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Barcoding Life: Classification of Insects

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Morphological and molecular techniques are complementary approaches for classifying organisms, each with their own merits and limitations. Students first classify an insect using a traditional taxonomic key based on comparative anatomy, which requires expertise, relies on examination of the adult stage of the life cycle and requires a whole, intact individual. Then students use a DNA barcoding technique, which requires little expertise, works for any life cycle stage, and does not require a whole intact individual. However, it is most useful for already-sequenced organisms. Students gain an appreciation of insect diversity using the two classification techniques.

Keywords: classification, taxonomic key, barcoding, PCR, DNA sequencing, insects

Introduction

This laboratory exercise introduces students to the methods systematists use to classify organisms, and gives students hands-on experience with how molecular biology is revolutionizing the identification of organisms. The lab was developed for an introductory biology course, but the general methods can be adapted and extended for a more advanced course in ecology and/or evolution. As written, the lab requires one full 3-hour lab session and about 30 minutes of a second laboratory session the following week to complete, not including approximately 30 minutes of homework once the DNA sequence is available.

Although this lab exercise emphasizes the comparison between traditional and molecular techniques to classify organisms, a number of different groups have described the use of DNA barcoding to involve students in discovery-based science. A 2013 ABLE Major Workshop described the use of DNA barcoding to examine biodiversity (Butler, 2013), and the Cold Spring Harbor Laboratory's NYC Urban Barcode Project involves NYC high school students in discovery-based projects such as checking for invasive plant or animal species, monitoring disease vectors, identifying exotic or endangered food products in markets, and detecting food mislabeling, in addition to examining local biodiversity (http://www.urbanbarcodeproject.org/). The Student DNA Barcoding Project is another example of how barcoding has been used to introduce high school and university students to biological research (Harris and Bellino, 2013). This is a course that has been developed and tested so far in New York City and in Belize (http://www.biobelize.org/dna-barcoding.html).

Although the technique of DNA barcoding is a great tool for identification of already-sequenced organisms, and for involving students in research and hypothesis-testing, the use of the technique to discover new species is controversial and has its critics (Collins and Cruickshank, 2013). With the rapid development of new techniques for sequencing entire genomes, there is a question of how DNA barcoding will adapt to this new technology (Taylor and Harris, 2012, Shokralla *et al.*, 2014). But these are also good lessons for students - that phylogenetic relationships between organisms are hypotheses that can change as new data become available, and that technology is advancing at such a fast rate, even a relatively new technique such as DNA barcoding can quickly become less relevant.

Student Outline

Introduction

Taxonomy is the practice of naming and classification of organisms. Traditionally, taxonomists have used mostly anatomical or morphological characteristics to classify an organism. For example, field ecologists may be interested in the diversity of species within a particular habitat. They use specific characteristics of each organism in the habitat to determine to what species, genus or order the organism belongs. In this laboratory exercise, you will use a taxonomic key to determine to which taxonomic order your insect belongs. Characteristics like number of wings and the number of segments within the distal part of the leg of an insect are used in this taxonomic key. As you will see, classification of an organism based on a taxonomic key is not always easy, and requires a certain amount of expertise. For this reason, taxonomists are specialists, and usually have the expertise to classify only a specific group of organisms.

In order to assign an organism to a particular group, such as species, genus or order, these groups of organisms must first be arranged into a hierarchical scheme. This Linnaean system of taxonomy is congruent with the evolutionary relationships between these groups of organisms. Systematics is the ordering of organisms based on their evolutionary relationships. Biologists make their best guess at evolutionary relationships between organisms based on shared derived characters, or homologies, and based on the fossil record. This is not always easy since it is sometimes difficult to determine whether characteristics are homologous or analogous. Analogous characters are similar traits that evolve independently in different phylogenetic lineages due to factors other than inheritance. The independent evolution of analogous characters is known as convergent evolution. Systematics involves developing hypotheses as to how groups of organisms are related over evolutionary time. These hypotheses are always being tested, and revised if necessary, as new evidence from living organisms or fossils comes to light.

Since the 1980s, DNA, RNA and protein sequences have also been used to classify organisms. Unlike anatomical or morphological traits, DNA sequences of certain common genes are highly conserved among widely divergent organisms. Since changes in heritable traits result from changes in DNA sequence, these changes should reflect evolutionary relationships between organisms. The more recently two species diverged from a common ancestor, the more similar their sequence of nucleotides should be. The availability of DNA sequence information from more and more genes from more and more organisms within databases on the internet has stimulated a surge in molecular systematics.

