Cataloging Species Richness of Insects & Plants at Davidson County Community College via DNA Barcodes

General Biology II

STUDENT INFORMATION &
PROTOCOL PACKET

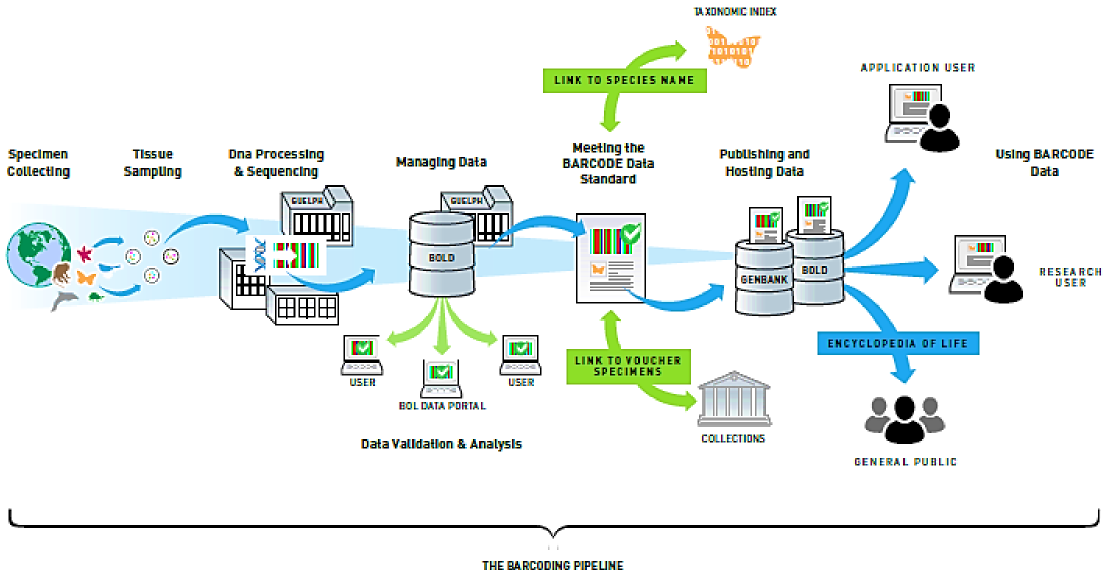
Pre-lab Barcoding I

Watch this quick intro to the history of DNA barcoding:
The History of DNA Barcoding

Visit the DNA Barcoding 101 Education Website and take a few minutes to explore.
http://www.dnabarcoding101.org/

Read this introduction, then proceed by downloading this step by step animation about barcoding. Finally, answer (type 10-12 pt. font) the following questions:
http://www.dnabarcoding101.org/introduction.html

1. What is a DNA barcode?
2. What genes are typically used? Why are they useful for barcoding?
3. Genes must be *haploid* to be useful for barcoding. What does haploid mean and why would this be more useful for barcoding?
4. Hypothesize as to why chloroplast and mitochondrial genes are haploid.
5. What is a trait?
6. Explain the relationship between DNA and traits.
7. Describe the structure of DNA and the structure codes for specific traits.



You may access and download a copy of the entire Barcoding Packet that includes: 1) an introduction, 2) protocols for specific phases of the project, divided over 4 weeks, 3) a data collection sheet, 4) suggested organism sampling techniques and sample documentation steps, 5) a dichotomous key to insect orders, and 6) an Electrophoresis Gel Tracking Sheet. Be sure that you save a copy to your own computer and if you print a copy as a resource for lab, that you keep up with it.

Pre-Lab Barcoding Parts II and II

Do the virtual labs on these sites

http://learn.genetics.utah.edu/content/labs/pcr/

http://learn.genetics.utah.edu/content/labs/extraction/

Type your answers to the following questions:

1. Describe how DNA is extracted and purified from cells.
2. Why is a detergent used?
3. What is PCR?
4. How does it work?
5. Why is it necessary?
6. What is a primer and what does it do?

Next week will do gel electrophoresis for a second time. To review, and these questions: What is gel electrophoresis and why do we do it after the PCR?

Do this simulated lab:

http://learn.genetics.utah.edu/content/labs/gel/

Post-Lab for DNA Barcoding

1. What is barcoding and how can it be used?
2. How is it useful in biodiversity studies?
3. What have you learned by participating in this lab?
4. Briefly describe the following processes and how they can be used:
    a. DNA extraction
    b. PCR
    c. Electrophoresis
    d. Sequencing (this link will help) http://seqcore.brcf.med.umich.edu/doc/educ/dnapr/sequencing.html

DNA BARCODING

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Using DNA Barcodes to Identify and Classify Living Things

LABORATORY

Using DNA Barcodes to Identify and Classify Living Things

OBJECTIVES

This laboratory demonstrates several important concepts of modern biology. During this laboratory, you will:

- Collect and analyze sequence data from plants, fungi, or animals—or products made from them.
- Use DNA sequence to identify species.
- Explore relationships between species.

In addition, this laboratory utilizes several experimental and bioinformatics methods in modern biological research. You will:

- Collect plants, fungi, animals, or products in your local environment or neighborhood.
- Extract and purify DNA from tissue or processed material.
- Amplify a specific region of the chloroplast, mitochondrial, or nuclear genome by polymerase chain reaction (PCR) and analyze PCR products by gel electrophoresis.
- Use the Basic Local Alignment Search Tool (BLAST) to identify sequences in databases.
- Use multiple sequence alignment and tree-building tools to analyze phylogenetic relationships.

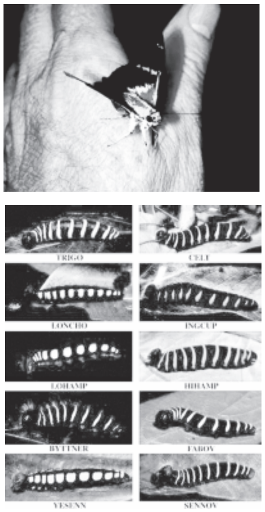
INTRODUCTION

Taxonomy, the science of classifying living things according to shared features, has always been a part of human society. Carl Linneas formalized biological classification with his system of binomial nomenclature that assigns each organism a genus and species name.

Identifying organisms has grown in importance as we monitor the biological effects of global climate change and attempt to preserve species diversity in the face of accelerating habitat destruction. We know very little about the diversity of plants and animals—let alone microbes—living in many unique ecosystems on earth. Less than two million of the estimated 5–50 million plant and animal species have been identified. Scientists agree that the yearly rate of extinction has increased from about one species per million to 100–1,000 species per million. This means that thousands of plants and animals are lost each year. Most of these have not yet been identified.

Classical taxonomy falls short in this race to catalog biological diversity before it disappears. Specimens must be carefully collected and handled to preserve their distinguishing features. Differentiating subtle anatomical differences between closely related species requires the subjective judgment of a highly trained specialist—and few are being produced in colleges today.

Now, DNA barcodes allow non-experts to objectively identify species—even from small, damaged, or industrially processed material. Just as the unique pattern of bars in a universal product code (UPC) identifies each consumer product, a "DNA barcode" is a unique pattern of DNA sequence that identifies each living thing. Short DNA barcodes, about 700 nucleotides in length, can be quickly processed from thousands of specimens and unambiguously analyzed by computer programs.



DNA Barcoding revealed that what was once thought to be one species of butterfly is really ten species with caterpillars that eat different plants.

The International Barcode of Life (iBOL) organizes collaborators from more than 150 countries to participate in a variety of "campaigns" to census diversity among plant, fungi, and animal groups—including ants, bees, butterflies, fish, birds, mammals, mushrooms, and flowering plants—and within ecosystems—including the seas, poles, rain forests, kelp forests, and coral reefs. The 10-year Census of Marine Life, completed in 2010, provided the first comprehensive list of more than 190,000 marine species and identified 6,000 potentially new species.

There is a surprising level of biological diversity, literally in front of our eyes. For example, DNA barcodes showed that a well-known skipper butterfly (*Astraptes fulgerator*), identified in 1775, is actually ten distinct species. DNA barcodes have revolutionized the classification of orchids, a complex and widespread plant family with an estimated 20,000 members. The urban environment is also unexpectedly diverse; DNA barcodes were used to catalogue 54 species of bees and 24 species of butterflies in community gardens in New York City.

DNA barcodes are also used to detect food fraud and products taken from conserved species. Working with researchers from Rockefeller University and the American Museum of Natural History, students from Trinity High School found that 25% of 60 seafood items purchased in grocery stores and restaurants in New York City were mislabeled as more expensive species. One mislabeled fish was the endangered species, Acadian redfish. Another group identified three protected whale species as the source of sushi sold in California and Korea. However, using DNA barcodes to identify potential biological contraband among products seized by customs is still in its infancy.

