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Chytridiomycota in Tree Bark

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CHYTRIDIOMYCOTA IN TREE BARK

by

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ABSTRACT

Chytridiomycota is a phylum of microscopic aquatic fungi that form motile spores that typically have a single posterior flagellum, thus they require water to disperse (James et al., 2000). Chytridiomycota, collectively called chytrids, have round shapes with structures called rhizoids that absorb nutrients and anchor them to their substrate (Mueller et al., 2004). Chytrids are typically found in aquatic environments and soils since zoospores require water to germinate (James et al., 2000), but they also have been found in a number of unexpected environments. Chytrids are difficult to find because they are microscopic and have time-sensitive life cycles (Mueller et al., 2004). Isolation is difficult because chytrid species require specific nutrients for growth and grow less rapidly than filamentous fungi, yeasts and bacteria. Because chytrids have been found in many habitats and an extensive amount of research on their preferred habitat is lacking, my question was could chytrids be observed and isolated from tree bark samples. In this study bark samples and soil at the base of the trees were collected from red maple (*Acer rubrum*) and amur cork (*Phellodendron amurense*) in Pennsylvania, Sunkhaze wildlife refuge in Milford, Maine, and the University of Maine campus in Orono, Maine. A teaspoon of bark or soil was put in gross cultures to bait chytrids. Every sample contained chytrids, abundantly on spruce pollen grain baits and sparsely on onion skin bait. The Internal transcribed spacer (ITS) regions of the ribosomal gene cassette were amplified from extracted DNA for sequencing and will be used to identify the genera of chytrids collected and isolated from samples. The ITS regions amplify highly variable gene sequences that are used to identify fungi. More research is needed, but these findings support that Chytridiomycota can be found on tree bark.

ACKNOWLEDGEMENTS

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INTRODUCTION

Although research has revealed much about plant and animal species and their ecological interactions, much is still unknown about the kingdom Fungi. One reason for the lag in knowledge about fungi is that researchers have problems accurately identifying some species of fungi, especially those in the Chytridiomycota, a phylum whose members are microscopic. Orders in this group are still being defined and rewritten as new information is discovered. Identification is particularly a problem for species in the Chytridiomycota, collectively called chytrids, because species have similar morphologies that require training to accurately differentiate. Mycologists still do not know how many chytrid species exist and what habitats and conditions chytrids can tolerate. Because of the rapid pace at which the world is changing, ecologists need to understand fungal species, especially chytrids. Microscopic organisms like chytrids may seem unimportant in the bigger picture of world problems but understanding these species better can give clues to other organism's habitats and life strategies. Chytrids are also important because they break down dead organic matter and continue to feed other life forms around them. Without fungi and bacteria, nothing in the environment would decompose and be beneficial for other life forms. Although some species may never be discovered before extinction, more research, especially in mycology, is needed to restore the natural world to a natural state and to have a better understanding of the environment. A better understanding of the environment and the complex interactions that happen there, will aid conservation and protection efforts aimed at helping an ecosystem, rather than just one species.

Many reasons contribute to the difficulty of identifying chytrids, mainly because they are microscopic and hard to isolate into pure cultures, which are needed to accurately document life stages to better understand and identify to species (Mueller et al., 2004). One of the main reasons that researchers are still identifying species and higher classifications of Chytridiomycota is the similarity in their morphology even though some species can be accurately defined based on thallus morphology, or how the main body of the chytrid looks and develops (Letcher et al., 2004). Identifying chytrids with accuracy without DNA analysis is very difficult. Despite difficulties, researchers have found ways to culture and observe chytrids in the lab using various time-sensitive practices.

Researchers perform a series of steps in order to isolate chytrids into pure culture. First, a sample from the environment is collected, whether the sample is soil, detritus from a tree canopy, or some other habitat (Longcore, 2005). Samples only need to be about a teaspoon. The sample is placed in what is called a gross culture, which entails covering the sample in deionized water and placing nutrient sources or baits on top. The most common baits are pollen grains from various coniferous tree species, onion skin, and shrimp skin (Mueller et al., 2004). These baits are used as a new source of nutrients for chytrids to colonize so that scientists can observe the organisms on a slide under the microscope. This process is time sensitive because it usually only takes one to two days for chytrids to colonize pollen grains, and about four to seven days to colonize cellulose baits such as onion skin (Mueller et al., 2004). Chytrids colonize different baits at different times because various species degrade specific biological compounds like chitin and cellulose and prefer one nutrient source over another. Once chytrids can be observed

on pollen grains, they can be transferred to an agar plate with antibiotics to obtain a culture. Antibiotics are necessary to suppress bacterial life that may overgrow the plate and destroy the chytrid culture (Mueller et al., 2004). To identify chytrids without DNA analysis, the developmental stages of growth need to be documented, including the zoospores, the morphology of the zoosporangium, the rhizoidal systems (filaments that anchor the main chytrid body into substrate), and the generation time. All of these factors together can help scientists and mycologists to identify chytrid samples (Mueller et al., 2004). Even with all of the tools and information to identify chytrids, researchers still need a highly trained eye to detect small differences in morphology. These methods are still used today to find chytrids from field samples, but because so few mycologists study this group, the number of chytrid species and the habitats where they can survive is unknown.

Since chytrids are difficult to identify by morphology, DNA identification is the widely accepted method for identifying chytrids (Joyce Longcore, personal communications). The large subunit of ribosomal DNA is accepted for identifying chytrids and the adjacent inter transcribed spacer regions (ITS1 and ITS2) with the 5.8S gene are widely used to identify fungi and is referred to as the "bar-coding region" (Schoch et al., 2012). ITS regions are called the "bar-coding" regions for fungi because they have the "highest probability of successful identification for the broadest range of fungi" (Schoch et al., 2012). ITS1 and ITS2 are highly variable which makes them useful for identifying fungi at species level. These ITS regions are helpful for identifying fungi, but they are not helpful for identifying what order a chytrid is in.

DNA sequencing has become the accepted method of chytrid identification because many aspects of chytrid morphology are similar and not easily described (Joyce Longcore, personal communication). The ITS region has highly conserved genes flanking the highly variable regions that are used to identify what genus or species the sample belongs to. DNA is amplified using primers in these conserved ribosomal genes because they help copy the "bar-coding region" used to identify fungal DNA. Most DNA sequences are stored in Genbank (GenBank ® is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences) so that researchers can compare future samples to known DNA.

Researchers have found evidence of chytrid populations all over the world including in ice from the Arctic and dung from domesticated animals such as horses (Simmons et al., 2012). Researchers Letcher and Powell, along with McGee (2004), have found chytrid species in forest soils from the mountains of Virginia as well as soils in South Wales in Australia, and these researchers have also described the distribution of chytrids in soil versus moss covered soil. These researchers used methods for chytrid collection similar to those described above and found interesting results. When examining samples from the Appalachian Mountains, scientists found 14 different chytrid species and only 8 of those could be classified from previous scientific findings (Letcher & Powell, 2001). Other species could be grouped into genus, but they lacked qualities to identify to species. Researchers also noticed that while distribution among sample sites was similar, all sample sites were not the same and that some chytrid species were in every sample while some were only in a few and others were only at one site (Letcher & Powell, 2001). Another study by the same researchers found 34 chytrid species in

samples from various soil types ranging from sub-tropical rainforest to dry evergreen or "sclerophyll" forests, of which 15 were new to Australian records (Letcher et al., 2004). Similar results of distribution have been found in other studies by the same researchers, but what exactly makes a habitat more suitable for different species of chytrid is still unclear. All of the studies mentioned here were conducted from samples of soil collected from various habitat types, but chytrids have also been reported from detritus collected from tree canopies. These findings are unique because chytrids have flagellated spores that require water to disperse, but tree canopies lack puddles or bodies of water for spores to move through (Longcore, 2005). Five species of chytrids were isolated from tree canopy samples from Australia and New Zealand that have been previously found in soil samples, but this habitat also contained other chytrids that could not be identified to species (Longcore, 2005). One of the chytrids, *Spizellomyces*, isolated from tree canopy detritus samples and previously found exclusively in soil, was the most common genus isolated from samples (Longcore, 2005). Longcore suggested that dormant resting spores may be blown by wind to the tree canopy waiting to germinate until conditions are favorable (Longcore, 2005). These unexpected findings were the inspiration for my research and directed my thoughts to possible chytrid habitats. Can chytrids be isolated from tree bark?

