

Identifying Mycorrhizas Present in Bigleaf Maple Canopy and Forest Floor Soils in a
Coastal Old-Growth Temperate Rainforest in Western Washington

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A thesis submitted in partial fulfillment of the Requirements for the degree of:

Master of Science

University of Washington

2015

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Program authorized to offer degree:

School of Environmental and Forest Sciences

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Abstract

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The temperate rainforests of Western Washington are known for their old-growth forests and unique ecosystem processes. In these stands of old-growth, it is common for trees to be 200-300 years old. On branches, Epiphytic mats form in the canopy. The bottom layer of these mats starts to decompose over time, forming a layer of organic matter known as 'canopy soil'. To better understand the role of canopy soil in the overall ecosystem, research needs to focus on canopy soil characteristics and the host tree relationships. The specific aims of this research were to attempt to 1) taxonomically identify mycorrhizal species in adventitious canopy and forest floor roots, and 2) compare the identified species between the canopy and forest floor levels. Twenty-four root samples were taken from four bigleaf maples (*Acer macrophyllum* Pursh) located in the Olympic National Rainforest in the Queets River Watershed, Washington State. Roots were washed and DNA was extracted for PCR, cloning, and DNA sequencing. The results were processed through the NCBI BLAST Database and EMBI ClustalW website to identify and analyze differences in genetic diversity for roots collected from the canopy and the forest floor. Mycorrhizas were successfully identified from both the canopy and forest floor roots. Due to a lack of vouchered references, some samples were only identified to class, order, or genus,

while others were placed in a group entitled 'unidentified ectomycorrhizal clones'. Three samples were identified to be from the class *Leotiomycetes*, one was narrowed to the order *Helotiales*, and another sample was identified as a *Cadophora* spp. All three groups have species that have been reported as being ectomycorrhizal. Although species weren't identified past these levels, sequence alignment still allowed analysis of their DNA. There appears to be several different mycorrhizal sequences present in both the canopy and forest floor roots. Further research will be needed using DNA extractions to better understand the coevolution of these species found in canopy soils.

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ACKNOWLEDGMENTS

I want to put out a sincere thank you, showing appreciation to my advisor, Daniel Vogt. Your willingness to show support and share all your knowledge throughout this project has not only taught me the expectations of academics, but has helped me grow as an individual. You are a very kind, enthusiastic mentor that has become a friend, as well. I look forward to continuing research with you. I would also like to send many thanks to Kristiina Vogt and Darlene Zabowski, two other members on my committee. Your teachings and support while I was an undergraduate gave me the enthusiasm to continue on in the field of ecology and soil sciences. As a graduate, both of you were always so welcoming and supportive when I had questions, and you have made a great impact on my education. The last member on my committee, but definitely not least, is Bruce Godfrey. I have so much appreciation for your patience and input while I was learning to work with DNA. Without your guidance and background knowledge of genetics and fungi, I doubt I would be standing where I am today.

I also very much appreciate the aide provided by Jerry Frielich and the Olympic National Park. Without the help and support, permits and research sampling for this project would not have been possible. Also, my gratitude for Camila Tejo-Haristoy is never ending. Without your willingness to mentor and introduce me to canopy soils and help me collect samples in the field, this project would have never happened. Your enthusiasm and love for nature shows deeply, and I cherish the friendship we have formed over the last 4 years.

I also thank Amanda Davis, Michelle Trudeau, and David Campbell. Your academic advising and support clarified many things, and made the road to graduation a lot smoother. Along with them, I send my appreciation to all of SEFS. Your programs, faculty, staff, and students have always been welcoming. Also, thanks to the Comparative Genomics Center and the Department of Biology for allowing use of the lab.

For financial support, I first thank Daniel Vogt for providing TA/RG positions when available. Those opportunities really helped. I also thank SEFS and Jon Bakker for helping me as an undergraduate, and allowing some research funds to transfer over. Lastly, my deepest gratitude goes to Teiko Mafune, my great aunt. Being affiliated with the University of Washington, she kindly chose to provide me with several private grants that supported this research and my education.

Working towards a Master's Degree has been a life-changing experience and difficult at times. All the support of my friends and family helped give me that extra push, when it was needed. Special thanks to Jason LaBay, Maya Lanham, Jessica Clark, and Jason Bartlett. Your friendships and support mean the world to me. To wrap it up, I would like to express my love for Scott Mafune, Beverly Mafune, Samantha Mafune, and Tamura Mafune. Without my family, this would be nothing.

CHAPTER 1

INTRODUCTION

The temperate rainforests near the coasts of Washington State make up part of the largest temperate ecoregion on the planet. Many distinctive characteristics define these ecosystems: fog and high rainfall make these areas highly productive; as well as multiple layers of habitat in the tree canopies acquiring resources through symbiotic associations (Nadkarni 1981). Adventitious roots develop in the canopy to help allocate nutrient resources; flora and fauna interact and form unique relations, and they host many endemic species (DellaSala *et al.* 2011; Nadkarni *et al.* 2001). Many of the characteristics found in the temperate rainforests of Western Washington resemble those found in mountainous cloud forest ecosystems in the tropics (Nadkarni, 1981), where the canopy processes aid in capturing nutrients from the atmosphere (Prescott, 2002), while providing most of the water consumed by lowland communities (Nadkarni and Su, 2004). It is important to determine how vulnerable these forests are to a changing climate or disturbance regimes, and how they have acclimated and adapted over time.

Early in ecological research, forest canopies were not studied and what they provide for these ecosystems was not considered. Over the last several decades, research has emphasized how important forest canopies are in the functioning and dynamics of these ecosystems (i.e., Prescott, 2002; Tejo *et al.*, 2014). Many of the old-growth trees have developed a thick layer of epiphytic and other organic material on their

branches, which increase in depth and decompose over time to form canopy soils (Orlovich *et al.*, 2013; Tejo *et al.*, 2014). The discovery of canopy soils forming on the tree branches of these forests suggests the need to further understand how the biotic communities living in the canopies might differ from those found in the forest floor.

Within these thick mats of canopy soils are adventitious roots that grow into the soils to help acquire nutrient resources for the host tree. Not only are the ground roots acquiring resources, but canopy roots also factor into the processes. When forest floor roots grow they often form a mutualistic symbiosis with mycorrhizal fungi. Mycorrhizas are prevalent in nature and perform a critical role in soil nutrient cycling and plant resource acquisition, by forming and extending fungal hyphae in the soils (Smith and Read, 2008; van der Heijden *et al.*, 1998). In this temperate rainforests, there has been research comparing growth of saprophytic and bacterial communities of canopy and forest floor soils (Rousk and Nadkarni, 2009), and there has been observations of arbuscular mycorrhizal hyphae on canopy roots (Nadkarni, 1981). However, there has not been an attempt to extract the DNA from these adventitious roots for taxonomic identification. Researching the mycorrhizal communities in these canopy soils will benefit our understanding how these unique ecosystems are structured and the potential importance of these unknown interactions.

There are two groups of mycorrhizal fungi that would be the most likely to form relationships with adventitious roots of bigleaf maples; arbuscular (Zygomycota) and ectomycorrhizal (Basidiomycota, Ascomycota, Zygomycota) fungi (Trappe, 1962); from this point on the Vesicular Arbuscular Fungi will be abbreviated as AMF and the Ectomycorrhizal Fungi will be abbreviated as EMF. Both are prominent in forest soils. It will be important to determine the abundance and composition of mycorrhizas, and if both groups can be taxonomically identified to exist in canopy and forest floor roots. This knowledge would provide information on symbiotic associations found in the canopy, and whether canopy associations might be adapted to a very different growth environment. This could open up more questions relating to plant physiology, canopy soil properties, and even evolutionary traits.

This research study is a small-scale project to better understand the evolution of mycorrhizas in an old-growth temperate rainforest. Rather than comparing and contrasting a mycorrhizal study in a greenhouse, a taxonomic understanding of potential mycorrhizal establishment was sought. The Olympic National Rainforest covers a large area, and it is hard to determine the diversity of mycorrhizas on a scale that large. Also, AMF and EMF will dominate certain areas depending on species composition of the vegetation, for example, it is expected that ericaceous plants will have ericoidal mycorrhizal affiliations (Trappe, 1962).

It is unlikely that a forested area would not have an abundance of both the AMF and EMF groups present in the soil (See Cairney, 2000). The focus of this research was to

develop new insights on canopy soils and to compare the mycorrhizal affiliations found in the adventitious canopy roots compared to that of forest floor roots. Recently, canopy soils have been studied for their role in decomposition processes, root turnover and nutrient uptake rates (See Matson *et al.*, 2014; Tejo *et al.*, 2014; Nadkarni, 1985). The ecosystem where this study took place has an abundance of canopy soil in the old-growth stands. Therefore, studies that don't consider the role of canopy roots and their symbionts would result in an incomplete understanding of forest nutrient cycles.

Hypothesis and Objectives

Hypothesis addressed in this research:

Mycorrhizas found colonizing adventitious canopy roots will differ from those found colonizing roots found growing in the forest floor.

The objectives of this study were to:

- 1) Taxonomically identify mycorrhizas colonizing canopy adventitious roots and forest floor roots of old-growth bigleaf maples, and
- 2) Determine if the mycorrhizas found colonizing roots in canopies differ from those colonizing roots found in the forest floor.

Chapter Roadmaps

Chapter 2 is a literature review separated into four sections: Temperate Rainforests, Soils and Nutrient Cycling, Mycorrhiza, and Canopy Soils. The purpose of this chapter is to provide a better understanding of the four topics that are relevant to this research. Chapter 3 provides the background, methodology and research findings of this project. Chapter 4 includes all DNA sequence results and a discussion on future research. Chapter 5 is a conclusive chapter, restating the question, hypothesis, and results.

CHAPTER 2

LITERATURE REVIEW

Temperate Rainforests

Temperate rainforests can be found all over the world, with the largest extent of forests located on the western coasts of North America, from Oregon to Alaska, that is over 2,000 km in length (See Fig. 1.1). The coasts of Chile, Australia, United Kingdom, Japan and New Zealand also host smaller areas of temperate rainforests that are also home to these unique forests. Temperate rainforests range from habitats dominated by deciduous species with warm summers and frosty winters (Rohrig and Ulrich, 1991) to areas dominated by evergreen species with mild winters (Ovington, 1983). In contrast, tropical rainforests have very high tree diversity, have higher rainfall inputs with warmer temperatures, and less seasonality year-round (Archibold, 1995).

