

University of Tennessee at Chattanooga

UTC Scholar

Honors Theses

Student Research, Creative Works, and
Publications

5-2020

A contribution to the characterization of the diversity of ectomycorrhizal fungi associated with American chestnut at the UTC Fortwood Street nursery

Colton Jones

University of Tennessee at Chattanooga, mjm783@mocs.utc.edu

Follow this and additional works at: <https://scholar.utc.edu/honors-theses>



Part of the [Plant Biology Commons](#)

Recommended Citation

Jones, Colton, "A contribution to the characterization of the diversity of ectomycorrhizal fungi associated with American chestnut at the UTC Fortwood Street nursery" (2020). *Honors Theses*.

This Theses is brought to you for free and open access by the Student Research, Creative Works, and Publications at UTC Scholar. It has been accepted for inclusion in Honors Theses by an authorized administrator of UTC Scholar. For more information, please contact scholar@utc.edu.

**A contribution to the characterization of the diversity of ectomycorrhizal fungi associated
with American chestnut at the UTC Fortwood Street nursery**

Colton Jones

Departmental Honors Thesis

The University of Tennessee at Chattanooga

Department of Biology, Geology, and Environmental Sciences

March 2020

Dr. Hill Craddock
Professor
Biology, Geology, and Environ. Sci.
Thesis director

Dr. Jose Barbosa
Associate Professor
Biology, Geology, and Environ. Sci.
Department Examiner

Dr. Margaret Kovach
Professor
Biology, Geology, and Environ. Sci.
Department Examiner

Table of Contents

I.	Abstract.....	2
II.	Introduction	
	Endo- vs Ectomycorrhizas.....	4-5
	Evolution of the mycorrhiza.....	5-6
	Ectomycorrhizal Morphology.....	7
	Plant Symbionts.	8
	Fungal Symbionts.....	8
	Benefits of Ectomycorrhizal Symbiosis.....	8-9
	American chestnut Background.....	10
	Project Outline.....	11-12
III.	Methods	
	Sampling.....	12-13
	DNA Extraction and Precipitation.....	13
	DNA Amplification.....	13
	PCR Product Purification.....	14-15
	DNA Sequencing.....	15
IV.	Results.....	15-18
V.	Discussion.....	18-21
VI.	References.....	22-27

I. Abstract

Ectomycorrhizas play several essential roles in the biosphere and have immeasurable implications on the ecosystems in which they exist. Much has been discovered about the relationships between ectomycorrhizal fungi and the trees with which they associate, but there is still much to learn. Due to the nature of ectomycorrhizal morphology, DNA analysis is frequently required in order to accurately identify the fungal partner. Some ectomycorrhizal fungi produce above-ground fruiting bodies that presumably contain the same DNA sequences as the fungi encapsulating corresponding plant root tips below the soil; these fruiting bodies have been frequently observed growing in nursery containers at the UTC Fortwood Street nursery. We hypothesized that DNA extracted from fruiting bodies found in these containers would match the DNA of fungi enveloping the tree's root tips. Additionally, we hypothesized that the variety of sequences produced by ectomycorrhizal root tips may display a greater diversity of mycorrhizal fungi than is represented by hypergeous fruiting bodies alone. In the course of this thesis, genomic DNA was extracted from mycorrhizal American chestnut root tips and fruiting bodies found in nursery containers at the Fortwood Street nursery; the DNA underwent PCR and was purified prior to sequencing and BLAST alignment. However, due to complications in the preparation of DNA for sequencing and the finite timeframe provided, the results of this project are limited. An ITS sequence from one fruiting body was successfully amplified and sequenced; the identity of this sporocarp was determined to be the obligate ectomycorrhizal fungus *Hebeloma hiemale s.l.*

II. Introduction

A mycorrhiza is a symbiotic relationship between a plant root and a mycorrhizal fungus. The term, coming from the Greek words *mykos* and *rhizon* literally means fungus root (Carlile et al., 1994). There are at least seven types of mycorrhizas described in scientific literature, each forming unique types of symbioses (Brundrett, 2002). Some of these relationships have existed for hundreds of millions of years; however, their significance has only recently been established and is still not fully understood (Strullu-Derrien et al., 2018). In the case of a mutualistic mycorrhiza, a soilborne fungus becomes physically and chemically associated with a plant's roots; the fungus radiates into the soil, increasing the surface area of the roots and transferring essential nutrients (e.g., phosphorus, nitrogen) back to the plant. In exchange, the plant transfers photosynthates to the fungus; the fungus uses the carbohydrates acquired as its primary source of energy (Carlile et al., 1994). The interactions between mycorrhizal plant roots and fungi involve complex cascades of gene expression and molecular mechanisms that are currently being researched worldwide (Hilbert & Martin, 1988). Benefits provided by mutualistic mycorrhizal symbioses are still being characterized not only in terms of the advantages provided to the host plant by the mycobiont, but also in potential ways mycorrhizas can be used in broader environmental restoration efforts (Blaudez et al., 2000). Distinguishing the species of mycorrhizal fungi colonizing particular plants is the first step to further research concerning the implications of mycorrhizal relationships. However, accurate identification of mycorrhizal fungi proves to be more difficult than identification of the host plant; for this reason, the modern characterization of mycorrhizal fungi often takes a molecular approach (Norris et al., 1994). The diversity of mycorrhizal fungi colonizing the American chestnut is of particular interest at the

University of Tennessee at Chattanooga, as the campus houses a breeding program that works towards re-establishing the vulnerable species in the wild.