Barcoding relies on short, highly variably regions of the genome. With thousands of copies per cell, mitochondrial and chloroplast sequences are readily amplified by polymerase chain reaction (PCR), even from very small or degraded specimens. A region of the chloroplast gene *rbc*L—RuBisCo large subunit—is used for barcoding plants. The most abundant protein on earth, RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) catalyzes the first step of carbon fixation. A region of the mitochondrial gene *COI* (cytochrome c oxidase subunit I) is used for barcoding animals. Cytochrome c oxidase is involved in the electron transport phase of respiration. Thus, the genes used for barcoding are involved in the key reactions of life: storing energy in carbohydrates and releasing it to form ATP. *COI* in fungi is difficult to amplify, insufficiently variable, and some fungal groups lack mitochondria. Instead, the nuclear internal transcribed spacer (*ITS*), a variable region that surrounds the 5.8s ribosomal RNA gene, is targeted. Like organelle genes, there are many copies of *ITS* per genome, and the variability in fungi allows for their identification.

This laboratory uses DNA barcoding to identify plants, fungi, or animals—or products made from them. First, a sample of tissue is collected, preserving the specimen whenever possible and noting its geographical location and local environment. A small leaf disc, a whole insect, or samples of muscle are suitable sources. DNA is extracted from the tissue sample, and the barcode portion of the *rbc*L, *COI*, or *ITS* gene is amplified by PCR. The amplified sequence (amplicon) is submitted for sequencing in one or both directions.

The sequencing results are then used to search a DNA database. A close match quickly identifies a species that is already represented in the database. However, some barcodes will be entirely new, and identification may rely on placing the unknown species in a phylogenetic tree with near relatives. Novel DNA barcodes can be submitted to GenBank® (www.ncbi.nlm.nih.gov).

FURTHER READING

Benson D.A., Cavanaugh M., Clark K., Karsch-Mizrachi I, Lipman D.J., Ostell J., Sayers E.W. (2013). *Nucleic Acids Res*. GenBank®. 41(D1): D36–D42.

Hebert P.D., Cywinska A., Ball S.L., deWaard J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270(1512): 313-21.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H., Hallwachs W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc Natl Acad Sci U S A*. 101(41):14812-7.

Hollingsworth P.M. et al (2009). A DNA barcode for land plants. *Proc Natl Acad Sci U S A* 106(31): 12794-7.

Ratnasingham, S., Hebert, P.D.N (2007). Barcoding BOLD: The Barcode of Life Data System. *Molecular Ecology Notes* 7(3): 355-64.

Stoeckle M. (2003). Taxonomy, DNA, and the Bar Code of Life. *BioScience* 53(9): 2-3.

Van Den Berg C., Higgins W.E., Dressler R.L., Whitten W.M., Soto-Arenas M.A., Chase M.W. (2009) A phylogenetic study of laeliinae (*Orchidaceae*) based on combined nuclear and plastid DNA sequences. *Annals of Botany* 104(3): 417-30.

OVERVIEW OF EXPERIMENTAL METHODS

I. COLLECT, DOCUMENT, AND IDENTIFY SPECIMENS

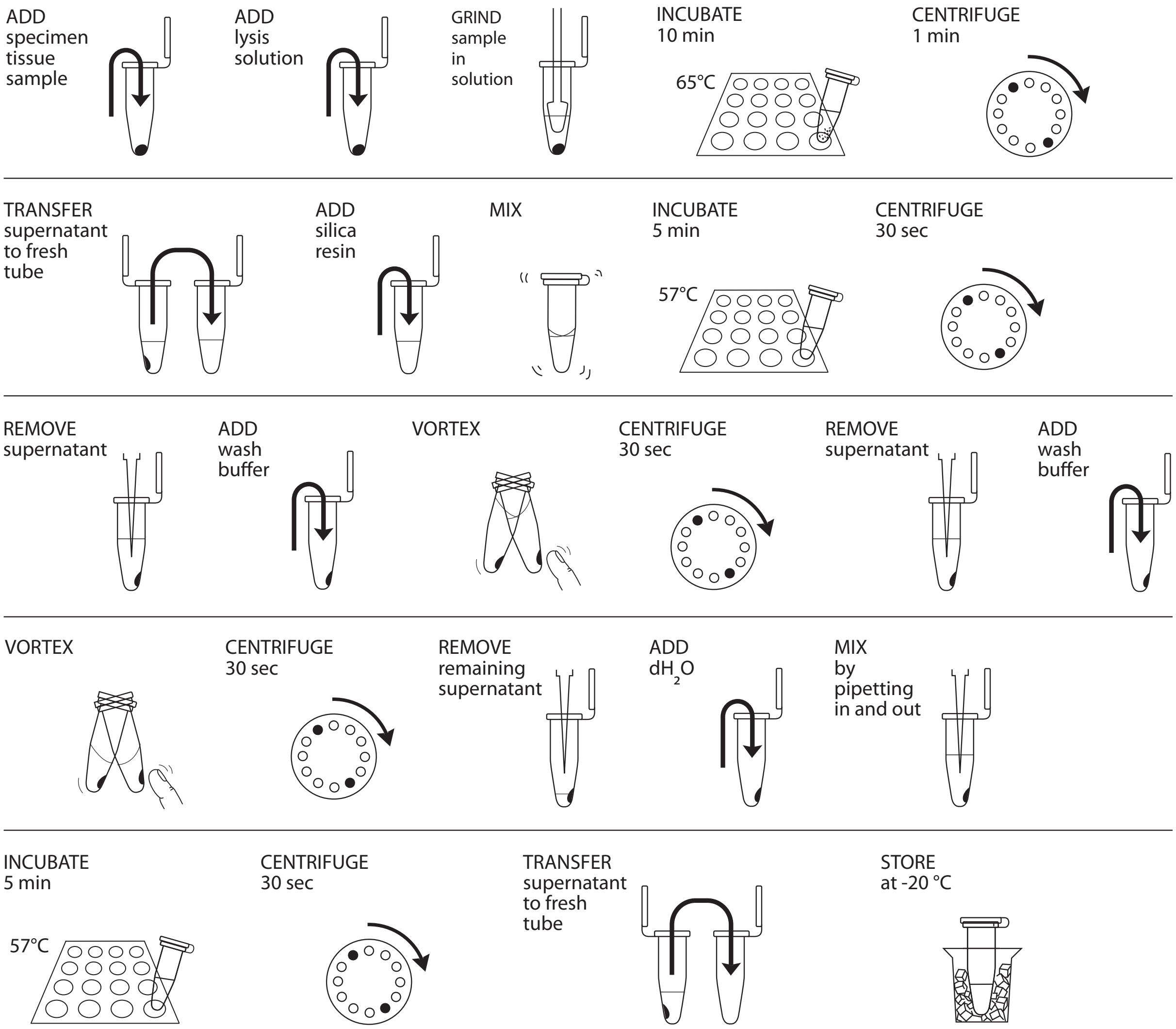
COLLECT specimen

DOCUMENT specimen

IDENTIFY specimen

COLLECT tissue sample

II. ISOLATE DNA FROM PLANT, FUNGAL, OR ANIMAL SAMPLES



ADD specimen tissue sample

ADD lysis solution

GRIND sample in solution

INCUBATE 10 min

CENTRIFUGE 1 min

TRANSFER supernatant to fresh tube

ADD silica resin

MIX

INCUBATE 5 min

CENTRIFUGE 30 sec

REMOVE supernatant

ADD wash buffer

VORTEX

CENTRIFUGE 30 sec

REMOVE supernatant

ADD wash buffer

VORTEX

CENTRIFUGE 30 sec

REMOVE remaining supernatant

ADD dH_2O

MIX by pipetting in and out

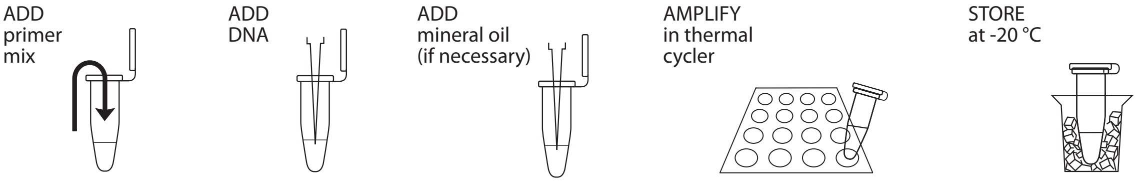
INCUBATE 5 min

CENTRIFUGE 30 sec

TRANSFER supernatant to fresh tube

STORE at -20 °C

III. AMPLIFY DNA BY PCR



ADD primer mix

ADD DNA

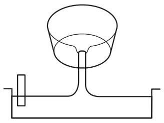
ADD mineral oil (if necessary)

AMPLIFY in thermal cycler

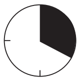
STORE at -20 °C

IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

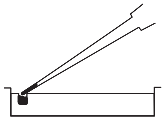
POUR
gel



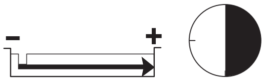
SET
20 min



LOAD
gel

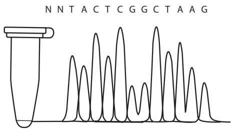


ELECTROPHORESE
130 volts
30 min

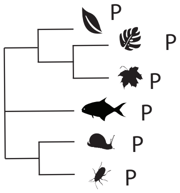


SEQUENCE PCR PRODUCT AND ANALYZE RESULTS

SEND
sample
for
sequencing



ANALYZE
results
using
bioinformatics



EXPERIMENTAL METHODS

I. Collect, Document, and Identify Specimens

The DNA isolation and amplification methods used in this laboratory work for a variety of plants, fungi, and animals—and many products derived from them.