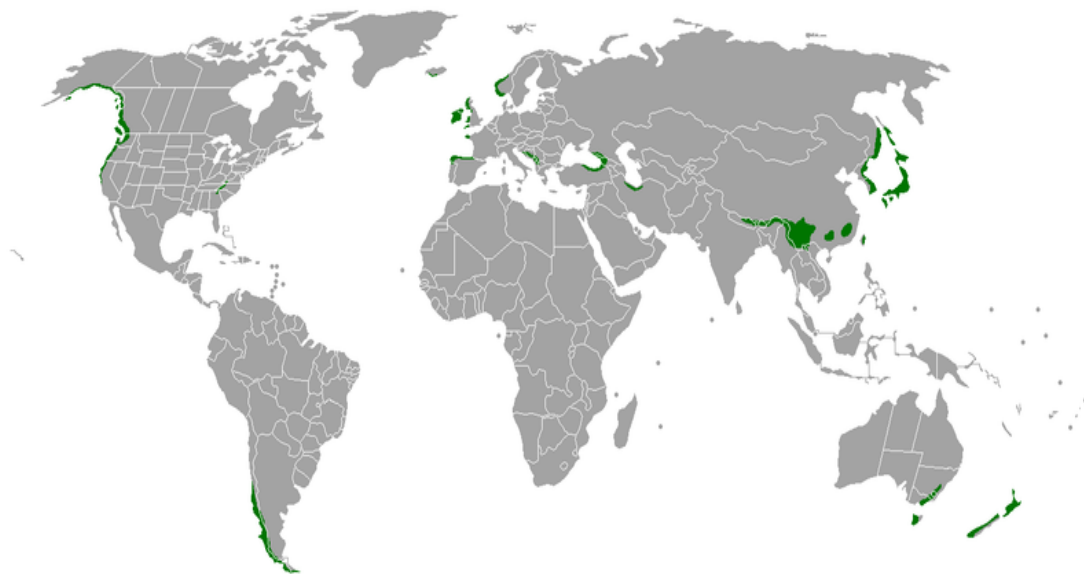


Fig. 1.1. A map representing the range of the world's temperate rainforests. The areas of rainforest are highlighted in green. Photo retrieved from Wikimedia.org.

In the past and still today, urban development and timber harvesting have decreased the area covered by these ecosystems. Until the mid-1970's, the amount of logging occurring in temperate rainforests was greater than what was reported for tropical rainforests (Malhi *et al.*, 1999). Deforestation rates in temperate rainforests have increased their contribution to total CO₂ emissions into the atmosphere and therefore their role in climate change, as well as destroyed habitat needed by many native and endemic species (Runyan *et al.*, 2012, Lomolino and Perault, 2004, Palmer and Engel, 2009, Prentice *et al.*, 2011).

The temperate rainforests located along the on the coasts of North America comprise the largest temperate rainforest ecoregion on the planet (Franklin *et al.*, 1981). Throughout this region, these old-growth forests are diverse with varying biodiversity levels and structural characteristics. They tend to be more heterogeneous than young stands, provide optimum habitat for many epiphytic and saprophytic species, and have high gross primary productivity and nutrient retention levels (Franklin *et al.*, 1981).

Located on the coast of Washington State is the Olympic National Rainforest, known for its old growth stands. Douglas fir, western hemlock, Sitka spruce, and bigleaf maple dominate many of the stands located in this area (Edmonds and Murray, 2002; Tejo *et al.*, 2014). Many studies have been conducted in these ecosystems to better understand their structural and functional attributes. A majority of this research has focused on litterfall inputs and nutrient returns (See Edmonds and

Murray, 2002; Klopatek, 2007). Recently, a study was conducted on litterfall rates and canopy soils in these forests (See Tejo *et al.*, 2014).

Soils and Nutrient Cycling

Forest soils provide nutrients, anchorage, and water for the plant species they host, while soil biota play a major role in the decomposition of organic matter (Brady and Weil, 2010; Trudell and Edmonds, 2004). The majority of soils found in temperate rainforests are high in organic matter and often wet due to their high precipitation levels. These conditions are a result of the low rates of litter decomposition and the loss of limiting nutrients from soils due to the higher precipitation levels (Edmonds and Murray, 2002; Liski *et al.*, 2003; Brady and Weil, 2010; Carpenter *et al.*, 2014). These conditions cause plants to be increasingly dependent upon symbionts to compete for the limiting nutrients as they are mineralized from decomposing litter.

A majority of the decomposition takes place on the surface of the forest floor, where litterfall accumulates (Bamforth, 2010). Litterfall input affects the rate of decomposition and nutrient turnover, and, as research shows, act as an important feedback mechanism in these forested ecosystems (Klopatek, 2007). The acquisition and supplies of limiting nutrients drive how these ecosystems function because they limit or promote the growth and development of plant species (Johnson *et al.*, 1982; Brady and Weil, 2010). The coniferous forests of the Pacific Northwest have been reported to have the largest carbon flux and storage capacity in soils (Klopatek,

2007, Vogt *et al.*, 1995), while nitrogen has been reported to be the most limiting nutrient impacting growth rates of plants and microbes (Johnson *et al.*, 1982).

In the Olympic National Rainforest, organic matter accumulation rates that form the forest floor are strongly related to litterfall input rates and nutrients available for microbes to produce the enzymes needed to decompose litter (Bray and Gorham, 1964; Abee and Lavender, 1972). Both factors vary across the landscape and determine the nutrient supply capacity found at any one location. Edmonds and Murray (2002) found that litterfall, near the study site of this research project (Hoh River Valley), had higher accumulation rates in the upper watershed than the lower watershed, which likely reflected the higher winds in the area (elevations ranging from 180m to 850m throughout that study). However, resorption of nitrogen and phosphorus by foliage was both higher in the lower watershed compared to the upper watershed (38% vs 24% and 57% vs 42%, respectively) suggesting nutrient limitations in the lower watershed. They suggested that this was likely due to diversity among the plant communities. The inputs of litter (i.e., green needles, senescent needles, woody litter) contain different nutrient concentrations, so it is important to consider how each component plays a role in the decomposition and nutrient cycling of the forest.

Litterfall, decomposition, and turnover rates affect the nutrient availability for plants in any given ecosystem. Over the years, mycorrhizal fungi have been recognized for their role in the acquisition of nutrients released during

decomposition and increasing the uptake of nutrients by plants from soils (See Vogt *et al.*, 1991; Hodges *et al.*, 2001; Finlay, 2008). The next section further describes these species and why they are important in the nutrient acquisition by plants.

Mycorrhizas

Mycorrhizas are widely recognized for their ability to form mutualistic relationships with a majority of vascular plants. They can be categorized into several major groups. Arbuscular mycorrhizas (AMF) form relationships with a majority of land plants (Rillig, 2004, Wang and Qui, 2006), and evidence suggests that they coevolved as plants first colonized land some 450-500 million years ago (Cairney, 2000). These fungal species penetrate through cortical cell walls and form arbuscules within these cells, i.e., highly branched, shrubby structures that form inside the root tip. Arbuscules act as the primary source for carbon and nutrient exchange between the plant and fungus (Smith and Read, 1997; Rillig, 2004). They also form intercellular and intracellular hyphae, and some produce a loose hyphal network in the surrounding soil (Gerdemann, 1968). These arbuscular species are also known as endomycorrhizas.

The second group of mutualistic fungi are known as ectomycorrhizas (EMF).

Ectomycorrhizas are also very prevalent in nature, often forming relationships with large woody trees, shrubs, and some herbaceous plants (Trappe, 1962; Smith and Read, 1997). This group of fungi forms an extensive sheath around the root tips and between root cortical cells of host plants (Cairney, 2000), but it does not penetrate

into plant cells. It grows an extensive mycelial network into the soil to obtain resources for the host plant.

Later down the line in the evolution of mycorrhizas, fungi coevolved to form relationships with certain families of plants creating additional mycorrhizal groups, such as ericoidal and orchidaceous mycorrhizas (Rasmussen and Rasmussen, 2014). Both these groups are unique in how they form relationships with plants and the ecosystem processes that they contribute to. For example, Orchidaceous mycorrhizas are often called seedling mycorrhizas because the host seedlings do not successfully germinate and develop without the presence of these fungi (Rasmussen and Rasmussen, 2014). However the Ericoid mycorrhizas are mostly affiliated with the Ericaceae family, infecting epidermal cells with less of a hyphal network than AMF and EMF (Cairney, 2000). But more recently it has been suggested that certain taxa in this group (e.g., Ericoid mycorrhizas) can also form relationships with other families (Cairney and Meharg, 2003). All four of these fungal groups hold very important roles in the range of ecosystems where they are found forming symbiotic associations with plants

Table 1.1. Characteristics of mycorrhizal groups mentioned in this chapter (ref needed).

Arbuscular Mycorrhiza	Ectomycorrhiza	Ericoid Mycorrhiza	Orchidaceous Mycorrhiza
<ul style="list-style-type: none"> • Growth and development within root cortical cells • Form vesicles 	<ul style="list-style-type: none"> • Growth and development between root cortical cells • Forms mycelial 	<ul style="list-style-type: none"> • Growth and development within root cortical cells • Forms hyphal 	<ul style="list-style-type: none"> • Growth and development within root cortical cells • Forms hyphal

and arbuscules <ul style="list-style-type: none"> • Extensive mycelial network, form runner hyphae • Forms relations with angiosperms, gymnosperms, mosses and ferns 	sheath on root-tip surface <ul style="list-style-type: none"> • Extensive branching mycelial network • Favors relationships with angiosperms 	coils <ul style="list-style-type: none"> • External hyphal network, not as extensive • Most often found in roots of Ericaceae family. 	coils <ul style="list-style-type: none"> • External hyphal network, not as extensive • Often needed for germination of Orchidaceous seedlings.
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Many nutrients are immobile in soil, often limiting plant growth. The mutualistic relationship formed with mycorrhizal fungi allows plants to access nutrients through the mycorrhizal hyphae that form on the roots, and in exchange mycorrhizas obtain photosynthate from the host (Trudell and Edmonds, 2004; Vogt *et al.*, 1989; Kluber *et al.*, 2010). It has been suggested, that along with obtaining nutrients for host plants, that mycorrhizas also play a role in the carbon and nitrogen storage mechanisms found in forests soils, and in facilitating organic matter decomposition (Vogt *et al.*, 1989; Averill *et al.*, 2014; Trudell and Edmonds, 2004). Over the last century, research has been furthering our understanding of these fungal groups and their role in ecosystem function. However, many studies that focus on mycorrhizal fungal research occur in greenhouses. To further our understanding of these organisms and their symbiotic relationships, it is important to follow through with more in-field studies (Vogt *et al.*, 1991).

Canopy Soil

In the last few decades, the important role of canopy soils as part of ecosystem level litterfall inputs, nutrient cycling, species diversity, and resource allocation has been recognized. Canopy soils are reported to occur in temperate, tropical, and montane ecosystems, where epiphytic biomass is high (Franklin, 1993; Nadkarni and Su, 2004). In these forests, epiphytes establish on tree branches, promoting the interception of organic materials and nutrients in leaf wash or stem flow. As the layer of epiphytic and organic materials accumulate in the canopy, the bottom layers start decomposing and forming what is being called 'canopy soil' (Matelson *et al.*, 1993; Vance and Nadkarni, 1990). These soils formed high up in the tree canopies have been categorized as arboreal Histosols (Enloe *et al.*, 2006). Recognition of their occurrence has fostered a considerable recent research to understand their role in ecosystem interactions and processes (See Fig. 2.1).



Fig. 2.1. (a) *on left* shows the top epiphytic layer of canopy soils, along the soil underneath with adventitious roots. (b) *on right* shows a side profile of canopy soil where the branch meets the trunk.