ENDO- VS ECTOMYCORRHIZAS

Endomycorrhizas, of which there are several types, make up the vast majority of mycorrhizal relationships in the biosphere; these types of mycorrhizas occur in more than 80% of vascular plants (Brundrett & Tedersoo, 2018). Implied by their name, endomycorrhizas are those that penetrate host root cells and form characteristic intracellular structures used to facilitate the chemical transactions between fungi and their hosts. Ectomycorrhizal (ECM) fungi do not penetrate host cell walls, but are capable of facilitating nutrient exchange through unique extracellular structures. Although only ~2% of vascular plants form ectomycorrhizas, this still accounts for over 6,000 species of plants colonized by 20,000-25,000 species of ECM fungi (Brundrett & Tedersoo, 2018; Christenhusz & Byng, 2016; Tedersoo et al., 2009).

The majority of ECM fungi can penetrate root cells as the roots begin to senesce or if equilibrium between the symbionts is disrupted (Smith & Read, 2017). However, it is rare for an ectomycorrhiza-forming fungus to penetrate host root cells; these types of relationships are only known to occur in *Pinus* and *Larix* spp. and are referred to as ectendomycorrhizas (Yu et al., 2001)

Though uncommon, a few tree genera and some shrubs and ferns are susceptible to colonization by both vesicular-arbuscular mycorrhizas (VAM) and ectomycorrhizas. Chilvers et al. observed an initial colonization of root tips by VAM with relatively high inoculum potential and rapid root colonization, followed by secondary infections by ECM fungi. The ectomycorrhizal fungi outcompeted the VAM later in terms of colonization of lateral roots and hyphal spread, and, while the existing VAM did not inhibit subsequent infection by

ectomycorrhizal fungi, the sheath of the latter formed a physical barrier to colonization by other VAM (Chilvers et al., 1987).

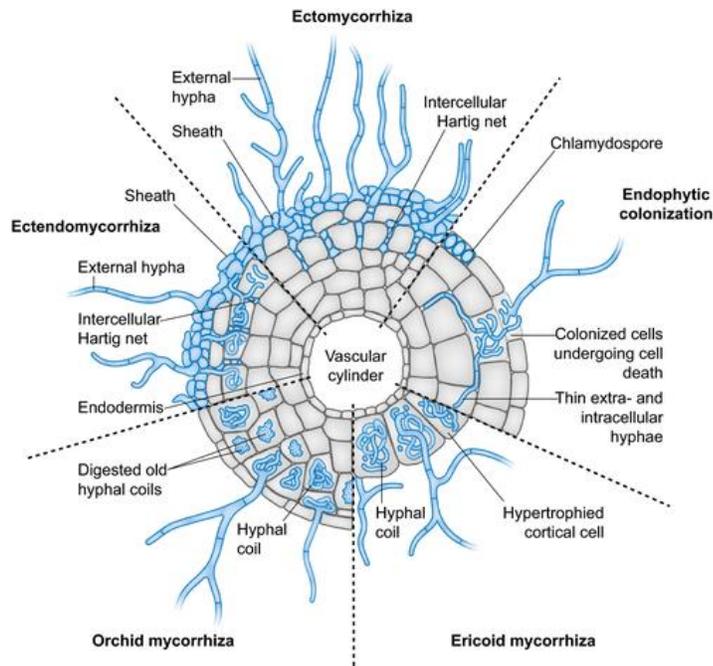


Figure A.
An illustrated cross section of root tip in which ectomycorrhizal and ectendomycorrhizal morphologies are compared with the morphologies of three types of endomycorrhizas (Weiss et al., 2016)

EVOLUTION OF THE MYCORRHIZA

Endomycorrhizas were the first real mycorrhizas to evolve following the colonization of land by early plants (Strullu-Derrien et al., 2018). In fact, it has been hypothesized that symbiotic relationships between aquatic plants and fungi made the transition to a terrestrial lifestyle possible (Pirozynski & Malloch, 1975). The first direct evidence concerning the evolution of mycorrhizas was found in the 407 million-yr-old Rhynie chert, in which paramycorrhizas (structures resembling mycorrhizas) were visible. These included the presence of members of the Mucoromycotina and Glomeromycotina in fossils of minute rootless stem vascular plants. By 315-300 million years ago, arbuscular mycorrhizas, or true mycorrhizas, had begun colonizing plant roots; Glomeromycetous arbuscules have been observed in lycopod root fossils from this era (Strullu-Derrien et al., 2018).

The Princeton chert of Canada contains the oldest known fossil of an ectomycorrhiza. This 48.7 million-yr-old chert shows evidence of *Pinus* roots with the essential features of an ectomycorrhiza: the fungal sheath and a Hartig net extending intercellularly to the endodermis. It is thought that ectomycorrhizal symbioses evolved independently more than 18 times in Angiosperms and ~78-82 times in fungi over at least 48Ma, indicating a relationship between the effects of the symbiosis and biological fitness (Strullu-Derrien et al., 2018).

Analyses of 12 ECM fungal genomes, some belonging to ectomycorrhizal fungi known to colonize *C. dentata*, suggest that some ECM fungi are the result of the convergent evolution of soil saprotrophs and of brown- and white-rot fungi. Many mycorrhizal fungi have exhibited decreases in genes encoding plant cell wall-degrading enzymes relative to their primarily saprotrophic ancestors; several genes encoding enzymes involved in the decomposition of various plant tissues and the cleavage of sucrose have been lost from ECM fungal genomes as well. Genes involved in the breakdown of soil particles, which may aid in the acquisition of organic nitrogen and phosphorus, have been preserved in these fungi. Consequently, ectomycorrhizal fungi have become more dependent on their hosts' carbohydrate reserves and more efficient in acquiring organic nutrients over time (Kohler et al., 2015; Strullu-Derrien et al., 2018).

Mycorrhizal symbioses are thought to have greatly impacted the evolution of both terrestrial plants and fungi. The benefits of these relationships have likely broadened the diversity and ranges of plant and fungal species due to their contributions in the expansion of available niches (Duplessis et al., 2001).

