AN ABSTRACT OF THE DISSERTATION OF

Cedar N. Hesse for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on May 17, 2012.

Title: Characterization of Fungal and Bacterial Communities Associated with Mat-forming Ectomycorrhizal Fungi from Old-growth Stands in the H.J. Andrews Experimental Forest

Abstract approved:

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Joseph W. Spatafora

Mat-forming ectomycorrhizal (EcM) fungi represent a prevalent constituent of many

temperate forest ecosystems and create dramatic changes in soil structure and

chemistry.  EcM mat soil have been shown to have increased microbial respiration

rates and have been hypothesized to harbor unique assemblages of fungi and bacteria.

The objectives of this dissertation were to characterize and examine the fungal and

bacterial communities associated with EcM mats in old-growth forests of the H.J.

Andrews Experimental Forest located in the Oregon Cascades.  Additionally, this

work assessed the application of traditional, emerging, and novel molecular sampling

techniques for determining microbial communities of environmental samples.  This

research investigated the microbial communities associated with two common EcM

mat genera found in old-growth Douglas fir stands in the Pacific Northwest;

*Piloderma* (Atheliales, Basidiomycota) and *Ramaria* (Gomphales, Basidiomycota).

Soil samples were collected from *Piloderma* and *Ramaria* mats and surrounding non-

mat soil for molecular analysis of nucleic acids.  First, a comparative study was

conducted to determine the most appropriate rDNA molecular sampling technique for microbial community characterization. Two next-generation sequencing methods, Roche 454 pyrosequencing and Illumina-based environmental sequencing, the latter developed by the author, were compared to a more traditional sequencing approach, i.e., Sanger sequencing of clone libraries. These findings informed the subsequent sampling of the fungal ITS and bacterial 16S rDNA fragment with 454 pyrosequencing to determine the microbial communities within mat and non-mat soils. Second, this work utilized a pyrosequencing approach to explore fungal community structure in EcM mat and non-mat soils. This work concluded that differences in microbial communities do exist between *Piloderma* mat, *Ramaria* mat, and non-mat soils, but the differences are largely quantitative with relatively few distinct taxonomic shifts in microbial constituents. *Piloderma*, *Ramaria* and *Russula*, in addition to being the dominant taxa found on mycorrhizal root tips, were found to be the most abundant taxa in bulk soils within their respective mat types or non-mat sample. The background fungal communities within the EcM mats in this study exhibited considerable taxonomic overlap with the exception of *Piloderma* vs. non-mat comparisons; *Russula* species dominated nonmat soils but tended to be excluded or significantly underrepresented in *Piloderma* mats. Lastly, this study explored the bacterial communities associated with *Piloderma* and *Ramaria* mats using lower-coverage 454-Jr pyrosequencing. Bacterial communities exhibited significant structure as a function of mat-type, soil horizon and pH, but this finding should be interpreted with respect to the nonrandom distribution of *Piloderma*-mats in the O-

horizon and the *Ramaria*-mats in the A-horizon, and the tendancy for EcM mats to be more acidic than surrounding soils. Nonetheless, the total microbial (bacterial and fungal) community was typically dominated by the mat-forming taxa, or *Russula*, in the case of non-mat soils. While the presence of *Piloderma* mats did enrich or restrict some bacterial groups, soil pH was also found to be a significant driver of bacterial richness and taxonomic diversity. Fungal and bacterial richness were also found to be positively related to one another, regardless of soil horizon or EcM mat type. This work, taken together, contributes to the understanding of hyperdiversity and heterogeneity of microbial communities of temperate forest soils and highlights the potential for fungal and bacterial communities to be influenced by the presence of EcM mats.

Characterization of Fungal and Bacterial Communities Associated with Mat-forming
Ectomycorrhizal Fungi from Old-growth Stands
in the H.J. Andrews Experimental Forest


by
Cedar N. Hesse



A DISSERTATION

submitted to

Oregon State University



in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy



Presented May 17, 2012
Commencement June 2012

Doctor of Philosophy dissertation of Cedar N. Hesse presented on May 17, 2012.

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ACKNOWLEDGEMENTS

CONTRIBUTION OF AUTHORS

S.M. Dunham was involved with the study design and field sampling associated with Chapter 2.  A.B. Leytem and S.M. Dunham conducted laboratory work including soil DNA extractions and clone library sequencing for Chapter 2.

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## CHAPTER 1 - INTRODUCTION

Cedar N. Hesse

**Ectomycorrhizal Fungal Diversity**

The great majority of terrestrial plant lineages form mutualistic associations with soil dwelling mycorrhizal fungi (Smith and Read, 2008). Although other mycorrhizal types exist, most mycorrhizal fungi are classified within two major groups defined by the mycorrhizal structures: the ectomycorrhizal (EcM) fungi and the arbuscular (AM) mycorrhizal fungi. Both groups are functionally similar in that they form close associations with plant roots at which nutrient exchange occurs, though their physiologies, host associations, and evolutionary paths differ. The arbuscular mycorrhizal association (also termed endomycorrhizae) represents one of the most common symbioses on Earth. Throughout the more than 500 million years since the origin of the Fungi, the evolution of the AM habit likely occurred only once. As such, all AM fungi represent a monophyletic grouping and are classified within the phylum Glomeromycota (Schüβler *et al.,* 2001). AM fungi are the predominant mycorrhizal associates of the majority of plants including most species of herbaceous plants and tropical trees, as well as numerous temperate woody gymnosperms (Smith and Read, 2008).

The ectomycorrhizal fungi (EcM) form associations with a relatively small proportion of plants when compared to AM fungi, however EcM associates occupy a disproportionally large percentage of land area and include many timber species important in silviculture. Dominant timber species within the Pinaceae, Myrtaceae, and Fagaceae have all been identified as predominantly ectomycorrhizal (Smith and Read, 2008). Ectomycorrhizal fungi, in contrast to AM fungi, are a polyphyletic

group representing multiple independent evolutionary origins of the EcM habit within the Zygomycota, Ascomycota, and Basidiomycota. Hibbett and Matheny (2009) provided a minimum estimate of at least eight separate evolutionary derivations of EcM within the Agaricomycotina, although with further taxon sampling that number will likely increase (Figure 1.1).

The artificial grouping of EcM taxa is due to common morphological and physiological attributes of the mycorrhizal structure (Figure 1.2). Root-tips of ectomycorrhizal associates are enveloped in a dense layer of fungal hyphae forming the mantle or sheath, which alters the morphologyof the roots. Extramatrical fungal mycelia emanate from the EcM mantle into the surrounding soil, facilitating mineral uptake through increased surface contact with soil resources. Fungal hyphae also grow into the fine root itself and colonize the intercellular spaces between root cortical cells, and more rarely epidermal cells, forming the Hartig net, which serves as the interface for nutrient transfers between symbionts. Photosynthetically derived sugars from the plant associate provide a high-energy carbon source to the fungus in return for essential nutrients (e.g., phosphates, nitrogen, essential minerals, etc.) transferred from the fungus to the host plant (Smith and Read, 2008).

**Mat-forming Ectomycorrhizal Fungi**

A subset of ectomycorrhizal fungi forms visually distinct aggregations of hyphae, mycorrhizal root-tips, and cord-like bundles of mycelia (rhizomorphs) in some forest soils. Commonly referred to as mycorrhizal "mats" due to the visually striking contrast to adjacent soil on the forest floor, fungal hyphae in mat soils can

constitute as much as 50% of the dry weight of soil (Ingham *et al.,* 1991). Although multiple studies have defined what qualifies as an EcM mat differently, most tend to converge on the concept that a mat is visually distinct from the surrounding soil, approximately 0.5-1.5m in diameter, and contains dense profusions of hyphae, root-tips, and rhizomorphs (Agerer, 2001; Dunham *et al.,* 2007; Griffiths *et al.,* 1991; Hintikka and Naykki, 1967). The abundance of EcM mats in forest soils has been shown to be higher in old-growth stands relative to younger forests, and in some old-growth Douglas fir forests EcM mats commonly occupy 25% of the soil surface area (Cromack *et al.,* 1979, Griffiths *et al.,* 1996). Geographically, this unique mat-forming habit has been documented from temperate and boreal forests throughout the northern hemisphere to subtropical forests in Australia, though particularly abundant in temperate and boreal coniferous forests.

Agerer (2001) proposed a classification scheme to identify the hyphal exploration type of ectomycorrhizal fungi which includes a "medium distance mat" exploration type to describe the mat-forming habit. Inspection of the Determination of Ectomycorrhizae database (http://deemy.de) for fungal taxa identified as having the medium distance mat exploration type shows the EcM mat habit is found in multiple EcM lineages and thus is not restricted to a single evolutionary origin (EcM lineages shown in red text of Figure 1.1). The breadth of taxonomic diversity of mat-forming EcM fungi is reflected in the variety of mat morphologies and physiologies seen in many forest ecosystems.

EcM mats have been the subjects of study in large part because they have been shown to alter the chemical and physical properties of the soils they inhabit (Griffiths *et al.,* 1994; Cromack Jr. *et al.,* 1979). Chemically, high levels of oxalic acid and other organic acids contribute to the average lower soil pH within EcM mats relative to adjacent non-mat soils (Cromack Jr. *et al.,* 1979; Griffiths *et al.,* 1990; Griffiths *et al.,*1991). This acidification of the soil is hypothesized to accelerate mineral weathering and increase nutrient availability (Cromack *et al.,* 1979; Griffiths *et al.,* 1994; Blum *et al.* 2002). Accordingly, EcM mat soils have been documented to contain higher concentrations of phosphate and sulfates, numerous metal ions including $Al^{3+}$, $K^+$, $Cu^{3+}$, $Zn^{2+}$, $Mn^{2+}$, and $Fe^{3+}$, and dissolved organic carbon (DOC).

Physical changes to mat soils are visually apparent due to the density of hyphae in the soil. Upon removing the humus layer from forest soils EcM mats can be visually identified by patches of white, yellow, and greenish hyphal aggregations. EcM mats are found in both organic and mineral soil horizons but some EcM mat species preferentially inhabit one or the other. A diversity of mat morphologies exists and likely reflects characteristics of different EcM taxa. One interesting morphology of some EcM mats commonly found in the mineral horizon results in soil that has a friable, ash-like texture and is strongly hydrophobic. The hydrophobic nature of these mats has been documented to reduce the overall soil moisture content and hinder penetration of rainwater (Unestam and Sun, 1995; Hintikka and Naykki, 1967).

EcM mat soils also represent areas of increased biological activity. Elevated $CO_2$ respiration rates relative to non-mat soils have been measured from EcM mats

indicating higher biological activity of aerobic organisms (Phillips *et al.,* 2012).

Cromack *et al.* (1988) found higher abundances of microarthropods and protozoans in

mat soils relative to non-mat soils.  The unique physical and chemical

microenvironments created by EcM mats have lead some to hypothesize unique

microbial communities could exist within EcM mats.  Knutson *et al.* (1980) found

increased abundance of calcium-oxalate degrading *Streptomyces* associated with the

ectomycorrhizae structure of some Douglas fir root-tips providing a basis for hypotheses

of unique oxalate-utilizing microbial assemblages in EcM mats (Jones *et al.,* 2001; van

Hees *et al.,* 2002).  Recently, a single ectomycorrhizal mat genus, *Piloderma*, has been

shown to harbor distinctly different fungal and bacterial communities than non-mat

soils with temporal variation in community size but not composition (Kluber *et al.,*

2011).

The EcM mats from Douglas-fir (*Pseudotsuga menziesii)* forests of the Pacific

Northwest, USA have been the subjects of continued research that together constitutes

much of our understanding of EcM mats.  The research presented in this dissertation

builds on the body of knowledge about EcM mats in the Pacific Northwest.  Much of

the research on EcM mats in the region has focused on two common and visually

distinct mat morphologies thought to be characteristic of different fungal genera.  The

first mat morphology is typically observed colonizing the organic soil horizon and has

conspicuous rhizomorphs that hold soil aggregates together.  Based on mycorrhiza

morphotyping, these organic horizon, rhizomorphic mats were thought to be formed

primarily by members of the genus *Hysterangium* (Hysterangiales, Phallomycetidae)

(Cromack *et al.,* 1979; Entry *et al.,* 1992), but in a recent phylotyping study by

Dunham *et al* (2007) it was found that many such mats in old growth forests were

actually more commonly formed by *Piloderma* (Atheliales, Agaricomycetidae) species

(Figure 1.3a).

The second common mat morphology is usually found in the mineral (A) soil

horizon and exhibits an ashy or friable appearance. These A-horizon mats are also

observed to be hydrophobic in nature, relative to the surrounding non-mat soils.

Initially identified as *Gautieria* (Gomphales, Phallomycetidae) mats (Griffiths, 1991),

the molecular methods employed by Dunham *et al.* (2007) revealed the genus

*Ramaria* (Gomphales, Phallomycetidae) is more commonly the mat former of the

hydrophobic mat (Figure 1.3b). It should be noted, however, that molecular

phylogenetic analyses have shown that *Gautieria* is nested within *Ramaria*,

representing one of the many independent origins of truffle-like fungi (Humpert *et al.,*

2001). Taken together, *Piloderma* and *Ramaria* EcM mats constitute approximately

80% of the EcM mats sampled in old-growth stands of the H.J. Andrews Experimental

Forest (Figure 1.4, Dunham *et al.,* 2007).

**Fungal diversity of soil**

Approximately 80,000 species of fungi have been formally described (Kirk and

Ainsworth, 2008), although the number of described species represents at most an

estimated 10% of total fungal diversity (Hawksworth, 2001; Schmit and Mueller,

2007). The soil ecosystem provides habitat for an astonishingly vast number of

organisms from all domains of life including a diverse array of macrofauna, protozoa,

prokaryotic microbes, and an enormous diversity of fungi. The ecologies of soil

dwelling fungi range from the beneficial symbionts of mycorrhizal fungi, to the

essential organic matter decomposing saprotrophs, to antagonistic pathogens of plants,

animals, and other fungi. Spanning all fungal phyla, the diversity of fungal species

present in a single gram of soil has been estimated to be in the hundreds or thousands

(Jumpponen *et al.,* 2009; Buée *et al.,* 2009).The attribute of a high density of species

richness in an environment has been termed hyperdiversity.

The number of fungi forming visible sporocarps at any given time in an

ecosystem is only a small fraction of the total belowground species richness, so visual

surveys provide limited understanding of belowground communities. Many fungi will

fruit at different times from one another, produce inconspicuous fruiting bodies, or

produce asexually resulting in inherent biases from visual surveys. Culture-based

methods have been used to explore fungal diversity in soil however they too suffer

from biases. Culture-based methods typically select for fast-growing saprobic fungi,

and are incapable of detecting most biotrophic and mutualistic fungi. Only recently,

molecular methods have allowed researchers to explore the unseen diversity of soil

fungi.

Molecular environmental sampling of fungi has primarily relied on DNA

sequence variation among the universally conserved ribosomal DNA (rDNA) genomic

regions to approximate fungal species. Variable sequences within the internally

transcribed spacer (ITS) region immediately adjacent to the conserved ribosomal

subunits provide sufficient sequence heterogeneity to distinguish many fungal species

(Schoch *et al.,* 2012). While not a formal designation, a phenetic species definition for fungi is commonly applied to the ITS rDNA sequence in environmental sampling studies, similar to the bacterial species definitions of the 16S rDNA region sequence (Rosselló-Mora and Amann, 2001; Stackebrandt *et al.,* 2002). Although between-species sequence variation of the ITS varies among taxonomic groups, common kingdom-wide thresholds are typically 2-5% sequence divergence defining the species boundary (Jumpponen *et al.,* 2009; U'Ren, 2010). Because much of the diversity of fungi is yet undiscovered, the database for which environmental sequences are compared is similarly lacking. It has become common practice to define operational taxonomic units (OTUs) based on sequence similarity thresholds that serve as approximations of fungal species (Schoch *et al.*, 2012).

Recently, molecular sampling of fungi in natural environments has revealed novel fungal lineages representing undescribed diversity at Phylum and Class levels. Phyl. nov. Cryptomycota represents a putative early diverging clade phylogenetically placed within Kingdom Fungi (Jones *et al.,* 2011b). Initially detected exclusively as DNA sequences from environmental samples, members of the Cryptomycota have subsequently been visualized using *in situ* fluorescent hybridization and are hypothesized to represent novel fungal diversity approaching half the breadth of the previously known Fungi (Jones *et al.,* 2011a). Intriguingly, the ecology of these fungi is still uncertain.

The new Class Archaeorhizomycetes also was first characterized from DNA sequences from environmental samples of high altitude soils. Initially reported by

Schadt *et al.* in 2003 as "soil clone group 1", or SCGI, Porter *et al.* (2008) identified

the enormous breadth of diversity represented by this previously unknown group.  By

searching Genbank for similar sequences, Porter and colleagues determined that taxa

very similar to SCGI were commonly encountered in environmental sequencing

studies from temperate forest soils.  Only recently has a cultured representative been

obtained facilitating the official classification and creation of a new fungal class, the

Archaeorhizomycetes (Rosling *et al.,* 2011).  While the representative culture of the

Archaeorhizomycetes, *Archaeorhizomyces finlayi*, has been shown to envelop tree

roots in a web of hyphae, it is still uncertain if members of the group function as

mycorrhizal symbionts.

**Bacterial diversity and associations with fungi**

Like fungal communities, bacterial communities exist in soil with an

astonishingly high degree of diversity.  An estimated $10^9$ prokaryotic cells (Whitman

*et al.,* 1998) representing somewhere between 6,400 and 10,000 (Curtis *et al.,* 2002;

Lunn *et al.,* 2004) unique bacterial species coexist in a single gram of soil.  The field

of molecular ecology of bacteria has closely paralleled that of fungi as the same

sampling techniques are employed in both fields. Like with the Cryptomycota and

Archaeorhizomycetes in fungi, novel bacterial lineages have been discovered and

described solely through the detection of rDNA sequence fragments.  Diversity within

the Acidobacteria, a phylum of Bacteria, has primarily been described through

molecular sampling of natural environments.  Members of the phylum have been

detected in nearly all surveys of soil bacteria; however the cultured representatives of

this group are few.  Despite their seemingly ubiquitous distribution in soil the

ecologies of most of the group are unknown, however recent genome sequencing of

the few cultured Acidobacteria available indicates the group may be generally slow

growing heterotrophs capable of tolerating low-nutrient conditions (Ward *et al.,* 2009).

Despite their intimate coexistence in most ecosystems the associations between

natural fungal and bacterial communities' remains understudied.  In the few studies

explicitly researching fungal-bacterial interactions in soil, rhizosphere bacteria have

been shown to elicit a variety of responses from soil dwelling fungi including

increasing hyphal growth, hyphal branching, and mycorrhization rate, as well as

antagonistic effects such as hyphal growth suppression (Tarkka *et al*., 2009; Maier *et

al.*, 2004; Frey-Klett *et al*., 2007).

In a review by Bending and others (2006) on microbial interactions in the

mycorrhizosphere, the authors present mechanisms by which mycorrhizal fungi might

influence the surrounding microbial communities.  They propose that mycorrhizal

fungi can directly affect soil microbial communities by changing the nutritional

availability relative to the surrounding soil.  Fungi explore their environments using

extracellular enzymes exuded from growing hyphae.  These exudates, along with the

hyphae themselves, may provide a food source for a variety of soil dwelling microbes

(Jones *et al.,* 2004).  Additionally, Bending and coauthors suggest mycorrhizal fungi

can indirectly affect the microbial communities in the mycorrhizosphere by modifying

the chemical properties of the soil environment.  Organic acids exuded by many

ectomycorrhizal fungi (Griffiths *et al.,* 1994; van Hees *et al.,* 2003), including mat-

forming genera, effectively acidify the surrounding soil and may correspondingly exclude or select for tolerant bacterial groups.  Likewise, the changes in nutrient availability caused by the acidic environment may select for microbial communities more able to utilize the resource (Bending *et al.,* 2006).

Probably the most studied fungal-bacterial interaction is that of the "Mycorrhization Helper Bacteria" (MHB) (Garbaye, 1994).  The bacteria identified as MHB are predominantly members of the genera *Bacillus* and *Pseudomonas*, however MHB have also been found in *Bradyrhizobium*, *Burkholderia*, *Paenibacillus*, *Rhodococcus*, and *Streptomyces* (Duponnois and Garbaye, 1991; Duponnois and Plenchette, 2003).  As their name suggests, MHB, when grown in co-culture with certain mycorrhizal fungi increase the rate of mycorrhizae formation on a host plant (Duponnois and Garbaye, 1991, Frey-Klett *et al*., 1999).  While the exact mechanisms behind the benefit of MHB remain uncertain multiple hypotheses have been proposed. It has been hypothesized that MHB may act to increase root receptivity by releasing the plant hormone indol-3-acetic-acid (IAA) (Gamalero *et al*., 2003), affect root-fungus recognition (Bending *et al.*2006), stimulate pre-symbiotic fungal growth (Duponnois and Garbaye, 1990), or act as spore germination promoters (Duponnois and Plenchette, 2003).

The hyperdiversity of fungal and bacterial communities makes studying their interaction in natural systems exceedingly difficult.  Accurate quantification of the fungal and bacterial constituents of a community enables correlative relationships between fungal and bacterial groups to be detected and hypotheses of their potential

interactions generated.  Environmental sequencing of fungal and bacterial communities occupying the same space has been limited, but may prove to be a valuable tool in exploring fungal-bacterial interactions.

**Next-generation sequencing in environmental sampling**

Molecular methods for environmental sampling of microbial communities have progressed greatly with the advent of next-generation sequencing technologies. Prior to 2006 the molecular methods used for characterizing both fungal and prokaryotic communities either provided coarse resolution ("fingerprinting" methods) or were labor intensive and costly (cloning and Sanger sequencing).  The relatively recent introduction of "next-generation" high-throughput sequencing methods from Roche/Life Sciences and Solexa/Illumina has spurred a multitude of microbial community studies.

Traditional community fingerprinting methods such as terminal restriction fragment length polymorphisms (T-RFLP) and length heterogeneity PCR (LH-PCR) have been used effectively to identify differences in the major constituents of microbial communities (Osborn *et al.*2000; Schütte *et al.,* 2008).  The limited taxonomic resolution of fingerprinting methods coupled with their inability to detect and quantify low-abundance taxa has prevented the methods from elucidating the true hyperdiversity of many microbial systems.  Similarly, DNA cloning and Sanger sequencing methods have provided needed insight into microbial communities, however they too suffer from limited ability to sample the hyperdiversity of microbial systems.  Although the quality of data from Sanger sequencing is optimal,

constructing and sequencing the large libraries needed to adequately sample a hyperdiverse microbial community is labor intensive and often prohibitively expensive. As a result of the technical challenges posed by traditional community profiling methods, many researchers have embraced next-generation sequencing platforms for studies of microbial community ecology.

Currently, two next-generation sequencing (NGS) platforms dominate the market-share: the pyrosequencing based 454 Roche Diagnostics platform and the clonal array based Illumina platform. Both methods rely on optical capture of fluorescent signals during or after nucleotide incorporation to determine the DNA sequence, but differ in the chemistries and processes used. Practically speaking, the difference in platforms ultimately is defined by the end-product of the data. When comparing the two platforms, read length, throughput, error-rate, and run-cost are most applicable. The current Roche Diagnostics (formerly Life Sciences) 454-FLX sequencing platform used in this study affords the read lengths nearing 500bp and approximately $10^7$ reads per run for a cost of around 8,000 USD. At the time of data collection for this study the latest Illumina platform was the Genome Analyzer I. Single ended reads of 36bp were produced at a throughput of 6 x $10^7$ reads per lane for approximately 2500 USD. In contrast, the current Illumina HiSeq 2000 platform produces paired-end reads of approximately 100bp with a throughput of greater than 100 x $10^7$ at an approximate cost of 2000 USD per lane.

Both next-generation sequencing platforms were designed primarily for whole genome resequencing and *de novo* genome sequencing, however molecular ecologists

have co-opted the technology for high-throughput sequencing of microbial rDNA amplicons.  The sample preparation for both platforms allows for sequencing of mixed templates without the need for a cloning step thereby reducing the cost and preparation time relative to clone-and-sequence approaches.  The sequence data obtained from next-generation sequencers is comparable to that obtained from Sanger sequencing although NGS reads are markedly shorter and tend to have higher per-base error rates (approximately 1% error).

Due to the increase in read length from the 454 sequencing platform, it has become favored for most microbial community studies.  The longer reads allow researchers to detect more polymorphisms in a 16S or ITS amplicon and are thus able to more confidently assign a taxonomy or operational taxonomic unit (OTU).  Robust methods for multiplex sequencing using unique oligonucleotide barcodes (Hamady *et al.,* 2008) have also allowed users to sequence tens or hundreds of samples in a single 454 run.  While the Illumina platform offers lower cost-per-base sequencing, the shorter read length has hindered its utility to microbial community profiling.  Nonetheless, recent work by Caporaso *et al.* (2011) has shown 16S amplicon sequencing on the Illumina platform is feasible for bacterial community profiling and successfully sequenced samples at a much greater read-depth than capable on the 454 system.

**Overview of dissertation research**

This dissertation represents the author's effort to characterize the microbial communities associated with *Piloderma* and *Ramaria* mat soils in the H.J. Andrews

Experimental Forest LTER site, Lane County, Oregon, USA. Due to their unique

physical and chemical properties a Microbial Observatory project, "*Structure and*

*function of mycorrhizal mat communities at the H.J. Andrews LTER Microbial*

*Observatory*," was initiated to examine, in part, the biological attributes of the two

most common EcM mat types in our study area. Initial hypotheses surmised that the

differences in the chemical profiles between *Piloderma* mat, *Ramaria* mat, and non-

mat soils may facilitate dissimilar microbial communities. As described previously in

this chapter, fungal and bacterial communities are intimately associated and compete

for the same resources and, in theory, fungi can directly and indirectly influence the

bacterial community composition in their immediate vicinity through chemical

modifications to the soil environment. Although additional work is needed,

mycorrhization helper bacteria may represent a third symbiont in the traditional

ectomycorrhizal mutualism. This work characterizes the fungal and bacterial

microbial communities using the most recent advances in DNA sequencing; next-

generation sequencing platforms.

Chapter 2 of this dissertation presents a comparison of three molecular

sampling methods, including one developed by the author, and their application to

describe fungal communities in soils. The three methods, cloning and Sanger

sequencing, 454-FLX Titanium amplicon sequencing, and Illumina-based

environmental signature sequencing, are compared in a systematic fashion to reveal

advantages, drawbacks, and potential biases of each. The subsequent findings inform

the methodologies for the following two chapters.

Chapter 3 of this dissertation presents the findings of a pyrosequencing-based analysis of fungal communities associated with *Piloderma*, *Ramaria*, and non-mat soils. Comparisons of fungal species richness, diversity, and taxonomic distributions highlight the heterogeneity of fungal communities in soil and reiterate the findings of fungal hyperdiversity in soil systems. In addition, differences in fungal communities are detected including diminished species richness in *Ramaria* mat soils and relative deficiency of the common non-mat ectomycorrhizal genus *Russula,* in *Piloderma* mat soils. Finally, Chapter 3 explores the concept that hyperdiversity in fungal systems is largely driven by the presence of a multitude of very rare taxa and that the functional diversity of a fungal community may be driven by few highly abundant taxa.

Chapter 4 explores the bacterial communities found within the same *Piloderma* mat, *Ramaria* mat, and non-mat soils from Chapter 3. This work represents one of the few studies to analyze both the bacterial and fungal components of a microbial community at the depth afforded by pyrosequencing. Although relatively shallow sequencing of the bacterial community was conducted, patterns of bacterial community composition are associated with *Piloderma* mat and non-mat soils, pH, and soil horizon. Additionally, when fungal community composition is considered in tandem with the bacterial community composition, predictable associations of bacterial families and fungal genera are found preferentially given certain environmental variables.

Finally, Chapter 5 contains the author's concluding remarks including primary

findings of this research and suggestions for future research on the microbial

communities associated with ectomycorrhizal mats.

