BSC 495: The Hidden World's Code

A Hands-On Fungal Genomics Lab

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Preface

This is a Quarto book created from the MycoEd curriculum. The Mycological Curriculum for Education and Discovery (Myco-Ed) is a Course-Based Undergraduate Research Experience (CURE) that features team-based, hands-on experiments in fungal biology and genomics. You will learn new laboratory techniques and data analysis by starting with sample collection, fungal isolation, and the characterization of the resulting data to improve fungal genomic resources. Genomic DNA will be extracted from samples for genome sequencing, assembly, and annotation. By isolating and sequencing new fungal species, your team will help produce high-quality fungal reference genomes. This course will prepare you with workforce training in fungal biology and genomics and empower you to be a contributor to fungal genomics! Importantly, you will practice lab and bioinformatics skills that are broadly applicable!

To learn more about Quarto books visit https://quarto.org/docs/books. To learn more about the MycoEd curriculum visit https://myco-ed.github.io/myco-ed/.

1 Introduction

This is a book created from the MycoEd Workbook from March 2026 (Source: https://dx.doi.org/10.17504/protocols.io.rm7vzqd5xvx1/v1) and adapted for the BSC 495 the Hidden World's Code, a course developed for students at North Carolina State University. The course is designed to provide students with hands-on experience in fungal biology and genomics, and to empower them to contribute to the field of mycology through research and discovery.

The Mycological Curriculum for Education and Discovery (Myco-Ed) is a Course-Based Undergraduate Research Experience (CURE) that consists of coordinated hands-on experiments across fungal biology teaching labs with the goals of

- Training students and professors in laboratory techniques and data analyses
- Compiling original data for improving fungal genomic resources
- Empowering students through isolation and genome sequencing of new fungal species

The content is organized into **modules**, each covering a specific topic related to mycology and education. The book is designed to provide comprehensive information and resources for educators, students, and anyone interested in learning about fungi and their role in the environment.

2 Module 0: Getting Started and Data Management [Instructor Instructions]

2.1 Module 0.1 – Lab Materials and Equipment Overview

This document lists the materials and equipment required for implementing the Myco-Ed program.

2.1.1 Module 1- Fungal sampling and Culturing

- 100x15mm Petri Dishes
- Agar, Bacteriological
- Autoclave
- Laminar Flow Hood (not necessary, if not available)
- Alcohol Lamp or Bunsen Burner
- Forceps
- Scalpel (make sure handle is metal, this will be flame sterilized, plastic will melt)
- 150ml Beaker (or similar, 2 per student)
- Parafilm
- Gloves
- Hole punch (all metal, for cutting leaf discs, scalpel will work in lieu of hole punch)
- Kimwipes
- Paper bags
- 10% bleach
- 70% ethanol
- Paper towels

2.1.2 Module 2- Fungal Isolation

- 60x15mm Petri Dishes
- Potato Dextrose Agar
- Autoclave
- Laminar Flow Hood (not necessary, if not available)
- Gloves

- Sharpie (fine tip is preferable)
- 70% ethanol
- Kimwipes

2.1.3 Module 3 - Phenotypic Assays

Materials and equipment will vary based on the phenotypic assay choice.

2.1.4 Module 4 – DNA Sequencing and Species Identification

This list may vary depending on your preferred extraction, amplification, and sequencing protocols. - Gloves - Sharpie - Thermal cycler - Vortex - Centrifuge - Heat block - 1.5mL microcentrifuge tubes - 0.2 mL PCR tubes - Micro and PCR tube racks - Label tape - Extraction Buffer (EB) - 3% BSA solution - Parafilm - 70% ETOH - 1-10 and 20-200 µL micropipettors - 1-10 and 20-200 µL micropipette tips - Kimwipes - PCR Primers (ITS1f, ITS4, 5.8SR, fITS7) - Taq polymerase mix - PCR water

2.1.5 Module 5 - Tissue Preparation for Genome Sequencing

- Cellophane
- 60x15mm Petri Dishes
- Potato Dextrose Agar
- Silica beads
- Gloves
- Transfer hood/Bunsen burner
- Incubator
- Balance
- Forceps
- Scalpel
- Small jar with lid
- Pure fungal culture

2.1.6 Module 6 – Sample Submission

- Shipping samples
- Sample form printout

2.1.7 Module 7 – Genomic Analyses

- Access to Mycocosm
- Computer with internet access

2.2 Module 0.2 – Create iNaturalist Traditional Project Instructions

2.2.1 iNaturalist Introduction

iNaturalist is a tool that we can use to document the fungal isolates in Myco-Ed courses. This tool allows instructors and students to record the metadata associated with the fungal isolates. Metadata will include plant species, geographic location, elevation, fungal DNA sequences, growth assays, and any other data relevant for this project. This platform will also enable us to track growth of the MycoEd project. The goal of this long-term research project is to develop annotated fungal genomes to better understand fungal biology.

To begin, make sure that you have an iNaturalist account.

2.2.2 Project Overview

There are three kinds of projects: 1) Umbrella Projects 2) Collection Projects 3) Traditional Projects

The umbrella project for MycoEd is the MycoEd Fungal Genomics Education Project. Each course will establish a Traditional Project in iNaturalist. A traditional project allows us to control whose observations are included in the project, which enables better downstream data management. We ask that your class use the Traditional Project for MycoEd for only your MycoEd fungi. If your course includes a fungal collection using iNaturalist, please have a separate iNaturalist project for these fungal observations.

2.2.3 Make a MycoEd Traditional Project [Instructor Instructions]

1. Log into iNaturalist.
2. Go to Projects.
3. This will take you to Your Projects.
4. On the bottom right of the page, select Start a Project.
5. Start a Traditional Project. Scroll to the bottom of the page to find the Traditional Project link for this option.

**Note: If you do not have this option, please contact Amy Honan (amy.honan@oregonstate.edu) or Andrew Wilson (andrew.wilson@BotanicGardens.org) for assistance.

6. Complete the following criteria indicated in the lettered parts of the figure below:
    a. Add Title. Include Institution, term or semester, and year.
    b. Make this Invite Only. You will add your students to this project.
    c. Add images or logos as you see fit. This step is useful if you manage multiple projects.
7. Other fields to consider:
    a. Location: You may choose to limit the locations of your observations. If your project is set to invite only, this is likely unnecessary.
    b. Observation Fields: Here you can link the fungal endophytes back to the host plants. In the future, we may have further instructions for data to add here.
8. Complete the project by selecting the Create button.
9. You may add your MycoEd concierges to your project if you wish (Andrew_wilson, ahonan).
10. Email Andrew Wilson (andrew.wilson@BotanicGardens.org) to be added to the MycoEd Fungal Genomics Education Project.
11. Add your project name to the text highlighted in yellow in copies of the following documents:
    a. Module 1 – Using iNaturalist
    b. Module 1 – Sampling Fungal Endophytes and Culturing Student Instructions

Adding your name to the copies of these documents will enable you to use them directly in your courses.

2.2.4 Add Participants (=Students) To The Project

Because we've made the Project "Invite Only" you will need to manually invite each of the students to join the course. They are instructed to join the course in Module 1.2.

1. Open your Myco-Ed project.
2. Select the "Members" section on the lower right. In this section you can manage membership and participation for the project.
3. Click on "invite new members here" in the yellow box on the right.
4. Once you've received the iNaturalist User IDs, start adding your students to the blank space under "Invite people…". Their names/user ID should appear in the drop down menu.

If their names do not show up, it is because they have not completed their iNaturalist registration, or the User ID is different from what they've supplied.

5. Instruct the students to look for their invitation and join the project. They must now add their course's iNaturalist observations to the project for them to show up in the project.

Use this last step as a requirement for course credit to ensure the process is completed by students.

2.3 Module 0.3 – iNaturalist Checklist

2.3.1 iNaturalist Checklist

This checklist will help you track your progress in managing Myco-Ed project data using iNaturalist.

2.3.1.1 For Instructors

- Create an iNaturalist Account. (Module 0.2)
- Create an iNaturalist Traditional Project for your course. (Module 0.2) You may need to request the assistance of the Myco-Ed team to create this if you do not have the pre-requisite number of observations.
- Tell your students the name of the iNaturalist Project, and request their iNaturalist user IDs.
- Add the students to the course project once they've completed Module 1.2. (Module 0.2)
- Instruct students on iNaturalist data management obligations for course credit (at the instructor's discretion).

2.3.1.2 For Students/Participants

Homework Assignment 1 (Module 1.2) - Create an iNaturalist Account. - Get the iNaturalist App for your phone - Give your instructor your iNaturalist User Name. - Update your real name under your account settings. - Watch some videos and tutorials. - Join the course project once you receive an invitation from the instructor/TA.

Make an Observation (Module 1.2) - Take multiple images of the plant host (or habitat photos of the environment) and include at least one image with a unique field number. (e.g., MycoEd_004) - Add the observation to your course project. - Save and upload the observation to the cloud once you're connected to wifi. - Add Observation Field for MycoEd ID. - Add Observation Field for DNA Barcode ITS once you have DNA sequence data. - Update the name of fungal isolates using DNA sequence BLAST results. - Add Observation Field for Host Observation for fungal isolates linking to the original host on iNaturalist.

3 Module 1: Fungal Sampling

3.1 Module 1.1 – Lab Prep Overview

3.1.1 Instructor Laboratory Preparation Instructions

Materials: - Cellophane - 100x15mm Petri Dishes - 60x15mm Petri Dishes - Agar, Bacteriological - Alcohol Lamp or Bunsen Burner - Forceps - Scalpel (make sure the handle is metal, this will be flame sterilized, plastic will melt)

Lab Prep:

Sterility: If you don't have a laminar flow hood, you may create a similar environment by setting up two torches over your workspace. First, clean your work area with bleach, and/or ethanol. Set the torches facing each other with enough space to work underneath. Light the torches and pour media into plates under the flames. We will use clean benches and laminar flow hoods when available, but this is a good alternative for those without access to these resources.

> ⚠ Warning
>
> Note that we will be working with fungi, which are generally not harmful to humans, but can cause allergic reactions in some individuals. Always practice good sterile technique and handle all materials with care. If you have any concerns about allergies or sensitivities, please consult with your instructor before beginning the lab.
> Additionally, we may not be able to work in a laminar flow hood, which increases the risk of contamination. Be extra cautious with sterile technique and be prepared for some contamination in your plates. If you have access to a laminar flow hood, please use it for this lab.

Water Agar Plates: Students isolate fungal endophytes from leaves on water agar. We use one 100x15mm Petri dish to plate up to 6 leaf discs.

Water Agar Recipe: Suspend 39 grams Agar, Bacteriological in 1000 mL (1 L) distilled water. Heat to boiling to dissolve the medium completely (in most cases, this step can be skipped as autoclaving will dissolve media). Sterilize by autoclaving at 15 lbs pressure at 121 °C for 15 minutes.

When the media is cool enough to handle with autoclave gloves, pour 100x15mm plates in a sterile environment. Do not let it cool too long as the media will solidify. Each 100x15mm plate holds approximately 20 ml media. If plates will be used immediately smaller amounts of media can be poured into plates. If plates will be stored, use larger amounts. Store plates in the original petri dish sleeve in a refrigerator (~4 °C) until use.

Disposal: Place all petri dishes in an autoclavable bag and sterilize at 15 lbs. pressure at 121 °C for 15 minutes. Follow institutional protocol for post-sterilization disposal. Be sure that the disposal of any heavy metals or other chemicals is properly handled.

3.2 Module 1.2 – Metadata Directions

3.2.1 Metadata

Metadata is the data providing information about other data. In this project, metadata refers to information related to the fungi you will study, including information about the substrate (e.g. plant host for fungal endophytes), location (latitude and longitude), elevation, images, fungal species, growth rates, assay information, images, etc. This document guides you through capturing this data and where to document it.