My hypothesis is that chytrids can be isolated from tree bark. This is a new research area, and to test this hypothesis of chytrids in tree bark, I followed methods similar to those described above to retrieve chytrids from bark samples, isolated samples into pure culture when possible, and extracted DNA from cultures.

MATERIALS AND METHODS

Sample Collection

To collect samples, I went out into the field with a pocket-knife, plastic sandwich bags, a notebook, pencil, and measuring tape. I recorded the diameter of the tree sampled as well as the approximate height of where I sampled. I used a pocket-knife to shave off pieces of bark without causing much damage to the tree and collected about two teaspoons of bark. I also used the pocket-knife to move leaf litter at the base of the tree and to loosen up soil for collection, and again collected about two teaspoons of soil. Soil was collected as a positive control since past soil studies have found an abundance and diversity of chytrid presence in soils. Between sample collections I cleaned the pocket-knife blade by wiping it with a cloth. Each sample went into a separate bag labeled with the date and sample type (Table 1).

Table 1: Date, location, type of sample and height collected, tree diameter and chytrid presence of samples collected

Date collected	Location	Type of tree[1]	Chytrid Presence in bark	Chytrid Presence in soil	Tree Diameter (cm)[2]	Height of sample (cm)[3]
1/20/20	Easton, near Morgan Hill, Pennsylvania	N/A	yes	N/A	N/A	~95
3/11/20	Oak point trail, Sunkhaze Wildlife Refuge, Milford, Maine	Red maple, *Acer rubrum*	yes	N/A	54	~95

Table 1 continued						
Date collected	Location	Type of tree[1]	Chytrid Presence in bark	Chytrid Presence in soil	Tree Diameter (cm)[2]	Height of sample (cm)[3]
6/21/20	Carter Meadow Rd trail, Sunkhaze Wildlife Refuge, Milford, Maine	Red maple, *Acer rubrum*	yes	yes	33	~95
7/9/20	Carter Meadow Rd trail, Sunkhaze Wildlife Refuge, Milford, Maine	Red maple, *Acer rubrum*	yes	yes	74.5	~95
7/25/20	Carter Meadow Rd trail, Sunkhaze Wildlife Refuge, Milford, Maine	Red maple, *Acer rubrum*	yes	yes	40.5	~95
8/19/20	Carter Meadow Rd trail, Sunkhaze Wildlife Refuge, Milford, Maine	Red maple, *Acer rubrum*	yes	yes	43	~51
9/11/20	Outside Littlefield Garden, University of Maine, Orono, Maine	Amur cork, *Phellodendron amurense*	yes	yes	>152	~51

Table 1 continued						
Date collected	Location	Type of tree[1]	Chytrid Presence in bark	Chytrid Presence in soil	Tree Diameter (cm)[2]	Height of sample (cm)[3]
10/8/20	Outside Littlefield Garden, University of Maine, Orono, Maine	Amur cork, *Phellodendron amurense*	yes	yes	>152	~51
10/8/20	Near Doris Twitchell Allen Village buildings, University of Maine, Orono, Maine	Red maple, *Acer rubrum*	yes	yes	38	~51

[1]N/A means not available
[2] > symbol means the diameter was greater than 152cm, restricted by measuring-tape length
[3] ~ symbol means approximately, sample heights were not measured directly but waist and knee measurements were used to approximate sample heights

Baiting Samples

After collecting samples, I made gross cultures to bait for chytrids. To start gross cultures, I put bark and soil samples in separate glass fingerbowls and covered them with distilled water, so that samples were submerged (Figure 1). Each fingerbowl was labelled with the date it was established, the sample type and what type of tree it came from. Next a light sprinkle of spruce pollen on top of the water as well as two to three small pieces of boiled onion skin in the water acted as bait for chytrids. I covered the top of the fingerbowl with another bowl and let this sit for about three to four days at room temperature before examining the pollen grain baits and examined the onion skin baits after about ten days.

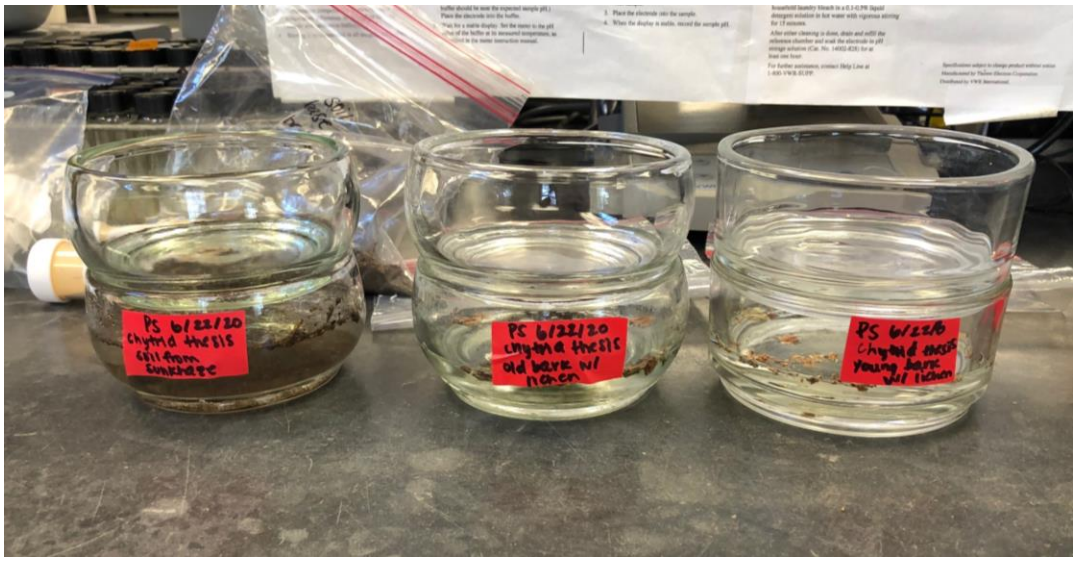

Figure 1: Gross cultures in glass fingerbowls from samples collected in June

Sample Examination

I prepared a slide of pollen by taking a coverslip and gently touching it to the top of the water at an angle to pick up pollen on the water surface. If this did not work, I used a pipet to pick up pollen from the water surface. I examined pollen grains under the compound microscope and inspected them for any chytrid bodies, either inside or outside the pollen grain. I recognized chytrids either by a walled, round body inside a pollen grain or spherical bodies on the pollen surface, especially in the middle of the pollen grain (Figures 1, 2).

