DIVERSITY AND RATE OF INFECTION OF ERICOID MYCORRHIZAL FUNGI THAT COLONIZE *RHODODENDRON MAXIMUM* ALONG AN ELEVATIONAL GRADIENT AND THEIR POTENTIAL TO DEGRADE POLY-AROMATIC HYDROCARBONS USING LIGNIN DEGRADING ENZYMES

A Thesis
by
DANIEL CHASE PARKER

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Abstract

Diversity and Rate of Infection of Ericoid Mycorrhizal Fungi that Colonize *Rhododendron maximum* along an Elevational Gradient and Their Potential to Degrade Poly-aromatic Hydrocarbons Using Lignin Degrading Enzymes

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Elevational differences in moisture, temperature, and edaphic conditions in mountainous areas may drive changes in the diversity and composition of fungi associated with the roots of plants. We explored these relationships with *Rhododendron maximum,* an endemic shrub species found at all elevations in the Southern Appalachians. This study focuses on the dynamics of ericoid mycorrhizal (ErM) fungal colonization levels and diversity in *R. maximum* roots along elevational gradients in the southern Appalachian Mountains. First we hypothesized that overall ErM colonization levels in fine roots would increase with an increase in elevation due to an increased need for nutrient acquisition by the fungal symbionts for the plant. We also hypothesized that community composition of the fungi would change along an elevation gradient because some fungi are likely to be better adapted to potentially more xeric and nutrient poor conditions on the tops of mountains or other associated factors. Our last hypothesis was that ErM species at higher

elevations will have an increased ability to degrade lignin and make additional organic nitrogen sources in the soil available to the plant. To address this question we sampled roots and soils from three iso-elevational transects from high to low elevations at two locations. Root associated fungi were cultured and the DNA amplified from these cultures was typed by restriction fragment length polymorphism (RFLP) analysis of the ITS regions. Integrity of the RFLP types was confirmed by sequencing multiple representatives from each group. Root colonization was quantified by counting mycorrhizal hyphae at random locations within the root using light microscopy. Colonization was shown to increase at both sites at higher elevations. Differences in edaphic properties were observed across the elevational gradient, and these changes coincided with changes in the fungal community structure. Changes in abundance, frequency and species composition of the fungal communities tracked elevational differences at the two locations tested in this research. Differences in species composition at Unaka Mountain were clearer, showing separation of fungal communities by elevation. The communities on Hawksbill Mountain were not as distinct at each elevation, possibly because of a valley effect causing cold temperatures to settle in the gorge which can change microbial metabolism. Lignin degrading capacity was found in approximately half of the fungal taxa but was not linked to elevational changes in fungal community composition. Future directions for this research include greater sampling of the fungal communities at additional sites, experimental approaches to explore the relationships between edaphic factors and community composition, and greater characterization of the lignin degrading ability of the fungi.

Dedication

This work is dedicated to the loving memory of Lois and Devon Hill, known to me more affectionately as Maw-Maw and Paw-Paw. Without my time spent with them working in the garden I may have never found my passion for the environmental sciences.

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I want to express my gratitude first and foremost to my committee chair, Dr. John Walker, who has been there for me as a mentor and a friend. Without his guidance this thesis would not have been possible.

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Table of Contents

Foreword

The research detailed in this thesis will be submitted to *Mycologia*, a peer reviewed journal owned and published by the Mycological Society of America. The thesis has been prepared according to the style guide for the journal.

INTRODUCTION

Ericoid Mycorrhizae

Mycorrhizal fungi are a group of diverse soil organisms that associate with nearly all plants, aiding in the uptake of water and nutrients for the host plant in exchange for photosynthetically derived sugars (Martino et al. 2007). There are different types of mycorrhizal associations identified by the type of connection the fungus makes with its host plant. One association of particular interest in the Southern Appalachians Mountains is ericoid mycorrhizae (ErM). These fungi associate with plants in the family Ericaceae (Martino et al. 2007). In ErM associations the fungi typically have spiraled hyphae that are located intracellularly in the cortical cells found in the fine "hair" roots of the host plant (Martino et al. 2007). Ericaceous plants include *Rhododendron, Vaccinum*, and other plants that are commonly found in areas of poor nutrient availability and low pH, conditions which are often due to low temperatures and slow decomposition rates (Bougoure and Cairney 2005).

Rhododendron maximum

Rhododendron maximum is a naturally occurring understory shrub in the southern Appalachians that has become nearly ubiquitous in the mountains. A nutrient feedback loop between *R. maximum* and ErM fungi exists, which is a potential driver for increased

abundance of rhododendron thickets. *Rhododendron maximum* plants are evergreen with tannin and lignin rich foliage, which makes their litter difficult to break down. The ability of ErM fungi to decompose lignin allows them to reclaim the nutrients (particularly N) locked up in the leaf matter and to avoid competition from other mycorrhizal fungi in the soil. The reclaimed nutrients are then transferred to the host plant in exchange for carbohydrates. More nutrients are thus recouped by the plant which facilitates higher survival and success of the host plant (Wurzburger and Hendrick 2009). However, the ability of ErM fungi to degrade polyaromatic hydrocarbons (PAHs) such as lignin has not been characterized across the diversity of fungi associated in this relationship.

The range of *R. maximum* extends from Nova Scotia to Georgia with populations found as far west as southern Ontario, Ohio, and Kentucky (Munns 1938, Anderson 2008). To the east, a population has been found in the coastal plain near Fredericksburg, Virginia (Iltis 1956). However, *the* main range is continuous in the Appalachian Mountains from southern Vermont to northern Georgia (Munns 1938, Anderson 2008)and at elevations ranging from sea level (Iltis 1956, Anderson 2008) to 1800m (Anderson 2008).

Rhododendron maximum is generally more abundant in cool, moist areas whereas elevation does not seem to have a significant impact on its distribution (Parker 2004, Anderson 2008, Vose 2012). They are most dominant on north facing slopes under a thick overstory canopy, but can also be found on south facing slopes as well as on eastern and western facing slopes. Southern slopes and mountain ridges are usually drier and the understories are often dominated by more xeric adapted species such as *Kalmia latifolia*.

Historically, the loss of chestnut trees, fire suppression, and extensive logging practices created three important disturbance events that caused drastic changes in the community structure of southern Appalachian forest. One change of great concern for forest management during the recovery of these forests was the spread of *R. maximum*. *Rhododendron maximum* is a native ericoid shrub that previously grew mainly in riparian zones in the Appalachian forests (Van Lear et al. 2002). After the aforementioned disturbances in the early 1900's *R. maximum* become a dominant understory cover at all elevations (Dobbs and Parker 2004).

Rhododendron maximum has a shallow root base that is very susceptible to forest fires. Forest fires were an important control keeping *R. maximum* from spreading into new areas (Dobbs and Parker 2004, Van Lear et al. 2002). The demise of *Castanea dentata* and logging practices opened up areas for *R. maximum* to spread (Anagnostakis 1987; Van Lear et al. 2002). Unlike forest fires, the chestnut blight and logging opens up canopy gaps without an immediate flux of nutrients. This allowed *R. maximum* to overcome the normal seral species. Studies have shown that the spread of *R. maximum* coincides with these disturbance events (Elliott and Vose 2012), however, *R. maximum* is still most dominant around riparian zones and in moist environments. Increased distribution is most abundant on north facing slopes where the temperature and direct sunlight is low (Plocher 1987).

Tree seedling reproduction is affected by *R. maximum* (Lei et al. 2002). It has been shown that seeds of canopy trees will reach the forest floor within a *R. maximum* thicket, but seedling mortality increases five-fold over that of seedlings that land outside of a

thicket. Furthermore, although *Quercus* and *Prunus* survivors in *R. maximum* thickets grew taller than saplings not in thickets, overall the seedlings had fewer leaves, less leaf area, and less leaf and stem mass (Lei et al. 2002). Sporophore abundance and diversity of ectomycorrhizal fungi, which associate with canopy trees but not ericoid shrubs, was found to be unaffected inside *R. maximum* thickets (Walker et al. 2002) and no differences were observed in fungal communities sampled by direct DNA extractions from colonized root tips (Walker et al. 2005). However, colonization levels of the seedlings were lower in the thickets (Walker et al. 1999).

In addition to the reduced colonization by these mutualistic fungi, various other factors are thought to be involved with the observed seedling mortality. *Rhododendron maximum* forms dense thickets and has broad evergreen leaves that shade out seedlings underneath (Lei et al. 2002). Nutrient availability is also lower within the thickets. Taken together, these results show that both competition above and belowground likely contribute to the inhibition of canopy trees beneath *R. maximum* thickets (Nilsen et al. 2001).

Ericoid Mycorrhizal Fungi

The effects of elevation on the diversity of ErM associations in eastern temperate forests have not been examined. However, previous research by Walker et al. (2011) in cold, Alaskan heathland, found that out of 303 cultures sampled, 148 were found to fit into six clades within the Helotiales, a large Ascomycete order containing many common ericoid symbionts. Interestingly, five of the cultures had blast affinities for *Irpex lacteus*, a polypore in the family Basidiomycota that is known more for its saprobic ecological niche. Members

of the Sebacinales, a group of Basidiomycetous mycorrhizal species which form a wide variety of different types of mycorrhizal associations with various hosts (Walker et al. 2011), were also seen in the Arctic in some of the direct root extractions. Sebacinales comprises a group of fungi that are commonly seen in direct extractions of ErM but are not culturable. Other researchers have sampled community structure of ErM with *Vaccinium membranaceum* along an elevation gradient in the western Canadian mountains from alpine zones to lower elevations. They found two dominant taxa: the first, *Rhizocyphus eric*ae, showed an affinity for high elevations in contrast with *Phialocephala fortinii*, which was abundant only at the low elevations (Gorzelek et al. 2012).

Edaphic Changes with Elevation

When moist air reaches the windward side of mountains it is forced up higher in the atmosphere causing it to cool and condense, a term known as orographic lift. Orographic lift results in precipitation on the windward sides and tops of mountains, while on the leeward side, air sinks and the compression caused by the increased atmospheric pressure causes it to heat up. Since warm air can hold more moisture than cool air, this usually results in less precipitation on this side (Price 1986).

In dry environments the adiabatic lapse rate is about 9^{o}C/km but in the Appalachian Mountains where moisture is high the lapse rate is between 3-7^{0}C/km (Bolstad et al. 1998). The amount of heating that comes from the mountain depends on the area of the mountain and on the area of mountains it is in close proximity too. A mountain of volcanic origin stands alone and will have a steeper cooling gradient compared to the Appalachian range with a lot more land area at the higher elevations (Price 1986).

During the day the temperature will increase but this heat will be lost quickly as soon as the sun sets in the higher elevations with less atmospheric pressure. The same effect accounts for the loss of moisture at higher elevations. Orographic lift causes more precipitation at higher elevations but the moisture is easily lost as the bulk of rain gravitates downhill and the remainder is quickly evaporated into the drier atmosphere at the higher elevations (Price 1986).

Aspects of the mountains play an important role on the amount of solar radiation the landscape receives. The Appalachian Mountains are in the northern hemisphere and the sun's radiation comes from the south. The southern slopes receive the bulk of the solar radiation and are heated to a greater extent than the northern slopes. This also means the south facing slopes are more exposed to evaporation effects of solar heating and will be drier than the north facing slopes in the warmer months.