Obviously some genes are much more highly conserved among organisms than others. The rate of change of nucleotides within a gene over evolutionary time varies considerably from gene to gene. The most highly conserved genes among all organisms are those that retain the same function in all organisms over evolutionary time. These include genes that encode RNA molecules found in ribosomes. These RNA-protein complexes synthesize proteins in all prokaryotes and eukaryotes. Therefore, the sequence of nucleotides within these genes changed very slowly over time. Comparison of these rRNA gene sequences is useful to study groups of organisms that diverged long ago. For example, these sequences have been used in conjunction with morphological and fossil evidence to revise relationships among the animal phyla.

In eukaryotes, genes encoding rRNA that are used for molecular systematics are found in the nucleus. In contrast, genes encoding mitochondrial proteins are found within the mitochondria. Mitochondrial genes are also highly conserved among eukaryotes since mitochondria evolved long ago from endosymbiotic prokaryotes, and their function is essential for all eukaryotes. However the rate of change of nucleotides within mitochondrial genes over evolutionary time is much faster than the rate of change of nucleotides within nuclear rRNA genes. This is probably due to a higher rate of DNA damage (i.e. mutations) associated with the oxidative environment in the mitochondria. Therefore, in contrast to nuclear rRNA genes, mitochondrial genes are useful for distinguishing among groups of organisms that have recently diverged, such as related species.

Grouping organisms based on the sequence of a single gene can be problematic. If you are only comparing the sequences of a few species, the number of different ways to construct trees diagramming their relatedness is not too large. However, if you are comparing 100 species, this quickly becomes too complicated even for sophisticated computer programs. Another complication of molecular systematics is that there are so many genes within the genomes of organisms. Using a single gene to assess relationships among organisms is subject to error just as using a single morphological trait would be subject to error. The strongest hypotheses are those supported by the most evidence, be it molecular, morphological, the fossil record, or all of the above!

One of the major problems encountered among both scientists and laypeople in classifying organisms based on morphological or anatomical characteristics is a lack of taxonomic expertise. Depending on the group of organisms, there may be only a few scientists worldwide that possess the expertise to differentiate between members of the group, or between species, based on these characters. Furthermore, this type of classification sometimes relies on examination of an adult stage of the life cycle, and often requires a whole, intact individual. A classification scheme that is based on DNA sequences gets around these problems since there is little expertise required to extract DNA from an individual, and since large DNA sequencing laboratories provide DNA sequencing as a service. Furthermore, DNA-based classification does not rely on examination of certain life cycle stages, and only requires a small sample rather than a whole, intact individual.

The “Consortium for the Barcode of Life” is a group of scientists that are promoting “DNA barcoding” as a system for identification of species based on their DNA sequence (http://www.barcodeoflife.org). In DNA barcoding, a portion of a single gene is used for species identification. The Consortium hopes that DNA barcoding will provide a fast, inexpensive means of

identifying the estimated 10 million species of eukaryotic life on Earth. Although only a portion of a single gene is used for species identification, this data will contribute to efforts using multiple genes to determine evolutionary relationships between organisms. There are many practical applications to DNA barcoding including identification of pathogens, agricultural pests, endangered species, and invasive species. Dr. Dan Janzen, here in Penn Biology, is a member of the Consortium, and one of the key promoters of DNA barcoding. He envisions a day when even a layperson can use a portable inexpensive device smaller than a GPS unit to scan the DNA barcode from the leg of a beetle or a bit of a leaf in order to get a species name. Someone unfamiliar with the species name may simply need to Google that name to learn more about the organism. Dr. Janzen, in collaboration with Dr. Paul Hebert at the University of Guelph, has used DNA barcoding to distinguish new species of butterflies and moths in Costa Rica (Hajibabaei *et al.*, 2006), and has written about integrating DNA barcodes into the ongoing inventory of complex tropical biodiversity (Hebert *et al.*, 2003, Janzen *et al.*, 2011).

The DNA "barcode" is a portion of the mitochondrial gene, cytochrome c oxidase I (COI). There are a number of reasons COI was chosen for this purpose. As mentioned earlier, mitochondrial gene sequences are highly conserved due to the highly conserved function of their gene products in eukaryotes, yet among conserved genes, the rate of nucleotide change is relatively fast over evolutionary time. Therefore, mitochondrial genes are better than nuclear genes for distinguishing between relatively recently diverged groups of organisms. Another reason is that, because the mitochondrial genome lacks introns, mitochondrial genes such as COI have limited recombination compared to nuclear genes. One of the reasons that COI was chosen as the mitochondrial gene of choice, as opposed to other mitochondrial genes, is that insertions and deletions are rare in COI since most would lead to a shift in the reading frame. The use of some other mitochondrial genes is limited for this purpose due to the insertions and deletions that complicate sequence comparison.