Once you have selected the plant(s) that you will isolate fungi from, upload an image of each plant to your course's iNaturalist Project page. This part of the larger MycoEd Fungal Genomics Education Project. The fungi you isolate from this plant and the data associated with the fungal isolates will be linked back to this observation. These data will then be freely available to you and any other researchers interested in investigating fungal endophyte ecology and genomics.

Record metadata on the Endophyte Excel spreadsheet. Below are descriptions for each category where necessary.

3.2.2 iNaturalist plant observation:

Provide the url link for your iNaturalist plant observation.

3.2.3 Sample Naming:

Your individual fungal isolates will be identified using several identifiers. The first will be the unique Host ID number that identifies the host for the fungi you isolate. Second will be your initials. Please use at least 3 initials since some large courses may have students with shared initials. Third, each individual leaf you cultured fungi from will be a separate number. If you

take multiple samples from the same leaf, they will be identified by letters. For example, if you have one leaf or needle with 3 different sections, they would be 1a, 1b, and 1c.

EXAMPLE: You have leaves collected from a maple tree on your campus. This tree was logged in iNaturalist and given a unique field number MycoED_004 (or whatever format the field numbers are provided in by your instructor/TA) (See the next module 1.2 for details). Let's assume your initials are ABC. From this collection, you have three leaves that you culture from: leaf 1, leaf 2, and leaf 3. From each of these leaves, you take six segments, so these are letters a-f. So, if you isolate a fungus from the second leaf, third section, and your initials are ABC, the name of the isolate would look like MycoED_004_ABC_2c.

With this naming scheme, anyone can trace the 1) plant host, 2) the researcher, 3) and the leaf and section the fungal isolate came from. NO other fungus will have this precise labeling unless additional fungi were isolated from this leaf section. After that step you will need to add additional identifiers.

3.2.4 Sample location:

Record latitude and longitude in decimal format. On a computer, go to iNaturalist observation. On the map, click on details at the bottom.

From the dropdown menu. The Lat/Long is now shown. Click the copy button and paste it into the Excel Spreadsheet.

You can also find Lat/Long coordinates on Google Maps. For Android phones: tap location and copy and paste in a text and it's automatically in decimal degree format. For the iPhones: 1. Open the Google maps app. Tap the "black triangle in a circle" icon to zoom to your location. 2. Long touch on your location. This should open a tab at the bottom of the screen. 3. Scroll up. You should see the latitude and longitude coordinates of that position, looking something like this: (45.1234567, -122.1234567). 4. Long touch the coordinates, tap copy and then paste into an email or text.

3.2.5 Elevation:

Record the elevation of the plant in meters. On a computer, go to iNaturalist observation and again, click on details at the bottom. 1. On a computer, go to iNaturalist observation and again, click on details at the bottom. 2. At the bottom of the details window, click macrostrat. 3. The next window will display elevation in meters in the upper right. Record this elevation.

3.2.6 Canopy:

Record the portion of the canopy where the leaf was collected (L-lower 1/3 of plant, M-middle 1/3 of plant, or U-upper 1/3 of plant).

3.3 Module 1.3 - Fungal Sampling and Culturing

3.3.1 Purpose

This lab will introduce you to sterile/aseptic technique, culturing fungi, isolating cultures, and documenting fungal growth.

3.3.2 Introduction

Every species of plant surveyed to date hosts a multitude of fungal **endophytes**. These symbiotic fungi exist in all plant tissues, including leaves, bark, roots, and reproductive structures such as flowers and seeds. All lineages of fungi contain endophytic species, however the majority of fungal endophytes described to date are Ascomycetes. Research has shown that fungal endophytes impact both plant species fitness and plant community fitness. Fungal endophytes can confer abiotic and biotic stress tolerance, influence biomass and water consumption, and alter resource allocation. The fungi live either between the plant cells, between the cell wall and the cell membrane, or sometimes even within the cell membrane. This project is part of the MycoEd Fungal Genomics Education Project. The goal of this long-term research project is to produce fungal reference genomes to better understand their biology.

3.3.3 Learning Goals

1. Documentation using iNaturalist
2. Practice sterile technique
3. Culture and isolate fungi
4. Investigate fungal endophytes
5. Characterize morphology of fungal cultures

3.3.4 Student Lab Supplies

- 3 Large Water Agar Petri Plates - these are for initial isolation
- Sharpie
- Parafilm
- Paper Towels
- Gloves
- Flame (alcohol lamp or Bunsen burner)
- Bleach
- Ethanol
- Scalpel
- Metal hole punch

- Forceps

3.3.5 Instructions

3.3.5.1 Isolation of Fungi from Leaves

Before you begin, read through the entire instructions. Be sure to consult your instructor if you have any questions.

Formulate your Research Question You will attempt to culture fungi from a total of 18 leaf sections. You may use any combination of plant species, individual plants, leaf portions, or leaf cuttings in your setup. Choose a combination that interests you. Are you interested in capturing the diversity within an individual plant? If so, are you interested in looking at the diversity of endophytes within the canopy of individual plants? For this question, you would plate leaves from lower, middle, and upper canopies. Are you interested in documenting the fungal endophyte diversity within a plant species? For this investigation, collect leaves from multiple individuals of the same species. Perhaps you want to compare species diversity between different plant species. To investigate this question, collect plant leaves from a variety of plant species.

Design your Experimental Setup Once you have decided what endophyte communities you would like to explore, obtain green leaves from your target host plants. These can be from deciduous or evergreen plants, including needles from conifers. You should plate your leaves within 24 hours of collection. Refrigerate your leaves if you need to store them between collection and plating.

Metadata Upload an image of the plants you have chosen to your course's iNaturalist Project page. Follow the instructions in Module 1.1 - Metadata Directions to be sure you have documented all necessary metadata.

Sampling Endophytes Materials needed: - Your phone with iNaturalist. - Sample labels with unique ID numbers. - Paper bags. - Clippers, scissors, or a knife to sample leaves.

Protocol: 1. Find a tree, or other plant to sample. Ideally, one that has healthy leaves. 2. Make an iNaturalist observation of the plant on your phone. a. Take multiple images. i. At least one of the entire plant. ii. Multiple closer pictures of the leaves, bark, any flowers or fruit, etc. iii. At least one observation must include one sample label with an ID number. b. Go to the projects section on iNaturalist. i. Select "Your iNaturalist Project". c. Save the observation on iNaturalist. i. Follow instructions in Module 1.2 Using iNaturalist 3. Place the label in a paper bag. 4. Sample up to three leaves using your cutting instrument. 5. Put the leaf samples into the bag.

Culturing Fungi From Leaves You have 3 larger (100x15mm) Petri dishes that contain water agar. Water agar media is more clear (whitish to translucent) than the smaller petri dishes which contain Potato Dextrose Agar (PDA). These water agar plates will be used for

Part I of this exercise. Section each of these into 6 regions as depicted in Figure 1. This will provide you space to culture 18 leaf sections.

Gather all supplies including sterilized water, 70% EtOH, and 10% bleach solution. Label the Petri dishes and read through procedures before starting. Place bleach solution and sterile water in a container with enough space to dip leaf tissue. Use the provided scalpel or metal paper hole punch from your lab kit to take leaf subsamples.

1. Sanitize/sterilize work surfaces. If available, wipe surfaces with ethanol. Choose a working location with the least amount of airflow to prevent contamination. If available, set up torches on either side of your work area with the flames aimed toward the middle of your workspace. The heat generated from the torches creates an upward airflow which decreases the potential of airborne contaminants falling onto your workspace or into your petri dishes.
2. Sterilize tools (scalpel or hole punch and forceps)
    a. Heat your tool using a flame (candle, alcohol lamp, torch, or something similar).
    b. Let cool – you can dip the tool (scalpel or hole punch) into the bleach solution to cool.
3. Cut leaf tissues. You may take as many cuttings from each leaf as desired. Be sure to label cuttings appropriately as described above. Cuttings should be ~1 cm.
4. Surface sterilize leaf tissue. Using sterilized forceps (heat and cool as above), immerse each cutting in 10% bleach solution for 10 seconds.
    a. To prepare the bleach solution, sterilize water by boiling water for 10 minutes. You will need enough water for the bleach solution and rinse step. Distilled water is preferred. If unavailable, use tap water. Let water cool to room temperature before coming into contact with plant tissue.
    b. Mix 1ml (1/4 teaspoon) household bleach with 500 ml (2 cups) sterilized water.
5. Rinse leaf tissue. Transfer leaf cutting from bleach solution using forceps and swirl in sterile water or %70 EtOH to rinse. Let excess water drip off leaf cutting or place on clean kimwipes to dry.
6. Add tissue to water agar. Place up to 6 leaf cuttings per plate of water agar labeled as described above (Figure 1). The water agar plates are the larger plates that contain clear to whitish media. Use sterile technique. Be sure to sterilize forceps (heat with flame and cool) in between each leaf transfer.
7. Seal Petri dish with Parafilm. This is a stretchy material similar to Saran Wrap. Cut a strip a little wider than the petri dish. Remove the paper backing. Hold one end of the Parafilm strip on the lip of the petri dish so that the gap between the lid and base is covered by the Parafilm. Keeping one end fixed with one hand, stretch and wrap the parafilm around the petri dish, sealing the gap between the lid and base of the petri dish. The stretching of the Parafilm will facilitate the seal, you will not need any tape to keep the Parafilm in place.
8. Incubate at room temperature in the dark and examine for growth each day.

3.4 Module 1.4 – Using iNaturalist

3.4.1 Using iNaturalist

For this semester we will be sampling plant tissue and isolating endophytic fungi for an experiment in fungal genomics. Part of this research will include the collection of important meta-data which includes latitude and longitude information, sampled material/host, and taxonomic information. To facilitate this we are using iNaturalist.

Homework Assignment 1: Get set up to use iNaturalist. - Go to https://www.inaturalist.org/ and make an account if you don't already have one. - Download the iNaturalist App to your phone. - Give your iNaturalist user ID to your course instructor or TA. They will need this to add you to the course project. Be sure to ask your instructor for the name of the course project. - Join your course project. Within your iNaturalist account, go to: Community>Projects Assuming you're invited you should see the name of your course project. Join the project. If you don't see it, send your iNaturalist user ID to your Instructor or TA to receive an invitation. - Add your Real Name to iNaturalist - For your name to display properly you need it spelled out in your profile. - Log into iNat. - Go to your profile icon in the top right. - Select Account Settings. - Type your real name into the space under Display Name. - iNaturalist Application Settings on your phone. - Go to settings on your phone. - Turn off "Automatic Upload" (recommended). - Adjust settings as they suit you.

Learn how to use iNaturalist to make observations for scientific research. iNaturalist makes documenting samples easy through observations. We will use this to help collect the metadata on our specimens in the upcoming lab. To prepare yourself, please review the following resources.

Homework Assignment 2: Review videos on how to document fungi. - Getting Started: https://www.inaturalist.org/pages/getting+started - iNaturalist Videos Page: https://www.inaturalist.org/pages/video+tutorials - NAMA/FunDiS How To Collect Research Specimens: https://youtu.be/EgZw37NjxkA - FunDiS Photography: https://fundis.org/get-started/photograph

Our protocol will be specific to our project, but will borrow from any/all of the video guides shown above.

3.4.2 Making observations for your mycology course (Myco-Ed)

To make the best use of iNaturalist as a tool for this course, and the broader MycoEd Project, you need to make quality observations of plant hosts, and the fungal endophyte cultures that come from these plants.

