the DNA. Different insects should have different sizes of PCR products which are discriminated using simple agarose gel electrophoresis.

Level of Presentation: The lab may be offered at any level although parallel lecture material in evolution, DNA, and PCR is helpful. The most difficult experimental step is the use of a 0-2 µl micropipette.

DNA analysis in systematics: During the last 20 years DNA analysis has contributed greatly to the knowledge of evolutionary relationships between organisms. DNA differences between species are measurable with confidence. Species can be identified from DNA sequences alone. Many current evolutionary trees have been constructed entirely from DNA data although DNA and morphology complement each other. Two of many examples of tree making using both DNA and morphology are in insects (Whiting et al, 1997) and in cichlid fishes (Stassney and Meyer, 1999). A general biology text (Purves et al, 1997) we use in introductory courses mentions both approaches.

Evidence that two species are related but not the same would be that comparable DNA sequences are similar but not identical. For example, distantly related species might have 50% identity in a particular DNA sequence, while more closely related species might have 90% identity in the same sequence. Finding the right DNA sequence to compare species is important; some DNA sequences are too different to make measurable comparisons, and others are too similar to have differences. Fortunately, there are several possible DNA sequences available to use in making comparisons. Sometimes just the lengths of particular DNA fragments can be used to discriminate between candidate species and that is what we will do in this lab. DNA sequencing is beyond the scope of this lab but could be done in undergraduate labs with a little more work.

Mutation survival and evolutionary clocks: Different DNA sequences in the genome undergo spontaneous random mutations at about the same rate, but whether or not a mutation survives depends on where it occurs. Mutations in essential genes are usually lethal; the mutant organism dies so the mutation does not survive. A few mutations in essential genes do survive but they accumulate slowly because it takes a long time by spontaneous random mutation to hit upon the few DNA changes which do not inactivate the essential gene. The rate of forming surviving mutations in an essential gene is slow for these reasons; it makes a slowly running evolutionary clock. By contrast, mutations in non-essential genes or non-expressed DNA sequences have fewer consequences so they survive more often; a non-essential DNA makes a more rapidly running evolutionary clock. The slowly running clock is useful to discriminate between distantly related species and the rapidly running clock is useful to discriminate between closely related species.

rDNA, rRNA, ribosomes, and ITS: We use two evolutionary clocks here, fast and slow. They are both in ribosomal DNA, (rDNA) which codes for ribosomal RNA, (rRNA). Ribosomes are complexes of RNA and protein which perform essential functions in protein synthesis. The structure of ribosomes depends on rRNA molecules which hold the ribosome together. The rRNA is more structural than informational; rRNA is not mRNA, messenger RNA.

In eukaryotes, rRNA is transcribed from rDNA into a single long RNA molecule, the *initial transcript,* which later is chopped up or *processed* into three smaller rRNA molecules found in ribosomes called 18S, 5.8S, and 28S. The initial rRNA transcript, a direct copy of rDNA, is 14,000 nucleotides long in humans but the three processed rRNA pieces in ribosomes add up to only 8000

nucleotides. That means 6000 nucleotides of the initial transcript are not conserved; they are thrown away. The ITS, intervening transcribed sequences, are parts of the thrown-away or non-conserved rRNA (White et al., 1990). A crude map showing spatial relations of the ITS to the conserved rDNA is shown below in Figure 3.1.

The human initial rRNA transcript is called 45S rRNA and the finished or conserved rRNA's are called 18S, 5.8S, and 28S rRNA. The S values, sedimentation coefficients in Svedbergs, measure the sedimentation velocity, how fast molecules move in a high speed centrifuge. The Svedberg equation which relates S values to molecular weights is not linear, so the sum of S values of the RNA fragments, 18S, 5.8S, 28S, and the ITS, does not add up to 45S.

rDNA contains fast and slow clocks: The parts of rDNA coding for mature, conserved rRNA (18S, 5.8S, and 28S) accumulate mutations slowly because the mature rRNA is essential. Surviving mutations are so few that there are recognizable similarities in rDNA sequence between the three major branches or domains of life. Because surviving mutations in conserved rDNA are infrequent, differences in conserved rDNA are best used to distinguish distantly related species.

The ITS non-conserved parts of rDNA do not code for essential functions, so mutations in them have fewer consequences for survival of the organism, and ITS mutations accumulate relatively rapidly. Differences in the ITS are best used to distinguish closely related species.

The processed rDNA and the ITS are conserved or not conserved in two senses, in RNA processing and in evolution. The rDNA conserved in processing is also conserved in evolution because mutations in it are rarely retained. The ITS are not conserved in processing and are less conserved in evolution; that is, their sequences are more divergent because mutations in them are more frequently retained.

Still faster clocks: There are even faster clocks which we will not use here. An example is DNA "microsatellite" sequences which are not related to rDNA, which have repeated DNA sequences which stimulate mutation. The mutation rate of microsatellite sequences is fast enough to make detectable differences between individuals within a species. Microsatellite sequences are now used routinely in forensics. For example, they were used, with varying success, to distinguish human individuals in the OJ Simpson trial and in "Monicagate".

Kinds of mutations in the ITS: The spontaneous mutations in the ITS can be deletions, duplications, insertions, and single nucleotide substitutions. The first three kinds of mutation can change the length of the ITS considerably, enough to be detected by the simple low resolution agarose gel electrophoresis which we can do easily. If mutations changed the sequence but not the length, we would have to do DNA sequencing to detect the mutations. Sequencing takes more work but is not impossible to do in undergraduate labs.