ECTOMYCORRHIZAL MORPHOLOGY

An ectomycorrhiza consists of three essential structures: the Hartig net, the mantle, and a network of hyphae radiating from the mantle into the surrounding soil. These structures function in the collection and transfer of water and nutrients between fungi and plants. The Hartig net serves as the extracellular junction of nutrient exchange between symbionts.

The formation of an ectomycorrhiza begins with stimulation of fungal growth by plant metabolites in the soil. As hyphae radiate outward and come into contact with roots, they envelop the root tips with denser hyphal networks. ECM fungi cultured on agar media have been observed growing significantly faster along root tips than across the media itself. Once hyphal envelopes have formed, specialized hyphae penetrate dead root cap cells and colonize intercellular spaces within the root tip, forming the Hartig net. The wedge-like hyphal tips expand in the plant tissue, separating plant cells and filling the newly-created spaces; hyphae may extend to the endodermis or be confined to the outermost layers of the root tip. These hyphae envelop individual cortical cells but do not disrupt intercellular plant communication, as plasmodesmata are not damaged in the process. Secretion of fibrillary polymers at the fungus-plant interface facilitates the adhesion of fungal and plant cell walls (Carlile et al., 1994). Subsequently, the true mantle is established as specialized hyphae form layer after layer of tissue enclosing the root tip. A hyphal network radiates outward from the mantle and spreads throughout the soil (Nylund & Unestam, 1981; Kendrick, 2017)

Colonization of a root tip by an ECM fungus slows rates of cell division at the root tip and root hairs and leads to radial elongation of cortical cells; this results in a relatively stumpy appearance of the colonized root (Carlile et al., 1994). However, if a root cap penetrates the mantle, the root may be subject to colonization by other ECM fungi (Kendrick, 2017).

PLANT SYMBIONTS

Although a relatively small percentage of seed plant species are ectomycorrhizal, these plants occupy a disproportionately large land area and dominate in the production of timber. Thus, ECM plants play a significant role in the ecosystems of boreal, temperate, and tropical forests, as well as in the infrastructures and economies of countries worldwide (Smith & Read, 2017; Alexander & Hogberg, 1987).

FUNGAL SYMBIONTS

Ectomycorrhizal fungi belong to the phyla Basidiomycota, Ascomycota, and Zygomycota. The most diverse orders include Agaricales, Cantharellales, Helotiales, Boletales, and Pezizales (Tedersoo et al., 2010).

BENEFITS OF ECTOMYCORRHIZAL SYMBIOSIS

The carbohydrates produced from photosynthesis, primarily hexose sugars, are delivered to the fungus where they are converted into sugar alcohols, e.g., mannitol, erythritol. This creates a hexose gradient that continually translocates sugars towards the fungus via passive diffusion. Fungi are able to excise the sugars from root tips by selectively increasing the permeability of plant cell membranes (Carlile et al., 1994).

In addition to increasing water and nutrient uptake in plants, ectomycorrhizal associations may confer resistance to a myriad of biotic and abiotic stresses. Some ectomycorrhizal fungi are capable of the uptake and compartmentalization of the heavy metal cadmium from soil; this reduces uptake by plant roots (Blaudez et al., 2000) and could also provide an effective mechanism for the phytoextraction of heavy metals from contaminated soils in land reclamation efforts (Sell et al., 2005). Ectomycorrhizal inoculation has also been noted for its role in

increasing plant tolerance to stresses inflicted by hyper-saline soils (Bandou et al., 2006) and drought conditions. (Wang & Gui-jie, 2013)

The benefits associated with ectomycorrhizal symbioses are not limited to the symbionts directly involved in the relationship; the presence of the ectomycorrhizal fungus *Pisolithus tinctorius* in soil may play a significant role in mitigating the effects of acid rain on below-ground microbial communities. (Maltz et al., 2019) In addition to decreasing biodiversity, acidic conditions can also increase the solubility of metals e.g., aluminum; dissolved aluminum can react with phosphorus in the soil, forming $AlPO_4$ and decreasing phosphorus availability in the soil. *P. tinctorius* can extract P from $AlPO_4$, allowing its host plant to benefit from the otherwise inaccessible nutrient (Cumming & Weinstein, 1990)

Underground hyphal connections formed by VAM fungi can serve as a means of plant-to-plant signaling and can enhance plant resistance to herbivory. Hyphae of a VAM fungus may connect multiple plants within a community; when attacked by aphids, a plant may send chemical messages through this junction which stimulates the production of chemicals that repel aphids and attract aphid parasitoids. Thus, plants not yet under attack can launch a preemptive defense to minimize predation (Babikova et al., 2013). The potential for communication between plants via ectomycorrhizal connections is supported by modern evidence, but this is an area that requires further research (Wagner et al., 2015).

VAM fungi are also thought to enhance plant resistance to pathogens in the soil. In the formation of an endomycorrhiza, the plant's immune defenses are modified, leading to a moderate systemic activation of the plant's immune system. The stimulation of primary immune responses may aid in the plant's defense against parasitic bacteria, fungi, plants, and nematodes (Jung et al., 2012). This topic also requires further research with respect to ectomycorrhizas.

AMERICAN CHESTNUT BACKGROUND

American chestnut populations have declined by approximately 90% in the past 115 years. This can be attributed primarily to *Cryphonectria parasitica*, the pathogenic fungus that causes chestnut blight. This necrotrophic parasite likely spread to American chestnuts from infected Japanese chestnut trees, which were first imported to North America as nursery stock in 1876. Japanese and Chinese chestnut trees have developed resistance to chestnut blight through natural selection. Contrarily, American chestnut trees had not been previously introduced to *Cryphonectria parasitica* and therefore carried no resistance to the pathogen. Phytophthora root rot, caused by the soil-borne oomycete *Phytophthora cinnamomi*, is another introduced disease that has contributed to declines in numbers and ranges of American chestnut. *P. cinnamomi* may be responsible for the permanent retraction of *C. dentata* from the southern portion of its native range and is now spreading to northern regions as temperatures warm (Dagleish et al., 2015).