Scientists have been collecting data, mainly morphological or anatomical, about thousands of different organisms, both living and extinct, over the past few centuries. Researchers have been doing their best to arrange these organisms into groups that depict their evolutionary relationships. However, systematics is not easy, and hypotheses continue to be revised. The advent of molecular systematics has added another type of data to these analyses - DNA sequence information. In many cases the DNA sequence information confirms the evolutionary relationships predicted from morphological data or fossil evidence. However, the evolutionary relationships predicted by the molecular data do not always correspond with relationships predicted from morphological data or fossil evidence. Many biologists put more faith in the fossil evidence and question the validity of the new molecular data. More research, and the analysis of more DNA is required to resolve these debates. Healthy debates and animated discussions are a good sign that evolutionary biology is a strong and lively field.

Time will tell whether or not DNA barcoding catches on, and whether or not it is an accurate means of species identification. In this laboratory, you will help resolve the ongoing debate about barcoding, comparing your taxonomic results from a morphology-based key to the results obtained from DNA barcoding.

Overview of Laboratory Exercises

This laboratory focuses on insect identification and classification, not phylogeny. Portions of this lab will span two lab sessions, with a homework assignment after the DNA sequence information is available. The lab consists of the following procedures:

Part A (Session 1): Each student will collect a single insect from the Biopond garden (in the summer or Fall semester) or obtain a frozen insect from their TA (for the Spring semester)

Part B (Session 1): Each student will use a taxonomic key based on morphology to try to determine to what order their insect belongs.

Part C (Session 1): Each student will extract DNA from their insect.

Part D (Session 1): Each student will set up a reaction that will amplify a portion of the cytochrome c oxidase I (COI) gene by the polymerase chain reaction (PCR).

Part E (Session 2): Each student will treat their PCR product with EXOsap-IT to clean up the DNA for sequencing and submit their PCR product for sequencing.

Part F (homework): Students will obtain DNA sequence information from their insect from the course website. Students will enter their sequence data into a bioinformatics search engine to determine to what taxonomic order their insect belongs. Students will compare their results from the morphological identification with their results from identification via "barcoding."

Experimental Protocol

Part A (Session 1): Collection of Insects from Biopond Garden (Summer and Fall) or Obtaining a Frozen Insect (Spring)

Obtain a small plastic jar and an insect net from the lab cart. Each student should have their own jar, but students will share insect nets if necessary. Go out to the Biopond garden, and try to find an insect. Make sure to stay on the paved or mulched trails, and do not enter any of the flower gardens!! Carefully collect an insect from a tree, bush, flower, rock, the air (in flight), on the ground, etc. Try not to touch the insect with any part of your body, as you may contaminate the insect with your own DNA. If the insect is not in flight, you may not need the insect net. You can try to capture the insect in your jar directly. If you need an insect net, wait until one is available after another student has collected their insect. Catch the insect in the net, and then carefully transfer it to your jar. Make sure to note where you found your insect (i.e. on a growing plant, in the soil, etc.). This will help you with the identification using the key on the following pages.

To freeze your insect, put your insect jar in the dry ice/ethanol bath in the fume hood. Use the thermal gloves, and be careful not to burn yourself in the dry ice/ethanol bath. After 5 minutes, use a pair of sterile forceps to transfer your frozen insect from your insect jar into a small plastic Petri dish. Keep this pair of forceps sterile by leaving it in the sterile Petri dish as you're going through the following taxonomic Key. Don't allow the forceps to become contaminated by touching anything other than the insect or the sterile Petri dish.

Part B (Session 1): Identification of Insect based on Morphology, Using a Taxonomic Key

One method that systematists use to classify animals based on morphology is using a "taxonomic key", such as the key you will use below. The following key is modified from keys that can be found in books by Chinery (1993) and Imes (1992). This key will only identify insects to a specific taxonomic order - it does not identify to the species level. There are 30 orders of insects, all with different observable characteristics. The key will only work for adult insects, and is not intended for juvenile insects and larvae/caterpillars.