Valleys between mountains can be sinks for cold air. Cold air is denser than warm air. As air is cooled at higher elevations it will fall into the surrounding valleys and become trapped. In some areas the air in the valley can be as cool as the air on top of the mountain. The warm air is displaced out of the valleys and is pushed up to higher elevations. As the air rises further in elevation it will decrease in temperature. The area of warmer air in the middle elevation is called the thermal belt. The thermal belt is often utilized for agriculture (Price 1986). This belt of warm temperature may affect elevational distributions of ErM fungi in this study as it commonly occurs in the elevations being investigated.

Nitrogen Inputs Along Elevational Gradients in Southern Appalachian Mountains

Nitrogen inputs come mostly from the process of deposition. Deposition is where rain or fog from clouds deposit nitrogen on the tops of mountains. Nitrogen has been seen as high as 18kg N/ha higher at high elevations than lower elevations due to deposition from rainfall amounts at higher elevations (Knoepp and Swank 1998). Research on nitrogen deposition shows increased levels of inorganic nitrogen at high elevation spruce-fir sites (Shubzda et al. 1995, Pauley et al. 1996, Barker et al. 2002) . However, in one of these studies there was a 30% decrease in nitrogen deposition from 1740 m in elevation to 1920 m (Shubzda et al. 1995).

In areas where ericoid plants are found nitrogen is more prevalent in organic pools and inorganic N is not readily available (Walker et al. 2011). Ericoid mycorrhizal fungal saprotrophic ability and the chemistry of *R. maximum* plant leaf litter support a closely coupled N cycle between Ericoid host plants and the soil in which they are located. The tannin rich leaf matter from *R. maximum* plants promotes the retention of protein-tannin N in soil. This complexed N is largely only accessible to the saprotrophic ErM symbionts of the plants and is less available to the co-occurring ecto- and arbuscular mycorrhizal symbionts and their hosts (Wurzburger and Hendrick 2009). A similar feedback loop was found in heathland soils where N was locked up in polyphenol tannic acids in the soil where ErM and not the co-occurring ECM fungi could gain access to it (Bending and Read 1996).

Ericoid mycorrhizal fungi are variably saprotrophic. Their ability to degrade organic substrates gives them access to organic sources of nitrogen and other nutrients. The ErM are likely utilizing lignin degrading enzymes in order to take up the bound organic N sources,

such as from ericoid plant litter. Lignin degradation has not been well characterized in the literature for ErM fungi but is well understood in the white rot basidiomycetes. White rot basidiomycetes use laccases and manganese peroxidases and are among the few organisms able to break down lignin and other organic aromatic structures like tannins (Chupungarset al. 2009). Lignin is a complex structure composed of many aromatic rings and enzymes that attach to the polyaromatic rings break them apart. These enzymes are also capable of breaking down a wide variety of poly-aromatic structures other than lignin (Muncnerova and Augustin 1994). Lignin peroxidase is an example of a lignin degrading enzyme and is found in *Phanerochaete chrysosporium* (Wang et al. 2009).

Goals of this Thesis

This study will focus on elevational differences in ErM diversity from the host plant *R. maximum* in the Southern Appalachian Mountains. The decrease in soil quality and temperature with elevation would likely lead to a higher dependence of host plants on associations with their mycorrhizal symbionts.

I tested three hypotheses in this study: (1) that overall ErM colonization levels in fine hair roots will increase with an increase in elevation due to an increased need for nutrient acquisition by the fungal symbionts for the plant; (2)that fungal diversity would change along an elevation gradient because of changes in habitat quality and quantity, and (3)that ErM species at higher elevations will have an increased ability to degrade lignin and release additional organic nitrogen sources into the soil.

MATERIALS AND METHODS

Field Sites

Rhododendron maximum roots were sampled on Hawksbill Mountain, Linville Gorge State Park, NC, and on Unaka Mountain, Unicoi County, TN during the first week of August 2010 and 2011, respectively. Linville Gorge and Unaka Mountain are each wilderness areas in the southern United States. Despite extensive logging throughout the region during the past two centuries, Linville Gorge was never clear cut because the steep terrain made it unprofitable to extract lumber. The wilderness was purchased in 1952 with funds donated by John D. Rockefeller and later designated as a wilderness area in 1964 through provisions of the Wilderness Act. Unaka Mountain was logged up until the early 1900's and was established as a wilderness area in 1986 (Frome 1994) .

Three isoclinal transects were located at each site. At Hawksbill Mountain transects were established at elevations of 746 m (N35^{o}54'51.76", W81^{o}53'53.04") at the bottom, 899 m (N35^{o}55'19.68", W81^{o}53'39.25") in the middle, and at 1,141 m (N35^{o}55'7.26", W81^{o}53'13.57") at the top. At Unaka Mountain the three transects were located at elevations of 708 m (N 36^{o}10'20.0", W82^{o}17'39.6"), 1127 m (N36^{o}8'7.3", W82^{o}16'28.4"), and 1,466 m (N36^{o}8'3.7", W82^{o}18'35.9"). All transects at both sites were on north facing slopes where *R. maximum* was more abundant. Locality data were retrieved with a GPS

(Garmin Etrex, Garmin International Inc., Olathe Kansas). Ten plants were selected along each transect by randomly placing points 4 m to 10 m apart, randomly choosing direction (above or below the transect), and then choosing the nearest *R. maximum* stem.

Root Collection

From each *R. maximum* plant three root samples were obtained by the following method: main roots were traced from the base of the shrub to the fine roots located near the distal ends of the roots, and approximately 500 cm of the fine roots were collected. There were three transects per site, 10 plants per transect and three samples per plant, for a total of 90 samples per site. The fine root samples were transported to the lab in plastic ziplock bags, and three 1 cm segments of fine roots were cut and cultured from each sample within 24 hours, for a total of 270 root segments per site. The root segments were cultured for the isolation of root endophyte and symbionts as described below. Five additional 1 cm fine root segments per sample were stained, then stored in glycerol and refrigerated for quantification of root colonization.

Staining / Light Microscopy / Root Tip Colonization Assay

To quantify fungal colonization of the *R. maximum* roots the basic method described by McGonigle et al. (1990) was employed. However, the original method was used to quantify arbuscular mycorrhizal colonization and had to be modified as described below to account for the structural differences found in ericoid mycorrhizae. The 1 cm sections of roots described in the sampling section above were cleared by autoclaving at 121^{o}C for 3 min in 5% KOH. The roots were then rinsed in water and acidified in 1% HCl for 24 hours. The endomycorrhizal and endophytic fungi in the roots were stained with acidic

glycerol/trypan blue for 3 min at 121^{o}C. After staining, root samples were kept refrigerated in glycerol and examined within 60 days. Three of the five cleared and stained root sections from each plant was then quantified for colonization with an Olympus BX51 light microscope by the following method: a 10 mm line with 10 randomly selected points was marked on a glass slide. The root segment was mounted, under a coverslip in water, periclinal to the line. At the 10 random points, anticlinal transects across the root segment were examined under 400 X magnification, and the number of internal hyphal elements that were encountered in the first 200 µm were counted. The sum of three root segments per plant were totaled for 10 plants across each transects. With three elevations per location there was a total of 30 points per location.

Mycorrhizal Fungal Culturing

Three 10 mm fine root sections from each of the three root samples per plant were washed in dH_2O, surface sterilized in 30% H_2O_2 for 30 to 60 seconds, and placed on a single sterile culture plate. The culture plates were plastic 15 mm Petri plates containing solid malt agar medium (18 g Bacto^{TM} Agar and 18 g Bacto^{TM} Malt extract dissolved in dH_2O for 1 L total volume). After fungal cultures emerged from the fine root a small section from each colony was cut from the outer margin and transferred to a separate plate of malt agar to establish pure cultures. All cultures were parafilmed to prevent desiccation and stored in large Tupperware containers with a lining of petroleum jelly around the edges to prevent insect infestation.

Sequencing and Restriction Fragment Length Polymorphism

A combination of restriction fragment length polymorphism (RFLP) typing and sequencing of the internal transcribed spacer (ITS) nuclear rDNA was used to type the fungi in the cultures at approximately the species level and to quantify the abundance and frequency of each type. All of the colonies were morphotyped according to their appearance prior to any molecular work to help organize the RFLP analysis. Five mm sections of hyphae taken from the outer edge of the colonies were placed in 50 µL of cetyl trimethylammonium bromide (Ctab) and were agitated by freezing in liquid nitrogen and thawing two times in a dry block at 65^{o}C. The samples were further macerated by manually grinding the samples with a micro-pestle. An additional 250 µL of Ctab was added for a total of 300 µL for each sample. Then 300 µL of chloroform were pipetted into each sample and vortexed for 10 seconds. The homogenized samples were centrifuged at 13,200 rpm for 10 min. Two hundred µL of the top aqueous layer was removed and pipetted into a new tube along with 600 µL ice cold isopropanol and then homogenized and stored for 24 hours in the freezer. Each sample was then centrifuged for 15 min at 13,200 rpm and the aqueous layer was carefully poured off. The precipitate was rinsed with 80% ice cold ethanol twice and all liquid was poured off. The remaining pellet was dried for approximately 15 min in a vacufuge (Vacufuge^{tm} Eppendorf, Hamburg-Eppendorf, Germany) and re-suspended in 50 µL sterile H_2O. The samples were then diluted by a factor of 1:50.

Polymerase chain reaction (PCR) was utilized to amplify nuclear DNA spanning the ITSI and ITSII regions from the extractions with the primers ITS1 and ITS4 (Gardes and Bruns, 1993) synthesized by Eurofins mwg Promega. The PCR cycle parameters were as follows:

for the initialization step temperature was held at 95^{o}C for 2 min, followed by 35 cycles of denaturation (30 sec, 95^{o}C), annealing (45 sec, 50^{o}C), and elongation (1 min, 72^{o}C), and a final single elongation step at 72^{o}C for 7 min.

RFLP analysis was used to initially assess conspecificity of all morphotypes. A double digest was performed for RFLP analysis with Alu1 and Hinf1 fast digest restriction enzymes and was carried out in 40 µL reactions. This reaction consisted of 2 uL of each enzyme, 4 uL of buffer, and 12 uL of molecular grade water along with 20 uL of the PCR product. Digestions were incubated in a dry heat block at 65°C for 2 hours. The digest was then dehydrated in a vacufuge for 30 min to concentrate the digest to half of the original volume. The product was then run out on a 1% agarose and 2% nusieve gel stained in ethidium bromide and visualized in ultraviolet light. From each type determined by RFLP at least half of the cultures were sequenced (Eurofins mwg Promega). All sequenced cultures were grouped together into contigs that represent one taxonomic group with 97% identity (Sequencher). RFLP types for which all sequenced cultures were assigned to one contig were considered conspecific. For RFLP types with members that were not assigned to a single contig, all of the cultures were sequenced and the RFLP type was not considered to be conspecific.