Your collection of specimens may support a census of life in a specific area or habitat, an evaluation of products purchased in restaurants or supermarkets, or may contribute to a larger "campaign" to assess biodiversity across large areas. It may make sense for you to use sampling techniques from ecology. For example, a quadrat samples the plant and/or animal life in one square meter (or ¼ square meter) of habitat, while a transect collects samples along a fixed path through a habitat.

Use common sense when collecting specimens. Respect private property; obtain permission to collect in non-public places. Respect the environment; protect sensitive habitats, and collect only enough of a sample for barcoding. Do not collect specimens that may be threatened or endangered. Be wary of poisonous or venomous plants and animals. Consult your teacher if you are in doubt about the safety or conservation status of a potential specimen. You will also need a small sample for classical taxonomic analysis and to act as a reference sample if you plan to submit your data to GenBank®.

Do not take more sample than you need. Only a small amount of tissue is needed for DNA extraction—a piece of plant leaf about ¼ inch in diameter or a piece of animal or fungal tissue the size of a pencil eraser.

Minimize damage to living plants by collecting a single leaf or bud, or several needles. When possible, use young, fresh leaves or buds. Flexible, non-waxy leaves work best. Tougher materials, such as pine needles or holly leaves, can work if the sample is kept small and is ground well. Dormant leaf buds can often be obtained from bushes and trees that have dropped leaves. Fresh frozen leaves work well. Dried leaves and herbarium samples are variable.

Avoid twigs or bark. If woody material must be used, select flexible twigs with soft pith inside. As a last resort, scrape a small sample of the softer, growing cambium just beneath the bark. Roots and tubers are a poor choice, because high concentrations of storage starches and other sugars can interfere with DNA extraction.

For fungi, obtain fruit bodies (such as mushrooms) when possible, as DNA is easier to obtain from fruiting bodies than mycelia. Only include multiple fruiting bodies in the same sample when they are clearly growing together and appear similar, and avoid contamination by other fungi. Fresh samples work well for DNA isolation, while dried samples give variable results. Fungal fruiting is weather and climate dependent, so their abundance will vary.

Small invertebrate animals, such as insects, can be collected whole and euthanized in a kill jar by placing them in a freezer for several hours. Samples of muscle tissue can be taken from animal foods—such as fish, poultry, or red meat. Blood, internal organs, and bone marrow are all good sources of DNA. Fresh and frozen samples, and those recently preserved in ethanol, work well. However, bone, skin, leather, feather, dessicated, and processed samples are challenging.

REAGENTS, SUPPLIES, & EQUIPMENT

To Share

Collection tubes, jars, or bags
Tweezers, scalpel, and scissors
Smartphone with camera or digital camera with GPS (optional)
Field guide or taxonomic key

1. Collect specimens according to a strategy or campaign outlined by your teacher. "Field Techniques Used by Missouri Botanical Garden" has many good methods for collecting and preparing plant specimens: http://www.mobot.org/MOBOT/molib/fieldtechbook/pdf/handbook.pdf.

If a camera is not available, make sketches of the location and sample.

2. Use a smartphone or digital camera to photograph your specimen in its natural environment, or where it was obtained or purchased.
   a. Take wide, medium, and close-up views.
   b. Include a person for scale in wide and medium shots. Include a ruler or coin for scale in close-ups.

Please be aware that details described in steps 3 and 4 may change as the devices, software, and websites develop over time.

3. A global positioning system (GPS)-enabled phone or camera stores latitude, longitude, and altitude coordinates along with other metadata for each photo. Visualize or extract this geotag information:
   a. In Apple *iPhoto*, click on "i" (image properties) to plot the photo on a map. Click on "Photo," then "Show extended photo info" to find GPS coordinates.
   b. *GeoSetter*, photo metadata freeware for PCs, will plot your photo on a map.
   c. In Google *Picasa* photo editor, click on "i' to find GPS coordinates.
   d. Your smartphone's manual should explain how to use the GPS feature to obtain coordinates.
   e. Many smartphones also have apps that make it easy to harvest GPS coordinates.

A smartphone app can continuously record your location, making it easy to document a collection trip or a sampling transect.

4. Share your collection location by dropping a pin on a Google map.
   a. Sign in to or create a *Google Maps* account.
   b. Create and name a new map.
   c. Zoom in as much as possible on the collection location.
   d. Click on the pin icon to create a pin, then click the collection location.
   e. Give a title to the pin, and add any collection notes in the description field.
   f. To add a link to a photo or other url, click on the picture icon under the "Rich text" option.
   g. Click on "Done" to save your pin drop.
   h. Click on "Collaborate" or "Share" to share your map with others.
5. Use a field guide or taxonomic key to identify your specimen as precisely as possible: kingdom > phylum > class > order > family > genus > species. Taxonomic keys for local plants, fungi, or animals are often available online, at libraries, or from universities, natural history museums, and botanical gardens.

6. Check to see if your specimen is represented in the Barcode of Life Database, BOLD (www.boldsystems.org) or GenBank®® (www.ncbi.nlm.nih.gov):
   a. Search by entering genus and species names in the search bar at top right. If the species is represented in the database, the "Taxonomy Browser" will list the number and sources of specimen records.
   b. Click on "Download Public Sequences" for a fasta file of available barcode sequences.
   c. Click on "Taxonomy Browser" at top left to explore barcode records by group.
7. Use tweezers, scalpel, or scissors to collect a small sample of tissue.
8 Freeze your sample at -20°C until you are ready to begin Part II.

II. Isolate DNA from Plant, Fungal, or Animal Samples

REAGENTS, SUPPLIES, & EQUIPMENT

For each group
(volumes for isolating DNA from 2 samples)

Distilled water (350 µL)
Lysis solution [6 M Guanidine Hydrochloride GuHCl] (700 µL)
Silica resin (10 µL)
Specimen tissue sample(s) (from Part I)
Wash buffer (2.5 mL)

To share

Container with cracked or crushed ice
Microcentrifuge
Microcentrifuge tube rack
6 microcentrifuge tubes (1.5 mL)
Micropipettes and tips (1–1000 µL)
Permanent marker
2 Plastic pestles
Vortexer (optional)
Water bath or heating block at 65°C and 57°C

This standard DNA extraction method is inexpensive and has the advantage of working reproducibly with almost any kind of plant, fungus, or animal specimen.

The large end of a 1000 µL pipette tip will punch leaf disks of this size. Animal tissue should be about ¼ the size of a pencil eraser. Using more than the recommended amount can inhibit the DNA extraction or amplification.

Lysis solution dissolves membrane-bound organelles including the nucleus, mitochondria, and chloroplast.

Grinding the tissue breaks up the cell walls and other tough material. When fully ground, the sample should be liquid, but there may be some particulate matter remaining.

1. Obtain plant, fungal, or animal tissue ~10–20 mg or ¼ inch diameter from your sample. If you are working with more than one sample, be careful not to cross-contaminate specimens. (If you only have one specimen, make a balance tube with the appropriate volume of water for centrifuge steps.)
2. Place sample in a clean 1.5 mL tube labeled with an identification number.
3. Add 300 µL of lysis solution to each tube.
4. Twist a clean plastic pestle against the inner surface of 1.5 mL tube to *forcefully* grind the tissue for 2 minutes. Use a clean pestle for each tube if you are doing more than one sample.
5. Incubate the tube in a water bath or heat block at 65°C for 10 minutes.
6. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for one minute at maximum speed to pellet debris.
7. Label a clean 1.5 mL tube with your sample number. Transfer 150 µL of the supernatant (clear solution above pellet at bottom of tube) to the fresh tube. Be

careful not to disturb the debris pellet when transferring the supernatant. Discard old tube containing the debris.