As organic matter forms under the epiphytic mats on these branches, it creates a habitat that is not completely understood. In the Olympic National Rainforest, the depth of canopy soil in Sitka spruce and bigleaf maple species ranged from 11-16cm and 13-48cm, respectively (Tejo *et al.*, 2014). As the depth of canopy soils increases, the host trees start to develop adventitious roots that grow into the canopy soil. The amount of canopy soil needed to start growth and development is unknown, but is expected to vary among tree species and ecosystem. The anatomy of adventitious roots differs from primary and lateral rooting systems. Instead of growing off of the primary rooting system as lateral roots do, they develop from aerial cells and tissue (Bellini *et al.*, 2014). Nadkarni (1981) reported the first evidence of adventitious

roots in canopy soils in 1981, and since then their role has been considerably expanded to address questions related to plant nutrient allocation and water retention/uptake.

Research has suggested that the species interactions that occur in canopy soils, ranging from fungal to animal to insect interactions, affect the ecosystem as a whole. There is evidence showing that a portion of litterfall is intercepted and retained on canopy branches, holding potential to alter the inputs that affect biogeochemical cycling within forest soils (Heitz *et al.*, 2002, Matson *et al.*, 2014). It has also been found that some microbial communities in canopy soils differ from those of the forest floor (See Rousk and Nadkarni, 2009).

Recognizing the potential role of canopy soils in forest soil nutrient cycling is important, but to understand this at a large-scale of an ecosystem, more research needs to focus on the characteristics of canopy soils. Like soils on the forest floor, canopy soil characteristics vary among host species. Tejo *et al.* (2014) compared canopy soils of Sitka spruce and bigleaf maple, within the study site of this research project. They found that spruce canopy soils were at a more advanced state of decomposition, and that nutrient and chemical properties of canopy soils differed. This study emphasizes why it is important to broaden the research of individual canopy soils and gather more information before applying this information to the ecosystem as a whole

In the Olympic National Rainforest, several studies have been conducted on canopy soils (See Fig. 3.1 and 3.2), even though most of the research has focused on studying forest floor soils. Mycorrhizas in forest soils are widely recognized, but a paucity of information about their role in canopy soils suggests that more research is needed to better understand how they contribute to nutrient cycling and plant nutrient allocation patterns.



Fig. 3.1 and 3.2. 3.1 (left) shows the end of an adventitious root, with fine root-tips and potential mycorrhiza. 3.2 (right) shows a close up of a root tip and fungal sheath. Moss near root tip for scale. Photo: Korena Mafune

CHAPTER 3

MYCORRHIZAL FUNGI IN CANOPY AND FOREST FLOOR ROOTS OF *ACER MACROPHYLLUM* IN THE QUEETS TEMPERATE RAINFOREST OF WESTERN WASHINGTON, USA

Introduction

The temperate rainforests of Western Washington make up part of the largest temperate rainforest ecoregion on the planet. These areas are recognized for their old-growth forests, high gross productivity, and ability to host epiphytic and saprophytic species (Franklin *et al.*, 1981). Studies have researched the importance of litterfall, decomposition, and nutrient cycling in these forests (See Edmonds and Murray, 2002; Klopatek, 2007), and in the last several decades, research revealing the role of canopy soils in these ecosystems has been published (See Nadkarni, 1981; Orlovich *et al.*, 2013; Hertel, 2011). Canopy soils form on host tree branches as a result of the accumulation and decomposition of plant litter, foliage, and other epiphytic materials (Tejo *et al.*, 2014; Perez *et al.*, 2005), and have been categorized as arboreal Histosols (Enloe *et al.*, 2006). These Histosols create a habitat with characteristics differing from those found in the forest floor (e.g., Rousk and Nadkarni, 2009; Aubrey *et al.*, 2013). Host trees are capable of developing and growing adventitious roots into canopy soils to aid in host-tree resource allocation, but the plant-soil relations these roots form within canopy soil is not yet well understood (See Hertel, 2011).

Mycorrhizas are a group of fungi forming mutualistic relationships with plant roots, and are found in almost all terrestrial ecosystems (Smith and Read, 2008). There

have been few studies reporting evidence of mycorrhiza in adventitious canopy the absence of mycorrhizal inoculation, even though the ground roots were heavily colonized (Hertel, 2011). These studies suggest that evolution of mycorrhizas in adventitious canopy soil roots may have adapted over time, and could be unique to host-trees and ecosystems.

With a limited amount of research focusing on the mutualistic relationships of adventitious canopy roots, this project aims to better understand these ecosystems at this level. In the Queets Temperate Rainforest, evidence of arbuscular mycorrhiza (AMF) was observed in cortical cells of bigleaf maple root-tips, but genetic analysis was not completed to identify the fungi (Nadkarni, 1981). The objective of this project is to extract and identify mycorrhizas from bigleaf maple adventitious and forest floor root tips.

Performing a genetic analysis holds the potential to return identities of mycorrhizas, which allows for diversity comparison. This is of interest because it would contribute to the understanding of how these canopy soils play a role in the adaptations of these old-growth forests.

Materials and Methods

Study Site

The study site is a pre-designated research plot on the west side of the Olympic National Rainforest. The plot is located in an old-growth forest stand in the upper

Queets River Watershed (47.34N, 124.09W). The temperate rainforests in this area are known for their frosty, wet winters, and warm dry summers (Rohrig and Ulrich, 1991). The dominant hardwood and coniferous species are bigleaf maple and Sitka spruce, respectively. Red alder (*Alnus rubra* Bong.), Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco], western hemlock [*Tsuga heterophylla* (Raf.) Sarg.], and vine maple (*Acer circinatum* Pursh.) are also established in this stand (Tejo *et al.*, 2014). The dominant understory species are redwood sorrel (*Oxalis oregana* Nutt.) and sword fern [*Polystichum munitum* (Kaulf.) C. Presl] (Tejo *et al.*, 2014).

In-Field Sampling

Four bigleaf maples are rigged for climbing into the canopies to facilitate sampling of canopy roots. Three root-tip samples from the canopy and the forest floor roots were collected from each of these four trees, totaling 24 root-tip samples. Single-rope climbing techniques (Perry, 1978) were used to access canopy branches. On each sampled branch, a saw and trowel were used to gently loosen the epiphytic mats to access the canopy soil underneath. Adventitious roots were then identified within canopy soil and traced back to host-tree branch for confirmation. Once proved to be adventitious roots from the host tree, root-tips were collected, labeled with sample and plot #s, and placed in a cooler until returned to the lab. From the same tree that adventitious roots were collected from, forest floor roots were also sampled. Similar techniques were used to obtain forest floor root tips. Ground root tips were collected, labeled with sample and plot #s, and placed in a cooler until back in the lab.

In-Lab Analyses

Once back in the lab, any loose debris was removed from adventitious and lateral root samples. Roots were washed in a 0.05% Tween 20 solution, followed by an ethanol rinse and sterile water rinse (Provided by the UW's Comparative Genomic Center). Clean root tips were then placed in the lyophilizer for 16 hrs, to remove any excess moisture. DNA was then extracted from each root-tip using QIAGEN's protocol for the DNEasy plant DNA extraction kit. From extractions, 1µl of pure DNA from each individual root-tip was used in individual PCR reactions. PCR was performed in 20µl reactions, by making a PrimeStar GXL Taq mastermix, and adding 0.5µl (10µM working stock) of primers ITS-1F and ITS-4 to amplify the fungal internal transcribed spacer (ITS) region of the nrDNA (White *et al.*, 1990). The PCR protocol used with Primestart GXL Taq was 35 cycles of 98°C for 10 seconds, 55°C for 15 seconds, and 68°C for 1 minute. After the 35 cycles, samples were incubated at 68°C for 10 minutes.

The resulting PCR reactions all resulted in double and triple bands in gel electrophoresis (See Fig. 4.1), signifying the presence of multiple genomic sequences within each root-tip. In order to identify fungi, the resulting PCR reactions were cloned using the protocol for QIAGEN's DNA Cloning Kit Plus. Blue and white screening was used to select up to 12 successful cloned root colonies per root-tip (See Fig. 4.2), and another PCR reaction was performed. Following the second round of PCR, DNA was cleaned up using the PEG precipitation protocol provided by the University of Washington's Comparative Genomics Center. Clean DNA was

sequenced, reactions were carried out as specified by the manufacturer (Applied Biosystems). Directly after sequencing, reactions were cleaned up using an ethanol precipitation protocol also provided by the Comparative Genomics Center. To analyze samples on the ABI 3730 genetic analyzer, 10 µl of HiDi formamide was added to the cleaned DNA.

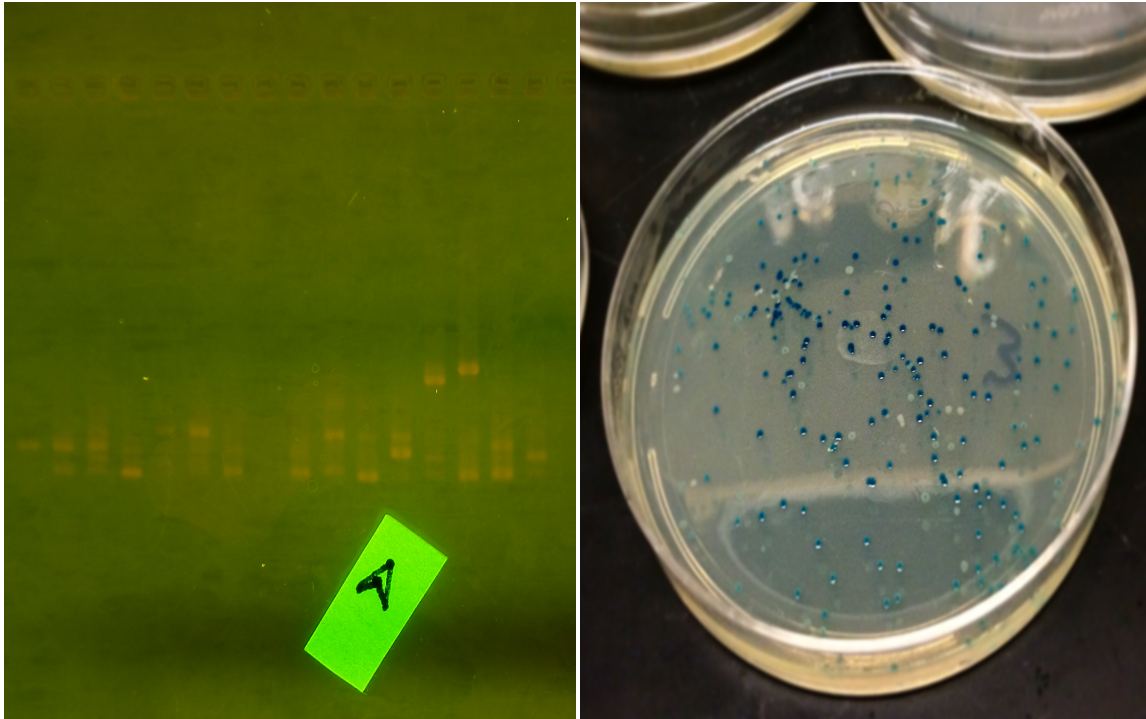


Fig. 4.1 and 4.2. 4.1 (left) shows gel electrophoresis that returned two to three bands, indicating more than one fungal species present in root tip extraction. Figure 4.2 (right) is a plate of LB agar with successful cloned colonies. The white dots contain fungal DNA extracted from root-tips. Photo: Korena Mafune

Sequenced samples were entered into the NCBI BLAST database (See Altschul *et al.*, 1990) for identification. BLAST, the Basic Local Alignment Search Tool, is provided by the National Center for Biotechnology Information. It is an extensive database of biological sequences, and uses statistics to find the best matches for query sequences. It returns the best matches, including identity score and an Expect value

(E-value). The closer the E-value is to zero, the more significant the match is to the query. After selecting the best matches, a master DNA FASTA file was submitted to the EBI ClustalW program (See Larkin *et al.*, 2007), provided online by the European Bioinformatics Institute. This runs a multiple sequence alignment algorithm on all of the sequences submitted.

