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Nguyen, Nhu Huynh

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Community Ecology of Bacteria Associated with the Digestive Structures of Insects,
Ectomycorrhizal Plants, and Fungi

By

Nhu Huynh Nguyen

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Thomas D. Bruns, Chair

Professor Mary K. Firestone

Professor Ellen L. Simms

Spring 2013

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Ectomycorrhizal Plants, and Fungi

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By Nhu Huynh Nguyen

Abstract

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Nhu Huynh Nguyen

Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Thomas D. Bruns, Chair

This dissertation explores symbiotic microbial community ecology. Symbiosis is a prominent, and yet relatively under-studied phenomenon between two or more organisms. The scope of symbiosis is wide ranging, occurring between macro-macro organisms, micro-micro organisms, macro-micro organisms and everything in between. The span of symbiosis is great, from parasitic to mutually beneficial relationships between the organisms involved. The three chapters presented here comprise an initial look into symbiotic ecosystems using cutting edge sequencing technology. The studies aimed to discover interactions between bacterial microbes and their eukaryotic partners.

Chapter 1 describes the microbes that live in the gut of the passalid beetle, *Odontotaenius disjunctus*. This beetle feeds only on wood, which is low in nutrients, particularly nitrogen. Parallel to the termite-microbe system, I hypothesized that these microbes assist the beetles in digestion of the wood. PhyloChip microarray technology was used to characterize the microbial communities. I found that each gut region (there are 4) of the beetle contained a different microbial community, and that the anterior hindgut of the beetle contained strong anaerobic signals whereas other parts of the gut were more aerobic. The microbes that live in each of these gut regions reflect the oxygen availability of that environment. There was also a signal of potential nitrogen fixation in the anaerobic anterior hindgut.

In chapter 2 and 3, I explored fungal-bacterial interactions. Fungal-bacterial interactions have been sparsely characterized, appearing sporadically in the literature. Some studies describe bacteria found on the outside of the fungal hyphae, other times on the inside. One remarkable study found that a pathogenic fungus was not pathogenic without its toxin-producing endosymbiont. Other studies focused on the interaction between leaf-cutting ants, fungi, and bacteria associates. Of the estimated 1.5 million species of fungi, this interaction must be tremendously widespread and waiting to be characterized.

In chapter 2, I explored the microbial communities that live with various ectomycorrhizal fungi. Mycorrhizal fungi exchange mineral nutrients with plants for photosynthetic carbon. The experiments were designed to test for species and/or community specificity of bacteria to their

fungus hosts. Several studies have aimed to answer similar questions, but the difference between the experiments presented in this chapter and others is that this involved temporal component, many more samples, and 454 sequencing that produced many orders of magnitude more sequences. The results showed that fungal species strongly determines the bacterial community. *Burkholderia* and members of the Rhizobiales were the most commonly encountered bacteria. Some of these have been found by other researchers, indicating a tight relationship between them.

And finally, in chapter three, I explored the interactions between saprobic fungi that form fungal mats in the soil and the bacteria that live with them. I tested for differences in bacterial community between fungal mat and non-mat leaf litter, whether fungal species has an influence on the bacterial community, and whether any distinct taxonomic group of bacteria were associated with the fungi. 454 pyrosequencing technology was employed for this purpose. The results showed that there is a strong difference in bacterial community composition between mat and non-mat litter. The Actinobacteria, particularly a *Pseudonocardia* species, showed strong preference for fungal mats. *Pseudonocardia* species are known for their antibiotic production and have been reported growing with other organisms, the most famous being the interactions between leaf-cutting ants, fungi, and *Pseudonocardia*.

Together, these three chapters provide different windows in which to peer into the world of microbial symbiosis, particularly those of bacteria with animal, plants, and fungi. It can be concluded that different symbiotic environments will select for different communities of bacteria, such as different gut sections and the presence of different species of fungi. When high throughput sequences of these environments are examined carefully, they can reveal clues into the important organisms that persist and participate in the ecology of said environments, priming for more extensive studies in symbiosis.

This dissertation is dedicated to something heartfelt, for without it,
these works would have been worthless.

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Although true Dutch I have yet learned
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Who fed me Cheez-It in a bowl
Like a docile puppy on the ground below

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Whose name now is Schechter
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And always sarcastically strong

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Brings laughs and cheers and jokes
Especially ones that made Tom choke

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For without the conversations we've had
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To my committee
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And surely without thee
Graduation will not come to me

To thousands of pines made sacrificed
Come not to me in the afterlife
For I am grateful to the dead
When comes to me my future bread

To those many whose paths I've crossed
To miss your names is truly a loss
Alas my mind is now without
Even strong coffee cannot help me out

Table of Contents

Abstract	1
Dedication	i
Acknowledgements	ii
Table of Contents	iv
Introduction	v
Chapter 1: Compartmentalization of microbial composition in the gut of wood-feeding passalid beetles (<i>Odontotaenius disjunctus</i>)		1
Table	14
Figures	15
Chapter 2: Bacteria within ectomycorrhizal root-tips and their potential for nitrogen fixation from within		20
Tables	32
Figures	33
Chapter 3: Bacterial communities associated with saprobic fungal mats with the potential for antagonistic and mutualistic symbioses		41
Tables	56
Figures	61

Introduction

Symbiosis – it's roots came from the Ancient Greek *σύν* "together" and *βίωσις* "living". The current usage of the word describes the relationship between two or more organisms that coexist on a sliding scale of interactions from negative, to neutral, to positive. Organisms enter into symbiosis relationships because they want something. Some organisms only take and we call these parasites, whereas others will give and take, and we call these mutualists, and others will interact but have no observable effects and we call them commensals. The mathematical possibility of all organisms on earth that could interact with each is immense and at this point incalculable because we have yet to find all the organisms that exist. Despite this shortfall, we have been able to characterize a number of interesting symbioses, some of which are directly relevant to the work presented in this dissertation.

The digestive structures of organisms are good places to look for symbiotic relationships. As the host organism digests the food, making recalcitrant compounds more available, others will be there to advantage of that resource. In return, however, these other organisms may also provide something else in exchange. Animals digest their food by secreting enzymes into their stomachs and then absorbing the nutrients. However, for certain groups of animals, such as those that feed on nutrient-poor plant xylem, digestion for the most part depends on microbial symbionts, particularly bacteria and flagellates, that live inside their guts. Some of the more well-characterized gut symbiotic relationships of wood-feeding organisms involve termites (Ohkuma 2003, Tokuda & Watanabe 2007), wood roaches (Slaytor 1992; Klass *et al* 2008), and bivalve ship-worms (Sipe *et al* 2000). These microbes function as an important component of gut communities, with each functional group providing different enzymes that break down the wood, synthesize or transform inert elements (like dinitrogen) into components usable by the host.

Bacteria are also involved in extremely important symbiotic relationships with plants, some of which are well-studied because these symbioses have a direct impact on human agriculture. The best-studied of these are the nodulating, nitrogen-fixers that associate with legumes. These soil bacteria can dwell within protected plant organs called nodules that protect their fragile nitrogenases from oxygen. Within these structures they fix atmospheric nitrogen, which is then passed on to the plants (Vincent 1982). Less well-known and well-studied are the bacteria that live with plants and their ectomycorrhizal associates. These are rhizospheric bacteria (bacteria that live in the soil area influenced by roots) that can influence the establishment of mycorrhizal fungi and their plant hosts (Garbaye 1994 and Frey-Klett *et al* 2007). These are difficult systems to study due to the less specific relationship and the many organisms involved. Nevertheless, a number of studies examining the relationship between bacteria, ectomycorrhizal fungi and plants have discovered patterns of specificity between certain groups of bacteria with ectomycorrhizal root-tips (Bending *et al* 2002, Izumi *et al* 2007, 2008, Burke *et al* 2008, Tanaka & Nara 2009, Izumi *et al* 2013).