PCR and universal ITS primers: (See Figure 3.1) The polymerase chain reaction (PCR), invented by the 1993 Nobel Prize winner Kary Mullis, amplifies a short *target sequence* of DNA (typically about 500 nucleotides) between the two shorter sequences which hybridize to DNA segments (typically about 25 nucleotides) called *primers*. Primers are made easily by automated machines. The two primers must hybridize to complementary DNA strands at the ends of the target sequence. The complementary strands are anti-parallel so the sequences of the two primers point at each other. ITS primers are actually not within the ITS but are in the conserved rDNA sequences near the ITS junctions; ITS primers point at each other from conserved rDNA across a non-conserved ITS. Because ITS primers are in conserved DNA sequences, they will produce PCR products from the DNA of many species; generally we do not have to make custom primers for each organism.

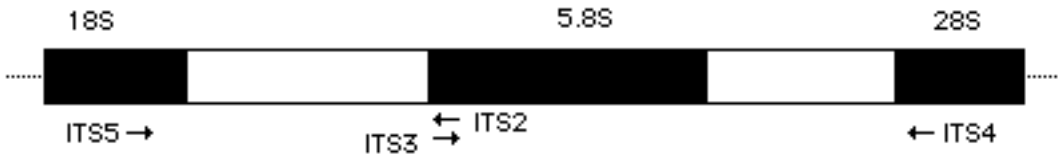

Figure 3.1. Map (not to scale) of a fragment of ribosomal DNA (rDNA) containing the two ITS in eukaryotes. The white regions are the two ITS and the black regions are conserved rDNA: all of the rDNA coding for 5.8S rRNA and the ends of rDNA coding for 18S and 28S rRNA. Positions of four universal ITS primers (ITS 2, 3, 4, and 5) for PCR are shown. It is possible to make PCR products between any two primers pointing toward each other. We usually use the combination ITS 3 and 4 or ITS 2 and 5 although we could use ITS 4 and 5. The ITS 4 and 5 PCR product including all of the conserved 5.8S sequence, is 600–1000 nucleotides long in insects, while the ITS 4 and 5 PCR product in mammals is much larger--over 2000 nucleotides. The nucleotide sequences of the primers, from White et al. (1990), are in Appendix A.

Problem with universal primers: The universality of ITS primers has a disadvantage if there is more than one organism represented in the DNA. As relevant examples, larvae of flies grown in rotting fruit or carrion and larvae of bat fleas grown in guano are contaminated with DNA from microorganisms such as bacteria, yeast, and molds. The ITS primers do not recognize bacterial DNA, but they do recognize fungal DNA. If we do PCR on insect DNA contaminated with fungal DNA we will get multiple PCR products representing insect and fungi. Without more information we would not be able to distinguish insect and fungal DNA. Fortunately there is a large database of sequence information which can help us distinguish the two. (See Figure 3.2 legend.)

Materials

The materials are given for a lab class of twenty-four students, in 12 teams of 2.

Agarose for gel electrophoresis, 12 g
Balance, top loading
Deoxyribonucleotide triphosphates, mixture of all four each with 10 mM, 12 tubes each with 100 µl, stored at -20°C
DNA size markers, BRL/Life Sciences "1 kb markers" or home-made M13 RF cut with Taq I
Electrophoresis power supply, 0-150 volt, adjustable, with leads
Electrophoresis slab gel box, 12 x 20 cm gel, two, with combs
Ethanol, 70% in water, chilled on ice, twelve 1 ml aliquots in microfuge tubes
Ethanol, 95%, 100 ml in one bottle and twelve 1 ml aliquots in microfuge tubes
Ethidium bromide, 10 mg/ml in formamide, four tubes each with 20 µl. Store at 4°C.
CAUTION: Dangerous. Wear gloves.
Forceps, fine tipped, twelve
Freezer, -20°C, or refrigerator freezing compartment
Gloves, latex, one box each small, medium, and large.
Glycogen, Boehringer-Mannheim, diluted to 1 mg/ml in water, stored frozen, 12 tubes each with 50 µl
Goggles or face shields to prevent UV exposure of eyes from UV light box, six
Graph paper, millimeter, 12 sheets, or computer with graphing program
Hand lenses for observing small insects, 12