Edaphic and Environmental Sampling

Soil collected within a 1 m circumference around each *R. maximum* stem was pooled together for 0.5 L sample at 10 transect for soil analysis (30 aggregate samples per site). The samples were frozen prior to analysis. Samples were amalgamated by drying, passing through a sieve (850 µm) and mixed together. Approximately 240 mL of soil was sent to the

NC Cooperative Extension for estimation of humic matter (HM), weight per volume (WV), cation exchange (CE), base saturation (BS), acidity, pH, P, K, Ca, Mg, S, Mn, Zn, ZnAl, Cu, and Na. For each sample an additional 25 mL was further ground and aliquots ranging between 20 and 30 mg per sample were analyzed for percent carbon and nitrogen (Thermo Electron Flash EA1112 CN analyzer, RKI Instruments, Inc., Union City, California).

Mn and Lignin Peroxidase Assay

A representative culture for each isolate was used to test for lignin degradation ability. A nutrient limited lignin modifying enzyme basal medium (LBM) with agar was used to grow fresh cultures (Pointing 1999). Two indicators, azure blue and α-napathol, were used to identify the presence of peroxidase enzymes or lignase enzymes, respectively. A copy of each of the isolate representatives was plated on LBM with azure blue both with and without Mn to be able to distinguish between Mn and lignin peroxidases. A third culture was made with LBM and α-napathol. If the fungal culture produced lignase, a green halo formed around the culture on the α-napathol plate as the lignase oxidized the indicator. Azure blue is degraded by peroxidase enzymes so if peroxidase enzymes were produced by the fungal isolate a clear decolorization zone was observed around the culture. Isolates on plates grown with azure blue but without Mn were not be able to produce Mn-peroxidase, so any decolorization that occurred was the result of lignin peroxidase.

Data Analysis

All soil characteristics and root colonization data were compared among and between sites (n = 10 plots per transect) using Analysis of Variance after checking for normality and equality of variances. The colonization sum of three root segments per plant

was totaled for 10 plants across each transects. Spearman correlations were utilized to determine which soil parameters were correlated with each other. Analyses of Variance and correlations were performed using Sigmaplot V.12 (Systat Software Inc., city, state). Significance for all statistical analyses was assumed with a $p \leq 0.05$.

Frequency and abundance of each taxon (ITS-type from the molecular analysis) were evaluated for all transects to uncover any changes in diversity over the elevational gradients. Frequency was defined as the number of plants from which that taxon was recovered. Abundance was the number of cultures obtained for each taxon per plant. I used indicator analysis (Indicator Analysis: PC-ORD version 6.0, MjM Software 2011) to find individual species that were suggestive of any of the three elevations at each location. Shannon's diversity index, richness, and evenness were calculated for each plot and transect (Row and Column Summary: PC-ORD version 6.0, MjM Software 2011) and then compared between transects and sites using a two-way Analysis of Variance.

We used Principle Component Analyses (PCA: PC-ORD version 6.0, MjM Software 2011) to explore relationships among fungal symbiont diversity on *R. maximum* across the elevational gradients at both locations by looking for grouping within the three elevational transects. We used a centered variance/covariance. Significant axes were determined by having an eigenvalue greater than the broken-stick eigenvalue for that axis. To further explore relationships among fungal symbiont diversity we used Canonical Correlation Analysis (CCA: PC-ORD version 6.0, MjM Software 2011) to assess correlation of differences in diversity with edaphic properties across the same gradient by plot. The rows and columns scores were standardized by Hill's (1979) method while ordination scores were

optimized by variable. Plot scores for graphing were derived from the variable as well. The null hypothesis for CCA is that there are no relationships between matrices. The percent of variance explained was calculated by dividing the eigenvalue for each axis by the total variance in the species data.

Results

Edaphic Properties and Colonization: Differences in Elevations and Locations

Edaphic properties differed noticeably among elevations and locations (Table 1 and 2). At Unaka Mountain humic matter, cation exchange capacity, P, Mg, Zn, Al, N and Ca all significantly increased with elevation. At Hawksbill Mountain humic matter was highest at the middle elevation and lowest at the lowest elevation while the high elevation was not significantly different then either other site. Cation exchange capacity, Mg, Zn, Al, N, and Ca did not change among all three elevations at Hawksbill Mountain. Phosphorus increased significantly with increased elevation at both locations. The C to N ratio was significantly lower at the high elevation on Hawksbill Mountain and did not change at any elevation on Unaka Mountain. There was no consistent elevational change in Cu, Mn, S, or K at either location. Hydrogen ion concentration decreased with an increase in elevation on Unaka Mountain but stayed constant on Hawksbill Mountain.

Table 1: Edaphic properties by elevation on Unaka Mountain. (LE = low elevation, ME = middle elevation, and HE = high elevation). Means ± 1 SE are presented from 10 plants per transet. Transect means within a location having different superscripts and bolded are significantly different ($p < 0.05$).

	LE	ME	HE
% Humic Matter (HM) g/100 cm^3	**1.34 ± 0.25^a**	**2.95 ± 0.23^b**	**3.99 ± 0.23^c**
Cation Exchange Capacity (CEC) meq/100 cm^3	**8.57 ± 0.50^a**	**10.13 ± 0.48^b**	**13.69 ± 0.48^c**
% Base Saturation (BS)	18.44 ± 1.60^a	17.60 ± 1.52^a	22.70 ± 1.52^a
Total Nitrogen (N)	**0.46 ± 0.081^a**	**0.52 ± 0.07^a**	**0.77 ± 0.07^b**
Hydrogen ion Concentration [H^+]	**0.60 ± 0.01^a**	**0.59 ± 0.01^{ab}**	**0.57 ± 0.01^b**
Phosphorus Index (PI)	**16.00 ± 2.26^a**	**15.00 ± 2.14^a**	**32.00 ± 2.14^b**
Potassium Index (KI)	45.44 ± 5.77^a	39.40 ± 5.47^a	52.40 ± 5.47^a
% Calcium (Ca)	10.78 ± 1.30^a	11.70 ± 1.23^a	14.20 ± 1.23^a
% Magnesium (Mg)	**5.33 ± 0.36^a**	**4.10 ± 0.34^b**	**6.40 ± 0.34^c**
Sulfer index (SI)	75.44 ± 5.95^a	64.00 ± 5.65^a	57.00 ± 5.65^a
Manganese Index (MnI)	91.56 ± 14.87^a	68.50 ± 14.11^a	70.80 ± 14.11^a
Copper Index (CuI)	179.67 ± 37.10^a	169.30 ± 35.20^a	141.10 ± 35.20^a
C:N	20.06 ± 1.07^a	19.13 ± 1.02^a	21.35 ± 1.02^a

Table 2: Edaphic properties by elevation on Hawksbill Mountain. (LE = low elevation, ME = middle elevation, and HE = high elevation). Means ± 1 SE are presented from 10 plants per transet. Transect means within a location having different superscripts and bolded are significantly different ($p < 0.05$).

	LE	ME	HE
% Humic Matter (HM) g/100 cm^3	**1.18 ± 0.23^a**	**2.09 ± 0.23^b**	**1.79 ± 0.23^{ab}**
Cation Exchange Capacity (CEC) meq/100 cm^3	8.24 ± 0.48^a	8.29 ± 0.48^a	8.11 ± 0.48^a
% Base Saturation (BS)	15.10 ± 1.52^a	11.60 ± 1.52^a	16.70 ± 1.52^a
Total Nitrogen (N)	0.39 ± 0.07^a	0.47 ± 0.07^a	0.44 ± 0.07^a
Hydrogen ion Concentration [H^+]	**0.56 ± 0.01^a**	**0.56 ± 0.01^a**	**0.59 ± 0.01^b**
Phosphorus Index (PI)	**11.60 ± 2.14^a**	**15.70 ± 2.14^a**	**24.50 ± 2.14^b**
Potassium Index (KI)	40.00 ± 5.47^a	51.10 ± 5.47^a	48.50 ± 5.47^a
% Calcium (Ca)	8.60 ± 1.23^a	6.40 ± 1.23^a	9.50 ± 1.23^a
% Magnesium (Mg)	3.80 ± 0.34^a	3.40 ± 0.34^a	3.80 ± 0.34^a
Sulfer index (SI)	88.00 ± 5.65^a	73.10 ± 5.65^a	74.30 ± 5.65^a
Manganese Index (MnI)	27.40 ± 14.11^a	15.40 ± 14.11^a	64.20 ± 14.11^a
Copper Index (CuI)	36.10 ± 35.20^a	51.20 ± 35.20^a	59.60 ± 35.20^a
C:N	**30.42 ± 1.02^a**	**31.10 ± 1.02^a**	**24.70 ± 1.02^b**

Shannon's index, richness, and eveness were not significantly different at any elevation on Hawksbill Mountain (p = 0.093, 0.659, and 0.131 respectively). Shannon's index, richness, and eveness were not significantly different at any elevation on Unaka Mountain (p = 0.403, 0.0.516, and 0.271 respectively). The frequency of hyphal elements in the roots increased with elevation at both Unaka and Hawksbill Mountain (Figure 1). The frequency more than doubled from the low elevation to the middle elevation and was more than 5 X higher at the high elevation at the Unaka Mountain site, and 2.4 X greater than the low elevation site at Hawksbill Mountain.

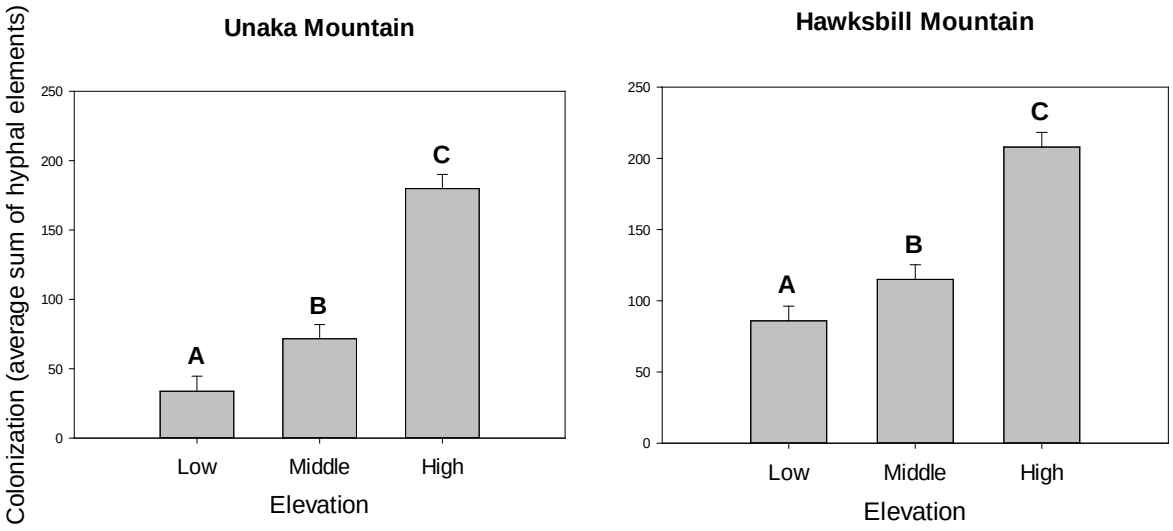

Figure 1: Colonization averages by transect at Unaka and Hawksbill Mountains. Colonization represents average of 10 plants per elevation. Significant changes are indicated by different letters above bar.