First, take a photograph of your insect. This will be useful when comparing your insect to photos you find on the internet later. Then, wearing gloves, place your Petri dish containing the insect under a dissecting microscope, and use your pair of sterile forceps to hold and observe your insects, following the key to identify the order to which your insect belongs. Use a ruler to measure its size. Terms that may be unfamiliar to you are underlined, and explained in a glossary at the end of the key. Your TA will also have pictures that may help you identify insect body parts.

Key to Insect Orders

Start at # 1 and follow the links until you have identified your insect.	Insect Order
1. Insect has wings	Go to 2
Insect wingless or with poorly developed wings	Go to 30
2. One pair of wings	Go to 3
Two pairs of wings	Go to 7
3. Body grasshopper-like with enlarged hind legs and pronotum extending back over abdomen	Orthoptera
Insects not like this	Go to 4
4. Abdomen with cerci ("tails")	Go to 5
Abdomen without cerci	Go to 6
5. Insects < 5 mm long, with relatively long antennae and wing with only one forked vein	Hemiptera
Larger insects with short antennae, many wing veins, and long cerci	Ephemeroptera
6. Forewings forming club-shaped halteres	Strepsiptera
Hindwings forming halteres (may be hidden)	Diptera
7. Forewings hard or leathery	Go to 8
All wings membranous	Go to 14
8. Forewings hard or leathery apart from membranous tip	Hemiptera
Forewings of uniform texture throughout	Go to 9

9. Outer forewings hard and veinless, meeting in center line	Go to 10
Forewings with many veins, overlapping at least a little and often held roof-wise over the body	Go to 11
10. Abdomen ending in a pair of forceps; hard outer wings always short	Dermaptera
Abdomen without forceps; hard outer wings commonly cover whole abdomen	Coleoptera
11. Insects with piercing and sucking beaks (mouthparts)	Hemiptera
Insects with chewing mouthparts; cerci usually present	Go to 12
12. Hind legs modified for jumping	Orthoptera
Hind legs not modified for jumping	Go to 13
13. Body dorso-ventrally flattened	Blattaria
Body not dorso-ventrally flattened; elongated thorax with fore-legs modified for grasping	Mantodea
14. Tiny insects covered with white powder	Go to 15
Insects not like this	Go to 16
15. Wings held flat at rest; mouthparts adapted for piercing and sucking	Hemiptera
Wings held roof-wise over body at rest; biting mouthparts	Neuroptera
16. Small, slender insects with narrow, hair-fringed wings; often found in flowers	Thysanoptera
Insects not like this	Go to 17
17. Head extending downwards into a beak	Mecoptera
No such beak	Go to 18
18. Wings more or less covered scales; coiled proboscis usually present	Lepidoptera
Wings usually transparent, although often hairy	Go to 19
19. Wings with a network of veins, including many cross veins	Go to 20
Wings with relatively few cross veins	Go to 24
20. Abdomen with long terminal threads	Go to 21
Terminal appendages short or absent	Go to 22
21. Forewings much larger than hind wings; wings held vertically over body at rest; two or three terminal threads	Ephemeroptera
Wings more or less equal in size, or hind wings larger; wings folded close to body at rest; two terminal appendages	Plecoptera
22. Antennae very short; body at least 25 mm long	Odonata
Antennae longer - greater than width of head	Go to 23
23. Tarsi three-segmented	Plecoptera
Tarsi five-segmented	Neuroptera
24. Wings noticeably hairy	Go to 25
Wings not noticeably hairy	Go to 26

25. All wings more or less alike; front tarsi swollen	Embioptera
Hind wings usually broader than forewings; front tarsi not swollen	Trichoptera
26. Tarsi with four or five segments	Go to 27
Tarsi with 1-3 segments	Go to 28
27. All wings alike	Isoptera
Hind wings much smaller than forewings	Hymenoptera
28. Hind wings similar to or larger than forewings; abdomen with cerci	Plecoptera
Hindwings smaller than forewings; no cerci	Go to 29
29. Tiny insects with at least 12 antennal segments	Psocoptera
Never more than 10 antennal segments; piercing and sucking beak	Hemiptera
30. Insects with slender, twig-like body	Phasmatodea
Insects not like this	Go to 31
31. Insects with grasshopper-like body and long back legs	Orthoptera
Insects not like this	Go to 32
32. Small, soft-bodied insects living on plants, often under protective shield or scale	Hemiptera
Insects not like this	Go to 33
33. Minute, soil-living insects, < 2 mm long without antennae	Protura
Insects not like this	Go to 34
34. Insects with cerci or other abdominal appendages	Go to 35
Insects without other appendages	Go to 42
35. Abdominal appendages long and conspicuous	Go to 36
Abdominal appendages short or hidden under body	Go to 39
36. Abdominal appendages forming pincers	Go to 37
Abdominal appendages not forming pincers	Go to 38
37. Tarsi three-segmented	Dermaptera
Tarsi one-segmented	Diplura
38. Abdomen with three long terminal appendages	Tysanura
Abdomen with only two terminal appendages	Diplura
39. Tiny jumping insects, head points downwards forming a beak	Mecoptera
No sign of beak	Go to 40
40. Small or minute insects with a forked “tail” under rear of abdomen, which enables them to spring out of harm’s way; generally found in soil or decaying vegetation	Collembola
Insects not like this	Go to 41
41. Tarsi usually four-segmented	Isoptera
Tarsi three-segmented; front tarsi swollen	Embioptera
42. Parasites in fur or feathers; insects generally flattened side-to-side	Go to 43