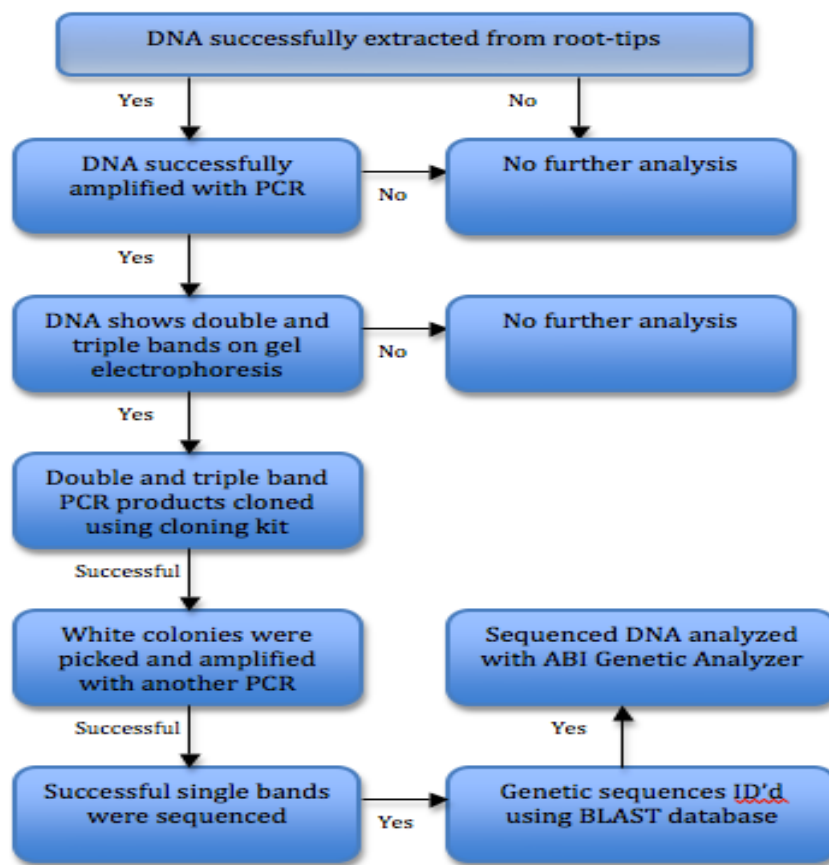


Figure 4.3. Flow chart of steps followed in lab methods and analyses.

Results

Adventitious canopy roots and forest floor roots extracted and the fungal ITS region of these samples was amplified in efforts to identify mycorrhizal DNA. From each root-tip 10-12 clones were sampled, and a single unique mycorrhizal DNA sequence

was identified in 13 out of the 24 root-tip extractions (12 adventitious and 12 ground roots). Out of the 13 root-tips that returned results suggesting the existence of mycorrhizal DNA, seven samples were from the forest floor and six were samples from the canopy. These results suggest that 54% of forest floor roots and 46% of adventitious roots collected in the canopy confirmed the presence of mycorrhizal DNA. Plot one returned three out of six mycorrhizal sequences for adventitious roots collected in the canopy soil, and plots two and four returned a single mycorrhizal sequence, respectively. Adventitious roots in plot three did not return any mycorrhizal DNA result. Mycorrhizal DNA was extracted from forest floor roots of all plots, except plot three. Successful mycorrhizal DNA extraction was independent to root type, and results were not statistically different ($P = 0.68$, $\alpha = 0.05$, Fischer's exact test).

Four out of the five mycorrhizas found in adventitious canopy roots are 'Uncultured EMF clones', and the remaining sample returned DNA for 'vouchered mycorrhiza', all of which have 97-99% identity match to sequences logged in GenBank except S9 which has 89% identity match (See Table 1.1). All adventitious root samples returned an 'uncultured EMF clone' identity, except a sample from plot one, which returned a 'vouchered mycorrhiza'.

Out of seven successful base root extractions, two samples returned EMF DNA from the class *Leotiomycetes*, one returned EMF DNA from the order *Helotiales* in the class *Leotiomycetes*, and another returned EMF DNA to the genus *Cadophora*. The

DNA that returned identities to these levels were all extracted from the base roots sampled in plot two.

The other three samples (two from plot one and one from plot four) were not identified further beyond the 'Vouchered mycorrhiza' designation, due to lack of vouchered references. Although a genus level was unable to be assigned to the majority of mycorrhizal DNA, ClustalW sequence alignment allowed further analysis of these samples.

ClustalW2 (Larkin *et al.*, 2007) was used to perform a multiple sequence alignment on the data, returning a percent identity matrix with alignment scores (See Table 1.2a and 1.2b) and a phylogenetic tree (See Fig. 5.1). The matrix measures similarity and calculates pairwise scores between each sequence by returning the percent of identical nucleotides, divided by the length of the shortest sequence. Those values were then divided by 100 and subtracted from 1 to calculate the distance matrix. The distance matrix is computed using a fast pairwise alignment algorithm (See Lopez *et al.*, 2004), and all sequences are clustered in hierarchical order to create the tree.

DNA sequences of clones from the base roots of plot one, S3 and S4, has a 99% alignment score in the percent identity matrix (Table 2.2 and 2.3). Clones from adventitious roots from plot one returned an alignment score of 99% for S6 and S13. Also, S5 and S10 scored 97%, but S5 was sampled from a branch in plot one

and S10 was from a base root in plot four. As displayed on the phylogenetic tree (Fig. 5.1), these mycorrhizas are more closely related to one another within their group, respectively. When comparing S3 and S4, S5 and S10, and S6 and S13, there is only about 30%-40% alignment score. The high scores among the sequence alignments were only returned for those 6 samples.

The other seven samples, identified as ‘uncultured ectomycorrhizal clones’ (two in the class of *Leotiomycetes*) do not show as much similarity (See Table 2.1). All these alignment scores return under 80%; several score between 30-40%, supporting that there is higher diversity among these sequences within and among plots (See Table 2.2 and 2.3). BLAST results suggest that all of these seven samples are ectomycorrhizal. Common regions within the DNA sequences are expected because they are related, but the gaps and non-alignment of nucleotides supports that they could be from different groups of species and genus.

Table 2.1. BLAST results showing identity for each sample. E-value and identity percent are included.

Plot	Root	Sample ID	Genbank Accession #	Closest Genbank Match	E-Value	Identity %
1	Base	S3	EF026068.1	Vouchered Mycorrhiza Clone	0	473/480 (99%)
1	Base	S4	EF026067.1	Vouchered Mycorrhiza Clone	0	460/465 (99%)
1	Branch	S5	EF026068.1	Vouchered Mycorrhiza Clone	0	447/457 (97%)
1	Branch	S6	KF879492.1	Uncultured EMF Clone	0	602/608 (99%)
1	Branch	S13	KF879492.1	Uncultured EMF Clone	0	616/617 (99%)
2	Base	S2	FJ553339.1	Uncultured EMF Clone (<i>Leotiomycetes</i>)	0	648/658 (98%)
2	Base	S7	FJ553339.1	Uncultured EMF Clone (<i>Leotiomycetes</i>)	0	600/610 (98%)
2	Branch	S8	JX042911.1	Uncultured EMF Clone	0	456/463

2	Branch	S9	IX043193.1	Uncultured EMF Clone	0	(98%) 454/510
2	Base	S11	IX243949.1	Cadophora sp.	0	(89%) 542/547
2	Base	S12	FJ553766.1	<i>Heliotales clone</i>	0	(99%) 485/507
4	Branch	S1	IX042840.1	Uncultured EMF Clone	0	(96%) 429/430(9
4	Base	S10	EF026065.1	Vouchered Mycorrhiza	0	9%) 498/505 (99%)

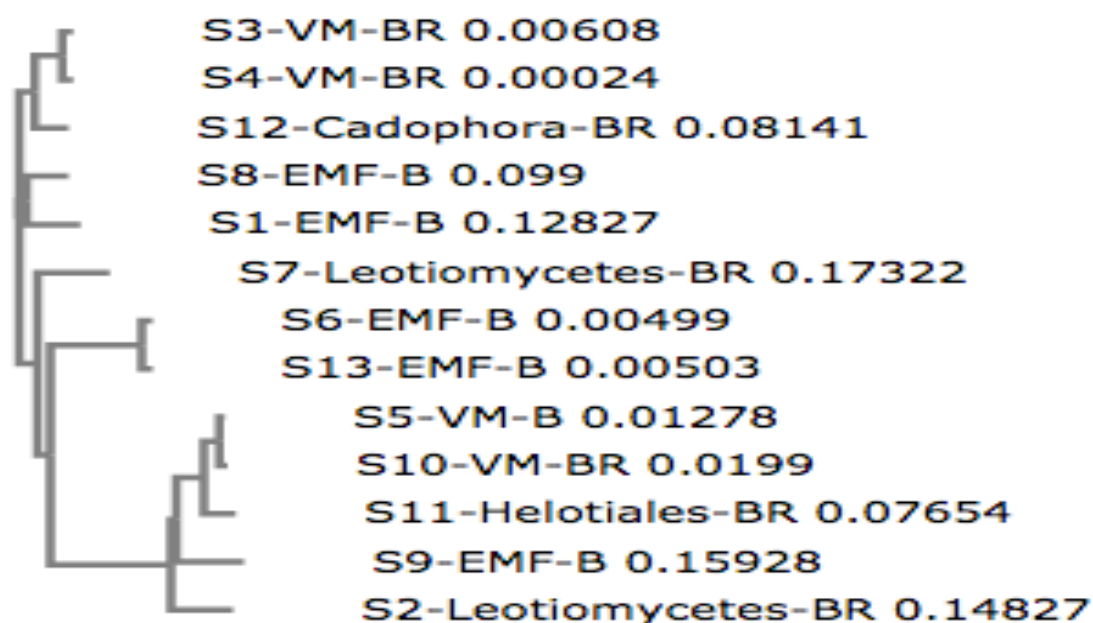


Fig. 5.1. Phylogenetic tree generated by ClustalW2. Samples can be identified by Sample #-EMF or Vouchered Mycorrhiza-Branch or Base Root. Values to the right display evolutionary distances, calculated from the by taking the percent identity scores, dividing by 100 and subtracting from 1, to give number of differences per site.

Table 2.2. Percent identity matrix calculated using ClustalW by taking the percent of identical nucleotides and dividing it by the length of the total sequence. Bold values indicate where the same sample aligns returning 100% identity match, and an asterisk (*) indicates any samples that have more than 90% identity match.