Hand lotion or Vaseline™ in case of skin exposure to phenol (See PCI.)
Hazardous waste container for used PCI, bottle with tight-fitting lid
HCl, 1 N, twelve 0.5 ml aliquots in 1.7 ml microfuge tubes
CAUTION: Dangerous. Wear gloves.
Ice buckets, 6
Ice machine or crushed ice stored in refrigerator
Kimwipes™ or equivalent tissues, six boxes
KOH-Joy™: 0.5 N KOH, 0.1% (v/v) Joy™, twelve 0.5 ml aliquots in microfuge tubes,
CAUTION: Dangerous.
Lab coats
Light box, ultraviolet, transilluminator, for ethidium bromide stained DNA
CAUTION: Dangerous.
Low Tris buffer: 10 millimolar Tris-HCl pH 8.0, 0.1 millimolar EDTA, 12-1 ml aliquots in microfuge tubes
Marking pens, Staedtler Lumicolor 313, black, permanent, twelve
Microfuge tubes, 0.5 ml, non sterile
The Eppendorf brand of 0.5 ml tubes can be used in the MiniCycler PCR machine.
Microfuge tubes, 1.7 ml, non sterile, bag of 1000, Eppendorf or Intermountain brand, not Baxter (Baxter 1.7 ml tubes have a narrow tip which prevents draining of alcohol from precipitates.)
Microfuges, 2, Eppendorf brand or equivalent, with adapters for 0.2, 0.5, and 1.7 ml tubes
CAUTION: Using the wrong size of tube adapter will cause tubes to break.
Microwave oven for melting agarose
Needles, 18 gauge, twelve, used as dissecting needles to cut insects
Oil, mineral, to use if PCR machine does not have a heated lid, 10 ml
Parafilm, one roll
PCI: Phenol, chloroform, isoamyl alcohol in the proportions 9:9:0.1, twelve aliquots of 0.5 ml in microfuge tubes made from stock on the day of the class. Use high quality phenol for nucleic acids. Store PCI stock at 4°C under Low Tris buffer supplemented with 10 millimolar beta-mercaptoethanol.
CAUTION: Dangerous. Wear gloves; keep tubes upright.
PCR buffer, 10x, 10 aliquots of 10 µl in microfuge tubes, stored at 4°C, supplied with enzyme.
If necessary, supplement with $MgCl_2$ to give 2 millimolar final concentration in the PCR reaction.
PCR enzyme, *e. g.* Klentaq1 (A B Peptides, St. Louis, Mo, 800-383-3362), twelve 0.5 ml tubes containing 2 µl each, store at -20°C
PCR machine MJ Research (800-729-2165) PTC-150 MiniCycler (≈$2500 US) or dual block PTC-200 (≈$8000 US)
Using a thermocycler with a heated lid saves time and trouble, but we give instructions for a thermocycler without a heated lid.
PCR primers, ITS 2, 3, 4, and 5 each with 10 µM. Five aliquots of 10 µl each. Store at 4°C. The primer sequences are in Appendix A.
PCR stop: 10 millimolar EDTA pH 8, 20% glycerol, 0.05% bromphenol blue, 12 tubes each with 20 µl.
PCR tubes, 0.2 ml or 0.5 ml (depending on the PCR machine used) Be sure tubes fit the machine.
Pipetman tips, non sterile, 0-1000, 0-200, 0-10 µl size, bag of 1000 for each of three sizes
Pipetmen or equivalent, P1000, P200, P20, P2, six of each size
Polaroid 667 film, 2 packs of 10 exposures each, or digital camera and computer
Polaroid camera (or digital camera) with filter for ethidium bromide fluorescence
Racks for PCR tubes, six

Racks for 1.7 ml microfuge tubes, seven, including one pre-chilled in freezer
Refrigerator
Rulers, millimeter, six
Scissors to cut Parafilm, one
Squeeze bottle containing 95% ethanol in case of PCI exposure
TBE buffer, 10X concentration, 1 liter, containing:
    121 g Sigma Tris Base or "Sigma 7-9", Sigma Chemical Co., 314-771-5750
    61.8 g boric acid
    7 g Na_4EDTA
Tris-HCl, 1 M, pH 7.2, twelve 1 ml aliquots in 1.7 ml microfuge tubes
Vortex mixers, six

Notes to Instructors

Time allotment: This lab is broken down into seven lab activities and six waiting periods. The activities can be done quickly, but the waiting periods make it impossible to teach everything in one afternoon. If taught as an integral part of a course, it would be best to intersperse segments of this lab with other labs. Alternatively, the lab might be an "independent project" for extra credit; students could work intermittently at their own paces. The lab activities are 1) preserving insects, 2-4) three steps in DNA extraction, 5) assembling and running the PCR plus beginning the gel electrophoresis of the PCR products, 6) finishing the gel electrophoresis plus gel photography, and 7) PCR product size analysis. The waiting periods are 1) fixation of insects in alcohol for overnight or longer, 2) alkali-detergent extraction and solubilization of DNA for overnight or longer, 3) DNA precipitation for at least one hour, 4) DNA drying and re-dissolving for at least one hour, 5) PCR for about 70 minutes, and 6) gel electrophoresis for 3–24 hours. Waiting periods 1, 3, and 4 could each be several days.

Hazards: The most dangerous reagents are, in order of decreasing danger, PCI, ethidium bromide, KOH-Joy™, and 1 N HCl. Stocks should be stored separately and dispensed to students in small volumes to minimize spills and the toxic vapor of PCI. Students and TA's should wear gloves and lab coats when using the dangerous reagents. A fume hood is probably not required for using these tiny volumes of PCI. A squeeze bottle of ethanol and hand lotion should be on hand while using PCI. Students should wear goggles, coats, and gloves. If PCI gets on the skin, rinse the skin repeatedly with ethanol until the phenol smell is gone. Dry the skin with a paper towel, and put on hand lotion or Vaseline™.

Consider the danger of electric shock from the electrophoresis equipment. If the gel boxes do not have safety interlocks, the instructors or TA's should be responsible for turning the power on and off. Alternatively, the power could be turned on and off by an appliance timer while the lab is empty.

Be sure that students have gloves and goggles when using the UV transilluminator.