Required Ingredients

Observations of Plant Hosts 1. Open your iNaturalist Application. 2. Make an observation of one host plant by taking a picture of it. a. Find the camera icon in the app and take a picture of your plant. b. Include one field label in an image of the plant. (see examples) c. Take images from multiple perspectives: whole plant, leaves, flowers+fruits, branches, trunk, etc. 3. Alternatively, take images on your phone and upload them to iNaturalist. Again, make sure you are including a field label in your images. 4. Before saving the observation, add it to your course project using the app. a. Go to "Projects" b. Select the course project. NOTE: You must join the course project before this step. See the third bullet point at the beginning of these instructions. 5. Click Done to save your observation. 6. Assuming you've turned "automatic upload" off, you will need to manually upload your observation. This will appear green on your phone until it is uploaded. 7. Double-check your observation online. a. It will appear on your account if it was uploaded correctly. 8. Add Myco-Ed ID. This will attach the field label ID number to this host observation. a. Under Observations Fields, on the lower right portion of the page, type "MycoEd" b. Select "MycoEd ID" from the dropdown that appears. c. Add the field label ID number assigned to this host in the blank field under MycoEd ID. d. Click "Add".

Under Project you should see the name for your course project. If not, click on the open field and the course project's name should appear in the drop-down menu. Select the

Observations of Fungal Cultures Protocols for making observations of fungal cultures that follow the same steps as above with some added metadata that will go in as "Observation Fields".

9. Open your iNaturalist Application.
    a. Alternatively, take images on your phone and upload them to iNaturalist.
10. Make an observation of the fungal isolate by taking a picture of it.
    a. Take pictures from the top and bottom, including the label.
11. Before saving the observation, add it to your course project using the app.
    a. Follow the instructions detailed above.
12. Click Done to save your observation.
13. Manually upload your observation. This will appear green on your phone until it is uploaded.
14. Double-check your observation online.
    a. It will appear on your account if it was uploaded correctly.

Adding iNaturalist Observation Fields 15. Add MycoEd ID. This will attach the field label ID number to this host observation. a. Follow the above directions. b. The format of the MycoEd ID of fungal isolates should be in the form of: MycoEd Host ID_Your Initials_Leaf and Fragment number. EXAMPLE: MycoEd_004_ABC_2c See: Myco-Ed Module 1.1 -

Metadata, under the Sample Naming section 16. Add other iNaturalist Observation Fields a. For ITS sequence data from fungal isolates, use DNA Sequence ITS b. To link to the host iNaturalist observation for this fungus, use the Host Observation Observation Field and add the url to the host observation.

4 Module 2: Fungal Isolation

4.1 Module 2.1 – Lab Prep Overview

4.1.1 Instructor Laboratory Preparation Instructions

Materials: - Cellophane - 100x15mm Petri Dishes - 60x15mm Petri Dishes - Agar, Bacteriological - Potato Dextrose Agar - Alcohol Lamp or Bunsen Burner - Forceps - Scalpel (make sure the handle is metal, this will be flame sterilized, plastic will melt)

Lab Prep:

Sterility: If you don't have a laminar flow hood, you may create a similar environment by setting up 2 torches over your workspace. First, clean your work area with bleach, and/or ethanol. Set the torches facing each other with enough space to work underneath. Light the torches and pour media into plates under the flames.

> ⚠ Warning
>
> Note that we will be working with fungi, which are generally not harmful to humans, but can cause allergic reactions in some individuals. Always practice good sterile technique and handle all materials with care. If you have any concerns about allergies or sensitivities, please consult with your instructor before beginning the lab.
> Additionally, we may not be able to work in a laminar flow hood, which increases the risk of contamination. Be extra cautious with sterile technique and be prepared for some contamination in your plates. If you have access to a laminar flow hood, please use it for this lab.

Potato Dextrose Agar (PDA) Plates: Fungi cultured from leaves on water agar is transferred to nutrient Potato Dextrose Agar (PDA) plates. We use one 60x15 mm Petri dish for each fungal isolate.

Potato Dextrose Agar Recipe: Suspend 39 grams Agar, Bacteriological in 1000 ml (1 L) distilled water. Heat to boiling to dissolve the medium completely (in most cases, this step can be skipped as autoclaving will dissolve media). Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

When the media is cool enough to handle with autoclave gloves, pour 60x15mm plates in a sterile environment. Do not let it cool too long as the media will solidify. Each 60x15mm plate holds approximately 8 ml media. If plates will be used immediately smaller amounts of media can be poured into plates. If plates will be stored, use larger amounts. Store plates in the original petri dish sleeve in the refrigerator (~4 °C) until use.

Option: Adding Antibiotics Once your PDA has cooled, you may choose to add antibiotics to the media. Streptomycin (streptomycin sulfate) is commonly used at a concentration of 50mg/L in agar. Add the appropriate amount to your media once cool to the touch and before pouring plates.

Cellophane Plates: Fungal tissue can be harvested from cellophane plates. Cut cellophane discs slightly smaller than the petri dish so that they lay flat on the PDA media. Using the bottom of the petri dish as a template, draw a circle on the cellophane and cut out discs on the inside of the line.

Place cellophane discs into an autoclavable container with water. Place each cellophane disc individually into the water and try to stagger them in the water. This staggering step ensures that you can more easily remove the discs from the water. Cover your container with foil and sterilize by autoclaving at 15 lbs. pressure at 121 °C for 15 minutes.

Working in your sterile environment, place one cellophane disc onto cooled PDA agar in 60x15mm plates. Use sterile forceps to transfer each cellophane disc onto the PDA plate. If you can't easily remove single discs, try the "slide" technique. Dip your forceps into the container with the discs until you touch a disc. Using the forceps as a probe (not pinched) slide the disc to the side of the container and up the side of the container. Often, you'll pull out one disc. Sometimes more than one disc will slide up, but it's usually easy to see the individual discs at this point. You can then use your forceps to pull out one disc. If you grasp one disc with your forceps, you can swirl it in the water in the container to dislodge any other discs that might be attached. Open the lid of the petri dish and lay the cellophane onto the solid agar.

Disposal: Place all petri dishes in an autoclavable bag and sterilize at 15 lbs pressure (121°C) for 15 minutes. Follow institutional protocol for post-sterilization disposal. Be sure that the disposal of any heavy metals or other chemicals are properly handled.

> **!** Important
>
> Please use the red biohazard bags for disposal of all petri dishes and materials that have come into contact with fungi. This includes gloves, paper towels, kimwipes, and any other materials used during the lab. Do not dispose of these materials in regular trash or recycling bins.

4.2 Module 2.2 - Fungal Isolation

4.2.1 Purpose

This lab will introduce you to sterile technique, culturing fungi, isolating cultures, and documenting fungal growth.

4.2.2 Introduction

In the previous lab, we started the process of sampling fungal endophytes. We documented the host plant using iNaturalist and then sampled foliar tissues from the plants. These foliar tissues were transported to the lab, surface sterilized to remove any external contaminants, and dissected. The dissected tissues were plated onto water agar plates, sealed, and labeled.

Today, we will isolate, or subculture, these fungi onto PDA agar (Potato Dextrose Agar). This nutrient agar will allow for fungal growth. We will then measure the growth rate of each individual fungal isolate.

This project is part of the Myco-Ed Fungal Genomics Education Project. The goal of this long-term research project is to develop annotated genomes of fungal endophytes to better understand their ecology and how they impact their host plants.

4.2.3 Learning Goals

1. Practice sterile technique
2. Culture and isolate fungi
3. Investigate fungal endophytes
4. Characterize morphology of fungal cultures
5. Create data spreadsheets
6. Isolate fungi for molecular extraction and analysis
7. Create Growth Curves

4.2.4 Student Lab Supplies

- Fungal Endophyte Sample Plates
- PDA Plates (60x15mm)
- 70% ethanol
- Kimwipes and Paper Towels
- Parafilm
- Fine Tip Sharpie
- Flame Source for Sterilization (alcohol lamp or Bunsen burner)

- Scalpel
- Ruler
- Forceps

4.2.5 Instructions

4.2.5.1 Part I - Subculturing Fungal Endophytes into Fungal Isolates

Fungal cultures usually emerge as white fuzz from your leaf discs, however, the hyphae might be pigmented, such as a brown or greenish color. Figure 1 depicts the fungal hyphae in brown and blue emerging from the green leaf tissue. The fungi are utilizing the nutrients available in the leaf tissue for growth as water agar does not contain nutrients. To promote the growth necessary for downstream analyses, it is essential to transfer the fungi to a growth medium that contains nutrients. For our exercise we are using Potato Dextrose Agar (PDA) as nutrient media. These are smaller petri dishes and the media has a yellowish tint (compared to the larger petri dishes containing the clear, or whitish, water agar from your initial isolation). This transfer step also allows for the separation of each fungal isolate to ensure pure cultures.

Fungal growth from the leaf cuttings may occur over the span of a few weeks, so you may have to transfer several times. Your fungal cultures will be most successful if you transfer them within a few days (1-3) from emergence or detection.

> **!** Important
>
> Read all directions and gather all supplies before beginning.

1. Practice Sterile Technique. Wear gloves. Clean work surface with disinfectant. If available, wipe surfaces with 70% ethanol. Choose a working location with the least amount of airflow to prevent contamination. If available, use a laminar flow hood. You may also set up torches or Bunsen burners on either side of your work area facing each other. The heat generated from the torches creates an upward airflow which decreases the potential of airborne contaminants falling onto your workspace or into your petri dishes.
2. Label a PDA petri dish with the sample number and date for each isolate (described in Module 1.1 - Metadata). Always label along the bottom edge. This ensures the label stays with the fungus, even if the top lid falls off. Label the edge of the plate because you don't want to obscure light transmission through the plate. Also, mark lines on the bottom of the plate as shown in Figure 2 to record the growth rate. Be sure to transfer only one fungal isolate to each PDA petri dish.
3. Sterilize the scalpel using flame and let cool. You can touch your scalpel to the agar in the petri dish to cool.

4. Make a "plug". Cut a small chunk of water agar containing hyphae of target isolate using a sterilized scalpel. Transfer this plug of agar and hyphae to the PDA plate. Ideally, you will have the least amount of agar transferred to the new petri dish.

    a. Work quickly and cleanly and consider your technique to prevent contamination. Remove Parafilm from the water agar plate.
    b. Tilt or lift the lid of the water agar petri dish as little as possible, try to keep the lid covering or hovering over the plate to prevent airborne contaminants. Work under flames if using flames.
    c. Using a cooled sterile scalpel remove the chunk of agar from the target fungal isolate.
    d. Replace the lid of the water agar.
    e. Open the PDA petri dish lid by slightly tilting the lid, or lifting the lid straight up. Place the agar chunk into the middle (or as mid-plate as possible) of the PDA petri dish. The agar will likely stick to the scalpel, so do your best to remove the chunk without contamination.
    f. Close the lid of the PDA petri dish lid as quickly as possible. Seal with parafilm.

4.2.5.2 Part II – Measuring Growth Rates

1. Design a data sheet to record growth.
2. On the bottom of the plate, trace the edge of your fungal culture using a fine-tip sharpie (Figures 3 and 4).
3. Measure, in mm, new growth at each of the eight edges along the lines (Figures 3 and 4)
4. Average the eight measurements to get the average growth (Table 1).

Table 1: Daily Growth Average

Table 4.1: Table 1. Daily Growth Average

Measurement	Value
1	3 mm
2	2.5 mm
3	2.7 mm
4	1.8 mm
5	3.1 mm
6	2.2 mm
7	2.6 mm
8	2.1 mm
Average	2.5 mm

5. Continue to measure average growth in mm 7 days from plating (Figure 4).
6. Calculate the average growth rate (distance/time).

7. Plot a graph of your fungal isolate(s) growth rate.
8. Submit images and your data sheet and a graph of growth rates in your lab blog post for this project. Please take images to add to your blog post.

5 Module 3: Phenotypic Assays

5.1 Module 3.1 – Phenotypic Assays

5.1.1 Instructor Laboratory Preparation Instructions

Materials and Equipment: - Autoclave - Laminar Flow Hood (if available) - 60x15mm Petri Dishes - Agar, Bacteriological - Potato Dextrose Agar - Zinc Sulfate - Alcohol Lamp or Bunsen Burner - Forceps - Scalpel (make sure the handle is metal as the scalpel will be flame sterilized and plastic will melt)

Sterility: If you don't have a laminar flow hood, you may create a similar environment by setting up 2 torches over your work space. First, clean your work area with bleach, and/or ethanol. Set the torches facing each other with enough space to work underneath. Light the torches and pour media into plates under the flames.