Silica resin is a DNA binding matrix that is white. In the presence of the lysis solution the silica resin binds readily to nucleic acids.

8. Add 3 µL of silica resin to tube. Mix well by pipetting up and down. Close and incubate the tube for 5 minutes in a water bath or heat block at 57 °C.

Centrifugation pellets the silica resin, which is now bound to nucleic acid. The pellet will appear as a tiny teardrop-shaped smear or particles on the bottom side of the tube underneath the hinge.

9. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the resin. Use a micropipette with fresh tip to remove all supernatant, being careful not to disrupt the white silica resin pellet at the bottom of the tube.

Wash buffer removes contaminants from the sample while nucleic acids remain bound to the resin. The silica resin is not soluble in the wash buffer. The silica resin may stay as a pellet or break up during the washing.

10. Add 500 µL of ice cold wash buffer to the pellet. Close tube and mix well by vortexing or by pipetting up and down to resuspend the silica resin.
11. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the resin. Use a micropipette with fresh tip to remove all supernatant, being careful not to disrupt the white silica resin pellet at the bottom of the tube.

Washing twice is much more effective than washing once with twice the volume.

12. Once again, add 500 µL of ice cold wash buffer to the pellet. Close tube and mix well by vortexing or by pipetting up and down to resuspend the silica resin.
13. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the resin.
14. Use a micropipette with fresh tip to remove the supernatant, being careful not to disrupt the white pellet at the bottom of the tube. Spin the tube briefly to collect any drops of supernatant and then remove these with a micropipette.

In the presence of water or TE buffer, nucleic acids are eluted from the Silica resin.

For long-term storage it is recommended DNA samples be stored in TE buffer (Tris/EDTA). Tris provides a pH 8.0 environment to keep DNA and RNA nucleases less active. EDTA further inactivates nucleases by binding cations required by nucleases.

15. Add 100 µL of distilled water (or TE buffer) to the silica resin and mix well by vortexing or by pipetting up and down. Incubate the mixture at 57°C for 5 minutes.
16. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the resin.

Sample DNA eluted in TE may require a 1:10, 1:20 or 1:50 dilution in water prior to PCR, if the initial amplification of the target gene from the eluted DNA is unsuccessful (this may occur particularly in plant samples).

17. Label a clean 1.5 mL tube with your sample number. Transfer 90 µL of the supernatant (clear solution) to the fresh tube. Be careful not to disturb the pellet when transferring the supernatant. Discard old tube containing the resin.
18. Store your sample on ice or at -20°C until you are ready to begin Part III.

In Part III, you will use 2 µL of DNA for each PCR reaction. This is a crude DNA extract and contains nucleases that will eventually fragment the DNA at room temperature. Keep the sample cold to limit this activity.

III. Amplify DNA by PCR

To amplify a DNA barcode region, choose the most appropriate set of primers for each sample. The table below lists available primer sets, the type of organism they target, and the PCR protocol for each set. For detailed information on the primer sets, go to Primer Sequences & References.

REAGENTS, SUPPLIES, & EQUIPMENT

For each group	*To share*
Appropriate primer/loading dye mix (25 µL)* per reaction	Container with cracked or crushed ice
DNA from specimen(s) (from Part II)*	Micropipettes and tips (1–100 µL)
Ready-To-Go PCR Bead in 0.2- or 0.5-mL PCR tube per reaction *OR* NEB *Taq* 2X Master Mix (12.5 µL)* per reaction	Microcentrifuge tube rack
	Permanent marker
	Thermal cycler

**Store on ice*

1. Obtain PCR tube containing Ready-To-Go PCR Bead. Label the tube with your identification number.
2. Use a micropipette with a fresh tip to add 23 µL of one of the following primer/loading dye mixes to each tube. Allow the beads to dissolve for 1 minute.

   Plant cocktail: *rbc*L primers (rbcLaF / rbcLa rev)

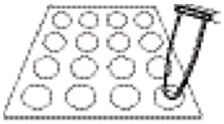
   Fungi cocktail: *ITS* primers (ITS1F/ITS4)

   Fish cocktail: *COI* primers (VF2_t1/ FishF2_t1/ FishR2_t1/ FR1d_t1)

   Vertebrate (non-fish) Cocktail:
   (VF1_t1/VF1d_t1/VF1i_t1/VR1d_t1/VR1_t1/VR1i_t1)

   Invertebrate cocktail: (LCO1490/ HC02198)
3. Use a micropipette with fresh tip to add 2 µL of your DNA (from Part II) directly into the appropriate primer/loading dye mix. Ensure that no DNA remains in the tip after pipetting.
4. Store your sample on ice until your class is ready to begin thermal cycling.
5. Place your PCR tube, along with those of the other students, in a thermal cycler that has been programmed with the appropriate PCR protocol.

If the reagents become splattered on the wall of the tube, pool them by pulsing the sample in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.



Primers	Profile
Plant cocktail (rbcLaF / rbcLa rev) **Vertebrate (non-fish) cocktail** (VF1_t1/VF1d_t1/VF1i_t1/ VR1d_t1/VR1_t1/VR1i_t1)	Initial step: 94°C 1 minute 35 cycles of the following profile: Denaturing step: 94°C 15 seconds Annealing step: 54°C 15 seconds Extending step: 72°C 30 seconds One final step to preserve the sample: 4°C *ad infinitum*
Fish cocktail (VF2_t1/ FishF2_t1/ FishR2_t1/ FR1d_t1)	Initial step: 94°C 1 minute 35 cycles of the following profile: Denaturing step: 94°C 15 seconds Annealing step: 54°C 15 seconds Extending step: 72°C 30 seconds One final step to preserve the sample: 4°C *ad infinitum*

Primers	Profile
Invertebrate cocktail (LCO1490/ HC02198)	Initial step: 94°C 1 minute 35 cycles of the following profile: Denaturing step: 95°C 30 seconds Annealing step: 50°C 30 seconds Extending step: 72°C 45 seconds One final step to preserve the sample: 4°C *ad infinitum*
Fungi cocktail (ITS1F/ITS4)	Initial step: 94°C 1 minute 35 cycles of the following profile: Denaturing step: 94°C 1 minute Annealing step: 55°C 1 minute Extending step: 72°C 2 minutes One final step to preserve the sample: 4°C *ad infinitum*

6. After thermal cycling, store the amplified DNA on ice or at -20 °C until you are ready to continue with Part IV.

IV. Analyze PCR Products by Gel Electrophoresis

REAGENTS, SUPPLIES, & EQUIPMENT

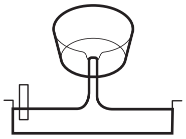
For each group

2% agarose in 1x TBE (hold at 60°C) (50 mL per gel)
pBR322/*Bst*NI marker (20 µL per gel)*
PCR products from Part III*
SYBR Green DNA stain (6 µL per group)
1x TBE buffer (300 mL per gel)

*Store on ice.

To share

Container with cracked or crushed ice
Gel-casting tray and comb
Gel electrophoresis chamber and power supply
Latex gloves
Masking tape
Microcentrifuge tube rack
3 Microcentrifuge tubes (1.5mL)
Micropipette and tips (1–100 µL)
Digital camera or photodocumentary system
Microwave
UV transilluminator <!> and eye protection
Water bath for agarose solution (60°C)



Avoid pouring an overly thick gel, which makes visualization of the DNA more difficult.

The gel will become cloudy as it solidifies.

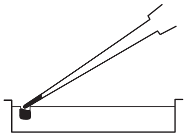
Do not add more buffer than necessary. Too much buffer above the gel channels electrical current over the gel, increasing running time.

1. Seal the ends of the gel-casting tray with masking tape, or other method appropriate for the gel electrophoresis chamber used and insert a well-forming comb.
2. Pour the 2% agarose solution into the tray to a depth that covers about one-third the height of the comb teeth.
3. Allow the agarose gel to completely solidify; this takes approximately 20 minutes.
4. Place the gel into the electrophoresis chamber and add enough 1x TBE buffer to cover the surface of the gel.
5. Carefully remove the comb and add additional 1x TBE buffer to fill in the wells and just cover the gel, creating a smooth buffer surface.
6. Use a micropipette with a fresh tip to transfer 5 µL of each PCR product (from

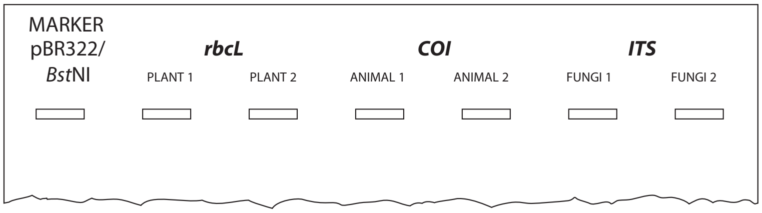
part III) to a fresh 1.5 mL microcentrifuge tube. Add 2 µL of SYBR Green DNA stain to tube.