I checked onion skin baits after about ten to fourteen days because chytrid species that colonize cellulose substrates have a longer life cycle and growing time than those that colonize pollen grains (Mueller et al., 2004). I examined the onion skin by picking up the onion skin with forceps, placing a few drops of water on a clean slide and putting the onion skin on the water droplets. Then I covered the onionskin and water with a coverslip and looked at it under the compound microscope for any chytrids (Figure 2 and 3).

Chytrid Isolation

Depending on how the rest of the pollen grains looked, meaning more or less chytrid growth or if pollen was overgrown with yeast and bacteria, I either placed pollen directly on agar isolation medium, or tried to establish a sub-culture with similar steps as a gross culture. For a sub-culture I used a plastic petri dish and transferred pollen from the slide into the dish with distilled water by using a squirt bottle, then filled the petri dish about one third with distilled water and put a fresh pinch of pollen on top for chytrids to infect. In two or three days I checked pollen under the compound microscope to check if more chytrids grew and for any reduction in unwanted growth such as yeast, bacteria, or filamentous fungi. If the pollen from the subculture looked better than previously, I plated pollen on PmTG medium (0.5g peptonized milk, 0.5g tryptone, 2.5g glucose, and5g agar in 500mL distilled water with 200mg/L Penicillin, and 200mg/L Streptomycin sulfate added after autoclaving). To plate pollen, I used a dropper with distilled water to wash pollen from the slide and coverslip onto the medium and tried to disperse pollen around the plate by gently tipping the plate in a circular motion so that water spread over the medium. I labeled each plate with the date and sample information, then sealed the plate with parafilm around the edges. For onionskin, I set up subcultures of distilled water in plastic petri dishes that contained only the onionskin and small pieces of boiled, sterilized cellophane. These dishes were also labelled with the date they were established and the sample they came from, and again these subcultures require more time for chytrid growth because they have different lifecycles. Once chytrids colonized the cellophane, cellophane was plated on the same medium described above and followed the same isolation and transferring method described below.

After plating pollen on PmTG medium, I checked on the growth of anything on the plate every day for two to three days by using the dissecting microscope. I checked plates every day in order to isolate viable chytrid growth before any other mold grew over the plate. If I observed chytrids growing on the medium, I isolated them by flaming a needle to sterilize it, waited for it to cool down slightly, then cut out chytrid colonies while looking under the dissecting microscope and placed them on a new plate of PmTG medium. After checking the new isolate plate to make sure that there was only chytrid growth and no bacteria in the sample, I transferred chytrids to an mPmTG medium slant tube. The medium recipe for mPmTG slant tubes is slightly different, it contains 0.2g peptonized milk, 0.2g tryptone, 1.0g glucose, 6g agar in 500mL distilled water. I stored tubes at room temperature to allow chytrids to grow on the medium. Once I could see new growth on the mPmTG medium, I refrigerated slant tubes to slow growth and maintain samples for future examination.

DNA Extraction

For DNA extraction, I added approximately 25mL of liquid PmTG broth into 50mL plastic tubes and inoculated the broth by transferring 1mL of broth with growing chytrid cultures using a 5mL sterile pipet under a hood from chytrid cultures that were previously inoculated by Joyce Longcore. I also inoculated broth by flaming a needle, cutting out pieces of medium with growing chytrid cultures and transferring them to broth tubes. Once I saw grainy substances in the liquid broth, the culture was put in a centrifuge to separate chytrids from broth for DNA extraction.

DNA Extraction Procedure

Once grainy substances were visible, I centrifuged the 50mL tubes with liquid broth and chytrid cultures at 4000 RPMs at 4°C for 20 minutes. After this was completed, I discarded most of the liquid leaving less than 1.5mL. This liquid was transferred to microcentrifuge tubes and centrifuged again at 13000 RPMs for 5 minutes. Liquid was removed again, and I put in a pestle in the tube before freezing samples in liquid nitrogen to make grinding easier. Sample tubes were held in liquid nitrogen until the liquid and chytrid culture material froze and the samples were ground with the pestle. I added 500µL of 2X CTAB extraction buffer (2% (w/v) CTAB, 100mM Tris, 20mM Na-EDTA, 1.4M NaCl) to the tubes and vortexed until mixed. The tubes were incubated at 65°C for 60 minutes and vortexed once during the incubation. After 60 minutes, I centrifuged tubes at 13000 RPMs for one minute to remove any condensation from tube lids. After this, I added equal amounts of chloroform to the tubes, about 500µL, and gently shook tubes to form an emulsion. DNA dissolves in the water partition and chloroform separates quickly in tubes, so they needed to be inverted a few times to maintain the mixture. The tubes were centrifuged at 13000 RPMs to form a clear supernatant, which was a clear layer of liquid on top of the tube. The supernatant was transferred to new 1.5mL microcentrifuge tubes without disturbing the interphase between layers. Then chloroform was added again, centrifuged again and supernatant was transferred to new microcentrifuge tubes two more times. Once these steps were completed, I added 2/3 of the volume of cold isopropyl alcohol and inverted tubes to mix them. Once they were mixed, I put the tubes in the freezer to incubate at -20°C for 60 minutes. After incubation tubes were centrifuged again at 13000 RPMs for 7 minutes to form a pellet of DNA at the

bottom of the tube. When pellets were visible, the alcohol was poured off into a waste container and liquid that was left was pipetted out without disturbing the pellet. Then 1mL of cold 70% ethanol was added to each tube to wash pellets. Ethanol was also poured off and pipetted out like isopropyl alcohol, then left to air dry in the hood for a few minutes. Lastly, 50µL of TE buffer was added to resuspend DNA and concentrations were checked using a Nanodrop. Samples were stored in the -20°C freezer.

PCR DNA Amplification and Gel Electrophoresis

For PCR, or Polymerase Chain Reaction, I removed microcentrifuge tubes from the freezer and let them defrost on ice. Reactions were set up with a 200µL total volume master mix using components in Table 2. This table describes the volume the stock solutions to add to make the Master Mix components for the eight PCR reactions that were performed. To calculate these amounts, I multiplied the standard amount for one PCR reaction from the procedure by eight, since there were 7 PCR reactions: six DNA samples and one negative control.

Table 2: Volume and list of components added to PCR Master Mix to equal 200µL volume

Component	Volume for Master Mix (µL)
H_2O	132.4
Taq Buffer	40
dNTPs	4
$MgCl_2$	6
Primer 1	8
Primer 2	8
Taq	1.6

I pipetted 20µL of master mix and then 5µL of DNA into each tube to make a 25µL PCR reaction in 500µL microcentrifuge tubes. The negative control used 5µL of sterile Milli-Q water instead of DNA. The primers used were ITS1 and ITS4 (White et al., 1990) to amplify part of the 28S, ITS1, 5.8S, ITS2 and part of the 18S ribosomal RNA cassette. The PCR reaction ran for two hours and eight minutes to amplify DNA. The primers ITS1 and ITS4 were used because these primers are called universal primers and are widely used in mycology to identify fungi to genus and even species. These primers copy the "bar-coding region" of genes that consist of highly variable and conserved regions of DNA that aid in species identification.

I created a 1.2% agarose gel by mixing 0.36g agarose with 30mL TBE buffer and microwaving the mixture for 30 seconds, then intervals of 7 seconds to diffuse agarose and create a homogeneous mixture. Once all agarose particles were dissolved, the gel mixture was poured into the gel electrophoresis apparatus with 10 wells formed and allowed to cool. 1µL of DNA staining dye was mixed with 4µL of each PCR reaction. A total of 5µL of each sample was loaded into the gel. A low mass base pair ladder, the six different DNA samples (Table 4) and negative control (sample of water with master mix) were loaded on the gel. The gel was covered with TBE buffer and run at 97V for 40 minutes. The gel was photographed under high UV light (Figure 9), but the gel was unusable for isolating the DNA bands because the UV light intensity could have affected the quality of DNA.