Potato Dextrose Agar (PDA) with Zn Plates for Zn Stress Assay: Suspend 39 grams Potato Dextrose Agar in 1000 ml (1 L) distilled water (or follow the directions on the bottle label). Add the appropriate amount of Zn sulfate to obtain concentrations of 1 mM Zn and 10 mM Zn in the media bottle. Heat to boiling to dissolve the medium completely (in most cases, this step can be skipped as autoclaving will dissolve media). Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

(PDA) Plates for Temperature Assay: Suspend 39 grams Potato Dextrose Agar in 1000 ml (1 L) distilled water (or follow the directions on bottle label). Heat to boiling to dissolve the medium completely (in most cases, this step can be skipped as autoclaving will dissolve media). Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

When the media is cool enough to handle with autoclave gloves, pour 60x15 mm plates in a sterile environment. Do not let it cool too long before pouring as the media will solidify. Each 60x15 mm plate holds approximately 8 mL of media. If plates will be used immediately smaller amounts of media can be poured into plates. If plates will be stored, use larger amounts. Store plates in original Petri dish sleeve in refrigerator (~4 °C) until use.

Working in your sterile environment, place one cellophane disc onto cooled PDA agar in 60x15mm plates. Use sterile forceps to transfer each cellophane disc onto the PDA plate. If you can't easily remove single discs, try the "slide" technique. Dip your forceps into the container with the discs until you touch a disc. Using the forceps as a probe (not pinched) slide the disc to the side of the container and up the side of the container. Often, you'll pull out one disc.

Sometimes more than one disc will slide up, but it's usually easy to see the individual discs at this point. You can then use your forceps to pull out one disc. If you grasp one disc with your forceps, you can swirl it in the water in the container to dislodge any other discs that might be attached. Open the lid of the petri dish and lay the cellophane onto the solid agar.

5.2 Module 3.2 – Phenotypic Assays

5.2.1 Phenotypic Assays

Objectives: 1. Determine how fungal isolates react to environmental stressors

Materials: - Student fungal isolates

Procedures: - Observe your fungal cultures and determine if they are pure cultures - We will use these cultures to set up stress tests (Zn assay and temperature assay) and for molecular identification.

5.2.2 Zn Stress Assay

In this lab, we will be investigating the tolerance and growth response of foliar fungal endophytes to varying concentrations of zinc (Zn). Zinc is an essential trace element for many biological processes, but in elevated concentrations, it can become toxic to fungi and other microorganisms. To understand how these fungi cope with different levels of Zn, we will isolate fungal endophytes from plant leaves and subject them to media containing 0 mM, 1 mM, and 10 mM of Zn. By measuring their growth rates over several weeks, we aim to determine the impact of Zn on their growth and survival.

Testing foliar fungal endophytes for Zn tolerance is particularly relevant as these fungi play crucial roles in plant health and ecosystem functioning. Endophytes can enhance plant growth, provide resistance to pathogens, and contribute to nutrient cycling. Understanding their tolerance to heavy metals like Zn is important for ecological studies, especially in areas affected by pollution or where plants are used in phytoremediation efforts. By assessing their ability to thrive in environments with varying Zn concentrations, we can gain insights into their resilience and potential applications in sustainable agriculture and environmental management.

Objectives: 1. Determine if the fungal isolates are tolerant of Zn. 2. Measure how different concentrations of Zn influence fungal growth rates.

Materials: - Pure fungal cultures - PDA 0 mM Zn + antibiotics - PDA 1 mM Zn + antibiotics - PDA 10 mM Zn + antibiotics - Fine tip Sharpie - Culture cutting tool - Burner - Ethanol jar

Procedures: - Label your plates with identifying information - On the bottom of each plate, draw four lines that intersect in the center of the plate (Figure 1) - Cut out three 0.5 cm^2 chunks from the edge of your pure culture and place one in the center of each of the Zn plates - Close and parafilm your plates - Carefully, flip the plates over and trace the cube using a fine tip Sharpie - Place the plate (unflipped) at room temperature in the dark and observe growth over the next few weeks

5.2.3 Temperature Assays

In this lab, we will explore the effects of temperature on the growth and survival of foliar fungal endophytes. Temperature is a critical environmental factor that influences fungal physiology and ecological dynamics. To investigate how these fungi respond to different thermal conditions, we will isolate fungal endophytes from plant leaves and cultivate them at three distinct temperatures: 4°C, room temperature (approximately 20°C), and 28°C. By measuring their growth rates over several weeks, we will better understand their tolerance to different temperatures.

Objectives: 1. Determine optimal growth temperature. 2. Measure how different temperatures influence fungal growth rates.

Materials: - Pure cultures (2 per student) - PDA with antibiotics (6 per student) - Fine tip Sharpie - Culture cutting tool - Burner - Ethanol jar

Procedures: - Label your plates with identifying information and growth temperatures (2 per temp.) - On the bottom of each plate, draw four lines that intersect in the center of the plate (Figure 6.1) - Cut out three 0.5 cm chunks from the edge of your pure culture and place one in the center of each of the temperature plates - Close and parafilm your plates - Carefully, flip the plates over and trace the cube using a fine-tip Sharpie - Bring your plates to the front of the class and place them (unflipped) in the correct box for incubation

5.2.4 Measuring Fungal Growth

Objectives: 1. Measure growth for Zn stress assay 2. Measure growth for temperature assay

Materials: - Fine tip Sharpie - Three Zn culture plates - Three temperature culture plates - Ruler to measure growth in mm (mm ruler)

Procedures: - On the bottom of the plate, trace the edge of your fungal culture - Measure, in mm, new growth at each of the four edges along the lines - Average the eight measurements to get the average growth and determine the average growth rate (distance/time)

6 Module 4: DNA Sequencing

6.1 Module 4.1 – DNA Sequencing and Species Identification Instructor Resources

6.1.1 Instructor Laboratory Preparation Instructions

Materials needed: - 3 x 250 mL Beakers - 100 mL graduated cylinder or 100 mL pipettes. - Distilled water - PCR pure water - 1.5 mL microcentrifuge tubes - Glassware with screw cap lids - Stock primers - 1-10 and 20-200 µL micropipettors - 1-10 and 20-200 µL micropipette tips - Magnetic stir bars - Hotplate and magnetic stir plate - Tris (need 100 mL of 1M Tris stock solution) - KCl (need 18.6 g) - EDTA (need 3.7 g) - Gloves - Sharpie

DNA Extraction Buffer (ES)

1M Tris Stock Solution (1 mole (M) of Tris = 121.14 grams) 1. Add 121.14 grams of Tris to 1000 mL of water (adjust quantity to lab needs) 2. Add a magnetic stir bar to the beaker and put the beaker on a hot plate/stir plate and set the heat and stirring to medium-low to facilitate the dissolving of the Tris.

Prepare Extraction Buffer (ES) 1. Add 10 mL of Tris stock solution to a 250 mL beaker. 2. Weigh and add 1.86 g KCl to the beaker. 3. Weigh and add 0.37 g EDTA. 4. Top up with 80 mL of distilled water. 5. Place on a hot/stir plate with a magnetic stir bar over medium-low heat and speed. Wait until dissolved. 6. Top up to 100mL total with distilled water and stir briefly. 7. Dispense 1000 µL into 1.5 mL microcentrifuge tubes and label the tubes "ES" for Extraction Solution. 8. Place tubes in a rack labeled "Mycology DNA Extraction Reagents" and store in the Freezer.

Dilution Solution (DS) (3% BSA) Prep 1. Add 3 g BSA (Bovine Serum Albumin) to 250 mL beaker. 2. Add 100 mL of distilled water. 3. Shake/stir to dissolve the BSA into solution. DO NOT HEAT! 4. Dispense 1000 µL into 1.5 mL microcentrifuge tubes and label the tubes "DS". 5. Place tubes in a rack labeled "Mycology DNA Extraction Reagents" and store them in the Freezer.

Diluting and prepping PCR primers 1. To dilute the vial to a stock concentration of 100 picomols/microliter first read the number of µmols present in the tube. a. Using this number, divide by 10, and add that many µL of PCR pure water to the tubes. This will give you a concentration of 100 pmol/µL 2. To make PCR ready primers we need to dilute the

concentration to 10 pmol/µL. a. Add 180 µL PCR pure water to a 1.5 mL microcentrifuge tube. b. Label the tube according to the primer being added. c. Add 20 µL of the 100 pmol/µL concentration primer.

6.2 Module 4.2 – DNA Sequencing and Species Identification

6.2.1 Purpose

The purpose of this module is to molecularly identify fungal isolates using the Internal Transcribed Spacer (ITS) rDNA region, also known as the fungal barcode. You will extract genomic DNA (gDNA) from your isolates, amplify the ITS rDNA using polymerase chain reaction (PCR), clean PCR product, Sanger sequence PCR product, and use DNA sequence to obtain a name for your isolate.

6.2.2 Learning Goals

1. Practice extracting fungal DNA
2. Successfully PCR amplify fungal ITS
3. Learn to BLAST ITS sequence to identify fungal isolates

6.2.3 Part I: DNA Extraction

6.2.3.1 Introduction

Identification of fungal cultures requires knowledge and techniques beyond the scope of this course. Luckily, we can use DNA sequencing of the fungal barcode region (ITS rDNA region) to obtain an identification for our isolates. To do this, you must (1) extract gDNA from a pure culture of your fungal isolate; (2) specifically amplify (make millions of copies) a portion of the ITS rDNA region using polymerase chain reaction (PCR); (3) sequence the PCR product using Sanger sequencing; and (4) submit your sequences for comparison against an online gene database (NCBI's GenBank).

In this part of the module, you will extract DNA from your fungal isolates. There are many ways to isolate DNA from fungi. Here we will use a very simple kit that uses an alkaline (pH 9.5-10) extraction solution at high heat to disrupt cells and release gDNA.

6.2.3.2 Materials:

- Fungal isolates
- 0.2 mL PCR tubes
- P20 micropipette with tips
- DNA extraction solution (ES)
- DNA dilution solution (DS)
- Ethanol jar
- Loop, pick, and forceps

6.2.3.3 Procedures:

1. Using the micropipette, aliquot 20 µL of ES into 0.2 mL snap-cap PCR tubes. We will run DNA extraction in duplicates, so fill two PCR tubes per culture. NOTE: Label your tubes before adding ES. Label on both the cap and side of the tubes, so you can identify the samples as yours and can differentiate to which culture they belong.
2. Place the tissue sample into the ES. Submerge and smash the sample against the tube wall if possible. NOTE: be careful not to add any agar to the ES as this will impact the pH of the solution and the extraction of gDNA.
3. Close the snap-caps and incubate at 65 °C for 10 minutes followed by 95 °C for 10 minutes.
4. Using the micropipette, aliquot an equal volume of DS (20 µL) to your extracts. Ensure that you can still read the labels and store in the freezer until next week. We will use these extractions to amplify the fungal ITS rDNA region using PCR.

6.2.4 Part II: Polymerase Chain Reaction (PCR)

6.2.4.1 Introduction to PCR

The polymerase chain reaction (PCR), developed in the mid-1980s, amplifies a specific DNA region by creating millions or billions of copies by 'in vitro DNA replication'. It uses a thermostable form of bacterial DNA polymerase, the enzyme that copies DNA, isolated from Thermus aquaticum, a thermophilic bacterium isolated from a Yellowstone hot spring. This enzyme, 'Taq polymerase', is active at 72 °C and can withstand brief heating to 95 °C. Thus, the PCR reaction is an example of how environmental microbes have benefited biotechnology and revolutionized biology.