or dorso-ventrally	
Insects not parasitic and not usually flattened	Go to 47
43. Jumping insects flattened from side-to-side	Siphonaptera
Insects flattened dorso-ventrally	Go to 44
44. Insects of moderate size; head partly withdrawn into thorax	Go to 45
Small, minute insects; head not withdrawn into thorax	Go to 46
45. Antennae very short; very "leggy" insects with strong claws well-suited to clinging to a host mammal	Diptera
Antennae long; body somewhat circular with less prominent legs and claws	Hemiptera
46. Prothorax distinct; biting mouthparts	Mallophaga
Thoracic segments fused into one unit; sucking mouthparts	Anoplura
47. Abdomen with pronounced "waist"; antennae often elbowed	Hymenoptera
No such features	Go to 48
48. Body > 5 mm long, with flattened hairs and scales; vestigial wings	Lepidoptera
Body usually < 5 mm long, bald or occasionally scaly; vestigial wings rarely present	Go to 49
49. Head as wide or nearly as wide as body; biting mouthparts; insects often found among dried materials	Psocoptera
Head narrower than body; sucking mouthparts; abdomen often with a pair of tubular outgrowths near hind end; insects found on growing plants	Hemiptera

Insect Identification Glossary

Abdomen: The third or hind-most region of the body of an insect, containing the insect's stomach.
Anterior: of or near the head end, or towards the front plane of the body.
Cerci ('tails'): segmented paired appendages located at the tip of the abdomen.
Dorsal: referring to the back or upper side of an organism.
Dorso-ventrally: extending from the back to the belly.
Forceps: "pincers", or structures shaped like forceps ("tweezers"), at the tip of the abdomen, which are used in defense and courtship to capture and hold prey.
Halteres: from the Greek word dumbbells, small knobbed structures homologous to wings and flapped to maintain stability while flying.
Proboscis: a mouthpart of an insect, usually long and skinny. Mosquitos have a proboscis to suck blood; butterflies and moths have a coiled up proboscis which they unravel to suck nectar from flowers.
Pronotum: the dorsal plate of the prothorax.
Prothorax: the anterior division of the thorax of an insect, bearing the first pair of legs.
Tarsi: the last (distal) part of the leg of an insect, usually divided into segments, corresponding to a jointed "foot."
Thorax: The second or middle region of the body of an insect, between the head and the abdomen, bearing the true legs and wings (if present).
Vestigial: not fully developed in mature animals.

Results from Morphology-Based Identification

To what order does your insect belong, based on the morphology-based taxonomic key?
During step 4 of the DNA extraction (Part C), you will have time to do a Google search on the insect order you have identified to find out more about your insect. What are some unique characteristics of this insect order?
How do the photos from your Google search compare to the photo that you took of your insect?

Part C (Session 1): Extraction of DNA from your Insect

1. Wearing gloves, use sterile forceps to transfer all or a portion of your insect (depending on the size of the insect) to a sterile 1.5 mL microcentrifuge tube. If your insect is 3 mm or less in size, transfer the entire insect to the tube. If your insect is greater than 3 mm in size, use a sterile razor blade to remove the legs of the insect. For a small (5 mm or less) insect, use several of the legs. For a larger insect, use one or a portion of one leg, depending on the insect size. For a housefly, for example, a single leg will suffice. Before the next step, check with your lab instructor to make sure you use an appropriate amount of your insect.