Although approximately four billion American chestnut trees have been afflicted by chestnut blight thus far, the future outlook is hopeful. Scientists are currently working towards breeding blight-resistant American chestnuts by crossing susceptible American chestnut trees with naturally resistant Chinese chestnut trees. The resulting hybrids are backcrossed with American chestnut, producing trees that are essentially American but possess genes for blight resistance. Many scientists breeding for blight resistance are now selectively breeding for Phytophthora resistance as well (Rellou, 2002). The American Chestnut Foundation (TACF) was founded in 1983 in an effort to combat the demise of the American chestnut. Since its foundation, TACF has worked towards breeding disease tolerant chestnut trees in order to renew decimated native populations (Steiner et al., 2017).

PROJECT OUTLINE

Dr. Hill Craddock runs an American chestnut breeding program on the University of Tennessee at Chattanooga campus. Coordinating with TACF, Dr. Craddock breeds pathogen-resistant chestnut trees in an effort to restore the fungus-ravaged species to its former glory. The chestnuts are initially planted in 2-gallon containers containing well fertilized potting medium and grown for one year in the nursery before outplanting into experimental orchards and forest plots within *C. dentata*'s natural range. Dr. Craddock has long noticed sporocarps from various fungal species fruiting in the nursery containers. The fruiting bodies can be identified with reasonable confidence and as most of these fungi are obligate symbionts, they are likely products of mycorrhizal relationships within the pot. However, identification of these fruiting bodies may not provide a real estimate of ectomycorrhizal biodiversity within the nursery; not all ectomycorrhizal fungi produce hypergeous fruiting bodies and those that are produced may be ephemeral. Another option in assessing ectomycorrhizal diversity is through visual identification of ectomycorrhizal fungi based on mantle and hypha morphologies. This would likely be an arduous, if not impossible, task; many species of ectomycorrhizal fungi are indistinguishable morphologically, cannot be cultured in vitro, or vary in morphology depending on sexual state. Consequently, identification of ectomycorrhizal fungi typically requires genetic analysis (Norris et al., 1994). In this project, the internal transcribed spacer (ITS) region was used for fungal species identification.

The ITS region of fungal DNA has been referred to as the universal DNA barcode for the identification of fungi. Fungal ITS regions are relatively short (420-825 base pairs) (Manter & Vivanco, 2007) and are repeated up to thousands of times in the fungal genome. Additionally, the sequences are highly variable between closely related organisms due to their relatively fast

evolution (Raja et al., 2017). High rates of amplification via PCR can be partially attributed to well-conserved flanking sequences, which allow for one set of primers to be nearly universal (Baldwin et al., 1995). The primers ITS1F and ITS4 are particularly effective in priming the amplification of the ITS regions of ECM fungi, e.g., Basidiomycetes, Ascomycetes, and Zygomycetes (Manter & Vivanco, 2007).

Although still concerning ECM fungi identification, the scope of this project has changed significantly over the past year. The initial proposal for this thesis was ambitious and involved multiple sampling locations in a several-hundred-mile radius and the extraction of DNA from mycorrhizal root tips and fruiting bodies at each location in an effort to distinguish the mycorrhizal diversity of American chestnut in different growing conditions. However, due to time constraints and unforeseen difficulties in the extraction and amplification of mycorrhizal DNA, the scope of this research was narrowed. This paper serves as a contribution to the characterization of the mycorrhizal diversity of the American chestnut at the University of Tennessee at Chattanooga Fortwood Street nursery.

III. Methods

(Modified from Palmer et al., 2008; Sahu et al., 2012)

Sampling:

Mycorrhizal fruiting bodies were collected from pots containing *C. dentata* saplings located at a container nursery on the UTC campus. The fruiting bodies were dried at 26.7°C for approximately 24 hours and, once dry, were brushed gently with a small paintbrush to remove adhering soil particles. Each sample was packaged independently in a ziplock bag and frozen at -80°C. Mycorrhizal root tips were collected from pots bearing fruiting bodies, isolated and

brushed gently to remove soil particles, and frozen at -80°C . The root tips and fruiting bodies underwent the same treatment after freezing.

DNA extraction and precipitation:

Approximately 70mg of dried fruiting body or 100mg of root tips were taken directly from the -80°C freezer and immediately ground to a powder with a -80°C mortar and pestle. The powdered tissue was transferred to a 2.5mL microcentrifuge tube and 500 μL of cell lysis buffer (CLB) was added. CLB consisted of 1.4M NaCl, 0.1M Tris-HCl 20mM ethylenediaminetetraacetic acid, and 2% hexadecyltrimethylammonium bromide. Each tube was agitated for 20 seconds and then heated at 65°C for 1 hour. The samples were then centrifuged for 5 minutes at 21,130 xg and the supernatants were transferred to clean tubes. This was followed by addition of one volume of -20°C isopropanol and placement in a -80°C freezer for 10 minutes. Samples were centrifuged for 15 minutes at 21,130 xg and the supernatants were discarded. Pellets were washed three times with 100 μL of 70% ethanol and dried in a DNA concentrator before resuspension in 25 μL of RNase-free water.

DNA amplification:

Polymerase chain reactions were carried out using the fungal ITS primers ITS1F and ITS4, diluted to 10 μM with deionized RNase-free water. Ratios of PCR reagents were as follows: 0.5 μL DNA : 25 μL LongAmp master mix : 1 μL forward primer : 1 μL reverse primer: 22.5 μL water. The thermocycler was programmed with the following specifications: initial denaturing at 94°C for 2 min; 30 cycles of denaturing at 94°C for 40 s, annealing at 53°C for 40 s, and extension at 72°C for 5 min.

The PCR product was then used as template for four additional PCR reactions using the same primer/buffer ratios.