The PCR reaction consists of mixing your template DNA with two primers: short sequences of DNA that are complementary to the ends of the region you want to amplify. The forward primer binds to one strand at the beginning of the region, and the reverse primer binds to the complementary strand at the end of the region. Primers are designed using computer databases of gene sequences, and synthesized chemically. In addition to the primers, Taq polymerase (or a synthetic polymerase enzyme), and your DNA, the PCR reaction also needs a buffer containing

MgCl2 and dNTPs (the four bases of DNA). The reaction is run on a machine called a thermal cycler, which automatically heats the reaction first to 95 °C to melt the double-stranded DNA (break H-bonds between strands), then to about 53 °C to allow the primers to anneal to the template DNA, and finally to 72 °C to allow the polymerase to add dNTPs and extend the product. Typically, scientists run 25-35 cycles of this reaction, producing 225 - 235 copies of the target region.

Figure 1: rDNA region containing the variable regions ITS-1 and ITS-2. Our forward primer (ITS-1F) will target a portion of the SSU and our reverse primer (ITS4) will target a portion of the LSU. Thereby, amplifying both variable regions and the 5.8S subunit.

6.2.4.2 Materials:

- DNA extractions with DS
- Control DNA sample
- 0.2 mL PCR tubes
- P10 micropipette with tips
- P20 micropipette with tips
- P200 micropipette with tips
- P1000 micropipette with tips
- Master mix containing buffer (MgCl2, dNTPs, primers, polymerase enzyme)
- PCR tube centrifuge
- Thermal cycler

6.2.4.3 Procedures:

We will run 20 µL PCR reactions 1. Add 18 µL of PCR master mix (contains PCR buffer, polymerase enzyme, dNTPs, and primers) to one PCR tube per sample. You can use the same pipette tip to aliquot master mix to PCR tubes. NOTE: Cap all PCR tubes when not in use to avoid cross-contamination. 2. Dilute DNA 1:10 (2 µl of DNA + 18 µL of DS) 3. Transfer 2 µL of your 1:10 diluted DNA to one PCR tube containing master mix. NOTE: Only one sample per tube and make sure to use a fresh tip for each sample to avoid cross-contamination. 4. Spin down your tubes before placing them in the thermal cycler. Run the reaction and observe the temperature cycles. After the reactions are finished, we will store your PCR products at -20 °C.

6.2.4.4 PCR Troubleshooting after Gel Electrophoresis

1. No amplification on any samples: check primers, polymerase, and other reagents.

2. Positive control OK, but samples show no amplification: Try dilutions of samples to check for inhibitors. Try a control with a primer set for common organisms to check that you can PCR something. Check the DNA amount.
3. Weak or multiple bands, smear: Mispriming from the incorrect program. Check primer concentration: an imbalance of primers can lead to multiple bands.

6.2.5 Part III: Gel Electrophoresis Introduction

Gel electrophoresis is a technique used to separate and analyze DNA based on fragment sizes and to verify the success of PCR. Gel electrophoresis separates DNA molecules based on size by applying an electrical current to either side of the gel. Since DNA is negatively charged, we load our DNA on the positive side of the gel to allow it to run toward the positive side of the gel when power is applied. The agarose gel allows the separation of DNA fragments based on size as the polysaccharide matrix acts as a sieve allowing small fragments to migrate faster and larger fragments slower.

Today, we will use gel electrophoresis to verify the success of our PCR reaction. We are looking for PCR product bands between

6.2.5.1 Prep 1% agarose gels for gel electrophoresis

To give enough time for the gel to cool and solidify, this task should be done in the first hour of the lab.

6.2.5.2 Materials needed:

- Agarose
- 1X TAE (or TE, TBE, or SB) buffer to make gels and running buffer Note: Use the same buffer for making and running the gel
- SYBR Safe to visualize DNA
- 250 mL Erlenmeyer Flask
- P20 micropipette with tips
- PCR products
- 100 bp Ladder
- Digital Balance
- Weigh Boats
- Gel rigs
- Gel comb
- Power source

6.2.5.3 Gel Preparation Protocol (Prep gel at least 20 minutes prior to use)

1. Measure 100 mL of 1x TE buffer in an Erlenmeyer flask.
2. Add 1 gram of agarose to the buffer.
3. Microwave for 30-second intervals.
4. Remove and swirl after each interval. If you still see un-melted agarose, continue in 30-second intervals until completely melted. If the agarose starts to boil, stop the microwave immediately to prevent it from boiling over.
5. Once all the agarose is melted, let sit on the counter until it cools enough to handle. Don't let it cool too long as it will solidify.
6. Set up the agarose mold and add a comb.
7. Once the agarose has cooled to the touch. Pour into the mold. Make sure the agarose is approximately 1/3 to 1/2 way up the combs.
8. If bubbles are present, use a micropipette tip to draw them to the sides of the gel.
9. Allow approximately 20 minutes for the gel to solidify and become somewhat translucent.
10. Gently remove the comb.
11. Remove gel from casting mold and place in gel rig. The gel rig is oriented to "run to red." Place the wells created from the comb towards the black electrode, opposite the red electrode.
12. Gels can be wrapped in saran wrap and stored for approximately 1 week.

6.2.5.4 Gel Loading:

1. Fill the gel rig with 1X TAE (or equivalent) buffer until it covers the surface of the gel.
2. Make a map of your samples in your gel.
3. Load Gel

    a. PCR product without loading dye: Using a micropipettor add drops (less than 1 L) of loading dye to the plastic side of parafilm. Add 5 L PCR product to loading dye. Swirl with pipette tip. Draw up the product and proceed to step 4.
    b. PCR product with loading dye: Draw up 5 L of PCR product using micropipette.

4. Dip pipette tip into the submerged well very carefully, without piercing through the gel. Depress the pipette slowly. Too much pressure or speed will force the sample out of the well. Without releasing the plunger, draw out the pipette. If you release the plunger, you will draw your sample back into the pipette.
5. Be sure to reserve one well per row for a standardized DNA ladder.
6. Run at 300 volts for about 15 minutes

    a. Keep an eye on the migration of your PCR product across the gel to ensure it doesn't run off the other end of the gel.

7. Visualize with UV light.

6.2.6 Part IV: PCR Cleanup

In order to sequence PCR products, the primers and other contaminants must be removed. 1. Remove ExoSAP-IT™ reagent from –20 °C freezer and keep on ice throughout this procedure. 2. Mix 5 µL of a post-PCR reaction product with 2 µL of ExoSAPIT™ reagent for a combined 7 µL reaction volume. When treating PCR product volumes greater than 5 µL, simply increase the amount of ExoSAP-IT™ reagent proportionally. 3. Incubate at 37 °C for 15 minutes to degrade remaining primers and nucleotides. 4. Incubate at 80 °C for 15 minutes to inactivate ExoSAP-IT™ reagent. 5. The PCR product is now ready for use in DNA sequencing. Treated PCR products may be stored at –20°C until required

6.2.7 Part V: Sanger Sequencing

Your cleaned PCR product will be sent to an external facility for Sanger sequencing.

6.2.8 Part VI: Identifying Species using DNA Barcode

6.2.8.1 Identify your sequence data using BLAST

To identify the sequence you've produced and determine if it represents the specimen you've sequenced and not some other contaminant, you can compare the sequence to a database of reference sequences. This is from the National Center for Biotechnology Information's (NCBI) Genbank database.

1. Go to NCBI: https://www.ncbi.nlm.nih.gov/.
2. Click on "BLAST" in the right-hand column under "Popular Resources"
    - BLAST = Basic Local Alignment Search Tool
3. Click on the Nucleotide BLAST image.
    - There are numerous BLAST options depending on the data you are working with. The two basics are nucleotides and amino acid (=Protein) sequence BLASTS. The blastx and blastn options allow you to convert these sequences into the complementary format.
4. Paste your sequence data into the big space under "Enter Query Sequence".
    - Open your FASTA file and select your sequences. This is the DNA sequence from the beginning of the ">" symbol, all the way to the end (e.g., the beginning of the next ">" symbol).
5. Under Choose Search Set you can keep it under "Standard databases (nr etc.)".

- You can come back and re-perform the BLAST using the "rRNA/ITS databases". After selecting this, choose "Internal transcribed spacer region (ITS) from Fungi type and reference material". This will be a much narrower database and set of comparisons, but the confidence in the results providing a correct ID will be *MUCH* higher.

6. Select the "BLAST" button at the end.

    - Genbank will then begin to query your sequence against sequences in the database. The top hit that comes back will be the sequence that is most like your sequence based on several stats.
    - Percent Identity = What percent of your sequence (query sequence) is identical to the matched sequence.
    - Query Cover = How much of the query sequence covers the matched sequence.
    - E value = The probability that this match occurred by chance in the provided database.

7. Review the results.

    - What is the "top hit"?
    - How much support is provided for this hit given the current database used?
    - What is the taxonomic ID of this result? What kind of fungi are these?
    - You may google the names and search under Wikipedia to get a better understanding of these organisms and what is known.

6.2.9 Part VII: Data Management - Spreadsheets and iNaturalist

[Content for this part would continue from the protocols, but it's cut off in the text]

7 Module 5: Tissue Preparation

7.1 Module 5.1 – Tissue Preparation for Genome Sequencing

7.1.1 Instructor Laboratory Preparation Instructions

Materials and Equipment: - Autoclave - Laminar Flow Hood (if available) - Cellophane - 100x15mm Petri Dishes - 60x15mm Petri Dishes - Agar, Bacteriological - Potato Dextrose Agar - Alcohol Lamp or Bunsen Burner - Forceps - Scalpel (make sure the handle is metal, as it will be flame sterilized and plastic will melt)

Sterility: If you don't have a laminar flow hood, you may create a similar environment by setting up 2 torches over your work space. First, clean your work area with bleach, and/or ethanol. Set the torches facing each other with enough space to work underneath. Light the torches and pour media into plates under the flames.

Potato Dextrose Agar (PDA) Plates: Fungi cultured from leaves on water agar are transferred to nutrient Potato Dextrose Agar (PDA) plates. We use one 60x15 mm Petri dish for each fungal isolate.

Potato Dextrose Agar Recipe: Suspend 39 grams Agar, Bacteriological in 1000 ml (1 L) distilled water. Heat to boiling to dissolve the medium completely (in most cases, this step can be skipped as autoclaving will dissolve media). Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

When the media is cool enough to handle with autoclave gloves, pour 60x15mm plates in a sterile environment. Do not let it cool too long as the media will solidify. Each 60x15mm plate holds approximately 8 ml media. If plates will be used immediately smaller amounts of media can be poured into plates. If plates will be stored, use larger amounts. Store plates in the original petri dish sleeve in the refrigerator (~4 °C) until use.

Cellophane Plates: Fungal tissue can be harvested from cellophane plates. Cut cellophane discs slightly smaller than the petri dish so that they lay flat on the PDA media. Using the bottom of the petri dish as a template, draw a circle on the cellophane and the cut out discs on the inside of the line.

Place cellophane discs into an autoclavable container with water. Place each cellophane disc individually into the water and try to stagger them in the water. This staggering step ensures

that you can more easily remove the discs from the water. Cover your container with foil and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Working in your sterile environment, place one cellophane disc onto cooled PDA agar in 60x15mm plates. Use sterile forceps to transfer each cellophane disc onto the PDA plate. If you can't easily remove single discs, try the "slide" technique. Dip your forceps into the container with the discs until you touch a disc. Using the forceps as a probe (not pinched) slide the disc to the side of the container and up the side of the container. Often, you'll pull out one disc. Sometimes more than one disc will slide up, but it's usually easy to see the individual discs at this point. You can then use your forceps to pull out one disc. If you grasp one disc with your forceps, you can swirl it in the water in the container to dislodge any other discs that might be attached. Open the lid of the petri dish and lay the cellophane onto the solid agar.

7.2 Module 5.2 – Tissue Preparation for Genome Sequencing

7.2.1 Purpose

This protocol guides you through the culturing and harvesting of fungal tissue for molecular applications.