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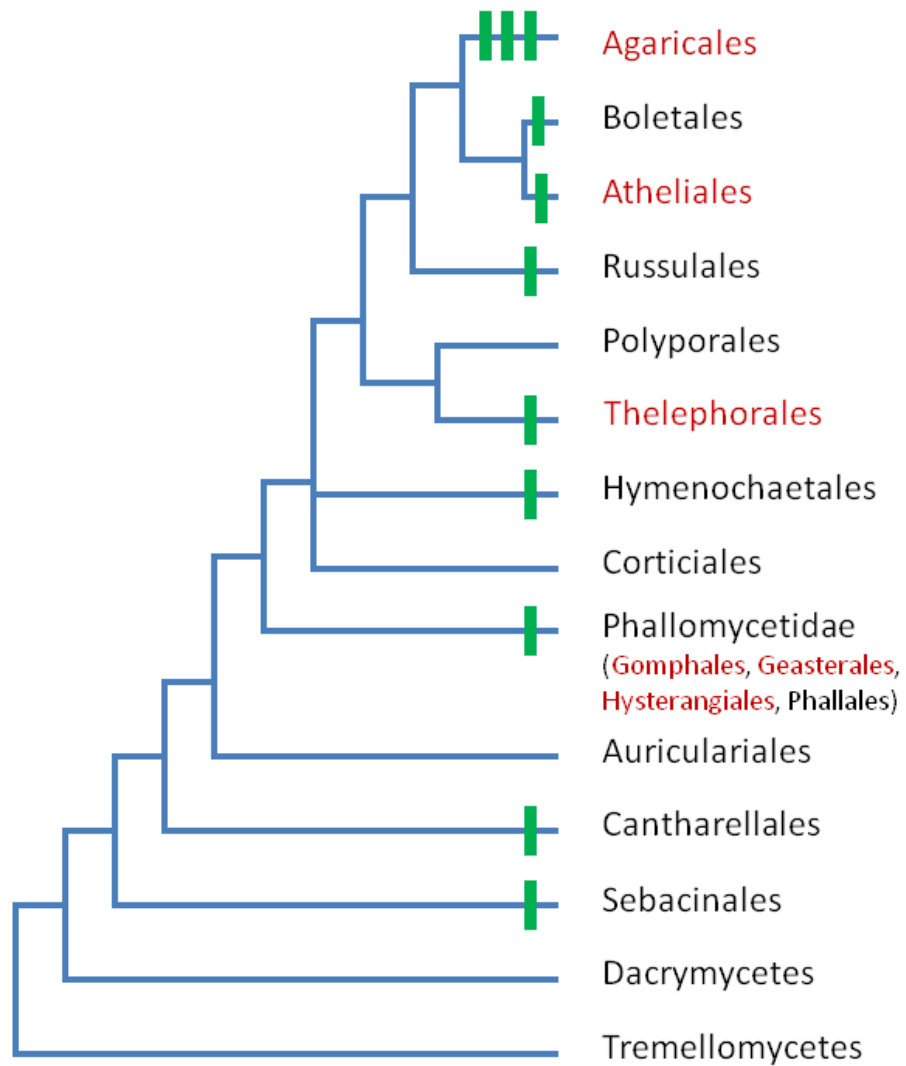
Figure 1.1 – Evolution of Ectomycorrhizae in the Agaricomycotina. Green bars represent independent derivations of the EcM habit among major orders of Basidiomycota. Red text indicates fungal orders in which mat-forming ectomycorrhizal fungi have been documented. Figure adapted from Hibbett and Matheny (2009) and Binder and Hibbett (2006).
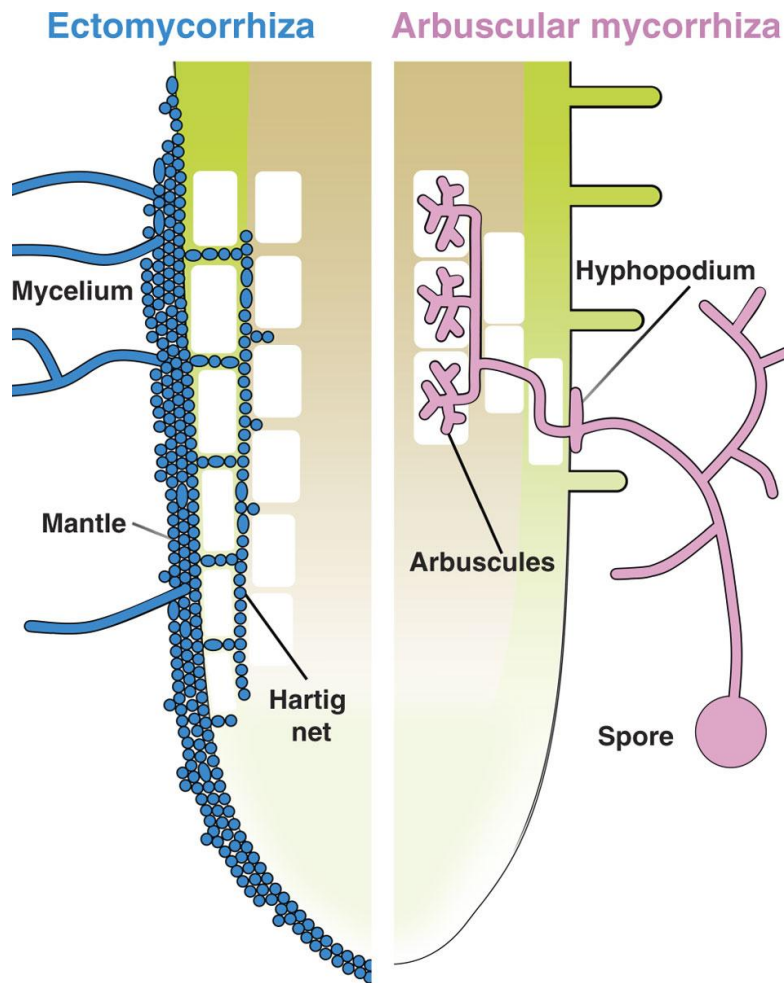
Figure 1.2 – Diagram comparing ectomycorrhizal and arbuscular mycorrhizal root tip structures. Reprinted by permission from Macmillan Publishers Ltd: [Nature Communications] Bonfante, P., & Genre, A. (2010). Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nature Communications*, *1*(4), 1–11. doi:10.1038/ncomms1046 Copyright 2010.
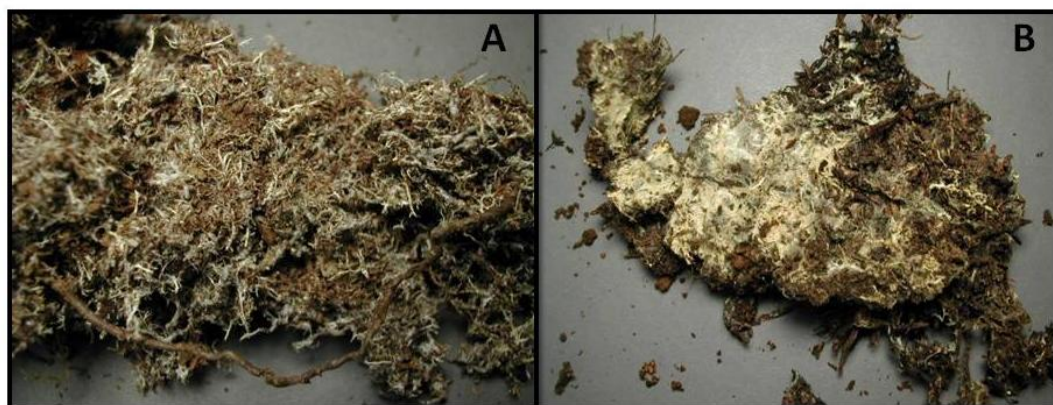
Figure 1.3 – Photographs of collected *Piloderma* and *Ramaria* mat soils.
Ectomycorrhizal mat soils from (A) *Ramaria* mat and (B) *Piloderma* mat. *Ramaria*
mat type exhibits ashy appearance, typically inhabiting the A-horizon, hydrophobic.
*Piloderma* mat aggregates soil with rhizomorphs and typically found in O-horizon.
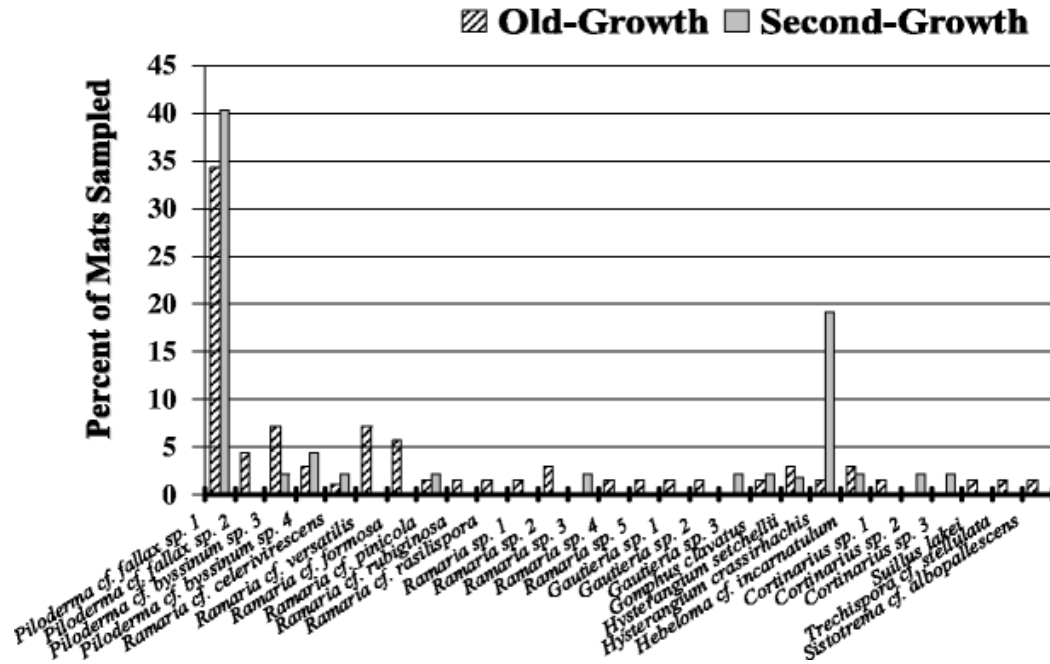
Figure 1.4 – Frequency histogram showing relative encounter rates of mat forming EcM taxa from old-growth (hatched bars) and second-growth (grey bars) stands from the H.J. Andrews Experimental Forest.  Used from Springer and Mycorrhiza, Volume 17 Number 8, 2007, Page 639, *Species richness and community composition of mat-forming ectomycorrhizal fungi in old- and second-growth Douglas-fir forests of the HJ Andrews Experimental Forest, Oregon, USA*, Susie M. Dunham, Karl-Henrik Larsson and Joseph W. Spatafora, Figure 2b, Copyright Springer-Verlag 2007) with kind permission from Springer Science and Business Media.

# CHAPTER 2 - A COMPARISON OF THREE ENVIRONMENTAL MOLECULAR SAMPLING TECHNIQUES AND THEIR APPLICATION TO DESCRIBE FUNGAL SOIL COMMUNITIES.

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**Abstract**

Recent advances in next-generation sequencing (NGS) technologies have resulted in numerous studies aimed at describing the hyperdiverse fungal communities found in many natural systems. Most studies to date have primarily utilized the 454 Life Sciences (Roche) pyrosequencing platform. Here we introduce a methodology for sampling the ITS1 ribosomal DNA region of an environmental sample using the Illumina sequencing platform. Additionally, this study provides a comparison of the two common NGS platforms (Roche/Life Sciences and Illumina) and traditional clone-library sequencing, as used for quantifying fungal communities sampled from *Piloderma* and *Ramaria* ectomycorrhizal (EcM) mats, as well as neighboring non-mat soils. All methodologies used in this study indicated EcM mats are typically dominated by a single fungal taxon representing the mat-forming individual of *Piloderma* or *Ramaria*. Species richness was generally lower in *Ramaria*-mat mineral horizons relative to the *Piloderma* mat and non-mat treatments. We conclude that both NGS technologies provide an increased resolution to the overall fungal community, as compared to a traditional clone-and-sequence approach. While potential systematic biases can be detected between NGS platforms, both provide remarkably similar broad-scale taxonomic distributions of the major fungal community constituents.

**Introduction**

Ectomycorrhizal (EcM) fungi are found throughout the terrestrial soils of the world and form symbioses with a diverse assemblage of plant species (Smith & Read

2008). Some of the most prolific and diverse EcM assemblages occur in temperate coniferous forests, a system typified by the *Pseudotsuga menziesii* (Douglas-fir) / *Tsuga heterophylla* (western hemlock) forests of the Pacific Northwest (PNW) of North America. The close association of tree roots and fungal hyphae in an ectomycorrhiza mutually benefits both organisms, allowing the plant to buffer water stress and nutrient scarcity, while the fungus receives photosynthetically derived sugars in return. For the H.J. Andrews Microbial Observatory we have used the unique growth forms of two common ectomycorrhizal genera representing separate evolutionary origins of EcM (Hibbet and Matheny, 2009): the genus *Piloderma* (Atheliales), and the genus *Ramaria* (Gomphales).

Ectomycorrhizal mats have been defined similarly by different groups of researchers (Hintikka and Naykki 1967, Entry *et al.,* 1991, Dunham *et al.,* 2007) and converge on the idea that an EcM mat is characterized by dense profusions of ectomycorrhizal root tips and fungal rhizomorphs that aggregate the soil and alter its appearance. Extensive work on EcM mats in Western Oregon has shown that EcM mats are characterized by lower pH and higher oxalate concentrations (Cromack *et al.,* 1979, Malajczuk and Cromack 1982, Griffiths *et al.,* 1994), higher inorganic ion concentrations (Entry *et al.,* 1992, Griffiths *et al.,* 1994), and higher carbon to nitrogen ratios than the adjacent non-mat soils (Griffiths and Caldwell 1992). These drastic differences in soil chemistry have led some to hypothesize that EcM mats may play a significant role in mineral weathering and nutrient cycling (Cromack *et al.,* 1979, Blum *et al.,* 2002).

Species of both *Piloderma* and *Ramaria* frequently form hyphal mats in coniferous forest soils (Dunham *et al.,* 2007). While the physical and chemical properties of *Piloderma* and *Ramaria* mats have been studied extensively, less is known about the biological communities associated with EcM environments. Cromack *et al.* (1988) observed an increase in microarthropods and protozoans within mats, and along with Entry (1991), documented increased microbial biomass and respiration relative to adjacent non-mat soils. Knutson *et al.* (1980) found increased abundance of calcium-oxalate degrading *Streptomyces* with ectomycorrhizae providing a basis for hypotheses of unique oxalate-utilizing microbial assemblages in EcM mats (Jones *et al.,* 2001, van Hees *et al.,* 2002). Recently, molecular techniques have been used to show hyperdiverse fungal communities in bulk soil not associated with mats (O'Brian *et al.,* 2005), but these types of studies within EcM mat systems have been limited to one clone library study (Kluber *et al.,* 2011) and root-tip phylotyping (Dunham *et al.,* 2007).

The EcM mats considered in this study represent two distinct mat morphologies that have been shown to differ in how they alter the biochemistry of forest soils and their co-occurrence within our study area provide a useful system for comparison. *Piloderma* species that form mats typically inhabit the upper organic soil horizon and produce dense profusions of white to yellow rhizomorphs that aggregate soil. *Ramaria* species that form mats tend to colonize the upper mineral horizon or the interface between organic and mineral horizons and typically exhibit a powdery or ashy morphology with a high density of mycorrhizal root tips and hydrophobic

properties. Both types of mats provide a unique microenvironment relative to adjacent non-mat soils and likely represent a niche habitat for soil organisms.

Recent advances in high-throughput DNA sequencing technologies have fueled an interest in profiling studies of microbial communities. Where a traditional 16S prokaryotic or fungal Internal Transcribed Spacer (ITS) clone library study would commonly yield hundreds to a few thousand sequence tags, new high-throughput methods provide tens-of-thousands to hundreds-of-thousands of tags. Dubbed "pyrotagging", the 454-FLX sequencing platform (Roche) has been almost exclusively the instrument of choice for fungal and bacterial community profiling studies. While other next-generation sequencing platforms exist, the 454-FLX has been preferred due primarily to the longer read-length (>400 bp) afforded by the platform. Only recently has another sequencing platform, the Illumina Genome Analyzer, been shown to be effective for 16S community profiling (Caporaso et. al. 2011). While the Illumina GA read lengths are shorter in length (40-150 bp), the platform provides the added benefit of sequencing depth with over 80 million reads per lane at a reduced cost. In this article we present a method for fungal community profiling of a soil biome using the Illumina sequencing platform and compare the results with that of a traditional clone-and-sequence approach and 454-FLX pyrotagging.

**Materials and methods**

***Study area and sampling design***

*Study area* – Fourteen study plots dominated by old-growth Douglas-fir (*Pseudotsuga menzensii)* were sampled from within the H.J. Andrews Experimental

Forest LTER site, Lane County, Oregon, USA. The selected sites were a subset of old-growth sites previously sampled in Dunham *et al.* (2007), that were identified as containing both *Piloderma* and *Ramaria* EcM mat types. For a more detailed description of the study site see Dunham *et al.* (2007).

   *Sampling design* – EcM mat sampling for this study relied on mat identities previously described by Dunham *et al.* (2007). Briefly, sampled in late fall 2005, each circular study plots (900m$^2$) was systematically searched by two individuals for thirty minutes or until thirty EcM mats were located. Mats were then randomly chosen and visually inspected for our sampling criteria (>0.5m$^2$ in area, presence of mycorrhizal root-tips and rhizomorphs) until three-to-five mats were selected and cored. Subsequent phylotyping identified representative *Piloderma* and *Ramaria* mats to be sampled for further study. Three soil cores from each representative mat were taken to a depth of approximately 10cm below the organic horizon-mineral horizon interface. Each soil core was split along the visually identified organic horizon-mineral horizon interface. Non-mat soils, identified by the lack of EcM rhizomorphs and conspicuous hyphae, were similarly collected at three randomly selected positions within the plot. This sampling resulted in six unique "treatments" which are herein to be referred by the following treatment identifiers: RO (*Ramaria* mat organic horizon), RA (*Ramaria* mat mineral horizon), PO (*Piloderma* mat organic horizon), PA (*Piloderma* mat mineral horizon), NO (non-mat soil organic horizon), and NA (non-mat soil mineral horizon). In total, 20 *Piloderma* mat samples, 11 *Ramaria* mat samples, and 17 non-

mat samples, all of which were split in to organic- and mineral-horizons, were used in this study.

   *DNA extraction* – Soon after collection, the three replicate samples for each mat and horizon sample were pooled together, soil sieved to remove any rocks, fine roots, and mycorrhizal structures, and homogenized using a mortar and pestle.  DNA was extracted from three 0.3g subsamples from each sample using the BIO101 Soil DNA Extraction kit and eluted in 40μL EB following manufacturer's protocols.

 **Clone library sampling**

   *PCR and clone library preparation* – Each DNA sample was amplified across the ribosomal subunit locus using two different universal eukaryotic rDNA primer sets.  The first primer set (NSF1179 and ITS4) amplified an approximately 1.2kbp region from ~600 bp from 3-prime end of the small subunit (18S), through the internally transcribed spacer (ITS) and 5.8S regions, to the beginning of the large subunit (28S).  A second set of primers (NLR512 and ITS1) were used to amplify an approximately 1.2kbp region from the 5-prime end of the small subunit, through the ITS and 5.8S, and the first 600bp of the large subunit.  These two amplicons will herein be referred to as SSU_ITS and ITS_LSU, respectively.  DNA amplification was conducted using high-fidelity *DNA*-polymerase under standard PCR conditions. Triplicate DNA extracts for each mat sample were amplified for each of the two primer sets, quantified for DNA concentration and the replications for each amplicon were pooled in equimolar concentrations.  Subsequently, amplified DNA was pooled by treatment across the entire study site (e.g., all *Ramaria* mat organic-horizon

ITS_LSU amplicons were pooled, etc.). The result of this final pooling yields twelve

sample units (3 treatment types X 2 horizons X 2 primer sets).

Promega Pgem-T vector systems were used to clone the amplified DNA

fragments from each sample unit into *Escherichia coli* DH5-alpha cells. *E. coli* cells

were streaked on antibiotic containing media allowing only those cells with a

transformed DNA fragment and resistance gene to grow. Colonies were allowed to

grow for a 24h period after which 96 colonies from each treatment library were

randomly selected for sequencing. Cloned DNA was reamplified using the

recommended 24bp pUC/M13 forward and reverse primers, then sequenced on the

ABI 3130 automated sequencer. Clone sequences are deposited in Genbank under

accession numbers JX006790 - JX007290 (ITS-LSU) and JX007291 - JX007765

(SSU-ITS).

*Data curation and manual identification of cloned sequences* – DNA

sequences were screened for poor sequence read quality and trimmed to delete vector

sequence in BioEdit V7.0.9.0 (Hall, 1999) before being checked for chimeric

recombinations using Bellerophon (Huber *et al.,* 2004). Contig assembly of the

resulting high-quality forward and reverse reads was done using CAP3 (Huang and

Madan, 1999). Contigs were clustered based on a 95% level of sequence similarity

using the OTUgroup tool from the USEARCH software package (Edgar, 2010) across

the ITS region to define operational taxonomic units (OTUs). Clusters were identified

to species when possible using BLAST (Altschul *et al.,* 1997) to query the ITS region

of the sequences against the Genbank database of known sequences. BLAST results

were inspected by eye and species designation was determined while considering potential database errors, database sources, and query-hit coverage.

*Automated identification of cloned sequences* – To facilitate the comparison to next-generation sequencing techniques a second OTU identification was determined without manual inspection for accuracy. BLAST results were parsed in the software package MEGAN (Huson *et al.,* 2007) using the default parameters (min support = 5). OTUs were placed within the NCBI taxonomy at the most terminal node that contains all significant BLAST hits.

**Illumina-based environmental signature sequencing (IBESS) sample preparation**

*Primer design* - The ITS1 region of the rDNA was amplified using custom primers designed to be universal for fungi. Conserved sequences on the 3-prime side of the commonly used ITS1F priming site and the 5-prime side of the ITS2 priming site were used to identify two new primer sequences. *In silico* testing of the new primer pairs using custom PERL scripts and the NCBI genbank database provided reasonable indications that they would allow for amplification of the ITS1 region for nearly all previously sequenced fungi. The final primer design contains the rDNA annealing site, a recognition site for the MME1 restriction enzyme, and a 10bp M13 overhang (Figure S1).

*ITS1 amplification and primer removal* - Amplification of the ITS1 region from environmental DNA extractions was carried out on a gradient-capable BioRad MyCycler (BioRad; Hercules, CA). Individual samples were amplified in eight 20ul reactions. Annealing temperatures for each reaction were constant within each

reaction and ranged, in even intervals, from 45C to 60C across the eight reaction

replicates.  The eight reactions for each sample were subsequently pooled and cleaned

on a Qiagen PCR purification column.  The recognition site incorporated into the

amplification primer is recognized by the Type III restriction enzyme *MMEI*.

Digestion by *MMEI* cleaves the dsDNA 18-20bp to the 3'-end of the recognition site.

The result of this digestion is the effective removal of the amplification primers from

both sides of the ITS1 amplicon.  Conditions for *MMEI* digestion of our PCR products

was as recommended by the enzyme supplier (New England Biolabs, Ipswich, MA); 2

hours at 32C.  Purification using the Qiagen PCR purification column stopped the

digestion and removed small digestion products and enzyme.

*Illumina sample preparation and sequencing* – Restriction digested samples

were prepared using the standard Illumina genomic DNA protocol with few

modifications.  As the ITS1 amplicon is roughly 180bp and within the suggested

Illumina library size range, the first step of the Illumina protocol (fragmentation) was

omitted. Subsequent sample preparation was as directed in the standard Illumina

protocol.  Six samples, representing pooled amplicons of each treatment, were run on

individual lanes of the Illumina GAI sequencing platform.  For a single soil extraction

(NO116) 76bp paired-end reads were generated on one lane of the Illumina GAII.

Illumina sequences are available from http://spatillumina.cgrb.oregonstate.edu.

*Data curation* – Sequences generated by the Illumina Genome Analyzer were

filtered for low-quality reads, keeping only sequences with Phred-equivalent Q-values

greater than 20 across the entire read.  On average approximately 550,000 single-end

40bp reads were generated in each lane that met the quality thresholds. Identical and nearly identical (>98% similarity) sequences from each sample were dereplicated using USEARCH to ease the computational load in subsequent analyses and to account for sequencing errors. Individual sequences were queried using BLAST against the database of Fungal ITS sequences described in Nilsson *et al.* (2009), which contains only fully identified and named fungal taxa. BLAST results were parsed using custom PERL scripts to incorporate the abundance distributions previously omitted during dereplication. The software package MEGAN was used to visualize BLAST results on the NCBI Taxonomy tree topology and export putative taxonomic identifications of OTUs.

### *454-FLX titanium sampling*

*Primer design and sample preparation* – Amplicon primers were designed following the guidelines set forth in the Roche manual for amplicon sequencing. The fungal ITS region was targeted using the ITS1F forward primer and the ITS4 reverse primer. Twenty-four unique error-correcting 8-bp nucleotide sequences (Hamaday, 2008) were incorporated into the amplification primer allowing for multiplexing of 96-samples across a 4-region picotiter plate. The final forward amplification primer contained the 454-FLX Fusion Primer A sequence, an 8-bp multiplex ID sequence, and the ITS1F annealing site sequence. The final reverse amplification primer contained the 454-FLX Fusion Primer B sequence and the ITS4 annealing site sequence.

Ninety-six samples were amplified in a single 96-well microtiter plate using one of the twenty-four unique barcoded forward primers and the universal reverse primer.  High fidelity polymerase (Phusion HF, New England Biolabs) was used to reduce the frequency of nucleotide substitution errors during amplification.  PCR conditions began with an initial denaturing step of 2 minutes at 92C followed by 25 cycles of 92C for 10 seconds, 50C for 30 seconds, and 72C for 40 seconds.  A final extension for 5 minutes at 72C concluded the amplification program.

All 96 amplified samples were individually purified using Qiagen PCR purification columns with a final elution volume equal to that of the initial PCR reaction (30ul).  Template concentrations of individual samples were quantified using the Qubit fluorometer and the Qubit BR reagent kit (Invitrogen).  Four groups of twenty-four samples, each with unique multiplex ID tags, were pooled in equimolar concentrations and gel purified using the Qiagen Gel Purification kit to remove any primer-dimer from the PCR reaction.  Samples were sent to the Duke Institute for Genome Sciences and Policy at Duke University for sequencing. 454-FLX amplicon sequences are deposited in NCBI Short Read Archive (accession SRA051397).

*Data curation* – The four multiplexed samples were sequenced in a single run on a four-region picotiter plate on the 454-FLX Titanium sequencing platform. Sequences were computationally de-multiplexed, trimmed for quality and primer sequences, and binned into treatment type using the MOTHUR software package (Schloss *et al.,* 2009).  For the following analyses, samples from the same treatment

(*Ramaria* O-horizon, *Piloderma* A-horizon, etc.) were pooled to allow for comparison to the pooled clone library and pooled Illumina 32-bp sequencing.

Sequences were clustered into 95% similarity groups defining OTUs using OTUpipe in the USEARCH package and custom Perl scripts. Putative taxonomic identities were placed on OTUs by BLASTing against the Fungal ITS sequence database and parsing the output in the program MEGAN (min support = 5).

### *Statistical methods*

*Rarefaction analyses* – Rarefaction analyses were conducted for each dataset using the VEGAN package (Oksanen *et al.,* 2011) and the "Rarefaction.R" script from (www.jennajacobs.org/R/rarefaction.html) in the statistical package R (R Development Core Team, 2010) with 500 randomizations. The OTU abundance matrices were created in Microsoft Access 2007 prior to analyses. Rarefaction curves were visualized using Microsoft Excel 2007.

*Rank abundance* – OTU frequency counts were relativized by sampling unit to account for differences in sequencing depth among samples. Rank abundance distributions were obtained in Microsoft Excel by ordering relativized frequency for each sampling from highest to lowest. Figure 1 shows only the first 25 most abundant OTUs across all treatments.

*Diversity indices* – Diversity indices were calculated using the VEGAN package in R. Shannon index (Shannon, 1948), Fisher's alpha (Fisher *et al.,* 1943), and Simpson index (Simpson, 1949) (expressed as 1-D), were determined to quantify

within treatment diversity while the Sørensen index of similarity describes pairwise
between-treatment similarity.

*Blocked multiple response permutation procedure* - A statistical test of the
hypothesis of no differences in communities determined between the two primer sets
was conducted using blocked multi-response permutation procedures (MRPP).  The
blocked MRPP test was used to indentify potential primer biases associated with the
technical replication of the two clone library amplicons (SSU-ITS and ITS-LSU).  A
primary matrix was prepared that contained the twelve sample units with abundance
counts relativized by sample unit and a secondary matrix contained the sample units
with categorical variables to distinguish primer set of the clone library and was
analyzed in the software package PC-ORD v6.51.  In the blocked MRPP analyses
Euclidean distances were used, however a considerable proportion (87%) of the
species matrix contained zeros.

### Taxonomic Distributions

*Pooled-sample taxonomic comparisons* – For each of the datasets (Clone
library, Illumina 32bp single-end, and 454-FLX), a table of taxonomic distributions
for each treatment was compiled.  Read counts were normalized to 100,000 reads per
treatment for comparison.  Column graphs depicting summarized counts at different
taxonomic levels, as determined by the NCBI taxonomy tree, were created in
Microsoft Excel 2007.

*Illumina paired-end and 454-FLX single sample comparisons* – The single
non-mat O-horizon sample (NO116) was sequenced on both the 454-FLX platform

and the Illumina GAI as paired-end 76bp reads.  Taxonomic distributions were
obtained from MEGAN for each direction of the Illumina paired-end sequencing and
compared to the 454-FLX read taxonomic distributions.  Comparisons of taxonomic
assignments were visualized in Microsoft Excel 2007.

**Results**

***Clone library***

Rarefaction analyses of clone library sequences indicate our clone library
sampling effort does not adequately capture the entire diversity of the fungal
community.  The rarefaction curves (Figure S2) for all six samples fail to approach an
asymptote and indicate undersampling of hyperdiverse communities.  The composite
rarefaction curve (Figure S3) of all treatments follows roughly the same trajectory as
individual treatment curves suggesting that not only is alpha diversity high within
treatment type, but beta diversity is high as well.  The trajectories of all curves were
statistically no different from one another at 95% confidence interval (CI), although
the *Ramaria*-mat mineral-horizon was significantly less than all others at 90% CI.

Rank-abundance distributions (Figure 2.1) show *Piloderma*-mat and *Ramaria*-
mat soils harbor single dominant taxa representing between 8-30% of the entire library
for that treatment, with a precipitous decline in the next most-abundant taxa.  Non-mat
soils, however, show a considerably more even distribution of fungal taxa.  All
treatments can be characterized as containing few highly abundant taxa and a long tail
of infrequently encountered taxa, at our sampling.  As expected, the most abundant
taxon in both mat treatments was the mat-forming species itself.  In *Piloderma*-mat

soils the "*Piloderma fallax group*" represented 14.7% of the total sequences in the treatment, while a group of closely related *Ramaria* species ("*Ramaria celerivirescens group*") represented 20.5% of all *Ramaria*-mat soil clones. The four most abundant taxa in the non-mat soils were all members of the Russulales, together representing 19.0% of all non-mat clone sequences.

The manually assigned taxonomic distribution of clone sequences for all samples was most abundant in members of the Basidiomycota, which represent on average 71.8% of all clones across all treatments and 58%-86% of samples within each treatment. Members of the Ascomycota were the second most commonly encountered representing on average 21.4% of all clones in this study and 10%-30% of clones within a treatment. Non-fungal sequences represented 6% of all clones in this study and early diverging fungal lineages (chytridiomycous and zygomycetous fungi) constituted less than one percent of all cloned sequences. Ordinal level diversity was high across treatment type with 22 orders within the Basidiomycota detected, nine of which were represented in five or more of the six treatments. Sixteen orders of the Ascomycota were detected, of which five were represented in five or more of the treatments. For consistency in taxonomic comparisons among sequencing methods, the automated taxonomic classifications as determined by MEGAN were used for clone sequences in all subsequent analyses.

Blocked multiple response permutation procedure was used to test the hypothesis of no difference in communities between groups defined by primer set.

We found no significant difference in communities when assessed by the two different primer sets (Blocked MRPP: A=-0.033, p=0.679).

Shannon diversity indices (Table S1) for the clone library ranged from 2.7-4.2, with the highest diversity in both horizons of the Non-mat soils and the lowest diversity in the *Ramaria* A-horizon. Similarly, Fisher's Alpha statistic (Table S2) showed the same pattern with non-mat soils having higher diversity and *Ramaria*-mat A-horizon having less diversity. Simpson's diversity indices (expressed as 1 - D) for clone library communities (Table S3) are very close to 1, indicating a very high amount of heterogeneity within plots. Simpson's index for the *Ramaria*-mat A-horizon, while still near 1 (at 0.85) is substantially less than those of the other treatments (group mean = 0.96).