To extract the bands, I made a 1% agarose gel by mixing 0.6g agarose with 60mL of TBE buffer using the same method described above. The gel mixture was poured into the gel apparatus with 20 wells instead of 10 and allowed to cool to a solid. 1µL of DNA

staining dye was mixed with 10µL of each PCR reaction and 11µL of each sample were loaded into the gel, with two wells loaded for each PCR reaction with an empty well in-between. A low mass base pair ladder, the six different DNA samples (Table 4) and negative control (sample of water with master mix) were loaded on the gel. The gel was run at 97V for 45 minutes. To clean up the DNA bands and prepare for DNA sequencing, bands of DNA were cut out under low UV light (Figure 10) and cleaned up using QIAEX II Gel Extraction Kit. Once DNA was cleaned up using the gel extraction kit, the concentrations were checked using a Nanodrop Spectrophotometer (Table 4).

DNA Sequencing

The cleaned-up DNA will be sequenced using the ITS1 and ITS4 primers to sequence forward and reverse DNA strands. Once the DNA is sequenced, the sequences will be put into BLAST to compare against DNA in GenBank (GenBank ® is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences) to see if any of the chytrids from my samples are already described, or if there are new species of chytrids found on tree bark.

RESULTS

Chytrid Presence

The majority of the field work was completed in the summer months between July and August, but the initial sample was collected in January (Table 1). I collected samples from a wide range of locations, the first was in Pennsylvania near my hometown and the rest were in locations around Maine. For the most part samples were collected from Sunkhaze wildlife refuge that sees little human activity. I only ever saw other cars driving on the road in the wildlife refuge and no one on foot near sample sites. However, other samples collected on campus and in Pennsylvania were close to homes and roads that get a lot of traffic and activity (Table 1). The height of the sample is included in Table 1 to demonstrate importance of where samples were collected on the trees. Sample tree diameter ranged from 33cm in diameter to over 152 cm (Table 1). Despite differences of sample sites, collection times, tree diameters, tree species, and sample heights, chytrids were observed in every sample collected (Table 3). Chytrid growth in pollen grains were observed in all samples collected (Figure 2 and 3) but chytrid growth on onion skin was less common from bark and soil samples (Table 3). A variety of chytrids grew inside and outside of pollen grains from soil and bark samples (Figure 2, 3, 4). I observed different chytrids on onion skin in samples collected after 9/11/20 than previously collected samples where chytrids were only observed on spruce pollen (Figure 5 and 6).

Table 3: Chytrid presence in bark and soil samples baited with pollen and onion skin seen in samples collected at different times.

		Presence of Chytrids in Bark Sample		Presence of Chytrids in Soil Sample[2]	
Date	Tree Type[1]	Pollen bait	Onion skin bait	Pollen bait	Onion skin bait
1/20/20	N/A	Yes	No	NC	NC
3/11/20	*Acer rubrum*	Yes	No	NC	NC
6/21/20	*Acer rubrum*	Yes	No	Yes	No
7/9/20	*Acer rubrum*	Yes	No	Yes	No
7/25/20	*Acer rubrum*	Yes	No	Yes	No
8/19/20	*Acer rubrum*	Yes	No	Yes	No
9/11/20	*Phellodendron amurense*	Yes	Yes	Yes	Yes
10/8/20	*Phellodendron amurense*	Yes	Yes	Yes	Yes
10/8/20	*Acer rubrum*	Yes	Yes	Yes	Yes

[1] N/A means not available
[2] NC means not collected

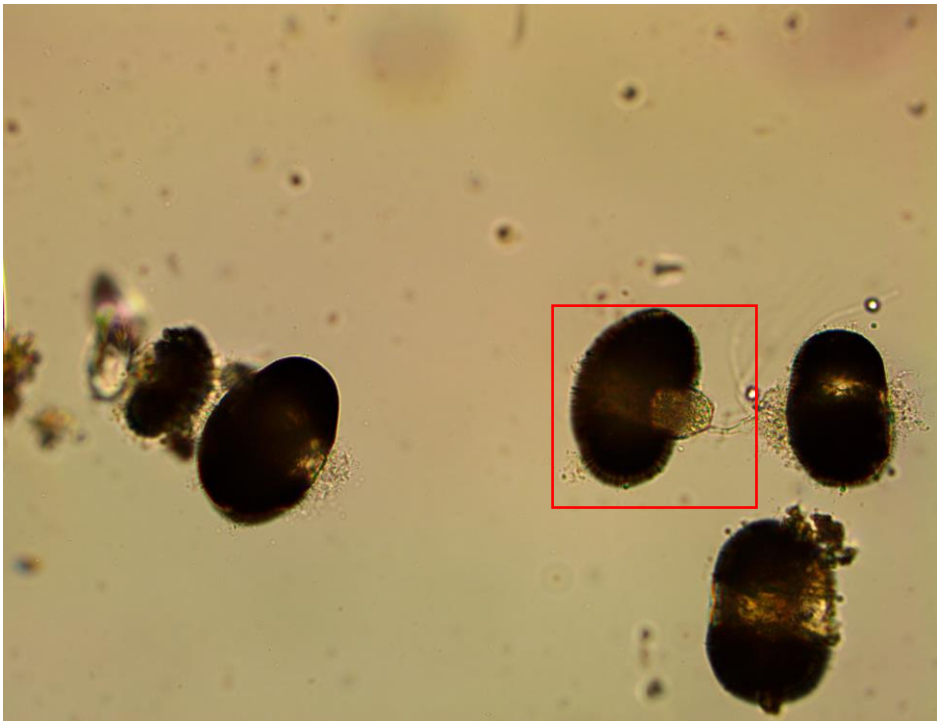

Figure 2: Chytrids growing on pollen grains used as bait for a red maple soil sample collected in July. 100X. The main chytrid is in the pollen grain towards the middle right of the picture, seen growing out of the middle of the pollen grain and a rhizoid attached at the middle of the chytrid body, indicated with a red box.