Fungi are the least well studied of the aforementioned three groups of organism that have bacterial symbionts. Evidence of strict symbioses are few (and most likely due to the interest of researchers) but those that have been found stand out as spectacular examples in the world of symbiosis. One such example involves the endohyphal bacteria of the genus *Burkholderia* and the pathogenic fungus *Rhizopus microsporus* (Partida-Martinez & Hertweck 2005). For a long

time it was thought that *R. microsporus* produced toxins that killed rice seedlings post-infection. It turned out that bacterial symbionts were the actual toxin producers. Furthermore, it seems that these two organisms have had a long evolutionary history because when the fungi were deprived of bacteria, they lost the ability to produce asexual spores and could only be cured by reintroduction of the symbiont (Partida-Martinez *et al* 2007). Other less spectacular examples include isolation of bacteria that live within the spores of arbuscular mycorrhizal fungi (Bianciotto 2003) and isolation of bacteria on fungal hyphae (Lim 2003). Characterizing what the bacterial symbionts do in association with these fungi is necessary to understand their symbiotic relationship.

The current thinking in symbioses have moved beyond the interactions between two species and expanded to include whole communities (i.e. multi-partner symbioses). Traditionally, organisms have been isolated, identified, and eventually tested against/with each other and the outcome observed. Now, powerful methods such as 454 pyrosequencing have allowed researchers to sequence deeply into the environment of interest to find organisms that would be masked from identification due to their low abundance. In two of the three chapters in this dissertation, I used this technology to identify the bacteria that would otherwise not be found using more traditional sequencing or culturing methods. In Chapter 1, I used PhyloChip microarrays to identify bacteria and archaea in the gut of wood-feeding beetles. In Chapters 2 and 3, I used 454 pyrosequencing to discover the bacteria that live with ectomycorrhizal and saprobic fungi. This work contributes to the understanding of bacteria and their symbiotic relationships in less-studied systems of wood-feeding beetles and fungi.

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CHAPTER 1

Compartmentalization of microbial composition in the gut of wood-feeding passalid beetles
(*Odontotaenius disjunctus*)

Abstract

Coarse woody debris represents an important amount of biomass in forest ecosystems. Different groups of insects have evolved to take advantage of this resource, including the wood-feeding beetle *Odontotaenius disjunctus* (family Passalidae). During its life cycle, the passalid beetle consumes large amounts of nutrient-poor wood and survives this diet through symbiotic interactions with its gut microbiome. To characterize the passalid beetle gut microbiome, PhyloChip microarray technology was used to identify the microbes that live in each morphologically differentiated gut region. The results showed that each gut region contained a different microbial community, which is correlated with oxygen availability. The anterior hindgut region stands out as an important region in which anaerobic processes such as nitrogen fixation and methane production could occur. This study contributes to the initial body of data, which will lead us towards the understanding and eventual production of a biofermenter with the ability to convert cellulosic material into ethanol.

Introduction

Coarse woody debris makes up a tremendous amount of biomass in forest ecosystems. As a result, a number of insect groups have evolved to take advantage of this abundant resource, forming specialist wood-feeding guilds. Among the most prominent groups of insects that have specialized in this manner are the termites (order Isoptera) and specific groups of beetles (order Coleoptera). These insects are economically important due to the destructiveness of some of their members, for example subterranean termites, powderpost beetles (family Bostrichidae), bark beetles (subfamily Scolytinae), and longhorn beetles (family Cerambycidae). The ability of such insects to perform mechanical and enzymatic breakdown of coarse woody biomass is of critical importance to the ecology of these ecosystems (Kaufman *et al* 2000; Moran 2007).

The ability to subsist on woody biomass is due to symbiotic associations between these insects and their gut microorganisms (Ohkuma 2003; Tokuda & Watanabe 2007). Much of the work in this area so far has focused on termites, which are divided into two groups, the lower and higher termites (Wood 1986). The lower termites are composed of six phylogenetically distinct families that are dependent on both gut flagellates and bacteria to aid in digestion. In contrast, the higher termites, which make up approximately 85% of all termites, belong to a single family Termitidae (Ohkuma 2003). These termites rely solely on symbiotic bacteria and archaea to aid in digestion. Despite their ability to break down woody biomass to obtain carbon and energy, the high carbon(C):nitrogen(N) ratio of this substrate results in N limitation. To alleviate N limitation, wood-feeding termites harbor nitrogen fixing microorganisms in anaerobic gut regions (e.g. reviewed in Brune & Ohkuma, 2011). Despite this very important biomass recycling process and the diversity of insects involved, most work to date has focused on termites. Even though wood-roaches have gained some attention (Slaytor 1992; Klass *et al* 2008), only limited attention has been paid to the gut microbiome of wood-feeding beetles (Suh *et al* 2003, Zhang *et al* 2003, Nardi *et al* 2006).

A representative of the beetle wood-feeding guild is *Odontotaenius disjunctus* (family Passalidae), a large beetle that can reach ~30mm in length and is common and widespread in the eastern United States from Florida, west to the Rocky Mountains and north to Southern Canada. They spend their entire lives (14 -16 months) within decayed wood of class III or above (visual and physical signs of decay present) where they feed and raise their larvae in subsocial communities (Krause & Ryan 1953; Pyle & Brown 1998). Adult beetles feed larvae a mixture of macerated wood and frass to replenish the microbes lost while shedding of the sclerotized hindgut during larval development (Nardi *et al* 2006). Observations of this behavior provided initial indications of the importance of microbial symbionts to these beetles.

It is surprising that even with their large size, widespread distribution, and common occurrence in decaying logs, this species had not been studied extensively. The first reported gut microorganism from this beetle was the protozoan *Gregarina passali-cornuti* (Leidy 1852). Its communities of phoretic arthropods and gut organisms were more extensively reported by Pearse *et al* (1936); followed a decade later by life history studies by Gray (1946). More recently, many yeasts were cultured from the gut (Nguyen *et al* 2006; Suh *et al* 2003). One of these yeasts is the commercially important species, *Scheffersomyces stipitis* (syn. *Pichia stipitis*), which has the

ability to ferment xylose, a significant component of hemi-cellulose (Shi *et al* 2010). Zhang *et al* (2003) identified a number of yeasts and other gut eukaryotic organisms using vector cloning techniques. Subsequently, in a detailed microscopic study of the gut morphology and gut inhabitants, Nardi *et al* (2006) observed that the gut inhabitants (which included bacteria, flagellates and fungi) seemed to partition themselves within each of the four morphologically distinct sections.

The range, gut morphology, microbiology, and potential industrial importance of this beetle make it an attractive organism to study, with the overreaching goal of developing a biofermenter system based on the beetle gut system. However, even initial details of the microbial community that resides within each gut region are still missing. This work characterized the microbial community structure within the digestive tract of this beetle to test whether morphological and physiological differences among the four gut regions (Figure 2, Nardi *et al* 2006) allow the existence of distinct microbial communities within. In addition, phylogenetic data was also used to determine if the microbial community within each gut section clustered together, indicating that perhaps habitat filtering (biotic and abiotic conditions within a habitat that determines the existence of a group of organism) was important in shaping the microbial communities of the gut.