The Sørensen index of similarity (Table S4), a measure of pairwise similarity between communities, showed on average higher amounts of similarity between horizons of the same mat-type than between communities from different mat types. While all values are relatively small, indicating low levels of similarity overall, the *Piloderma*-mat O-horizon appears to be least similar to most other communities with the exception of the *Piloderma*-mat A-horizon community.

***Illumina-based environmental signature sequencing***

High-throughput sequencing of fungal ITS amplicons on the Illumina GA1 platform yielded on average approximately 540,000 (range from 397,000 – 787,000) high-quality sequences per treatment. After clustering sequences at 95% similarity approximately 2% of sequences in each treatment were determined to be singletons or

doubletons.  Total numbers of OTUs detected per treatment range from 5593 to
18,483.  It should be noted that both *Piloderma*-mat horizons showed considerably
more OTUs than any of the other treatments.  Counts of the most abundant OTUs that
represent 90% of the data from each treatment are represented in Table S5.

Rank-abundance distributions (Figure 2.1) of the OTUs from Illumina
sequencing showed a pattern of very few highly abundant taxa with a long distribution
of rarely encountered taxa.  This pattern was less evident in both soilhorizons within
*Piloderma* mats, where the most abundant taxon only represented 4.3% and 3.5% (PO
and PA respectively) of the total library followed by a more gradual decline in
abundance for subsequent ranks.

Diversity statistics for Shannon index, Fisher's Alpha, and Simpson's index
are presented in Tables S1, S2, and S3, respectively.  Together, these statistics show a
pattern of high diversity and heterogeneity within plots, especially in *Piloderma*-mat
treatments.  Again, the *Ramaria*-mat A-horizon shows markedly less diversity when
compared to the other treatments.  Beta diversity represented by the Sørensen index
(Table S6) shows strongest similarities between horizons of the same mat-type or non-
mat soil.  Additionally, the *Piloderma*-mat A-horizon shows more similarity to non-
mat soils than most other treatments.

Rarefaction curves generated from Illumina 32bp single-end sequencing
(Figure 2.2) do not appear to be asymptotic and indicate the continued accumulation
of new OTUs with additional sequencing.  The relative positions of the non-mat O-
and A-horizons and *Ramaria*-mat O- and A-horizons appear similar to those found in

the clone library, however the positions of both *Piloderma*-mat horizons follow a significantly higher trajectory.

### 454FLX Fungal ITS sequencing

Multiplex sequencing of fungal ITS amplicons on the 454-FLX Titanium platform yielded approximately 90,000 – 150,000 high-quality sequences for each of the six treatment types (Table S7). 480 to 799 OTUs were detected per treatment when clustered at 95% sequence similarity. When only the most abundant taxa representing 90% of the total data were considered the number of OTUs ranged from 26-111.

Rank-abundance distributions of the 25 most abundant OTUs in each treatment all showed a similar pattern. All have one dominant taxon followed by a steeply sloping distribution of less abundant taxa. All exhibit long tails of rarely encountered OTUs extending beyond the 25th rank visualized in Figure 2.1.

Rarefaction curves generated from the 454-FLX amplicon data (Figure 2.3) appeared to level off and possibly reach an asymptote. This suggested that if one were to sample more deeply within a given treatment, new OTUs would be encountered less often compared to the Illumina and clone library analyses. Like the rarefaction curves from the clone library communities, the 454 amplicon data show the *Ramaria*-mat A-horizon may harbor fewer taxa.

Diversity statistics for the 454-FLX amplicon data are presented in Tables S1, S2, and S3 for Shannon, Fisher's Alpha, and Simpson indexes, respectively. These measures of alpha diversity suggested relatively similar species richness in most

treatments with the exception of the *Ramaria*-mat A-horizon, which showed relatively less richness. Of the six treatments, Non-mat O-horizon and both *Piloderma*-mat soil horizons show the greatest levels of alpha diversity. Between treatment diversity (beta diversity) again showed the highest level of pairwise similarity between horizons of the same mat-type or non-mat soil. In contrast to beta diversity measures from the clone library and Illumina sampling, average pairwise similarity between all treatments is higher (0.61) relative to clone library (0.21) and Illumina sampling (0.34) averages.

### *Taxonomic distributions of pooled samples*

Phylum-level distributions of OTUs in pooled samples from all methodologies (Figure 2.4) were predominantly Basidiomycota, which represented 56%-89% of sequences in all treatments and sampling methods. Phylum Ascomycota represented the next most frequently encountered at between 6% and 29% of all classified sequences. In all treatments and sampling methods a small percentage of reads could only be classified as "Fungi" or "Dikarya", accounting for 2.5% and 5.4% of all treatments, on average. In general, relative proportions of the total libraries showed similar distributions between the two most common phyla.

Class-level distributions (Figure S4) reflect the dominance of the Agaricomycetes in all treatments and sampling methods, representing 45-83% of classified sequences. Within the Ascomycota, the distribution of classes was more even with common representation from the Dothidiomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes, and Pezizomycetes. Considering sampling methods,

both next-generation sequencing methods detected low-levels of numerous classes not represented in the automated classification of clone sequences.

Ordinal-level distributions within fungal phylum Basidiomycota (Figure 2.5) begin to show differences in treatment type and possible systematic bias of different methodologies. Both the clone library and 454-FLX sequencing show Gomphales as the most commonly encountered Agaricomycetes order in *Ramaria* mat soils (~45-85%) while the Illumina 32bp sequencing indicates Agaricales as the most common (~65-80%) with a relatively small abundance of the Gomphales (~4-18%). Similarly, relative abundance of the Russulales in non-mat soils was high (~50-70%) in the clone library and 454-FLX sequencing but considerably diminished in the Illumina sequencing (~8-10%), again replaced by an abundance of the Agaricales (~60-68%). *Piloderma*-mat ordinal distributions provided the most agreement among methodologies. Members of the Atheliales tended to be the most common order within the Agaricomycetes in most *Piloderma*-mat samples, although Agaricales was relatively abundant as well. As with the Class-level distributions, both next-generation sequencing techniques detected many low-abundance orders that were undetected by the clone library classification.

***Taxonomic distributions of a single sample***

Like the single-end Illumina sequencing, paired-end 76bp reads compare similarly to 454-FLX sequencing of a single non-mat soil sample. Phylum-level distributions show Basidiomycota as the most commonly encountered followed by Ascomycota in both methodologies and both forward and reverse Illumina reads.

Interestingly, a higher proportion of reads in the 454-FLX library were unable to be assigned to a phylum level and were identified only as "Fungi" or "Dikarya".

Ordinal distributions of Basidiomycota (Figure S5) show remarkably similar patterns between methodologies. The most commonly found orders in both methodologies were Agaricales, Russulales, and Thelephorales. Ordinal distributions within the Ascomycota (Figure S6) are similarly congruent showing the most common orders as Chaetothyriales, Helotiales, and Pezizales.

The increased read length of the paired-end Illumina data allowed for more thorough family level comparisons when compared to the single-end 40bp Illumina reads. The distribution of Basidiomycota and Ascomycota families are presented in Figure 2.6 and Figure S7, respectively. In general, family-level taxonomic distributions were similar between methodologies for the most commonly encountered groups of both Basidiomycota and Ascomycota. The diversity of Basidiomycota families is comparable between technologies, although the relative abundance of the rare families is not. The diversity of Ascomycota families is higher within the Illumina sampling, although the higher diversity consists of many very rarely encountered groups.

**Discussion**

The majority of microbial communities sampled from natural ecosystems have shown remarkably high species diversity. As microbial ecologists have attempted to decipher the composition of these complex communities through the use of molecular techniques, the "problem" of hyperdiversity and sampling effort has grown in

importance. Hyperdiverse community assemblages have been described in arthropod communities (Shultz, 2003 and Smith *et al.,* 2005), bacterial communities (Fierer, 2006; Jones *et al.,* 2009), and fungal communities (Jumpponen, 2009; Walker, 2005). Inherent in molecular sampling of hyperdiverse communities is the likelihood of undersampling, thereby missing the rare species and underestimating the overall diversity of a community.

This study represents the application of three distinct methodologies to describe and compare the same fungal communities at varying degrees of sequencing depth and quality. These data provide a unique opportunity to compare and contrast the consistency and biases of each method in a systematic fashion. Ten years ago the most advanced method for fungal community description was a clone-and-sequence approach. While the sequence data obtained from this method is superior, in terms of read-length and quality, to next-generation sequencing platforms, the cost per sequence and labor commitment made such approaches infeasible for many researchers studying hyperdiverse communities. The advent of Roche 454 (pyrosequencing) and the Illumina sequencing platforms now allow sequencing of fungal barcodes at orders-of-magnitude more coverage and lower cost per sequence. The trade-off of next-generation sequencing platforms is shorter read lengths and higher per-base error rate relative to Sanger sequencing methods, but the increased depth of sequencing proves useful to elucidating the structure of hyperdiverse communities.

*Comparison of techniques*

Of the three methodologies used to sample the fungal communities in forest soil, the clone library and 454-FLX amplicon sequencing appeared to provide similar representations of the microbial community. In general, the Shannon and Simpson measures of diversity were congruent across all sampling methods. Although the Shannon index was markedly higher for Illumina sampling, the relationship among treatments within the Illumina sampling was consistent with the other techniques. Across all methodologies, the *Ramaria*-mat A-horizon exhibited lower diversity than other treatments. Fisher's Alpha index provided the most drastic difference between samples. While the clone library and 454-FLX measures are comparable, Fisher's Alpha for all Illumina sampling showed an order-of-magnitude increase. This difference may be attributable to the anomalous nature of our Illumina sampling, discussed below.

Rarefaction curves generated by subsampling the data at different sequencing depths suggested that our clone library sampling was not adequately deep to capture the diversity of fungi in the soils sampled. In comparison, the rarefaction curves of the 454-FLX sampling showed that, while still potentially undersampling the community, additional sampling would likely yield fewer yet-undetected OTUs. The rarefaction curves generated from the Illumina sampling contrast with those of the 454 method, showing that even as the sampling expands into the hundreds-of-thousands of reads new OTUs were still being detected at a rapid rate. It is important to note, however, that while the magnitude of the diversity differs immensely between Illumina

sampling and the other two methodologies, the relative position of the curves to one another is maintained, particularly between the *Ramaria*-mat A-horizon and the *Ramaria*-mat O-horizon, and non-mat samples.  For reasons described below (section 4.3), and through comparisons with other studies, it is reasonable to suspect the diversity estimates from the Illumina platform to be anomalously high, particularly for the *Piloderma*-mat treatments.

Rank-abundance distributions across all treatments and methodologies tended to show a Log-like distribution with few highly abundant taxa and very many rare taxa.  Four exceptions to this generalization existed.  Both *Piloderma*-mat O- and A-horizons, when sampled using the Illumina methodology, have less skewed distributions of abundant taxa.  Similarly, both non-mat O- and A-horizons exhibited a similar tendency toward more even distributions when sampled with the traditional clone-and sequence approach.  The more even distribution of taxa in the non-mat soils may be explained by the absence of a mat-former and the random effects of sampling non-mat soil.  The absence of a single dominant taxon in the Illumina sampling, although possibly biologically true, may also be due to artifacts associated with sequence filtering during post-processing or primer bias of the newly created Illumina ITS amplicon primers.

*Taxonomic analysis and potential biases*

In general, the phylum- and class-level taxonomic distributions were consistent between sampling methodologies.  As expected, the clone-library samples exhibited characteristic artifacts of undersampling, as they were unable to detect the numerous

low-abundance fungal classes found by the next-generation sequencing techniques.

Notably, in all treatments and methodologies, early-diverging fungal phyla

(Chytridiomycota and Zygomycota) and the Glomeromycota were very rarely

encountered.  While their absence may be explained by primer biases the use of

different primer sets for all methodologies yielded similar results.

Ordinal-level taxonomic distributions showed conspicuous discrepancies

within the Illumina sampling, as compared to the clone-library and 454-FLX

sampling.  Primers for Illumina sampling were designed specifically for this

experiment and had not been widely used prior to this study.  It is likely that the

Illumina ITS amplification primers, while effective *in silico* at universal targeting of

all Fungi, were less cosmopolitan in practice.  Compared to the clone library and 454-

FLX sampling, the Illumina sampling may be bias against equal amplification of

members of the Gomphales and Russulales, while showing a propensity to

disproportionately amplify Agaricales and Geoglossales.

While both next-generation sequencing techniques were able to detect

numerous rarely encountered groups, it is interesting to note the differences in

abundance of the rare taxa.  Comparing Basidiomycota family distributions of the

single soil sample paired-end Illumina data and the 454-FLX data, it can be seen that

while both detect most of the same families, the relative abundance of rare-to-common

groups differs.  In the 454-FLX sampling, the three most common families

(Russulaceae, Thelephoraceae, and Atheliaceae) represent approximately 65% of all

Basidiomycota families; while in the Illumina sampling the same three families (also

the most common) represent nearly 90% of all Basidiomycota families.  The

remaining "rare" families represented 35% of the 454-FLX library and only 10% of

the Illumina library.  This pattern may be explained by the repeated PCR

amplifications required for the Illumina library preparation.  While both the Illumina

preparation and the 454-FLX preparation involve initial amplification of the ITS

region and subsequent PCR enrichment of the final sequencing library, the 454-FLX

utilizes emulsion PCR for the second amplification while the Illumina procedure uses

traditional PCR.  It may be possible that subjecting Illumina samples to two

exponential amplifications using traditional PCR results in bias that alters the most-

abundant and least-abundant templates detected in the sample.

### *Anomalies in Illumina-based short read sampling*

As demonstrated in the diversity statistics, rarefaction analyses, and rank-

abundance distributions, the abundance data generated from the Illumina GAI

platform appeared to be outliers as compared to the clone and 454-FLX data.  There

were potentially many sources for error in the technique including issues related to

sample preparation, sequencing and quality filtering, and informatics.

In order to obtain a taxonomically informative region of the ITS rDNA

sequence the restriction enzyme MME1 was used to digest off the amplification

primers and enabled annealing of sequencing adapters directly to the hypervariable

ITS region.  One side-effect of the restriction digestion was restriction site wobble and

mild exonuclease activity.  While much of the site wobble can be alleviated during

sequence post-processing, the exonuclease activity results in numerous reads differing

in sequence but belonging to the same amplicon. Our clustering procedure cannot detect the low-level tiling of exonuclease digested amplicons and, as a result, designated many clusters, or OTUs, to a single biological sequence.

Inherent in single-end Illumina sequencing was the lack of information about read direction. As a result, for every unique amplicon representing a single biological sequence two unique 32-bp tags were generated corresponding to the 5' and 3' end of the amplified sequence. Without a complete database of all possible ITS sequences, it was impossible to pair one tag with another. Thus, at a minimum the number unique OTUs detected in our single-end Illumina sampling was twice what may be biologically true.

Finally, the per-base error rate of the Illumina GAI is known to be between 1-2%. While this study attempted to minimize the effect of mis-reads by filtering data at a stringent quality threshold (Q20) it is conceivable that some proportion of read errors received a quality score greater than Q20. Clustering reads at 95% sequence similarity, as done in this study, will correct single-base misreads in a 32bp read, reads containing two or more errors will be placed in unique clusters and counted as a new OTU. It may be advisable to discard those reads which were encountered only one or two times (singletons or doubletons) in the sequencing run, but due to the depth of sequencing we cannot rule out that any particular sequence error is generated more than twice.

The generation of additional OTUs from exonuclease activity or read errors will certainly inflate the diversity estimates that are calculated from OTU counts,

including most diversity indices and rarefaction analyses. However, if diversity is measured based on a lower-level taxonomic assignment (Order, Family, etc) as identified by the BLAST algorithm, the erroneous reads will likely be classified correctly. As a result, the diversity indices and rarefaction curves presented in this study show marked differences in diversity between the two next-generation sequencing techniques, yet the relative taxonomic distributions of many groups were considerably more congruent.

**Conclusions**

This study investigated the applicablility of next-generation sequencing techniques to describe soil fungal communities, as compared to traditional clone-and-sequence approaches. Additionally, this study presents the first use of the Illumina sequencing platform to capture Fungal ITS sequences from environmental samples. In both cases where next-generation sequencing technologies were used, the estimated diversity of fungi within the system was greatly increased relative to traditional clone-and-sequence methods. These data reinforce the assertion that fungal communities in natural systems are commonly hyperdiverse and that deeper sampling will provide a more detailed view of the total community. Lastly, it is important to note the differences and similarities between communities as determined by the different next-generation sequencing platforms. Both platforms congruently determined the major taxonomic contributors to the fungal community, however less prevelant groups appeared to be systematically missed by one or the other platform. This highlights the

need for additional studies utilizing alternative sequencing platforms and primer sets in order to identify the true biases in these techniques.

## Acknowledgements

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  fungus assemblages on oak seedlings in mixed forests in the southern
  Appalachian Mountains," *Molecular Ecology* 14, no. 3 (2005): 829–838.
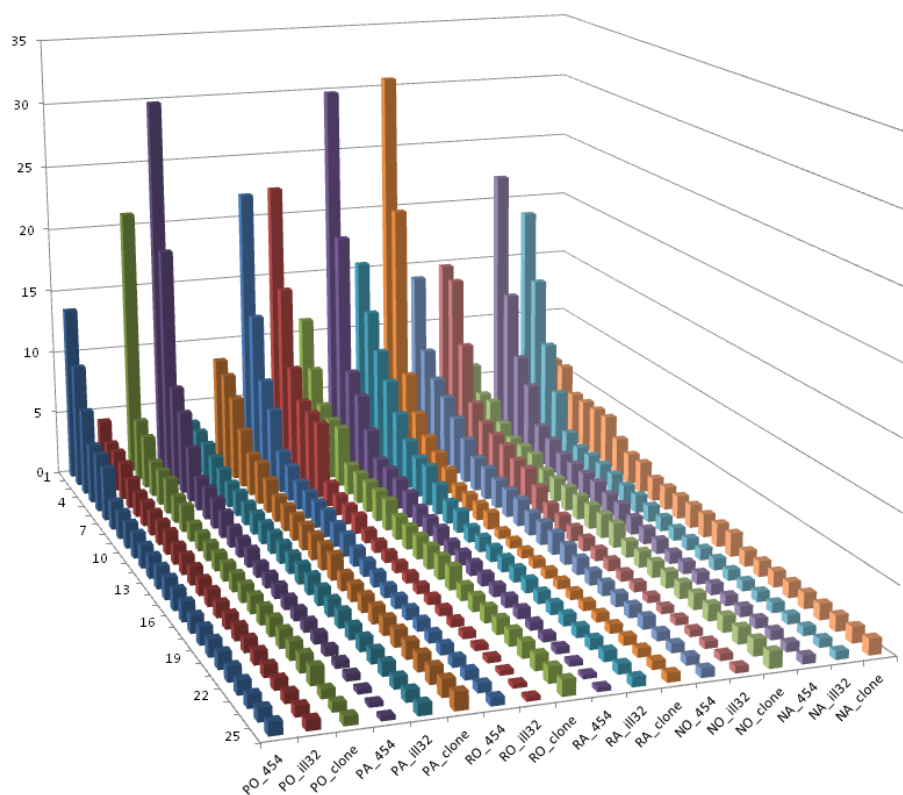


Figure 2.1 – Rank-abundance distributions of pooled samples.  Top 25 ranked-
abundances for each treatment and sampling method.  Shown as percentage of total
library (vertical axis).  Naming convention indicates treatment type (PO = *Piloderma*-
mat organic horizon, PA = *Piloderma*-mat mineral horizon, RO = *Ramaria*-mat
organic horizon, RA = *Ramaria*-mat mineral horizon, NO = Non-mat organic horizon,
NA = Non-mat mineral horizon) and sequencing method (454 = Roche/Life Sciences
454FLX-Titanium, ill32 = Illumina Genome Analyzer 32bp single-end reads, clone =
sanger sequencing of SSU-ITS and ITS-LSU clones).  Identical naming conventions
used in subsequent figures.
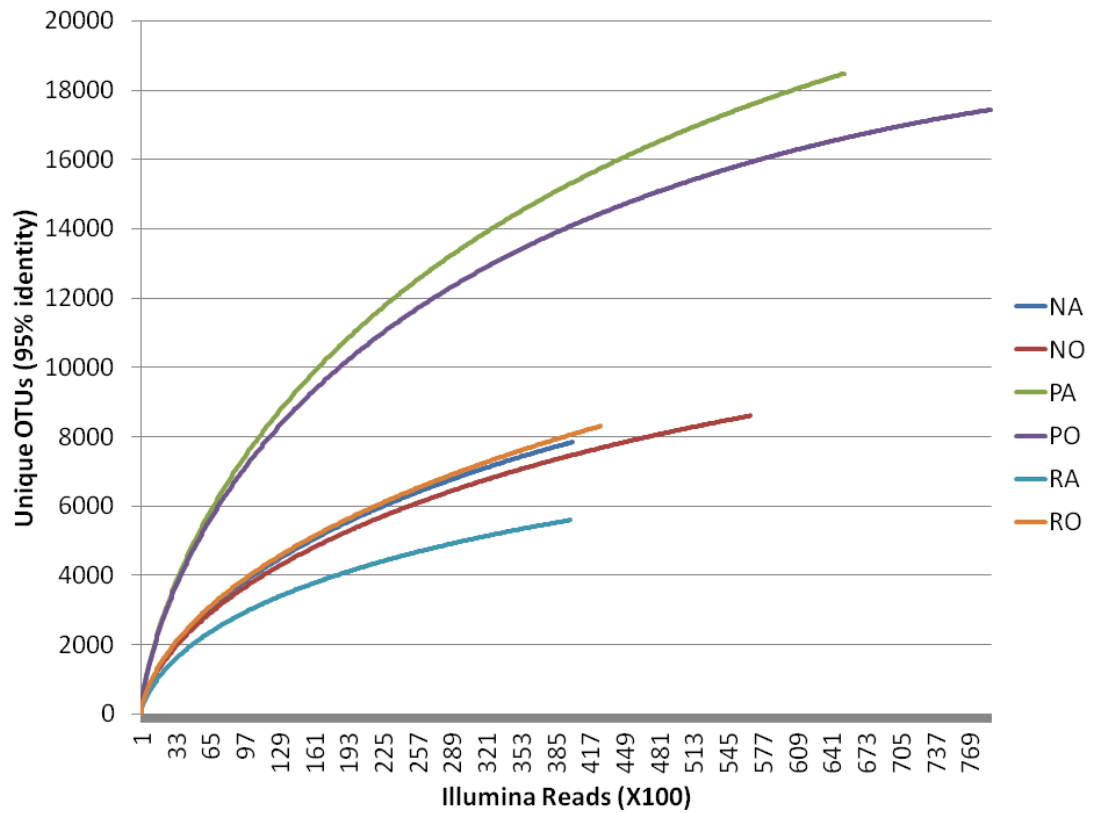
Figure 2.2 - Rarefaction curves for single-end 32bp Illumina sequencing of pooled samples. Curves represent accumlation of 95% OTUs by sampling without replacement across 500 randomizations. Horizontal axis label represents 100s of reads.
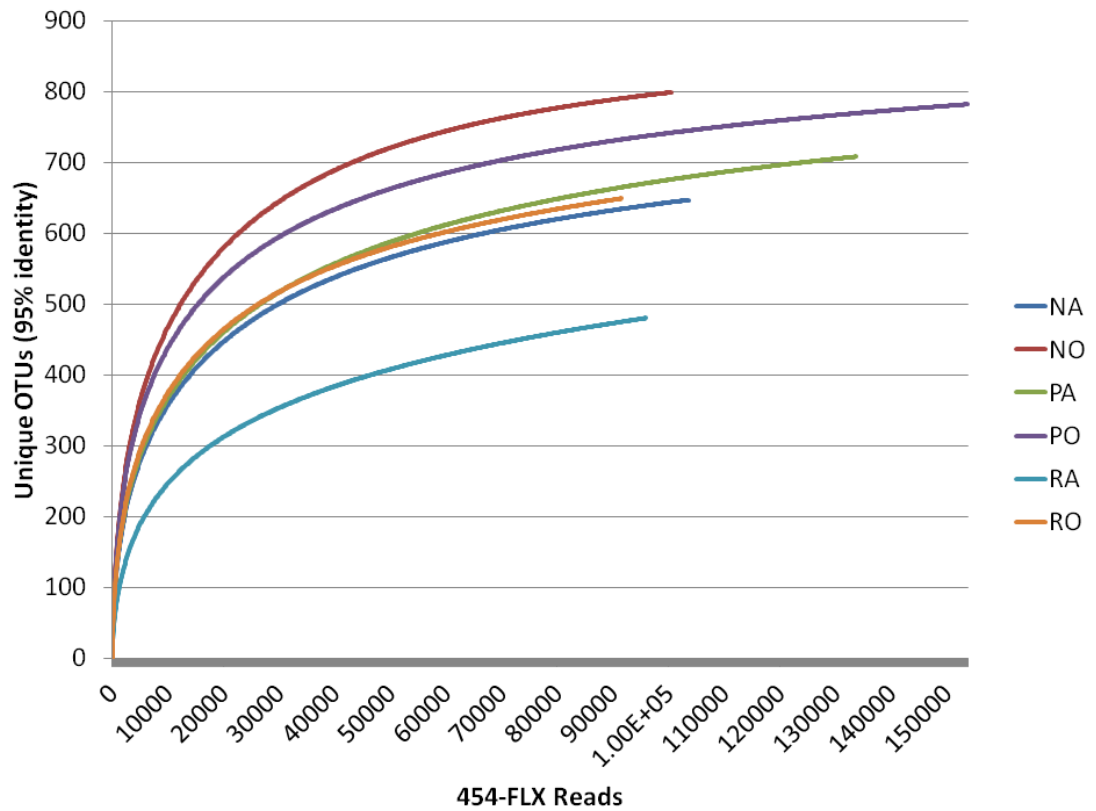
Figure 2.3 – Rarefaction curves for 454-FLX sequencing of pooled samples. Curves represent accumlation of 95% OTUs by sampling without replacement across 500 randomizations.
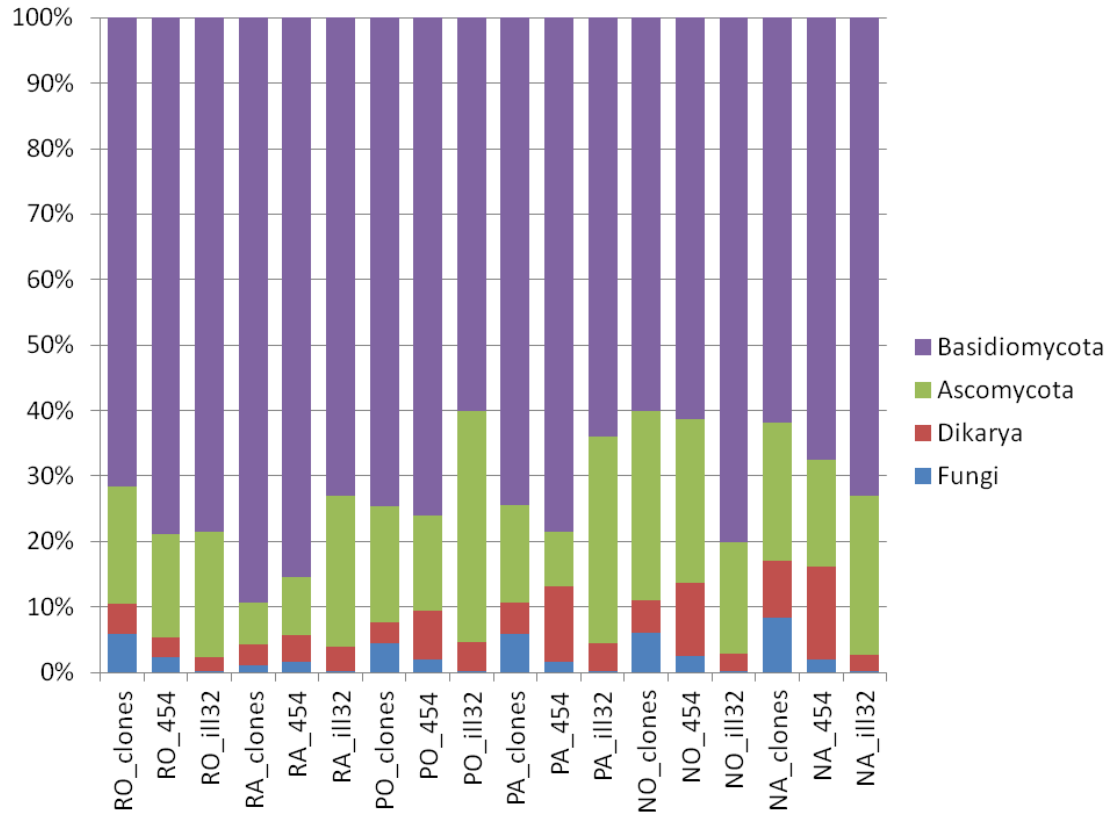
Figure 2.4 – Phylum level taxonomic distribution across all treatments and sampling methods. Vertical bar represents 100% of the seqeunces identified as Basidiomycota, Ascomycota, Dikarya, or Fungi. Zygomycota, Chytridiomycota, and Glomeromycota represended less than 1% of all libraries and were omitted from the analysis.
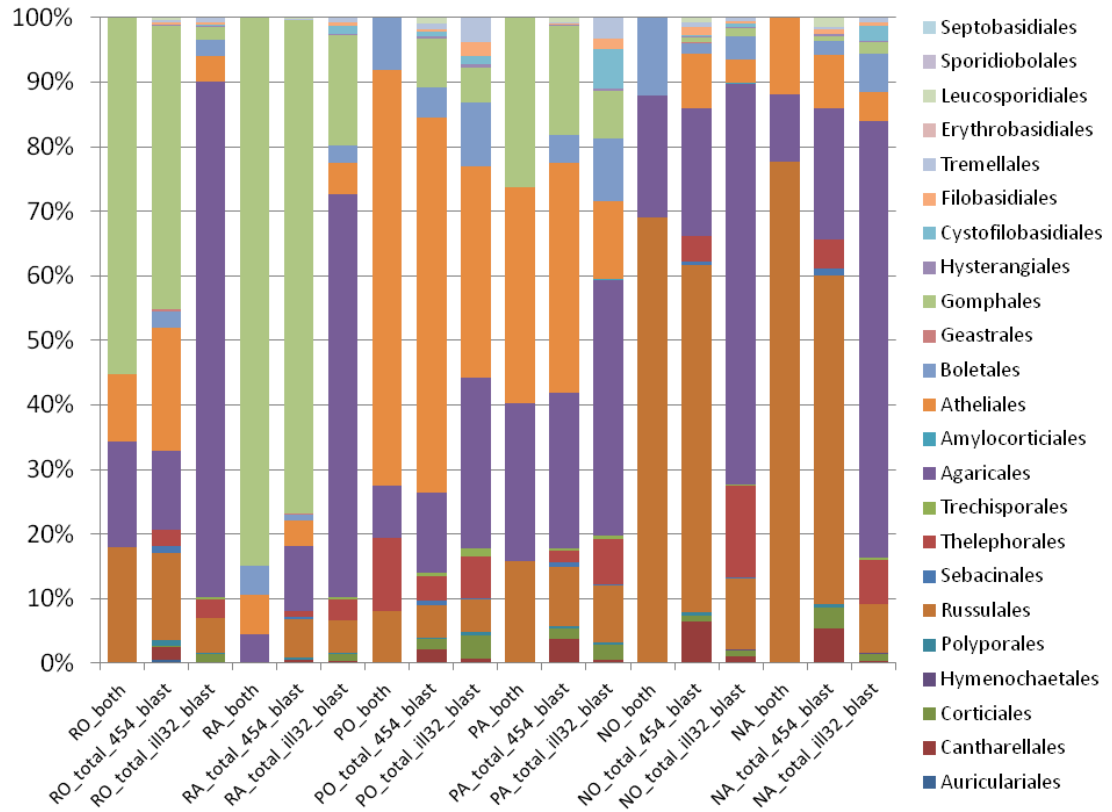
Figure 2.5 – Ordinal-level distribution within the Basidiomycota. Vertical bars represent 100% of the reads classified to at least the order level within the Basidiomycota. Suffix "_both" represents clone library data from both SSU-ITS and ITS-LSU amplicons.
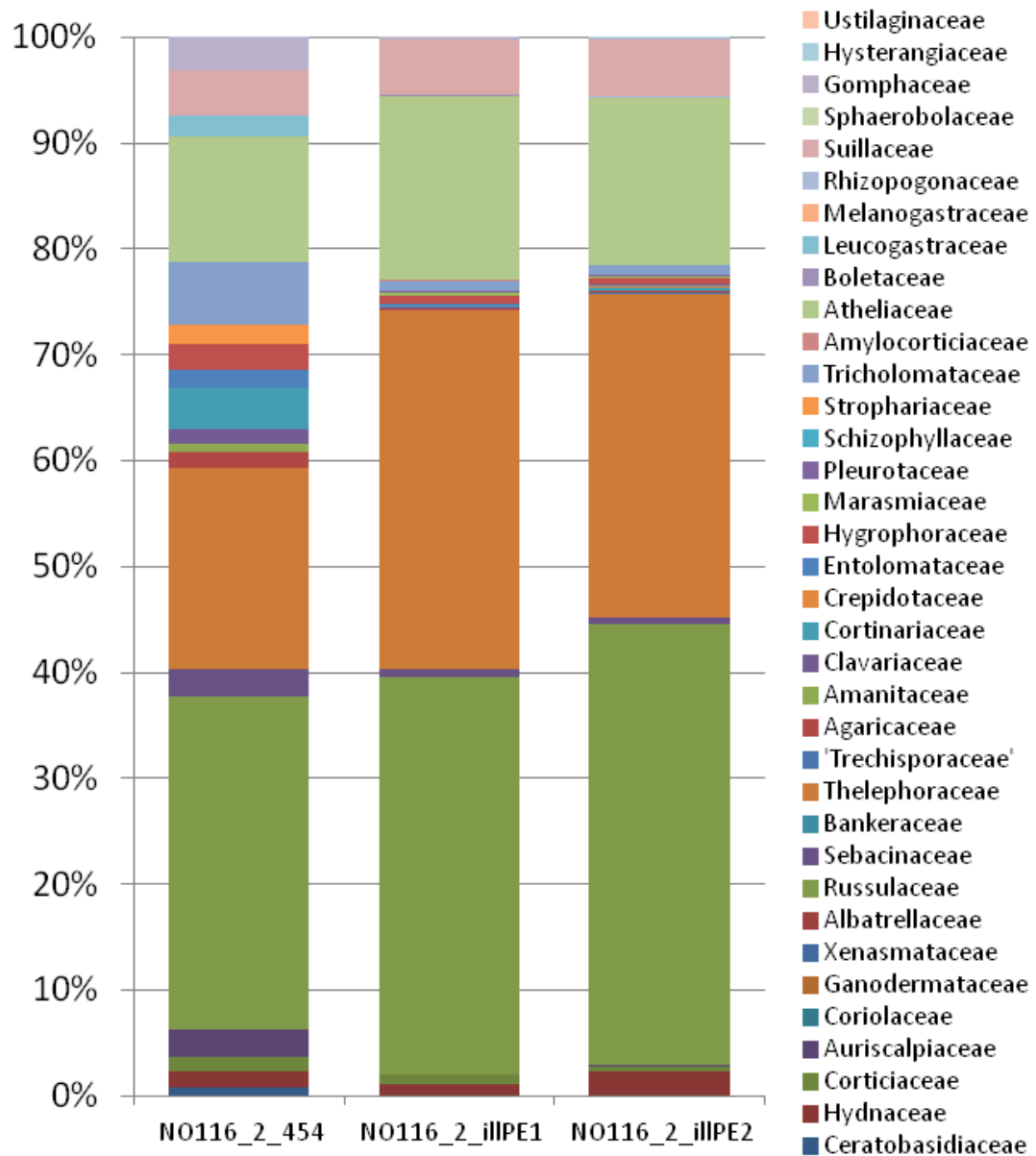
Figure 2.6 – Family-level distribution within the Basidiomycota. Vertical bars represent 100% of the reads classified to at least the Family level within the Basidiomycota for the single sample NO116-2. Suffix "_illPE1" and "_illPE2" represent the first and second read of the paired-end 76bp Illumina sampling, respectively.