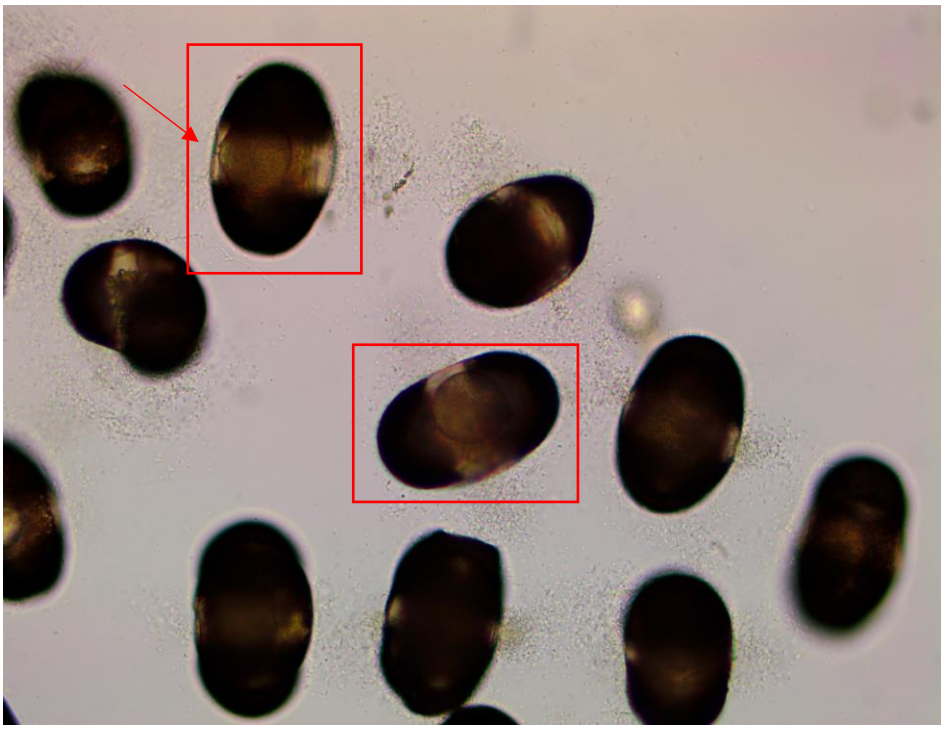

Figure 3: Chytrids inside pollen grain bait from gross culture of maple bark collected in October. 100X. Two different individual chytrids can be seen, indicated with red boxes around pollen grains. In upper left hand corner pollen grain, its discharge papillae is visible, pointing to the left side of the picture, indicated with an arrow.

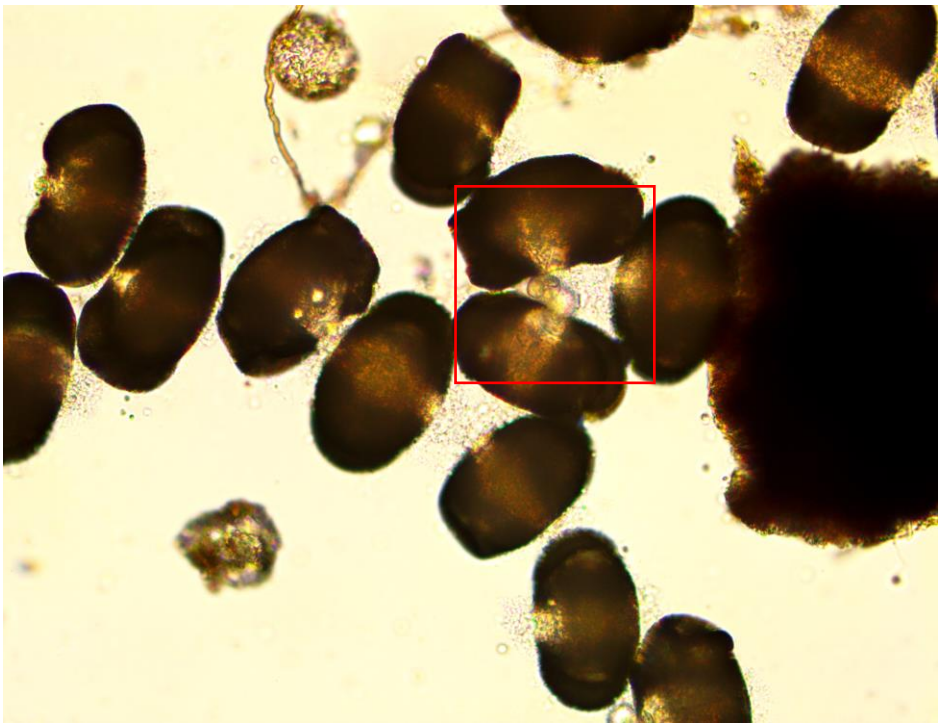

Figure 4: Chytrids growing outside of pollen grain bait for red maple soil sample collected in July. 100X. A few possible chytrids are seen growing on pollen, indicated with a red box.

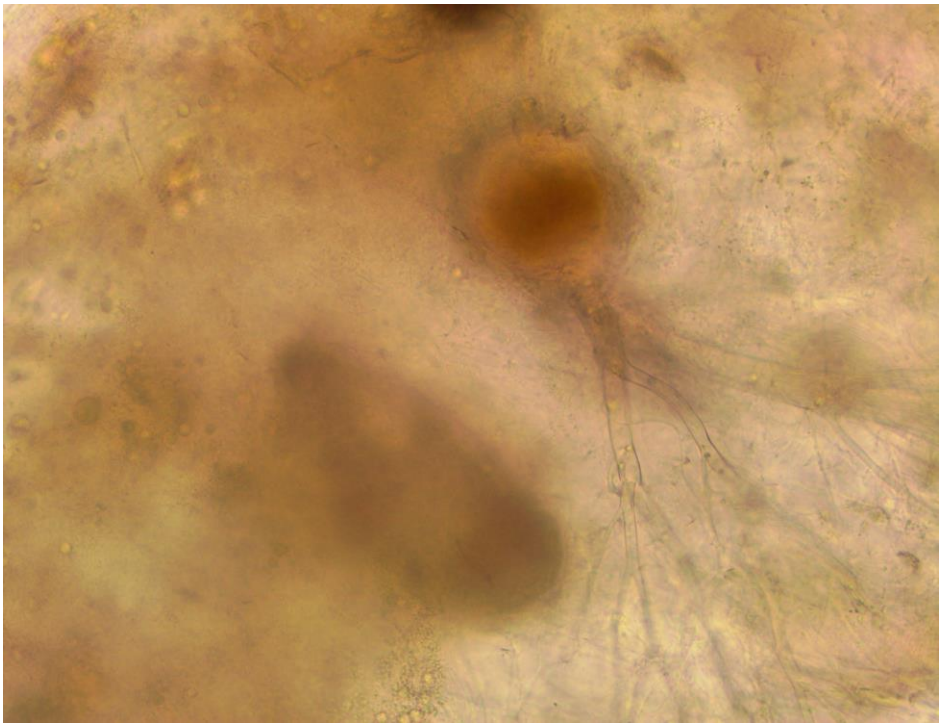

Figure 5: Chytrid in onion skin used as bait with Amur cork bark collected in September. 100X. Joyce Longcore identified this chytrid as a possible *Rhizophlyctis rosea.* The chytrid is large and orange with thick branching rhizoids growing toward the bottom right-hand corner of the picture. An inner section of orange can be seen within the main body of the chytrid closer to the middle of the picture.

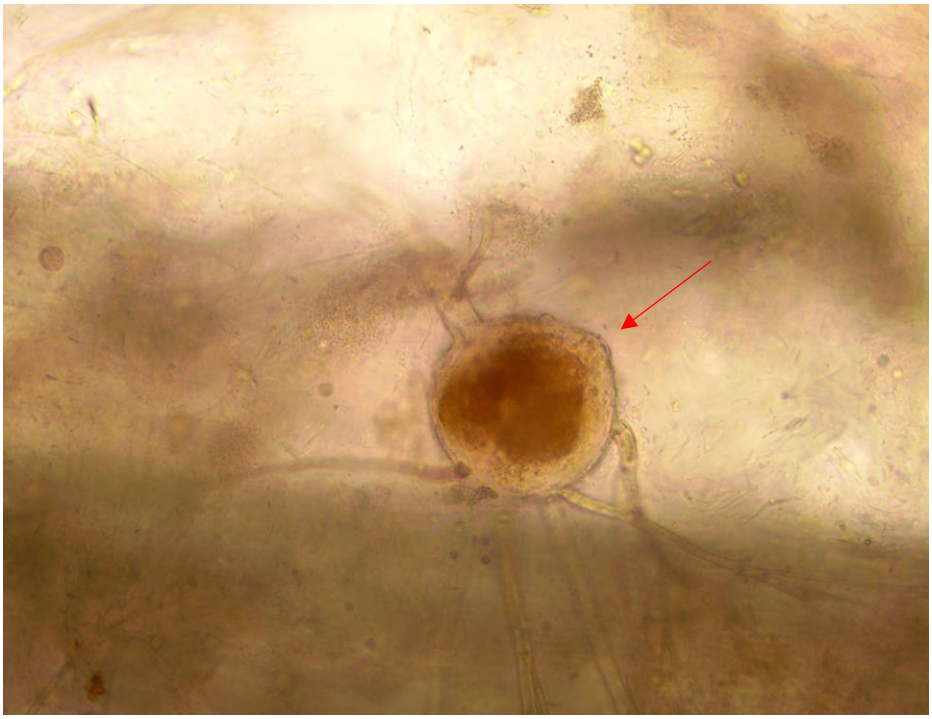

Figure 6: Chytrid in onion skin used as bait with Amur cork bark collected in September. 100X. This picture shows a different individual where its' discharge papilla can be seen pointing towards the upper right corner of the picture, indicated with an arrow. This chytrid has rhizoids branching in all directions and an inner section of orange can be seen within the main body.