Identification of Taxa

Among three transects on Hawksbill Mountain 166 cultures were successfully grouped into 26 taxa with 97% identity (Table 3). Two taxa, *Cryptosporiopsis diversispora* and *C. ericae1*, made up 57% of all cultures found at this location. Eight taxa had between 3 to 8 representative cultures, and 16 taxa were considered rare with less than two cultures found for each. All of the cultures fell into 7 different orders. Sixteen of the taxa were matched with species in the order Helotiales. Four taxa were found to be Basidiomycetes in the order Agaricales.

Among three transects on Unaka Mountain 191 cultures were successfully grouped into 17 taxa with 97% identity (Table 4). The two most dominant taxa were *Dermea acerina* and *Cryptosporiopsis diversipora* making up 30% of the cultures. Unaka Mountain is represented with greater evenness than the cultures identified at Hawksbill Mountain. Eight taxa have between 2 to 5 cultures and the remaining 7 taxa had between 11 to 19 cultures. All taxa were grouped into 5 different Orders. Helotiales was the most abundant Order with 11 taxa.

Table 3: Fungal taxa listed by best match to BLAST search species for Hawksbill Mountain. Percent match is the percent of contig sequence for Hawksbill Mountain. Percent match is the percent of contig sequence for the ITS-type that matches the BLAST named sequence. Classifications were found in either NCBI or GBIF databases as indicated. Abundance values are given for each taxa.

Closest BLAST Search	% Match	Phylum	Class	Order	Family	# of Cultures
Acephala macrosclerotiorum	99	Ascomycota	Leotiomycetes	Helotiales		3
Coleophoma eucalyptorum	93	Ascomycota	Dothideomycetes	Botryosphaeriales	Botryosphaeriaceae	1
Cryptosporiopsis californiae	93	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	2
Cryptosporiopsis diversispora	97	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	48
Cryptosporiopsis ericae	97	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	47
Cryptosporiopsis ericae2	100	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	4
Cryptosporiopsis radicicola	96	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	2
Dermea viburni	100	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	2
Hyphodiscus hymeniophilus	100	Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	2
Lobaria macaronesica	97	Ascomycota	Lecanoromycetes	Lecanorales	Lobariaceae	2
Meliniomyces variabilis	97	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	6
Mollisia cinerea	93	Ascomycota	Ascomycetes	Helotiales	Dermateaceae	2
Mycena vitilis	96	Basidiomycota	Basidiomycetes	Agaricales	Mycenaceae	1
Myxocephala albida	93	Ascomycota	Sordariomycetes			4
Oidiodendron maius	98	Ascomycota	Eurotiomycetes	Onygenales	Myxotrichaceae	2
Penicillium urticae	98	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	2
Pezicula carpinea	97	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	5
Pezicula cinnamomea	97	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	5
Pezicula sp	94	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	2
Pezicula sporulosa	94	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	2
Phialocephala fortinii	99	Ascomycota	Leotiomycetes	Helotiales		8
Phialocephala scopiformis	97	Ascomycota	Leotiomycetes	Helotiales		7
Russulaceae sp1	94	Basidiomycota	Basidiomycetes	Agaricales	Russulaceae	2
Russulaceae sp2	96	Basidiomycota	Basidiomycetes	Agaricales	Russulaceae	2
Talaromyces striatus	94	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	1
Tricholoma portentosum	93	Basidiomycota	Basidiomycetes	Agaricales	Triholomataceae	2

Table 4: Fungal taxa listed by best match to BLAST search species for Unaka Mountain. Percent match is the percent of contig sequence for Unaka Mountain. Percent match is the percent of contig sequence for the ITS-type that matches the BLAST named sequence. Classifications were found in either NCBI or GBIF databases as indicated. Abundance values are given for each taxa.

Closest BLAST Search	% Match	Phylum	Class	Order	Family	# of Cultures
Colpoma quercinum	99	Ascomycota	Leotiomycetes	Rhytismatales	Rhytismataceae	2
Cryptosporiopsis diversispora	94	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	30
Cryptosporiopsis ericae	99	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	14
Dermea acerina	99	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	28
Dermea viburni	97	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	3
Hypocrea minutispora	100	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	4
Lecythophora mutabilis	97	Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	19
Leptodontidium sp.	94	Ascomycota	Leotiomycetes	Helotiales		12
Mollisia cinerea	100	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	2
Neonectria radicicola	97	Ascomycota	Sordariomycetes	Hypocreales		3
Pezicula cinnamomea	97	Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	11
Phialocephala fortinii	100	Ascomycota	Leotiomycetes	Helotiales		16
Phialocephala sphaeroides	97	Ascomycota	Leotiomycetes	Helotiales		14
Phialocephala turiciensis	97	Ascomycota	Leotiomycetes	Helotiales		19
Phialocephala virens	97	Ascomycota	Leotiomycetes	Helotiales		4
Xylaria frustulosa	97	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	5
Xylaria laevis	99	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	5

Frequency and Abundance of Cultures

At Hawksbill Mountain two species made up 57% of the cultures from all elevations (Table 3). *Cryptosporiopsis ericae1* had the highest frequency of all cultures (Figure 2) and was present at all elevations. It showed up in 8 of 10 plots at the low elevation, and 5 of 10 plots at both the middle and high elevations. *Cryptosporiopsis diversispora* had the second highest frequency of all cultures collected on Hawksbill Mountain. It was present at 9 of 10 plots at the high elevation but only 4 of 10 plots at the middle and low elevations. The low elevation had a higher frequency of rare species compared to the high elevation.

At Unaka Mountain *Dermea acerina* had the highest frequency over all transects. It was found in all 10 plots at the high elevation, 3 of 10 plots at the middle elevation, but only in one plot at the low elevation (Figure 2). *Neonectria radicicola* had the second highest frequency and was present at both the high and low elevation transects in 6 of 10 plots but only in 1 of the 10 plots at the middle elevation. *Cryptosporiopsis diversispora* frequency was found to decrease with elevation at Unaka, which is the opposite of what was seen at Hawksbill Mountain. It was present in 6 of 10 plots in the low elevation, 4 of 10 plots on the middle elevation, but only in 2 of 10 plots at the high elevation. Again, low elevation sites had a higher frequency of rare species.

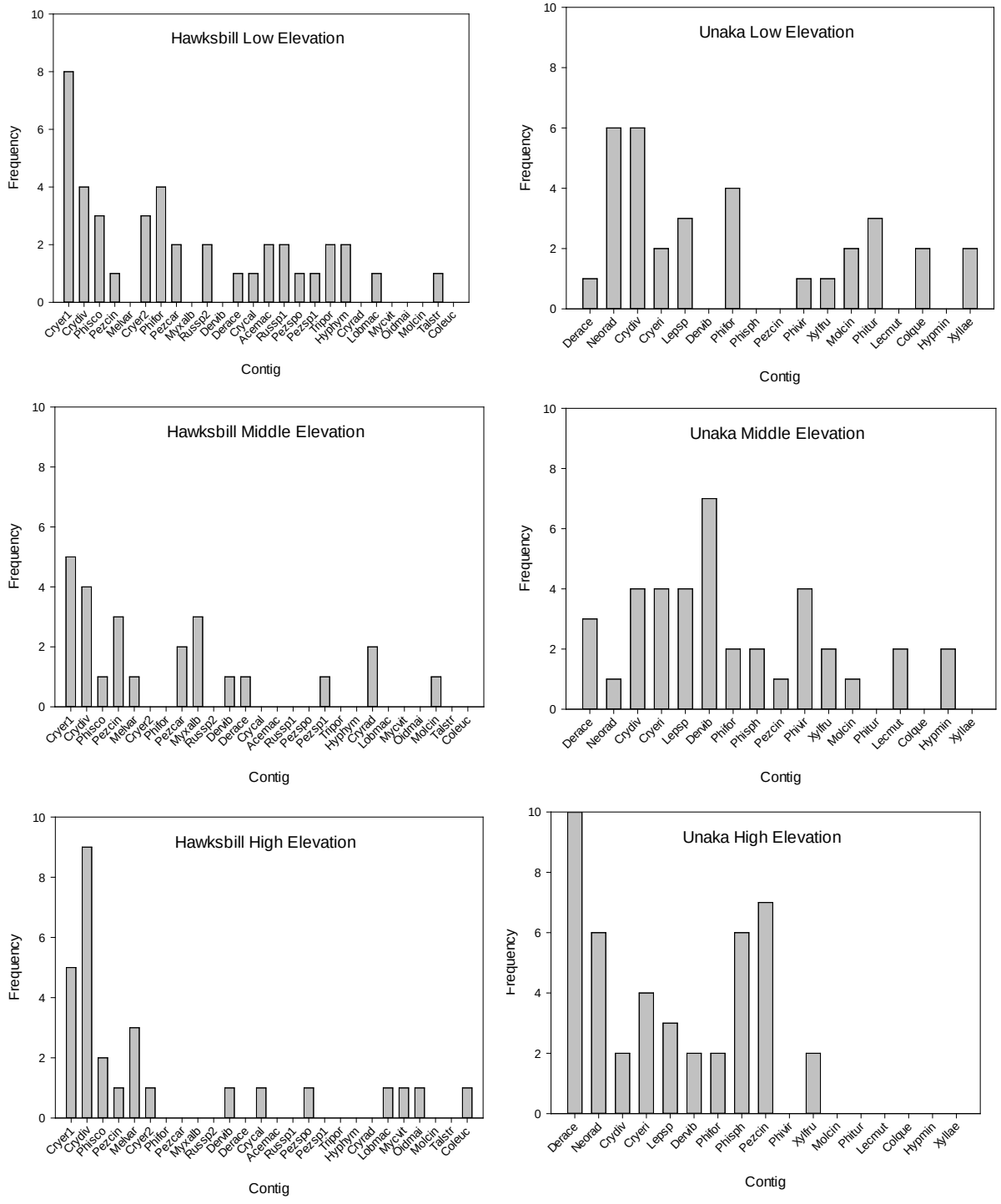

Figure 1: Frequency of each taxon for low, middle, and high elevations at Hawksbill Mountain and Unaka Mountain. X axis is arranged by highest overall frequency (left) to lowest overall frequency (right) for all three elevations. Taxa are abbreviated by using only the first three letters of genus and specific epithet.

Indicator Analysis

Hawksbill Mountain exhibited one significant indicator species among the three sites only at the low elevation (Table 5). *Phialocephala fortinii* had an indicator value of 0.555 for the low elevation site (p = 0.0188). *Cryptosporiopsis diversipora* had a significant indicator value of 0.476 for the high elevation site (p = 0.0338). *Myxocephala albida* showed an affinity towards the middle elevation with an indicator value of 0.471 (p = 0.0858) but this was not significant.

Unaka Mountain exhibited 5 significant indicator species among the three different elevations sampled (Table 6). *Phialocephala turiciensis* had an indicator value of 0.05 for the low elevation site (p = 0.0248). *Dermea viburni* had an indicator value of 0.610 for the middle elevation site (p = 0.0054). Three species, *Dermea acerina*, *Phialocephala sphaeroides*, and *Pezicula cinnamomea* had indicator values of 0.745, 0.525, and 0.689 respectively for the high elevation site (p = 0.0002, p = 0.0232, p = 0.0016 respectively).