PCR product purification:

All PCR products derived from a particular DNA solution were mixed together and initially purified using phenol:chloroform:isoamyl alcohol. One volume of phenol:chloroform:isoamyl alcohol was added to the PCR product and shaken by hand for 20 seconds. This was centrifuged at room temperature at 16,000 xg for 5 minutes. The top layer was then extracted and placed in a clean microcentrifuge tube. One half volume of ammonium acetate (5M) and 2.5 volumes of 100% ethanol were added to the sample and the tube was placed at -80°C for 1 hour. The solution was centrifuged at 21,130 xg for 15 minutes and the supernatant was discarded. 150µL of 70% ethanol was added and the sample was centrifuged at 16,000 xg for 2 minutes; the supernatant was discarded and this step was repeated at 21,130 xg for 1 minute. The pellet was dried in a Centrivap DNA concentrator and resuspended in 300µL of TE (10 mM Tris-HCl, 1mM EDTA, pH 8).

The entirety of the resuspended DNA was electrophoresed in a 1% agarose gel. Prominent bands ~750bp were excised from the gel and the DNA within was purified with a Thermo Scientific GeneJET Gel Extraction Kit. Gel slices were weighed and placed in individual microcentrifuge tubes; one volume (volume: weight) of binding buffer was added to each. Tubes were incubated at 60°C for 10 minutes and then vortexed for 5 seconds. All melted gel solutions from a given sample were transferred to a single GeneJET purification column and centrifuged for 1 minute at 14,000 xg; the flow-through was discarded. An additional 100µL of binding buffer was added to the column, the sample was centrifuged for 1 minute at 14,000 xg, and the flow-through discarded. This was followed by the addition of 700µL of wash buffer, centrifugation for 1 minute at 14,000 xg, and discarding of flow-through. An additional centrifugation of the sample was undertaken to remove residual ethanol. The column was placed

into a clean microcentrifuge tube and 30 μ L of elution buffer was added to the center of the purification column membrane. This was centrifuged for 1 minute at 14,000 xg. The DNA solution was subsequently concentrated to a volume of approximately 15 μ L.

DNA sequencing:

Sequencing was performed by Psomagen, Inc. via Sanger sequencing, or the chain-termination method. The primers ITS1F and ITS4 were used to sequence both strands of the PCR product for confirmation. Two tubes were sent for each ITS sequence extracted. Each tube contained 5 μ L of the purified PCR product and 5 μ L of the forward or reverse primer (diluted to 5 μ M). The resulting sequences can be seen in Figure 3. and Figure 4.

IV. Results

Over the course of this project, genomic DNA was successfully extracted from nine fruiting bodies and nine mycorrhizal root tip samples (Figure 1.). Each sample of genomic DNA was prepared for PCR and submitted to thermocycling as described above. Of these 18 samples, one ITS sequence was successfully amplified. Overall, seven different methods of genomic DNA isolation were tested for their quality and reliability with respect to downstream PCR amplification. The initial protocol involved freezing ECM root tips in CLB and using a plastic pestle to grind the sample in a plastic microcentrifuge tube. This resulted in a thick brownish solution. Genomic DNA could be observed in agarose immediately following extraction, but not following PCR. A second method of cell-lysing, bead beating, was used on following samples and produced genomic DNA that was not successfully amplified via PCR. PCR of another DNA sample purified with phenol:chloroform:isoamyl alcohol was also unsuccessful. Additional extractions utilized a mechanical tissue homogenizer; using this method to grind the cells also produced a viscous brown solution. This approach resulted in degraded DNA fragments.

Additionally, a yeast DNA extraction kit (Masterpure MPY80200) was used along with the mechanical cell grinding approach; this also resulted in degraded DNA. The most successful method for the lysing of fungal cell walls involved grinding a frozen sample using a -80°C mortar and pestle. Disruption of cell walls was most effective in the absence of CLB; lysing each sample without adding reagents produced powdered tissue which was kept relatively cool due to the chilled mortar and pestle. Using this method for cell lysis with the remainder of the original protocol for DNA isolation, an ITS sequence of a single fruiting body was successfully amplified (Figure 2.).

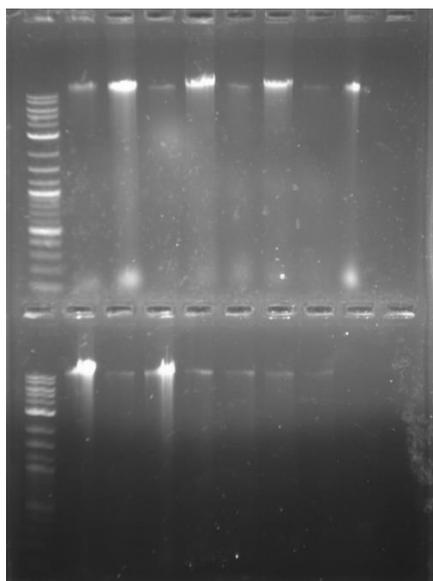


Figure 1. Genomic DNA representing fourteen of eighteen extractions
 Top row left to right: 2log ladder, fruiting body, fruiting body, root tips, fruiting body, root tips, fruiting body
 Bottom row left to right: 2log ladder, root tips, fruiting body, root tips, root tips, root tips, root tips

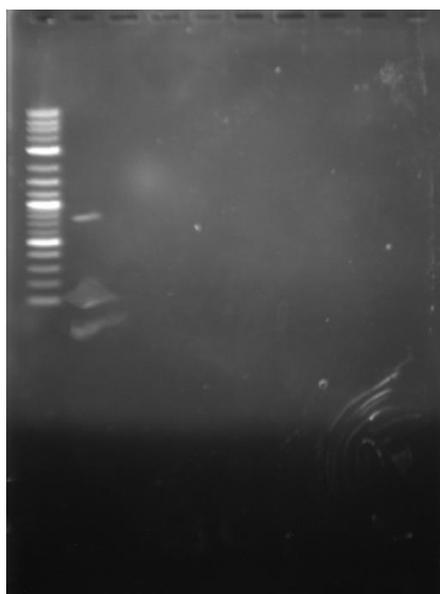


Figure 2.
 Left column: 2log DNA ladder
 Right column: ITS sequence of fruiting body amplified with the primers ITS1F and ITS4 and sent for sequencing