Materials and Methods

Specimens

Eight specimens of uniform size - measuring approximately 30mm in length - were collected from a single oak log in Baton Rouge, LA, USA (30° 24.98'N 91° 7.18'W). Each beetle was kept separately in individual containers with the wood pieces that they were found with. Dissection and bacterial community identification were made approximately two weeks after the collection date.

Beetle dissection and DNA extraction

All the beetles were surface sterilized by immersing in 95% EtOH for 2 minutes followed by a wash in sterile phosphate buffer saline (PBS). Individual beetles were dissected in sterile phosphate buffer saline solution by first removing the elytra to expose the membranous dorsal side. Subsequently, the wings were removed and the cuticular membrane was dissected to expose the natural gut alignment within the abdominal cavity (Figure 1). The whole gut was then removed, stretched out and cut into four sections (foregut – FG, midgut – MG, anterior hindgut – AHG, posterior hindgut – PHG, Figure 2). Each section was placed in 1 ml RNALater (Qiagen, CA, USA) and stored overnight at 4 °C before extraction. Crude extracts were prepared by bead beating each gut section in 750µl RLT Buffer (Qiagen) and Lysing Matrix E (Qbiogene Inc., CA, USA) for 30 s at 5.5 m/s, cooling for 1 minute and repeating the process for another 30 s. Nucleic acids were separated from the mixture by standard phenol-chloroform phase separation techniques and precipitated with ethanol. Crude nucleic extracts were further purified using the AllPrep DNA/RNA kit (Qiagen) according to manufacturer's instructions to simultaneously separate the DNA and RNA fractions.

pH measurement

In a different set of 4 beetles, I measured crude pH values of each gut segment by dissecting out each portion and homogenizing the gut contents in 30% water (weight by volume) pH was measured for each gut region using the Satorius Basic pH Meter PB-11.

PCR amplification for PhyloChip analyses

For this experiment, 4 beetles (replicates) and 4 gut sections per beetles (n = 16) were chosen. PCR reactions were performed three times per sample with three different annealing temperatures using 5 μ l 1X Takara ExTaq PCR buffer with MgCl₂, 300 pM of primers 27F (GTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) for bacteria, 1492R and 4fa (TCCGGTTGATCCTGCCRG) for archaea, 1 μ M BSA, 200 μ M dNTPs, 2.5 U ExTaq DNA polymerase (Takara Mirus Bio Inc., WI, USA), 5 ng template, and milliQ H₂O to complete 50 μ l volume. The complete PCR cycle included an initial denaturation at 95°C for 3 min, followed by 25 cycles at 95 °C for 30s, annealing at 48 °C, 53 °C and 56 °C for 25s, extension at 72 °C for 2 min and a final extension of 72 °C for 10 min. Products from the three different annealing temperatures (150 μ l) were combined and concentrated using ethanol precipitation (15 μ l 3M sodium acetate and 300 μ l 100% ethanol). Pellets were resuspended in TE buffer and purified using a PCR purification kit (Qiagen) before quantification by gel electrophoresis.

PhyloChip hybridization and sample detection

Microarray construction, methods for labeling, hybridizations, detection, and quantification are described in detail by Brodie *et al* (2006). The G2 PhyloChip contains more than 300,000 probes that target 8741 bacterial and archaeal taxa. From the combined PCR reactions, 200 ng of bacterial and 50 ng of archaeal PCR products were fragmented with DNase I, biotin labeled, hybridized, washed and stained according to the manufacturer's recommended protocol. In total, 16 microarrays were analyzed. Each PhyloChip was scanned and recorded as a raster graphics image, and initial data acquisition and intensity determination were performed using standard Affymetrix software (GeneChip microarray analysis suite, version 5.1). A taxon/OTU was considered present in the sample when over 90% of its assigned probe pairs were positive in at least 3 of the 4 replicates per gut region.

Statistical analysis of PhyloChip data

All statistical analyses were carried out in the R programming environment (R Development Core Team 2008). The corrections for variation associated with quantification of amplicon target and downstream variation associated with target fragmentation, labeling, hybridization, washing, staining, and scanning were performed as detailed in Brodie *et al* (2006). The relative abundances (normalized fluorescence units) for each taxon were compared across the 4 gut regions using ANOVA. The resulting p-values were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) procedure (Benjamini & Hochberg 1995). The resulting data was a list of OTUs which were significant within each gut region. Using these data, I calculated the Shannon Diversity Index (H') for each gut region and a comparison of community similarity using PERMANOVA. I also used these data to determine the presence of certain taxa between different gut regions. For instance, I compared the FG vs. MG & MG vs. FG and MG vs. AHG & AHG vs. MG. Only pairwise-adjacent gut regions were analyzed because I view the gut as a linear space with a uni-directional flow of gut content and thus only comparisons between regions adjacent to each other are meaningful. I also generated a

phylogenetic tree for the detected bacterial taxa as detailed in Goldfarb *et al* 2011 and visualized and annotated using the interactive tree of life (ITOL) web server (Letunic & Bork 2007).

Phylogenetic community analysis

Phylogenetic community analyses (also known as PhyloCom) were performed using the Picante R package (Kembel *et al* 2010). This test uses phylogenetic information to understand how related taxa are distributed among ecological communities. A maximum likelihood (RAxML, Stamatakis *et al* 2005) tree of all sequences targeted by the G2 PhyloChip was used as the initial tree. Taxa not detected in all gut regions were trimmed from the tree, resulting in a tree with 874 taxa. I used the tip shuffling model (model 1) running 10,000 replicates (Webb *et al* 2008). In this model, the tips (individual taxa) are shuffled throughout the phylogeny and the resulting metrics were compared to the observed value using $\alpha = 0.05$. The tip shuffling model was determined to be robust for PhyloCom analysis with a low level of Type I error (false positives; Hardy, 2008). I used the NRI (net relatedness index) metric to measure phylogenetic clustering of microbial communities across the distinct gut regions. I also calculated total phylogenetic diversity (PD), which is the sum of all the branch lengths of a tree (Faith 1992).

Results

Beetle internal anatomy and physiology

Figure 1 shows the dorsal gut alignment within the abdominal cavity of *O. disjunctus*. The foregut (FG) resides within the head and part of the thorax (not dissected). The convoluted midgut (MG), the anterior hindgut (AHG), and the posterior hindgut (PHG) reside within the abdominal cavity. The midgut is visible as the longest section, where many white tracheal tubes are attached. Both sections of the hindgut lie directly above part of the midgut. The anterior hindgut lies above a layer of tracheal tubes (not visible) whereas the posterior hindgut lies immediately adjacent to part of the midgut that is surrounded by tracheal tubes. The whole abdominal cavity is covered by a thick cuticle layer protected by a pair of wings (removed) that lock together and only open during flight, which rarely occurs in the species.

The typical length of the whole gut measured 12 cm with the FG being the shortest section and the MG being the longest section (Figure 2). Each gut section is morphologically differentiated overall internal and external morphology (Nardi *et al* 2006, Figure 2). The FG contains freshly macerated wood material, which presumably is aerobic.