2. Using a p200 Pipetman and a sterile yellow tip, transfer 100 µL of DNA extraction buffer into your tube with the insect part(s). After pipetting the DNA extraction buffer, use the yellow tip to push your insect part to the bottom of the tube. Make sure your insect part is covered in DNA extraction solution. Cap your 1.5 mL tube.

3. Vortex your tube, and then use a sterile yellow tip if necessary to make sure all insect parts are immersed in the DNA extraction buffer.

4. Cap your 1.5 mL tube and write your initials on TOP of the tube using a sharpie marker. Place a 1.5 mL tube cap-lock on your tube to prevent it from popping open in the heat block. Place the tube (with the cap-lock on) in the 95˚C heat block for 10 minutes.

5. After 10 minutes, remove your tube from the heat block. Wait at least 2 minutes before removing the cap-lock from the tube. After at least 2 minutes at room temperature, remove the cap-lock, and add 100 µL of DNA dilution buffer. Cap and vortex your tube once again to mix.

Part D (Session I): Amplification of the COI gene from your insect DNA extract using the Polymerase Chain Reaction (PCR)

1. Put gloves on! This will prevent any DNA from your skin from entering the PCR reaction.

2. Obtain a thin-walled 0.5 mL PCR tube from the lab cart. Label the TOP of this tube with your initials. Add the reagents indicated in the table below to the tube, using a new sterile tip for each reagent. The “extracted insect DNA” is the contents of the tube containing an insect part that you boiled in order to extract the DNA in Part C. Add the solution in this tube, not part of the insect itself. The two primers and PCR Reaction Mix are on the ice bucket in the center of each table. Make sure to add both primers. The PCR Reaction Mix contains dATP, dTTP, dCTP, dGTP, Taq polymerase, and pink dye.

Reagent	Volume
Extracted Insect DNA	4 µL
Primer # 1490	1 µL
Primer # 2198	1 µL
Sterile Water	4 µL
PCR Reaction Mix	10 µL

3. Mix the contents of the tube by tapping and microfuge for about 10 seconds using the special PCR tube adaptors. Use another student’s sample to balance the microfuge.

4. When indicated by your TA, place your tubes in the thermal cycler. Your TA will demonstrate how the thermal cycler works. It has been programmed to run as follows:

PCR Program for COI Amplification:

Cycles	Action	Temperature	Duration
1	Denaturation	94˚C	1:00
2-7	Denaturation	94˚C	0:30
	Annealing	45˚C	1:30
	Primer Extension	72˚C	1:00
8-40	Denaturation	94˚C	0:30
	Annealing	51˚C	1:30

	Primer Extention	72˚C	1:00
41	Primer Extention	72˚C	5:00
42	HOLD	4˚C	?

The cycles will be complete in about 3 hours. Your TA will remove your lab's tubes from the thermal cycler and store them at 4˚C until the next lab session.

Part E (Session 2): Clean-up of PCR product and preparation for DNA sequencing

Prior to DNA sequencing, PCR products must be "cleaned up" to remove the excess oligonucleotide primers and dNTPs that didn't get incorporated into the amplified product during PCR. If these primers and dNTPs remain, they will interfere with the DNA sequencing reaction, and your sequencing will not be successful.

EXOsap-IT is a product developed by the USB Corporation for cleaning up the PCR DNA product. It makes use of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, to digest all the unconsumed dNTPs and primers. These enzymes are active at 37˚C, but are quickly deactivated at 80˚C. After EXOsap-IT treatment, the PCR product is pure enough for DNA sequencing.

1. Obtain your PCR product in the PCR tube from the last lab session, and microfuge this tube for 1 min at the highest speed.

2. Transfer 6 µL from the top of the supernatant into a new 0.5 mL PCR tube, and label this new tube (on the TOP).

3. Add 1 µL of ExoSAP-IT to the tube, tap the tube with your fingers to mix, and pulse for
10 sec in the microfuge. Place this tube in the thermal cycler. Start the ExoSAP program. This program is just one cycle of 15 min at 37˚C and 15 min at 80˚C and a hold at 4˚C. This should take about 30 min to finish.

4. After the program is finished (reading HOLD at 4˚C), remove your tube from the thermal cycler.

5. Your TA will prepare the DNA sequencing "tube strips" by labeling the first tube on the tube strip "Robinson", and putting their name on the second tube on the tube strip. Your TA will also number the tubes on the tube strip. Finally, your TA will add 3 µL of COI primer # 1490 to each tube in the tube strip. This primer is used for DNA sequencing of your PCR product.