Figure 5. partial BLAST alignment of the forward ITS sequence amplified in the lab at UTC (Query) and the ITS sequence of *H. vaccinum* (Sbjct) (NCBI)

```

Query  199  AGGTCTATGTTTTTCATATACCCCATTGTATGTAACAGAATGTATCATTGGGCCTTGTGC  258
      |||
Sbjct  210  AGGTCTATGTTTTTCATATACCCCATTGTATGTAACAGAATGTATCATTGGGCCTTGTGC  269
Query  259  CTATAAACCTTATAACAACCTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACG  318
      |||
Sbjct  270  CTATAAACCTTATAACAACCTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACG  329

```

V. Discussion

Chestnut trees are known to form mycorrhizas with a variety of fungi; some of which are choice edibles and have economic value. Chanterelle mushrooms, for example, are highly sought-after fruiting bodies of obligate ectomycorrhizal fungi belonging to the genus *Cantharellus*. There is some evidence suggesting that widespread cultivation of these mushrooms may be plausible (Pilz et al., 2003). Some mycorrhizal fungi associated with chestnut trees may be poisonous, as is the case with all species of *Hebeloma*. These mushrooms, if ingested, can cause extreme gastrointestinal distress in humans (Miller & Miller, 2006). This provides some context as to why it is important to know what species of fungi form mycorrhizas with chestnut trees; some fungi may provide food and industry in thriving forests, and others may cause harm if ingested.

There has been some research done on the mycorrhizal fungi associated with chestnut trees at the UTC Fortwood street nursery. In 2012, M. Taylor Perkins studied the effects phosphite has on the colonization of chestnut root tips by ectomycorrhizal fungi. The chestnut seedlings sampled were separated into four main groups; three of which were inoculated with spores from ECM fungi. The species of fungi used to inoculate chestnut seedlings included *Pisolithus tinctorius*, *Scleroderma geaster*, and *Scleroderma citrinum*. Each group consisted of

two subgroups; one received phosphite treatment and the other did not. The plants inoculated with *P. tinctorius* and *S. citrinum* and those that were not inoculated formed significantly fewer mycorrhizas when treated with phosphite (Perkins, 2012).

In 2014, J. Miles Jorgensen tested the effects that inoculating chestnut seedlings with *P. tinctorius* and administering different levels of phosphite have on seedling survival. Inoculation with *P. tinctorius* was associated with a significant increase in seedling survival, regardless of phosphite concentration. Lower phosphite levels were associated with significant increases seedling growth but not with significant changes in mycorrhiza formation, while higher phosphite levels were associated with decreased seedling growth, survival, and mycorrhiza formation (Jorgensen, 2014). The experiments carried out by Perkins and Jorgensen provided valuable information with respect to chestnut breeding, but there was no confirmation that the fungi used to inoculate the seedlings was the same species that formed the mycorrhizas observed.

Despite the successful extraction of genomic DNA from mycorrhizal fungi found at the UTC Fortwood Street nursery, the final results of this thesis were limited by failures in consistently amplifying target DNA sequences via PCR. One possible cause of a failed polymerase chain reaction is contamination of the DNA solution with secondary metabolites. Certain polysaccharides have been shown to hinder the efficacy of eukaryotic DNA polymerases and increase the viscosity of the solutions in which DNA molecules are dissolved (Shioda & Murakami-Murofushi, 1987). Additionally, the oxidation of polyphenols (due to exposure to atmospheric oxygen during cell lysis) can lead to their binding to DNA molecules; this can cause the DNA to appear brownish in color (Sahu et al., 2012). Phenolic compounds present in plant tissues play a role in resistance to pests and pathogens, and they can also inhibit amplification reactions (Barakat et al., 2009). Fragmentation of DNA samples from tissues homogenized via

bead-beating was likely due in part to overbeating. The root tips were never fully homogenized; this may be attributed to their submersion in CLB, which warmed the root tips and decreased their rigidity. Similarly, the DNA extracted from mechanically-ground root tips was likely fragmented for the same reason; failed attempts to fully homogenize samples led to the shearing of DNA from already lysed cells. Grinding the samples with a -80°C mortar and pestle was likely more successful due to the sustained low temperature of the sample. Colder temperatures decrease the rates of oxidation reactions, therefore minimizing the opportunities for these reactions to occur. Utilization of liquid nitrogen in the cell-lysing stage may significantly increase the success rate of the protocol outlined above. Due to the obstacles outlined above, impending DHON deadlines, and expected graduation at the end of the Spring 2020 semester, the results of this thesis were limited. One fungal fruiting body was identified via DNA sequencing and BLAST alignment. Due to most members of *Hebeloma* being obligate symbionts, it is reasonable to suspect the sporocarp sampled to be an ectomycorrhizal fruiting body (Hacskeylo & Bruchet, 1972).