Among the four beetles dissected for our array analysis, each gut type of segment for the four beetles dissected for our array analysis was quite uniform in weight and averaged as follows: FG = 13.3 ± 2.1 mg, MG = 263.5 ± 37.9 mg, AHG = 125.5 ± 21.4 mg, and PHG = 5 ± 11.8 mg. All individual gut segments yielded abundant DNA/RNA for amplification. Bacterial 16S rRNA gene amplification was successful for all gut regions. However, archaeal 16S rRNA genes were only successfully amplified in the anterior hindgut region.

General pH values were measured for each gut region from a different set of four beetles (Table 1). The pH of the MG was more alkaline than the all other regions ($p < 0.01$ for midgut vs. all other region) whereas the posterior hindgut was more acidic than either the foregut or the midgut ($p < 0.05$, Table 1).

Community composition of gut regions

For OTU/taxon detection, a taxon was considered present in a gut region when the florescent probe threshold ($pf \geq 0.9$) was found in at least 3 of the 4 replicate beetles. This stringent criterion further minimizes false positives. Based on these criteria, I determined the combined total bacterial and archaeal OTUs of all gut regions to be 1521 (1498 Bacteria, 23 Archaea). The foregut contained the highest richness with 1179 bacterial OTUs, the midgut contained 825 bacterial OTUs, the anterior hindgut contained 707 bacterial and 23 archaeal OTUs, and the posterior hindgut contained 937 bacterial OTUs (Table 1). The phylogenetic diversity (PD, Faith 1992; Kembel *et al* 2010) and the Shannon diversity index (H') are also reported in Table 1.

Members of the Phyla Proteobacteria and Firmicutes make up the highest diversity of bacteria inhabiting all four gut regions. The Proteobacteria made up of 41% and Firmicutes made up 23% of the total diversity. These phyla also comprised the major diversity in the gut of the wood-feeding beetle *Anoplophora glabripennis* (family Cerambycidae) and were the sole inhabitants of the gut of the Southern Pine Beetle *Dendroctonus fontalis* (family Curculionidae, Schloss *et al.* 2006, Vasanthakumar *et al.* 2006), suggesting that at least these two phyla are important in the gut microbiota of wood-feeding beetles. The next most abundant groups (each makes up about 5-9% of the total diversity) are the Bacteroidetes and Actinobacteria.

The anterior hindgut was unlike the other gut regions. In all other regions, the Proteobacteria were most diverse, followed by Firmicutes, Actinobacteria and Bacteroidetes. However, in the anterior hindgut, the pattern changes to Firmicutes being the most diverse, followed by Proteobacteria, Bacteroidetes and Actinobacteria.

Figure 3 visualizes taxa that are significantly present from at least one gut region when compared to all other regions. These taxa are indicated by a black bar. The majority of taxa detected (75%) showed significant differences in distribution across gut region. As a general pattern, these differences tend to be found in the foregut and the anterior hindgut. This overall pattern can be compared to the hierarchical clustering of gut sections below.

Hierarchical clustering (Figure 4) of the bacterial communities within each of the 16 gut sections (four regions of four different beetles) demonstrated that the community of the foregut and anterior hindgut formed two distinct clusters (clusters A and D). These were distinct from the clusters that comprised the midgut and posterior hindgut community (clusters B and C). The latter two regions were not resolved, rather clustering together by beetle (cluster B). When the communities of the different gut regions were compared to each other through PERMANOVA, the results showed that different gut regions contained statistically different bacterial communities ($p = 0.005$) and that these differences explained 39.5% of the variation within the dataset ($R^2 = 0.395$).

To determine which groups of bacteria significantly differed in abundance between gut regions, I compared linearly adjacent gut regions to each other (columns 1-6, Fig. 5). Then metabolic functional groups were clustered for a better visualization (groups A-H, Fig. 5). The general results showed that each region contains a metabolically different group of organism than the adjacent region. For example, the foregut contains more aerobic organisms (groups A, E, H) than are contained in the adjacent midgut (column 1) and the midgut contains more aerobic organisms than are contained in the anterior hindgut (column 3). The anterior hindgut is composed almost

entirely of anaerobic organisms (groups C, D, F) when compared to both the midgut and the posterior hindgut (columns 4 and 5). And the anterior hindgut contains more anaerobic organisms (groups A, E, H) than the posterior hindgut (column 5). The overall pattern of the anterior hindgut is that it is composed almost entirely of anaerobic organisms.

To test for phylogenetic clustering of microbes in each gut region, I used Phylogenetic Community Analysis (PhyloCom). A quantitative result of this analysis is indicated by the net relatedness index (NRI), where a positive NRI represents communities that are clustered by phylogenetic relatedness and negative NRI represents communities that are not phylogenetically clustered (=dispersed). Our results show that the community within each gut region is significantly clustered, with the more aerobic regions (foregut, midgut, posterior hindgut) showing the greatest clustering ($p < 0.001$, Table 1). The anterior hindgut (presumably the most anaerobic) showed the least clustering.

Discussion

Odontotaenius disjunctus is one of the largest arthropod wood-feeders, with a broad distribution throughout eastern North America (Pearse *et al* 1936). These beetles inhabit decaying wood and survive on a low nutrient diet. Other insects such as termites also belong to this feeding guild, where the gut contain symbiotic organisms such as bacteria and flagellates that contribute with lignocellulose digestion and nitrogen fixation. The beetles show a subsocial behavior (similar to faculty at major universities), where they exhibit a crude form of parental care, feeding their young macerated wood well mixed with their own fresh feces.

The wood-feeding habit, microbial associations, reinoculations, and morphologically differentiated gut segments that are linked with different bacterial groups provide an interesting environmental model to study microbial community assembly processes. Some factors that can affect community assemblages include pH, availability of oxygen, redox potential, biotic interactions amongst the organisms, etc (Dillon & Dillon 2004). I made crude pH measurements of the gut contents suspended in water (comparable to the methods used to determine termite gut pH by Bignell & Eggleton 1995). Unlike the higher termites, in which at least one gut region (hindgut or paunch) is extremely alkaline (as high as 10.5), the beetle gut environment measured near neutral in most cases, the exception being the midgut, with an average pH of 8.38 (Table 1). This is in contrast to pH conditions found in many other insect and mammalian guts, where extremely alkaline or acidic environments are required for proper food digestion (Bignell & Eggleton 1995, Hongoh 2011). At the moment, I do not wish to correlate community differences with crude pH measurements. More precise measurements using microelectrodes must be performed in order to determine how pH and other chemistries correlate with the patterns observed in this study.

Community partitioning among the gut regions

Our results showed that each of the four gut regions had significantly different communities of microbes. The communities that reside in the foregut were different from those in other gut regions. This outcome is not unexpected, since the foregut is only a small region that allows for the passage of freshly macerated food particles. It would not be surprising if the community of

microbes in this aerobic gut region were similar to the ones living in the gallery (holes in which the beetles make in a log) walls of the beetles as preliminary data indicate (data not shown).

The similarity of community profiles between the midgut and posterior hindgut could perhaps be due to food material passing through transiently. The walls of these gut regions do not have any specialized structures (pockets, caecum, etc.) to support the establishment of microbial reservoirs like the anterior hindgut (Nardi *et al.* 2006). However, these gut regions are more anaerobic than the foregut, which could explain why their community composition differs from the foregut (Fig. 5).