# CHAPTER 3 - SOIL FUNGAL COMMUNITIES ASSOCIATED WITH THE MAT-FORMING ECTOMYCORRHIZAL GENERA *PILODERMA* AND *RAMARIA* DETERMINED BY ITS AMPLICON PYROSEQUENCING

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**Abstract**

The ectomycorrhizal (EcM) genera *Ramaria* and *Piloderma* represent the two most commonly encountered genera of mat-forming fungi in old-growth forests of the Pacific Northwest, USA.  The chemical and physical attributes of these EcM mat soils are markedly different than their neighboring non-mat EcM soils and likely represent unique habitats for soil microbes.  This study utilized a pyrosequencing approach to compare the fungal communities associated with the common EcM mat soils and non-mat soils in the HJ Andrews Experimental Forest LTER site.  Forty-eight soil cores (20 *Piloderma*-mat, 11 *Ramaria*-mat, and 17 Non-mat) were divided into organic (O) and mineral (A) horizons for a total of 96 samples.  An average of 7521 sequences across the rDNA internal transcribed spacer (ITS1) region were generated per sample and rarefaction analyses of the individual samples indicated heterogeneity in the overall species richness and differences in abundance distributions within and among mat types and horizons.  Species-accumulation curves of pooled samples supported *Piloderma* and non-mat O-horizons as the most species rich communities followed by *Piloderma* and non-mat A-horizons and *Ramaria* O-horizons; *Ramaria* A-horizons were the least diverse.  In all samples hyperdiverse communities were detected, but most samples were dominated by one-to-few taxa.  These findings demonstrated that the mat-forming taxa were highly abundant in the bulk soil of EcM mats and that *Russula* species were the dominant taxa in non-mat bulk soils.  In addition to being the primary mycorrhizal associates on root-tips, these results indicate the three phylotypes also represent significant components of bulk soil fungal biomass of their in respective

EcM niches, and that community structure was driven by the high abundance of relatively few individual phylotypes. Finally, we present evidence to support the relative exclusion of *Russula* species from the EcM mats of *Piloderma.*

**Introduction**

  Soil microbial communities have been identified as integral constituents of many natural processes including key biogeochemical processes of the carbon and nitrogen cycles. Fungi represent a major component of below-ground biomass in most natural systems in addition to bacteria, plant roots, and micro-eukaryotes. Mat-forming ectomycorrhizal (EcM) fungi represent a prevalent constituent of many temperate forest ecosystems (Hintikka and Naykki, 1967; Fisher, 1972; Cromack *et al.,* 1979) and create dramatic changes in soil structure and chemistry (Cromack *et al.,* 1979, Malajczuk and Cromack 1982, Griffiths *et al.,* 1994). Ectomycorrhizal mat soils have been shown to have higher rates of cellular respiration when compared to adjacent non-mat soils indicating EcM mats may be hot-spots of biological activity (Cromack *et al.,* 1988; Högberg and Högberg, 2002; Phillips *et al.,* 2012). While EcM mat soils also have been demonstrated to have greater microbial biomass than adjacent soils (Ingham *et al.,* 1991), the exact differences in fungal community structure have yet to be revealed. The recent Microbial Observatory 2 project in the H.J. Andrews Long Term Ecological Research (LTER) site in Western Oregon explored the chemical and biological aspects of the two dominant mat-forming EcM genera, *Ramaria* and *Piloderma*. This study presents the findings of the survey of fungal communities associated with these EcM mats.

EcM mats have been described from many temperate coniferous ecosystems throughout the northern hemisphere (Hintikka and Naykki, 1967; Fisher, 1972; Cromack *et al.,* 1979).  One of the most well documented regions of EcM mat diversity has been in the Pacific Northwest region of the United States (Cromack, 1979; Griffiths, 1996).  Coniferous forests in this region are dominated by *Psuedotsuga menziesii* (Douglas-fir) and *Tsuga heterophylla* (western hemlock) as the major overstory trees.  The diversity of mat-forming EcM lineages spans many genera within the Basidiomycota, but in the old-growth forests of the Cascade Range of the PNW two genera, *Piloderma* and *Ramaria*, are the most commonly observed (Dunham *et al.,* 2007).  These two genera form visually distinct mat morphotypes and soils associated with *Piloderma* and *Ramaria* mats differ in their physical and chemical properties.

*Piloderma* mats are typically found inhabiting the upper organic soil horizon and characterized by dense profusions of white to yellow rhizomorphs and a high degree of soil aggregation.  Mats formed by *Ramaria* species tend to grow in the upper mineral horizon or at the interface between organic and mineral horizons and typically exhibit a powdery or ashy morphology with a high density of mycorrhizal root tips and hydrophobic properties.  EcM mat soils tend to be more acidic than surrounding soils and have been documented to contain higher accumulations of oxalate (Cromack *et al.,* 1997), organic acids, and metal ions (Al, Fe, Cu, Mn, Zn), suggesting EcM mats may be sites of accelerated mineral weathering (Griffiths *et al.,* 1994). Both types of mats provide a unique microenvironment relative to adjacent non-mat soils,

particularly in the organic horizon of *Piloderma* mats and the mineral horizon of

*Ramaria* mats, and likely represent a niche habitat for soil organisms (Kluber *et al.,*

2011).

The recent advent of high-throughput molecular sampling techniques has

spurred an influx of studies in soil microbial community ecology.  Fungal

communities in soil have been described using next-generation sequencing methods

from numerous sources including the *Quercus* rhizosphere (Jumpponen *et al.,* 2010;

Jumpponen and Jones 2009), forest successional gradients (Blaalid *et al.,* 2011),

grasslands (Lekberg *et al.,* 2011), and broadleaf forests (Buée *et al.,* 2009).  To date,

all surveys of soil fungal communities indicate very high degrees of species richness,

diversity, and spatial heterogeneity.  Most studies also observe a characteristic

distribution of few very highly abundant taxa and very many rarely encountered

species in any given sample (Unterseher *et al.,* 2010).  This observation of

hyperdiverse heterogeneous community structure in many fungal systems has

prevented the application of many ecological theories (niche partitioning, priority

effect, competitive exclusion, etc.) from being applied to next-generation sequencing

surveys.

This study describes the fungal soil communities associated with individual

*Piloderma* and *Ramaria* EcM mats sampled from the H.J. Andrews Experimental

Forest LTER site.  For comparison, mat fungal soil communities were contrasted to

fungal communities from adjacent non-mat soils.  Previous work has shown that,

within ectomycorrhizal mats, the mat-forming species dominate the mycorrhizal

association with nearby tree roots (Dunham *et al.,* 2007).  It has been demonstrated, however, that the relative abundance of ectomycorrhizal taxa found on root-tips may not reflect mycelial abundance in bulk soil(Kjøller, 2006).  By investigating the fungal communities within bulk soil, while excluding mycorrhizal and rhizomorph structures, we investigated the background fungal communities associated with EcM mats and attempt to identify patterns of exclusion or facilitation within mat soils.

**Materials and Methods**

*Study area and sampling design*

Fourteen old-growth Douglas-fir (*Pseudotsuga menzensii)* plots from within the H.J. Andrews Experimental Forest LTER site, Lane County, Oregon, were sampled for this study.  To ensure we captured a high degree of EcM mat diversity we intentionally selected sites known from previous studies by Griffiths *et al.* (1991) to contain high abundances and diversity of EcM mats.  The selected sites were a subset of those old-growth sites previously sampled in Dunham *et al.* (2007), which were identified as containing both *Piloderma* and *Ramaria* EcM mat types.  For a more detailed description of the study site see Dunham *et al.* (2007).

EcM mat sampling relied on the previously described mat identities from Dunham *et al.* (2007).  Briefly, sampled in late Fall 2005, each circular study plot (900m$^2$) was systematically searched by two individuals for thirty minutes or until thirty EcM mats were located.  Mats were then randomly chosen and visually inspected for our sampling criteria (>0.5m$^2$ in area, presence of mycorrhizal root-tips and rhizomorphs) until three to five mats were selected and cored.  Subsequent

phylotyping identified representative *Piloderma* and *Ramaria* mats to be sampled for further study. Three soil cores from each representative mat were taken to a depth of approximately 10cm below the organic horizon-mineral horizon interface. Each soil core was split along the visually identified organic horizon-mineral horizon interface and the three replicate samples for each mat and horizon were pooled into a single bag. Non-mat soils were similarly collected at three positions within the plot by randomly selecting an angle from North and a distance from the center of the plot and verifying the absence of an EcM mat at the sampling location. This sampling resulted in six unique "treatments" which will herein be referred by the following treatment identifiers: RO (*Ramaria* mat organic horizon), RA (*Ramaria* mat mineral horizon), PO (*Piloderma* mat organic horizon), PA (*Piloderma* mat mineral horizon), NO (non-mat soil organic horizon), and NA (non-mat soil mineral horizon). In total, 20 *Piloderma* mats, 11 *Ramaria* mats, and 17 non-mats were sampled as part of this study.

### *454-FLX Titanium sequencing of ITS amplicons*

Soon after collection, soil samples were sieved to remove any rocks, fine roots, rhizomorphs and mycorrhizal structures, and homogenized using a mortar and pestle. Three 0.3g subsamples from each sample were subjected to DNA extraction using the BIO101 Soil DNA Extraction kit and eluted in 40μL EB following manufacturer's protocols. Ninety-six DNA extractions representing each of the two soil horizons of the 48 mat and non-mat samples were amplified for the fungal internal transcribed spacer (ITS) region of the ribosomal subunit using custom primers. Amplification

primers were designed according to the guidelines for amplicon sequencing on the 454-FLX Titanium platform. The forward primer contained the 454-FLX Fusion A sequence, an 8-bp error-correcting multiplex ID sequence (Hamaday, 2008) and the universal fungal primer ITS1F. The reverse primer contained the 454-FLX Fusion B sequence and the universal fungal primer ITS4.

All samples were amplified simultaneously in a 96-well microtiter plate using one of the twenty-four unique barcoded forward primers and the universal reverse primer. High fidelity polymerase (Phusion HF, New England Bio) was used to reduce the frequency of nucleotide substitution errors. PCR conditions began with an initial denaturing step of 2 minutes at 92C followed by 25 cycles of 92C for 10 seconds, 50C for 30 seconds, and 72C for 40 seconds. A final extension for 5 minutes at 72C concluded the amplification program.

All 96 amplified samples were individually purified using Qiagen PCR purification columns with a final elution volume equal to that of the initial PCR reaction (30ul). Template concentrations of individual samples were quantified using the Qubit fluorometer and the Qubit BR reagent kit (Invitrogen). Four groups of twenty-four samples, each with unique multiplex ID tags, were pooled in equimolar concentrations and sent to the Duke Institute for Genome Sciences and Policy at Duke University for sequencing. Raw sequencing files available through the NCBI Short Read Archive (SRA051397).

*Data curation*

The four multiplexed samples were sequenced in a single run on a four-region picotiter plate on the 454-FLX Titanium sequencing platform. Sequences were computationally de-multiplexed, trimmed for quality and primer sequences, and binned into treatment type using the MOTHUR software package (Schloss *et al.,* 2009).

*OTU-based clustering* - Sequences were clustered into 95% groups defining operational taxonomic units (OTUs) as recommended by Jumpponen and Jones (2009) and Jumponnen *et al.* (2010) using OTUpipe in the USEARCH package and custom Perl scripts. For these analyses only OTUs consisting of 4 or more sequences in the entire library were considered. Putative taxonomic identities were placed on OTUs by BLASTing against the Fungal ITS sequence database and parsing the output in the program MEGAN (min support = 5).

*Phylotype clustering* – All sequences in a given sample were classified to the Genus-level, where possible, using the software MEGAN to interpret BLAST comparisons to the Fungal ITS sequence database. Individual reads were assigned to a taxonomic level within the NCBI taxonomy tree (minimum support = 5). Tips were collapsed to the Genus level and normalized to 100,000 reads per treatment for comparison. A final abundance matrix was exported from MEGAN, which contained normalized counts for assignments to Genus and below.

*Statistical Analysis*

*Rarefaction analyses* – Rarefaction analyses were conducted for each dataset using the VEGAN package (Oksanen *et al.,* 2011) and a custom modification of the "Rarefaction.R" script from (www.jennajacobs.org/R/rarefaction.html) in the statistical package R (R Development Core Team, 2010) with 500 randomizations. The data matrices were created in Microsoft Access 2007 prior to analyses. Rarefaction curves were visualized using Microsoft Excel 2007.

*Community ordination analyses* - Non-metric multidimensional scaling (NMS) ordination methods (Kruskal and Wish, 1978) were used throughout these analyses to visualize differences in communities as these data are not assumed to be linear nor normally distributed. The data were formatted in Microsoft Access prior to importing to the PCORD 6.05 program (McCune and Mefford, 2009). NMS techniques were chosen to avoid the "zero truncation problem" due to a high proportion of the species matrix containing zeros. As recommended for molecular sampling studies, all ordinations were calculated using Sørensen distances allowing for a fixed maximum value for sample units containing no shared species. All NMS ordinations were done in autopilot mode on the "slow and thorough" setting. Visual inspection of the ordination plots and overlay of environmental variables and primary matrix abundances were used to identify patterns in the dataset.

The data matrix was manipulated in multiple ways to achieve the most appropriate comparisons of community composition. Analyses were conducted using the full dataset, the dataset trimmed of mat-forming fungi (those OTUs identified as *Piloderma* or *Ramaria*), and the dataset trimmed of mat-forming fungi and rarely

encountered taxa (OTUs that were detected in fewer than 5% of the plots). In all cases

after the removal of taxa from the species matrix the sample units were relativized to

one (sum to one in the row) to account for any differences in sequencing effort

between samples.

A secondary environmental matrix was constructed containing categorical

variables to identify mat-type, horizon, site, and aspect and continuous variables for

soil pH and elevation. This secondary matrix was used to identify groupings in

subsequent PerMANOVA tests and to determine if any environmental variables

correlated strongly with NMS ordination axes.

*Permutation-based multivariate analysis of variance -* PerMANOVA analyses

were conducted to determine the amount of variation between communities that could

be explained by the categories of the environmental matrix. PerMANOVA requires

balanced groups for comparisons so the primary matrix was randomly sampled to

create balanced datasets for each analysis using the "stratified sampling" method. The

random sampling was done via batch operation in PC-ORD and iterated 20 times for

each analysis. Published statistics represent averages of the 20 independent sub-

samplings of the dataset.

**Results**

*Sequencing output*

After quality filtering, multiplex binning, forward primer removal and

detection of ITS2 priming site a total of 714,530 sequences were obtained from the

454-FLX Titanium run. Of the 96 multiplexed samples one sample (RA13_2) did not

obtain any reads and was omitted from subsequent analyses. The average number of reads for the remaining samples was 7521, with the highest number for any one sample at 22,267 and the lowest at 2687.

These data were interpreted using two common approaches for molecular amplicon data: OTU-based and phylotype-based. OTU-based analyses relied on groups of sequences clustered at 95% similarity across the ITS1 region. This clustering level results in OTUs roughly equivalent to phylogenetic "species" and likely therefore represents a more accurate estimation of true species richness. Phylotype-based analyses, in contrast, group sequences based on shared similarity to a known sequence. In this study phylotype identifications were constrained to the Genus level in an effort to minimize the errors introduced from using an incomplete database for comparison.

### *OTU community statistics*

All sequences clustered at 95% identity across the ITS1 region yielded 1298 unique OTUs (data matrix available at http://spatillumina.cgrb.oregonstate.edu). A summary of the average richness, evenness, and diversity for each treatment type is presented in Table 3.1. Average skewness and kurtosis of each treatment is also given in Table 3.1. The skewness metric measures the asymmetry of a distribution and kurtosis describes the "peakness" of the distribution. All treatments appear to be very similar in all metrics with the exception of the *Ramaria*-mat A-horizons, which on average have lower species richness, evenness, and diversity than the other five

treatments.  Skewness and kurtosis are higher in *Ramaria* mat A-horizons relative to the other treatments.

### *Rarefaction curves*

Sampling effort curves, as depicted by the accumulation of unique OTUs with increasing numbers of reads (Figure 3.1), indicate heterogeneity in the overall species richness and differences in abundance distributions between samples.  The ability to saturate sequencing, such that previously undetected OTUs are rarely encountered, appears to be uncorrelated with mat-type or horizon.  In most samples, rarefaction curves indicate undersampling of the community, but many appear to be beginning to approach saturation. The five most species rich and subsequently undersampled samples are all non-mat soils from the O-horizon.  By comparison, rarefaction curves generated from data pooled by treatment (Figure 3.2) show higher overall richness in pooled samples but with a greater degree of saturation.  The *Ramaria*-mat A-horizon pooled sample shows less overall richness than the other treatments, while the non-mat O-horizon is the most species rich.

### *Phylotype taxonomic distributions*

Genus-and-below distributions of reads are depicted for each sample in Figure 3.3.  The three most commonly encountered genera, *Russula*, *Piloderma*, and *Ramaria*, are colored red, green, and blue, respectively.  An unknown fungal group classified only as "Basidiomycota" in the MEGAN classification was the fourth most commonly encountered group and is colored in yellow in Figure 3.3.  The 5th-8th next most common groups are highlighted in pastel shades of blue, pink, green, and purple

representing *Byssocorticium*, *Inocybe*, *Cenococcum*, and *Hygrophorus*, respectively. All other groupings are depicted in grayscale and represent less commonly encountered groups.

Visual inspection of the phylotype taxonomic distributions show that, in most samples one or two taxonomic groups represent most of the total abundance, however, there is considerable variation in the co-dominant taxa between samples. In non-mat soils *Russula* is frequently one of the dominant taxa, while *Piloderma* and *Ramaria* are common in *Piloderma*-mat and *Ramaria*-mat soils, respectively. Co-dominant taxa include many ectomycorrhizal groups including *Byssocorticium*, *Inocybe*, *Cenococcum*, *Hygrophorus*, *Tricholoma, Hygrocybe*, *Hydnum*, and *Gautiaria*. The unknown group classified as "Basidiomycota" commonly represents greater than 10% of total abundance in a given sample and is detected in all samples.

The mat-identifications used in this study were determined by direct sequencing of previously sampled root-tips and rhizomorphs. Although this study attempted to sample only mats identified as *Piloderma* or *Ramaria* and avoid mixed mats containing both *Piloderma* and *Ramaria*, our analysis of bulk soil phylotypes indicates the possibility for misidentified and mischaracterized mats. Higher abundances of *Ramaria* than *Piloderma* are found in *Piloderma* mat samples 41_1 and 41_3, suggesting the possible misidentification of the mat from root-tip/rhizomorph sequencing. Similarly, *Ramaria* mats 29_2 and 37_2 show a very low to absent concentration of *Ramaria*, but instead harbor an abundance of *Piloderma* and *Gautieria*, respectively. *Gautieria* is a genus of basidiomycetous false-truffles that

forms morphologically similar ECM mats as *Ramaria* (Griffiths *et al.,* 1991).

Phylogenetically, it is nested within *Ramaria*, representing one of the many origins of

truffles among fleshy fungi (Humpert *et al.,* 2001) and is considered congeneric with

*Ramaria* in these analyses.  Additionally, *Piloderma*-mats 147_1 and 116_1 appear to

be mixed mats, as they both have a high abundance of *Piloderma* in the O-horizon but

a high abundance of *Ramaria* or *Hygrocybe* in the A-horizon (samples 147_1 and

116_1, respectively).  Mixed mats have been observed in other studies (Kluber *et al.,*

2010), and all samples flagged as misidentified or mixed species mats were excluded

from ordination and PerMANOVA analyses.

Unlike the mat samples, non-mat samples were randomly sampled without any

*a priori* knowledge of the fungal community.  Even with the random sampling,

*Russula* appears as one of the dominant groups in most non-mat samples.  In few non-

mat samples *Russula* was not the dominant genus.  These samples were instead rich in

*Inocybe*, *Hygrophorus*, *Cenococcum*, *Hydnum*, and *Tricholoma* among others.

### *NMS ordinations*

Non-metric multidimensional scaling ordination of the Genera phylotype

matrix, relativized by plot, yielded a three-dimensional solution (final stress = 14.11,

final instability < 0.00).  The first two axes of the solution are presented in Figure 3.4,

with vectors denoting continuous variables with significant correlations with the first

two axes ($r^2 > 0.200$).  Samples, coded by mat-type, are oriented in 'genus-space' in a

manner such that variance is minimized.  Groupings by mat-type are strong with

minimal overlap of mat-types and non-mat samples.  Significant correlations with the

main matrix are the three most-commonly encountered OTUs in the library,

corresponding to the genera *Russula, Piloderma,* and *Ramaria*. Segregation of mat-

types correlates with the species vectors suggesting that *Piloderma* mats are

predominantly characterized by an abundance of *Piloderma*, *Ramaria* mats are

characterized by *Ramaria*, and non-mat samples are characterized by *Russula*. pH is

the single environmental character strongly associated with any of the ordination axes

at the 0.2 $r^2$ cutoff. The biplot vector for pH indicates more basic soils (higher pH) are

correlated with ordination axis 1 and subsequently correlated with non-mat samples

and the presence of *Russula*. Measures of soil pH ranged from 4.53 to 6.34.

In an effort to compare fungal communities without consideration of the mat-

forming individual, all OTUs with taxonomic identifications of *Ramaria* or *Piloderma*

were removed from the dataset and new NMS ordinations were created. The first two

axes of the three dimensional solution are shown in Figure 3.5 (final stress = 16.94,

final instability < 0.000). In contrast to the ordination with the mat formers included,

considerable overlap exists between mat-types and non-mat samples when the mat-

forming groups are excluded. Vectors associated with the ordination axes are *Russula*

positively correlated with axis 1, the unknown "Basidiomycota" group, *Lecythophora*,

and *Umbelopsis* positively correlated with axis 2, and *Hygrophorus* negatively

correlated with axis 2. Additionally, *Tricholoma* was positively correlated with the

third ordination axis (not shown).

***Permutation-based multivariate analysis of variance***

PerMANOVA tests were used to determine the amount of variation in the phylotype dataset that could be explained by the categorical groupings of samples by horizon, mat-type, or an interaction between horizon and mat-type. When the entire dataset was considered, including the mat-formers, the grouping of mat-type or non-mat soils explained on average 39% of the variation within the dataset (p=0.0002). After the primary mat-forming taxa were removed from the dataset only 7% of the variance was explained (p=0.0004). Regardless of mat-type, soil horizon explained approximately 2.1% to 2.6% of the variation (p=0.0728 and p=0.0168) when including the mat-former or excluding the mat-former, respectively. Including or excluding the mat-forming taxa in either dataset, exhibited a non-significant interaction term between soil-horizon and mat-type (p=0.89 and p=0.95, respectively).

**Discussion**

This study utilizes the power of pyrosequencing to compare the fungal community structure of unique microenvironments created by two common mat-forming EcM taxa, *Piloderma* and *Ramaria*, with that of neighboring non-mat soils in old-growth forests of the H.J. Andrews Experimental Forest. Results of this study reveal that, like many previous studies of soil fungal communities, EcM mat and non-mat soils harbor hyperdiverse assemblages of taxa and exhibit very high degrees of spatial heterogeneity. While technically quantifying the relative abundance of rDNA copies within a sample, these data are used as a proxy for relative abundance of fungal biomass in the soil cores. As such, our sampling indicates that in addition to dominating the mycorrhizal association on root-tips, the overall fungal biomass found

within the bulk soils of *Piloderma* and *Ramaria* mats is commonly the mat-forming

genus.  Additionally, when non-mat soils are randomly sampled within the study area,

members of the EcM genus *Russula* are commonly found to be the most abundant

constituent of the fungal community.  Taken together, the three EcM genera,

*Piloderma, Ramaria,* and *Russula,* constitute a large proportion of the fungal

community in soils of the old-growth forests of the H.J. Andrews.  Finally, the unique

physical and chemical properties of the mineral horizon in *Ramaria* mat soils correlate

with a lower overall richness of fungal species relative to non-mat and *Piloderma* mat

soils.  In contrast, *Piloderma* mat soils have similar levels of species richness relative

to their non-mat counterparts, yet are qualitatively different in their taxonomic

distribution as they tend to harbor proportionally less *Russula* species.

### *Species diversity and richness*

The OTU-based analyses used in this study provide a minimum estimate for

fungal diversity in old-growth forest soils within the H.J. Andrews Experimental

Forest.  While certainly not capturing all unique habitat niches, the sampling

conducted for this work represents one of the most complete survey of below-ground

fungal diversity in the region.  This study detected nearly 1300 unique 95% OTUs,

which were found at least 4 times in our pyrosequencing library.  As the highest

richness detected in any pooled treatment was 799 (Non-mat O-horizon, Figure 3.2),

the sampling of unique EcM mat niches increased the overall diversity in the system

by nearly two-thirds.

Average species richness, diversity, and evenness were higher in the organic horizon of both mat and non-mat soils (Table 3.1). Average richness, diversity, and evenness was less in the mineral horizon for all treatments, but more so in both mat soils. The higher degrees of skew and kurtosis in the *Ramaria* mat A-horizon relative to the other treatments suggest that the difference between the most abundant taxa and the very rare taxa (rank abundance distribution) is greater in the RA treatment. While the methods used in this study cannot determine differences in overall biomass between samples, this observation suggests that in *Ramaria* mats the mat-former represents a higher proportion of the total fungal community, relative to *Piloderma* mats and non-mat soils.