The ITS sequence of *H. vaccinum* is reported to be distinct from all known species of *Hebeloma* except for *H. cavipes*; these species must be distinguished by their ecology and morphology. *H. vaccinum* is primarily found in arctic habitats and dunes; it is usually associated with trees belonging to the genera *Populus* and *Salix*. *H. cavipes* is said to be capable of associating with most trees; it is typically found with broadleaf trees but has also been observed with some conifers. This fungus can live in most soil types and has been found in the continental United States (Eberhardt et al., 2015). However, it is not known to occur in Tennessee. *H. helodes* has been used as a synonym to *H. cavipes* in some literature, and both species fall under the more general species *Hebeloma hiemale s.l.* (Grilli, 2007). The mushroom in question was a

little brown mushroom (LBM). These kinds of mushrooms are notoriously difficult to identify; for this reason, consumption of LBMs is generally not advised. Furthermore, the taxonomy of these mushrooms is frequently debated as species overlap and are often described independently by multiple researchers. It is not plausible to assign definite, lasting taxonomic labels to many of these organisms, i.e., the taxonomy is confused. The taxonomic intricacies/ debate of these fungi are beyond the scope of this research; therefore, identification as *Hebeloma hiemale s.l.* is adequate for this report.

VI. References

- Alexander, I. J., & Hogberg, P. (1986). Ectomycorrhizas Of Tropical Angiospermous Trees. *New Phytologist*, 102(4), 541–549. doi: 10.1111/j.1469-8137.1986.tb00830.x
- Babikova, Z., Gilbert, L., Bruce, T. J. A., Birkett, M., Caulfield, J. C., Woodcock, C., . . . Johnson, D. (2013). Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. *Ecology Letters*, 16(7), 835-843. Retrieved from <https://proxy.lib.utc.edu/login?url=https://search-proquest-com.proxy.lib.utc.edu/docview/1366720299?accountid=14767>
- Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., & Donoghue, M. J. (1995). The ITS Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny. *Annals of the Missouri Botanical Garden*, 82(2), 247. doi: 10.2307/2399880
- Bandou, E., Lebailly, F., Muller, F., Dulormne, M., Toribio, A., Chabrol, J., . . . Bâ, A. M. (2006). The ectomycorrhizal fungus *Scleroderma bermudense* alleviates salt stress in seagrape (*Coccoloba uvifera* L.) seedlings. *Mycorrhiza*, 16(8), 559–565. doi: 10.1007/s00572-006-0073-6
- Barakat, A., Diloreto, D. S., Zhang, Y., Smith, C., Baier, K., Powell, W. A., . . . Carlson, J. E. (2009). Comparison of the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) in response to the chestnut blight infection. *BMC Plant Biology*, 9(1), 51. doi: 10.1186/1471-2229-9-51
- Blaudez, D., Botton, B., & Chalot, M. (2000). Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. *Microbiology*, 146(5), 1109–1117. doi: 10.1099/00221287-146-5-1109

- Brundrett, M. C. (2002). Coevolution of roots and mycorrhizas of land plants. *New Phytologist*, 154(2), 275–304. doi: 10.1046/j.1469-8137.2002.00397.x
- Brundrett, M. C., & Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist*, 220(4), 1108–1115. doi: 10.1111/nph.14976
- Carlile, M. J., Watkinson, S. C., & Gooday, G. W. (1994). *The fungi*. London: Academic Press.
- Chilvers, G. A., Lapeyrie, F. F., & Horan, D. P. (1987). Ectomycorrhizal Vs Endomycorrhizal Fungi Within The Same Root System. *New Phytologist*, 107(2), 441–448. doi: 10.1111/j.1469-8137.1987.tb00195.x
- Christenhusz, M. J., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa*, 261(3), 201. doi: 10.11646/phytotaxa.261.3.1
- Cumming, J. R., & Weinstein, L. H. (1990). Utilization of AlPO₄ as a phosphorus source by ectomycorrhizal *Pinus rigida* Mill. seedlings. *New Phytologist*, 116(1), 99–106. doi: 10.1111/j.1469-8137.1990.tb00514.x
- Dalgleish, H., Nelson, C., Scrivani, J., & Jacobs, D. (2015). Consequences of Shifts in Abundance and Distribution of American Chestnut for Restoration of a Foundation Forest Tree. *Forests*, 7(12), 4. doi: 10.3390/f7010004
- Eberhardt, U., Beker, H. J., Vesterholt, J., & Schütz, N. (2016). The taxonomy of the European species of *Hebeloma* section *Denudata* subsections *Hiemalia*, *Echinospora* subsect. nov. and *Clepsydroidea* subsect. nov. and five new species. *Fungal Biology*, 120(1), 72–103. doi: 10.1016/j.funbio.2015.09.014
- Grilli, E. (2007). Type studies in *Hebeloma*. Unravelling a taxonomic-nomenclatural problem: what is *Hebeloma leucosarx*?. *Micologia e vegetazione mediterranea*. 22. 133-176.

- Hacsckaylo, E., & Bruchet, G. (1972). Hebelomas as Mycorrhizal Fungi. *Bulletin of the Torrey Botanical Club*, 99(1), 17. doi: 10.2307/2484235
- Hilbert, J. L., & Martin, F. (1988). Regulation of gene expression in ectomycorrhizas. I. Protein changes and the presence of ectomycorrhiza-specific polypeptides in the *Pisolithus-Eucalyptus* symbiosis. *New Phytologist*, 110(3), 339–346. doi: 10.1111/j.1469-8137.1988.tb00270.x
- Jorgensen, J.M. (2014). The Effects of Different Concentrations of Phosphite on Ectomycorrhiza Formation by *Pisolithus tinctorius* in *Castanea dentata*. University of Tennessee at Chattanooga, Biological and Environmental Sciences.
- Jung, S. C., Martinez-medina, A., Lopez-raez, J., & Pozo, M. J. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *Journal of Chemical Ecology*, 38(6), 651-64. doi:http://dx.doi.org.proxy.lib.utc.edu/10.1007/s10886-012-0134-.6
- Kendrick, B. (2017). *The fifth kingdom: an introduction to mycology*. Hackett Publishing Company, Inc.
- Kohler, A., Kuo, A., Nagy, L. G., Morin, E., Barry, K. W., Buscot, F., ... Martin, F. (2015). Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nature Genetics*, 47(4), 410–415. doi: 10.1038/ng.3223
- Maltz, M. R., Chen, Z., Cao, J., Arogyaswamy, K., Shulman, H., & Aronson, E. L. (2019). Inoculation with *Pisolithus tinctorius* may ameliorate acid rain impacts on soil microbial communities associated with *Pinus massoniana* seedlings. *Fungal Ecology*, 40, 50–61. doi: 10.1016/j.funeco.2018.11.011
- Manter, D. K., and Vivanco, J. M. (2007). Use of the ITS primers, ITS1F and ITS4, to characterize fungal abundance and diversity in mixed-template samples by qPCR and