The anterior hindgut is the most morphologically differentiated gut region with pockets, folds, and internal projections (Nardi *et al.* 2006), which could allow for a diversity of niches. Greater inferred niche diversity was expected to support higher OTU diversity based on competitive exclusion, but our results showed that this gut region contained the lowest diversity. However, the microbial community in this morphologically distinct region is distinguished by high concentrations of organisms specialized for anaerobic living (columns 4 & 5, microbial groups D & F in Fig. 5). Their metabolism is dependent on an anaerobic environment. It is only within this gut region that archaeal methanogens such as *Methanosarcina* and *Thermococcus* species were detected. Since these microbes produce methane, it was not surprising to find them complemented by methane oxidizers (in group F, Figure 5). Thus, even if there is a broader inferred niche diversity, these niches are only available to anaerobic organisms, which are much less diverse than aerobic organisms in our study system.

The anterior hindgut was the only region in which there was a very high relative probe intensity of nitrogen fixers (G & H in Fig. 5). These organisms, including the spirochetes, have been shown to be important nitrogen fixers in termite guts and other environments (Lilburn *et al.* 2001, Ueki *et al.* 2006). The anaerobic environment of the anterior hindgut and low nitrogen availability in wood make this gut region very favorable for nitrogen fixation. Since wood is very poor in nitrogen, it is likely that a large portion of the nitrogen taken up by the beetles came from fixed nitrogen through their microbial symbionts.

These results taken together indicate that chemical and physical environments within each gut region have the ability to influence the microbial community therein. Based on the strong presence of certain groups within each gut region, I predict that oxygen is likely an important factor that contributes to community partitioning within each gut region. Future work should measure oxygen gradients within the gut and correlates that to microbial communities.

Community phylogenetic structure

Phylogenetic clustering in communities is interpreted as evidence of habitat filtering where a group of species possesses certain traits that allow them to persist in a particular environment (Webb *et al.* 2002, Horner-Devine & Bohannan 2006). For example, nitrogen fixing *Rhizobium* tend to be found in association with legumes and different species may even be found in the same habitat. They cluster together phylogenetically, but they also cluster together based on the traits of living within plant nodules. Alternatively, this clustering can also be interpreted as the ability of a group of organisms to disperse and/or colonize an environment or due to an adaptive radiation event. I used this method to determine if the gut environment causes phylogenetic

clustering, which in turn would indicate that the gut may serve as a filter for certain groups of bacteria. This analysis uses 16S rRNA genes to determine a cluster of species. Because of the slow rate of evolution in this gene it is unlikely that any group could have radiated into a species cluster that can be detected by the 16S rRNA gene over a 2-3 year lifetime of the beetles. Therefore adaptive radiation cannot be used to explain the patterns observed in the overall clustering of the beetle gut community. The opposite of phylogenetic clustering is phylogenetic overdispersion, which is interpreted as results of negative interactions such as competition and competitive exclusion (Ackerly *et al.* 2006, Cavender-Bares *et al.* 2006).

I used the metric NRI in the software package picante (PhyloCom) to measure phylogenetic clustering and overdispersion in the gut community. Positive NRI values indicate phylogenetic clustering and negative NRI values indicate phylogenetic overdispersion within a community. A complementary p-value to NRI of ≤ 0.05 indicates that the clustering or overdispersion did not result from stochastic processes. The results show positive NRI values and significant p-values for each gut region (Table 1), indicating that the clustering of bacterial communities within each region may be due to habitat filtering. While hierarchical clustering (Fig. 4) was unable to distinguish the midgut community from the posterior hindgut community, phylogenetic NRI data showed that the organisms in each gut region do cluster closely together. The differences here are due to the method that was used. Hierarchical clustering only takes into account presence and absence while PhyloCom takes into account phylogenetic information and thus is generally accepted to be more informative.

There are parallels between microbial community composition of the beetle gut with those of the termite gut. Termites require bacteria and archaea, sometimes with the addition of flagellates to help them digest their wood substrates which have a high C:N ratio. The passalid beetle gut does not appear to require the same type of termites (Nardi *et al.* 2006) and perhaps the main digesters of wood are bacteria and archaea. Nitrogen fixation through gut symbionts is the main process by which termites obtain their nitrogen (Brune & Ohkuma, 2011). The results presented here showed that the anaerobic regions correlated with potential nitrogen fixers (and thus fixed nitrogen) may be the mechanism in which beetles get their nitrogen. Future work in *nifH* expression from different gut regions, followed by tracing that fix nitrogen into the tissue of the beetles will test this hypothesis.

The results presented here showed that the gut of the passalid beetle represents a highly compartmentalized environment with well-stratified composition of microbial communities that seems to correlate with oxygen availability. I showed that the microbial composition in each gut regions differed significantly from each other, and that despite the flow of food linearly through the gut, at least two of the four regions maintained their microbial signature. The regions that were thought to be more anaerobic contained greater signals of microbes that require an anaerobic environment. Future works should explore functional aspects of gut organisms, with a focus on wood degradation, oxygen-dependence, and nutrient cycling within the gut environment. Understanding how these compartmentalized populations interact to deconstruct cellulosic materials could aid the optimization of industrial biofuel production processes.

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Table 1. Diversity, pH, and phylogenetic community metrics, within each gut segments of the passalid beetle gut. H' = Shannon's diversity index, PD = phylogenetic diversity, NRI = net related index, p_{NRI} = p-value of NRI metric. Positive NRI values indicate phylogenetic clustering. The letters a, b, c indicates statistically significant means for pH measurements from each gut segments. FG = foregut, MG = midgut, AHG = anterior hindgut, PHG = posterior hindgut.

Gut region	OTU observed	pH	H'	PD	NRI	p_{NRI}	indicates
FG	1179	7.44± 0.21 ^a	7.08	208.9	7.34	0.0001	clustering
MG	825	8.38± 0.12 ^c	6.72	172.5	6.50	0.0001	clustering
AHG	730	7.21 ± 0.05 ^{ab}	6.56	169.8	3.99	0.0002	clustering
PHG	937	6.89± 0.18 ^b	6.85	183.9	5.52	0.0001	clustering

Figure 1. Dorsal view of a dissected passalid beetle showing gut placement within the abdominal cavity. MG = midgut, AHG = anterior hindgut, and PHG = posterior hindgut. Bar = 5mm.