Isolation

I was able to isolate the sample PS01 with the help of Joyce Longcore in March from the sample collected in Pennsylvania. I was unable to isolate from later samples on my own, but with duplicate samples given to Longcore, she was able to isolate chytrids from samples on medium and established growth in broth medium. I inoculated broth for DNA extraction from her isolates and the isolate I obtained in March from the sample collected in Pennsylvania (PS01). Isolation of chytrids is difficult even for experts, Longcore has been attempting to isolate the same chytrid from onion skin (similar to ones in Figures 5 and 6) for over a month. Sample isolates used for DNA extraction were from Amur cork tree bark and soil, and PS01. The Amur cork bark and soil samples were

collected in October while the bark sample from which PS01 was isolated was collected in January. Chytrids of various stages were observed in broth from all isolate samples (Figure 7 and 8). All broth samples had a mixture of individual chytrids (Figure 7) and colonies of chytrids (Figure 8).

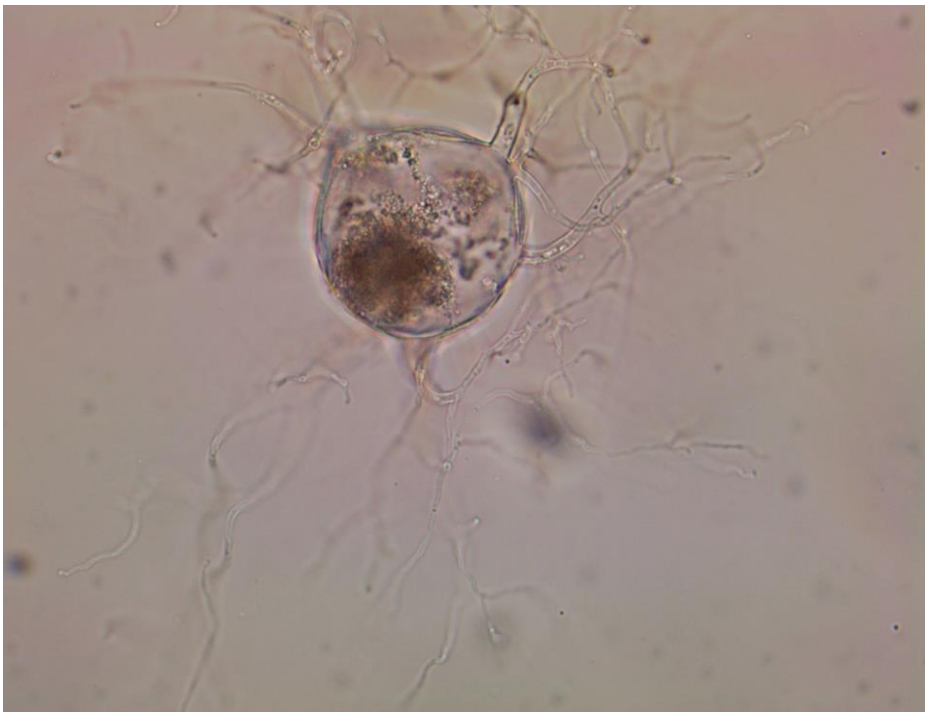

Figure 7: Chytrid from PS01 sample growing in PmTG broth. 400X. An individual chytrid is shown isolated from the first sample collected in Pennsylvania. There are many rhizoids visible in this picture. It also appears as though there are materials moving through the rhizoids to distant regions of the body.

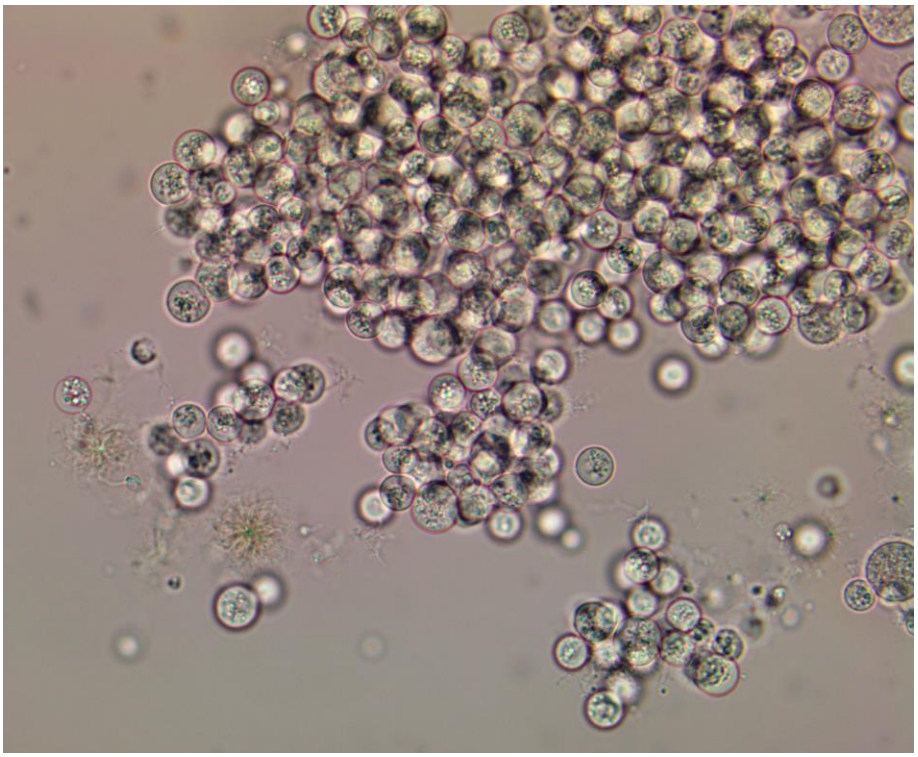

Figure 8: Colonies of the chytrid isolate from Amur cork soil sample growing in PmTG broth. 400X. Large colony of chytrids seen in the top of the picture with smaller colonies and some individuals growing towards the bottom of the picture.

DNA Extractions and PCR

DNA concentrations of the extractions were high, and most samples were over 1000ng/µL before dilution, but 260/280 ratios remained largely the same after dilution (Table 4). Before dilutions the highest average DNA concentration was 2675.9ng/µL from an isolate from Amur cork soil sample and the lowest concentration was 158.5ng/µL from a PS01 sample. After dilutions, the highest average DNA concentration was 35.9ng/µL from an isolate from Amur cork soil sample and the lowest was 12.8ng/µL from a PS01 sample.