A 100-bp ladder may also be used as a marker.

7. Add 2 µL of SYBR Green DNA stain to 20 µL of pBR322/*Bst*NI marker.
8. Orient the gel according to the diagram below, so the wells are along the top of the gel. Use a micropipette with a fresh tip to load 20 µL of pBR322/*Bst*NI size marker into the far left well.
9. Use a micropipette with a fresh tip to load each sample from Step 6 in your assigned wells, similar to the following diagram:



Expel any air from the tip before loading, and be careful not to push the tip of the pipette through the bottom of the sample well.



The samples you load may not be exactly the same as those shown.

10. Store the remaining 20 µL of your PCR product on ice or at -20°C until you are ready to submit your samples for sequencing.
11. Run the gel for approximately 30 minutes at 130V. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.
12. View the gel using UV transillumination. Photograph the gel using a digital camera or photodocumentary system.

Transillumination, where the light source is below the gel, increases brightness and contrast.

RESULTS AND DISCUSSION

I. Think About the Experimental Methods

1. Describe the effect of each of the following steps or reagents used in DNA isolation (Part I of Experimental Methods):

   i. Collecting fresh or dried specimens

   ii. Using only a small amount of tissue

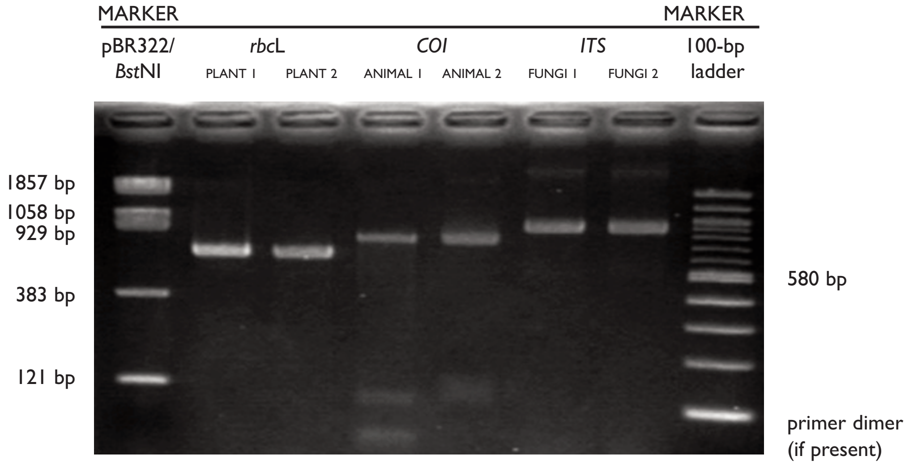
   iii. Grinding tissue with pestle

   iv. Lysis solution

   v. Heating or boiling.

II. Interpret Your Gel and Think About the Experiment

1. Observe the photograph of the stained gel containing your PCR samples and those from other students. Orient the photograph with the sample wells at the top. Use the sample gel shown on the next page to help interpret the band(s) in each lane of the gel.
2. Locate the lane containing the pBR322/*Bst*NI markers on the left side of the gel. Working down from the well, locate the bands corresponding to each restriction fragment: 1857, 1058, 929, 383, and 121 bp. The 1058- and 929-bp fragments will



be very close together or may appear as a single large band. The 121-bp band may be very faint or not visible.

Additional faint bands at other positions occur when the primers bind to chromosome loci other than the intended locus and give rise to "nonspecific" amplification products.

3. Looking across the gel at the PCR products, do the bands all appear to be the same bp size and intensity?
4. It is common to see a diffuse (fuzzy) band that runs ahead of the 121-bp marker. This is "primer dimer," an artifact of the PCR that results from the primers overlapping one another and amplifying themselves.

If you have a very faint product or none at all, your teacher will help you decide if your sample should be sent for sequencing.

5. Which samples amplified well, and which ones did not? Give several reasons why some samples may not have amplified; some of these may be errors in procedure.
6. Generally, DNA sequence can be obtained from any sample that gives an obvious band on the gel.

BIOINFORMATICS

I. Use BLAST to Find DNA Sequences in Databases (Electronic PCR)

1. Perform a BLAST search as follows:
   a. Do an Internet search for "ncbi blast."
   b. Click the link for the result BLAST: *Basic Local Alignment Search Tool*. This will take you to the Internet site of the National Center for Biotechnology Information (NCBI).
   c. Under the heading "Basic BLAST," click "nucleotide blast."
   d. Enter the primer set you used into the search window. These are the query sequences. (See box at top of next page.)
   e. Omit any non-nucleotide characters from the window because they will not be recognized by the BLAST algorithm.
   f. Under "Choose Search Set," select "NCBI Genomes (chromosome)" from the pull-down menu.
   g. Under "Program Selection," optimize for "Somewhat similar sequences (blastn)."

The following primers were used in this experiment:

Plant *rbc*L gene	
rbcLa f	5'- ATGTCACCACAAACAGAGACTAAAGC-3' (forward primer)
rbcLa rev	5'- GTAAAATCAAGTCCACCRCG-3' (reverse primer)
Vertebrate (non-fish) *COI* gene	
VF1_t1	5'-TCTCAACCAACCACAAAGACATTGG-3' (forward primer)
VR1d_t1	5'-TAGACTTCTGGGTGGCCRAARAAYCA-3' (reverse primer)
Fish *COI* gene	
VF2_t1	5'-CAACCAACCACAAAGACATTGGCAC-3' (forward primer)
FishR2_t1	5'-ACTTCAGGGTGACCGAAGAATCAGAA-3' (reverse primer)
Fungi *ITS*	
ITS1 F	5'-TCCGTAGGTGAACCTGCGG-3' (forward primer)
ITS4 R	5'-TCCTCCGCTTATTGATATGC-3' (reverse primer)
Invertebrate *COI* gene	
LCO1490_F	5'-GGTCAACAAATCATAAAGATATTGG-3' (forward primer)
HC02198_R	5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (reverse primer)

h. Click "BLAST." This sends your query sequences to a server at the National Center for Biotechnology Information in Bethesda, Maryland. There, the BLAST algorithm will attempt to match the primer sequences to the DNA sequences stored in its database. A temporary page showing the status of your search will be displayed until your results are available. This may take only a few seconds or more than a minute if many other searches are queued at the server.

2. The results of the BLAST search are displayed in three ways as you scroll down the page:

   a. First, a *Graphic Summary* illustrates how significant matches, or "hits," align with the query sequence. **Why are some alignments longer than others?**

   b. This is followed by *Descriptions of sequences producing significant alignments*, a table with links to database reports.

      - The accession number is a unique identifier given to a sequence when it is submitted to a database, such as Genbank®. The accession link leads to a detailed report on the sequence.
      - Note the scores in the "E Value" column on the right. The Expectation, or E, value is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the E value, the higher the probability that the hit is related to the query. For example, an E value of 1 means that a search with your sequence would be expected to turn up one match by chance.
      - **What is the E value of your most significant hit, and what does it mean? What does it mean if there are multiple hits with similar E values?**
      - **What do the descriptions of significant hits have in common?**

   c. Next is an *Alignments* section, which provides a detailed view of each primer sequence (*Query*) aligned to the nucleotide sequence of the search hit (*subject*). Notice that hits have matches to one or both of the primers:

	Forward Primer	*Reverse Primer*
Plant	nucleotides 1-26	nucleotides 27-46
Vertebrate (non-fish)	nucleotide 1-25	nucleotides 26-53
Fish	nucleotides 1-25	nucleotides 26-51
Fungi	nucleotide 1-19	nucleotides 20-39
Invertebrate	nucleotides 3-25	nucleotides 26-51

3. Predict the length of the product that the primer set would amplify in a PCR reaction (*in vitro*).
   a. In the *Alignments* section, select a hit that matches both primer sequences.
   b. **Which nucleotide positions do the primers match in the subject sequence?**
   c. The lowest and highest nucleotide positions in the subject sequence indicate the borders of the amplified sequence. Subtracting one from the other gives the difference between the coordinates.
   d. However, the PCR product includes both ends, so add 1 nucleotide to the result that you obtained in Step 3.c. to determine the exact length of the fragment amplified by the two primers.
   e. **What value do you get if you calculate the fragment size for other species that have matches to the forward and reverse primer? Do you get the same number?**
4. Determine the type of DNA sequence amplified by the primer set:
   a. Click on the accession link (beginning with "*ref*") to open the data sheet for the hit used in Question 3 above.
   b. The data sheet has three parts:
      - The top section contains basic information about the sequence, including its basepair (bp) length, database accession number, source, and references to papers in which the sequence is published.
      - The bottom section lists the nucleotide sequence.
      - The middle section contains annotations of gene and regulatory FEATURES, with their beginning and ending nucleotide positions ("xx..xx"). These features may include genes, coding sequences (CDS), regulatory regions, ribosomal RNA (rRNA), and transfer RNA (tRNA).
   c. Identify the feature(s) located between the nucleotide positions identified by the primers, as determined in 3.b. above.

