eDNA Lab

Smithsonian-Mason School of Conservation

Collection

~1 hour

Overview of activities:

You will individually collect eDNA samples from Leach Pond as well as several habitat characteristics from the site. We will process the samples in the lab and correlate your results with the environmental data as well as examine the bullfrog distribution in the pond in the context of previous data:

Preparation of eDNA collection equipment:

1. Faculty have prepared 50 mL centrifuge tubes (blue lids) to bring into the field by adding 1.5 mL of 3M sodium acetate and 20 mL of 100% ethanol.
2. Label your tube and the lid for the location you will be visiting "Leach Pond-*your name*." When we go into the field, you should bring this tube and we'll bring graduated cylinders for the water samples.

Collection of eDNA samples:

1. At the field location, collect 15 mL of water from your designated collection site and add it to the tube for that site. Invert the sample several times to mix it thoroughly
2. Collect environmental information for your site (see data sheet at end) as described in class.
3. You will use these samples today, but they could be stored at -20 ^{o}C at this point to preserve the DNA

The DNA sample could be stored here for several weeks or months if necessary

Extraction (eDNA)

~1.5 hours

Overview of activities:

You will extract DNA from your water samples using the Qiagen DNA mini kit. You will perform the detailed procedure as outlined below:

eDNA spin column extraction:

You will be provided a tube rack with the following materials:

- One 2 mL tube labeled LP containing 20uL of Qiagen Proteinase K
- One 1.5 mL tube with 300 µL of Buffer AL
- One 1.5 mL tube with 300 µL of 100% ethanol
- One 1.5 mL tube with 600 µL of Buffer AW1
- One 1.5 mL tube with 600 µL of Buffer AW2
- One 1.5 mL tube with 300 µL of Buffer AE
- One Qiagen Spin column in 2 mL tube
- Four empty 2 mL tubes (3 without lids)

1. Invert the 50 mL centrifuge tube you used in the field several times to mix your sample.
2. Add 200 µl of your sample to the 2 mL microcentrifuge tube with Proteinase K. The tube is labeled "LP"**.** Add your initials to the lid as well.
3. Add 200 µL of Buffer AL to the LP labeled tube. Vortex for 15 seconds
4. Incubate at 56 ^{o}C for 10 min. **Be sure your tube has your initials**.
5. Briefly centrifuge the tube using the table-top centrifuge to remove drops from inside the lid.
6. Add 200 µL of 100% ethanol to the tube. Vortex for 15 sec.
7. Briefly centrifuge the tube using a table-top centrifuge (#1R, 6000 rpm, USA Scientific minifuge) to remove drops from inside the lid.
8. Carefully apply the mixture from step 6 to the QIAamp Mini spin column in a 2 ml collection tube without wetting the rim (there should be ~620 µL of material). Close the cap, label the spin column with LP and your initials, and centrifuge at 8000 rpm for 2 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate **UNLESS** there is material still above the filter. If material is still present, centrifuge again at higher speed until the filter is empty.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 8000 rpm for 2 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate

UNLESS there is material still above the filter. If material is still present, centrifuge again at higher speed until the filter is empty.

10. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (14,000 rpm) for 3 min.
11. Place the QIAamp Mini spin column in a new 2 ml collection tube (provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 3 min.
12. Place the QIAamp Mini spin column in a clean 2 ml microcentrifuge tube (provided) labeled with LP and your intials, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE.
13. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 8000 rpm for 2 min.
14. Discard the QIAamp Mini spin column and store the filtrate on ice.

The DNA sample could be stored here for several weeks or months if necessary at -20 °C

PCR (eDNA)

~1 hour

PCR (polymerase chain reaction) is a technique for amplifying specific pieces of DNA. It makes many copies of a section of DNA using primers, enzymes normally found in cells to duplicate DNA and changing temperature regimes to denature and anneal DNA strands. The reactions are set-up in a special small-volume plastic tube and processed on a machine called a **thermocycler** that changes temperature in a repeated and precise fashion.