Table 5: Taxon maximum identity to an elevation at Hawksbill Mountain. Max group gives the elevation the taxon is most associated with. The observed indicator value gives a numerical value for how well it represents the maximum group as an indicator species, where the closer it is to one the greater the indicator value. Significant indicator values represented with a *p* value of less than 0.05 are shown in bold.

Species	Max Group	Observed Indicator value (IV)	*p*
Acephala macrosclerotiorum	Low	0.37	0.31
Coleophoma eucalyptorum	Middle	0.26	1.00
Cryptosporiopsis californiae	Low	0.09	1.00
Cryptosporiopsis diversipora	High	0.47	**0.03**
Cryptosporiopsis ericae1	Low	0.28	0.34
Cryptosporiopsis ericae2	Low	0.34	0.29
Cryptosporiopsis radicicola	Middle	0.37	0.30
Dermea acerina	Low	0.09	1.00
Dermea viburni	Middle	0.09	1.00
Hyphodiscus hymeniophilus	Low	0.37	0.31
Lobaria macaronesica	Low	0.09	1.00
Meliniomyces variabilis	High	0.34	0.28
Mollisia cinerea	Middle	0.26	1.00
Mycena vitilis	High	0.26	1.00
Myxocephala albida	Middle	0.47	0.08
Oidiodendron maius	High	0.26	1.00
Pezicula carpinea	Low	0.14	1.00
Pezicula cinnamomea	Middle	0.25	0.57
Pezicula sp	Low	0.09	1.00
Pezicula sporulosa	Low	0.09	1.00
Phialocephala fortinii	Low	0.55	**0.01**
Phialocephala scopiformis	Low	0.18	0.84
Russulaceae sp1	Low	0.38	0.30
Russulaceae sp2	Low	0.38	0.31
Talaromyces striatus	Low	0.26	1.00
Tricholoma portentosum	Low	0.38	0.31

Table 6: Taxon maximum identity to an elevation at Unaka Mountain. Max group gives the elevation the taxon is most associated with. The observed indicator value gives a numerical value for how well it represents the maximum group as an indicator species, where the closer it is to one the greater the indicator value. Significant indicator values represented with a *p* value of less than 0.05 are shown in bold.

Species	Max Group	Observed Indicator value (IV)	*p*
Colpoma quercinum	Low	0.40	0.08
Cryptosporiopsis diversipora	Low	0.35	0.13
Cryptosporiopsis ericae	High	0.08	1.00
Dermea acerina	High	0.74	**>0.01**
Dermea viburni	Middle	0.61	**>0.01**
Hypocrea minutispora	Middle	0.37	0.31
Lecythophora mutabilis	Middle	0.37	0.30
Leptodontidium sp.	Middle	0.08	1.00
Mollisia cinerea	Low	0.26	0.29
Neonectria radicicola	Low	0.30	0.22
Pezicula cinnamomea	High	0.68	**>0.01**
Phialocephala fortinii	Low	0.25	0.30
Phialocephala sphaeroides	High	0.52	**0.02**
Phialocephala turiciensis	Low	0.50	**0.02**
Phialocephala virens	Middle	0.44	0.09
Xylaria frustulosa	Middle	0.05	1.00
Xylaria laevis	Low	0.40	0.09

Principle Components Analysis

The first two axes defined by PCA explained 75% of the variation between 26 taxa found on Hawksbill Mountain. The first axis separated all three elevations linearly (Figure 3). The middle elevation was overlapped by both low and high elevations. The low elevation was also slightly overlapped by the high elevation. Phosphorus, pH, Zn, Al, and colonization vectors are increasing parallel to axis one along with an increase in elevation.

The first two axes defined by PCA explained 52% of the variation between 17 taxa found on Unaka Mountain. All three elevations were separated across the second axis (Figure 4). The middle elevation is between the low and high elevation groupings with overlap in each and is predominantly on the positive values for axis one. The low elevation group and the high elevation group do not overlap. Humic matter and colonization is positively correlated to axis two and slightly negatively correlated to axis one.

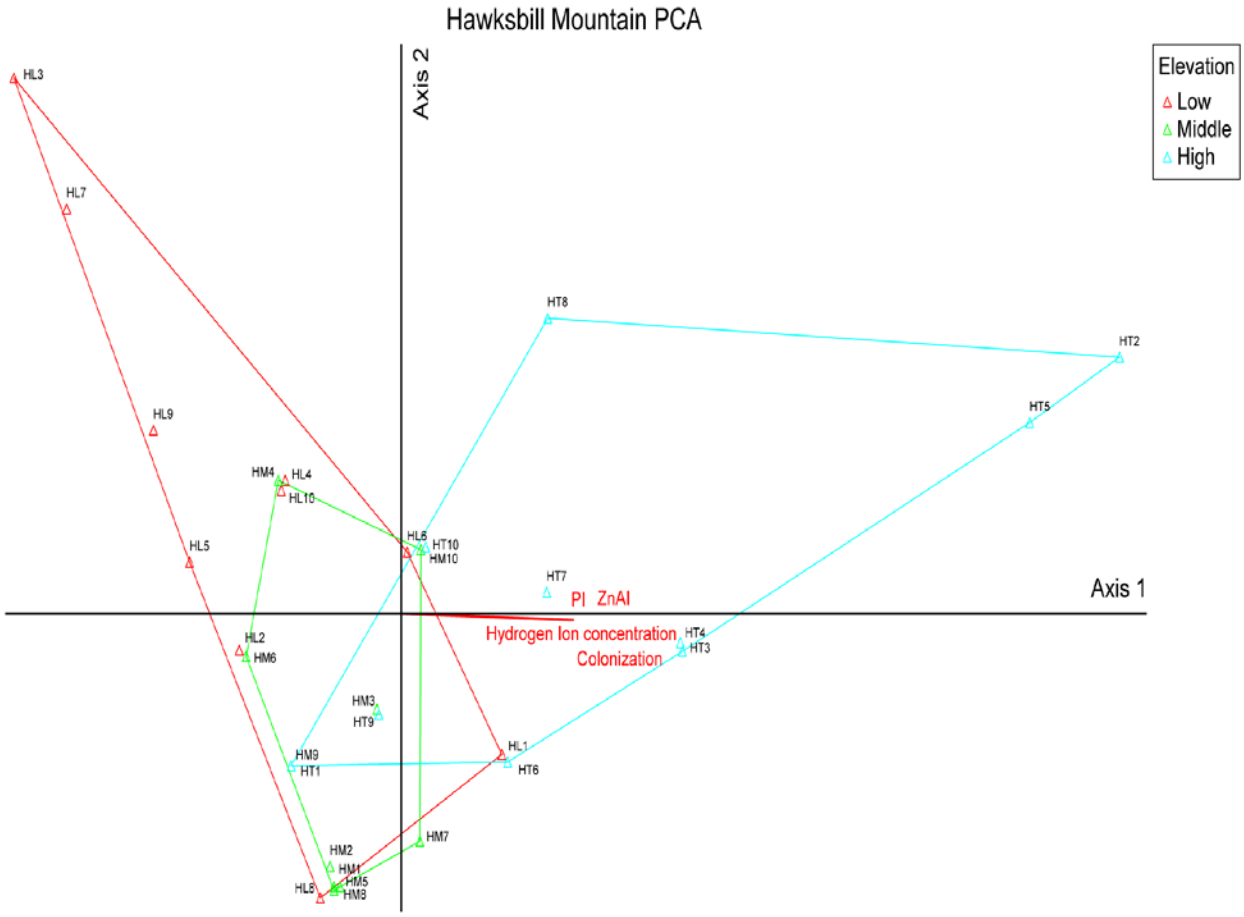

Figure 2: Principal components analysis (PCA) of plots at Hawksbill Mountain. Axes were defined by abundance & frequency values of taxa at each plot. All 10 plots at each elevation (low, middle, and high) were outlined to show how well each transect grouped together and their spatial composition to each other. Edaphic properties were plotted in a second matrix and values were overlain over the distance measures made by the main matrix.

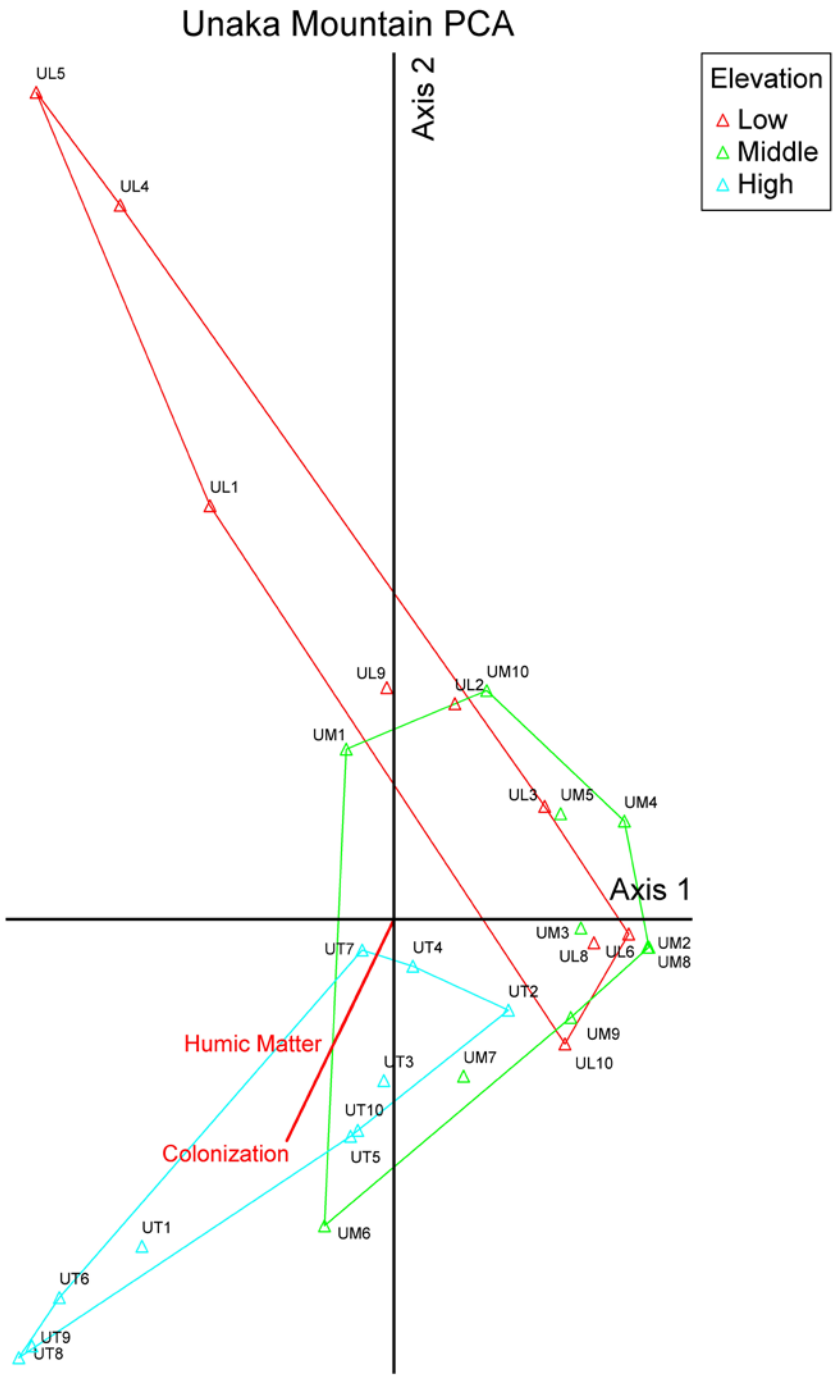

Figure 3: Principal components analysis (PCA) of plots at Unaka Mountain. Axes were defined by abundance & frequency values of taxa at each plot. All 10 plots at each elevation (low, middle, and high) were outlined to show how well each transect grouped together and their spatial composition to each other. Edaphic properties were plotted in a second matrix and values were overlaid over the distance measures made by the main matrix.