II. Determine Sequence Relationships Using the Blue Line

The following directions explain how to use the Blue Line of *DNA Subway* to analyze novel DNA sequences generated by a DNA sequencing experiment. If you did not sequence your own DNA sample, you can follow these directions to use DNA sequences produced for other students. You can find supplementary instructions by clicking on the "manual" link on the *DNA Subway* homepage.

DNA Subway is an intuitive interface for analyzing DNA barcodes. Generally, you progress in a stepwise fashion through the button “stops“ on each “branch line." An “R" indicates that analysis is available. A blinking “R" indicates an analysis is in process. A “V" means that results are ready to view.

You can analyze relationships between DNA sequences by comparing them to a set of sequences you have compiled yourself, or by comparing your sequences to others that have been published in databases such as GenBank® (National Center for Biotechnology Information). Generating a phylogenetic tree from DNA sequences derived from related species can also allow you to draw inferences about how these species may be related. By sequencing variable sections of DNA (barcode regions) you can also use the Blue Line to help you identify an unknown species, or publish a DNA barcode for a species you have identified, which is not represented in published databases like GenBank® (www.ncbi.nlm.nig.gov/genbank).

1. Create a *DNA Subway* Project and Upload DNA Sequences
   a. Log in to *DNA Subway* at www.dnasubway.org. If you do not have an account, you will need to register first to save and share your work.
   b. Select “Determine Sequence Relationships” (Blue Line) to begin a project.
   c. Select “*rbc*L” or “*COI*” from the “Select Project Type” section. (*rbc*L (plant) sequences must be analyzed separately from *COI* (animal) sequences.) If you are analyzing a barcode region that is not listed, select “DNA."
   d. “Select Sequence Source” provides several ways to obtain sequences for barcode analysis:
      - *Upload sequence(s) in ab1* (files ending with .ab1) *or FASTA* format. Click “Browse” to navigate to a folder on your desktop or drive containing your sequence(s). Select a sequence by clicking on its file name. Select more than one sequence by holding down the ctrl key while clicking file names. Once you have selected the sequences you want, click “Open”.
      - *Enter a sequence in FASTA format*. Below is an example of this format. The “>” symbol demarcates the sequence name. The sequence is started on the next line.

        >sequence name
        atcgccccttaatattgcctt…..
      - *Import a sequence/trace from the DNALC*. Click your tracking number. Select one or more files from the list. Click to “Add” selected files.
      - *Select a sample sequence*.
   e. Provide a title in the *Name Your Project* section.
   f. Write a short description of your project in the *Description* section (optional).
   g. Click “Continue” to load the project into *DNA Subway*.
2. View and Build Sequences

   There are many plants, animals, and fungi which do not have a documented barcode sequence. For instance, there are an estimated 350,000 species of angiosperms (flowering plants), but as of June 2013 there were only about 74,000 *rbc*L angiosperm sequences in GenBank®. For other species, diversity in the barcode sequences are not well characterized. This means that there are opportunities to submit novel sequences and contribute to the global barcoding effort. Only samples that have high quality sequence for both the forward and reverse reads are good enough to ensure a low error rate and can be published to GenBank®, so the sequence quality must be checked. Sequences for which there is only one high quality read are not considered high enough quality to publish. These sequences and those with no high quality sequence can still be analyzed even though the results are not publishing quality.

a. On the *Assemble Sequences* branch line, Click “Sequence Viewer” to display the sequences you have input in the project creation section. If you did not upload trace files, you can scroll to see the sequence. If you uploaded trace files, click on the file names to view the trace files.

- The DNA sequencing software measures the fluorescence emitted in each of four channels—A, T, C, G—and records these as a trace, or electropherogram. In a good sequencing reaction, the nucleotide at a given position will be fluorescently labeled far in excess of background (random) labeling of the other three nucleotides, producing a “peak” at that position in the trace. Thus, peaks in the electropherogram correlate to nucleotide positions in the DNA sequence.
- A software program called *Phred* analyzes the sequence file and “calls” a nucleotide (A, T, C, G) for each peak. If two or more nucleotides have relatively strong signals at the same position, the software calls an “N” for an undetermined nucleotide.
- *Phred* also examines the peaks around each call and assigns a quality score for each nucleotide. The quality scores corresponds to a logarithmic error probability that the nucleotide call is wrong, or, conversely, to the accuracy of the call.

***Phred* Score**	**Error**	**Accuracy**
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

- The electropherogram viewer represents each *Phred* score as a blue bar. The horizontal line equals a *Phred* score of 20, which is generally the cutoff for high-quality sequence. Thus any bar at or above the line is considered a high-quality read. **What is the error rate and accuracy associated with a *Phred* score of 20?**
- Every sequence “read" begins with nucleotides (A, T, C, G) interspersed with Ns. In “clean" sequences, where experimental conditions were near optimal, the initial Ns will end within the first 25 nucleotides. The remaining sequence will have very few, if any, internal Ns. Then, at the end of the

read the sequence will abruptly change over to Ns.

- Large numbers of Ns scattered throughout the sequence indicate poor quality sequence. Sequences with average *Phred* scores below 20 will be flagged with a "Low Quality Score Alert." You will need to be careful when drawing conclusions from analyses made with poor quality sequence. **What do you notice about the electropherogram peaks and quality scores at nucleotide positions labeled "N"?**
- **Note:** The exclamation icon (!) indicates poor quality sequence.

b. Use the "X" and "Y" buttons to adjust the level of zoom. You can undo zooming by pressing the "Reset" button.

c. Examine the quality of the sequence(s). Any sequence for which the forward or reverse has the warning icon indicating a low quality score is not of good enough quality to publish and any determination of novelty will be tentative as sequencing errors could appear to be novel polymorphisms.

d. Click "Sequence Trimmer" to trim your sequences; this automatically removes Ns from the 5' and 3' ends of selected sequences. Click again to view the trimmed sequences. **Why is it important to remove excess Ns from the ends of the sequences?**

e. If you wish to view trimmed sequences, click on the file name.

3. Pair and Build Consensus for Forward and Reverse Reads

a. If you have two reads for a sample, pair the sequences by checking the box to the right of each read for the sample. By default, *DNA Subway* assumes that all reads are in the forward orientation, and displays an "F" to the right of the sequence. If any sequence is not in that orientation, click the F to reverse complement the sequence. The sequence will display an "R" to indicate the change.

b. After checking the second read, a dialogue box will appear asking if you wish to designate the sequences as a pair. Alternatively, Click "Try auto pairing" to pair sequences which have identical sample names, but appended with an F or R based on sequencing direction.

c. Click "Save" to save your pair assignments.

d. Once you have created sequence pairs, click "Consensus Editor" to make a consensus sequence from both sequences in the selected pairs. To examine the consensus sequence click "Consensus Editor" again, and then click on the link to the pair you wish to examine. How does the consensus sequence optimize the amount of sequence information available for analysis? Why does this occur?

e. If there are any mismatched nucleotides between the first and second sequence, these will be highlighted yellow in the consensus editor window. **Do differences tend to occur in certain areas of the sequence? Why?**

f. Large numbers of yellow mismatches—especially in long blocks—may indicate that you have incorrectly paired sequences from two different sources (organisms), or that you failed to reverse complement the reverse strand.

- Return to *Pair Builder* to check your pairs and reverse complements.
- Click the red "x" to redo a pairing, and toggle "F" and "R" settings, as needed.

h. A large number of mismatches in properly paired and reverse complemented sequences indicate that one or both sequences is of poor quality. Often, one of the sequencing reactions produces a high quality read that can be used on its own. To determine this:

- Examine the distribution of Ns to see if they are mainly confined to one of the two sequences.
- Examine the electropherograms to see if one of the two sequences is of good quality.
- If one of the sequences seems of good quality, return to *Pair Builder*, and click the red x to undo the pairing.
- Continue on to Step 4.

i. Few or no internal mismatches indicate good quality sequence from forward and reverse reads. If you like, you can check the consensus sequence at yellow mismatches and override the judgment made by the software:

- Click a highlighted mismatch to see the electropherograms and graphic summarizing *Phred* scores for each read.
- Click the desired nucleotide in the black rectangle to change the consensus sequence at that position. You should only change the consensus if you have a strong reason to believe the consensus is wrong.
- Click the button to "Save Change(s)."