- length heterogeneity analysis. *Journal of Microbiological Methods*, 71(1), 7-14.
doi:10.1016/j.mimet.2007.06.016
- Martin, F., Duplessis, S., Ditengou, F., Lagrange, H., Voiblet, C., & Lapeyrie, F. (2001). Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. *New Phytologist*, 151(1), 145–154. doi: 10.1046/j.1469-8137.2001.00169.x
- Miller, O. K., & Miller, H. (2006). North American mushrooms: a field guide to edible and inedible fungi. Guilford, CT: Falcon Guide.
- National Center for Biotechnology Information (NCBI). Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [cited 2020 March 21]. Available from: <https://www.ncbi.nlm.nih.gov/>
- Norris, J. R., Read, D. J. and Varma, A. K (1994). *Techniques for mycorrhizal research*. Academic Press, London
- Nylund, J. & Unestam, T. (1981). The process of mycorrhiza formation in Norway spruce in vitro. *Structure and Physiology of Ectomycorrhizae, Vol.(91)*, 63-79.
- Perkins, M.T. (2012). The Effect of Phosphite on Mycorrhiza Formation in American Chestnut (*Castanea dentata*). University of Tennessee at Chattanooga, Biological and Environmental Sciences.
- Pilz, D., Norvell, L., Danell, E., Molina, R. (2003). *Ecology and management of commercially harvested chanterelle mushrooms*. Portland, OR: U.S. Dept. of Agriculture, Forest Service, Pacific Northwest Research Station.
- Pirozynski, K., & Malloch, D. (1975). The origin of land plants: A matter of mycotrophism. *Biosystems*, 6(3), 153–164. doi: Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N.

- H. (2017). Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of natural products*, 80(3), 756–770.
<https://doi.org/10.1021/acs.jnatprod.6b01085> 10.1016/0303-2647(75)90023-4
- Rellou, J. (2002, March 15). Chestnut Blight Fungus (*Cryphonectria parasitica*). Retrieved February 9, 2020, from http://www.columbia.edu/itc/cerc/danoff-burg/invasion_bio/inv_spp_summ/Cryphonectria_parasitica.htm
- Sahu, S. K., Thangaraj, M., & Kathiresan, K. (2012). DNA Extraction Protocol for Plants with High Levels of Secondary Metabolites and Polysaccharides without Using Liquid Nitrogen and Phenol. *ISRN Molecular Biology*, 2012, 1–6. doi: 10.5402/2012/205049
- Sell, J., Kayser, A., Schulin, R., & Brunner, I. (2005). Contribution of Ectomycorrhizal Fungi to Cadmium Uptake of Poplars and Willows from a Heavily Polluted Soil. *Plant and Soil*, 277(1-2), 245–253. doi: 10.1007/s11104-005-7084-5
- Shioda, M., & Murakami-Murofushi, K. (1987). Selective inhibition of DNA polymerase α by a polysaccharide purified from slime of *Physarum polycephalum*. *Biochemical and Biophysical Research Communications*, 146(1), 61–66. doi: 10.1016/0006-291x(87)90690-5
- Smith, S. E., & Read, D. J. (2017). *Mycorrhizal symbiosis*. Amsterdam: Elsevier, Academic Press.
- Steiner, K.C., Westbrook, J.W., Hebard, F.V., Georgi, L.L., Powell, W.A., and Fitzsimmons, S.F. (2017). Rescue of American chestnut with extraspecific genes following its destruction by a naturalized pathogen. *New Forests* 48: 317-336.
<https://doi.org/10.1007/s11056-016-9561-5>

- Strullu-Derrien, C., Selosse, M.-A., Kenrick, P., & Martin, F. M. (2018). The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. *New Phytologist*, 220(4), 1012–1030. doi: 10.1111/nph.15076
- Tedersoo, L., May, T. W., & Smith, M. E. (2010). Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza*, 20(4), 217–263. doi: 10.1007/s00572-009-0274-x
- Wang, B. & Qiu, YL. *Mycorrhiza* (2006) 16: 299. <https://doi.org/10.1007/s00572-005-0033-6>
- Wang, Y. & Gui-jie, D. (2013). Influence of Ectomycorrhiza on nutrient absorption of *Pinus massoniana* seedlings under water stress. *Forest Research*, Vol. 26(2), 227-233.
- Wagner, K., Linde, J., Krause, K., Gube, M., Koestler, T., Sammer, D., . . . Kothe, E. (2015). 0RW1S34RfeSDcfkexd09rT2Tricholoma vaccinum1RW1S34RfeSDcfkexd09rT2 host communication during ectomycorrhiza formation. *FEMS Microbiology Ecology*, 91(11) doi:<http://dx.doi.org.proxy.lib.utc.edu/10.1093/femsec/fiv120>
- Weiss, M., Waller, F., Zuccaro, A., & Selosse, M. (2016). Sebacinales - one thousand and one interactions with land plants. *New Phytologist (Online)*, 211(1), 20-40. doi:<http://dx.doi.org.proxy.lib.utc.edu/10.1111/nph.13977>
- Yu, T.E., Egger, K.N. & Peterson, L.R. Ectendomycorrhizal associations – characteristics and functions. *Mycorrhiza* 11, 167–177 (2001). <https://doi.org/10.1007/s005720100110>