Canonical Correlation Analysis

Three axes were defined for Hawksbill Mountain that explained 25% of the variation with 26 taxa and 17 environmental factors. *Acephala macrosclerotiorum, Hyphodiscus hymeniophilus, Talaromyces striatus, Phialocephala fortinii, Tricholoma portentosum, Dermea acerina, Pezicula sp1. Cryptosporiopsis ericae, Russulaceae* sp1, and *Pezicula carpinea* are all associated with the low elevation and their points are all within the low elevation boundary (Figure 5). *Phialocephala scopiformis, Phialocephala sporulosa, Lobaria macaronesica, Russulaceae* sp 2 and *Cyptosporiopsis californiae* are also associated with the low elevation but are not located within the low elevation grouping. None of these outliers are significant indicator species of the low elevation. *Coleophoma eucalyptorum, Cryptosporiopsis radicicola, Mollisis cinerea, Myxocephala albida*, and *Pezicula cinnamomea* are associated with the middle elevation and are located within the middle elevation boundary. *Dermea viburni* is also loosely associated with the middle elevation but is not a significant indicator and falls outside of the middle elevation boundary. *Meliniomyces variabilis, Mycena vitilis*, and *Oidiodendron maius* are associated with the high elevation site and are located within or right outside the outer edge of the high elevation boundary. All three groupings slightly overlap each other. The middle elevation grouping overlaps about a third of the group created for the high elevation. The low elevation group is mainly encompassed in the quandrant with positive axis 1 values and negative axis 2 values. The middle elevation group is largely encompassed in the quadrant with positive axis 1 and 2 values. The high elevation site is largely encompassed in the quadrant with negative axis 1 values and positive axis 2 values. The three elevations are separated linearly on the first

axis and axis 2 further separates the low elevation group into a distinct grouping. Colonization, P, Mn, Hydrogen ion concentration, and Zn, Al are significant negative vectors along the first axis. C/N ratio is a significant vector positive on the first axis.

Canonical correspondence analysis defined 3 axes that explained 34% of the variation with 17 taxa and 16 environmental variables on Unaka Mountain. *Cryptosporiopsis diversispora, Phialocephala fortinii, Colpoma quercinum, Mollisia cinerea, Neonectria radicicola, Phialocephala turiciensis*, and *Xylaria laevis* are all associated with the low elevation site (Figure 6). All of these species share negative scores less than -0.751 on the first axis except for *N. radicicola*. They also all have positive scores on the second axis. *Cryptosporiopsis ericae, Dermea viburni, Leptodontidium sp., Lecythophora mutabilis, Hypocrea minutispora, Phialocephala virens*, and *Xylaria frustulosa* are all associated with the middle elevation and all have values on the first axis between -1.166 and 0.167. They all have negative values on the second axis. *Dermea acerina, Phialocephala sphaeroides*, and *Pezicula cinnamomea* are all associated with the high elevation site and all have positive scores on the first axis greater than 1.1 and scores between -0.22 and 0.79 on axis 2. The high elevation group only slightly overlaps with the middle elevation group but all three form distinctive groups mainly separated across the first axis and the middle elevation further separated across the second axis. Humic matter, P, colonization and cation exchange capacity all have significant positive vectors on axis 1.

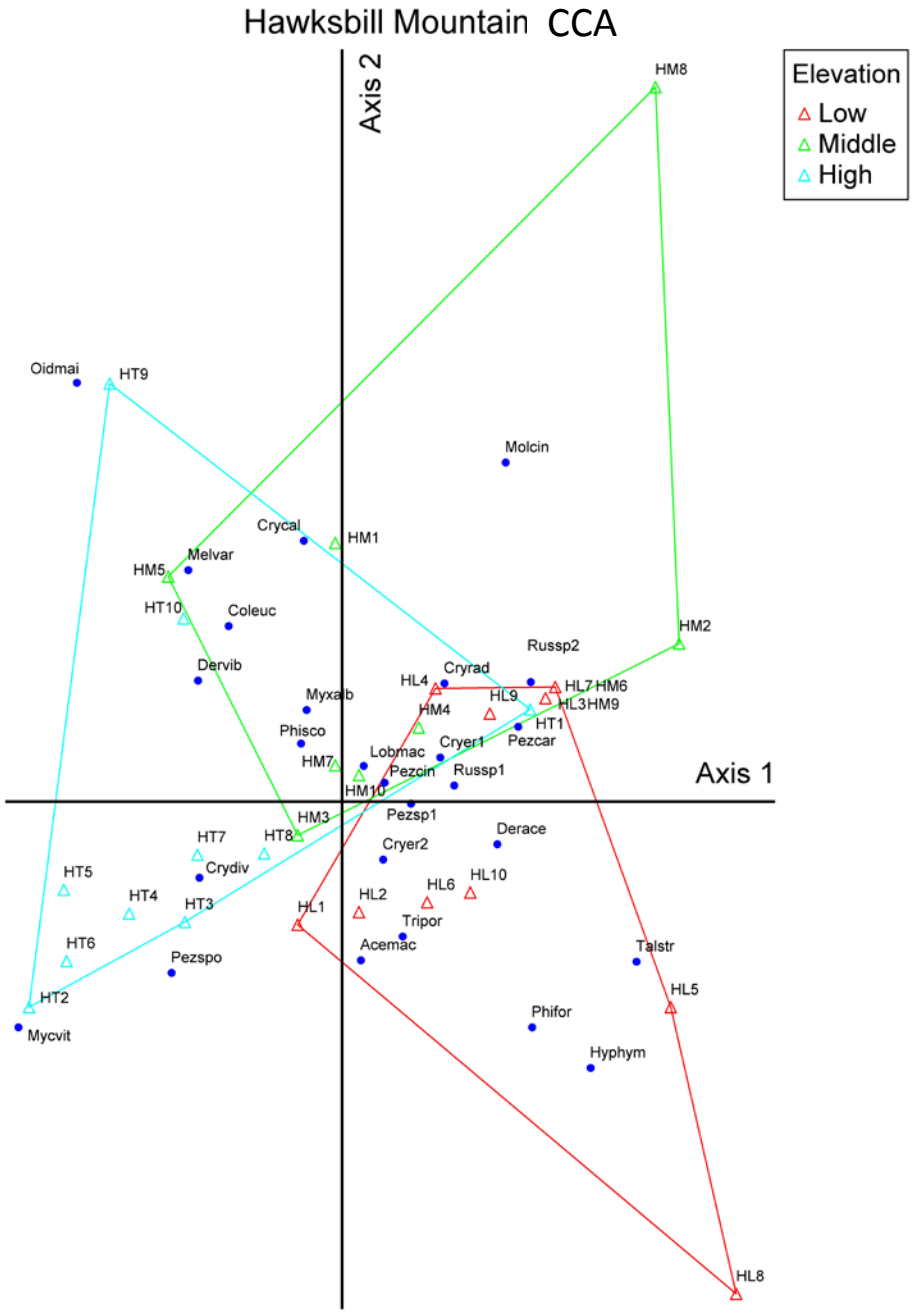

Figure 4: Canonical correlation analysis (CCA) of plots at Hawksbill Mountain. Axes were defined by an analysis constrained by two matrices, the first containing abundance values of taxa by plot and the second containing edaphic values by plot. All 10 plots at each elevation (low, middle, and high) were outlined to show how well transects at each elevation grouped together. Taxa were overlaid on the same axes to show their correlation to the plots or elevational groups.

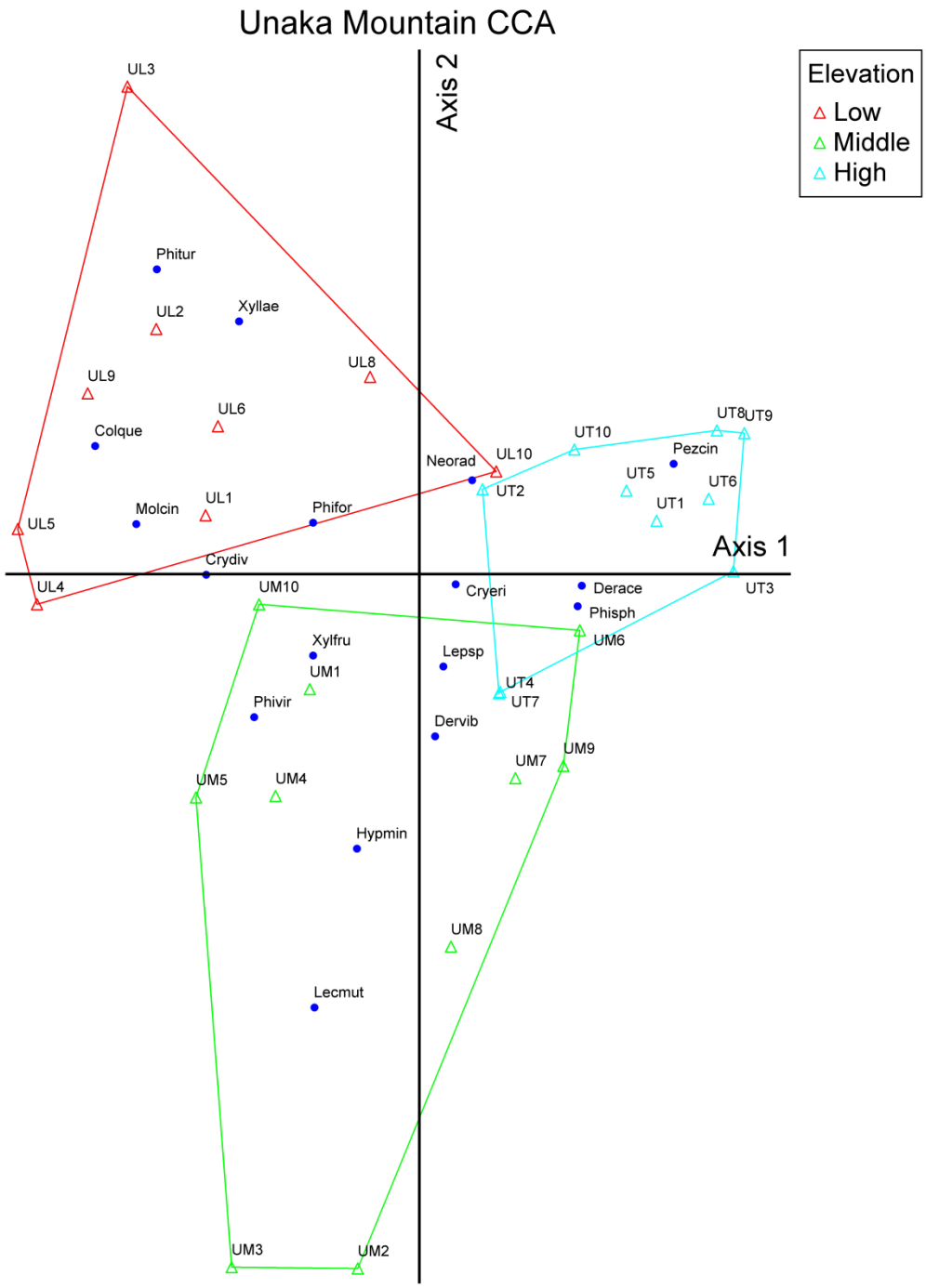

Figure 5: Canonical correlation analysis (CCA) of plots at Hawksbill Mountain. Axes were defined by an analysis constrained by two matrices, the first containing abundance values of taxa by plot and the second containing edaphic values by plot. All 10 plots at each elevation (low, middle, and high) were outlined to show how well the transect at each elevation grouped together. Taxa were overlaid on the same axes to show their correlation to the plots or elevational groups.