7.2.2 Introduction

The long-term goal of this endophyte isolation project is to develop a robust dataset of fungal genomes. We are piloting this project in our course to determine if whole genome extractions are possible using home laboratory techniques. If successful, the project will be expanded in several directions. Any data generated from this project will be made available on the Mycocosm Web Portal. These data can then be used by students and researchers to analyze fungal genomes. Beyond phylogenetic inferences and evolutionary reconstructions, a variety of research questions can be addressed using fungal genomic data. Genomic data can elucidate the functions of these fungi and contribute to an understanding of their biology. In addition, if this pilot project is successful, future studies will incorporate stress factors, such as salts, metals, or temperatures, to understand the morphological, physiological and genetic responses of fungi to environmental stressors.

This pilot project is being conducted in collaboration with mycologists and mycology students across North America. In addition to Oregon State University, students from the University of Colorado, both Denver and Boulder, Denver Botanic Gardens, Colorado State University, and Duke University, are collecting tissue and isolating fungal endophytes from plant hosts. Genomic data will be generated by the Joint Genome Institute. This is the first project of its kind and the most comprehensive project investigating endophytes on a large geographic and taxonomic scale.

To prepare your fungal tissue for full genome sequencing, you will transfer some mycelia to a petri dish with cellophane.

Once fungal endophytes have been cultured from host plants, these fungal cultures will be identified using ITS (the fungal barcode region) sequencing. The ITS sequence will provide some level of taxonomic placement of your isolate, perhaps even to species. This ITS identification step also allows the determination of which fungal cultures to submit for full genome sequencing.

7.2.3 Sub-Culturing on Cellophane

1. Clean your workspace as described previously. Work under flames or in a laminar flow hood if available.
2. Label your Cellophane lined Petri Dish appropriately.
3. Sterilize implements (forceps and scalpel).
4. Scrape mycelia from PDA plates that you have been growing. Be sure to avoid transferring any media as much as possible.
    a. Leaving the assay plate on the bench space, remove the parafilm of the inoculation plate and slightly crack open the lid using your non-dominant hand.
    b. Insert your forceps into the crack of the petri dish and attempt to sample some mycelium.
        i. If you are unable to remove mycelia with your forceps, use your scalpel to remove mycelia with the least amount of agar possible.
5. Transfer the mycelia to a petri dish with cellophane.
    a. Carefully remove the chunk of agar. Replace the lid. Slide the sample plate away. Slide a cellophane plate towards you.
    b. Crack open the cellophane plate using your non-dominant hand, and carefully insert the forceps with the agar and mycelium.
    c. To deposit the agar onto the PDA agar, submerge the forceps into the agar on the plate. This should force the agar and mycelium from your forceps, onto the PDA agar.
    d. Pull your forceps straight out to leave the agar and mycelium on the plate.
    e. The mycelia will be sticky, so you might need to scrape it off using sterile implements. If you're using forceps to transfer, use the sterilized scalpel to scrape mycelia off the forceps onto cellophane in the petri dish.
6. Seal petri dish with parafilm.
7. Allow fungi to fill the cellophane.
8. Repeat this process with all fungal isolates that will be sent for genome sequencing.

7.2.4 Harvesting Mycelia for Genome Sequencing Instructions

Full genome sequencing requires large amounts of tissue to be successful. The tissue grown on the cellophane plates will be harvested, dried, and sent for genome sequencing.

7.2.5 Student Lab Supplies

- 1.5 ml Eppendorf tubes
- Sharpie
- Scalpel
- Forceps
- Silica
- Probe

7.2.6 Overview

The goal of this supplement is to prepare fungal tissue for DNA extraction and sequencing. The cellophane overlay method was implemented to obtain pure tissue conducive to clean DNA extractions (Fig. 1).

Figure 1 - Using aseptic techniques (step 1), prepare agar plates and arrange a sterile location for transfer. Add one disk of sterile cellophane to each plate (step 2) and inoculate with the fungal isolate, avoiding the transfer of residual agar, in the center of the disk (step 3). Allow the fungus to grow on top of the cellophane in a dark incubator at 26°C(step 4). Take the initial weight of the empty Eppendorf tube. After the cellophane disk is covered, peel the tissue to remove it and place it in the empty Eppendorf tube. Place the open tube in a jar with silica gel in the bottom. Cover the jar. Allow the tissue to dry for 4-7 days (step 5). Once the tissue is dry, close the tube and take the final weight. 1-2 grams of tissue should be recovered. Store the sample in a clean place. It is now ready for in-house DNA extraction or shipping. Figure created in BioRender. Knight, C. (2025) https://BioRender.com/s61a377

7.2.7 Instructions

1. You will be harvesting fungal tissue from the cellophane PDA petri dishes.
2. Use a laminar flow hood or set up flames to create a sterile environment. Clean your sterile area.
3. Light your flame source. Dip your harvesting implement(s) (scalpel, forceps, and/or probe) into alcohol. Flame your implements and let it cool.

4. Once cool, open your petri dish lid, and lay this facing upward on your workspace. Remove fungal tissue by scraping from cellophane. In some cases, the entire fungal tissue will easily peel from the cellophane. In some cases, the mycelia will be stuck to the cellophane. In this case, you will need to use some combination of instruments to remove the fungi from the cellophane (Figs 2 and 3, Videos 1 and 2).
5. Place Eppendorf tubes into a jar filled with silica. The silica will allow you to stand up the tubes in the jar. Keep the lids of the Eppendorf tubes open to allow the tissue to dry.
6. Seal silica-filled jar with the lid. Allow the tissue to dry for 5-10 days.
7. Once the tissue is dry, remove the Eppendorf tubes from the silica jar and close the lids.
8. Proceed to the Sample submission module.

8 Module 6: Sample Submission

8.1 Module 6.1 – Myco-Ed Sample Submission SOP

8.1.1 Overview

Use this document to submit fungal samples to Arizona Genomics Institute (AGI) for DNA extraction.

8.1.2 Definitions

- JGI = Joint Genomics Institute
- AGI = Arizona Genome Institute

8.1.3 Associated Documents

MycoEd Metadata Form

8.1.4 Process Steps

1. Create a JGI Single Sign on Account
2. Email photos of the live tissue you intend to send AGI before collection to JTA-LAG@email.arizona.edu and kwbarry@lbl.gov.
3. Make a copy of the MycoEd Metadata Form
    a. Click on MycoEd Metadata Form link
    b. Under file, select Make a Copy
    c. Rename your new copy [University][Instructor Last Name][MycoEd Metadata][Date]
4. Fill out your copy of the MycoEd Metadata Form
    a. Professor is the instructor for the class and their email address
    b. Name of the University/Institution is your University/Institution
    c. Date submitted/shipped is the date you ship the samples to AGI
    d. JGI proposal name & number is DS 509937 (already provided)

5. Fill out Sample Table
    a. Enter requested metadata into the table
    b. Use one row per sample/tube
    c. Insert additional rows if needed
    d. Columns with gray field headers, B and D, will be filled out by JGI
    e. For Column C, Sample Name - please use below convention for naming: [University][Plant][Plant tissue] Metagenome [Sample ID]
    f. Column G is only needed if you are sending more than 22 samples
    g. Student is the full name of student that collected the sample and their email address
    h. Columns with Yellow Header, X-AF, will be filled out by AGI after DNA extraction
6. Email form to AGI & JGI
    a. Once all required metadata is provided, download a copy to excel
    b. Email a copy to Jayson at (jtalag@ag.arizona.edu) and Kerrie (kwbarry@lbl.gov).
    c. Include in the subject line of your email "MyCoEd 509937" [Professor's Name][University/Institution] "Sample Submission"
    d. Include in your email your name, your college/university, the exact name of your samples (column D) that are ready to ship to AGI (and the rows in this sheet).
    e. Wait for approval/confirmation from AGI & JGI.
        i. If needed make requested changed and email updated copy
7. Prepare samples for shipment
    a. A) Print out (in Landscape orientation) the paper copy of the Myco-Ed Metadata Form and put it with the shipment and also email the completed sheet to: JTALAG@email.arizona.edu, and kwbarry@lbl.gov
    b. Email FEDEX tracking # when your shipment is picked up.
    c. Ship samples to Jayson Talag 1657 E. Helen St. The University of Arizona Tucson, AZ 85721 United States Ph: 520-626-9596

8.1.5 High Molecular DNA Extraction Protocol

The AGI uses the following high molecular DNA extraction protocol:

High molecular weight DNA is extracted from (biomass) using the protocol of Doyle and Doyle (1987) with minor modifications. Flash-frozen biomass is ground to a fine powder in a frozen mortar with liquid nitrogen followed by very gentle extraction in 2% CTAB buffer (that includes proteinase K, PVP-40 and beta-mercaptoethanol) for 30min to 1h at 50 °C. After centrifugation, the supernatant is transferred to a new tube, treated with 200ul 50mM PSMF for 10 minutes at room temperature then gently extracted twice with 24:1 Chloroform : Isoamyl alcohol. The upper phase is transferred to a new tube and 1/10th volume 3 M Sodium acetate is added, gently mixed, and DNA precipitated with iso-propanol. DNA precipitate is collected by centrifugation, washed with 70% ethanol, air dried for 5-10 minutes and dissolved

thoroughly in elution buffer at room temperature followed by RNAse treatment. DNA purity is measured with Nanodrop, DNA concentration measured with Qubit HS kit (Invitrogen) and DNA size is validated by Femto Pulse System (Agilent).

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem Bull. 19:11–15.

9 Module 7: Genomic Analyses

9.1 Module 7.1 – Sexual Reproduction in Ascomycota Genomic Analysis

9.1.1 Introduction

Fungal sexual reproduction is complicated. Unlike animals, where usually only 2 opposite mates are observed, some fungi can have hundreds to thousands of different mates (Casselton & Olesnicky, 1998)! Beyond granting the ability to mix genetic material, sexual reproduction has additional importance in fungi since reproductive structures are often more hearty and capable of overwintering, meaning their presence can seriously complicate disease management practices. Undergoing sexual reproduction also provides an important cleaning mechanism for some fungi, enabling them to treat their genomes and remove transposons using processes like Repeat Induced Point mutations, or RIP (Cambareri et al., 1991). In this exercise, we will learn how to determine mating types in Ascomycota using MycoCosm. Compared to most other fungi, Ascomycetes have a relatively simple mating system, where only two types are observed. Here, we will focus on the genus Neurospora where the mating types are simply named "A" and "a" (Metzenberg & Glass, 1990).

What we know for this exercise: Several mating type-related genes have already been identified. These control the initiation of sexual reproduction. They are mostly transcription factors, which you can think of as highly specialized workers: each has a unique 'skillset' (genes) that they control (turn on or off) to perform some task (in this case, regulate reproduction-related machinery).

How are mating types determined in fungi? Are the same genes found in both mating types, but regulated differently? Or, are there genes unique to each mating type that are responsible for this? Using comparative genomic approaches, let's try and find out!

Beginning with known mating type-related genes collected from NCBI, we will use MycoCosm to branch out into related fungi, identify opposite mates, then formulate hypotheses and learn about the genetics underlying fungal sexual regulation. This approach can be directly applied to predict mating types in other fungi, or used to explore presence/absence of other types of genes!

9.1.2 References:

- Casselton, L.A., Olesnicky, N.S., 1998. Molecular Genetics of Mating Recognition in Basidiomycete Fungi. Microbiol Mol Biol Rev., 62(1): 55–70. doi: 10.1128/mmbr.62.1.55-70.1998
- Cambareri, E.B., Singer, M.J., Selker, E.U., 1991. Recurrence of repeat-induced point mutation (RIP) in Neurospora crassa. Genetics, 127(4):699-710. doi: 10.1093/genetics/127.4.699.
- Metzenberg, R.L., Glass, N.L., 1990. Mating type and mating strategies in Neurospora. Bioessays. 1990 Feb;12(2):53-9. doi: 10.1002/bies.950120202.