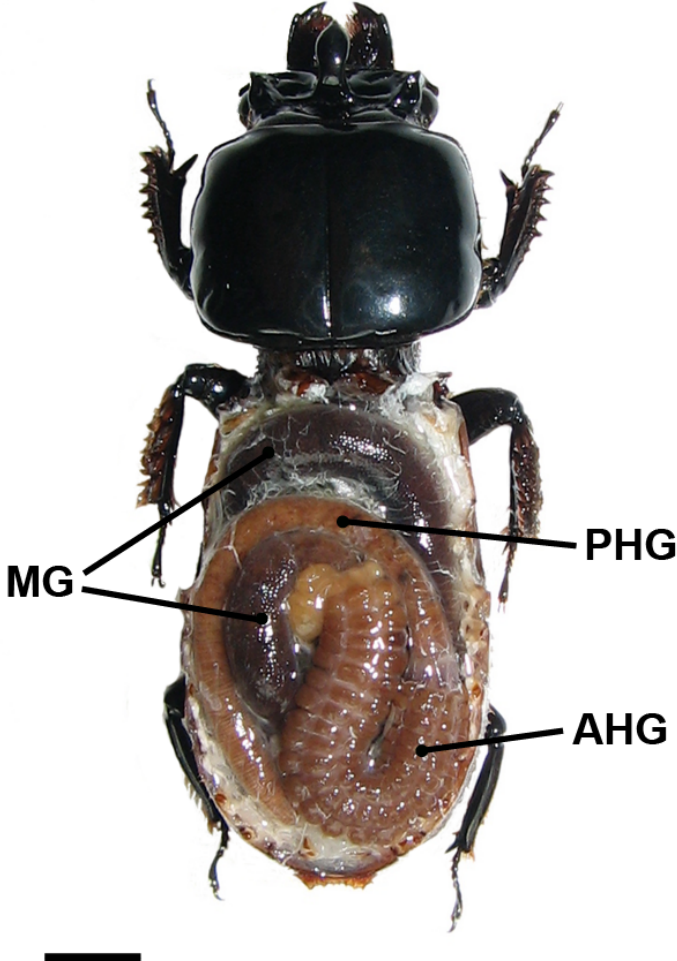


Figure 2. Dissected gut of a passalid beetle showing the morphology of each gut section. The total length of the gut is 12cm. FG = foregut, MG = midgut, AHG = anterior hindgut, and PHG = posterior hindgut. Bar = 10mm.



Figure 3. Phylogenetic tree of all detected taxa in the passalid beetle gut. Branches are colored by phylum. The four rings colored in various shades of red are dependent on intensity measures where the brightest red represents the strongest probe intensities. Starting from the inside, each ring represents different gut regions in order: foregut (FG), midgut (MG), anterior hindgut (AHG) and posterior hindgut (PHG). The outer most ring in black shows taxa that have statistically different intensities in any one of the gut regions (see Materials and Methods). Each phylogenetic group is labeled based on either Phylum or Class level. γ = γ -Proteobacteria, Clos = Clostridia, Spiro = Spirochetes, Cyan = Cyanobacteria, D = Deinococcus-Thermus, ϵ = ϵ -Proteobacteria, Acido = Acidobacteria, N = Nitrospira, Chloro = Chloroflexi, others = all other taxa, including Aquificae, Caldithrix, Chlamydiae, Chlorobi, Coprothermobacteria, Deferribacteres, Gemmatimonadetes, Lentisphaerae, Natronoanaerobium, Synergistes, Thermotogae, Thermodesulfobacteria, Verrucomicrobia, which make up 13.2 % of the tree.

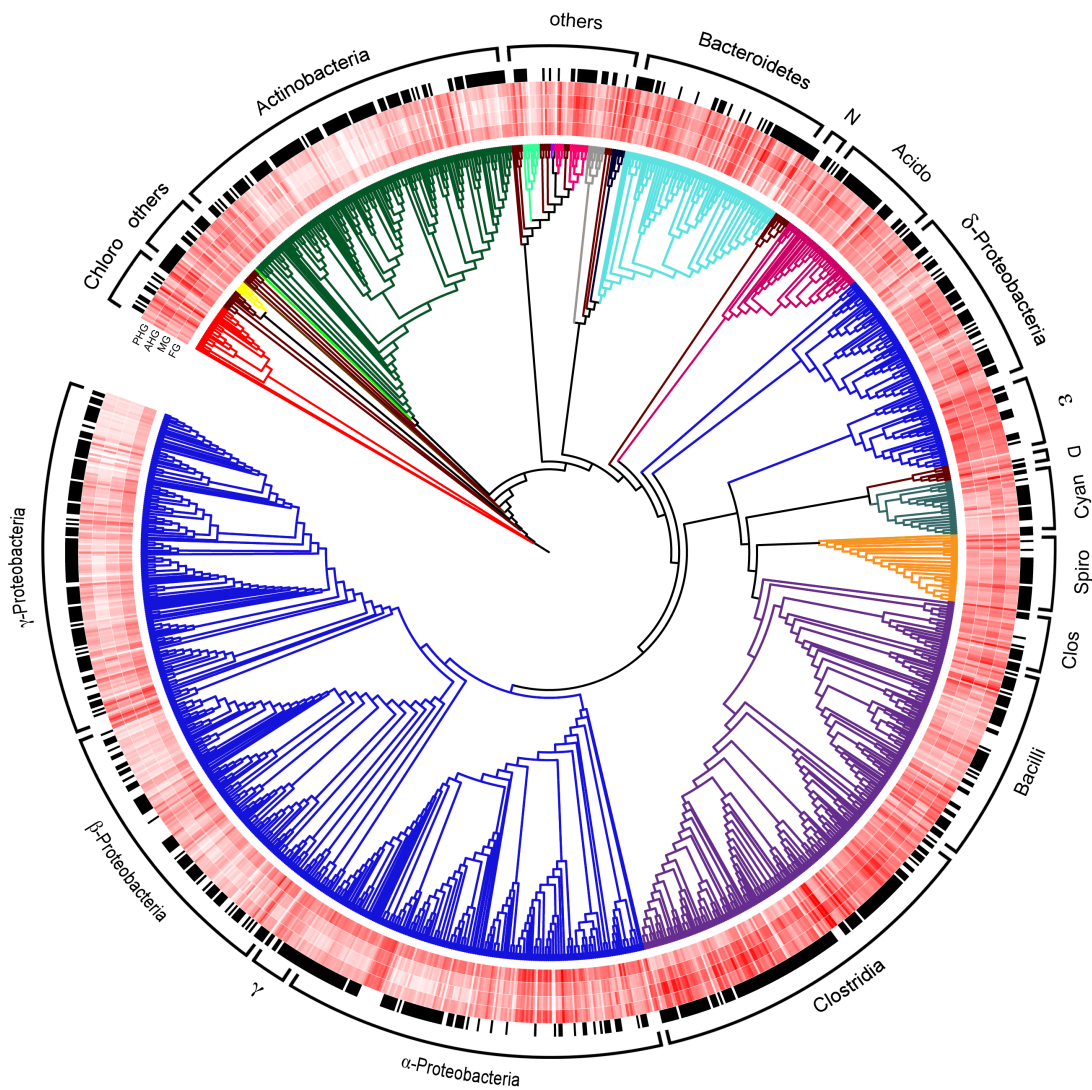


Figure 4. Hierarchical cluster analysis of the microbial community within the four gut sections from four beetles. Each terminal leaf represents an array from a single gut section. Numbers in parentheses correspond to an individual beetle (1-4). A, B, C, D shows the four clades of the tree. FG = foregut, MG = midgut, AHG = anterior hindgut, and PHG = posterior hindgut.