Lignin Assay

The following colorimetric assays give a qualitative identification of lignin degrading enzymes (Table 6). Three enzymes systems were used to degrade lignin and tested here. Three positive results (*Acephal macrosclorotorum, Phialocephala fortini*, and *Xylaria laevis*) were found in the α-napathol assay but the other two assays were also positive for those taxa. *Cryptosporiopsis ericae, Lecythophora mutabilus, Mollisia cinarea, Myxocephala albida*, and *Phialocephala scopiformis* were only positive for lignin peroxidase and no other enzymatic system. No significant differences were found for lignin degrading fungal taxa at any elevation (p = 0.134, 0.093 Hawksbill, Unaka Mountain, respectively)

Table 6: Lignin Assay. Colorimetric assays were performed for 34 fungal cultures on three different medias. A (+) identifies a positive result for each assay and a (-) identifies a negative result.

	α-Napathol	Azure B w/ Mn	Azure B w/out Mn
Acephala macrosclerotorum	+	+	+
Coleophoma eucalyptorum	-	-	-
Colphoma quercinum	-	-	-
Cryptosporiopsis diversispora	-	+	-
Cryptosporiopsis ericae	-	-	+
Dermea acerina	-	-	-
Cryptosporiopsis radicicola	-	-	-
Dermea verburni	-	-	-
Hyphodiscus hymeniophilus	-	+	+
Hypocrea minutispora	-	-	-
Cryptosporiopsis californiae	-	+	+
Russulaceae sp 2	-	-	-
Lecythophora mutabilus	-	-	+
Leptodontidium sp.	-	+	+
Lobaria mararonesica	-	-	-
Meliniomyces variabilis	-	-	-
Mollisia cinerea	-	-	+
Pezicula sporulosa	-	+	+
Russulaceae sp 1	-	-	-
Mycena vitilis	-	-	-
Myxocephala albida	-	-	+
Neonectria radicicola	-	+	+
Oidiodendron maius	-	-	-
Pezicula carpinea	-	+	+
Pezicula cinnamonea	-	+	+
Phialocephala fortini	+	+	+
Phialocephala scopiformis	-	-	+
Phialocephala sphaeroides	-	-	-
Phialocephala turiciensis	-	+	+
Phialocephala virens	-	+	-
Talaromyces striatus	-	-	-
Tricholoma portentosum	-	+	+
Xylaria frustulosa	-	-	-
Xylaria laevis	+	+	+

Discussion and Conclusion

In the past century *R. maximum* has spread extensively in the Appalachian mountain range. It once used to be predominantly a riparian zone species, but now can be found in a wide diversity of habitats and at all elevations. We expected *R. maximum* might have different mycorrhizal partners to assist in its ability to dominate the subcanopy in many areas over a broad elevational range in the Appalachian Mountains. Our results strongly indicate that *R. maximum* can in fact associate with different ErM assemblages that form a graded series across different elevations, and that it has higher colonization levels and very likely higher reliance on ErM fungi at higher elevations. We know that elevation can have large impacts on climate and edaphic properties over a short distance. Moreover, we expected to find greater needs for mycorrhizae where nutrients are not as readily available, which could be indicated by the density of root infection by mycorrhizal fungi.

Hawksbill and Unaka Mountain showed generally similar patterns with edaphic changes and ErM fungal communities. These changes along an elevational gradient might be explained by lower temperatures at higher elevations as indicated by the adiabatic lapse rate.. The dry adiabatic lapse rate is 9.8 ^{o}C/km, but in the southern Appalachians the adiabatic lapse rate is lower due to high moisture in the atmosphere (7 ^{o}C/KM). The decrease in temperature causes slower decomposition rates and changes edaphic

properties along an elevational gradient (Bolstad et al. 1998). We saw greater changes at the Unaka Mountain location. This is likely due to a valley effect at Hawksbill Mountain where the denser cold air drains into the valley and is trapped. This thermal belt effect causes colder air at the low elevation transect similar to that of the high elevation transect in contrast to Unaka Mountain which is located along a solitary mountain ridge where cold air is not trapped at the low elevation. Soil temperature and moisture measurements would be a valuable component for future investigations.

Most mountains will have limiting temperature and moisture at higher elevations (Shanks 1956, Price 1986, Knoepp and Swank 1998). Higher elevations receive an increased amount of rain due to orographic projection but the soil is porous and does not retain water as well as organic soils found at the lower elevations. At the highest elevations physical weathering becomes the dominant process for soil formation due to decreased micro fauna and decreased biological activity (Price 1986, Knoepp and Swank 1998). Litter fall is usually less at the higher elevations than lower elevations as well (Shanks 1956). Soil organic matter builds up at the higher elevations and on the northern facing slopes (Knoepp and Swank 1998).

We found that two edaphic properties increased significantly with elevation across all elevations at both sites (p and humic matter). However, six additional edaphic properties increased on Unaka Mountain (cation exchange capacity, Mg, ZnAl, N, and Ca). Several of these factors are generally though to influence distributions of fungi. Broader scale investigations would enhance our ability to interpret how these changes are

generalized across the region and better understand how they may be coupled with ErM community structure.

We found that on both mountains root colonization increases with elevation. Climatically an increase in elevation is analogous to an increase in latitude in that both produce a decrease in temperature. The difference in elevation shows climactic differences in a drastically smaller area compared to a change in latitude. In another study, abundance of Ericoid mycorrhizae in *Vaccinium* roots increased with increasing elevation (Väre et al. 1997). Similarly, on McBride's peak in east central British Columbia colonization tended to be slightly higher on alpine and mid-elevation transects than subalpine and low elevation transects, although the differences were not significant (Gorzelak et al. 2012). At higher elevations or higher latitudes where temperatures are cooler and microbial decomposition is slower there are less available nutrients in the soil because they are bound to organic substrates (Väre et al. 1997). At lower elevations nutrients are in soluble form and more easily taken up by plants. Plants are less likely to form extensive mycorrhizal relationships if the plant can access nutrients on their own because of the tradeoff of carbohydrates to maintain the symbiosis. One experiment showed that colonization decreases with increased levels of ammonium nitrate applied to *Calluna vulgaris* (Johansson 2000). Based on our results and these congruent results from other studies, we conclude that at high elevation areas where nutrients are bound in organic sources the plant likely relies on the fungal symbiont to gain access to the nutrients and readily forms a symbiosis. Studies have show an increase in nitrogen with elevation above 1700 m (Shubzda et al. 1995, Pauley et al. 1996, Barker et al. 2002). Our data does not show a significant difference in nitrogen at

any elevation on Hawksbill Mountain and a decrease in nitrogen on Unaka Mountain. It is possible that in our field sites locate between 700 and 1400 m nitrogen deposition is not at prevalent. However, our data does not distinguish organic and inorganic sources of N directly, and it is unclear what role N deposition at high elevations might have at the study sights. Additional research is needed in this area.

The majority of taxa discovered were in the order Helotiales, which are Ascomycetes. Various lineages within Helotiales have been commonly identified as symbionts of plants in the family Ericaceae. A few taxa cultured in this study were in the order Agaricales which are Basidiomycetes, which are not as common ericoid symbionts especially in cultured samples. Two of these taxa placed in the family Russulaceae are known to be strictly mycorrhizal but normally form ectomycorrhizal associations with other trees, with an exception found with another ericoid plant, *Monotropa* spp. which is parasitic on ectomycorrhizal symbioses (Bidartondo and Bruns 2002). One other Agaricales culture, sp. (in Tricholomatacea) may share these role, being commonly known as ectomycorrhizal with the exception of possible association with *M*onotropa spp. It would be a novel result for them to form ErM associations, and future experiments should explore this potential ability using resynthesis methods. The last Agaricales member was placed in the Mycenaceae, which are commonly observed as saprobes. Taxa in the order Xylariales were found and are also commonly observed as saprobes. Most ericoid mycorrhizal fungi are hypothesized to have evolved separately from other mycorrhizal fungal forms in numerous disparate lineages with varying saprobic capacity throughout the Ascomycetes and Basidiomycetes. Ericoid plants obtain nutrients such as N from organic sources that are

provided by associated fungi. Forming a symbiosis with fungi with strong saprobic capacity is advantageous to the plant and may become more important at higher elevation where organic matter accumulates and mineralization is slow. Frequency and abundance show how the presence of each taxon changes with elevation. Two of the most abundant taxa on Hawksbill Mountain (*Cryptosporiopsis ericae*1 and *C. diversispora*) were seen to shift inversely to each other across elevations. This is a possibly the result of specificity of each fungus for the different environments at each elevation. The same dynamic was observed at Unaka Mountain in the two most abundant taxa's (*Dermea acerina* and *Neonectria radicicola*). On both mountains rare species were more abundant at the low rather than the high elevation. Overall, Unaka Mountain had a more even distribution of taxa over all transects with less rare species (two or less representative cultures). High elevations may have an affinity to more specialized fungal symbionts that tolerate arid and colder environments.

Indicator analysis identified five significant indicator species on Unaka Mountain and two indicator species on Hawksbill Mountain. Unaka showed a greater separation in edaphic properties by elevation and had the greatest elevational range. The greater heterogeneity of soil nutrients among elevations could have led to the greater individuality of the fungal assemblages at each elevation reflected by more indicator species being present. For overall richness, Hawksbill Mountain had nine more species total. Community structure had more taxa overlapping among the three elevations compared to the Unaka Mountain location.