9.1.3 Finding mat A

9.1.3.1 Locate mat A locus in the Neurospora crassa OR74A genome

To guide our exploration, let's create a testable hypothesis. Being informative and straightforward, let's hypothesize that mating type is determined by the presence/absence of specific genes. Given what we know about mating in Neurospora, a good starting point would be the mat A locus. It is composed of 3 genes, mat A-1, mat A-2, and mat A-3.

1. Start by visiting NCBI (https://www.ncbi.nlm.nih.gov/) and collecting a reference sequence. Go to the nucleotide database and search for "Neurospora crassa OR74A mating-type protein A-1 (matA-1), mRNA".
2. Find the matching record, "Neurospora crassa OR74A mating-type protein A-1 (matA-1), mRNA" and collect the corresponding nucleotide sequence by clicking the 'FASTA' link (see below), then select and copy the sequence.
3. Let's find the location of this gene in the genome! Go to the Neucr2 MycoCosm portal (https://mycocosm.jgi.doe.gov/Neucr2) and search for it using the BLAST tool.
4. Click the BLAST tab, paste your sequence into the query sequence box. Using blastn, select the filtered model transcripts set and click the 'Run blastn' button.
5. You should see two hits, each shown in a separate row.

    - The first column shows the portal ID and transcript ID (since we are searching against nucleotides) of the hit. Portal IDs are unique identifiers assigned to each genome. For example, Neucr2 is the portal ID for Neurospora crassa OR74A, and it can be used to access the portal (e.g. https://mycocosm.jgi.doe.gov/Neucr2). This unique identifier is also displayed across various portal pages to label data from a particular organism.
    - The second column shows an alignment of that sequence against your query, which is helpful for seeing how well hits cover your query sequence.
    - The score and EValue columns are also useful when assessing hit quality. Higher scores are better, and conversely, lower e-values are better.

6. The first hit looks better. You can tell because the hit covers more of our query and the score is higher. Let's explore the protein page for the best hit and look for evidence that this is actually mat A-1. Click on the identifier in the first column to access this page.
7. Let's explore the genome browser by clicking on the genome coordinates at the top of the page, in the 'Location' section (Above image, red arrow).
8. After loading is complete (this might take a little while, lots of data are on this browser!), click on the -10x in the top middle portion of the page to get a bigger view (see image below).
9. The genome browser will show you information we have available for a particular stretch of DNA sequence in your assembly. It contains details on whether we observed the same (or similar) stretch of DNA in a related organism (Vista tracks), predicted genes in that region/locus, and any other information we have. Other information can include messenger RNA expression (mRNA) levels, locations of blast hits for proteins from other fungi, and more! All of these other tracks can provide valuable information to understand fungal biology as well as evidence for validating gene predictions.
10. You just found the mat A locus in N. crassa! In the position box in the upper left corner, you can see your location: Supercontig_1:1853928-1868957. Remember, if you are lost, every MycoCosm page has a HELP! tab you can access from the menu bar.
11. Now that we are at the mating type locus, let's look at the genes. In this case, genes were predicted at the Broad Institute instead of the JGI. Click on the small '+' to the left of the 'BroadModels' track to view them.
12. Note that some regions have 2 overlapping genes. These are called 'isoforms', which are variants of the same gene where RNA can be sliced in different ways to produce alternative transcripts and proteins (these are rare in fungi).
13. In this region of the genome, you can see 5 genes (2 with isoforms). To learn more about what these genes might do, we can look at their protein pages. You can get to the protein page by clicking on the gene, then selecting 'feature' web page (or center mouse click on the gene to open it in a new tab). For convenience, here are links to the protein pages for all 5 genes in this region: NCU01961T0, NCU01960T0, NCU01959T0, NCU01958T0, NCU01957T0.
14. Using the information on the protein page, can you find mat A-2 and mat-A3?
15. At the mating type locus, do you notice anything interesting when exploring the read depth coverage plots (pink tracks) from related Neurospora genomes?
16. Based on this information, let's revisit our hypothesis about how fungal mating type is determined. Given that many related Neurospora crassa genomes are completely missing this region, this appears to support our hypothesis that fungal mating type is determined by gene presence/absence, and specifically suggests that this locus might be what we are looking for! This would mean that the fungi without this locus are of the opposite mating type. Let's continue to test this hypothesis and see if it holds up to further scrutiny!

9.1.3.2 Exploring mating type in related Neurospora

Let's try to find mat A genes in related genomes. We can start by selecting a representative gene and then searching gene families present on the mcl clusters page.

1. Go to the MCL clusters tab. The MCL page displays information on gene families present across a set of related genomes. You can use it to filter gene families and identify those that fit specific criteria you are interested in.
2. We can also use this page to search for specific proteins of interest. Using the protein ID you gathered earlier from either mat A-2 or mat A-3, in the 'filter by keywords' field, type: portalID.proteinId (e.g. Neucr2.487), then hit enter.
3. You should see a single cluster appear. It looks like this gene is missing from one of these genomes! Actually, all 3 genes from the mat A locus are missing in this lineage. This is not surprising given what we saw earlier on the genome browser (Step 14 from the 'Finding mat A' section).
4. Hover over the lineage name and you will get a clue as to why this might be. Specifically, this strain is named Neurospora tetrasperma FGSC 2509 mat a, telling us that the organism lacking these genes is the opposite mate!
5. You can take a closer look at gene families by clicking on the cluster number to the left of the donut plots (see above figure). This page will show you details on all genes (rows) that are part of this gene family. Click on the synteny view and make some observations.
6. Genes rearrange all the time in fungi, leading to poor conservation of gene neighborhoods. As you can see from this view, the genes surrounding the mating type locus are an exception, where across all species they tend to keep the same order. Feel free to head back to the full mcl clustering run (here) and click around on some families to see how different it really is!

9.1.3.3 Finding mat a

Let's repeat this exercise with the mat a locus to confirm our suspicions, but instead of using nucleotide sequence, let's try another approach: using protein sequence.

1. Start by searching the NCBI protein database for "mating-type protein Mat a-1, partial Neurospora discreta" and collect the protein sequence.
2. Let's find it on the mat a genome portal you found earlier for N. tetrasperma strain FGSC 2509, (https://mycocosm.jgi.doe.gov/Neute_mat_a1).
3. Can you find the corresponding protein using blastp against the 'Neurospora tetrasperma mat a v1.0 filtered gene models (proteins)' set?
4. The best hit is to N. tetrasperma protein ID 124723, can you explain why?
5. Let's revisit the Neucr2 mcl clustering page and search for this, again by typing portalID.proteinID (e.g. Neute_mat_a1.124723) in the search field.

6. Do you notice anything different about this cluster compared to the mat-A cluster? Explore this family to learn more - click the on the cluster number (#771) and take a look at the synteny view again.
7. It looks like our mat a gene is found in a region that is not present in any of the others, which are all the opposite mating type (mat A)!
8. With this information, we can say that all our current evidence supports our hypothesis that fungal mating types are driven by presence/absence of specific genes, mat a and A in particular!

9.1.3.4 Annotating mating type loci in Myco-Ed (and related) genomes

Let's apply what you learned! Pick a MycoEd genome from our group portal - can you identify its mating type and find the mating type locus?

1. Identify mating type genes from a related fungus on NCBI and go through this exercise again on your own. Note that not all mating type genes will be the same as those from N. crassa. Consequently, using the mat a or A genes you identified earlier as bait might not work to fish out mating type genes in other groups of fungi. You will have to find these on your own!
2. If you cannot find a gene to use as bait from NCBI, try using the search function on MycoCosm! Try searching for something like 'mating-type' and see what sorts of annotations come up for the resulting list of genes.
3. Go through the same exercise to search mcl clusters. Do you see a similar pattern to what we observed in N. crassa?
4. For these MycoEd genomes, you can add your own text to describe what you discovered about any particular gene! This will be of great value to the scientific community, especially those studying fungal sexual reproduction.
5. Once you have identified the mating type locus, go to the corresponding protein page and click 'view/modify manual annotation'.
6. Next, add your description! You should see 'add/edit' buttons on the next page. For the 'Description' attribute, select 'add', then enter details on what you discovered about that gene and hit 'save item'. For example, if you found mat A-1, a useful description would be 'mating type protein A-1'.
7. Your name will be associated with your entry and by the following day, it will become searchable across the portal! This means anyone who visits this genome can easily find mating type loci, and know who to thank (you!) for this work.
8. Using your exploration of MCL clusters, you might also be able to characterize mating type loci in related fungi. If you do, please share the identified portal, protein ID, and your text description with your instructor. In addition, please share your name and email address so we can attribute these discoveries to you!

9.2 Module 7.2 – Fungal Natural Product Production Genomic Analysis

9.2.1 Introduction

Fungi are famous producers of natural products that become new drugs, including anticancer agents (Kumar et al., 2019), cholesterol-reducing products (Seenivasan et al., 2008), and a variety of diverse antibiotics. One of the most famous examples is penicillin, the first antibiotic. Discovered by Alexander Fleming in 1929 as a fungal contaminant on his bacterial cultures, his observation that the fungus (Penicillium rubrum; reclassified later as P. chrysogenum) could kill bacteria (Fleming, 1929) led to the development of penicillin, saving countless lives during World War II and beyond. Like most of these interesting fungal products, they are secondary metabolites. Secondary metabolites are typically produced by organisms to provide a competitive advantage in a given environment (e.g., antibacterials for protection, toxins for causing disease, etc).

Core to the production of any secondary metabolite is a 'backbone' gene. These backbone genes can be very strange, such as Non-Ribosomal Peptide Synthetases (NRPSs), which are specialized machines that enable the formation of non-standard products. This can even include amino acids outside of the standard 20 essential for life! NRPSs are large proteins that contain repeated modules, each including an Adenylation (A), Peptide Carrier Protein (PCP), and condensation (C) domain (Streiker et al., 2010). Combined, these allow the NRPS to add amino acids to a growing chain, producing unique molecules. These molecules are then tweaked by decorating enzymes (Keller, 2019) that modify whatever the backbone gene produces, allowing fungi to produce a multitude of bizarre molecules that can wreak havoc in other cells! Decorating genes are physically located next to backbone genes, allowing us to define Biosynthetic Gene Clusters (BGCs) responsible for the production of specific metabolites.

In this exercise, let's follow up on Alexander Fleming's discovery and find the BGC that controls penicillin production. We will then use comparative genomic approaches to discover other fungi that are likely producers of penicillin and variants thereof (which could be candidates for creating exciting new antibiotics), then apply our knowledge to characterize new, unexplored BGCs!

What we know for this exercise: From Fleming's work, we know penicillin is produced by Penicillium chrysogenum. An NRPS is responsible for penicillin production and in P. chrysogenum the penicillin BGC contains three genes: pcbAB, pcbC, and penDE. Which of these is the backbone gene? Can we find it in related Penicillium genomes? Can you identify any fungi that might produce novel penicillin variants? Using comparative genomics approaches, let's begin by identifying the penicillin BGC in P. chrysogenum, then formulate hypotheses and learn about its distribution across fungi. The same approach can be used to characterize diversity and distribution of other BGCs, even ones where we don't know the metabolite they produce yet!