	S3-VM-BR	S4-VM-BR	S12-Cadophora-BR	S8-EMF-B	S1-EMF-B	S7-Leotiomyces-BR
S3-VM-BR	100	99**	84.	76	75	69
S4-VM-BR	99**	100	85	76	75	70
S12-Cadophora-BR	84	85	100	77	74	64
S8-EMF-B	76	76	77	100	77	67
S1-EMF-B	75	75	74	77	100	64

S7-Leotiomycetes-BR	69	70	64	67	64	100
S6-EMF-B	61	62	59	58	53	57
S13-EMF-B	61	61	59	59	53	58
S5-VM-B	40	41	40	38	38	39
S10-VM-BR	39	40	39	38	36	38
S11-Helotiales-BR	38	39	37	35	35	35
S2-EMF-BR	38	39	37	38	35	35
S9-EMF-B	34	34	35	35	33	35

Table 2.3. Percent identity matrix calculated using ClustalW by taking the percent of identical nucleotides and dividing it by the length of the total sequence. Bold values indicate where the same sample aligns returning 100% identity match, and an asterisk (*) indicates any samples that have more than 90% identity match.

	S6-EMF-B	S13-EMF-B	S5-VM-B	S10-VM-BR	S11-Helotiales-BR	S2-EMF-BR	S9-EMF-B
S3-VM-BR	61	61	40	39	38	38	34
S4-VM-BR	62	61	41	40	39	39	34
S12-Cadophora-BR	59	59	40	39	37	37	35
S8-EMF-B	58	59	38	38	35	38	35
S1-EMF-B	53	53	38	36	35	35	33
S7-Leotiomycetes-BR	57	58	39	38	35	35	35
S6-EMF-B	100	99*	35	35	32	34	33
S13-EMF-B	99*	100	35	34	32	34	33
S5-VM-B	35	35	100	97**	88	73	73
S10-VM-BR	35	34	97**	100	86	71	73
S11-Helotiales-BR	32	31	88	86	100	72	69
S2-EMF-BR	34	34	73	71	72	100	67
S9-EMF-B	33	33	73	73	69	67	100

Discussion

This study was designed to taxonomically identify mycorrhizas colonizing canopy adventitious roots and forest floor roots of old-growth bigleaf maples. This study was able to provide evidence of ectomycorrhizal fungi forming symbiotic associations with adventitious canopy roots of bigleaf maple. Seven out of the twelve samples could not be identified past the point of 'Uncultured EMF clone'. The pairwise percent identity scores among the unidentified EMF clones showed variation among sequences. For example, S2 and S3 are located on opposite ends of

the phylogenetic tree, and have an alignment score of 38%. Although there is lack of genus comparison, the amount of variability between these two sequences supports the high possibility of them being different levels of species or genus. When observing scores among all 'Uncultured EMF clone' sequences, it becomes evident that there is diversity in the sequences of mycorrhizas associated with canopy and forest floor roots.

This study identified three samples with fungi in the order and class of *Leotiomyces* and *Helotiales* that are known to be ectomycorrhizal (Toju *et al.*, 2013). The only sample identified to genus level was a *Cadophora spp.*, which has also been reported as an ectomycorrhizal species (Smith and Read, 2008) found in the class *Ascomycetes*. *Cadophora spp.* do not commonly form mycorrhizal relationships with bigleaf maple, so to fully support the claim of it being mycorrhizal, microscopic morphological analysis would need to be undertaken. However, BLAST returns closely related to this particular sequence of *Cadophora spp.* were all ectomycorrhizal. Observation of the above determines that these sequences are different; suggesting that there is mycorrhizal diversity among canopy and forest floor roots.

Previous research had reported AMF in these bigleaf maples, this study had a goal to taxonomically identify the mycorrhizas reported to colonize these tree roots. Out of all the mycorrhizal samples, 69% identified are EMF, which suggests that both EMF and AMF are present in the roots of these bigleaf maples. Bigleaf maples are widely

known to form relationships with AMF (Bunnell and Dunsworth, 2009), so it was interesting to find that the majority of DNA sequenced was ectomycorrhizal. However, other angiosperms such as *Populus*, *Salix*, and *Eucalyptus* can be colonized by both AMF and EMF within the same root system (Wagg *et al.*, 2011; van der Heijden, 2001), so it is possible that both types of fungi could be present in the ground root system and the adventitious root system. Unfortunately, we cannot statistically determine if AMF and EMF colonization is dependent on type of root, due to small sample size.

Another objective of this study was to determine if the mycorrhizas found colonizing roots in the canopies of maple trees differed from those colonizing roots found in the forest floor for the same tree species. There were not enough samples identified to genus level to run statistical analyses to compare if the genus of mycorrhizas in adventitious roots found in canopy soils and forest floor roots were significantly different. However, after running Fisher's exact test (FET) on the data collected, it was possible to report that mycorrhizal DNA extraction was not dependent on whether the root was sampled from the ground or canopy.

One other study did report the presence of EMF in adventitious canopy roots of silver birch (*Nothofagus menziesii*) in a temperate rainforest in New Zealand (Orlovich *et al.*, 2013). In contrast, another study in Costa Rica reported endophytic fungal species, but an absence of mycorrhizas in the canopy roots of *Quercus copeyensis* (Fagaceae) (Hertel, 2011). This indicates that mycorrhizal inoculation of

canopy roots is not universal, and could depend on evolutionary traits and/or host tree species.

It has become apparent that with the evolution and adaptation of these ecosystems, mycorrhizas are not just prevalent in the forest floor. Nadkarni (1981) reported morphological evidence of arbuscular mycorrhizal hyphae in the adventitious roots of bigleaf maple in this same study area. But this initial report was not followed by further research verifying the abundance or species taxonomy to identify what fungi formed these symbiotic relationships.

A majority of the studies on canopy soils focus on litter input and decomposition, epiphytic plant diversity and associations, and the role of canopy soils in nutrient cycling (See Tejo *et al.*, 2014, Nadkarni *et al.*, 2001, Matson *et al.*, 2014). Knowing that mycorrhizas are associated with adventitious roots expands our understanding of how canopy soils may contribute to the ecosystem as a whole. The environment found in the canopy appears to favor some mycorrhizal types over others.

Adventitious roots in canopy soils of bigleaf maple could have evolved to favor relationships with EMF over AMF, due to the fact that EMF have the most active set of enzymes for foraging on complex organic materials and have evolved towards optimal nutrient uptake for the host plant (Olsson *et al.*, 2003). In contrast, AMF favor the uptake of soluble nutrients, and have evolved towards optimal search for an alternative host (Olsson *et al.*, 2003). On branches with canopy soil, there are

roots from the host tree, saplings, and epiphytic species forming in the soil.

Mycorrhizas have been reported in epiphytic canopy plants (Rains *et al.*, 2003), but there isn't much potential to form a mycelial network beyond the island of arboreal soils of that local branch. Continuing to research the relationship between adventitious canopy roots and canopy soil will benefit our understanding of how these ecosystems have evolved and adapted to potentially benefit ecosystem function.

SUMMARY

The Queets River Watershed is located in the Temperate Rainforests of Western Washington. In the old-growth stands, mats of epiphytic material form high up on canopy branches, collecting organic debris. With time, the organic materials decompose forming a humic substance known as 'canopy' or 'arboreal' soil. This material contains nutrients and water for established epiphytic communities. Certain host trees form adventitious roots, which penetrate into soil, to aid in resource allocation from these canopy soils. These canopy roots have the ability to form mutualistic relationships with mycorrhizal fungi, but the role of these associations in canopy roots is not yet well understood.

This study took place in an old-growth forest stand dominated by Sitka spruce [*Picea sitchensis* (Bong.) Carriere], and aimed to [1] taxonomically identify mycorrhizas present in canopy roots of bigleaf maple (*Acer macrophyllum* Pursh.),

and [2] compare mycorrhizal species identified in canopy roots to the forest floor roots of the host species. Adventitious and lateral root-tips, 12 from canopy and 12 from ground, were collected from four bigleaf maples and analyzed in the lab. The DNA was extracted from cleaned root-tips for PCR. The amplified fungi were cloned in bacteria. The cloned sequences in the resulting bacterial colonies were amplified by PCR for DNA sequencing. DNA sequences from the individual ITS sequence clones were submitted for BLAST, and aligned using Clustal analysis. The results provide evidence of ectomycorrhizas (EMF) in canopy and forest floor roots, but the majority of taxonomic results found by BLAST queries only provided identification as uncultured EM fungi. Multiple alignment analysis and a phylogenetic tree illustrate a diversity among sequences, suggesting that even though most ID's did not narrow down to family or genus, there is still an unexplored diversity.

CHAPTER 4

MORE THAN MYCORRHIZA

Other Findings

The objective of this research was to taxonomically identify mycorrhizas associated with canopy adventitious and forest floor roots. However, mycorrhizal fungi were not the only type of fungi found in root-tips. There were also several fungal endophytes, fungi, and pathogens found in both root-tip types (See Table 3.1). When genetic analysis is performed using universal fungal specific primers, it is common for results to show a diversity of groups because several types of fungi can colonize root-tips (See Qi *et al.*, 2011; Kernaghan and Patriquin, 2011). With evidence for the presence of EMF and AMF in both forest floor and canopy soils, another interesting finding was the presence of dark septate endophytes (DSEs) in adventitious and forest floor roots.

These dark septate endophytes are fungal endophytes belonging to a few of the orders of Ascomycota (Jumpponen and Trappe, 1998), and are the most wide spread type of root endophytes (Stoyke *et al.*, 1992; Jumpponen and Trappe, 1998). They colonize and develop hyphae in cortical cells and intracellular regions of root-tips, forming microsclerotia (Knapp *et al.*, 2012). It has been noted that the characteristics of the hyphal coils they form are very similar to those of ericoid mycorrhizas (Mundyam and Jumpponen, 2009). Due to this hyphal growth, certain DSE are suggested to be mycorrhizal, depending on their host and habitat

characteristics (Zijlstra *et al.*, 2005). Over the last several decades, the characteristics and affinities of DSE have become better understood, but research related to DSE being mycorrhizal is limited.

In the old-growth maples, several *Cryptosporiopsis spp.* were identified, and have been categorized as being in the DSE group (Zijlstra *et al.*, 2005; Tsuneda and Wang, 2009). *Cryptosporiopsis ericae*, *C. Radicola*, and *C. melanigena* were all identified in the base roots of maple trees, and *C. Radicola* was also found in an adventitious root samples collected in the canopy soils.

For the first time, this study reports evidence of a DSE being isolated from canopy adventitious roots of bigleaf maple (See Table 3.1). Where mycorrhizal DNA was successfully extracted, DSE DNA was also successfully extracted. The results suggest that mycorrhizas are often associated with DSEs, and there is a potential that these DSE may even be mycorrhizal. It is of interest to better understand the presence of these DSE in adventitious roots, in regards to mycorrhizas and overall ecosystem function.

Table 3.1. BLAST results showing identity for ALL samples. E-value and identity percent are included.