Principal components analysis separated all three elevations for both sites into distinct groups. The groups were calculated by the presence of fungal taxa at each elevation. Unaka showed a clearer separation between all three sites with just a little overlap of the high elevation and the low elevation sites with the middle site but not with each other. Hawksbill groupings showed the same pattern but groups were not as well separated as seen for Unaka Mountain. Canonical correspondence analysis included soil conditions along with frequency and abundance of individual taxa at each site. This analysis gives a more complete picture of how the edaphic properties measured in this study correspond with the changes seen in community structure. A higher percent of variation explained (34%) at Unaka Mountain compared to Hawksbill (25%) is possibly a result of more significant changes in edaphic properties along the elevational gradient on Unaka than Hawksbill. As seen in the PCA analysis which explained a much higher amount of the variation in the fungal assemblages, the boundaries made from the placement of each plot for the three elevations show distinct separation. The groupings of plots for each transect are spread along a gradient on axis one but a little overlap is present for all three elevations for Hawksbill Mountain. Transects were largely separated on the first axis for Unaka and the middle elevation grouping of plots only overlapped slightly with the high elevation plots. The low elevation plots did not overlap at all with either of the other two transects. Taxa that had greater identity with a given elevation according to indicator species analysis were more clearly defined within the plot boundaries for each elevation on Unaka compared to Hawksbill. The overall picture at both sites indicates that the community structure seems to be tracking elevation with more distinctly different high and low elevation groups and a

transitional middle elevation group. Further research is needed to test the hypothesis whether or not the valley effect is the significant difference in the results found between these two sites. We need to see if this pattern occurs in other gorges compared to other mountains that are more free standing.

The evidence found in this study supports the hypothesis that the diversity of ericoid mycorrhizae does shift over an elevational gradient along with corresponding shifts in edaphic properties. We hypothesized that different ErM taxa respond differently to different environmental stresses. One of these stresses would be to obtain nutrients from recalcitrant organic sources particularly at high elevation. Therefore, we expected to see mycorrhizae with the ability to breakdown lignin more so at the higher elevations. Some of the fungi did break down lignin but we did not find any significant results showing that lignin degrading mycorrhizal fungi were found more in more organic soils. More research is needed to see if these fungi have higher capacities to break down lignin using a quantitative assay. We can then see if a fungus with a higher capacity correlates to areas with higher organic matter.

White rot basidiomycetes have been clearly shown to have the ability to breakdown poly-aromatic hydrocarbons (PAH's) with lignin degrading enzymes (Chupungars et al. 2009). We have shown with a qualitative assay that ericoid mycorrhizal fungi do possess the same lignin degrading enzymes. After quantifying and comparing their ability to white rot basidiomycetes, we could potentially develop a new system for bioremediation in breaking down environmental contaminant PAH's. If ericoid mycorrhizal fungi can as efficiently breakdown PAH's their partnership could potentially function as a self-sustained

system to ameliorate contaminated sites with the phytobiont as a carbon source. We did not find any significant difference in the capability for degrading lignin by where fungi are located among elevations. However, a future study will quantify the rate of degradation for each taxon. By quantification we can see if the fungal symbionts with better lignin degrading activity are specific to different environments. We will also be able to compare activity to know saprobes already used in bioremediation and have a better idea of the efficacy of using this symbiosis in future bioremediation studies.

Colonization was shown to increase at both sites at higher elevations. Differences in edaphic properties were observed across the elevational gradient, and these changes coincided with changes in the fungal community structure. Changes in abundance, frequency and species composition of the fungal communities tracked elevational differences at the two locations tested in this research. Differences in species composition at Unaka Mountain were clearer, showing separation of fungal communities by elevation. The communities on Hawksbill Mountain were not as distinct at each elevation, possibly because of a valley effect causing cold temperatures to settle in the gorge which can change microbial metabolism. Lignin degrading capacity was found in approximately half of the fungal taxa but was not linked to elevational changes in fungal community composition. Future directions for this research include greater sampling of the fungal communities at additional sites, experimental approaches to explore the relationships between edaphic factors and community composition, and greater characterization of the lignin degrading ability of the fungi.

Bibliography

Anagnostakis SL. 1987. Chestnut Blight: The classical problem of an introduced pathogen. Mycologia 79: 23-37.

Anderson MD. 2008. *Rhododendron maximum*. In: Fire Effects Information System. F. S. U.S. Department of Agriculture. Rocky Mountain Research Station, Fire Sciences Laboratory.

Barker M, Van Miegroet H, Nicholas NS, Creed IF. 2002. Variation in overstory nitrogen uptake in a small, high-elevation southern Appalachian spruce-fir watershed. Can. J. For. Res. 32:1741- 1752.

Beier CM, Horton JL, Walker JF, Clinton BD, Nilsen ET. 2005. Carbon limitation leads to suppression of first year oak seedlings beneath evergreen understory shrubs in Southern Appalachian hardwood forest. Plant Ecol 176: 131-142.

Bending GD, Read DJ. 1996. Nitrogen mobilization from protein-polyphenol complex by ericoid and ectomycorrhizal fungi. Soil Bio Biochem 28: 1603-1612.

Bidartondo MI, Bruns TD. 2002. Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. Molec Ecol 11: 557-569.

Bolstad PV, Swift L, Collins F, Regniere J. 1998. Measured and predicted air temperatures at basin to regional scales in the southern Appalachian mountains. Agricultural and Forest Meteorology 91: 161-176.

Bougoure DS, Cairney JW. 2005. Assemblages of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae) as determined by culturing and direct DNA extraction from roots. Enviro Microbiol 7: 819-827.

Chupungars K, Rerngsamran P, Thaniyavarn S. 2009. Polycyclic aromatic hydrocarbons degradation by *Agrocybe sp*. CU-43 and its fluorine transformation. Int Biodeterioration and Biodegradation 63: 93-99.

Dobbs MM, Parker AJ. 2004. Evergreen understory dynamics in Coweta Forest, North Carolina. Physical Geography 25: 481-498.

Elliott KJ, Vose KJ. 2012. Age and distribution of an evergreen clonal shrub in the Coweeta basin: *Rhododendron maximum* L. Journal of the Torrey Botanical Society 139(2): 149 - 166.

Frome M. 1994. Strangers in high places: the story of the Great Smoky Mountains. University of Tennessee Press.

Gardes M, Bruns T. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Gorzelak MA, Holland TC, et al. 2012. Molecular approaches for AM fungal community ecology: A primer. Journal of Microbiological Methods 90(2): 108-114.

Hill MO. 1979. DECORANA – A FORTRAN program for Detrended Correspondence Analysis and Reciprocal Averaging. Section of Ecology and Systematics. Cornell University, Ithaca, New York. 52.

Iltis HH. 1956. Studies in Virginia Plants II. *Rhododendron maximum* in the Virginia Coastal Plain and Its Distribution in North America. Castanea 21(3): 114-124.

Johansson M. 2000. The influence of ammonium nitrate on the root growth and ericoid mycorrhizal colonization of Calluna vulgaris from a Danish heathland. Oecologia 123: 418 – 424.

Knoepp JD, Swank WT. 1998. Rates of nitrogen mineralization across an elevation and vegetation gradient in the southern Appalachians. Plant and Soil 204: 235-241.

Lei TT, Semones SW, Walker JF, Clinton BD, Nilsen ET. 2002. Effects of *Rhododendron maximum* thickets on tree seed dispersal, seedling morphology, and survivorship. Plant Sci 163: 991-1000.

Martino E, Murat C, Vallino M. 2007. Imaging mycorrhizal fungal transformants that express EGFP during ericoid endosymbiosis. Curr Genet 52: 65-75.

McGonigle TP, Miller MH, Evans DG, Fairchild GL. 1990. A new method which gives an objective measure of colonization of roots by arbuscular mycorrhizal fungi. New Phytol 115: 495-501.

Muncnerova D, Augustin J. 1994. Fungal Metabolism and detoxification of polycyclic aromatic hydrocarbons: A review. Bioresource Tech 48: 97-106.

Munns EN. 1938. The distribution of important forest trees of the United States. Gerald W. Williams Collection 287: 164.

Nilsen ET, Clinton BD, Lei TT, Miller OK, Semones SW, Walker JF. 2001. Does *Rhododendron maximum* L. (Ericaceae) reduce the availability of resources above and belowground for canopy tree seedlings? American Midland Naturalist 145: 325-343.

Nilsen ET, Walker JF, Miller OK, Semones SW, Lei TT, Clinton BD. 1999. Inhibition of seedling survival under *Rhododendron maximum* (Ericaceae): could allelopathy be a cause? American Journal of Bot 86: 1597-1605.

Parker MM. 2004. Evergreen understory dynamics in Coweeta forest, North Carolina. Physical Geography 25(6): 481-498.

Pauley EF, Nodvin SC, Nicholas NS, Rose AK, Coffey TB. 1996. Vegetation, biomass, and nitrogen pools in a spruce-fir forest of the Great Smoky Mountains National Park. Bulletin of the Torrey Botanical Club 123(4): 318-329.

Plocher AE, Carvell KL. 1987. Population dynamics of rosebay Rhododendron Thickets in the southern Appalachians. Torrey Botanical Society 114: 121-126.

Pointing SB. 1999. Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2: 17-33.

Price LW. 1986. Mountains and Man: A Study of Process and Environment, University of California Press.

Shanks RE. 1956. Attitudinal and microclimatic relationships of soil temperature under natural vegetation. Ecology 37(1): 1-7.

Shubzda J, Lindberg SE, Garten CT, Nodvin SC. 1995. Elevational trends in the fluxes of sulphur and nitrogen in throughfall in the southern Appalachian Mountains: Some surprising results. Water, Air, Soil Pollution 85:2265-2270.

Walker JF, Miller JR. OK. 2002. Ectomycorrhizal sporophore distributions in a southeastern Appalachian mixed hardwood/conifer forest with thickets of *Rhododendron maximum*. Mycologia 94: 221- 229.

Walker JF, Miller OK Jr, Horton JL. 2005. Hyper-diversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. Molecular Ecology 14: 829-838.

Walker JF, Aldrich-Wolf L, Riffel A, Barbare H, Simpson NB, Trowbridge J, Jumpponen A. 2011. Diverse Helotiales associated with the roots of three species of Arctic Ericaceae provide no evidence for host specificity. New Phytologist, 191(2), 515-527.

Wang C, Sun H, Li J, Li Y, Zhang Q. 2009. Enzyme activities during degradation of polycyclic aromatic hydrocarbons by white rot fungus *Phanerochaete chrysosporium* in soils. Chemosphere 77: 733-738.

Wurzburger N, Hendrick RL. 2009. Plant litter chemistry and mycorrhizal roots promote a nitrogen feedback in a temperate forest. Journal of Ecol 97: 528-536.

Van Lear DH, Vandermast DB, Rivers CT, Baker TT, Hedman CW, Clinton BD, Waldrop TA. 2002. American chestnut, Rhododendron, and the future of Appalachian cove forests. Proceedings of the eleventh biennial southern silvicultural research conference 622.

Väre H, Vestberg M, et al. 1997. Shifts in Mycorrhiza and Microbial Activity along an Oroarctic Altitudinal Gradient in Northern Fennoscandia. Arctic and Alpine Research 29(1): 93-104.

Vallino M, Drogo V, Abba S, Perotto S. 2005. Gene expression of the ericoid mycorrhizal fungus *Oidiodendron maius* in the presence of high zinc concentrations. Mycorrhiza 15: 333-344.

Vose KJ. 2012. Age and distribution of an evergreen clonal shrub in the Coweeta basin: *Rhododendron maximum* L. Journal of the Torrey Botanical Society 139(2): 149-166.

Biographical Sketch

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