PCR sample set-up:

You will be provided a tube rack with the following materials:

- Your 2 mL tube labeled LP containing 200 µL of extracted DNA
- One 2 mL tube labed +CON containing a known sample of bullfrog eDNA
- One 1.5 mL tube with 25uL of primer bullfrog 1
- One 1.5 mL tube with 25uL of primer bullfrog 2
- One 2 mL tube with AmpliTaq Gold PCR Master Mix
- One 1.5 mL tube with 360 Enhancer
- One 4-tube PCR strip

On the board in the classroom, the volume of each material you will need to add for your PCR reaction mix will be written. Record this information below. **Do this before pipetting anything!**

Record the volumes (in µL) in the table below:

	Negative Control (-Con)	Positive Control (+Con)	BF-A	BF-B
*Your primer*1				
Your primer 2				
360 optimizer				
DNA sample	Distilled water			
Master Mix				

1. Load the appropriate section of the 4-tube PCR strip using filtered pipette tips **except the Master Mix**. Be sure to change tips between each tube for all reagents. Label the tube tops with the column headings; **label the -Con tube with your initials too**.
2. Gently mix the 2 mL tube with Master Mix in it (avoid bubbles)
3. Pipette the Master Mix into each tube of the 4-tube PCR strip using filtered pipette tips and changing the tip between each tube.
4. Close the lid of each tube. **Important: Be sure the lid clicks securely.** If it is open, you will lose volume during the PCR reaction.
5. You will transport tube to the thermocycler and briefly spin the strip to make sure all materials are at the bottom of the tube.

We set up the thermocycler to run the following PCR cycle:

Bullfrog (From Ficetola et al. 2008):

10 minutes of denaturing (95 ^{o}C)
66 cycles of:
    Denature (95 ^{o}C) for 30 sec
    Anneal (61 ^{o}C) for 30 sec
    Extend: (68 ^{o}C) for 5sec
Hold (72 ^{o}C) for 10 min
Hold (4 ^{o}C) for infinity
Lid temperature (105 ^{o}C)

The DNA sample could be stored here for several weeks or months if necessary at -20 ^{o}C

Visualization

~2 hours

Overview of activities:

You will visualize the extracted and amplified DNA.

PCR Gel loading set-up:

1. Use the same materials you used to visualize your extracted DNA sample.
2. On a piece of parafilm, aliquot the loading dye into 3 separate drops of 5 µL each using filtered pipette tips. Add the 10 µL of PCR product to each drop. Be sure to change tips between each tube for all reagents. All numbers in the table below are in µL:

eDNA PCR				
	-Con	+Con	BF-A	BF-B
Loading dye	5	5	5	5
PCR product	10	10	10	10

1. You will load each of these 15 µL samples into an individual well in another gel prepared yesterday (stored in the 4 ^{o}C refrigerator overnight)
2. Put your initials on the sheets in the lab in the columns that correspond to your wells and **record your relative position on the gel here**. Also record any anomalies with your samples or loading:
3. We will run the gels for ~1 hr @ 95 V after everyone has loaded their sample.
4. After the gels have finished running, each one will be loaded into the UV light source and imaged using the Enduro software.

What did the results for your sample indicate? _____

_____

5. We will discuss the results in class and compare with previously published data from the literature and previous year's data.

Discussion Questions:

Submit your answers to the following questions separately:

1. Describe 2 advantages and 2 disadvantages of using eDNA for sampling cryptic species. For each disadvantage, suggest how it could be addressed.
2. Non-invasive genetic techniques like eDNA and barcoding have the potential to revolutionized how we sample for species in the field. Choose a species or system of interest to you and describe how you would use molecular sampling to answer a question about that species' distribution or behavior in the field. Be specific-how would you actually implement the study (e.g. what is your specific question and hypothesis?; what types of samples would you collect?; what patterns would you hypothesize to see in the data?) Include a description of why molecular monitoring would be preferable or necessary compared to traditional methods in this circumstance.

Reagent Recipes:

3M Sodium acetate

- 246.1 g sodium acetate
- 1 L deionized water

Qiagen buffers provided in the DNA mini kit (Product #: 51304)

2% agarose gel (with GelRed)
For one gel's worth of material:

- 2 g agarose
- 100 mL TBE buffer
- 10 µL GelRed DNA stain

TBE Electrophoresis buffer
Prepare as a 10X stock solution:

- 108 g Tris base (#4109-04, J.T. Baker, ThermoFisher Scientific)
- 55 g Boric acid (#BP168-500g, ThermoFisher Scientific)
- 40 mL of 0.5 M EDTA (pH 8) (#0322-500g, Amresco, Solon, OH)
- 1 L deionized water

Loading dye

- 50% glycerol
- 0.1 M EDTA (pH 8)
- 1% SDS
- 0.1% Bromophenol Blue

Leach Pond Data sheet

Date:
Time started:
Time finished:
Weather conditions:

Sketch the position along the pond where your sample was collected. Include important landmarks so that someone else could find the approximate location. Also, record the specific GPS coordinates of your sampling location with the specificity (see the “Satellite” option on the menu):