Rarefaction curves (Figure 3.1) derived from these data indicate undersampling is common in many samples. Considering that the mat-forming taxa can represent up to 80% of the reads for any sample, it may be less surprising that even with 7500 reads per sample, the tail of the species distribution is not adequately sampled. It should be noted that even in non-mat samples, the communities were dominated by one or few highly abundant taxa, similar to mat communities. Thus, the relatively random distribution of rarefaction curve trajectories between treatments reflects the heterogeneity in rank-abundance distributions regardless of treatment.

Pooled rarefaction curves (Figure 3.2) do not appear to be merely additive accumulations of single curves suggesting some proportion OTUs are shared among samples (beta-diversity), and that with the increasing sequencing depth sampling is nearing saturation. Differences in richness by treatment type can be detected at 95%

confidence intervals (Figure 3.2), indicating non-mat and *Piloderma* mat organic

horizons are more species rich than their mineral horizons and *Ramaria* mat organic

horizons.  Additionally, the pooled *Ramaria* mat mineral horizon harbors significantly

less species than all other treatments.  These findings are consistent with the

hypothesis that the microenvironments of *Ramaria* mat soils are characterized by

lower species diversity than its neighboring soils.  Whether this reduction in species

diversity is due to unique chemical and physical properties (e.g., pH, hydrophobicity)

or broad-scale competitive exclusion by *Ramaria*, however, is not discernable by these

analyses.

### *Taxonomic distributions*

Phylotype analyses of pyrosequencing data reveal that the bulk soil of

*Pildoderma* and *Ramaria* mats are commonly dominated by the mat-forming species.

In contrast to results found by Kjøller (2006), the root-tip mycorrhizal community

within *Piloderma* and *Ramaria* mats does generally reflect the relative abundance of

those species in adjacent bulk soil.  Although our sampling was designed to target only

previously identified *Piloderma* and *Ramaria* mats our results suggest that in few

cases, either the bulk soil fungal community within a mat is not dominated by the mat

forming individual or, perhaps more likely, mats were misclassified from the previous

root-tip and rhizomorph sequencing study (Dunham *et al.,* 2007).

Non-mat samples, though randomly selected throughout the sampling plot

without *a priori* knowledge of the fungal community, show the prevalence of *Russula*

species in bulk soil.  The relative abundance of *Russula* in coniferous forest systems

has been documented in various EcM root-tip studies (Avis, 2003), but the distribution

of *Russula* hyphae in bulk soil is not well documented.  Interestingly, the high

abundance of *Russula* in bulk soil detected in this study contradicts the "contact

exploration type" habit of *Russula* extramatrical hyphae proposed by Agerer (2001),

which presumes the physiology of *Russula* species to have limited emanating hyphae

that do not extend far from the mycorrhizal structure.  The argument may be made that

the abundance of *Russula* in non-mat samples could be attributed to a high abundance

of basidiospores rather than active mycelia.  While spores are likely detected in our

samples due to the depth of sequencing, the low abundance of other prolific spore

producers (*Rhizopogon*, for example (Kjoller and Bruns, 2003; Bruns *et al.,* 2009)) is

consistent with the majority of sequences being derived from hyphae.  Based on this

study, it should be considered that *Russula* may possess a more broad exploration

strategy than previously thought.

Phylotype distributions across all samples contain relatively few sequence

types of unknown fungal diversity.  Interestingly, of the fourteen phylotype groups

found in 90% of all samples, nine were classified at lower than the genus level

(including the "Basidiomycota" group, yellow bar in Figure 3.3).  While the failure to

classify a sequence to the genus level may be due to inadequate taxonomic resolution

within the ITS1 region, it may also be indicative of undescribed diversity not

represented in the Fungal ITS database.  The recent discoveries of two new class-level

groups of fungi, the Cryptomycota (Jones, et al. 2011a, Jones *et al.,* 2011b) and the

Archaeorhizomycetes (Rosling *et al.,* 2011), highlight the potential for yet-

undiscovered diversity in Fungi.  The phylotypes assigned to groups other than Genus

in this study certainly warrant future investigation.

### *Differences in EcM mat communities*

NMS ordinations show structuring of the dataset highly correlated with mat-

type, a pattern verified statistically using PerMANOVA.  When the phylotype dataset

with anomalous samples removed is considered, the groupings by mat-type are

considerably tight (Figure 3.4) showing much of the similarity between samples of the

same treatment is due to the presence of the mat-forming taxon.  When the mat-

forming taxa are removed from the analysis, thereby allowing a comparison of the

"background" communities, the groupings by mat-type become more dispersed

(Figure 3.5), although non-mat samples retain in a relatively tight grouping.  The

overlap of samples from *Ramaria* mats with all other samples indicates that *Ramaria*

mat-type is not a strong predictor of the underlying fungal community once the mat-

forming genera are removed.  *Piloderma* mat samples, while more dispersed than in

the ordination including the mat-former, tend to be negatively correlated with Axis 1

(Figure 3.5) and form a relatively independent group from non-mat samples.

Correlations with the three axes of the NMS ordinations from the main matrix

and secondary matrix show a handful of species are strongly associated with the

ordination axis, and that pH is also a predictor of community composition.  When the

mat-formers have been removed from the dataset, samples are oriented along Axis 1

with increasing abundance of *Russula* and positively correlated with non-mat samples.

Taken together, Axis 1 (Figure 3.5) suggests that not only do non-mat samples harbor

higher proportions of *Russula*, but also that background mat-communities, specifically within *Piloderma* mats, harbor less *Russula,* on average, than the surrounding soil. While these data are not conclusive, these findings would support a hypothesis that *Piloderma* mats may be acting to competitively exclude *Russula,* one of the most ubiquitous ectomycorrhizal genera in coniferous forests.

**Conclusion**

Our analyses indicate fungal community structure does differ between non-mat soils and the soils of *Piloderma* and *Ramaria* EcM mats, though the mat-communities are modified in contrasting ways. The mineral horizon of *Ramaria* mat soils tend to harbor fewer species than non-mat or *Piloderma* mat soils, however, once the mat-forming taxon is removed from the analysis, the background fungal community within *Ramaria* soils appears quite similar to non-mat soil fungal communities. In contrast, *Piloderma* mat soils harbor a statistically similar abundance of fungal species to non-mat soils, yet once the mat-former is removed from the analysis, the back-ground fungal community appears distinct to non-mat soil communities, largely due to the lower abundance of *Russula* species in the *Piloderma* mat soils.

These data reinforce the findings of hyperdiversity and heterogeneity that are common in studies of soil fungal communities. While nearly every sample in this study harbored over one hundred 95% OTUs, the majority of samples were dominated by one to few taxa. Though technically "hyperdiverse" (i.e., high species richness) the functional or active diversity of these samples may be simpler if restricted to the most abundant taxa. Ribosomal DNA sampling, as conducted in this study, measures, in the

strictest sense, relative rDNA copy abundance among taxa in a given sample. Most studies dismiss rDNA cassette copy number as an unavoidable source of bias and treat rDNA sequence abundance as a proxy for biomass. That is, fungi that are more frequently encountered are more abundant and represent a greater proportion of the biomass in a given environmental sample. What is unknown however, is whether the source of rDNA from a total soil DNA extraction is from biologically active mycelia or from biological inactive material such as resting structures (eg. spores, conidia, sclerotia) or aged non-functional mycelium. The abundance distributions observed in this study, and many others, may indicate that although hyperdiverse, the biologically active or functional component of a fungal community may be simpler, i.e., fewer taxa, than depicted by total species diversity as detected by ITS rDNA environmental sampling techniques.

**Acknowledgements**

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|  | Richness | Evenness | Shannons Diversity | Simpson diversity (1-D) | % empty cells | skew | kurtosis |
|---|---|---|---|---|---|---|---|
| NA | 118.4 | 0.474 | 2.269 | 0.7396 | 90.86 | 25.554 | 729.092 |
| NO | 158.9 | 0.536 | 2.724 | 0.8209 | 87.741 | 23.182 | 615.92 |
| PA | 103.2 | 0.48 | 2.203 | 0.7351 | 92.041 | 26.656 | 798.971 |
| PO | 126.9 | 0.539 | 2.583 | 0.8015 | 90.204 | 25.797 | 757.661 |
| RA | 100.6 | 0.351 | 1.638 | 0.5473 | 92.238 | 31.352 | 1053.889 |
| RO | 127.9 | 0.521 | 2.512 | 0.783 | 90.13 | 26.398 | 789.138 |

Table 3.1 - OTU-based Community Statistics.  NA = Non-mat A-horizon, NO = Non-mat O-horizon, PA = *Piloderma* mat A-horizon, PO = *Piloderma* mat O-horizon, RA = *Ramaria* mat A-horizon, RO = *Ramaria* mat O-horizon.  Values represent averages from all samples from similar treatments.  RA highlighted showing lowest levels of richness, evenness, and diversity.
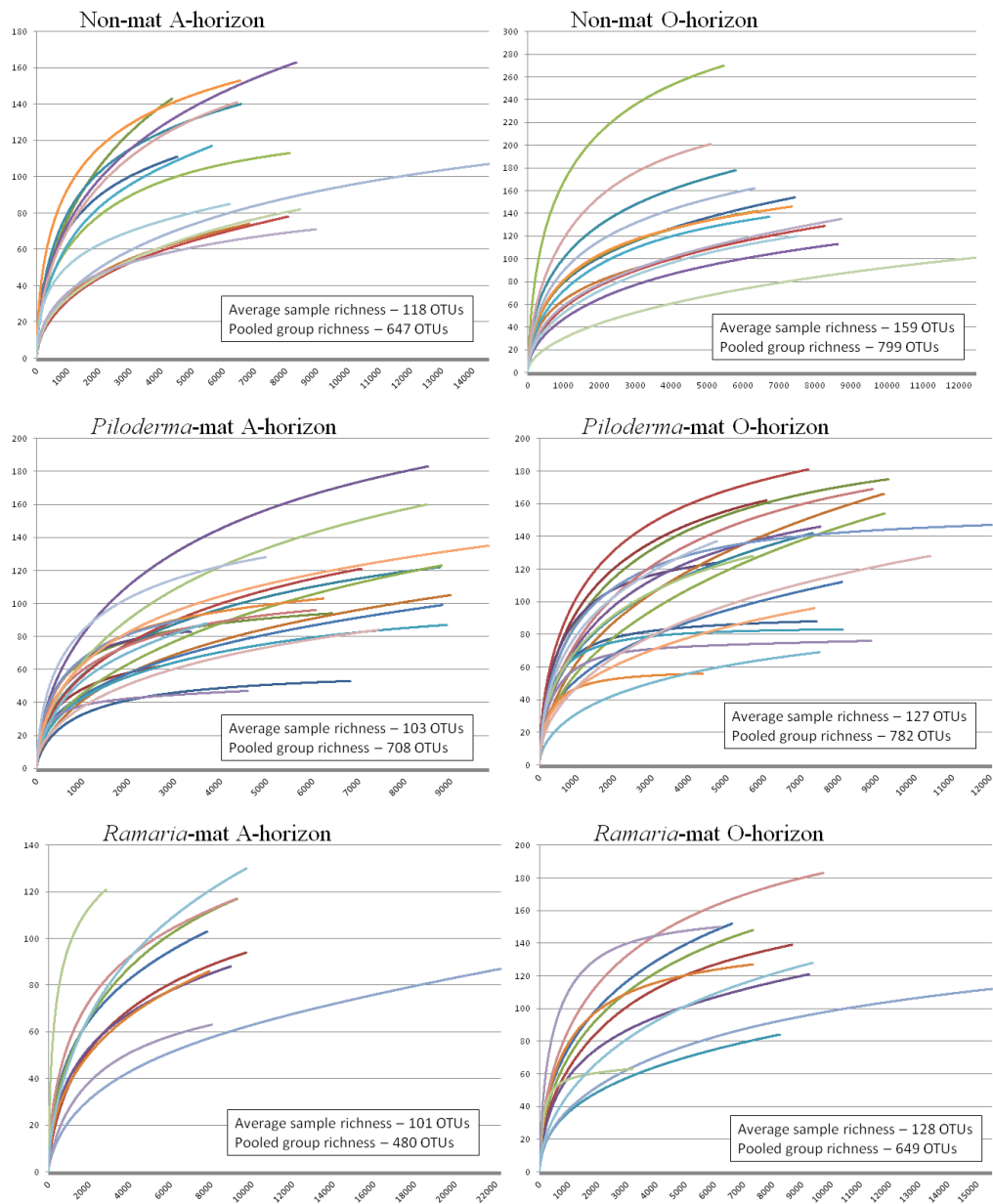
Figure 3.1 – Rarefaction curves for individual samples.  Curves represent accumulation of 95% OTUs by sampling without replacement across 500 randomizations.
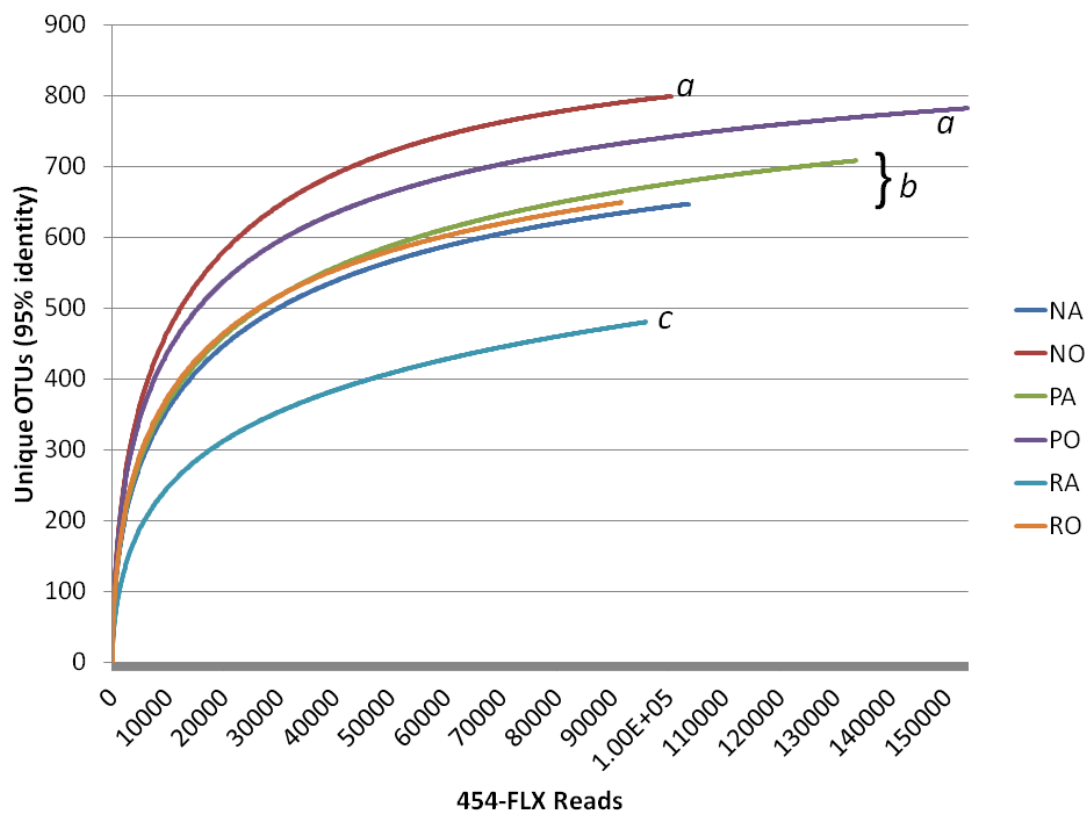
Figure 3.2 – Rarefaction curves for pooled samples.  Curves represent accumulation of 95% OTUs by sampling without replacement across 500 randomizations.  **a**, **b**, and **c** represent groups with overlapping 95% confidence intervals for the Mao Tao richness measure.
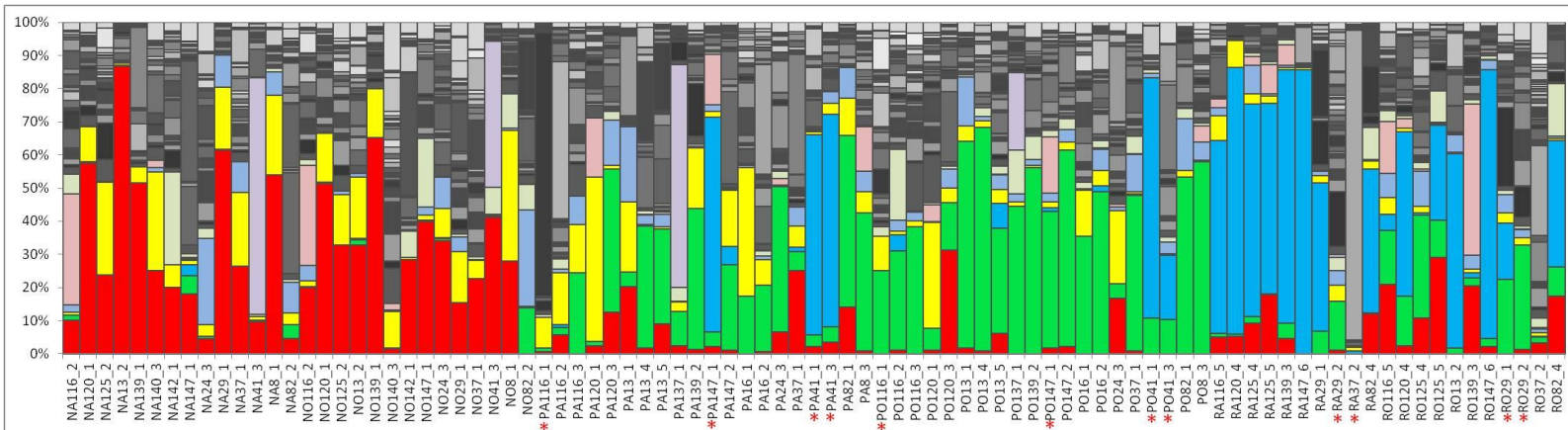
Figure 3.3 – Phylotype taxonomic assignments by sample. Vertical bars represent 100% of sequences classified by MEGAN into Genus-level groups or lower-level taxonomic assignments. *Russula* = red, *Piloderma* = green, *Ramaria* = blue, "Basidiomycota" = yellow, *Byssocorticium* = pastel blue, *Inocybe* = pastel pink, *Cenococcum* = pastel green and *Hygrophorus* = pastel purple. Greyscale bars represent less-commonly encountered genera. Red asterisk (*) denotes samples omitted from analyses due to mat misidentification or mixed mats.
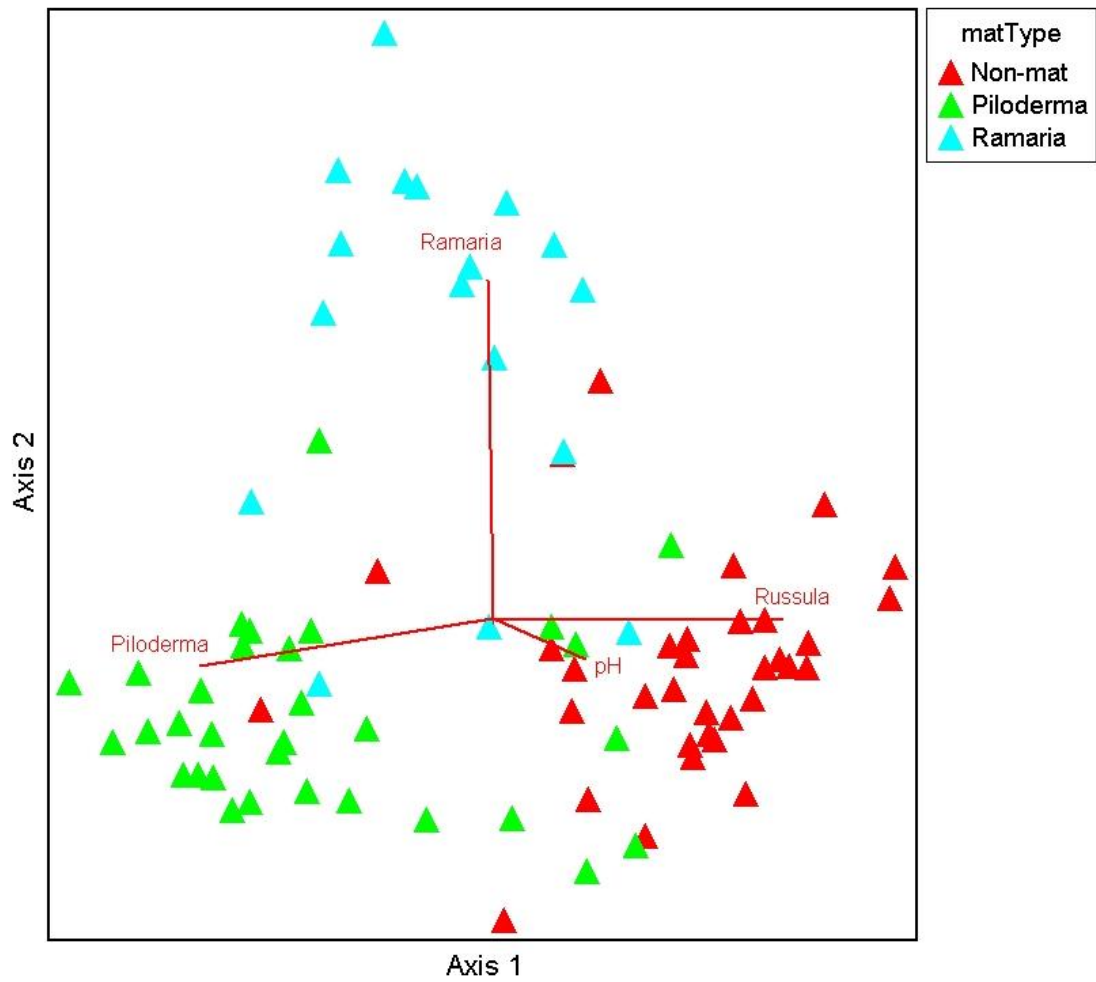
Figure 3.4 – NMS Ordination including mat-forming taxa.  Figure represents first two axes of a 3-dimensional solution.  Triangles represent samples oriented in "genus" space.  Biplot vectors displayed for $r^2 > 0.200$.
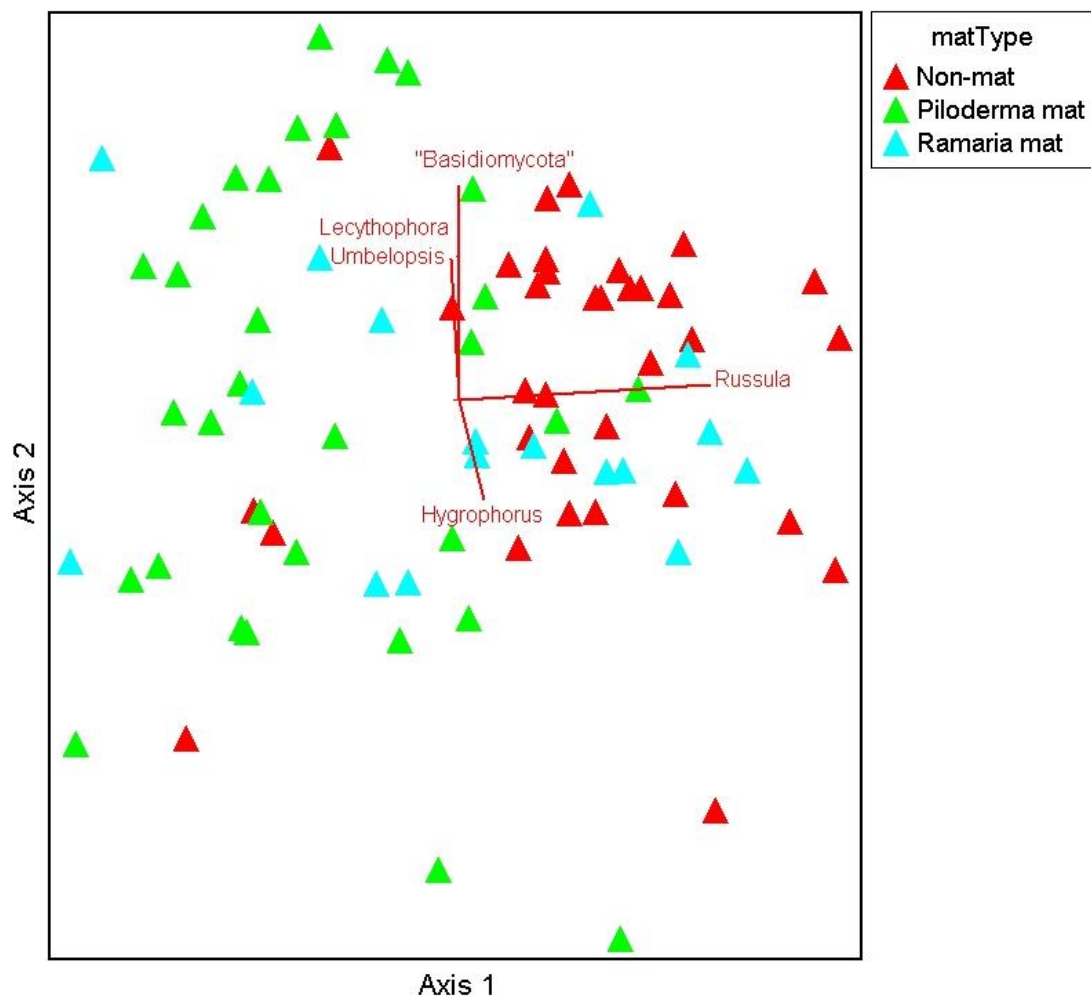
Figure 3.5 – NMS Ordination excluding *Piloderma* and *Ramaria*. Figure represents first two axes of a 3-dimensional solution. Triangles represent samples oriented in "genus" space. Biplot vectors displayed for $r^2 > 0.200$.

# CHAPTER 4 – CHARACTERIZATION OF BACTERIAL COMMUNITY STRUCTURE ASSOCIATED WITH ECTOMYCORRHIZAL MAT AND NON-MAT SOILS

Cedar N. Hesse, Joseph W. Spatafora

**Abstract**

Natural associations of bacteria and fungi in soil have been understudied despite the intimate association of the two taxa in soil habitats. Fungi have been hypothesized to affect bacterial community structure through a variety of direct and indirect methods; however empirical evidence from natural systems is limited. In this study we utilize 16S rDNA 454 pyrosequencing to investigate the bacterial communities associated with two common ectomycorrhizal mat-forming fungal genera: *Piloderma* and *Ramaria*, in forest soils. Shown to alter the chemical makeup of the soils they inhabit through acidification by organic acid exudates, ectomycorrhizal mats have been hypothesized to select for unique bacterial assemblages relative to adjacent non-mat soils. This study identified bacterial families showing disproportional abundance and scarcity in EcM mat soils relative to non-mat soils, however, soil horizon and pH gradients were strongly correlated with bacterial community, as well. Additionally, we concluded that *Ramaria* mat soils harbor fewer bacterial species relative to *Piloderma* mat and non-mat soils and those bacterial communities within *Piloderma* mats were qualitatively different than non-mat soils. This study also found modest evidence for enrichment of known mycorrhization helper bacterial families in *Piloderma* mat soils. These data corroborated the findings of other studies, which find bacterial community richness was highly influenced by soil pH, while fungal community richness was not. Finally, we present evidence that bacterial community richness and fungal community richness were directly related to one another regardless of pH or EcM mat type.

**Introduction**

  Bacterial communities in soil represent one of the most diverse and species rich assemblages on earth.  The complexity of soil microbial communities has been documented from a multitude of environments including forest and prairie soils (Fierer, 2006; Kuske, 2002), arable and rhizosphere soils (Smit *et al.,* 2001; Girvan *et al.,* 2003; Smalla *et al.,* 2001), soils polluted with heavy metals and other contaminants (Diaz-Ravina *et al.,* 1994; Flieβbach *et al.,* 1994), among many others. Relatively few studies have examined the microbial composition in soils associated with mycorrhizal fungi, or the mycorrhizosphere (Bending *et al.,* 2006).  Despite their intimate association within the soil habitat, fungi and bacteria have traditionally been studied separately from one another.  Studies of fungal-bacterial interactions in soil, though limited, have indicated a variety of interesting associations between the two. Among the interactions of fungi and bacteria in soil is the potential for exclusion and facilitation of bacterial groups in the presence of certain fungi (Bending *et al*., 2006), synergistic effects of some bacteria on the growth rate of fungal hyphae and rate of mycorrhization (Frey-Klett *et al*., 2007), and in the implication that mycorrhizal associations may represent tripartite symbioses involving bacteria in addition to the fungus and photobiont (Berliner *et al*., 1989; Li *et al.,* 1992).

  Soil fungi have been hypothesized to affect the surrounding bacterial communities both directly and indirectly (Bending *et al.,* 2006).  Fungi are theorized to directly affect the bacterial community by modifying the nutritional profile of the soil either through hyphal exudates or by serving as a food source for bacteria feeding

on fungal hyphae (Boer *et al.,* 2005).  Additionally, some fungi may actively exclude

certain bacterial groups through the production of antimicrobial or other inhibitory

compounds.  Soil fungi may indirectly influence the bacterial community structure

through modifications to the soil chemistry.  Many ectomycorrhizal (EcM) fungi

produce and excrete organic acids thought to aid in the dissolution and subsequently

the increased availability of mineral nutrients including P, K, Al, and Mg (Cromack *et al.,*1979; Jones, 2004).  As a result of organic acid release from fungal hyphae, the

surrounding soil is lower in pH.  Bacterial communities have been shown to shift

along pH gradients in soil either through the selection for acid tolerant bacterial groups

or through the increased mineral availability afforded by the acidic environment

(Lauber *et al.,* 2009).

In a comparison of soil bacterial communities across a contintental scale

representing a wide range of soil pH levels, Lauber *et al.* (2009) found soil pH to be a

relatively reliable predictor of course-level taxonomic distributions and phylotype

richness.  Near neutral pH soils were, on average,more species rich than highly acidic

or basic soils.  Similar findings were described by Rousk *et al.* (2010) across an

artificial pH gradient created by a long-term soil liming treatment.  The same study

also notes that overall fungal richness along the same pH gradient was relatively

unaffected.  The differential effect of soil pH on fungal and bacterial communities is

hypothesized to be a result of differences in optimal growth ranges (Rousk *et al.,*

2010).  In pure culture studies of the effect of pH on growth, many bacterial groups

exhibit relatively narrow growth optima (Rosso, *et al.* 1995) while fungal growth is largely unaffected by varying pH levels (Wheeler *et al.,* 1991; Nevarez *et al.,* 2009).