Sample ID	Plot	Root	Genbank Accession #	Closest Genbank Match	E-Value	Identity %
1	Base	S3	EF026068.1	Vouchered Mycorrhiza Clone	0	473/480 (99%)
1	Base	S4	EF026067.1	Vouchered Mycorrhiza Clone	0	460/465 (99%)
1	Branch	S5	EF026068.1	Vouchered Mycorrhiza Clone	0	447/457 (97%)
1	Branch	S6	KF879492.1	Uncultured EMF Clone	0	602/608 (99%)
1	Branch	S13	KF879492.1	Uncultured EMF Clone	0	616/617

						(99%)
1	Branch	S14	JF908439.1	<i>Mycena stylobates</i>	0	557/563
						(98%)
1	Base	S15	AF141193.1	<i>Cryptosporiopsis</i>	0	527/530
				<i>Radicola</i>		(99%)
1	Branch	S16	FJ168567.1	<i>Agrocybe erebia</i>	0	587/587
						(100%)
1	Branch	S17	KM248537.1	<i>Ilyonectria crassa</i>	0	469/496
						(100%)
1	Base	S18	KF428646.1	<i>Ilyonectria sp.</i>	0	472/474
						(99%)
2	Base	S2	FJ553339.1	Uncultured EMF Clone	0	648/658
				(<i>Leotiomyces</i>)		(98%)
2	Base	S7	FJ553339.1	Uncultured EMF Clone	0	600/610
				(<i>Leotiomyces</i>)		(98%)
2	Branch	S8	JX042911.1	Uncultured EMF Clone	0	456/463
						(98%)
2	Branch	S9	JX043193.1	Uncultured EMF Clone	0	454/510
						(89%)
2	Base	S11	JX243949.1	<i>Cadophora sp.</i>	0	542/547
						(99%)
2	Base	S12	FJ553766.1	<i>Heliotales clone</i>	0	485/507
						(96%)
2	Branch	S19	AY521246.1	<i>Agrocybe Erebia</i>	0	652/657
						(99%)
2	Branch	S19	DQ093725.1	<i>Mortierella sp.</i>	0	578/579
						(99%)
2	Branch	S20	U51968.1	<i>Monacrosporium</i>	0	539/547
				<i>cionopagum</i>		(99%)
2	Branch	S21	AF141193.1	<i>Cryptosporiopsis</i>	0	522/522
				<i>Radicola</i>		(100%)
3	Base	S22	KJ475469.1	<i>Ilyonectria crassa</i>	0	506/506
						(100%)
3	Base	S23	AF141196.1	<i>Cryptosporiopsis</i>	0	441/447
				<i>melanigena</i>		(99%)
3	Base	S24	FJ168567.1	<i>Agrocybe erebia</i>	0	507/507
						(100%)
3	Base	S25	KF850370.1	<i>Cryptosporiopsis ericae</i>	0	515/515
						(100%)
4	Branch	S1	JX042840.1	Uncultured EMF clone	0	429/430
						(99%)
4	Base	S10	EF026065.1	Vouchered Mycorrhiza	0	498/505
						(99%)

Future Research

The results from this project suggest further research on the topic of fungi in adventitious canopy roots. Although the sample size of mycorrhizas was not large enough to run a statistical analysis that could support the hypothesis, it did identify

genetic diversity among the mycorrhizal identities isolated from forest floor and canopy soil roots. For example, EMF and AMF were not the only fungal groups whose DNA was extracted from root-tips, DSE DNA was also extracted from forest floor and adventitious roots collected from the canopy soils. There is limited research on these mutualistic endophytes, and they have never been reported in adventitious canopy roots prior to this study. They have been suggested to have mycorrhizal characteristics, and it is not known if they are acting as mycorrhiza in canopy soils.

Future research is needed in this ecosystem to sample more adventitious and canopy roots from bigleaf maples to more robustly sample and identify fungal species forming symbiotic associations with tree roots. With more samples, it may be possible to statistically compare mycorrhizal diversity among adventitious canopy soil and forest floor roots. It would also be worthwhile to sample the roots of Sitka spruce trees growing in the same forests. The canopy soil characteristics between these tree species differ (Tejo *et al.*, 2014), and determining whether similar patterns of mycorrhizal colonization would be found in both forest floor and canopy soil root systems. Comparing the diversity of symbionts in a different tree species growing under the same climatic and edaphic conditions would complement the findings produced during this study. This is important because it will provide a better understanding of how these old-growth forests adapt, and if they are resilient to factors such as climate change.

To further comprehend how forests with canopy soils adapt to climate change are important to research. If climate change results in drier conditions, the role of the canopy soil roots and symbionts may be reduced and there is the potential of a loss of forest resilience. Once there is a more robust understanding of mycorrhizas and other endophytes in canopy soils within these old-growth ecosystems, the results from this study area may be expanded to other areas characterized by similar conditions. The overall goal would be to compare the fungal associations of canopy roots in this temperate old-growth rainforest to a New Zealand or Chilean Temperate rainforest, and then a cloud forest of Costa Rica. It is not expected that these ecosystems will share the same associations due to varying ecosystem characteristics but the functional roles of symbionts should be similar among these ecosystems. Researching mutualistic fungal associations in adventitious canopy roots among several ecosystems around the world will benefit our understanding of how canopy soils play a role in these areas.

CHAPTER 5

CONCLUSIONS

The research conducted for this Master's Thesis focused on DNA identification of mycorrhizal fungi in forest floor and canopy adventitious roots of old-growth bigleaf maple, located in the Queets River Watershed in Washington's Olympic National Park. This research is important to the field of ecological science because it is seeking a better understanding of how adventitious canopy roots contribute to forest resilience and acquisition of nutrients under conditions where the trees are less connected to soil nutrients. The objectives of this project were to 1) Determine if it was possible to identify mycorrhizas through root-tip DNA extraction, and 2) Compare the results among and between forest floor and adventitious roots. It was hypothesized that the mycorrhizas identified in adventitious roots would be different from those identified from forest floor roots, which relates to adaptations of the host tree.

Sampling took place in the Queets Temperate Rainforest, four bigleaf maples were climbed to collect adventitious roots. The samples were analyzed in the lab using DNA extraction, PCR, cloning, and DNA sequencing. Out of 24 root-tip samples, 13 returned with results suggesting the presence of mycorrhizal DNA. This study is also reporting evidence of EMF in adventitious roots growing in canopy soils of bigleaf maple. Due to sample size and lack of vouchered references, there was not enough data collected to statistically determine if mycorrhizal type was dependent on root type. However, quantitative analyses allowed determination that there is a diversity 37

of mycorrhizas among canopy and forest floor roots. This study also showed using FET to determine that mycorrhizal DNA extraction was not dependent on root type or where root samples were collected.

REFERENCES

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Abee, A., and Lavender, D. 1972. Nutrient cycling in throughfall and litter fall in 450-year-old Douglas-fir stands. *Proceedings: Research in Coniferous Forest Ecosystem—a Symposium*. Bellingham, WA. pp. 133-143.
- Archibold, O.W. 1995. *Ecology of World Vegetation*. Chapman and Hall, London, UK.
- Aubrey, D.A., Nadkarni, N.M., and Broderick, C.P. 2013. Patterns of moisture and temperature in canopy and terrestrial soils in a temperate rainforest, Washington. *Botany (formerly Canadian Journal of Botany)* 91: 739-744.
- Averill, C., Turner, T.L., Finzi, A.C. 2014. Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature* 505:543–545
- Bamforth, S.S. 2010. Distribution of and insights from soil protozoa of the Olympic coniferous rain forest. *Pedobiologia* 53(6):361-367
- Bellini, C., Pacurar, D.I., Perrone, I. 2014. Adventitious roots and lateral roots: similarities and differences. *Annu. Rev. Plant Biol.* 65 639–666
- Brady, N.C., and Weil, R.R. 2010. *Elements of the nature and properties of soils*. Upper Saddle River, N.J.: Pearson Prentice Hall.
- Bray, J.R., and Gorham, E. 1964. Litter production in forests of the world. *Advan. Ecol. Res.* 2: 101-157.
- Bunnell, F.L., Dunsworth, G.B., Huggard, D.H., and Kremsater, L.L. 2009. Sustaining forest dwelling species. In *Forestry and Biodiversity: Learning How to Sustain Biodiversity in Managed Forests*. Edited by F.L. Bunnell and G.B. Dunsworth. University of British Columbia Press, Vancouver, BC. pp. 173–215.
- Cairney, J.W.G. 2000. Evolution of mycorrhiza systems. *Naturwissenschaften*, 87:467–475
- Cairney, J.W.G., and Meharg, A.A. 2003. Ericoid mycorrhiza: a partnership that exploits harsh edaphic conditions. *European Journal of Soil Science*, 54: 735–740.

- Carpenter, D.N., Bockheim, J.G., and Reich, P.F. 2014. Soils of temperate rainforests of the North American Pacific Coast. Publications from USDA-ARS / UNL Faculty. Paper 1413.
- Clark, K.L., Nadkarni, N.M., and Gholz, H.L. 1998. Growth net production, litter decomposition, and net nitrogen accumulation by epiphytic bryophytes in a tropical montane forest. *Biotropica* 30:12-23
- DellaSala, D.A., Moola, F., Alaback, P., Paquet, P.C., von Wehrden, H., Nauman, R.S. 2011. Just what are temperate and boreal rainforests? In *Temperate and Boreal Rainforests of the World: Ecology and Conservation*. Edited by D.A. DellaSala. 2011. Island Press.
- Edmonds, R.L., and Murray, G.L.D. 2002. Overstory litter inputs and nutrient returns in an old-growth temperate forest ecosystem, Olympic National Park, Washington. *Canadian Journal of Forest Research* 32:742-750.
- Enloe, H.A., Graham, R.C., and Sillett, S.C. 2006. Arboreal histosols in old-growth redwood forest canopies, northern California. *Soil Science Society of America Journal* 70:408-418.
- Finlay, R.D. 2008. Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. *Experimental Botany*, 59:1115-1126.
- Franklin, J.F., Cromack, Jr., K., Denison, W., McKee, A., Maser, C., Sedell, J., Swanson, F., and Juday, G. 1981. Ecological characteristics of old-growth Douglas-fir forests. Gen. Tech. Rep. PNW-118, USDA Forest Service, Pacific Northwest Forest and Range Experiment Station, Portland, Oregon.
- Franklin, J.F. 1993. Lessons from old growth. *Journal of Forestry*. 91(12), 11-13.
- Gerdemann, J.W. 1968. Vesicular-arbuscular mycorrhizae and plant growth. *Ann Rev Phytopathology* 6:39
- Hertel, D. 2011. Tree roots in canopy soils of old European beech trees—An ecological reassessment of a forgotten phenomenon. *Pedobiologia* 54:119-125
- Hertel, D., Köhler, L. and Rillig, M.C. 2011. Mycorrhizal, Endophytic and Ecomorphological Status of Tree Roots in the Canopy of a Montane Rain Forest. *Biotropica*, 43: 401-404
- Hietz, P., Wanek, W., Wania, R., and Nadkarni, N.M. 2002. ¹⁵N natural abundance in a montane cloud forest canopy as an indicator of nitrogen cycling and epiphyte nutrition. *Oecologia* 131:350-355