4. BLAST Your Sequence

A BLAST search can quickly identify any close matches to your sequence in sequence databases. In this way, you can often quickly identify an unknown sample to the genus or species level. It also provides a means to add samples for a phylogenetic analysis.

a. On the *Add Sequences* branch, click "BLASTN". Then, click the "BLAST" button next to the sequence you want to query against DNA databases.

b. The returned list has information about the 20 most significant alignments (hits):

- Accession number, a unique identifier given to each sequence submitted to a database. Prefixes indicate the database name—including gb (GenBank®), emb (European Molecular Biology Laboratory), and dbj (DNA Databank of Japan).
- Organism and sequence description or gene name of the hit. Click the genus and species name for a link to an image of the organism, with additional links to detailed descriptions at Wikipedia and Encyclopedia of Life (EOL).
- Several statistics allow comparison of hits across different searches. The number of mismatches over the length of the alignment gives a rough idea of how closely two sequences match. The Bit Score formula takes into

account gaps in the sequence; the higher the score the better the alignment. The Expectation or E-value is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the E-value, the higher the probability that the hit is related to the query. For example, an E value of 0 means that a search with your sequence would be expected to turn up no matches by chance. **Why do the most significant hits typically have E-values of 0?** (This is not the case with BLAST searches with primers.) **What does it mean when there are multiple BLAST hits with similar E values?**

- Examine the last column in the report called "Mismatches." For barcodes, this is the informative column, with the best hits being those with the lowest number of mismatches. Note that hits with low numbers of mismatches can sometimes be lower on the list, as the bit scores are used to arrange the hits in the table. High bit scores can occur when the alignment length is longer, even when there are more mismatches than for other hits.
- If there are zero mismatches between your sequence and a BLAST result, it is unlikely that your sequence is unique. Instead, the identical sequences probably match because they are in the same taxonomic group as your sample. Check to see if the matching sequences are from species that seem reasonable for your sample. If your best matches include some mismatches, you may have identified a novel barcode. The more mismatches you find, the more likely that your sequence is unique, especially in regions of the sequence with high quality scores. However, sequencing errors could explain the difference, so it will be important to reexamine the trace files at any sites with mismatches to ensure that the consensus at those locations is of high quality.

c. Add BLAST sequence data to your phylogenetic analysis by checking the box(es) next to any accession number(s), then clicking on "Add BLAST hits to project" at the bottom of the BLAST results window.

5. Add Sequences to Your Analysis

a. Click "Upload Data" to add additional sequence data to your analysis without starting a new project. Use "Upload Sequence(s)" to upload *ab1* trace files or *FASTA*-formatted sequences stored locally on your computer; Use "Enter Sequences(s)" to paste or type sequences in *FASTA* format.

b. If you would like to import sequences from non-local sources you can use "Import Sequence" to search a sequence database using a sequence identifier. For GenBank® sequences you can search by identification number (GI or Version). Search BOLD by species name, or search the DNALC sequence database by tracking number for sequences you processed with GENEWIZ through the DNALC system.

c. If your sequence had no hits with zero mismatches, you may use NCBI BLAST to confirm that the sequence is novel. Click on the BLASTN button and then double-click on the sequence (the actual nucleotides) that you identified as possibly novel to select them. Right-click (PC) or command-click (Mac) and then select copy to move the sequence to your clipboard.

- In a web browser go to http://blast.ncbi.nlm.nih.gov. From this page click on “nucleotide blast.”
- Paste your sequence into the “Enter Query Sequence” window under “enter accession number(s), gi(s), or FASTA sequence(s).”
- Under “Program Selection” select “Highly similar sequences (megablast)”; next click “BLAST.”
- On the results page you will get a list of results very similar to what was returned by *DNA Subway*.
- Scrolling down the page, you will find alignments of your sequence (Query) to the sequences from the closest matches in GenBank® (Sbjct).
- Analyze the results of the BLAST search, which are displayed in three ways as you scroll down the page:
  - First, a graphical overview illustrates how significant matches (hits) align with the query sequence. Matches of differing lengths are indicated by color-coded bars. For barcoding results, it is likely that most matches will be red, indicating high scores, and cover most of the width of the table, showing matches that span the length of your query sequence.
  - This is followed by a table with “Descriptions of sequences producing significant alignments” much like the table for BLAST results in *DNA Subway*.
  - Next is an “Alignments” section, which provides a detailed view of each primer sequence (“Query”) aligned to the nucleotide sequence of the search hit (“Sbjct,” “subject”).
  - From the table, identify any matches that are 100% identical or any matches with high identity that appear to represent species or sequences you have not identified previously. Select these sequences by clicking on the box to the left of each hit. After selecting sequences, click Download, ensure *FASTA* (complete sequence) is selected, and then click Continue.
  - Open the resulting *FASTA* file (named seqdump). *Double-click* the sequences to select them all, then *right-click* (PC) or *command-click* (Mac) and *select* copy to move the sequence to your clipboard. Add these sequences to your project using the Upload Data function, as in step 1.
- Click “Sequence Viewer” back on *DNA Subway*, and view the trace file for the forward read of your query sequence. Locate the position on your table where the query sequence differed from the GenBank® match. Determine if the nucleotides you identified as different were of high quality (e.g. not sequencing errors). Because of sequence trimming, you may have to search for the polymorphic site, as the numbers from the BLAST alignment and in the trace file may not correspond.

d. You may also choose to search for your sequence at the International Barcode of Life (IBOL) database, BOLD (Barcode of Life Online Database); their records are not all in GenBank®.

- Click the BLAST button and then *double-click* the nucleotides for the sequence you are analyzing. *Right-click* (PC) or *command-click* (Mac) and then *select* copy to move the sequence to your clipboard.
- In a web browser go to http://boldsystems.org. From this page, click on "Identification."
- Select the tab that corresponds to the appropriate kingdom for the sample (animal, plant, or fungal).
- Under the Animal Identification [COI] tab, select "Species Level Barcode Records." On the Fungal Identification [ITS] tab, select "ITS Sequences." On the Plant Identification [rcbL & matK] tab, select "Plant Sequences."
- Paste the sequence into the search box labeled "Enter sequences in fasta format"; next click "Submit."
- Again, a results table is produced. The column labeled "similarity" indicates how similar your sequence was to the records in the BOLD, with a 100% match indicating they were exact matches. Some records in BOLD are not public, or are not accompanied by species-level identifications. Scrolling down the list of matches you will see a pairwise alignment of your sequence (Query) to the matched sequences (Subj). Once again, identify any new hits that may be identical to your sequence. For published hits, you can download the sequence by clicking the link to the right of "Published," then clicking "FASTA" and saving the file. This FASTA file can be uploaded, as described above, in step 1.

e. Click "Reference Data" (optional) to include additional sequences. Depending on the project type you have created, you will have access to additional sequence data that may be of interest. For example, if you are doing a DNA barcoding project using the *rbc*L gene, samples of *rbc*L sequence from major plant groups (Angiosperms, Gymnosperms, etc.) will be provided. Choose any data set to add it to your analysis; you will be able to include or exclude individual sequences within the set in the next step.

6. Analyze Sequences: Select and Align

Many unknown species can be rapidly identified by a BLAST search. In this case, a phylogenetic analysis adds depth to your understanding by showing how your sequence fits into a broader taxonomy of living things. If your BLAST search fails to identify your sequence, phylogenetic analysis can usually identify it to at least the family level.

a. Click "Select Data" to display all the sequences you have brought into your analysis, including "user data," BLAST hits, or reference data. Check off sequences you wish to include in an alignment. In general, to determine the relationship of your sequence to species with known barcodes, it is best to concentrate on similar sequences. For instance, you should align sequences from samples that you believe are the same species and any close matches from database searches. You may also use the "Select all" feature to include all sequences; to deselect all sequences, click "Select all" a second time. You may run new alignments or download different sequences at any time after selecting a new set of sequences.

- To download selected sequences to a FASTA file click the "Download" button and save the resulting file.
- To save your selections, click "Save Selections" in the blue dialog box that appears when you make any selections.

b. Click "MUSCLE" to run the MUSCLE multiple alignment software. This software will align all sequences that were included in the "Select Data" step. Click "MUSCLE" again to open the created multiple alignment. An alignment that is suitable for creating a phylogenetic tree will have an overall high Sequence Conservation Score (represented by the height of the gray bars along the Sequence Conservation row at the top).