The gel and the gel electrophoresis running buffer will become contaminated with ethidium bromide, so treat them accordingly. Do not pour contaminated gel buffer down the drain! We dry used gels for about 3 days at room temperature on Saran Wrap and give the dried gels to our Safety Services who dispose of them. Dried gels are much safer than wet gels. We re-use gel running buffer about 20 times before giving it to Safety Services.

Going further: See Appendix B. For PCR product analysis we do only the easiest version: Finding size differences on agarose gels. The different sizes of PCR products made with the same primers should allow the student to distinguish between several possible insect species but it will not allow

the computation of evolutionary distances between species. If you want to proceed farther in this subject, we would be glad to consult with you on more sophisticated analysis including RFLP, DNA sequencing, computer-assisted sequence comparisons, and database searching.

Student Outline

This lab will consist of seven brief lab activities interrupted by waiting periods. It might take three weeks of one lab per week to finish everything. The lab will more interesting if you cooperate with other students studying different insects. There will be a better chance of success with a group project that with working alone. It is always more satisfying to report on positive results than failures. Even if everyone succeeds, the results of a whole class project will be more interesting than solo results. There is room for at least 16 samples in the MiniCycler PCR machine and room for 12 or 24 PCR samples in each agarose electrophoresis gel box.

1. Preserving insects in alcohol

Fix insects in alcohol for at least 18 hours. Use small insects, the size of *Drosophila melanogaster* (3 mm) or smaller. We have had best results with small adult insects such as fleas, flies, mosquitoes, or gnats. If the insects are larger than 3 mm long, cut them up and work with pieces 2-3 mm long; for example just the legs of one large fly would be enough for this project. To prevent degradation of DNA in dead insects, live insects should be put in 95% ethanol. Put one whole insect or one 3 mm insect fragment per 1 ml of ethanol in a 1.7 ml microfuge tube. Dead insects can be used only if they have been freshly killed before being put in alcohol. If 95% ethanol is not available, 70% isopropanol (rubbing alcohol from the drug store or supermarket) will do. Insects can be stored for years in alcohol if the tubes do not leak. If the alcohol dries out, the procedure might fail. If you think the tube might leak, seal it by wrapping the top tightly with Parafilm™.

2. DNA extraction, first part

Cut the insect in half: Cutting the insect helps to extract DNA. Remove the insect from alcohol with cleaned forceps and blot it damp-dry on a Kimwipe™. Place the insect in a clean, dry, labeled 0.5 ml microfuge tube and cut it in half using a dissecting needle or the sharp beveled edge of an 18 gauge hypodermic needle as a knife. If your insect sample is a cut piece from a larger insect (legs are good) further cutting is not necessary. Keep the insect in the bottom of the tube to avoid losing flying fragments when the insect snaps in two. Insects fixed in 95% ethanol are more brittle than those fixed in 70% isopropanol. If you want to save the cut up chitin carcass for later morphological identification, cut the insect in a way that does not destroy morphological features; for example, cut the insect in the abdomen to avoid damaging the mouth parts and the genitalia.

Extract with KOH-Joy™: KOH is used traditionally in entomology to clear larvae for examination with the microscope. Joy™ dishwashing detergent (lemon-scented or not) is a mixture of non-ionic and anionic surfactants which help to solubilize the insects by acting as a wetting agent. Joy is used because it is readily available and because it does not precipitate in KOH. The detergent SDS, by contrast, makes a horrible precipitate. KOH-Joy™ solubilizes many things including DNA. KOH also degrades RNA into nucleotides and denatures DNA into single strands, but denaturation of DNA does not affect the PCR. PCR requires DNA denaturation as the first step.

Add 40 µl of KOH-Joy™ to the cut-up insect or insect pieces in the 0.5 ml tube. Make sure the insect is covered. We have had variable results with soaking whole insects in KOH-Joy™; sometimes it worked but at other times the DNA was not extracted. The insect pieces might float at first but should sink within a few hours. Let the insect pieces soak overnight at room temperature.

We found that three days of soaking was successful but as yet we have not tried longer soaking. If you wish to prepare slides for later morphological identification of your insect with the microscope, take care to keep the insect exoskeleton undamaged.

3. DNA extraction, second part

Neutralize the KOH: After soaking of the cut-up insect in 40 µl of KOH-Joy (overnight or longer), neutralize the KOH by adding 20 µl of neutralization mixture: three volumes of 15 µl 1 N HCl plus one volume of 1 M Tris-HCl, pH 7.2. Be careful not to contaminate the reagent bottles of HCl and Tris with insect DNA because PCR is extremely sensitive to contaminating DNA. One way to avoid contaminating the reagents is to make a small volume of the neutralization mixture and then throw away the unused mixture. While doing PCR you have to develop defensive strategies to avoid DNA contamination. We use an another strategy below for glycogen.

Prepare to use glycogen as a precipitation carrier: Label 1.7 ml microfuge tubes, one for each 0.5 ml tube containing an insect soaked in KOH-Joy™. Put 6 µl of 1 mg/ml glycogen in each clean 1.7 ml tube, using the same Pipetman tip for all of the tubes. (Vortex recently thawed reagents like glycogen before using.) The glycogen will later act as a precipitation carrier to aid the alcohol precipitation of tiny amounts of insect DNA which might not precipitate otherwise. You will later add insect DNA to the same 1.7 ml tubes but add the glycogen first to avoid contaminating the glycogen with insect DNA. We do not like to throw away expensive glycogen.