9.2.2 References

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9.2.3 Finding the Penicillin Biosynthetic Gene Cluster

9.2.3.1 Locate the known cluster in P. chrysogenum

1. To start, let's retrieve a sequence from NCBI that can help us locate the Penicillin BGC. We know there are 3 genes in this cluster: pcbAB, pcbC, and penDE. Go to NCBI (https://www.ncbi.nlm.nih.gov/) and search the 'Nucleotide' database for pcbAB
2. Select the first record, titled "Penicillium chrysogenum PcbC (pcbC) gene, partial cds; pcbC-pcbAB intergenic spacer, complete sequence; and PcbAB (pcbAB) gene, partial cds". This corresponds mostly to the DNA in between pcbC and pcbAB, which will be a great tool for identifying very similar regions in other genomes!
3. Click the 'FASTA' button to see the nucleotide sequence, then copy the sequence.
4. Now, let's find this region in the Penicillium chrysogenum genome in MycoCosm using BLAST. Here, we can browse the genome and conduct comparative analyses across related fungi. Go to the Penicillium chrysogenum genome portal (https://mycocosm.jgi.doe.gov/Pench1) and click on the BLAST tab.
5. Paste in your nucleotide sequence and conduct a blastn analysis against the assembly. Remember, we are using the sequence that mostly falls between two genes here to locate our region of interest, so we need to search the assembly instead of the available gene model sets.
6. You should see a single hit! Click on the coordinates to view this region of the genome.
7. Of course, we are searching for DNA that is mostly found in between these penicillin-related genes. Click -10x to get a bigger view of this locus.
8. Excellent! You just found the Penicillin Biosynthetic Gene Cluster (scaffold_1:6815459-6827128)! You will notice that there is no nucleotide conservation with the genomes

presented in the Vista tracks at the top of the page. Let's expand the GeneCatalog by clicking on the '+' next to the track and look at a few of these genes.

9. In this region of the genome, you can see 4 genes. To learn more about what these might do, we can look at their protein pages. You can get to the protein page by clicking on the gene, then selecting 'feature' web page (or center mouse click on the gene to open it in a new tab). For convenience, here are links to the protein pages for all 4 genes in this region, named by their protein IDs (which are the main identifiers used within MycoCosm): 67014, 32451, 67324, 22458
10. As the backbone gene is central to the production of a specific secondary metabolite, let's use it to discover other fungi that might make similar products! On the 22458 protein page, click on the green bar underneath the domain information section (see figure immediately above). Copy this sequence to your clipboard, we will use it in the next section.

9.2.3.2 Find candidate Penicillin BGCs in related fungi

1. With our NRPS bait in hand, let's try to fish out similar genes in related fungi! But before we do, do you have any hypotheses about how often it will be found?
2. On MycoCosm, you can view individual genomes, or analyze groups of them simultaneously. There are several group page categories, including: Project groups, which contain genomes associated with specific research projects. For example, our own MycoEd project group! EcoGroups, which contain genomes of organisms with shared ecology, e.g. plant pathogenic fungi. PhyloGroups, which contain genomes of taxonomically related organisms.
3. You can access groups either using the Groups pulldown menu on the MycoCosm homepage, or if you know the group of interest you can type it into the url. For this exercise, let's visit the Penicillium phylogroup page.
4. You can see we have over 70 Penicillium genomes available for comparative analysis! Genomes are colored differently based on their status, red = private, only available to you and your colleagues, white = publicly available, green = published.
5. With so many genomes available, we have a great dataset with which we can test our hypothesis that all Penicillium produce penicillin! There are several routes we can take to explore this, but for this exercise let's again use BLAST. On group pages, this tool allows us to search all these genomes simultaneously. Click on the BLAST tab to get started.
6. Paste the protein sequence you copied from the P. chrysogenum Penicillin NRPS into the query sequence box. Select the blastp alignment program and the Gene Catalog protein database. Confirm that your search criteria are correct, then click 'Run blastp'. Note that this is a large protein and we are searching a lot of genomes, so this might take a few minutes.
7. After a few minutes, you should see results. We get a lot of hits (1,929 of them!). The top hit should almost always be to the original backbone gene you selected. To assess quality take a look at the alignment, which shows how well the hits cover your query sequence. Also take a look at the 'Score'. The higher the score, the better.

8. Can you find where hit quality begins to drop off? You can see by just looking at the top 10 that quality drops off really fast!
9. Where to define cutoffs is somewhat arbitrary and depends on your research question. For this exercise, let's keep it simple and set this cutoff by calculating a Blast Score Ratio (BSR; Rasko et al., 2005). By dividing a given score by the score of the query against itself, we can get a feel for how good a match is compared to the best possible match.
10. With this new information, let's revisit our hypothesis. Given that we see only 7 really good matches to the penicillin NRPS out of the ~70 available genomes, it seems that our first hypothesis was incorrect, meaning not all Penicillium species produce penicillin.
11. Let's revise it! Maybe the Penicillium genus is too diverse and only a small subset of closely related fungi can produce this product! Perhaps a compelling new hypothesis would be that all penicillin producers are closely related evolutionarily.
12. Record the names in the organism column from our BLAST output for the top 7 hits. We can use the precomputed phylogenetic tree from the 'TREE' tab to continue our exploration and test whether they are closely related or not.
13. Open the 'TREE' tab, either in a separate tab or a new window so you can easily see both your blast results and the phylogeny.
14. Here, we will see a pre-computed phylogenetic tree showing evolutionary relationships within this PhyloGroup. The node colors on the tree indicate support values, green: 1.0, orange: 0.7, red: < 0.7, white: no support. This phylogenetic tree is generated based on amino acid sequence alignments from hundreds to thousands of proteins across these fungi. If a particular node is well supported, it means that a lot of the underlying sequence alignment agrees with the relationships shown in that part of the tree. In contrast, if support is low, we should be suspicious of the relationships depicted there.
15. At the top of the page is a search box where we can type in some of our organisms of interest to see where they are on the tree. Try typing in Penicillium chrysogenum. Hit enter (or the 'Filter' button) and see what happens!
16. You will see that the tree compresses and highlights the lineages that match your search, in this case, strains of P. chrysogenum. Everything else will be grayed out. Click 'Expand All' and you can see where they are in the larger Penicillium tree. Click the clear to restore everything to default colors.
17. Looking over this tree, can you find the 7 fungi from our BLAST results that contain our intact Penicillin NRPS? Are they found in the same part of the tree, or all over the place?
18. We can point to 3 clades where this NRPS is found, with most being present in Clade 2 (see above image). However, in all cases there are very close relatives which lack it. Overall, this suggests that the ability to produce penicillin is pretty variable across the Penicillium genus. While phylogeny can provide some clues, it alone cannot be used to predict the ability to produce penicillin.
19. This tells us that our hypothesis that penicillin producers are all closely related evolutionarily is incorrect (again!) - and that is OK! Based on our current results, we can conclude that penicillin production is both dispensable (as clearly not all Penicillium can make it) and not phylogenetically driven.
20. This knowledge raises a ton of new hypotheses, such as: i) The Penicillin BGC arose in

these fungi due to horizontal gene transfer, ii) The Penicillin BGC is associated with specific ecological niches, iii) The Penicillin BGC is restricted to specific geographic locations, iv) etc… Using MycoCosm, NCBI and other online bioinformatics tools, feel free to explore some of these ideas!

9.2.4 Characterize Myco-Ed BGCs

9.2.4.1 Find interesting BGCs using Secondary Metabolite (SM) clusters page

Penicillin is a famous example of a fungal natural product. However, fungi are some of the most prolific producers of secondary metabolites worldwide, and there is a lot to discover here! Let's do some exploratory work and characterize secondary metabolites produced by MycoEd fungi.

1. Pick a genome from our MycoEd group portal. For these MycoEd genomes, you can add your own text to describe what you discover about any particular gene. This will be of great value to the scientific community, especially those studying fungal natural product diversity!
2. Keep in mind, what you are doing here is completely new - you might be the first person ever to look at these fungal natural product genes! If you observe something unexpected or cannot find similar genes in related genomes that is OK, and could reveal something very exciting! Documenting your work, manually curating genes (see 'Annotate them!' section, below), and sharing it with your instructor will be a big help to future investigators.
3. Let's set a goal to describe the distribution of fungal BGC backbone genes, following the same workflow you just did for penicillin.
4. Each MycoCosm portal has a secondary metabolite clusters page, which can be used to study these BGCs in greater detail. On the menu bar for any portal, you will see an 'Annotations' pulldown. Click it, then select 'Secondary Metabolism Clusters'
5. The secondary metabolism clusters page shows a table of BGC counts across a set of closely related genomes. The columns separate different types of backbone genes and the rows are individual genomes. For your MycoEd genome, click on the number in the 'Total' column to see the BGCs found only in this genome, or click on the number within the backbone gene column you are interested in (e.g. NRPS).
6. The following page will show details on all BGCs found. This includes the type of cluster, the location on the genome and its architecture, including the backbone gene (in color) and nearby predicted decorating enzymes (gray). Note that some clusters might be missing decorating enzymes - this could be real, or we failed to predict them.
7. Hovering your mouse over the genes in the cartoon will provide information on those proteins. Pick a cluster and repeat our previous exercise to characterize its distribution. You can start by clicking on the backbone gene in the cartoon, which will bring you to the corresponding protein page. Keep this page open in a separate tab for the rest of the exercise, we will revisit it later.

8. Collect the protein sequence, then search a related MycoCosm Phylogroup. You can find a related group by going to the portal Info page. At the bottom of this screen is a 'Links' section. Select the PhyloGroup containing this organism's closest relatives, which is at the end of the list. If there are only a few genomes (less than 5), try going one taxonomic level up by returning to the info page and selecting the second to last link.
9. Start exploring by conducting a BLAST search! If you have a lot of good hits, make sure to increase the number of alignments shown until you see low-quality hits. Click 'Configure this screen' and increase the number of hits shown to a larger number (e.g. 100); see below.
10. Select any hit that passes the BSR threshold of 0.9 we defined earlier (see Step 9 in the 'Find candidate Penicillin BGCs' section)' and let's see where those fungi are on the interactive tree. The interactive tree is produced only once or twice a year, so your chosen MycoEd genome might not be there depending on when you conduct this analysis. That is OK since we are mostly interested in where these other fungi are found.

9.2.4.2 Annotate them!

1. Once you have completed your analysis, return to the protein page for your backbone gene and click 'view/modify manual annotation'.
2. Next, add your description. You should see 'add/edit' buttons on the next page. For the 'Description' attribute, select 'add' then enter details on what you discovered about that gene and click 'save item'.
3. Your name will be associated with your entry and eventually, it will become searchable across the portal! This means anyone who visits this genome can easily retrieve these details (via the search page), and know who to thank (you!) for this work.

10 Summary

In summary, this book covers the complete Myco-Ed curriculum for BSC 495: The Hidden World's Code. Students will gain hands-on experience in fungal biology and genomics, from sample collection and fungal isolation through DNA sequencing, genome assembly, and annotation. By the end of the course, students will have contributed to high-quality fungal reference genomes and developed broadly applicable skills in lab techniques and bioinformatics. More information and course resources are available at the BSC495-HWC GitHub organization.

11 Carlos C. Goller

Carlos C. Goller is a Teaching Professor at North Carolina State University in Raleigh, NC. He teaches microbiology, life science, public health, and other fun courses as part of the Department of Biological Sciences. When not playing with **Delftia acidovorans** or code, Carlos enjoys spending time with his family (Claire Gordy also at NC State, Amada, and Aurelio) and playing with his dog Sunshine.

11.1 Education

North Carolina State University, Raleigh, NC | Advisor: Dr. Sue Carson | Topic: Biotechnology Education and Curriculum Development

Duke University Medical Center, Durham, NC | Advisor: Patrick C. Seed | Topic: Bacterial Pathogenesis and Anti-infective Screening The Hartwell Foundation Postdoctoral Fellowship | September 2008 - July 2012

Emory University, Atlanta, GA | Advisor: Tony Romeo | Topic: Bacterial Biofilm Formation and Regulation Ph.D. in Microbiology and Molecular Genetics | August 2002 - September 2008

Worcester Polytechnic Institute | Worcester, MA B.S in Biology and Biotechnology | August 1998 - May 2002

11.2 Experience

Teaching Professor | North Carolina State University Department of Biological Sciences | 2024 - Present Associate Teaching Professor | North Carolina State University Department of Biological Sciences | 2019 - 2024 Assistant Teaching Professor | North Carolina State University Department of Biological Sciences | 2015 - 2019

References