- Hodge, A., Helgason, T., and Fitter, A.H. 2010. Nutritional ecology of arbuscular mycorrhizal fungi. *Fungal Ecology* 3:267-273.
- Johnson, D.W., West, D.C., Todd, D.E., and Mann, L.K. 1982. Effects of sawlog vs. whole-tree harvesting on the nitrogen, phosphorus, potassium, and calcium budgets of an upland mixed oak forest. *Soil Science Society of America Journal*, 46(6), 1304-1309.
- Jumpponen A, Trappe J.M., 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytol* 140: 295–310.
- Knapp, D.G., Pintye, A., and Kovács, G.M. 2012. The Dark Side Is Not Fastidious – Dark Septate Endophytic Fungi of Native and Invasive Plants of Semiarid Sandy Areas. *PLoS ONE*, 7(2), e32570.
- Kernaghan, G., and Patriquin, G. 2011. Host associations between fungal root endophytes and boreal trees. *Microbial ecology*, 62(2), 460-473.
- Klopatek, J.M. 2007. Litterfall and fine root biomass contribution to nutrient dynamics in second- and old-growth Douglas-fir ecosystems. *Plant and Soil* 294: 157-167.
- Kluber, L.A., Tinnesand, K.M., Caldwell, B.A., Dunham, S.M., Yarwood, R.R., Bottomley, P.J., Myrold, D.D. 2010. Ectomycorrhizal mats alter forest soil biogeochemistry. *Soil Biology and Biochemistry* 42: 1607-1613.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21): 2947-2948.
- Liski, J., Nissinen, A., Eerhard, M., and Taskinen, O. 2003. Climate effects on litter decomposition from arctic tundra to tropical rainforest. *Global Change Biology* 9:575-84.
- Lomolino, M.V., and Perault, D.R. 2004. Geographic gradients of deforestation and mammalian communities in a fragmented, temperate rainforest landscape. *Global Ecology and Biogeography* 13:55-64.
- Malhi, Y., Baldocchi, D.D., and Jarvis, P.G. 1999. The carbon balance of tropical, temperate and boreal forests. *Plant, Cell and Environment*, 22: 715–740.
- Matelson, T.J., Nadkarni, N.M., and Longino, J.T. 1993. Longevity of fallen epiphytes in a Neotropical montane forest. *Ecology* 74: 265–269.

- Matson, A.L., Corre, M.D., and Veldkamp, E. 2014. Nitrogen cycling in canopy soils of tropical montane forests responds rapidly to indirect N and P fertilization. *Global Change Biology*, 20: 3802–3813.
- Mandyam, K., and Jumpponen, A. 2008. Seasonal and temporal dynamics of arbuscular mycorrhizal and dark septate endophytic fungi in a tallgrass prairie ecosystem are minimally affected by nitrogen enrichment. *Mycorrhiza*, 18(3), 145-155.
- Nadkarni, N.M., Merwin, M.C., and Nieder, J. 2001. Forest canopies: plant diversity. In *Encyclopedia of Biodiversity*. Edited by S. Levin, 27-40, Academic
- Nadkarni, N.M., and Su, M. 2004. Old-growth forest canopy structure and its relationship to throughfall interception *Forest Science* 50:290-298.
- Nadkarni, N.M. 1981. Canopy roots: convergent evolution in rainforest nutrient cycles. *Science* 214:1023–1024,
- Nadkarni, N.M. 1985. Nutrient capital of canopy epiphytes in an *Acer macrophyllum* community, Olympic Peninsula, Washington State. *Canadian Journal of Botany* 77:136-42.
- Olsson, P.A., Jakobsen, I., and Wallander, H. 2003. Foraging and resource allocation strategies of mycorrhizal fungi in patchy environments. In *Mycorrhizal ecology*, 2003. Edited by Van der Heijden, M.G.A. and Sanders, R. Springer-Verlag Berlin Heidelberg. pp. 93-105
- Orlovich, D.A., Draffin, S.J., and Daly, R.A. 2013. Piracy in the high trees: ectomycorrhizal fungi from an aerial ‘canopy soil’ microhabitat. *Mycologia* 105:52-60
- Ovington, J.D. 1983. Temperate broad-leaved evergreen forests. *Ecosystems of the World*, Vol. 10. Elsevier, Amsterdam
- Palmer, C., and Engel, S. 2009. Introduction: reducing CO₂ emissions through avoiding deforestation, Ch. 1: Deforestation: Prospects for Mitigating Climate Change, Routledge, Oxford, pp. 1-8.
- Pérez, C.A., Guevara, R., Carmona, M.R., and Armesto, J.J. 2005. Nitrogen mineralization in epiphytic soils of an old growth *Fitzroya cupressoides* forest, southern Chile. *Écoscience* 12:210–215
- Perry, D.R. 1978. Method of access into crowns of emergence and canopy trees. *Biotropica* 10:155-157.

- Prentice, I.C., Harrison, S.P., and Bartlein, P.J. 2011. Global vegetation and terrestrial carbon cycle changes after the last ice age. *New Phytologist*, 189: 988–998
- Prescott, C.E. 2002. The influence of the forest canopy on nutrient cycling. *Tree physiology*. 22:1193.
- Qi, F., Jing, T., and Zhan, Y. 2012. Characterization of Endophytic Fungi from *Acer ginnala* Maxim. in an Artificial Plantation: Media Effect and Tissue-Dependent Variation. *PLoS ONE* 7(10): e46785
- Rasmussen, H.N., and Rasmussen, F.N. 2014. Seedling mycorrhiza: a discussion of origin and evolution in Orchidaceae. *Botanical Journal of the Linnean Society*.
- Rillig, M.C. 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecology Letters*, 7: 740–754.
- Rohrig, E., and Ulrich, B. 1991. Temperate Deciduous Ecosystems. *Ecosystems of the World*, vol. 7. Elsevier, Amsterdam.
- Rousk, J., and Nadkarni, N.M. 2009. Growth measurements of saprotrophic fungi and bacteria reveal differences between canopy and forest floor soils. *Soil Biology and Biochemistry*, 41(4), 862-865.
- Runyan, C.W., D'Odorico, P., and Lawrence, D. 2012. Physical and biological feedbacks of deforestation. *Reviews of Geophysics*, 50(4).
- Smith, S.E., Read, D.J. 2008. *Mycorrhizal Symbiosis*, Ed 3. Academic Press, New York
- Stoyke, G., Egger, K.N., and Currah, R.S. 1992. Characterization of sterile endophytic fungi from the mycorrhizae of subalpine plants. *Can J Bot* 70:2009-2016.
- Tejo, C.F. 2013. Canopy soils, litterfall and litter decomposition in a coastal old-growth temperate rainforest, Washington. Doctoral Dissertation. University of Washington.
- Tejo, C.F., Zabowski, D., and Nadkarni, N. 2014. Canopy soils of Sitka spruce and bigleaf maple in the Queets river watershed, Washington. *Soil Science Society of America Journal*, 78(1), 118-124.
- Toju, H., Yamamoto, S., Sato, H., and Tanabe, A.S. 2013. Sharing of diverse mycorrhizal and root-endophytic fungi among plant species in an oak-dominated cool-temperate forest.
- Trappe, J.M. 1962. Fungus associates of ectotrophic mycorrhizae. *Botanical Review* 28, 538-606.

- Tsuneda, A., Wang, W., Tsuneda, I., and Currah, R.S. 2009. Endomembrane system of aspen roots plays a key role in defense against a common fungal endophyte *Cryptosporiopsis radicola*. *Mycologia* 101 (2009): 182-189
- Trudell, S.A., and Edmonds, R.L. 2004. Macrofungus communities correlate with moisture and nitrogen abundance in two old-growth conifer forests, Olympic National Park, Washington, USA. *Can J Bot* 82:781
- Van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., and Sanders, I.R. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* 396: 69-72.
- Vance, E., and Nadkarni, N.M. 1990. Microbial biomass and activity in canopy organic matter and the forest floor of a tropical cloud forest. *Soil Biology and Biochemistry* 22:677-84.
- Vogt, K.A., Vogt, D.J., Moore, E.E. and Sprugel, D.G., 1989. Methodological considerations in measuring biomass, production, respiration and nutrient resorption for tree roots in natural ecosystems. In J.G. Torrey and L.J. Winship (Editors), *Applications of Continuous and Steady-State Methods in Root Biology*, Kluwer, London, pp. 217-232.
- Vogt, K., Publicover, D.A., Vogt, D. 1991. A critique of the role of ectomycorrhizas in forest ecology. *Agriculture, Ecosystems and Environment*, 35:171–190
- Vogt, K., Vogt, D., Brown, S., Tilley, J., Edmonds, R., Silver, W., and Siccama, T. 1995. Dynamics of forest floor and soil organic matter accumulation in boreal, temperate, and tropical forests. In R. Lal, J. Kimble, E. Levine, and B.A. Stewart eds., *Soil Management and the Greenhouse Effect, Advances in Soil Science*, CRC Press Boca Raton. Pages 159-178.
- Wagg, C., Antunes, P.M., Peterson, R.L. 2011. Arbuscular mycorrhizal fungal phylogeny-related interactions with a non-host. *Symbiosis* 53, 41–46
- Wang, B. and Qui, Y.L. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza*, 16: 299–363
- Zijlstra, J.D., Van't Hof, P., Baar, J., Verkley, G.J.M., Summerbell, R.C., Paradi, I., Braakhekke, W.G., and Berendse, F. 2005. Diversity of symbiotic root endophytes of the Helotiales in ericaceous plants and the grass, *Deschampsia flexuosa*. *Stud Mycol* 53: 147–162.

APPENDIX A

METHODOLOGY

Step-by-step explanation of field and lab techniques.

Canopy Soil Sampling (Repeat for each sample site)

Tools: Trowel, saw, sharpie, whirlpak sampling bags, small storage bag for sample bags, backpack, gloves.

1. Designate a branch that is thick in canopy soil to climb to.
2. Slightly loosen the epiphytic layer on top.
3. Use trowel to loosen canopy soil and designate adventitious roots that connect to branch.
4. Use trowel or saw to cut the adventitious roots.
5. Place sample in bag and label.
6. Repeat step 1-5 for branches at different heights in host tree, collecting 12 root-tips per tree.
7. When back on the ground, place labeled samples in cooler.

Forest soil sampling

Tools: Trowel, Shovel, Knife, whirlpak sampling bags, small storage bag for sample bags, backpack, gloves.

1. On the forest floor designate a root path for the host species that was just climbed.
2. Once this is designated, dig a small pit near the rooting system.
3. Trace the host roots until fine tips have been located.
4. Use trowel or saw to cut floor roots.
5. Place sample in bag and label.
6. Put bag in cooler.
7. Repeat steps 1-5 for several areas around host tree, collecting 12 root tips.
8. Repeat step 1-6 for each sample site of interest.

Root Storage and Clean-Up

Tools: Sieve, Sterile H₂O, 70% Ethanol solution, Tween 20, sterile containers

1. Root tips were stored at 4 °C in labeled bags until further analyses.
2. When ready for extraction, they were placed in sieve and rinsed with tap water, knocking loose coarse soil debris.
3. After surface cleaned with water, they were rinsed in a solution of Tween 20.
4. Following the Tween 20 rinse, they were rinsed with sterile H₂O.
5. After the sterile H₂O rinse, they were soaked in 70% Ethanol solution for 60 seconds and then rinsed again with sterile H₂O.
6. After the cleaning process, they were placed in a lyophilizer overnight (18 hrs) to completely dry root-tips.
7. After lyophilization root-tips are ready for DNA extraction.