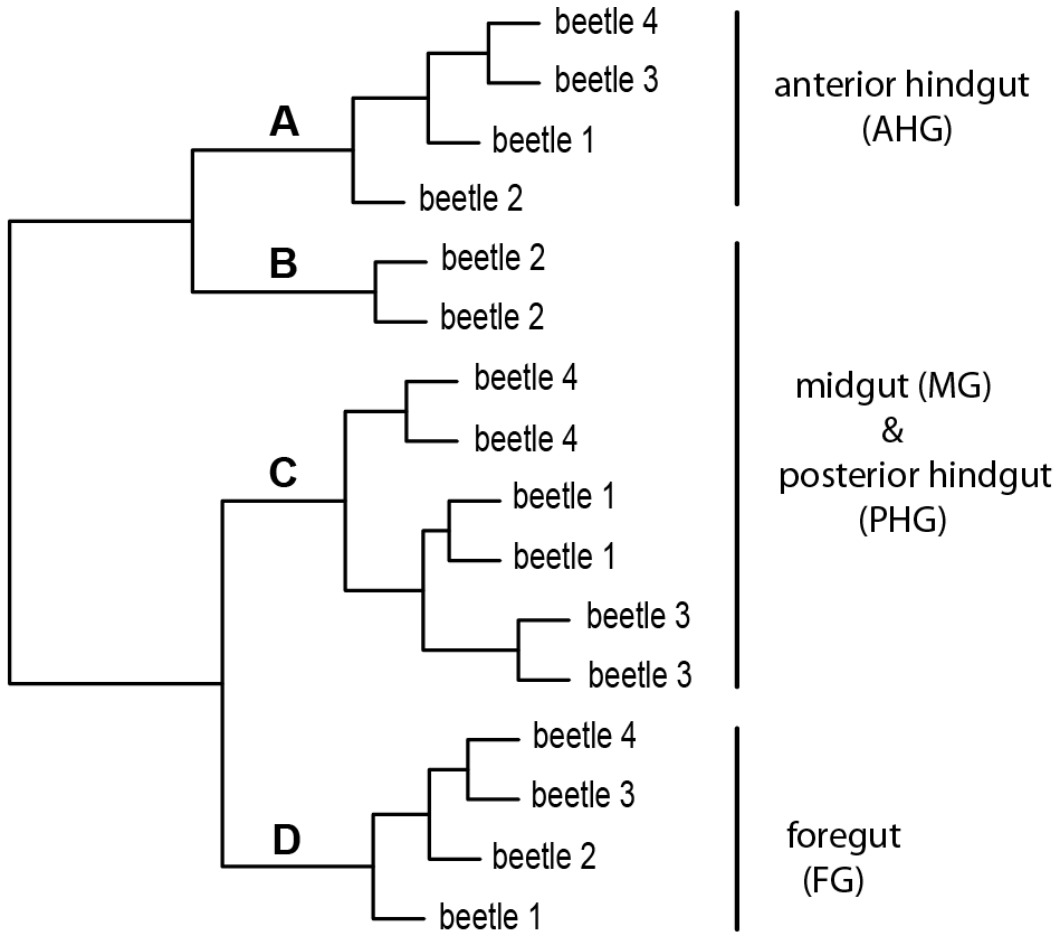
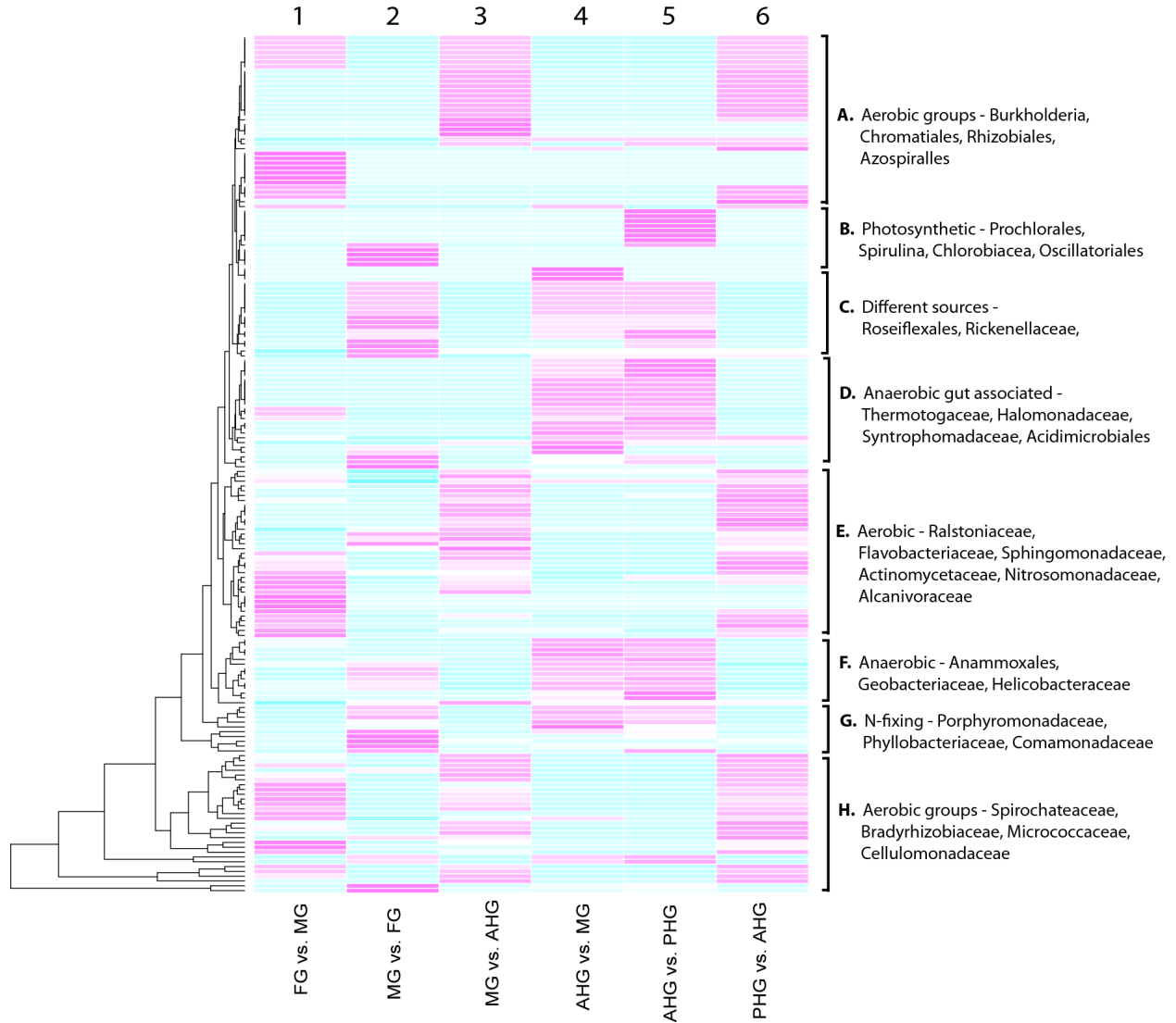


Figure 5. Cluster comparisons of detected bacterial groups between any two linearly adjacent gut regions. Cyan colored bars shows absence of a taxon whereas pink colored bars show presence of a taxon. The presence of a taxon was defined as presence of that taxon in three out of the 4 replicate beetles. The more intense the color, the stronger the probe intensity = amount of DNA in the sample. For example, in column 1, the foregut is compared to the midgut, and in this comparison, aerobic groups tend to be more present (pink) than absent (blue), which indicates that aerobic groups tend to be more present in the foregut. FG = foregut, MG = midgut, AHG = anterior hindgut, PHG = posterior hindgut. A-H = functional groups. The number on top of each column indicates column number.