PCI extraction: Add 60 µl PCI (phenol-chloroform-isoamyl alcohol) to the neutralized KOH-Joy™ in the 0.5 ml tube. Cap the tube tightly. Vortex the mixture vigorously in the closed tube for 10 seconds at room temperature. Wear gloves because the tube might leak, allowing PCI to get on your hands even when it is closed. The PCI helps to release sequestered DNA. Be sure to vortex hard enough to make a uniform white emulsion. Find microfuge adapters for the tubes and then microfuge the tubes for 10 seconds to break the emulsion into two phases: an upper aqueous phase and a lower PCI phase. A white layer of denatured protein may be visible between the phases. The DNA will be in the upper phase. Use a P200 Pipetman set at 150 µl to draw up all of the liquid, including both liquid phases but not the solid insect carcass, into the pipette tip. You should be able to see the separation between the two phases in the tip. Carefully put the lower phase and the white interphase layer back into the 0.5 ml tube with the carcass, leaving the upper phase in the tip. Put the upper phase in the labeled 1.7 ml tube containing the glycogen. Try to avoid getting PCI into the 1.7 ml tube. If too much PCI is added, you will see a little puddle of PCI after 5 seconds of microfuging the 1.7 ml tube and you can remove it. Too much PCI can ruin your DNA preparation.

Ethanol precipitation: Add 150 µl of 95% ethanol to the recovered aqueous phase and vortex the closed tube. You will probably see a clear mixture because there is usually too little precipitating material to make it cloudy. Put the tube in the freezer for 1 hour or longer to aid precipitation. Several days in the freezer is fine. It is a good idea to arrange the tubes in balanced pairs or triplets at this time, anticipating that you will need to balance tubes during centrifugation in the next step.

Recover insect carcass for microscopy: To save the chitin carcass pieces of the insect for later identification with a microscope, remove the PCI from the 0.5 ml tubes with a Pipetman and place the PCI into a hazardous waste container, leaving the chitin pieces in the tube. Add 200 µl of 95% ethanol to the tube. The carcass will be stable indefinitely in the 0.65 ml tube as long as the ethanol does not dry out. You may wish to seal the tube with Parafilm to prevent drying in long term storage.

PCI disposal: Never pour used PCI down the drain; put it in a hazardous waste container.

4. DNA extraction, third part

Concentrate and re-dissolve the DNA: After the DNA has precipitated for one hour or longer in cold ethanol, microfuge the tube for one minute (10,000-12,000 RPM) at room temperature. Decant and discard the liquid as soon as the microfuge stops. After decanting, do not right the tube but keep it upside down to drain it for 10 seconds or longer. The pellet of precipitated material is usually not visible. After draining, blot the lip of the tube with a Kimwipe™, right the tube, and add 0.5 ml of 70% ethanol. Vortex the tube briefly. Rinsing with 70% ethanol gets rid of tiny traces of PCI which can inhibit the PCR in the next step. Microfuge the tube again for 30 seconds. Decant and discard the supernatant solution immediately after microfuging. Keep the tube open and upside down for about 30 minutes at room temperature, until the tube is dry. Re-dissolve the DNA in 10 µl of low Tris buffer for at least 30 minutes at room temperature or overnight in the refrigerator, at +4° C. (DNA cannot dissolve in the freezer.) The DNA is stable for months in the refrigerator. You may freeze the DNA after it has dissolved but that is not necessary. The only good reason for freezing DNA is to avoid drying it out through small leaks in the tube during months of storage in the refrigerator. It is best to accumulate DNA samples in the refrigerator until you can do several PCR reactions at once.

Measure DNA concentration? Do not try to measure the concentrations of DNA because the concentrations will be too low. The evidence for the presence of DNA will be visible PCR products after electrophoresis, as compared with a control.

5. Assembling and running the PCR

Setting up the PCR machine: The PCR machine can hold 16 or more tubes, depending on the type of machine. A PCR volume of 10 µl is adequate but 10 µl reactions require careful pipetting.

Learn how to run your PCR machine before you use it. Most machines have a simple dialog presentation to coach you through the procedures. Set up the machine so you can start the right program quickly when you need it.

The program we use for insect DNA with primers ITS 3 and4 or ITS 2 and 5 is our BFLEA program in Table 3.1. The BFLEA program takes about 70 minutes of run time on MJ Research PCR machines.

Table 3.1. BFLEA program for ITS 3 and 4 or ITS 2 and 5

Step in PCR program	**What occurs during this step**
1. 94°C for 60 seconds	Denatures high molecular weight DNA.
2. 94°C for 10 seconds	Denatures low molecular weight PCR products.
3. 56°C for 10 seconds	Allows primers to hybrize to their complementary sequences on the denatured DNA.
4. 72°C for 40 seconds	DNA polymerase extends primed DNA up to 1000 nucleotides.
5. go to step two 29 times	Repeats steps two through four 29 times.
6. 72°C for 60 seconds	Finishes off incomplete strands.
7. 30°C for 30 seconds	Cools to near room temperature.
8. end	Stops, finished.