Note: The diversity of fungi identified from ITS PCR clones was different

when the ITS PCR was carried out on roots not washed with detergent.

Without detergent wash more yeasts and fewer mycorrhizal fungi were

found, whereas with the detergent wash no yeasts were found and the

proportion of mycorrhizal and endophytic fungi was higher. Therefore

the detergent wash was adopted as the standard procedure for this project

DNA Extraction

Tools: Sieve, various lab utensils such as pipettes and tips, DI and Sterile H₂O,

QIAGEN DNEasy 96-well Plate Extraction Kit

1. Keep lyophilized sampled store in a cool dry place until ready for extraction.
2. Follow procedures provided by QIAGEN DNEasy 96-well Plate Extraction Kit protocol.

PCR Reaction

Tools: Template DNA (root tip extractions), reverse and forward primers (ITS1-F and ITS-4), 96 well plates (skirted), PrimeStar TAQ (Both Max and GXL were used), sterile dH₂O, gloves, PCR thermocycler, pipette, pipette tips, .2 ml PCR tubes, centrifuge.

1. Set up PCR plate and take extracted DNA from root tips and use pipettor to transfer to PCR plate.

2. Add 10 μ l PrimeStar Mastermix, 8 μ l sterilized H₂O, 1 μ l forward primers, and 1 μ l reverse primers to each well that has a DNA sample (This can also be calculated and mixed as a overall master mix, then added to each well, instead of doing it individually).

3. While mixing, keep reagents on ice.
4. Gently tap tube, and slowly spin in centrifuge, moving the contents to the bottom.

5. Keep on ice until ready for thermo-cycle.

6. Cover with safety film, and place in thermocycler.

7. Run a cycle at 98 C for 10 seconds, allowing the DNA to separate.

8. Run a cycle at 55 C for 15 seconds, allowing primers to bind

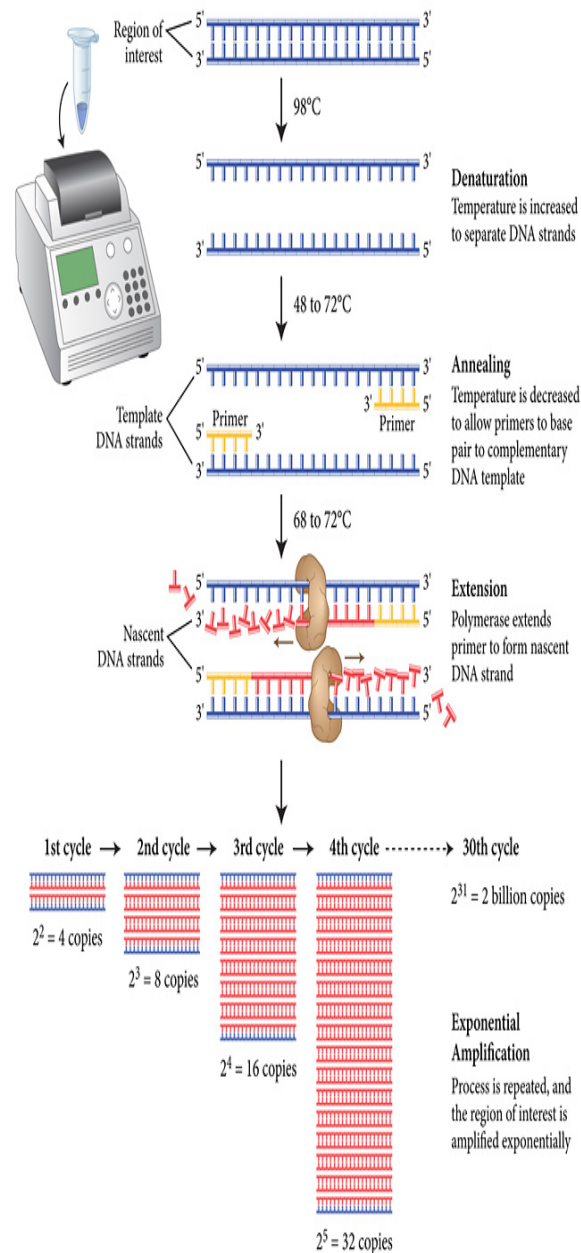


Figure A.1. Illustration of Polymerase Chain Reaction (PCR). Photo courtesy of Bioinformatics.

9. Run cycle at 72 C for 5 seconds, allowing TAQ polymerase to extend.
10. Program thermocycler to repeat steps 7-9, 35 times. Each cycle replicates the DNA segment.

(NOTE: Thermocycler settings will likely vary as implementation approaches)

Gel Electrophoresis

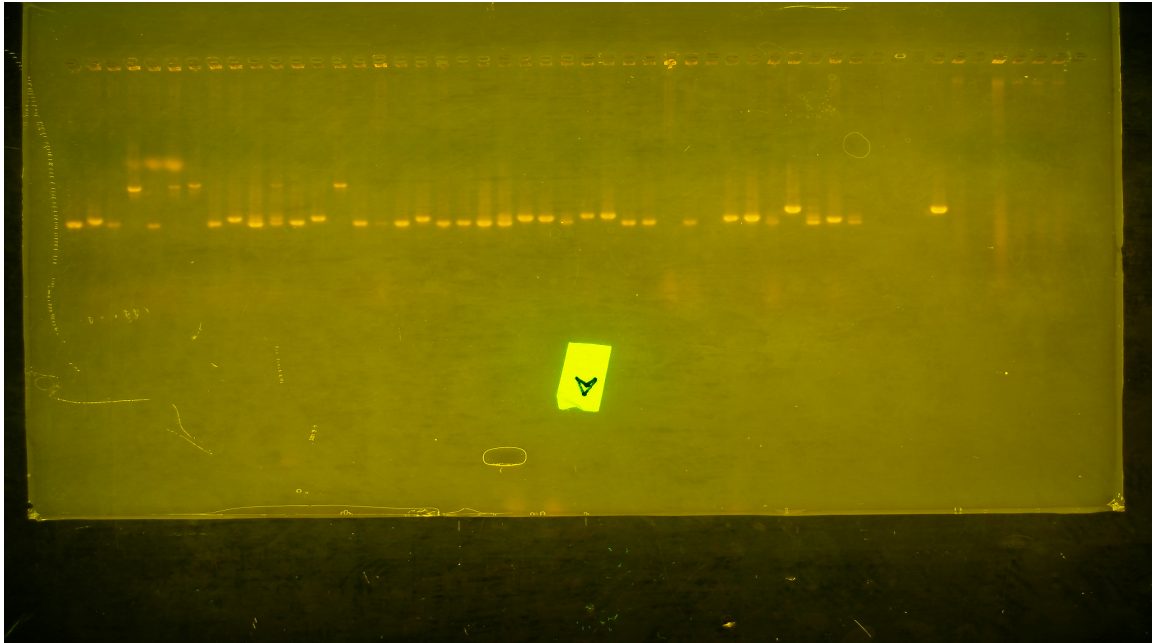


Fig. A.2. Visual representation of several successful PCR amplifications, shown in gel electrophoresis.

Tools: Electrophoresis machine, well comb, molecular ladder, agarose, TAE buffer, Ethylene Bromide, pipette and tips, blue dye, UV light.

1. Add 5 μ l blue dye to completed PCR samples.
2. Mix 1g agarose with 100ml TAE and microwave for about 3 minutes in microwave safe jar (will vary).
3. Put well comb in place and pour agarose into electrophoresis machine. Let solidify.
4. Fill surrounding area with TAE buffer.
5. Load ladder into first well, followed by one DNA sample per well, thereafter

6. Run the gel at 80-150V until the dye line is approximately 75-80% of the way down the gel.
7. Carefully remove the gel upon completion and place under UV light for observation of PCR success.

PCR Clean-Up (Provided by UW Comparative Genomics Center)

Tools: PEG 8000, NaCl, PCR reactions, sterile dH₂O, 100ml glass container, paper towel, micro-plate centrifuge, vortex.

1. Make a 20% PEG, 2.5 M sodium chloride stock solution by mixing with sterilized water, in a sterile container.
2. Add 20 µl of the PEG/NaCl solution to each well (equal to the volume of the PCR reaction).
3. Vortex the plate briefly and then place it in a 37°C water bath for 15 minutes.
4. Centrifuge at ~6000 x G, or maximum speed, in a swinging bucket plate centrifuge.
5. Invert the plate over the sink using a smooth circular motion to dump out as much of the supernatant as possible. Then blot the plate on a paper towel.
6. Place the plate upside down on a folded paper towel and centrifuge briefly at 600 x G to remove residual supernatant. Briefly means you use "Hold" for the time setting and just allow the centrifuge to reach 600 x G and turn it off.
7. Add 125 µL of cold 70% ethanol to each well, invert the plate using a smooth circular motion to dump as much ethanol as possible and then blot the plate on a paper towel.
8. Place the plate upside down on a folded paper towel and centrifuge briefly at 600 x G to remove residual supernatant.
9. Resuspend the samples in an appropriate amount of dH₂O.
10. The pure DNA is now ready for sequencing on the PCR block

DNA Sequencing on PCR Block

Tools: PCR Thermocycler, 2 µL of 2.5 x dilution buffer, 2 µL of BigDye 3.1 RRM, 4 pmoles of primer, cleaned-up DNA from PCR.
water + template + primer to 10 µL final volume, 36-well plate.

1. Mix DNA, buffer, and BigDye in a single well (for each DNA sample).

2. Run sequencing cycle on PCR block.
3. Once completed, plate is ready for BigDye clean-up.

BigDye Clean-up by Ethanol Precipitation(Provided by UW Comparative Genomics Center)

1. Make a premix of: 60 uL 3M NaOAc, pH 5.6, 290 uL H₂O, 1250 uL 100% EtOH
2. To each 10 uL sequencing reaction add 20 uL of the premix.
3. Vortex carefully to avoid splashing contents of wells into each other.
4. Hold at room temperature for 15 minutes.
5. Spin 20-30 minutes at full speed (5760 x G in our SIGMA plate centrifuge).
6. Invert the plate to drain it and place it upside down on a folded paper towel.
7. Add 125 uL of 70% EtOH to each well, mix by gently inverting the covered plate. Re-use the sealing film from the cycle sequence reaction as the cover.
8. Spin the plate at full speed for 10 minutes (right side up!)
9. Pour off the 70% ethanol and then briefly spin the plate inverted on top of a folded paper towel: Use the Hold setting for spin time. Let the speed just reach 600 x G, then stop the centrifuge.

DNA Sequencing on ABI Prism

Tools: ABI/PRISM 3130XL long array sequencer, 10uL HiDi

1. Add 10µl HiDi to each cleaned up sequenced well.
2. Set up in the ABI/PRISM 3130XL DNA sequencer, and run with short injection.
3. The ABI/PRISM 3130XL color codes the DNA nucleotides using the BigDye, providing the sequence that can be entered in the BLAST database for taxonomic identification.