Mycorrhizal fungi, common constituents of nearly all soil systems, have been shown to have increased hyphal growth rate, hyphal branching, and higher rates of mycorrhization in the presence of certain bacterial strains. The synergistic effect of these bacteria, dubbed mycorrhization helper bacteria (MHB) (Duponnis *et al.*1991), has been documented in numerous soil dwelling bacterial lineages but predominantly in members of *Bacillus* and *Pseudomonas* (Garbaye, 1994). While the exact mechanisms by which of MHB promote hyphal development is unclear, the potential for a tripartite mycorrhizal symbiosis is intriguing. Most MHB studies have been conducted under laboratory conditions with simple microbial communities and investigations of natural systems have yielded inconclusive results as to the importance of MHB in nature.

A subset of ectomycorrhizal (EcM) fungi form dense hyphal aggregations in soil termed EcM mats. EcM mats have been characterized from temperate and boreal forests throughout the northern hemisphere and have been documented to commonly inhabit as much as 25% of the soil surface area in some ecosystems (Cromack *et al.,* 1979; Griffiths *et al.,* 1996). The dense aggregations of fungal hyphae and mycorrhizal root-tips that define the EcM mat also result in drastic chemical changes in the surrounding soil, most prominently the acidification of the mycorrhizosphere soils. In the H.J. Andrews Experimental Forest in the Cascade Mountains of Oregon, USA, two distinct EcM mat types have been identified and determined to be formed

by different EcM genera: *Piloderma* (Atheliales) and *Ramaria* (Gomphales) (Dunham *et al.,* 2007). Although the chemical profiles of soils from both EcM mats are similar in the degree of acidification relative to non-mat soils, the physiology of the mats differ. *Piloderma* mats typically grow in the organic soil horizon and produce prolific rhizomorphs, which tend to aggregate the soil. In contrast, *Ramaria* mats grow predominantly in the mineral horizon and frequently give the soil an ashy or friable appearance and texture. Soils associated with *Ramaria* mats also commonly exhibit hydrophobic tendencies. Chemical analyses of both mat-forming genera show very high production of oxalic acid, which is likely a major contributor to the acidification of the soil within the mats (Griffiths *et al.,* 1994).

Previous work utilized a pyrosequencing approach to identify the fungal communities associated with *Piloderma* and *Ramaria* mat soils, as well as adjacent non-mat soils (Chapter 3). Non-mat soils were commonly dominated by the non-mat forming ectomycorrhizal genus *Russula*, while mat soils were dominated by the mat-forming genera. *Ramaria* mat soils were determined to harbor relatively fewer fungal species than non-mat or *Piloderma* mat soils. In contrast, *Piloderma* mat soils contained similar fungal species richness to non-mat soils yet the background fungal communities differed qualitatively between the two, including the lower relative abundance of *Russula* in *Piloderma* mat soils.

This study builds on the previous fungal community survey of EcM mats by conducting a low-coverage examination of the associated bacterial communities in the same samples. While the 454-Jr pyrosequencing platform used in this study does not

provide a comprehensive view of bacterial diversity in all samples, it is adequate for comparison between samples. Also, the application of 454-FLX to bacterial communities did not result in levels of sampling approaching saturation (e.g., Lauber et al. 2009), and thus the use of 454-Jr provided a cost effective sampling approach for comparison across numerous samples. The unique microenvironments created by EcM mats, along with the fungal community data, provide a useful system to explore fungal-bacterial interactions in a natural mycorrhizosphere system. The main goals of this study were to explore the microbial diversity within EcM mat soils and determine if the unique physical and chemical properties of EcM mats might harbor unique assemblages of bacteria relative to non-mat soils.

**Materials and Methods**

*Study area and sampling design*

The study site consisted of fourteen plots located within old-growth Douglas fir (*Pseudotsuga menzensii*) stands in the H.J. Andrews Experimental Forest LTER site, Lane County, Oregon. The study plots were selected as they had been used in prior EcM mat research (Griffiths *et al.,* 1991; Dunham *et al.,* 2007) and were known to harbor a diversity of EcM mat morphotypes. Plots selected for this study are a subset of those used in the Dunham *et al.* (2007) study for which the mat phylotypes were successfully determined to contain members of the EcM mat genera *Piloderma* or *Ramaria*.

Sampling of EcM mats within the 900m$^2$ circular study plots was described previously in Dunham *et al.* (2007). Briefly, in late Fall 2005 each plot was searched

and EcM mats flagged for an equivalent of 60 minutes by one individual.   Flagged

mats were chosen at random and visually inspected to verify adequate size ($>0.5m^2$)

and the presence of root-tips and rhizomorphs, until three to five mats were selected

and cored.  Three soil cores from each representative mat were taken to a depth of

10cm below the organic-mineral soil horizon interface.  Soil cores were split into the

two horizon layers and the cores from the same mat were pooled into a single bag.

Non-mat soils were randomly located from within the study plot by randomly

selecting an angle from North and a distance from plot-center, verifying the absence of

EcM mat, cored, and split along the horizons identically to the mat sampling.  This

sampling resulted in six unique "treatments" which will herein be referred by the

following treatment identifiers: RO (*Ramaria* mat organic horizon), RA (*Ramaria* mat

mineral horizon), PO (*Piloderma* mat organic horizon), PA (*Piloderma* mat mineral

horizon), NO (non-mat soil organic horizon), and NA (non-mat soil mineral horizon).

In total, 20 *Piloderma* mats, 11 *Ramaria* mats, and 17 non-mats were sampled as part

of this study for a total of 96 samples (48 mat/non-mat X 2 soil horizons).

### *Molecular methods and Pyrosequencing*

Soils obtained from field sampling were transported to the laboratory and DNA

was extracted in a timely manner.  Briefly, soils were sieved to remove any large

aggregates including rocks, fine roots, rhizomorphs, and mycorrhizal structures prior

to homogenization with mortar and pestle.  Three 0.3g subsamples were extracted for

DNA from each soil sample using the BIO101 Soil DNA Extraction kit, eluting in

40ul EB following manufacturer's protocols.  Triplicate DNA extractions from the same soil sample were pooled for all downstream applications.

Ninety-six samples were amplified for the 5-prime end of the bacterial 16S rDNA region using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG) (Lane *et al.,* 1991) and 519R (5-GWATTACCGCGGCKGCTG) (Turner *et al.,* 1999).  Amplification primers also contained the Fusion sequences for Life Sciences 454-based pyrosequencing and unique 12bp error-correcting barcodes on the 27F forward primer.  All samples were simultaneously amplified on a 96-well microtiter plate using a high-fidelity polymerase.  PCR conditions for amplification included initial denaturing step of 2 minutes at 92C followed by 25 cycles of 92C for 10 seconds, 50C for 30 seconds, and 72C for 40 seconds.  A final extension for 5 minutes at 72C concluded the amplification program.  All samples were verified for amplified product using gel electrophoreses, purified on Qiagen PCR purification columns, and final products were quantified using the Qubit fluorometer and the Qubit BR reagent kit (Invitrogen, Carlsbad, CA).

Prior to 454-Jr sequencing, samples were pooled in equimolar concentrations into a single tube containing equal templates of all ninety-six samples.  The pooled sample was sent to the Center for Genome Research and Biocomputing's Core Services Lab at Oregon State University (Corvallis, Oregon) for further preparation and sequencing on the Life Science 454-Jr pyrosequencing platform.  Sequences are deposited in the NCBI Short Read Archive (accession number pending).

*Computational methods*

Raw pyrosequencing data was processed in the software package Mothur (Schloss *et al.,* 2009) to trim low-quality sequences (average Q-value <35 within a 50bp sliding window), remove sequences with greater than 8bp homopolymeric runs, and to remove any sequences with more than one error in the multiplex barcode region.  For longer sequences only the first 225bp were retained for subsequent analyses to allow for more accurate clustering of reads varying in length.  The retained sequences were aligned to the Silva database alignment (Pruesse *et al.,* 2007) of the bacterial 16S rDNA and the subsequent alignment was trimmed to remove columns containing only gaps (final alignment length = 499bp).

Sequences were searched for potential chimeric artifacts using the UCHIME algorithm from within Mothur and putative chimeras were removed from subsequent analyses.  The aligned sequences were used to create a genetic distance matrix in which a string of gaps was considered to be a single insertion (Mothur option calc=onegap).  The distance matrix was used for nearest-neighbor clustering at 97% similarity to form the operational taxonomic units (OTUs) used in further analyses. Following sequence de-replication and clustering to reduce computational load, OTUs were classified within the Ribosomal Database Project (Cole *et al.,* 2009) taxonomy using the naïve Bayesian classifier (Liu *et al.,* 2008) within the Mother package and the RDP training dataset 6.  Taxonomic designations which were assigned with 75% confidence or greater in the naïve Baysian classifier were retained.  Phylotypes were

determined at the bacterial Class level and Family level by collapsing the RDP

taxonomy tree to the given level and tallying the sequences assigned to each grouping.

*Rarefaction, diversity, and statistical analyses*

Rarefaction curves were calculated in Mothur for individual samples and

pooled samples (by treatment) using 97% similar OTUs.  Randomized sampling for

rarefaction curves was determined at five-read intervals using 1000 randomizations.

Data were visualized in Microsoft Excel 2007.  Alpha (Shannon and Simpson indexes)

and Beta diversity statistics were calculated for individual samples and pooled samples

within the Mothur package using the 97% similarity threshold to define OTU

boundaries.  Chao richness estimates were calculated to incorporate sampling effort

into estimates of total OTU abundance.  PerMANOVA tests for no difference between

groups of bacterial OTUs were conducted in PC-ORD v6 (McCune and Mefford,

2009) for groupings by treatment and by soil horizon.  Linear regression models were

calculated for the relationship between Chao estimated richness in bacterial and fungal

communities (see Chapter 3 for fungal community data methods) and soil pH.  Linear

regression was also used to compare Chao estimated richness between fungal and

bacterial communities.   Data were plotted and regressions calculated using Microsoft

Excel 2007.

*Taxonomic distributions and ordinations*

Class level phylotypes, determined above, were visualized using Microsoft

Excel 2007.  All samples were relativized to account for differences in sequencing

effort and plotted as percentages of total identified diversity.  Phylotypes identified as

"unclassified" are taxonomic groups existing in the RDP database without assigned taxonomy.

Two primary data matrices were used for non-metric multidimensional scaling ordinations in this study. Some sampling points were omitted from these analyses due to insufficient read numbers from multiplex pyrosequencing. The first primary matrix consisted of phylotype abundances of 83 bacterial families and 120 fungal genera (determined previously, Chapter 3) for sixty-six individual sampling points. The second primary data matrix consisted phylotype abundances of only the 83 bacterial families for seventy-nine individual sampling points. A secondary environmental data matrix was used to provide explanatory variables for NMS ordinations. The secondary matrix contained coded designations for treatment, mat type, horizon, aspect, and site. Also included were other environmental variables including elevation, soil pH, and relative abundances of the fungal and bacterial phylotypes used in the main matrices.

Non-metric multidimensional scaling (NMS) ordinations (Kruskal and Wish, 1978) were conducted in the PC-ORD v6.05 software package (McCune and Mefford, 2009). NMS techniques were chosen to avoid the "zero truncation problem" due to a high proportion of the species matrix containing zeros. As recommended for molecular sampling studies, all ordinations were calculated using Sørensen distances allowing for a fixed maximum value for sample units containing no shared species. All NMS ordinations were done in autopilot mode on the "slow and thorough" setting.

Visual inspection of the ordination plots and overlay of environmental variables and primary matrix abundances were used to identify patterns in the dataset.

## Results

### *Rarefaction analyses*

Individual rarefaction curves generated from OTUs of 97% sequence similarity (Figure 4.1) indicate that all soil cores were not sequenced deeply enough to capture all of the diversity within the system. This result of undersampling is expected as the average sequencing depth per sample was approximately 590 reads, an understandably inadequate depth to capture the hyperdiversity of bacteria in soil. Nonetheless, the individual rarefaction curves indicate moderate heterogeneity between samples within a given treatment, with the number of OTUs detected per sample typically ranging between 200 and 450.

When samples are pooled by treatment, the rarefaction curves (Figure 4.2) again indicate undersampling despite some treatments (NO, NA, PO, PA) having more than 10,000 reads. The pooled data suggests that, in general, non-mat organic horizons tend to harbor more unique 97% bacterial OTUs than other treatments. The least OTU-rich treatments, as indicated by the pooled data, were *Piloderma* mat O-horizon and *Ramaria* mat A-horizon. The curves for both *Ramaria* mat horizons are considerably shorter than their non-mat and *Piloderma* mat counterparts as a result of fewer *Ramaria* mat samples overall in this study.

*Diversity statistics*

Alpha diversity of pooled samples (Table 4.1) reflects the increased OTU richness of the non-mat organic horizon observed in the rarefaction analyses. Within the non-mat O-horizon samples a total of 3092 OTUs were detected. The Chao richness estimator, taking into account the undersampling of the group, estimates the total richness of the non-mat O-horizon to be approximately 7300 OTUs. This estimate is significantly higher than all the EcM mat treatments (PA, PO, RA, RO) and marginally higher than the non-mat A-horizon sample (overlapping 95% confidence bounds). Similar results are found when comparing alpha diversity averages from individual samples of a treatment (Table 4.2).

The alpha diversity averages for individual samples (Table 4.2) show that diversity of pooled samples and individual samples are not always directly related. The *Ramaria* mat O-horizon, for instance, has an average estimated richness (Chao) of 837 OTUs in individual samples, greater than that of all other EcM mat averages (PO, PA, RA). This finding, taken along with the lower pooled diversity estimates for RO, suggests that individual *Ramaria* mats harbor highly diverse assemblages of bacteria in the organic soil horizon but that beta diversity among *Ramaria* mat O-horizons is relatively low when compared to other mat samples.

Pairwise beta diversity measures of pooled samples (Table 4.3) indicate higher degrees of similarity among non-mat and *Piloderma* mat samples. The six highest Chao shared diversity estimates represent all pairwise comparisons between non-mat and *Piloderma* mat samples. In contrast, *Ramaria* mat samples are shown to have

lower estimated relative similarity to all other treatments and show the lowest shared

OTUs between horizons of *Ramaria* mats.

Shared OTU distributions between horizons are visualized in the Venn

diagram of Figure 4.3. Relatively few OTUs are shared between groupings. Of the

8046 total OTUs detected, only about 24% were detected in both soil horizons.

PerMANOVA analyses, testing the hypothesis for no difference between bacterial

communities based on treatment and horizon, were rejected (p=0.0001 and p=0.0002,

respectively) but the groupings only explained 10% (treatment) and 5% (horizon) of

the variation in the dataset. 2-way PerMANOVA indicated no significant interaction

term between treatment and horizon.

### *Taxonomic distributions*

The higher level taxonomic designations (Class) for individual samples are

presented in Figure 4.4. The major groups found in all samples are defined in the

figure caption and represent the major bacterial lineages commonly found in soils

including multiple groups of Acidobacteria (primarily Groups 1 and 2),

Actinobacteria, Alphaproteobacteria, and Betaproteobacteria. Also commonly

encountered were multiple groups of unclassified bacteria. The distributions depicted

in Figure 4.4 appear relatively uniform among samples and between treatments.

Although the higher level (Class) distributions are uniform, the groupings quickly

break down as lower taxonomic levels are considered (Family, Genus) due to lower

confidence in the taxonomic designation and heterogeneity among samples.

### *Non-metric multidimensional scaling ordinations*

The NMS ordination methods used to visualize the phylotype abundance data for the total microbial community (bacterial families and fungal genera) of individual samples show plots strongly grouped by treatment. The first two axes of the 3-dimensional NMS ordination are depicted in Figure 4.5. Joint plot vectors show strongest correlations with primary axes are from the two mat-forming fungal taxa (*Piloderma* and *Ramaria*) and *Russula*. Bacterial family abundances that correlate with the ordination axes ($R^2 > 0.200$) include Acidobacteria Gp6 and an unclassified Bacterium associated with non-mat soils (negatively associated with Axis 1) and Sphingobacteriaceae, Burkholderiaceae, Microbacteriaceae, and Acetobacteriaceae associated positively with Axis 1 and *Piloderma* mat soils. No bacterial families are associated with *Ramaria* mat soils in this ordination. Increasing pH is correlated with both ordination axes and non-mat soils.

The NMS ordination presented in Figure 4.6 represents samples placed in "bacterial family space", without consideration of the fungal community. Many bacterial families are associated with the ordination axes and are identified in the figure legend. Horizon and pH are also significantly correlated with the ordination axes. Segregation of samples by treatment is not as pronounced as the previous ordination (Figure 4.5), although visual groupings of non-mat and *Piloderma* mat samples are evident along Axis 1. *Ramaria* mat samples are interspersed among samples from other treatment types and do not correlated with any of the ordination axes. Four predictable assemblages of bacterial families are indicated by the ordination correlating with soil horizon and pH gradients. The mineral soil horizon

harbors higher abundances of Hyphomicrobiaceae, Acidobacteria Group 7, and

Catenulisporaceae. The organic soil horizon has higher abundances of ten bacterial

groups (labels g-p in Figure 4.6), including Caulobacteriaceae, Xanthomonadaceae,

and unknown lineages within the Solirubrobacterales and Sphingomonadales. Lower

pH soils positively associated with Axis 1 show increased abundances of

Acidobacteria Group 1, Acetobacteriaceae, and Actinospicaceae. Higher pH soils are

positively associated with nine bacterial families (labels q-y in Figure 4.6) including

Acidobacteria groups 4, 6, and 17 and an unknown Bacterial group.

### *Relationships of fungal richness, bacterial richness, and pH*

Linear regressions of Chao estimated bacterial and fungal richness with soil pH

are presented in Figure 4.7. There is a strong positive correlation between estimated

bacterial richness and soil pH ($R^2 = 0.1486$) with an estimated 240 additional bacterial

OTUs encountered across a full unit pH, at the scale measured in this study. The

linear regression model of fungal richness and soil pH does not indicate a strong

relationship between one another ($R^2 = 0.0335$). Plotted sampling points, color coded

by mat-type or non-mat soils, show a clustering of non-mat samples at higher pH

levels, however introgression along the pH gradient among mat samples and non-mat

samples does occur.

The comparison of the estimated richness of fungal communities to bacterial

communities in the same samples is depicted in Figure 4.8. The linear regression

model shows a direct relationship between the richness of fungal communities and

richness of bacterial communities ($R^2 = 0.2768$), regardless of treatment type or soil

pH level.  The regression fit suggests that, within our sampling, approximately nine

bacterial OTUs are encountered for each additional fungal OTU encountered.

**Discussion**

As expected, the low-coverage sampling of hyperdivese bacterial communities

in this study did not capture the diversity of bacterial taxa to saturation (Figures 4.1

and 4.2).  This sampling, however, was sufficient to use non-parametric richness

estimators and ordination analyses to compare the richness and composition of

bacterial communities.  The patterns of diversity and richness observed in bacterial

communities associated with EcM mat soils suggest that *Piloderma* and *Ramaria* mats

affect bacterial community structure in similar ways that they influence the

background fungal community, in the same EcM mats (Chapter 3).  Quantitative

differences in bacterial richness were found in *Ramaria* mat soils relative to non-mat

and *Piloderma* mat soils, while qualitative differences in bacterial communities were

evident in *Piloderma* mat soils, relative to non-mat samples.  Although differences in

bacterial community structure are observed between mat and non-mat soils it is

important to note that soil pH and soil horizon are strongly correlated with the

community shifts and these environmental variables are non-independently related to

the presence of EcM mats.

*Alpha- and Beta-Diversity of Bacterial Communities*

Overall bacterial richness of pooled *Ramaria* mat soils (pooled alpha diversity)

was marginally lower than all other treatments (Table 4.1); however individual

*Ramaria* mat samples have, on average, similar numbers (individual alpha diversity)

of bacterial OTUs to other treatments (Table 4.2). Ordination analyses do not show unique bacterial family assemblages strongly associated with *Ramaria* mat samples (Figure 4.5) suggesting the differences in beta diversity between *Ramaria* mat soils and other treatments may be driven by exclusions at a lower taxonomic level (e,g., genus or species), rather than the family level.

Non-mat organic horizon soils harbored higher than average bacterial alpha diversity relative to the other treatment types while non-mat A-horizon and *Piloderma* mat soils contained statistically similar alpha diversity. Although estimated bacterial family richness was similar between non-mat and *Piloderma* mat communities, ordination analyses (Figure 4.6) suggest qualitative differences in the bacterial community constituents. An increase in abundance of numerous bacterial families associated with the higher pH levels indicates the more neutral chemistry of non-mat soils may be more habitable to a wider variety of bacterial groups.

Distributions of bacterial classes among treatments is relatively even (Figure 4.4) despite differences in overall richness between treatments. PerMANOVA analyses verify that bacterial communities differ by treatment when considering 97% sequence similar OTUs, suggesting that the differences in community structure is likely driven at taxonomic levels lower than Class (Family, Order, Genus, etc.). The Venn diagram presented in Figure 4.3 highlights the hyperdiversity and heterogeneity among samples showing that only 23% of all OTUs are shared between horizons. As a result of this extreme species-level heterogeneity and our understanding that the

bacterial communities are undersampled (Figure 4.1 and 4.2), these data are more readily interpreted at Family level phylotypes.

*Taxonomic microbial community structure*

When the entire microbial community (Bacteria and Fungi) is considered in an ordination analysis, samples segregate largely based on mat-type. This result may be expected as the mat-forming fungal taxon can represent as much as 80% of the overall fungal abundance thereby driving the ordination (Chapter 3). Despite the strikingly different chemical profiles of EcM mat soils, relatively few bacterial families segregate strongly with treatment type (Figure 4.5). Acidobacteria Group 6 and an unknown Bacterial family associate with non-mat soils while Sphingobacteriaceae, Burkholderiaceae, Microbacteriaceae, and Acetobacteraceae associate with *Piloderma* mat soils. As members of the Burkholderiaceae have been implicated as possible MHB, the co-abundance of the lineage and *Piloderma* may indicate a synergistic mycorrhizal association. Interestingly, despite the distinct physical and chemical properties of *Ramaria* mat soils no bacterial families associate either positively or negatively in the ordination.

Oxalates produced by *Piloderma* and *Ramaria* have been predicted to be utilized by oxalate-degrading bacteria in soils, specifically members of the Streptomycetaceae (Knutson *et al.,* 1980). Our analyses do not indicate any increased abundance of Streptomycetaceae within either EcM mat soil where increased oxalates would be present. The capacity for oxalate degradation, however, is not restricted to

the Streptomycetaceae and is present in numerous other bacterial lineages, though family-level ecologies in bacteria are tenuous preventing further interpretation.

### *Bacterial communities, fungal communities, and soil pH*

The NMS ordination based on only the bacterial family phylotypes (Figure 4.6) indicates two environmental gradients are strongly structuring bacterial community composition: soil horizon and pH. As *Piloderma* and *Ramaria* mats both tend to have lower soil pH levels than surrounding soil and preferentially segregate into different soil horizons, there is a non-independence of environmental variables to treatment type. Nonetheless, predictable assemblages of bacterial families are present based on soil horizon and pH, and subsequently treatment type. Numerous bacterial families were positively associated with increasing pH as well as with the organic soil horizon, while fewer families were positively associated with lower pH and the mineral horizon. Among those bacterial groups enriched in lower pH soils were Acidobacteria Group 1, Acetobacteraceae, and Actinospicaceae, all of which have members that have been identified previously as at least moderately acidophilic.

Other studies have shown that soil pH strongly influences bacterial community structure at large geographic scales or artificial gradients (Lauber *et al.,* 2009; Rousk *et al.*, 2010). In these studies, the driver of soil pH is presumed to be pedogenic and relatively constant at a local scale in the absence of anthropomorphic influences. This study demonstrates that through natural processes ectomycorrhizal mats serve to modify the soil environment and subsequently alter local bacterial communities. In this study, as with previous studies, soil pH influences bacterial communities both in

composition and overall richness.  Additionally, our findings corroborate the findings

of Rousk *et al.* (2010) that fungal community richness does not appear to be correlated

with soil pH to nearly the same degree as bacterial richness, even within the relatively

narrow pH range of soils in this study.  Interestingly, these data demonstrate that

fungal and bacterial richness are positively correlated with one another, independent

of soil pH or their EcM mat status.  Understanding the mechanisms by which

microbial richnesses are correlated will require further work, however, the

heterogeneous distribution of nutritional resources in soil (Schlesinger *et al.,* 1996;

John *et al.,* 2007) may serve to restrict richness of bacterial and fungal communities in

similar fashions.

**Conclusions**

The analyses presented here show bacterial communities differ between EcM

mat soils and non-mat soils.  The indirect effect of decreased soil pH within EcM mats

appears to be a very strong driver of bacterial community differentiation.  Differences

in soil horizon also serve to structure bacterial communities; however this may be

done independently from EcM mat type as indicated by the lack of a signficant

interaction term between horizon and treatment in the two-way PerMANOVA

analysis.  Importantly, the non-independence of EcM mat type, soil pH, and soil

horizon limit our ability to draw definitive cause-and-effect conclusions.  While these

analyses cannot address the potential for mycorrhizal fungi to directly modify the

bacterial community, the abundance of putative MHB lineages in *Piloderma* mats may

indicate an active facilitation effect.   Finally, this study presents the novel finding that

bacterial community richness and fungal community richness are directly correlated to one another, independent of soil pH or the presence an EcM mat.

Although considerable progress has been made in the ability to analyze bacterial community data, gaps persist in our understanding of bacterial ecologies. Family level groupings of bacterial families prove useful for identifying patterns in community data, however within-family ecologies can differ greatly and limit our ability for further interpretation. Nonetheless, this work provides a framework for generating hypotheses regarding fungal-bacterial interactions in natural systems and will benefit greatly from further research. Additionally, this work contributes to the understanding of mechanisms driving soil resource heterogeneity and, subsequently, microbial community heterogeneity in temperate forest ecosystems which may serve to inform future soil microbial surveys.

**Acknowledgements**

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**Non-mat A-horizon**

**Non-mat O-horizon**

*Piloderma*-mat A-horizon

*Piloderma*-mat O-horizon

*Ramaria*-mat A-horizon

*Ramaria*-mat O-horizon
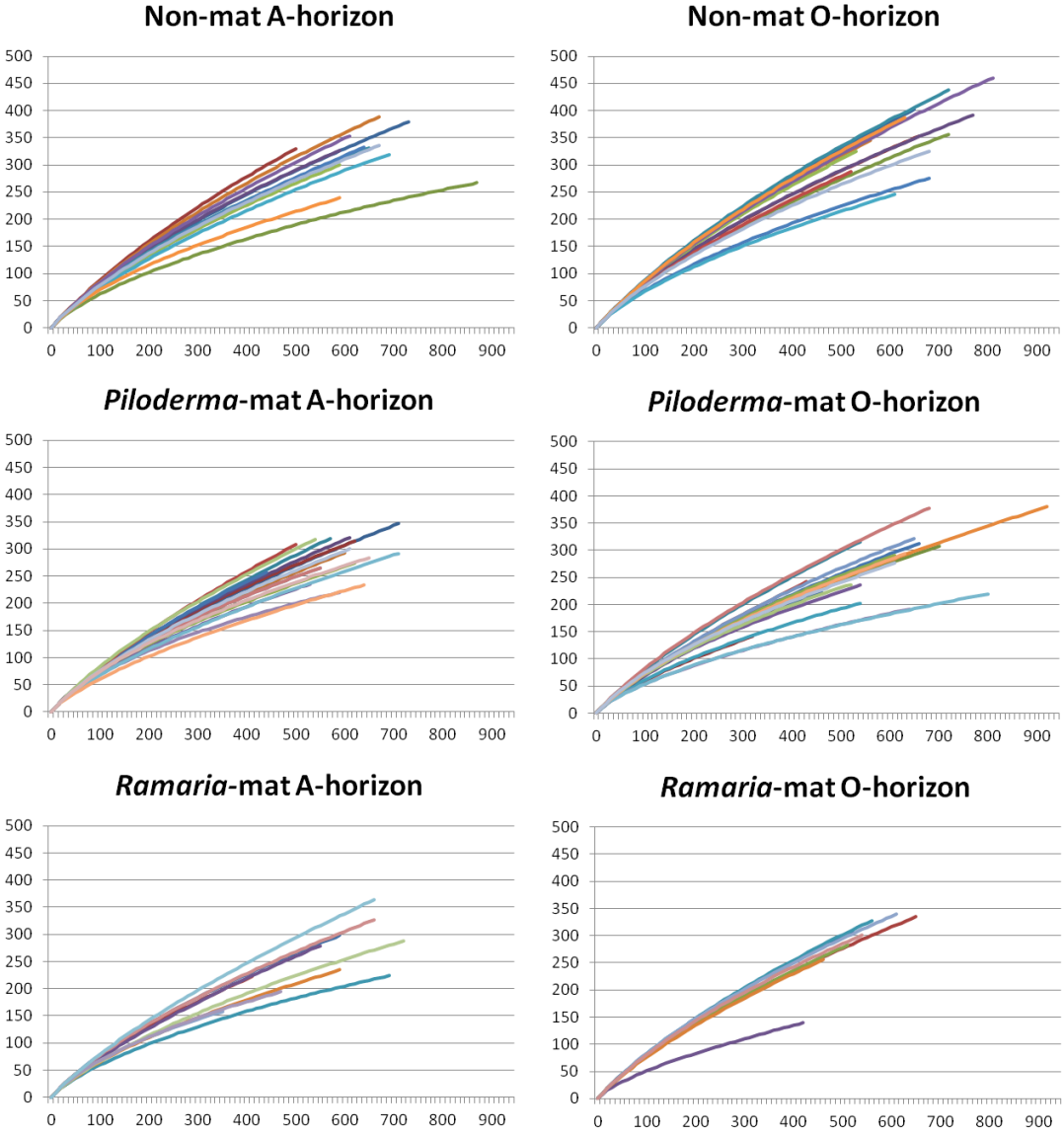
Figure 4.1 – Individual sample rarefaction curves of 97% similar OTUs representing bacterial species.
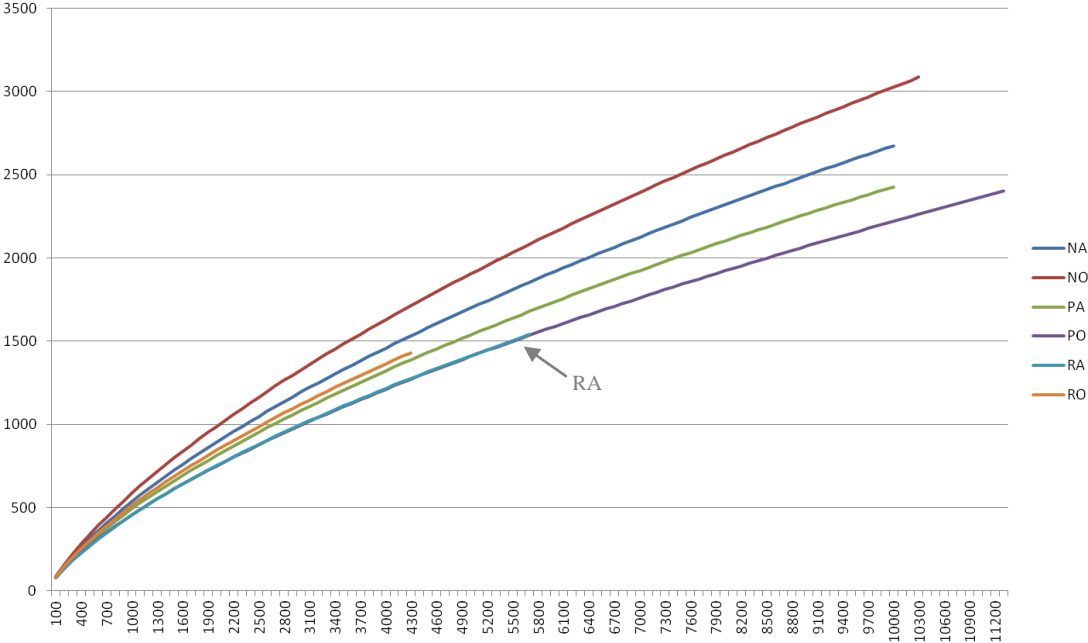
Figure 4.2 – Rarefaction curves of samples pooled by treatment and representing 97% similar bacterial OTUs.

| Treatment | 97% OTUs | Chao | Lower CI | Upper CI | Shannon | Lower CI | Upper CI | Simpson (1 – D) | Lower CI | Upper CI |
|---|---|---|---|---|---|---|---|---|---|---|
| NA[ab] | 2688 | 6284.64 | 5814.89 | 6824.97 | 6.60 | 6.56 | 6.63 | 0.9946 | 0.9950 | 0.9943 |
| NO[a] | 3092 | 7308.01 | 6795.44 | 7891.51 | 6.87 | 6.84 | 6.91 | 0.9962 | 0.9965 | 0.9960 |
| PA[b] | 2441 | 6022.71 | 5529.16 | 6595.15 | 6.35 | 6.32 | 6.39 | 0.9931 | 0.9935 | 0.9927 |
| PO[b] | 2411 | 5674.59 | 5225.52 | 6195.31 | 6.14 | 6.10 | 6.18 | 0.9900 | 0.9907 | 0.9895 |
| RA[c] | 1553 | 3944.83 | 3540.57 | 4431.33 | 6.01 | 5.96 | 6.06 | 0.9909 | 0.9916 | 0.9902 |
| RO[c] | 1435 | 3750.17 | 3345.83 | 4240.07 | 6.26 | 6.21 | 6.31 | 0.9944 | 0.9949 | 0.9940 |

Table 4.1 – Alpha diversity measures (Shannon and Simpson indices) of pooled samples based on 97% sequence similarity bacterial OTUs.  Superscripts indicate groups of non-overlapping 95% confidence intervals for Chao richness estimates.