6. Transfer 6 µL of your cleaned PCR product from your 0.5 mL PCR tube to one of the numbered tubes of a tube strip. Remember which numbered tube that you pipetted your PCR product into.

7. On the "Request for DNA Sequencing Services" form that your TA will pass around the lab, write your name in the position on the form that corresponds to the tube number in which you pipetted your PCR product.

8. Your TA will cap the tube strips, and send them, along with the "Request for DNA Sequencing Services" form, to the DNA Sequencing Core Facility here at Penn. Within a few days, the results will be back from the facility, and posted to the course website.

Part F (Homework): Analysis of Results

1. When the sequencing results are available, your TA will hand out the electropherograms of all of the sequencing reactions that worked, labeled with the sample number. Look through these electropherograms to see if there's a file with your sample number on it. If so, your sequencing reaction worked! If not, team up with another lab-mate, and help them analyze their results.

2. The electropherogram shows your sequence as indicated by a series of peaks of different colors, corresponding to the four nucleotides. Your TA can help you understand the meaning of your electropherogram, and interpret ambiguous results.

3. If your sequencing reaction worked, you can retrieve your DNA sequence as a Microsoft Word file by going to the course website. Go to "course documents", and look for a folder named "DNA sequencing results." Within this folder, look for your sample name. Clicking on this will open a "seq" text file with your DNA sequence. These files can be opened in Microsoft Word from within the application.

4. Copy your DNA sequence from the Microsoft Word file.

5. Go to the following website: http://www.ncbi.nlm.nih.gov/BLAST/

6. Under the "Basic BLAST" heading, click on "nucleotide blast."

7. On the BLAST page, paste your sequence in the box below the title "Enter Query Sequence."

8. To the right of "Database", make sure the "Others" heading is selected.

9. Click on the "BLAST" button at the bottom of the page. The BLAST program will search for similarities between the sequence you submitted and all the other sequences in its database. This may take some time, but you will usually get results within a minute.

10. The "results of BLAST" page will come up with your results from the search. As you scroll down, you will see the number of hits that the program found with similarities to the sequence that you entered (the "query sequence"). It will score these matches on the basis of sequence similarity. The Color Key for Alignment Scores and the e values refer to the amount of homology between the original sequence and the matches. The first entry under "sequences producing significant alignments" is the sequence with the greatest homology to the entered sequence.

11. Go to the first entry under "sequences producing significant alignments" that has a species name, and you will see a "Description." The "Description" will tell you more about this sequence. The column in this table labeled "Ident" shows the percentage of identities between your sequence and this sequence from the database. What is the percentage of identity between your sequence to the sequence with greatest homology?

12. Now click on this first row (on the "Description") to find out more about your sequence. Underneath the description of the organism, there will be a "Sequence ID." Click on the letters and numbers next to "Sequence ID:" to open a new page. Scroll down on this page until you see "ORGANISM" in the list of terms on the left. This should tell you the name of the organism (Genus species) that is most closely related to your insect. What is the name of the organism (Genus species)?

Open a new window on your web browser, and do a Google search on this organism to find out more about it. What is the common name of this organism?

The Google search may take you to a webpage with a picture of this organism, or a clear description. Compare this picture with the photo that you took. Based on this information, how similar is this organism to your insect?

What are some commonalities and some differences between this organism and your insect?

13. Go back to the BLAST webpage. The percentage of identical nucleotides from step #11 above gives you an idea of how close you are to actually identifying your insect to the species level. Underneath the word "ORGANISM", the classification of this organism is shown. The fourth entry tells you the phylum. Insects are arthropods, so if this entry is Arthropoda, you are on the right track. The sixth entry shows the class. If the class indicated is Insecta, then you know that the most closely related organism to your insect is actually an insect! The tenth entry shows the order. Write down the order of the most closely related insect to the one that you sequenced.

14. Is the order identified by barcoding the same or different than the order that you identified using the taxonomic key?

15. If the order is the same, you can be pretty sure that you've identified your insect to the order level. If the order is different from the order that you identified using the taxonomic key, what do you think might account for these different results?

