Molecular Barcoding of Fungal Unknowns:

Materials:

Your two unknown fungi in pure culture

Extract n’ Amp buffer, autoclaved toothpicks, dilution solution

PCR supplies

Master mix aliquot, your extracted fungal unknown’s DNA

Introduction:

In lecture we have previously talked about the transition of systematics and taxonomy from entirely based on morphological features to contemporary practices utilizing multiple molecular genetic loci to whole genomes to understand evolutionary relationships among fungi. While morphological characteristics are still useful for identification *sensu lato* and important to recognize, molecular barcoding of phylogenetically informative loci are standard protocols in taxonomy and systematics. Over the next two lab classes, you will engage in “molecular barcoding” your fungal unknowns to aid in species identification. Molecular barcoding utilizes sequencing of a specific locus or loci that are generally considered to be indicative of a species concept within a given field of study. For fungi, the Internal Transcribed Spacer Regions 1 & 2 of the Ribosomal DNA (rDNA) cassette are considered the species barcode locus (Fig 1.).

Fig 1. Diagram of the ITS region of the rDNA including the SSU, 5.8s, and LSU.

The ITS region was chosen in part because of several factors: it is a noncoding region which means mutations in nucleotide identity are not selected against and can be informative for shallow evolutionary relationships (i.e. relationships among species within a genus, some genera within a family), it contains the conserved 5.8s region that allows for an anchor point to serve as the root of the phylogenetic analysis (i.e. similar enough that we can generally group more distantly related organisms together here as a starting point before letting the variable regions show hypothesized paths of evolutionary divergence), and there are multiple copies within a single cell (compared to nuclear genes) thereby greatly enhancing our ability to replicate our loci of interest in polymerase chain reaction.

Your adventure in lab today…

Part 1: DNA Extraction

1. Take your pure cultures of your fungal unknowns and give them a barcode number (MM\_1\_1, MM\_1\_2, etc.) from the shared excel document.
2. Fill in the various metadata columns in the excel document (your name, collection date, isolation source, location)
3. Label your specific well on the 8-strip tube the same as on the spreadsheet.
4. At your bench, tear open your fresh fungus, exposing “sterile” tissue inside. Remove some of the trama that is approximately the size of a rice grain using flame sterilized forceps. For dried specimens, remove a few pieces of the hymenophore (some gills, a few teeth, a clump of pores, etc.) using flame sterilized forceps.

\*\*\*For Cultures\*\*\*

In the Bio Safety Cabinet, using a sterilized toothpick or other tool, scrape off some of the fungal tissue on the surface of the plate \*\*\*Be careful not to get any agar as it inhibits PCR\*\*\*.

1. Deposit and submerge the tissue in the corresponding, numbered strip tube, proceed to grind the tissue with a sterile toothpick. Close the cap and sterilize your tools.
2. Let the ground tissue incubate in the buffer for 10+ minutes, then incubate at 95 C for 10 minutes.
3. In the Biosafety Cabinet, open the tubes back up and using a fresh pipette tip, add 20 ul or a volume equal to the initial amount of extraction buffer of the dilution solution so that the final volume of Extraction:Dilution solution ratio is 1:1. Congratulations, your freshly extracted DNA that is now ready for PCR!

Part 2: Polymerase Chain Reaction (PCR)

Now that you have template DNA for your unknown fungal organisms, it is time to amplify our region of interest. Remember that PCR allows us to make copies of specific regions of interest in our DNA at a logarithmic pace. We essentially follow a cooking recipe that utilizes either a pre-made or in-house assembled cocktail termed the Master mix. This molecular cocktail contains: molecular grade water (free of inhibitors, DNA free), dNTPs (the nucleotides to copy our DNA region), forward & reverse primer (the starting point for our enzyme to assemble a copy of our region of interest), 10X Taq polymerase buffer (a mixture of water, MgCL, and proprietary elements that create favorable conditions for the Taq polymerase), and the Taq polymerase (the enzyme that assembles the copies of our region of interest). Now it is time to just add the DNA.

1. In the biosafety cabinet and using a clean pipettor tip, transfer 23 ul of Master mix to each tube you will use for PCR. Once everyone in your group has added the Master mix to the tubes, proceed to set 2.
2. Using the smaller volume pipettor and a clean tip, transfer 2 ul of template DNA from your En’A tube to the corresponding strip tube for PCR. Repeat for your other unknown fungal sample. \*\*\*Make sure not to cross contaminate or place your DNA in the wrong tube, only have the tube lids open 1-at-a-time\*\*\*
3. Once your group has combined all of their fungal unknown DNA samples into the correct strip tubes, double checked the information in the excel sheet and all the lids are closed, briefly flick your tubes to mix the DNA and Master mix, spin them down in a miniature centrifuge, and take them to the thermocycler to start the heating cycles that will activate the taq polymerase and begin amplifying the DNA.

Molecular Barcoding of Fungal Unknowns Part Two:

Materials:

TAE Buffer

2% Agarose Gel

Loading Dye w/ Syber green

1k bp ladder

Parafilm

PCR Products

Introduction: Now that we have done PCR, it is time to check if it was successful. We do this through visualizing our products via gel electrophoresis. We will take a small volume of the PCR product for each sample, add a loading dye to visualize the migration of our DNA fragments across the agarose gel under normal light, adding a chemical to stain the DNA so it fluoresces under UV light, and then add it to our 2% agarose gel. Then, we will add a ladder that has several DNA fragments of known sizes so we can estimate the size of our product (Fungal ITS 1F-4 is ~500-900bp). We will mix our PCR products with the dye and DNA stain, incubate them for a few minutes, then add them to a well in the agarose gel in the TAE buffer, connect the electrode ports, turn the gel rig on, make sure the current is running black to red (run to red), and wait ~45 minutes for our PCR products to separate on the gel.

1. Cut an approximately 3x 2 piece of parafilm and place it on the bench Infront of you, this is where you will mix the loading dye, DNA stain, and PCR product.
2. Transfer 1 ul of loading dye onto the parafilm for each of your samples (n=8). Make sure to place the 1 ul in a series of individual dots in a line.
3. Take 5 ul of PCR product from a single well of your strip tube, change the volume on the pipettor to 6ul, eject the PCR product onto the loading dye blob, mix the two by pipetting up and down a few times, expel the combined liquids onto the parafilm to incubate for 2 minutes, change tips and repeat for the remaining samples.
4. With your pipette set to 6 ul, uptake the 6 ul of combined PCR product+loading dye+DNA stain and add them to the wells of your gel in order according to your PCR strip tube. When you are done, Add 5 ul of your DNA ladder to the last well of your gel.
5. Make sure your gel and loaded wells are covered by the TAE buffer, replace the lid of the gel rig carefully, and connect it to the ports on the power source (red to red, black to black). Turn the machine on and make sure it is set for 140V. As it runs, make sure to check that bubbles are forming at the black end of your gel rig (this is a double check your electrodes are set up correctly and the current is running in the right direction (to red)
6. While we let the gel run for ~45 minutes, we will head back to the Medical Mycology Lab to check on our yeast tests, your fungal unknowns, and evaluate who is the winner in our bracket of unknown fungal champions!