Table 4.2 – Bacterial alpha diversity averages from individual samples averaged within treatment type.  OTUs based on 97% sequence similarity.

| | Ave. OTUs | Ave. Chao | Ave. Shannon |
|---|---|---|---|
| NA | 320.54 | 888.02 | 5.30 |
| NO | 354.92 | 982.44 | 5.45 |
| PA | 282.76 | 791.11 | 5.14 |
| PO | 267.95 | 738.87 | 4.95 |
| RA | 260.30 | 726.19 | 4.96 |
| RO | 288.25 | 836.96 | 5.19 |

Table 4.3 – Pairwise beta diversity statistics for all possible combinations of treatment types.  Shared OTUs and Shared Chao represent counts from samples pooled by treatment.  Rows sorted from largest to smallest Shared Chao value.

| Comparison | | Shared OTUs | Shared Chao |
|---|---|---|---|
| NO | PO | 965 | 1981 |
| NA | NO | 1068 | 1934 |
| NO | PA | 994 | 1912 |
| NA | PA | 959 | 1810 |
| PA | PO | 911 | 1721 |
| NA | PO | 839 | 1638 |
| PO | RA | 676 | 1550 |
| PA | RA | 739 | 1513 |
| NO | RO | 682 | 1508 |
| NO | RA | 725 | 1502 |
| NA | RA | 739 | 1429 |
| PO | RO | 633 | 1383 |
| PA | RO | 586 | 1261 |
| NA | RO | 558 | 1187 |
| RA | RO | 476 | 1160 |
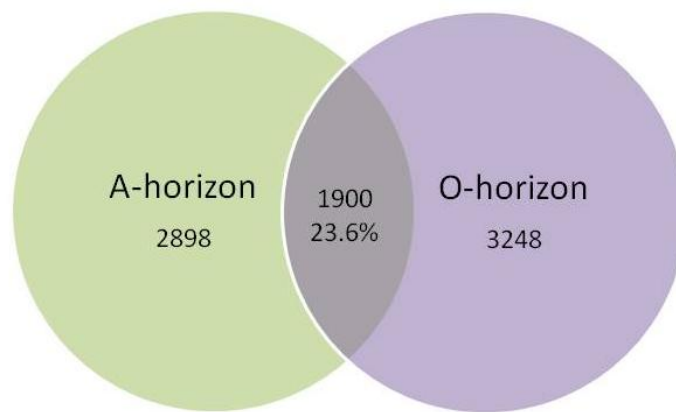
Figure 4.3 – Venn diagram representing shared and unique 97% sequence similarity OTU diversity of bacteria between soil horizons, regardless of treatment type.  A – Mineral Horizon, O – Organic Horizon.
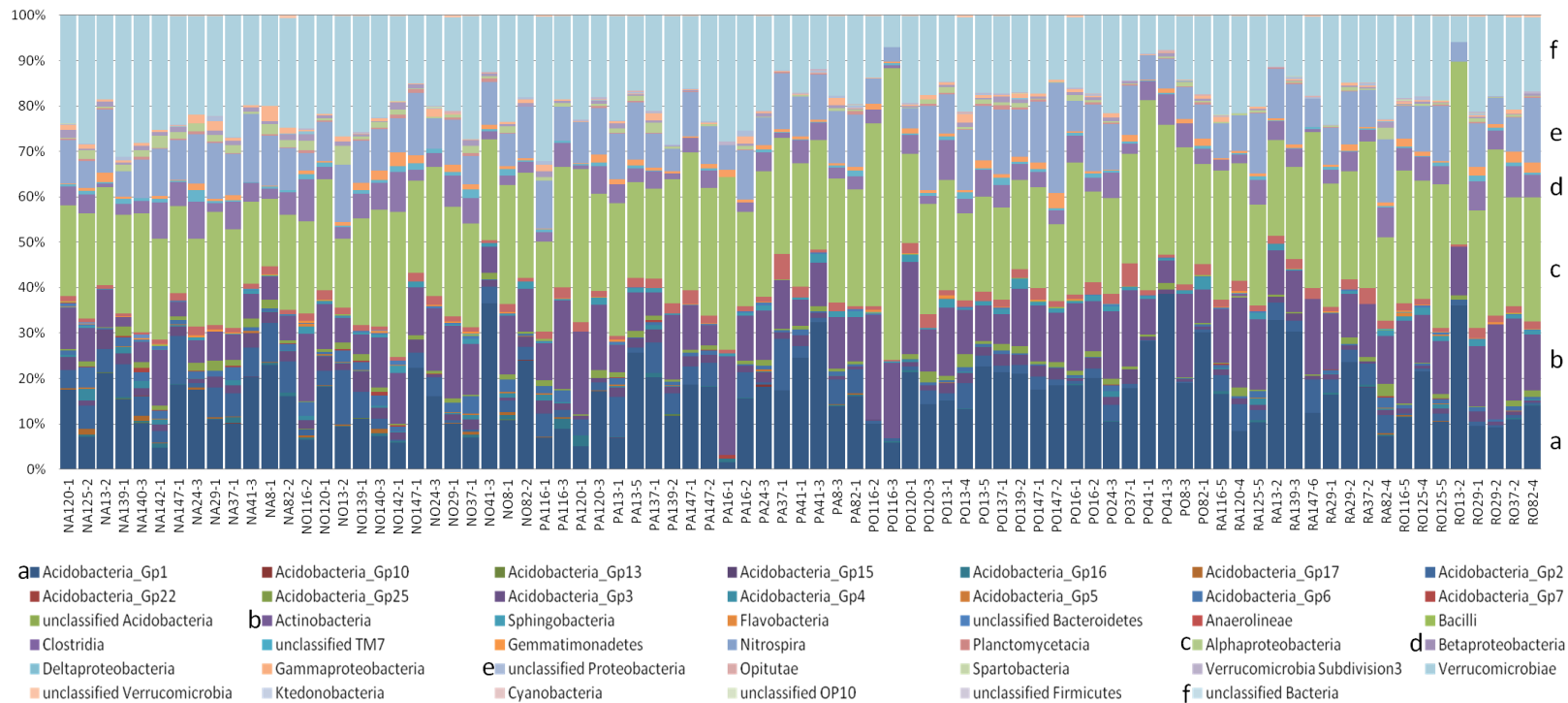
Figure 4.4 – Relative abundance of bacterial classes per sample as determined by 97% sequence similarity OTU classification to the RDP taxonomic hierarchy in Mothur. Vertical bars represent 100% of sequences classified to at least the class level. Bacterial families are ordered from bottom to top. Samples are designated by treatment as described in Materials and Methods (e.g., NA = nonmat) and sample number. Letters a-f denote abundant groups found in all treatments.
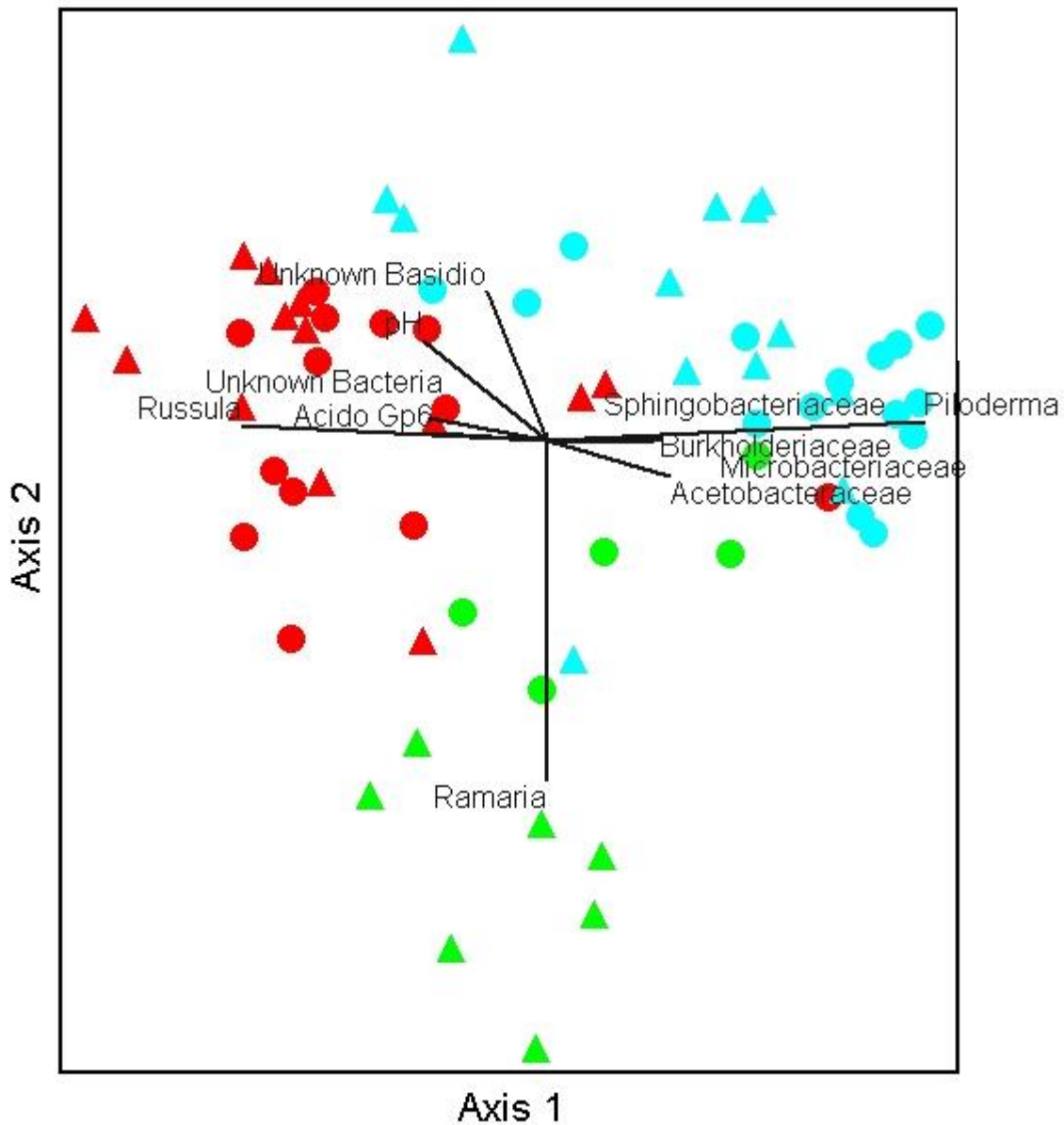
Figure 4.5 – First two axes of a three-dimensional NMS ordination of samples in bacterial family and fungal genus phylotype space Mat-type is coded as color (Red – non-mat, Green – *Ramaria* mat, Blue – *Piloderma* mat.  Soil horizon is coded by symbol shape (Circles – O-horizon, Triangles – A-horizon).  "Acido" abbreviates "Acidobacteria".  Joint plot vectors shown for variables with $R^2 > 0.200$.  Final stress = 15.71
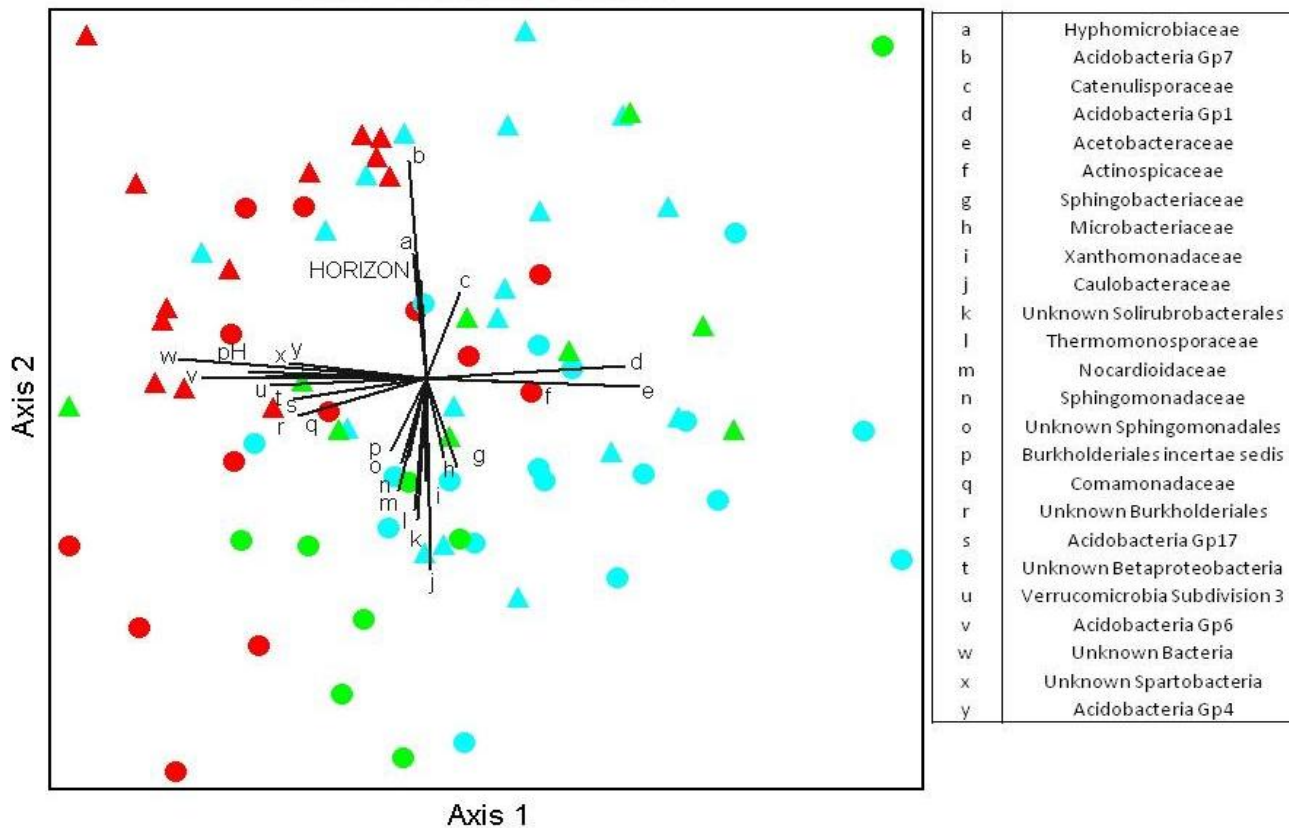
Figure 4.6 – First two axes of a three-dimensional NMS ordination of samples in bacterial phylotype space Mat-type is coded as color (Red – non-mat, Green – *Ramaria* mat, Blue – *Piloderma* mat. Soil horizon is coded by symbol shape (Circles – O-horizon, Triangles – A-horizon). Joint plot vectors shown for variables with $R^2 > 0.200$. Final stress = 18.90
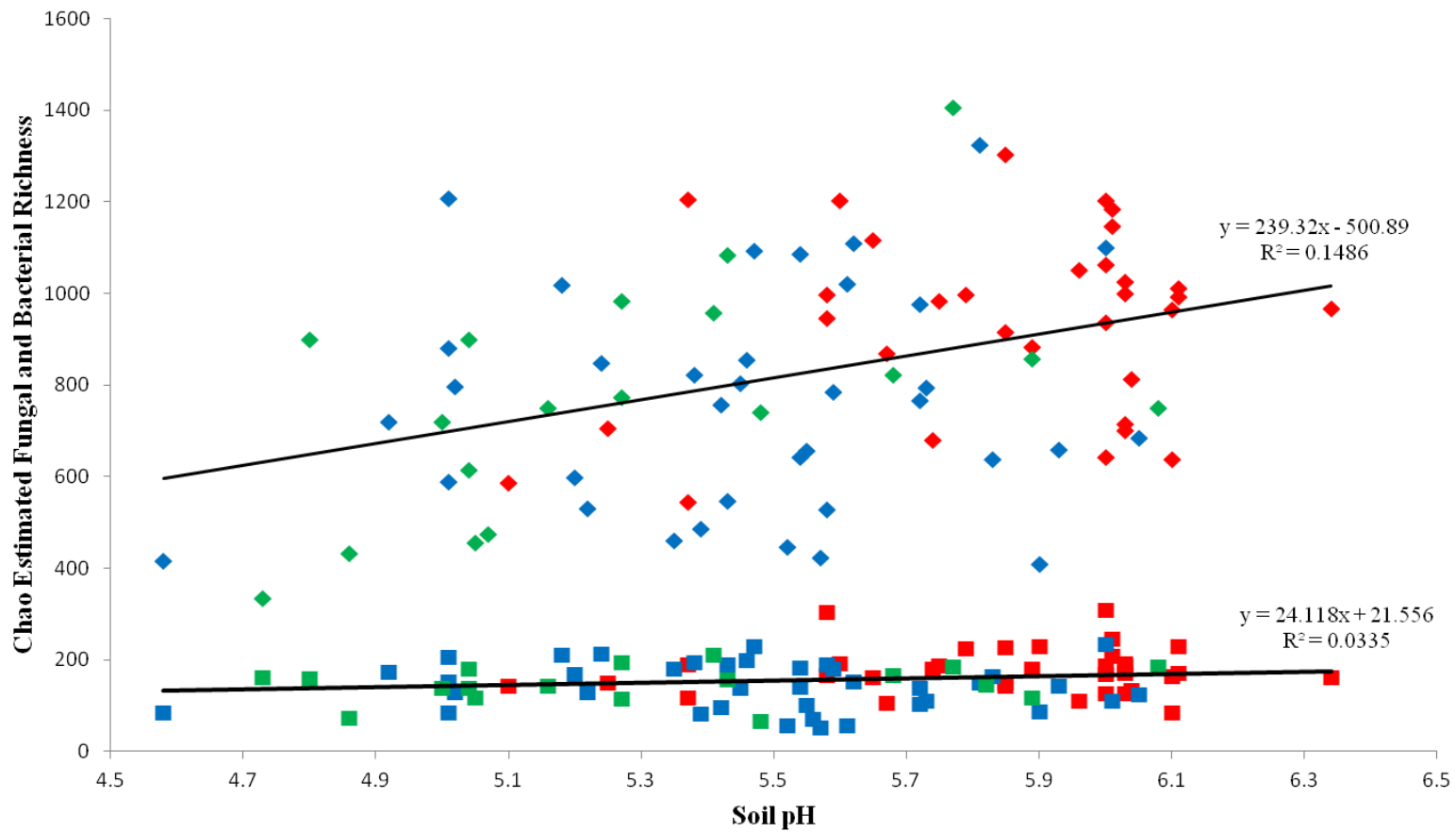
Figure 4.7 – Linear regression of estimated bacterial and fungal richness across a soil pH gradient.

Figure 4.7 – Linear regression of estimated bacterial and fungal richness across a soil pH gradient. Chao estimated richness for bacterial communities (diamonds), based on 97% sequence similar OTUs, and fungal communities (squares), based on 95% similar OTUs, for individual samples. Colors correspond to mat-type (*Ramaria* = Green, *Piloderma* = Blue) and non-mat soils (Red). Linear regression equation and $R^2$ noted on right of figure.
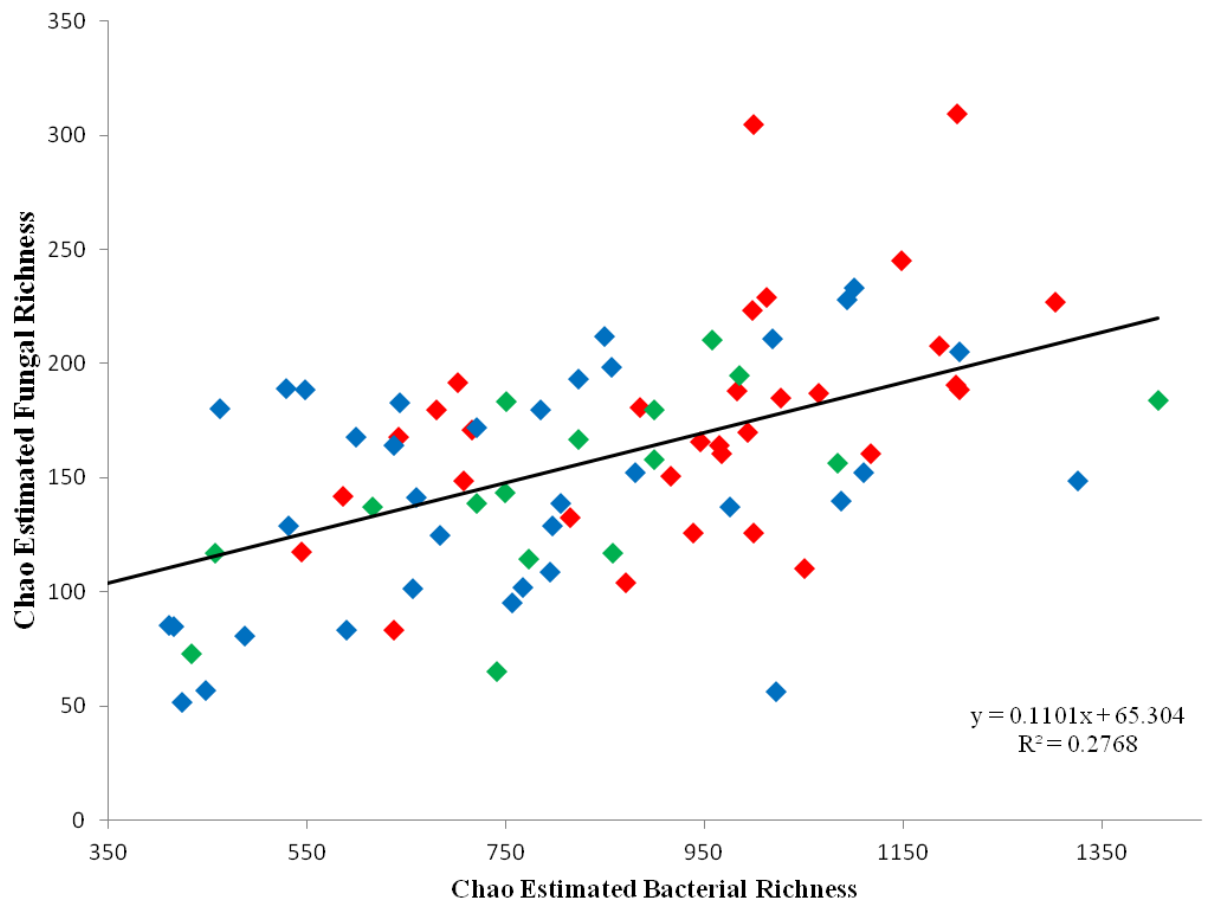
Figure 4.8 - Linear regression of estimated bacterial richness by estimated fungal richness.

Figure 4.8 - Linear regression of estimated bacterial richness by estimated fungal richness. Chao estimated richness for bacterial and fungal communities (determined by 97% and 95% sequence similar OTUs, respectively) colored by mat-type (*Ramaria* = Green, *Piloderma* = Blue) and non-mat soils (Red). Linear regression equation and $R^2$ noted on right of figure.

# CHAPTER 5 – GENERAL CONCLUSIONS

Cedar N. Hesse

**Summary of findings**

This dissertation presents a series of studies aimed at exploring the differences in fungal and bacterial communities associated with ectomycorrhizal mats in the H.J. Andrews Experimental Forest. As part of a Microbial Observatory project, this study was designed in conjunction with work by L.A. Kluber (Dissertation) to examine the enzymatic profiles of EcM mats and J.H. Blanchard (Dissertation) to explore the community shifts between mat birth and death. Although the contributions of this work are largely descriptive, the comparison of molecular sampling methods and the observations of the structure of hyperdiversity and heterogeneity of microbial communities are important to the understanding of molecular ecology in general.

This study utilizes the most advanced DNA sequencing techniques in to explore the microbial communities associated with the ectomycorrhizal mats in forest soils. The initial field sampling was conducted with the intent to use T-RFLP fingerprinting and low-coverage clone library methods to describe the microbial communities. The advent and proliferation of next-generation sequencing technologies allowed these samples to be analyzed using higher resolution methods with considerable less work.

Chapter 2 presents a comparison of three molecular sampling methods, including one developed by the author, and their application to describe fungal communities in soils. Of the three methods compared, both next-generation sequencing technologies provided considerably higher resolution of the fungal

community structure, relative to the traditional clone-and-sequence approach. Despite the differences in sequencing methods between the Illumina and 454 platforms the relative abundance of taxonomic groups were similar one another. Illumina-based ITS sequencing provided inflated overall abundance counts, relative to the 454-platform, likely due to the inability to pair single-end reads with one another and higher rates of sequencing error. Although the Illumina platform provides similar taxonomic distributions as the 454 platform, the shorter read length and more tedious sample preparation made it less desirable for the purposes of this study. It is worth noting, however, that since the initial Illumina sequencing for this study was conducted, the read length and sample preparation methods have improved significantly. Replicating this study with the current Illumina technology may provide alternative interpretations of the value of this method.

Chapter 3 of this dissertation presented the findings of a pyrosequencing-based analysis of fungal communities associated with *Piloderma*, *Ramaria*, and non-mat soils. This study demonstrates that in addition to being the most abundant taxa found on the root-tips within EcM mats, the mat-forming ectomycorrhizal genera *Piloderma* and *Ramaria* are also the most commonly encountered taxa in the bulk soil within their respective ectomycorrhizal mats. Similarly, the non-mat forming EcM genus *Russula* was found to be highly abundant in non-mat bulk soil corresponding to its prevalence on non-mat root-tips. Previously identified as having medium-distance

mat-forming exploration strategies this work indicates *Ramaria* and *Piloderma* species may have a more extensive exploration habit.

Comparisons of fungal species richness, diversity, and taxonomic distributions highlight the heterogeneity of fungal communities in soil and reiterate the findings of fungal hyperdiversity in soil systems. Differences in fungal communities are detected with diminished species richness in *Ramaria* mat soils relative to non-mat and *Piloderma* mat soils. While methodological and sampling biases cannot be ruled out, biological mechanisms contributing to the paucity of fungal taxa within *Ramaria* mats should be further explored. Also observed was the relative deficiency of the common non-mat ectomycorrhizal genera *Russula* in *Piloderma* mat soils. This finding provides a framework to investigate the possible competitive exclusion of *Russula* from forest soils inhabited by *Piloderma* mats.

Finally, Chapter 3 explores the concept that hyperdiversity in fungal systems is largely driven by the presence of a multitude of very rare taxa. The skewed distribution between abundant and rare taxa has led the author to hypothesize that while taxonomically diverse, the functional diversity of a fungal community may be driven by few highly abundant taxa. Our sampling within individual mats rather than pooling samples (as done in many studies) allowed for a more complete understanding of the heterogeneity of fungal communities within the soil system. This finding should be strongly considered when designing microbial community samplings of complex systems.

Chapter 4 explores the bacterial communities found within the same *Piloderma* mat, *Ramaria* mat, and non-mat soils from Chapter 3.  This work represents one of the few studies to analyze both the bacterial and fungal components of a microbial community at the depth afforded by pyrosequencing.  Although relatively shallow sequencing of the bacterial community was conducted, patterns of decreased diversity were observed in *Ramaria* mat soils relative to *Piloderma* mat and non-mat soils, similar to the fungal communities in *Ramaria* mat soils.  While the overall bacterial species richness across all *Ramaria* mat soils was less than the other treatments, within individual mats richness was equal to that of other treatments.  Taxonomic exclusions could not be detected at the bacterial family level used in this work; however it appears that bacterial communities within *Ramaria* mat soils are likely a subset of the diversity seen in non-mat or *Piloderma* mat soils.