The BFLEA program in Table 3.1 is for half-length ITS using ITS 3 and 4 or ITS 2 and 5. (See Figure 3.1.) If you want to run full-length ITS, with primer combinations ITS 4 and 5, increase step 4 from 40 seconds to 60 seconds. Since insect ITS sizes are usually 1000 nucleotides or less and

human ITS sizes are 2000 nucleotides or more, a short step 4 prevents accidental production of human PCR products from contamination of insect material with DNA from the experimenter.

If the PCR machine *does not* have a heated lid, put a drop of mineral oil in each of the wells.

Put insect DNA in PCR tubes: Label the PCR tubes with a permanent marker to identify them later. Place the marks so that they will not be rubbed off, on tops of 500 µl tubes or on sides of 200 µl tubes. Add 4 µl of the 10 µl of DNA to each tube. Include a no-DNA control tube which contains 4 µl of low Tris buffer. Close the tubes and store them on ice or in the refrigerator until you are ready to add the reaction mixture.

Add reaction mixture to PCR tubes: Prepare a reaction mixture of PCR reagents to add to the DNA shortly before use. Make enough reaction mixture for several DNA tubes at once; it is difficult to make fewer than four tubes-worth of mixture. The mixture will keep on ice for 30 minutes. Be sure to vortex the reagents thawed from frozen stocks before you use them. If you have n tubes with DNA, prepare enough reaction mixture for n + 1 tubes. The amount for an extra tube is to compensate for the material which will stay on the tube walls and pipette tips. The volumes for a single reaction mixture for a 10 µl PCR are in Table 3.2. Multiply the volumes in Table 3.2 by n + 1 for n PCR's. You will need to use a P2 Pipetman to measure sub-microliter volumes of enzyme.

Table 3.2. Reaction mixture (6 µl) to add to DNA (4 µl) to make one PCR sample

2.95 µl distilled water
1.0 µl 10 x PCR buffer containing 20 millimolar Mg^{++}
1.0 µl deoxyribonucleotide triphosphate mixture, each of the four @ 10 millimolar
0.5 µl primer ITS 3, 10 micromolar
0.5 µl primer ITS 4, 10 micromolar
0.05 µl Taq polymerase enzyme (Klentaq1 or Amplitaq) 5 enzyme activity units per µl

Be sure to vortex the mixture after you make it and keep it on ice. It should be active for at least 30 minutes on ice. Finally add 6 µl of the reaction mixture to each PCR tube already containing 4 µl of DNA. An easy way to do this is to have the open tubes sitting in the microfuge; spot 6 µl on the inside of each tube and then spin the tubes one second. Vortex each PCR tube after spinning. Put the tubes back on ice.

Use of oil or heated lids: If your PCR machine uses 0.5 ml tubes and *does not* have a heated lid, you must add 20 µl of mineral oil to each well of the PCR machine, between the PCR tube and the heated aluminum block, and also you must put 12 µl of mineral oil inside each tube. (Use the manufacturer's instructions if they are different from these.) The oil on the outside of the tube is to improve thermal contact with the aluminum block if you use 0.5 ml PCR tubes. The 0.2 ml thin wall polypropylene PCR tubes have an improved flexible design which can achieve good thermal contact without oil on the outside of the tube. The oil on the inside of the tube is to prevent refluxing of the PCR reaction mixture during the temperature changes; without oil, all of the liquid would condense on the top. On newer and more expensive PCR machines, a heated lid prevents refluxing. Accurate pipetting of oil turns out to be the hardest part of the whole lab for beginners. If you have never pipetted oil before, practice a little before you have to do it. Use a chopped-off Pipetman tip to make the pipetting of the viscous oil easier. You cannot avoid oil sticking to the inside of the tip, so coat the inside of the tip with two or three preliminary ups and downs and then dispense 12 µl portions of oil to all of your tubes using the same coated tip, by the "to deliver" method. Do not try to get all of the oil out of the tip; you have to live with the amount that delivers easily with one push of the Pipetman. Watch out

for oil adhering to the outside of the tip and running down into your tube. It is easy to add more oil from the outside of the tip than from the inside. To prevent that outside oil from running into your tubes, never insert the tip more than 1 mm deep into the oil when drawing up oil into the tip, or wipe the oil off the outside of the tip before dispensing oil to your tube. After adding oil, microfuge the PCR tubes again for a second or two. The final result should be a layer of about 2 mm of oil over each PCR reaction. If you are getting too much oil, get rid of oil on the outside of the tip or reduce the amount delivered. Keep the tubes on ice as much as possible until you can begin the PCR. It is the silly little details like this that can make the difference between success and failure.

If your PCR machine *does* have a heated lid, you will save yourself all of the oil headaches but you might need to microfuge the tubes again for 1 second to get all of the liquid down to the bottom of each tube before beginning the PCR. Keep the tubes on ice as much as possible until you can begin the PCR.

Run and stop the PCR: When you are finally ready to start the PCR press the start button on the PCR machine to start the program, plug the tubes into their wells, and close the lid on the machine until the program is done. During the hour that the run takes, make a gel for electrophoresis, unless you will not have time to run it. At the end of the PCR, spot 2 µl of the blue PCR stop mix on the inside top of each tube; spin 1 second, vortex, and spin 1 second again. The stopped reactions may be kept in the refrigerator overnight or longer if necessary. If you had to add oil, you will remove the oil at the time of loading the PCR products on the gel for electrophoresis.