Table 4: DNA concentrations, 260/280 ratios from Nanodrop, dilutions, and average concentrations before PCR reactions

Sample[1]	Concentration [ng/µL]	260/280 ratio	Dilutions Amount of stock DNA to add to water to get to 20µL of 10ng/µL	Average Concentration after dilution [ng/µL]	Average 260/280 ratio after dilution
1-Amur cork bark, Rep 1	1104.4	2.21	0.18µL	23.2	2.20
2-Amur cork soil, Rep 1	2675.9	2.18	0.07µL	16.5	2.24
3-Amur cork soil, Rep 2	1010.8	2.20	0.20µL	23.3	2.17
4-Amur cork soil, Rep 2	1369.5	2.18	0.15µL	35.9	2.20
5-PS01, Rep 2	556.8	2.21	0.36µL	18.6	2.14
6-PS01, Rep 1	158.5	2.18	1.26µL	12.8	2.13

[1] Rep 1 means the sample was isolated by Joyce Longcore. Rep 2 means the sample was isolated by Paige Strasko.

PCR reactions contained distinct bands of DNA between 400 and 500 base pairs (bp), and a second band between 300 and 400 bp (Figure 9 and 10). The initial gel was photographed under high UV light, which could have damaged PCR fragments. To

account for this damage, another gel of 1% agarose was made to extract PCR fragments for clean-up (Figure 10). Without clean-up the DNA fragments would not produce a clear gene sequence. Samples in the 1% agarose gel were loaded with an empty lane in-between samples to allow space for DNA fragments to spread and allow space for bands to be extracted. This second gel also had very distinct bands between 400 and 500 bp but the fragments were more spread out. There were also bands between 500 and 600 bp, as well as very faint bands between 100 and 200 bp (Figure 10).

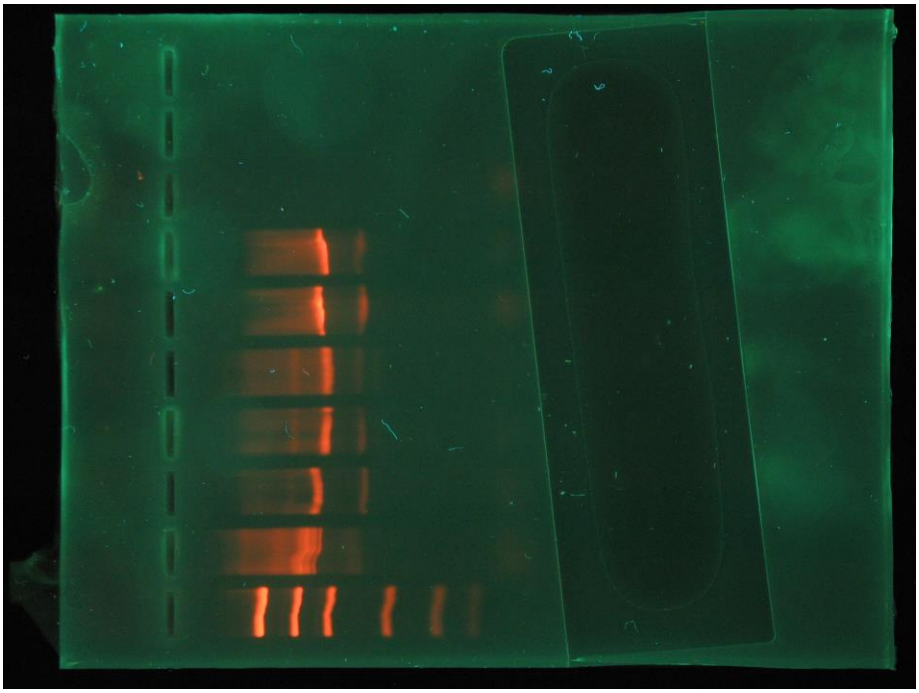

Figure 9: 1.2% agarose gel showing bands of DNA between 400 and 500 bp photographed under high UV light. Bands visible from PCR amplification of samples. First lane had a low mass ladder, lane two had sample 1, lane three had sample 2, lane four had sample 3, lane five had sample 4, lane six had sample five, lane seven had sample 6, and lane eight had negative control PCR reaction.

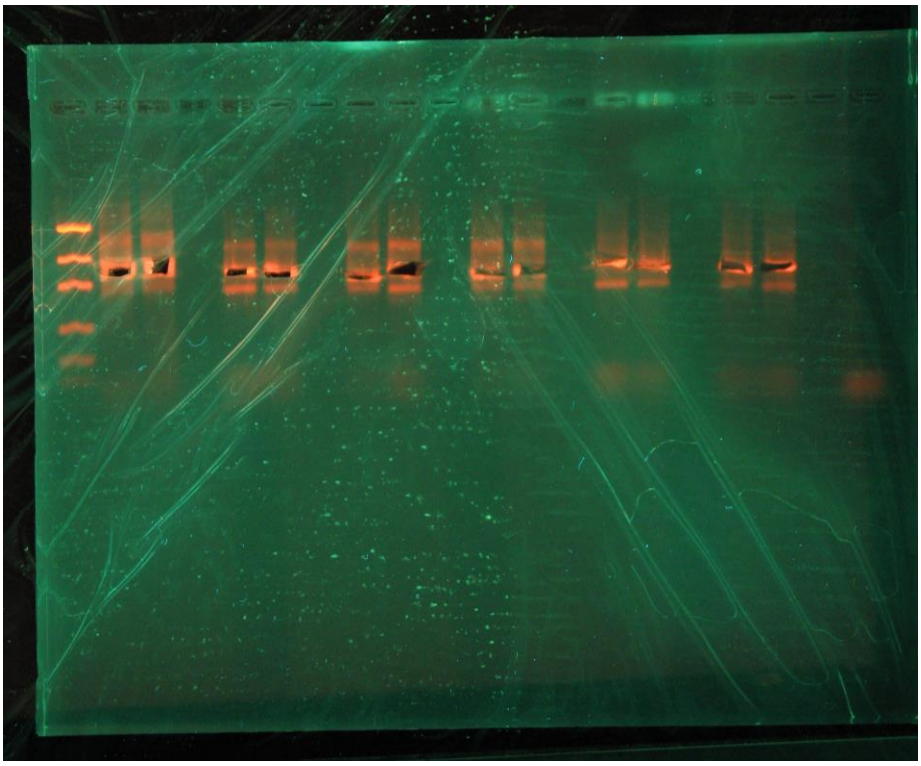

Figure 10: 1% agarose gel after bands of DNA were cut out for clean-up DNA bands cut out were between 400 and 500 bp. Clear bands can be seen where gel was cut out. Lane one had a low mass ladder, lane two and three had sample 1, lane five and six had sample 2, and so on like Figure 9. There was only one lane with negative PCR control reaction.

After clean-up with gel extraction kit, DNA concentrations and 260/280 ratios were all very similar to each other (Table 5). The range of concentrations was from 4.33ng/µL to 5.27ng/µL and range of 260/280 ratios was from 2.02 to 2.72.

Table 5: DNA concentrations of the PCR fragments after QIAEX gel extraction and clean-up

Sample[1]	Concentration [ng/µL]	260/280 ratio
1-Amur cork bark, Rep 1	4.37	2.02
2-Amur cork soil, Rep 1	4.33	2.65
3-Amur cork soil, Rep 2	5.27	2.72
4-Amur cork soil, Rep 2	5.23	2.53
5-PS01, Rep 2	4.60	2.57
6-PS01, Rep 1	4.60	2.40

[1] Rep 1 means Joyce Longcore isolated the sample. Rep 2 means Paige Strasko isolated the sample.