- Scroll through your alignments to see similarities between sequences. Nucleotides are color coded, and each row of nucleotides is the sequence of a single organism or sequencing reaction. Columns are matches (or mismatches) at a single nucleotide position across all sequences. Dashes (-) are gaps in sequence, where nucleotides in one sequence are not represented in other sequences.
- Note that the 5' (leftmost) and 3' (rightmost) ends of the sequences are usually misaligned, due to gaps (-) or undetermined nucleotides (Ns). **What causes these problems?**
- Note any sequence that introduces large, internal gaps (-----) in the alignment. This is either poor quality or unrelated sequence that should be excluded from the analysis. To remove it, return to Select Data, uncheck that sequence, and save your change. Then click "MUSCLE" to recalculate.

c. You will need to "trim" the alignment. To trim, click the "Trim Alignment" button on the upper-left of the Alignment Viewer. **Why is it important to remove sequence gaps and unaligned ends?**

7. Analyze Sequences: Create a Phylogenetic Tree

a. Click "PHYLIP ML" to generate a phylogenetic tree using the maximum likelihood method. Click "PHYLIP ML"again and a tree will open in a new window, and the MUSCLE alignment used to produce it will open in another window.

b. A phylogenetic tree is a graphical representation of relationships between taxonomic groups. In this experiment, a *gene tree* is determined by analyzing the similarities and differences in DNA sequence.

c. Look at your tree.

- The branch tips are the DNA sequences of individual species or samples you analyzed. Any two branches are connected to each other by a node, which represents the common ancestor of the two sequences.
- The length of each branch is a measure of the evolutionary distance from the ancestral sequence at the node. Species or sequences with short branches from a node are closely related, while those with longer branches are more distantly related.

- A group formed by a common ancestor and its descendants is called a clade. Related clades, in turn, are connected by nodes to make larger, less closely-related clades.
- Click on a node to highlight sequences in that clade. Click the node again to deselect the clade. **What assumptions are made when one infers evolutionary relationships from sequence differences?**
- Generally, the clades will follow established phylogenetic relationships ascending from genus > family > order > class > phylum. However, gene and phylogenetic trees do disagree on some placements, and much research is focused on "reconciling" these differences. **Why do gene and phylogenetic trees sometimes disagree?**

d. Find and evaluate your sequence's position in the tree.

- If your sequence is closely related to any of the reference or uploaded sequences, it will share a single node with those species.
- If your sequence is identical to another sequence, the two will diverge directly from the node without branches.
- If your sequence is distantly related to all of the species in your tree, your sequence will sit on a branch by itself—with the other sequences grouping together as a clade.
- Look at the scientific names of sequences within the most closely associated clade. If all members share the same genus name, you have identified your sequence as belonging to that genus. If different genus names are represented, check and see if they belong to the same family or order.

e. Return to the menu, and click on "PHYLIP NJ" to generate a phylogenetic tree using the neighbor joining method. **How does it compare to the maximum likelihood tree? What does this tell you?**

f. If neither tree places your sequence within an identifiable clade—or if that clade is only at order level—you will need to add more sequences that may increase the resolution of your analysis. Return to Step 5, and add more reference sequences or obtain sequences within the order or family clade that contained your sequence. Then repeat Steps 6-7 to select, align, and generate trees from your refined data set.

8. Exporting Sequences to GenBank®

If you do not identify any identical hits through searches in *DNA Subway*, GenBank®, and BOLD, and you have determined that your sequence is of high quality, you may have a novel sequence.

Once you have identified a potentially novel sequence there are additional steps that you can take, including publishing your sequence to GenBank® through *DNA Subway*. It is not required that a sequence be novel to publish it to GenBank®. However, discretion should be used, and sequences that are already present in GenBank® multiple times for a particular species or without vetted metadata (definitive species identification, collection information, etc.) should not be published.

Note: Only high quality consensus sequences that have been generated by a submitter, and which have not been previously submitted can be exported to GenBank®.

a. Click "Export to GenBank®" in the project window.

b. Click "New submission." (If you are working with an animal sample, you need to specify if it is from a vertebrate, invertebrate, or echinoderm) then Click "Proceed."

d. If you have already collected information of your samples in the DNALC Barcoding Samples Database, write the sample's code number. Its information will be retrieved automatically. If not, you can enter the sample information manually in the next step; click "Continue."

e. Verify and fill in the information required in the "Specimen info" window; click "Continue".

f. Add photos of the sample if you have any available.

g. Verify your submission information, make any appropriate changes if necessary, and finally click "Submit." You will receive a notification that your sequence has been submitted to NCBI and a specialist there will check it. If your submission passes NCBI's verification procedure, you will receive a notification that your sequence has been published in GenBank®.

DNA Barcoding Specimen Collection Protocol

Procedure:

1. Get the latitude and longitude coordinates for your campus collection site from instructor
2. Be sure to write down the entire number to the last decimal place provided
3. Within the "road boundary" that surrounds the forested areas in northeast part of campus (see map), Two areas were designated for point sampling of insects and have been designated as either: 1) Area ECU or 2) Area Haunted Trail. Area ECU is nearest the ECU Dentistry Building and is color-coded purple on colorized maps and Area Haunted Trail color-coded orange.
4. With the instructors help, if needed, locate 2-3 other peers who will be sampling in the same area who are closest to your sample point. You, as a group, should walk to your collection sites and share smart phones with GPS and camera capabilities if possible.
5. Gather the materials you will need for the collection of your specimen (zip lock bag, a glove (2 max), net, a brush to share, etc. for insects, clippers and trowels for plants).
6. Using a phone with GPS capabilities, enter your latitude and longitude coordinates into mapping app. Again, enter the ENTIRE number. Walk to your site.

Collecting Plant Material

1. Collect one plant sample from your sample point within a meter radius (if possible). For large plants like trees, a small branch with leaves should be collected, for small herbaceous plants; the whole plant can be collected. Take an image of the plant prior to sampling.
2. Use clippers to cut a voucher or trowel to dig it up. This specimen should include flowers or other reproductive structures if possible. An appropriate voucher should show leaf arrangement on the stem. An apical sample is preferred. Place in Ziploc bag along with label with the following information:
    a) Specimen ID: The ID should be your initials and the 4-digit ID number you were assigned. Example: JWF-6645.
    b) GPS Coordinates and Elevation.
    c) Date
    d) Habitat Type

   Other information such as location (city, state) can be included or determined based on GPS.
3. Bring all the collected materials back to the lab in Gee to be processed or stored until processing. Place your bag with specimen into the area designated by the instructor. Then be sure to return the rest of your supplies to their designated area and clean your lab table. Your specimen will be awaiting you next week for the DNA extraction.

Collecting Insects

1. Collect one insect (6 legs) from your sample point within a meter radius (if possible) based on your collection code (see codes below) and place in your zip lock bag. If you have to move any further than a couple of meters (≈ 6 feet), for example to the nearest log, make a note and get the exact latitude and longitude coordinates. Place in Ziploc bag along with label with the following information:

DNA Barcoding Specimen Collection Protocol

Specimen ID: The ID should be your initials and the 4 digit ID number you were assigned. Example: JWF-6645.

e) GPS Coordinates and Elevation.
f) Date
g) Habitat Type

Other information such as location (city, state) can be included or determined based on GPS.

2. Bring all the collected materials back to the lab in Gee to be processed or stored until processing.
3. In the lab, take an image of your specimen that is as clear as you can possibly get it.

Code	**Search Criteria**
TB1	Collect Insect from Tree trunk or branches
LR1	Collect Insect from beneath log or rock
SL1	Collect Insect from Surface level of Leaf Litter (Top)
BSL1	Collect Insect from below Surface level of Leaf Litter (turn small area of leaf litter over and search)
FN1	Collect Flying Insect (Get Net from Instructor)

4. Once you have returned to the lab, fill out a small label with your name, sample ID and date and place inside the bag. Then, collect a cotton ball and using forceps; partially submerge it in the labeled acetone and place inside your zip lock bag to be humanely euthanize your specimen. Place your bag with specimen into in the area designated by the instructor. Then be sure to return the rest of your supplies to their designated area and cleaning your lab table. Your specimen will be awaiting you next week for the DNA extraction..

For All Students

1. Using your phone (and the iNaturalist app) or a computer, upload the image to iNaturalist. It will attempt to ID the specimen as best as it can to the most exclusive level of classification. We will use it as the temporary identification and compare that to the DNA barcoding identification. Once you have done this, be sure to fill out the data spreadsheet online before you leave or you will receive no credit for lab.

DNA BARCODING TIMELINE

Week 1

Collection and processing of insects and plants.
Storage

Week 2

DNA Extraction
Set up PCR
PCR runs and is placed in freezer for next week

Week 3

Run PCR products on gel
PCR clean up kit
Samples are sent to sequencing by technician or instructor

Week 4

Sequencing analysis