Materials

Supplies for 25 Students:

gloves (small, medium and large)	25 pairs
60 mm Petri dish containing dead insect	25
sterile disposable razor blade	25
sterile 1.5 mL microcentrifuge tube	25
1.5 mL tube cap-locks	25
sterile yellow pipet tips	6 boxes

DNA “extraction buffer”	3 mL (3 tubes of 1 mL)
DNA “dilution buffer”	3 mL (3 tubes of 1 mL)
sharpie markers	12
microcentrifuge tube rack	12
0.5 mL PCR tube	50
COI primer # 1490 (10 pmoles/µL)	60 µL (3 tubes of 20 µL)
COI primer # 2198 (10 pmoles/µL)	60 µL (3 tubes of 20 µL)
PCR Reaction Mix	300 µL (3 tubes of 100 µL)
sterile water	300 µL (3 tubes of 100 µL)
ExoSAP-IT	30 µL
DNA sequencing “tube strips”	3
caps for tube strips	3
COI primer #1490 (10 pmoles/µL)	90 µL

Equipment:

Dissecting microscopes	25
p200 Pipetman	12
p20 Pipetman	12
sterile forceps	25
95˚C heat block	1
stopwatch	2
microcentrifuge	1
ice bucket	3
thermal cycler	1

Notes for the Instructor

Students often have difficulties classifying their insect based on the taxonomic key. For example, they sometimes miss the second pair of transparent wings underneath the outer hard wings in beetles. Even with the dissecting microscopes, it's difficult to tell how many segments are found in the tarsi. Students also have trouble making calls about whether body segments or wings are hard or soft or membranous, or whether veins in wings have many or few cross veins. Halteres are also difficult to visualize. It is helpful to distribute a handout with photographs and diagrams indicating insect body parts. We emphasize to students that these are difficulties that all but the most expert of taxonomists have, and that they just have to do their best to follow the key. This is part of the lesson of this lab exercise - that morphological classification takes expertise, whereas barcoding does not.

During the DNA extraction procedure, students often have trouble deciding how much of their insect to use for the extraction. Students should check with their instructor because too much insect material will inhibit the PCR and sequencing reactions. Instructors should guide their students toward using about 3 mm of insect material if possible. This corresponds to only a portion of a leg from a large bee, or to two legs from a ladybug, for example. The remainder of the barcoding experiment is straightforward, and approximately 70% of students obtain a sequence that when BLASTed, results in the correct identification of the insect to the species level. Sometimes the sequence returned is of an insect closely related, but not identical to the student's insect. This also shows a limitation of barcoding since only already-sequenced organisms can be identified this way. We instruct the 30% or so of students who don't obtain readable sequences to share a readable sequence with another student in the lab, and to find out to what order this student's insect was classified using the taxonomic key.

For instructors that wish to barcode organisms other than insects, the "Barcode of Life" website (http://www.barcodeoflife.org/sites/default/files/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf) provides protocols and primer sequences to use for different organisms.

If your campus does not have DNA sequencing facilities, cleaned PCR products can be mailed to a variety of facilities that carry out DNA sequencing. One example is GenScript at: http://www.genscript.com/sequencing.html?src=google&gclid=CP7IzrS7_cICFfLm7AodGyEAXg

Safety Concerns

Students collecting live insects should use caution freezing them in the dry ice/ethanol bath. Safety goggles and thermal gloves MUST be worn when freezing insects in the dry ice/ethanol bath, and this should be done in a fume hood. Students should use caution when cutting insects with razor blades, and they should always cut away from themselves onto the Petri dish. Students should wear gloves when working with the extraction and dilution buffers in the Extract-N-Amp Plant PCR Kit.

Sample Results

About 50% of students who obtain readable sequences find out that they classified their insect to the same order using the taxonomic key as the order they identified after BLASTing their sequence. The remaining 50% of students discover that, although their BLASTed sequence results in the identification of an insect that shares some features of their insect (when they Google image the genus and species identified), this insect is in a different order than the order which they identified using the taxonomic key.

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Lori Spindler received a PhD in Biology from the University of Pennsylvania. Since 2008, Lori has been a lecturer and full time lab instructor for Introductory Biology courses in the Biology Department at the University of Pennsylvania. Lori has taught Environmental Science and Ecology courses and organizes a professional development program for Philadelphia High School biology teachers

Appendix
Sources and Suppliers for Materials

Primer Sequences:
CO1 Primer #1490: 5' -GGT CAA CAA ATC ATA AAG ATA TTG G -3'
CO1 Primer # 2198: 5' -TAA ACT TCA GGG TGA CCA AAA AAT CA -3'

Sources for Materials:
Sigma's Extract-N-Amp (or REDExtract-N-Amp) Plant PCR kit (Catalog # XNAP or XNAP2) is the source for the DNA extraction buffer (Extraction Solution), DNA dilution buffer (Dilution Solution), and PCR reaction mix (Extract-N-Amp or REDExtract-N-Amp PCR Reaction Mix).

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