6. Electrophoresis of PCR products

Preparing the gel: The following is a recipe for a 13 x 20 cm horizontal slab gel (260 sq cm), containing 150 ml of 0.7% agarose in TBE buffer. Scale the recipe up or down according to the area of your gel box. Put all of the PCR reactions in wells on the gel. Use a 20 or 24 tooth comb for PCR reactions of 10 µl.

Level the gel box using a carpenter's level. Attach the end pieces or tape to the flat bed to contain the melted agarose.

Put on gloves and a lab coat because the next steps involve ethidium bromide, a mutagen.

Weigh out 1.05 g of agarose in a 250 ml Erlenmeyer flask.
Add 135 ml of distilled water.
Heat to boiling in the microwave, full power for 1.5 minutes.
Simmer at 30% power for 1.5 minutes.
Check to be sure the agarose has all melted; if not, simmer the solution longer.
Put the flask of melted agarose in a large beaker of room temperature water to cool it.
Add 15 ml of 10 X TBE and swirl the flask to mix the solution.
Add 13 µl of 10 mg/ml ethidium bromide in formamide and mix again.
Pour the agarose-ethidium bromide into the gel box within 1 minute after beginning the cooling or put the flask in a 45°C water bath to keep the agarose melted until you can pour it.
After pouring the gel, rinse the flask with water before the residual agarose sets.

The agarose gel should set within 20 minutes. After it has set, remove the end pieces and add enough 1 X TBE buffer to give 3 mm of buffer depth over the slab. Do not pull out the comb until just before loading samples.

Enough ethidium bromide stays in the gel during electrophoresis so there is no point in adding more ethidium bromide to the 1 X TBE running buffer; it would just make more hazardous liquid to spill.

Loading and running PCR products: Attach the leads, turn the power on, and adjust the voltage before loading the PCR products. Then turn the voltage off until after loading. You will want to run a 20 cm gel for 450- 500 volt-hours which can be as fast as 150 volts for 3 hours or slow as 21 volts for 24 hours. It is convenient to have the power supply running off an appliance timer which can turn the power on automatically after you finish loading the gel and turn it off in case you cannot get back to the lab on time.

Now it is time to pull out the combs which made the wells in the agarose gel. The PCR products at this time should be still in the PCR tubes and should be blue from the addition of stop mix; if not, add 10% v/v stop mix now. The stop mix contains dense glycerol which is necessary to make the samples dense so they sink into the wells. Using a P20 Pipetman load 10 µl of DNA size markers in one well and then load the PCR products in the other wells.

If you used oil because the PCR machine does not have a heated lid, you can use the "Parafilm principle" of hydrophobic interactions to separate oil from aqueous PCR product. Fold accordion pleats in a 10 x 10 cm piece of Parafilm and prop it against something at a 45° angle. With a P20 Pipetman draw up all the blue aqueous material from one PCR tube and apply it to the top of one Parafilm groove. Let the 10 µl of blue solution roll down the groove for about 5 cm; then recover the PCR product with a clean Pipetman tip and load it on one slot in the gel. The oil should stick to the Parafilm and the aqueous material should not.

Slow overnight running of a gel results in some diffusion and broadening of low molecular weight DNA (less than 1000 nucleotides), but unless you are trying to separate very closely spaced bands, the convenience of overnight running outweighs this disadvantage.

7. Size analysis of PCR products

Put on gloves, a lab coat, and eye protection to avoid exposure to ethidium bromide and UV light.

After running the gel, slide it out onto the UV light box. Turn on the light box and look for PCR products with protected eyes. Look only for a few seconds. It is much safer for your eyes to look at a film, so use the camera. We expose Polaroid 667 film 1 second at f 5.6 using a special filter to block excitation wavelengths for ethidium bromide fluorescence and we develop the film for 40 seconds at room temperature.

Instead of Polaroid film you can use a digital camera to make a TIFF file of the image with a computer. The digital camera needs an excitation-wavelength blocking filter too.

You can use a millimeter ruler to measure distances from the top of the photo to the bands in the size marker lane and in the PCR product lanes. Or you can scan the Polaroid photo into a TIFF file and measure distances with a computer as you would for an image made with a digital camera. We use the free program "NIH Image" (http://rsb.info.nih.gov/nih-image/index.html) to measure distances (in pixels) on TIFF representations of fluorescent gels. Using either method, make a table of molecular weights of the standard markers versus the distance the markers moved in the gel, either in millimeters or in pixels. Plot DNA size in nucleotides versus distance moved either on millimeter graph paper or in a graphing program (DeltaGraph or CricketGraph) to curve-fit your data and get an equation of size as a function of distance. See Figure 3.2 for an example of data to be used in size analysis.