When considering the entire microbial community, few bacterial families were segregated based on treatment type. Four bacterial family phylotypes were positively correlated with the *Piloderma* mat samples including Sphingobacteriaceae, Burkholderiaceae, Microbacteriaceae, and Acetobacteriaceae, while no bacterial phylotypes were correlated with *Ramaria* mat soils.  When the data were analyzed using bacterial family phylotype abundances alone strong structuring of communities was observed by soil horizon and along a soil pH gradient.  The concept that soil pH influences the structure bacterial communities is not new, however the observation that soil acidification by biological entities (ectomycorrhizal mat), rather then

pedogenic pH determinants, can cause bacterial community shifts at a fine-scale is somewhat novel and is demonstrated here using next-generation sequencing for the first time.

Bacterial communities largely appear to be influenced by soil pH, while fungal communities were less responsive. The non-independence of mat-type, soil pH, and horizon make further interpretation of these results a bit tenuous. Linear regression models, however, show direct relationships between the OTU richness of bacterial communities and fungal communities in the same sample. Unfortunately, the inability to assign accurate ecologies to large microbial groups (e.g. bacterial families) restricts our understanding of these data at a more functional level. Nonetheless, the results of this work greatly inform our understanding of how hyperdiversity and heterogeneity of microbial communities can confound meaningful analyses and highlight the challenges and importance of sampling design and replication.

**Future directions**

This work, along with the other studies from the H.J. Andrews Microbial Observatory project, provides an excellent framework for future work on ectomycorrhizal mats and microbial community ecology. The author envisions that future directions of this work will include additional explorations of microbial community composition through quantitative PCR estimates of community size, rRNA sequencing to discern metabolically active community constituents, and true metagenomic sequencing to further understand the functional roles of microbial

community members.  This work, as well as other soil microbial community studies, would benefit from an understanding of how the heterogeneity of soils systems demands thoughtful and deliberate sampling to ensure the most powerful and comparable analyses can be utilized.

Understanding microbial community structure requires knowing not only the constituents of the community, but also the size of the community.  While pyrosequencing approaches used in this study are a useful tool in deciphering community composition, they lack information about population size.  The relationship between the fungal community and the bacterial community inhabiting the same niche can only be understood if the relative abundance of fungi to bacteria is known.  While numerous techniques exist to estimate fungal to bacterial biomass ratios, all are only rough approximations.  Of the three most common techniques (phospholipid fatty acid (PLFA) analysis, isotopic carbon labeling, and quantitative-PCR) a quantitative-PCR approach will give us the most consistent and tractable results without the need for additional field sampling.

The community survey conducted in this study independently measures relative rDNA abundances fungi and Bactieria within individual soil samples.  What is not measured is overall abundance between samples.  That is, two samples could appear identical in microbial community composition in our sampling, however, it cannot be known if the overall biomass differs between samples.  Likewise, among individual samples, differences in fungal-to-bacterial biomass ratios could conceivably

exist and contribute to different interpretations of the microbial community as a whole. It would be beneficial for future work in this system to quantify the overall biomass of fungi and bacteria for comparison between samples. Quantitative PCR using fungal specific and bacterial specific primers would likely be the most straightforward approach to determining differences in fungal and bacterial abundances. Relative activities could also be considered, as it remains unclear as to the contributions of metabolically inactive biomass to rDNA sampling.

Activity within soils could be determined by detecting the presence of rRNA transcripts rather than rDNA. The presence of rRNA is a result of active transcription within an organism and, by proxy, could be used as a relative measure of metabolic activity. Multiple methods exist for quantifying rRNA including RT-qPCR, however using a next-generation sequencing platform would allow for higher resolution detection of metabolic activity. Quantifying rRNA may inform the hypothesis that, in fungal communities, only the most abundant taxa, as determined by rDNA abundance, are active, while the long tail of rarely encountered taxa represents resting structures such as spores, conidia, or other resting bodies.

Finally, the interpretation of community data is limited by our understanding of the ecological and metabolic roles of individual taxa or groups. In the absence of comprehensive ecological data, true metagenomic sequencing of environmental genomic DNA or RNA could help elucidate functional patterns associated with EcM mats. Although the methodologies for true metagenomic studies in soil systems are

still being developed, the further advancement of next-generation sequencing will undoubtedly enable the practice in the near future.

One theme of this work has been the highly heterogeneous nature of microbial communities. Samples within a site and between soil horizons of a single sample are often as different from one another as to any other sample. Further investigation into the differences between mat and non-mat microbial communities may benefit from a more fine scale transect-based sampling. Rather than comparing samples from sites across a large geographic region, such as the H.J. Andrews Experimental Forest, a sampling within a 1 m$^2$ grid around an EcM mat may provide a deeper understanding of how microbial communities differ. Furthermore, the heterogeneity of soil communities confounds the concept of experimental replication. In this work, the definition of "non-mat soils" was simply the absence of an EcM mat. While non-mat soil samples did provide meaningful comparisons to mat communities in this study, the variability among non-mat samples was a confounding variable. Even the sampling of multiple "non-mat" soil cores within a 900m$^2$ sampling plot likely fails to characterize the microbial diversity of non-mat soils. Pooling of samples, while often employed in similar studies serves only to dilute the heterogeneity of communities and often oversimplifies comparisons among sampling units.

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**APPENDIX**

Figure S1 - Illumina ITS1 primer design schematic
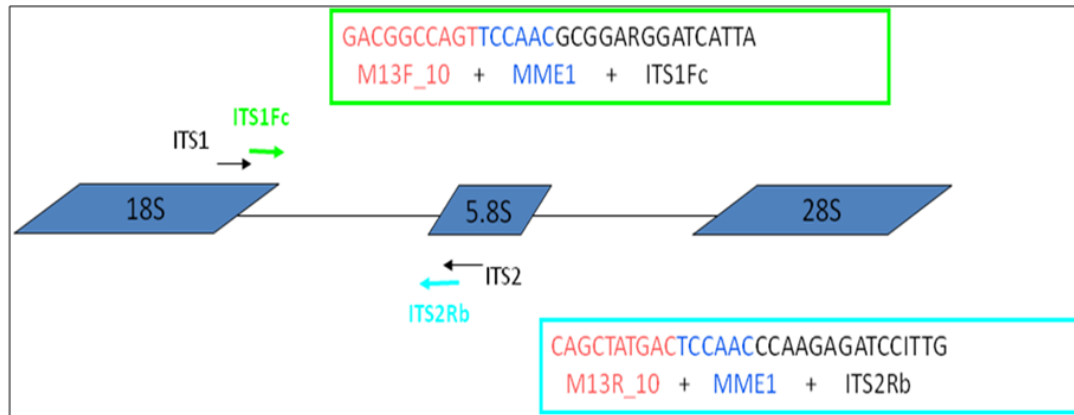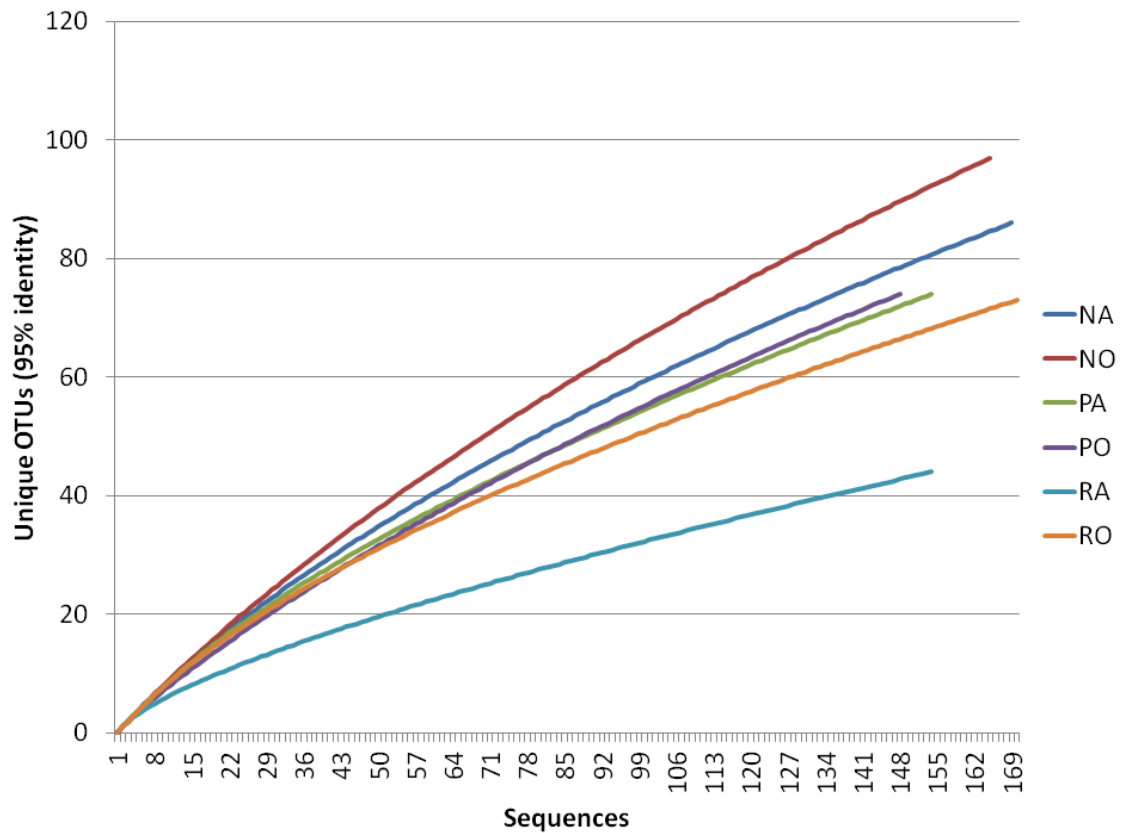


Figure S2 - Rarefaction curves from clone library by treatment

Figure S3 - Rarefaction curves from all clones in library (pooled)

Figure S4 - Class-level distributions across pooled samples

Figure S5 - Ordinal level distributions within the Basidiomycota for single-mat sample NO116-2

Figure S6 - Ordinal level distributions within the Ascomycota for single-mat sample NO116-2
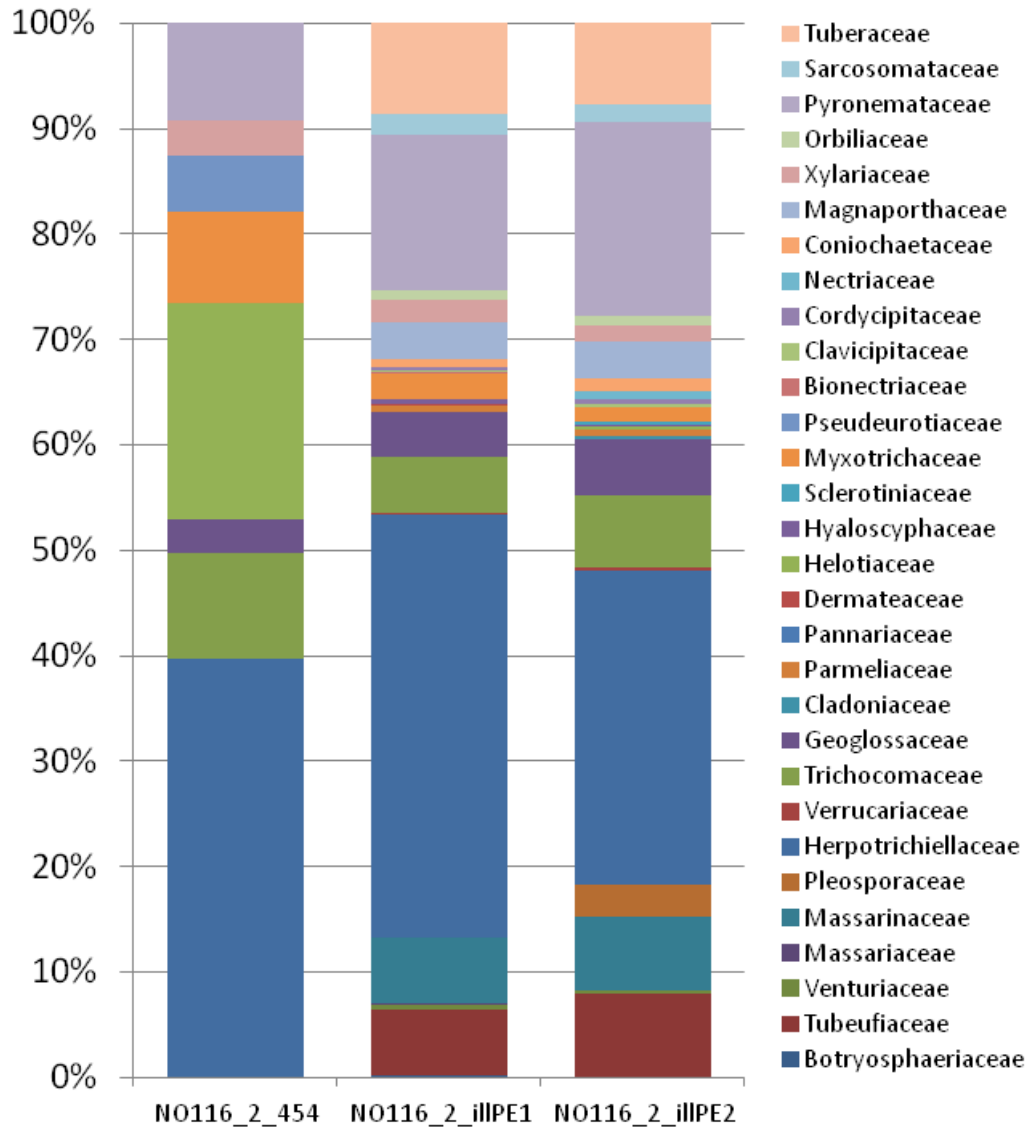
Figure S7 - Family level distributions within the Ascomycota for single-mat sample NO116-2

Table S1 – Shannon Diversity of pooled samples from all treatments and sequencing methodologies.

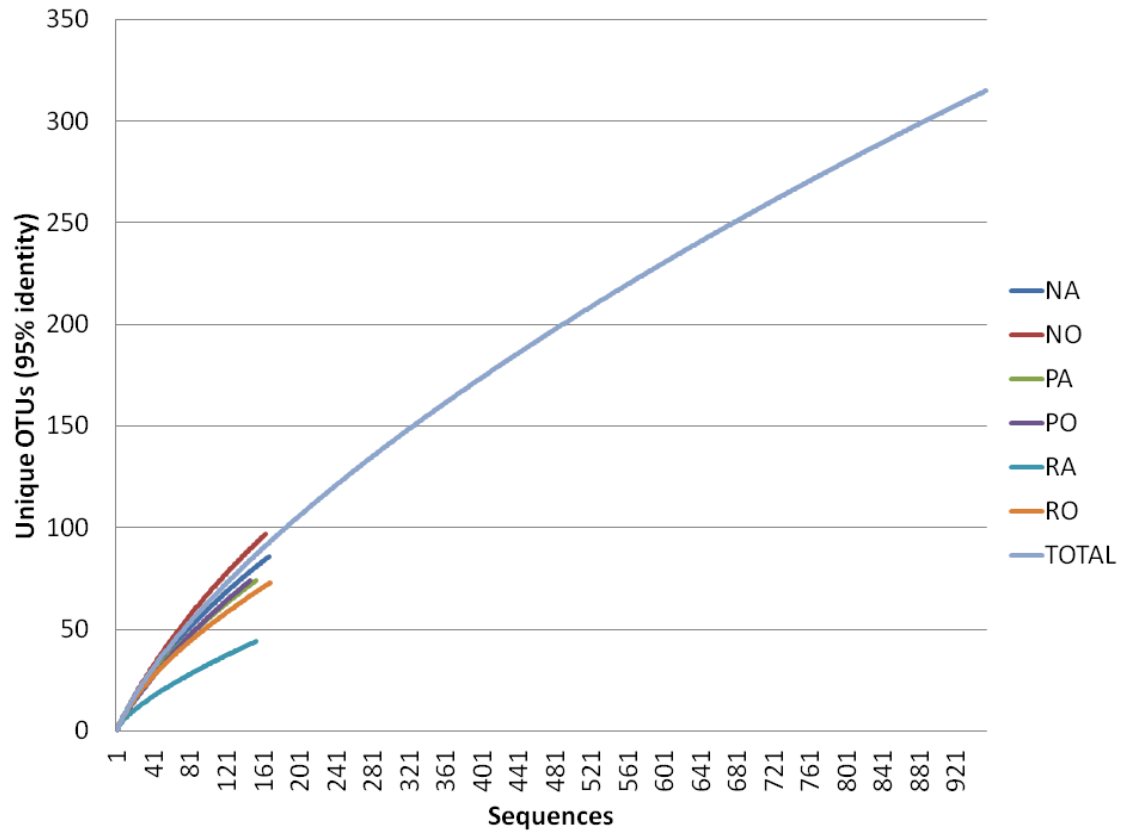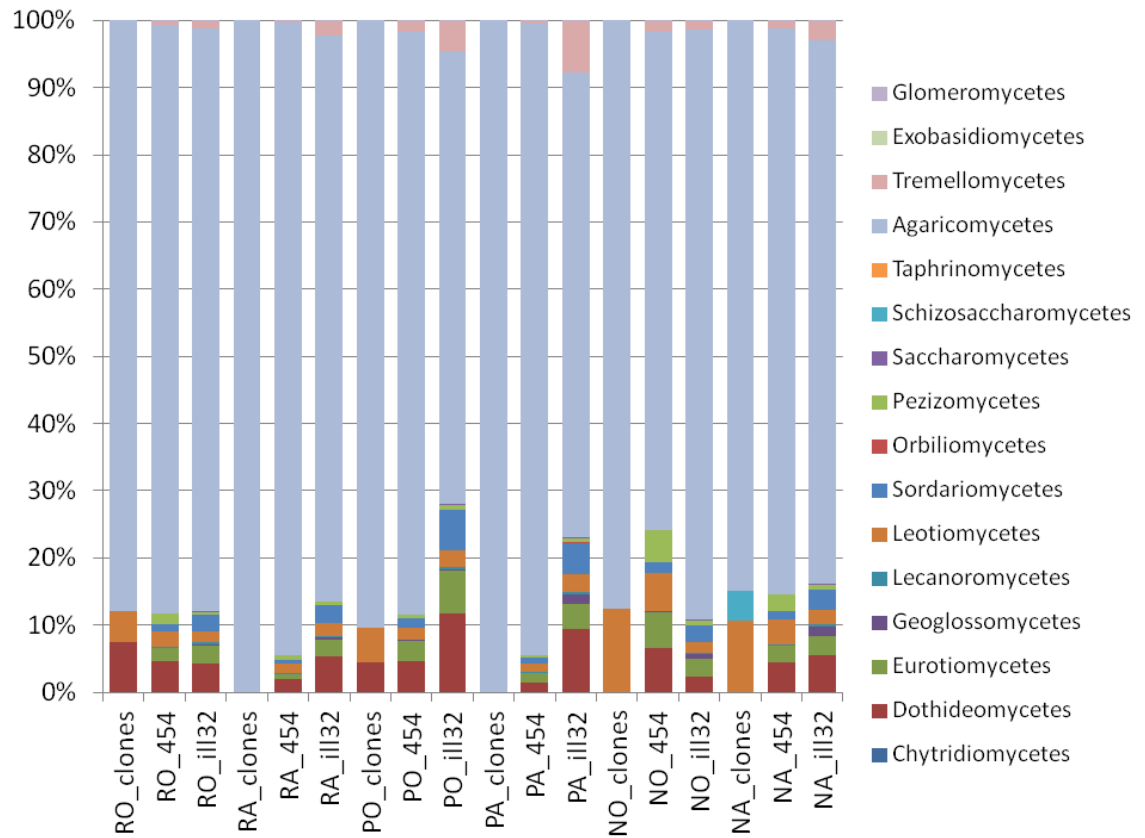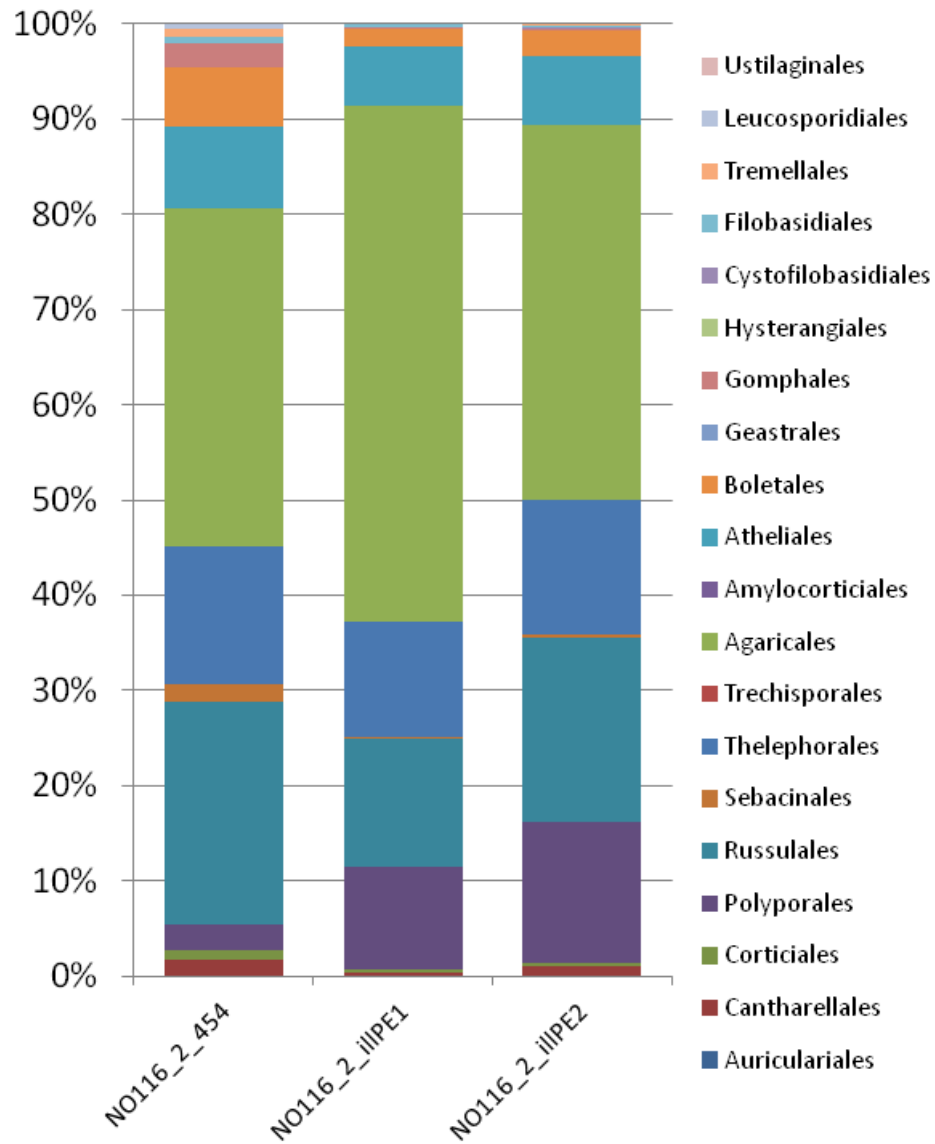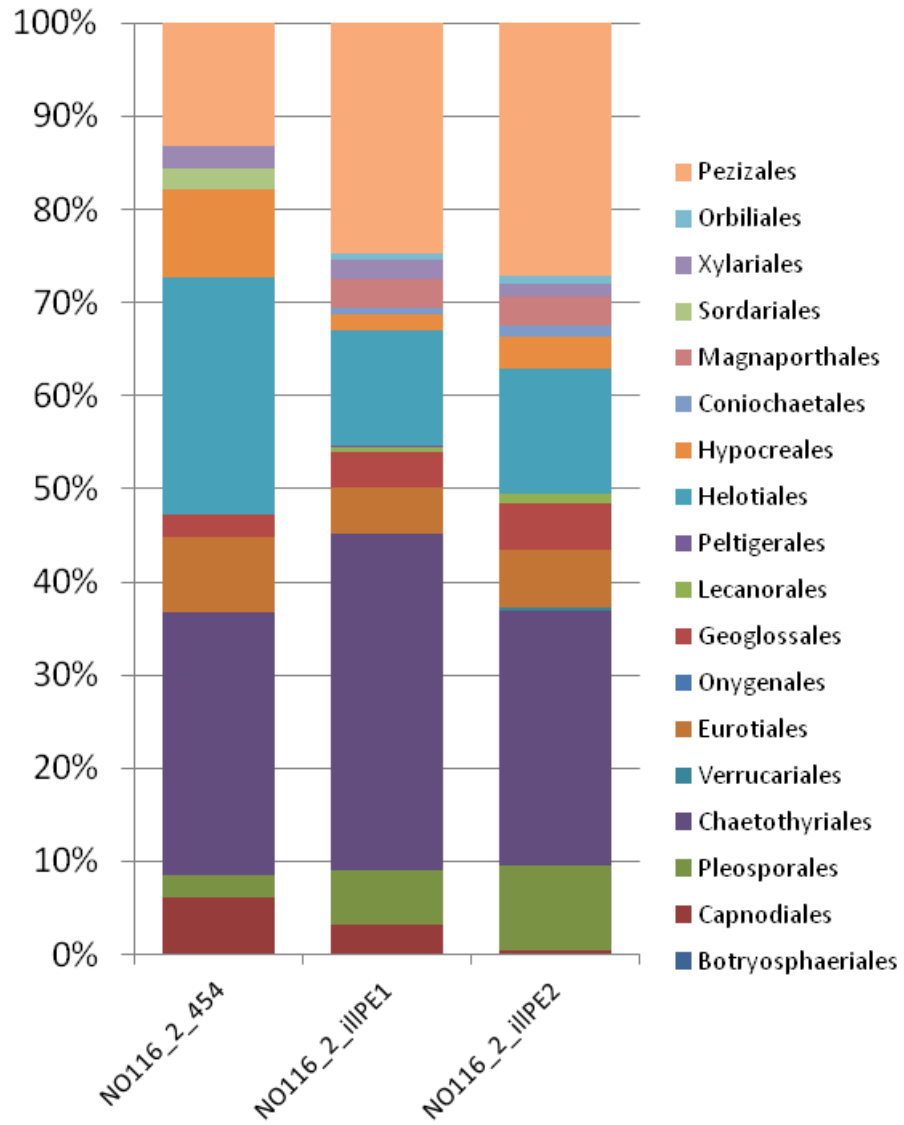|       | ill32 | 454  | Clone |
|-------|-------|------|-------|
| NA    | 4.35  | 3.68 | 4.04  |
| NO    | 4.27  | 4.22 | 4.23  |
| PA    | 6.07  | 4.07 | 3.87  |
| PO    | 6.04  | 3.99 | 3.63  |
| RA    | 3.99  | 2.91 | 2.69  |
| RO    | 3.93  | 3.89 | 3.80  |
| TOTAL | 5.80  | 4.62 | 4.83  |

Table S2 - Fisher's Alpha of pooled samples from all treatments and sequencing methodologies.

|       | ill32   | 454    | Clone  |
|-------|---------|--------|--------|
| NA    | 1383.63 | 92.09  | 70.65  |
| NO    | 1440.23 | 118.48 | 99.78  |
| PA    | 3539.95 | 98.11  | 56.43  |
| PO    | 3156.79 | 107.66 | 59.43  |
| RA    | 921.63  | 65.92  | 20.67  |
| RO    | 1463.21 | 94.37  | 48.80  |
| TOTAL | 4855.84 | 153.17 | 164.20 |

Table S3 - Simpson Index (expressed as 1-D) of pooled samples from all treatments and sequencing methodologies.

|       | ill32 | 454  | Clone |
|-------|-------|------|-------|
| NA    | 0.93  | 0.92 | 0.97  |
| NO    | 0.94  | 0.96 | 0.97  |
| PA    | 0.99  | 0.96 | 0.97  |
| PO    | 0.99  | 0.92 | 0.92  |
| RA    | 0.94  | 0.86 | 0.85  |
| RO    | 0.91  | 0.95 | 0.96  |
| TOTAL | 0.99  | 0.97 | 0.98  |

Table S4 - Sørensen index of similarity for all pairwise combinations of clone library sequences.

|  | NA | NO | PA | PO | RA | RO |
|---|---|---|---|---|---|---|
| NO | 0.32 | | | | | |
| PA | 0.25 | 0.22 | | | | |
| PO | 0.15 | 0.15 | 0.24 | | | |
| RA | 0.22 | 0.17 | 0.15 | 0.14 | | |
| RO | 0.26 | 0.24 | 0.15 | 0.19 | 0.27 | |
| TOTAL | 0.43 | 0.47 | 0.38 | 0.38 | 0.25 | 0.38 |

|      | QC'd   | total OTUs | singletons | % singleton | doubletons | % doubletons | 90% of data |
|------|--------|-----------|------------|-------------|------------|--------------|-------------|
| NO   | 564706 | 8604      | 3279       | 0.58        | 1779       | 0.63         | 161         |
| NA   | 399285 | 7843      | 3374       | 0.85        | 1482       | 0.74         | 168         |
| RO   | 425412 | 8305      | 3778       | 0.89        | 1458       | 0.69         | 176         |
| RA   | 397289 | 5593      | 2129       | 0.54        | 1044       | 0.53         | 79          |
| PO   | 787012 | 17434     | 3966       | 0.50        | 4272       | 1.09         | 829         |
| PA   | 651943 | 18483     | 6231       | 0.96        | 3975       | 1.22         | 814         |

Table S5 - Illumina 32bp sequencing read statistics.  Singletons are OTUs encountered only once in the sequencing, doubletons are encountered only twice.  90% of data column represents the number of the most abundant OTUs that account for 90% of the total sequencing reads in a given treatment.

Table S6 - Sørensen index of similarity for all pairwise combinations of illumina 32bp sequencing treatments

|       | NA   | NO   | PA   | PO   | RA   | RO   |
|-------|------|------|------|------|------|------|
| NO    | 0.58 |      |      |      |      |      |
| PA    | 0.45 | 0.43 |      |      |      |      |
| PO    | 0.25 | 0.28 | 0.59 |      |      |      |
| RA    | 0.24 | 0.22 | 0.31 | 0.27 |      |      |
| RO    | 0.21 | 0.20 | 0.30 | 0.31 | 0.42 |      |
| Total | 0.40 | 0.43 | 0.74 | 0.71 | 0.30 | 0.42 |

Table S7 – 454-FLX ITS read statistics.  90% of data column represents the number of the most abundant OTUs that account for 90% of the total sequencing reads in a given treatment.

|     | QC'd   | total OTUs | 90% of data |
|-----|--------|------------|-------------|
| NO  | 100446 | 799        | 71          |
| NA  | 103539 | 647        | 111         |
| RO  | 91415  | 649        | 74          |
| RA  | 95771  | 480        | 26          |
| PO  | 153602 | 782        | 103         |
| PA  | 133514 | 708        | 75          |