DISCUSSION

Sample Collection

Samples collected earlier in the summer were sampled at greater heights, closer to my waist height than knee height (Table 1). These results demonstrate that chytrids are deposited on tree bark, or are persisting on tree bark, by means other than splash back from rain in soil. Sample heights collected were too high for rain to splash resting spores from the soil onto tree bark. While this was not necessarily the case in later samples collected on University of Maine campus, it is interesting because all samples collected had chytrid growth regardless of height sampled or location. The range of dates that samples were collected from tree bark and observed to grow on pollen grain baits indicates that chytrid resting spores are present at any time of year on bark and if they are in the right environment, the spores germinate readily. This can be seen in the fact that samples used for DNA extraction procedures were collected nine months apart. Tree bark may also be a regular habitat for chytrids since samples collected from two states all had resulting chytrid growth.

Another interesting factor of collection was the wide range of sites where I collected samples. Even though the initial sample was collected in January from a sapling in a parking lot in Pennsylvania and the sample was not placed in gross culture for over a month, a chytrid was still observed and isolated. Water is needed for chytrid zoospores to disperse, but the initial sample from Pennsylvania and samples collected on the University campus were not near water, not even puddles of rainwater. Although I did not observe puddles of water at some of the sample sites, bark would still get intermittently wet from precipitation. This raises more questions as to how chytrid resting spores are

deposited on these locations and further research is required. The range of time that samples were collected, the various locations where I collected samples, and differing heights of sample collection all support the hypothesis that chytrids can be observed on tree bark. As the season progressed, increasing amounts of yeasts confounded efforts to isolate and I tried sampling other tree species. I thought that different tree species might affect results, but they did not. Other researchers have found 15 different chytrid species in soils associated with birch, oak and dogwood tree species (Letcher & Powell, 2001). At the beginning of the experiment, I thought tree species might affect results because of differences in bark growth and chemical composition of tree species, but this research further supports my findings that chytrids are present across a range of conditions and more research is required to understand the relationship between chytrids and tree bark.

Baiting and Examining Samples

Baiting and examining samples posed their own issues because the two baits used, spruce pollen and onion skin, attract different species of chytrids that have different spore germination and growth periods. Chytrids that colonize pollen normally accumulate within two to four days while chytrids that colonize onion skin appear in ten to fourteen days (Mueller et al., 2004). Because of these differences, and the time sensitivity of chytrid growth cycles, I may have examined the onion skin bait too soon and too late for some of the pollen grain baits. Giving too much time before examining pollen grain baits may have caused the overgrowth of bacteria, fungi, and yeast that have faster growth cycles than chytrids.

Isolation

Isolation was very difficult for samples collected after June because of growth of yeast and bacteria. When samples collected in July, August and September experienced higher growth of yeast and chytrids, it was more difficult to determine whether or not pollen grains had chytrids at all. This was because the yeast growth I observed was very similar to chytrid growth, but in much larger quantities. Both have circular bodies that grow on pollen grains, but yeast is much smaller, and the small circular fragments of the yeast were visible in the surrounding water on the slide. When I did see chytrids on or inside pollen grains I used distilled water to wash the pollen from the slide into the isolation plate, which may have directed bacteria from the gross culture onto the plate. The amount of water used to wash pollen grains onto medium also affected the amount of yeast growth on plates. More water produced a greater amount of yeast growth that spread over the plate in a shorter amount of time.

I was not able to successfully isolate any chytrids from samples after June because of the difficulty of the isolation process. One of the main problems I encountered while trying to isolate chytrids was mold and yeast growth infecting pollen grains instead of chytrids. Since yeast and chytrids are in the Kingdom Fungi, any antibiotics that could be added to medium to kill yeast would also deter chytrids, so I tried to subculture samples periodically to kill off the unwanted growth. This rarely worked and when I sub-cultured samples, I often saw less chytrid growth than before I established the subculture.

The isolation process itself is difficult because if I did observe a chytrid colony growing on medium, I needed to look under the dissecting scope while marking where the colony was, then continue to look under the dissecting scope while extracting the

chytrid colony. One of the most common ways to isolate pure chytrid samples are by scraping sporangia from baits onto agar medium ("How to Find and Isolate Chytrids - Maine Chytrid Laboratory - University of Maine," n.d.). This involves looking under the dissecting microscope (usually using 40X magnification) using a sterile, sharpened needle to move sporangia onto agar medium. Chytrid sporangia can be cleaned by dragging them through agar or by re-isolating them on new agar plates ("How to Find and Isolate Chytrids - Maine Chytrid Laboratory - University of Maine," n.d.). Without training, it is difficult to keep a steady hand to cut out tiny pieces of medium to transfer. As previously stated, even experts have difficulties isolating chytrids because this process requires cooperative samples, patience, practice, and a very steady hand to keep samples clean of contaminants.

DNA Extraction and PCR

One of the most difficult aspects of this project was the DNA extraction and PCR reactions since I have never worked with DNA before. It was difficult to measure out such small amounts of liquid, and stressful to make sure it was 1µL and not 100µL. For my first attempt at DNA extraction, I did obtain a high amount of DNA with concentrations over 1000ng/µL before PCR reactions. All of the PCR reactions worked the first time and gave clear results on agarose gel. The bands between 400 and 500bp are the typical size of bands in the ITS region used to identify many fungi (Seanna Annis, personal communication).

DNA identification is required for chytrids and other fungi because there is still a lack of knowledge on genetic relationships and classification of chytrid species, especially in the Chytridiales, which is the largest order (James et al., 2000; D. R.

Simmons et al., 2020). The main reason for use of DNA in chytrid identification is due to similarities in morphology. Researchers normally compare sections of rDNA sequences to data in databases like the Collection of Zoosporic Eufungi at the University of Michigan (CZEUM) by aligning 28S and 18S genes that are highly conserved sections of ribosomal genes (D. R. Simmons et al., 2020). By comparing the DNA fragments isolated in this experiment with those in CZEUM and Genbank, I will be able to identify what species of chytrids are found on tree bark and compare those results with findings in the soil.

Related Findings

Similar studies of Chytridiomycota in soil have shown that chytrids are found in very surprising places. Even where plant life cannot survive, like high elevation soils in the Arctic, researchers have found an abundance and diversity of chytrids (Freeman et al., 2009). One variable that may improve findings would be pH of soils at the base of sample trees. In past research, pH of soils was collected as a possible indicator of chytrid growth, or a useful variable to measure, based on previous studies describing factors that influence chytrid growth (Letcher & Powell, 2001). This study observed chytrids in many different soil samples collected over a year, despite that the soil habitats vary widely in elevation, slope, and pH (Letcher & Powell, 2001). Another studied demonstrated that chytrids are present in soils from four distinct vegetation types despite differences in nutrient and soil composition (Letcher et al., 2004). The majority of chytrid findings are related to their existence in soil, but this research does not include information of chytrid presence on tree bark. Research in the field of Chytridiomycota needs to be expanded to gain understanding of their habitats and fill in gaps of knowledge in the genetic tree.

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AUTHOR'S BIOGRAPHY

Paige Strasko was born and raised in Easton, Pennsylvania on December 5, 1998. She graduated from Easton Area High School in 2017 with academic excellence and two different local scholarships. Paige majored in ecology and environmental science with a concentration in natural history and environmental studies. She received many scholarships throughout her academic career including the Class of 1923 scholarship and the Racino scholarship.

Upon graduation, Paige plans to take time to spend with family while pursuing a career in the National Park Service and acquiring additional training for field work.