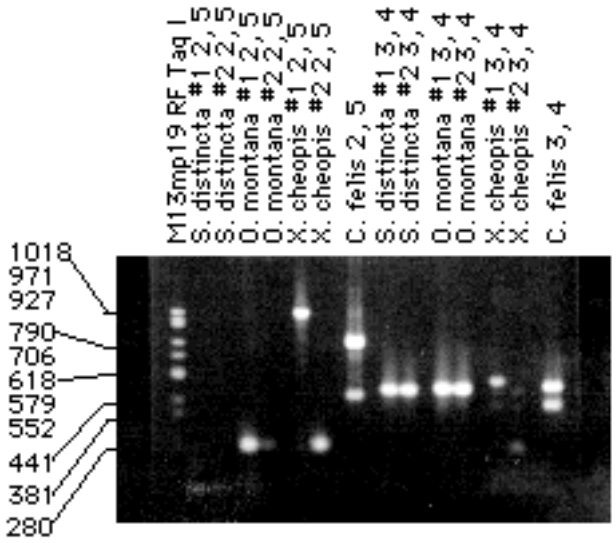

Figure 3.2. Photograph of agarose gel with ITS PCR products of DNA extracted from single fleas. The numbers on the left are sizes in nucleotides of DNA fragments in the marker lane, M13 bacteriophage replicative form cut with Taq I. The bat flea *S. distincta* always gave a PCR product with ITS 3&4 but never with ITS 2&5, for unknown reasons. *S. distincta* and *O. montana* could not be distinguished by the sizes of their ITS 3&4 products (because the sizes were the same) but they could be distinguished by ITS 2&5 products; *O. montana* had a product and *S. distincta* did not. Our *X. cheopis* population of fleas was mixed with *O. montana* which we discovered by PCR of individual fleas. Larvae of the cat flea *C. felis* gave two products with each set of primers. The DNA sequence of the smaller *C. felis* product from each primer combination closely matched the Gen-EMBL database sequence for baker's yeast, *S. cerevisiae*. The larger *C. felis* product had no significant matches to yeast but did have similarities to the equivalent sequences from the other fleas. We made yeast-specific primers from the published yeast ITS sequences to aid identification of yeast PCR fragments.

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We wish to acknowledge support from the University of Utah Research Committee. We had a very helpful discussion on the use of ITS primers with Prof. William C. Black IV of Colorado State University. We thank Dr. Ken Gage, CDC Ectoparasite Lab, Fort Collins, CO for sending insects to us.

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Appendix A

DNA sequences of ITS primers, from White et al. (1990).
ITS 2: GCTGCGTTCTTCATCGATGC
ITS 3: GCATCGATGAAGAACGCAGC
ITS 4: TCCTCCGCTTATTGATATGC
ITS 5: GGAAGTAAAAGTCGTAACAAGG

Appendix B

"Now that I have PCR products, how do I construct a phylogeny?": A brief introduction to numerical taxonomy and phylogenetics

Robert Bossard

A phylogeny is a hypothesis about evolutionary relationships among a set of taxa. To use molecular data (or any other characteristic) we assume that species sharing similar characteristics are more closely related than other species not sharing those similarities. Ideally, similarity would involve all the characteristics of the taxa, but in reality we only obtain a fraction of a taxon's characteristics. The assumption that similarity equals relatedness is invalidated with convergent or parallel evolution, horizontal gene transfer, or indirect feedbacks through the environment.

The PCR products obtained in the ABLE laboratory exercise are genetic fragments of varying sizes and the sizes are themselves taxa characteristics. Unfortunately, to construct phylogenies PCR fragment size is usually not informative enough; the PCR products must be sequenced (Hoy, 1994).

Usually we cannot associate phenotypic effects with sequence differences, so the approach of numerical taxonomy is to consider each character state as varying independently and randomly. In the case of DNA sequences, the character state is the nucleotide. We must line up the sequences of the various taxa so they match as much as possible. Though this can be done by hand, it is often difficult because of genetic insertions or deletions. Instead a computer program is used.

Now we calculate the similarity between every pair of taxa. The simplest formulas are based on: Similarity = Number of matching characters / Total number of characters. Formulas with different definitions of matching and non-matching, or with more complicated forms, are available that may be more useful with certain assumptions and taxa.

The traditional way to show the similarities is to place similar taxa close together and dissimilar taxa farther apart. This forms the famous evolutionary tree. But where is the "root" of the tree? If we have an "outgroup" taxon in our data, in other words a taxon that is quite different from any of the other taxa in the group, we may wish to place the root of the tree near this taxon (Strauss, 1993). There are usually a large number of different trees that can be formed from a set of phylogenetic data depending on assumptions about the relation between distance and similarity, parsimony (i.e. the extent to which simpler hypotheses are preferred), and the most likely evolutionary processes thought to be involved (Swofford and Olsen, 1990).

Finally, we may place a time scale on the branching. The "molecular clocks" mentioned at the beginning of the handout, or fossil evidence, can supply times at which branching may have occurred. With these inferences, we have a phylogenetic hypothesis that can be compared with other phylogenies constructed for these taxa.

Various software with the capability of analyzing and graphing data from molecules or other characteristics for taxonomy include HENNING86, MacClade, PAUP, and PHYLLIP (Hoy, 1994), and general purpose software such as SAS and SPSS. You can also calculate taxa similarities with a spreadsheet and, though spreadsheets do not have tree graphs, x-y graphs can be substituted. Most

usefully, these numerical taxonomic approaches can be used to group and analyze a variety of data in addition to molecular phylogenetics, such as behavioral and ecological attributes including mating systems and geographical variation (Hoy, 1994).

Here is a little summary of the literature cited below. Hoy's book is a comprehensive introduction to the subject with methods and a chapter on insect molecular systematics and evolution. Strauss's article give a broad perspective on computer-aided methods for systematic biology. The article by Swofford and Olsen gives more perspectives and the other articles in Hills and Moritz discuss several protocols in molecular taxonomy including analysis of proteins, nucleic acids, immune reactions, and cytogenetics.

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