CHAPTER 2

Bacteria within ectomycorrhizal root-tips and their potential for nitrogen fixation from within

Abstract

Bacteria have been observed to grow with fungi, and those that associate with ectomycorrhizal fungi have often been thought of as symbionts that may either increase or decrease mycorrhizal formation rate. The work in this area so far has not shown that ectomycorrhizal fungi could influence the bacterial community nor has it shown convincing evidence that certain groups of bacteria associate with ectomycorrhizal fungi. Despite this lack of knowledge, earlier works did show that bacteria affected the initial colonization of roots by ectomycorrhizal fungi. To understand this symbiosis from a community ecology perspective, I sampled ectomycorrhizal root-tips over a three year period and used 454 pyrosequencing to identify the bacteria that live inside the mycorrhizal root-tips. The data showed that fungal species had a stronger effect on the bacterial community than either year or site. Members of the Burkholderiales and Rhizobiales were major players in the system, suggesting that they may serve as symbiotic diazotrophs within the ectomycorrhizal root-tip.

Introduction

Ectomycorrhizal fungi (EMF) form widespread and important symbioses with multiple families of primarily trees and shrubs. Ectomycorrhizal trees often dominate temperate forests in northern latitudes, whereas their dominance is typically more limited in the tropics and southern hemisphere (Smith and Read 2008). Traditionally, it is thought that the ectomycorrhizal symbiosis involves only two groups of organisms, the fungi and their plant hosts. However, evidence arose in the 1980's that bacteria may also play important roles in this symbiosis system. The term "mycorrhiza helper bacteria" (MHB) was coined to describe rhizospheric bacteria that can help establish mycorrhiza formation between fungi and plants. Although described as "helpers", these bacteria actually had a wide range of interactions ranging from positive to negative (reviewed in Garbaye 1994 and Frey-Klett *et al* 2007).

Both molecular and cultural methods have provided a slightly better understanding of the identities of the bacteria found around or in the EMF root-tips of plants from. For example, bacteria have been isolated and/or identified from the root-tips of Scots pines (*Pinus sylvestris*) in Europe, Japanese red pine (*P. densiflora*) in Japan, and Douglas fir (*Pseudotsuga menziesii*) in Oregon, USA (Bending *et al* 2002, Izumi *et al* 2007, 2008, Burke *et al* 2008, Tanaka & Nara 2009). Generally, the results from these studies showed little to no specificity of bacteria to fungal hosts. However, there is an emerging pattern associated with these discoveries - bacteria in the genera *Burkholderia*, *Bacillus*, *Paenibacillus*, *Clostridium*, *Azospirillum*, *Pseudomonas*, and the Order Rhizobiales have been repeatedly isolated from root-tips.

One of the most obvious and interesting characteristics of these bacteria is that they have the ability to fix nitrogen, either in symbiotic structures such as nodules or as "free-living" microbes (Ludwig 1984, Santos 2001). These observations suggested that such microbes living in the EMF root-tips could provide fixed nitrogen to the fungi and their plant partners. This system could parallel the legume nodule model, in which symbiotic bacteria live inside a thickened host structure that shelters their fragile nitrogenases from oxygen, and the high cost of N fixation is paid with carbon from the host. In fact, there has been scattered evidence that nitrogen fixation does occur within the ectomycorrhizal root-tips in the presence of bacteria (Li 1992, Paul 2007).

The lack of specificity between bacteria and fungi and the sparse evidence from the studies cited above may in part be due to past technological limitations. In this work, I greatly increased sampling by using 454 pyrosequencing to study bacterial communities associated with EMF root tips in monoculture stands of Bishop Pines (*Pinus muricata*) and ask the following two questions: 1) Do root-tips of different EMF species harbor different bacterial communities? 2) What groups of bacteria are important associates of Bishop Pine EMF root-tips? These two basic questions will help to bolster the basic information needed to continue exploring these multi-partner symbioses.

Materials and methods

Sampling and sample processing

The sampling site was Point Reyes National Seashore where monoculture stands of Bishop pines (*Pinus muricata*) occur. The three sampling sites were located along Limantour Road: Site 1 (38.058620° -122.849529°), Site 2 (38.054417° -122.852805°), and Site 3 (38.046778° -

122.867500°). Sites 1 and 3 are separated by 2.1 km and site 2 is located between Sites 1 and 3. At each site, three cores (10cm in diameter, 10cm deep) were taken in April 2009, April 2010, January 2011, and March 2011. The cores were approximately 15m from each other at each site. At all sites, the soil was covered by approximately 10cm of pine needles, which were lifted away before coring. The cores were kept at 4°C and processed within 36 hours of sampling. Soil from each core was washed away to expose EMF root-tips that were then sorted and grouped by morphotypes. A single root-tip (= one sample) was selected from each morphotype for further analyses. Since my interest was only in the community of microbes inside of the root-tips, they were surface sterilized in 30% H₂O₂ for 20 seconds and washed three times in sterilized water to remove surface bacteria (modified from Izumi *et al* (2007), which showed sufficient surface degradation of DNA). The root-tips were stored in 250 µl of CTAB buffer + 2% PVPP and DNA was extracted using standard ethanol precipitation protocol. Typically, root-tips are large enough that a single root-tip was considered one sample. In addition, three uncolonized root-tips, four colonized root-tips, and soil surrounding 2 mm of these roots from the January 2011 sampling were also sequenced for comparison. In total, 105 colonized root-tips, 3 uncolonized root-tips and 4 soil samples were processed.

Molecular methods

The fungi were identified by Sanger sequencing the internal transcribed spacer (ITS) region using primers ITS1F and ITS4 (White *et al.* 1990, Gardes & Bruns 1996). Nine samples with mixed signals, indicating colonization by multiple fungi were discarded; thus only 94 root samples were pyrosequenced to identify the associated bacteria.

Bacteria were identified by 454 pyrosequencing the V4-V7 region of the 16S rRNA gene using the chloroplast exclusion primer 533f (5'-GTGCCAGCAGCCGCGTAA-3') and the degenerate reverse primer 1185r (5'- GAYTTGACGTCATCCM-3') designed by Hodkinson & Lutzoni (2010). These primers are able to exclude chloroplasts and mitochondria but are still broad enough to recover a good representation of bacterial diversity. The forward primer was attached to 454 adapters and 8bp multiplex barcodes: A-adaptor + MID barcode + primer (533f) and used in combination with B-adaptor + primer (1185r). Each PCR reaction contained 1.25 units of HotStarTaq polymerase (Qiagen), PCR buffer (containing 50 mM KCL, 10 mM Tris, 2.5 mM MgCl₂, and 0.1 mg/ml gelatin), 0.2 mM dNTPs, 25µg BSA, 2 mM MgCl₂, 0.3 µM of each primer and H₂O to a final concentration of 25 µL. Touchdown thermocycle conditions were: initial denature for 15 min at 95 °C; 95 °C for 30 s; 78 °C for 30 s, -0.7 °C per cycle; 72°C for 1 minute + 2 seconds per cycle; repeat 29 times; 1 min at 72°C, and final extension for 10 min at 72°C. Each PCR reaction was conducted in triplicate to minimize PCR bias. The three PCR products were pooled and magnetically cleaned using the Agencourt AMPure XP kit (Beckman Coulter, Inc.) according to manufacturer's instructions. Purified PCR products were quantified using the Invitrogen Qubit Fluorometer (Invitrogen, Carlsbad, CA). Each barcoded sample was then pooled in equimolar concentration. The pooled sample was cleaned once again through sodium borate agarose gel purification to select for the expected product size. Samples were sent to the University of Illinois Keck Center for sequencing on ½ of a plate with the Roche GS FLX+ system with 2.8 software, Flow B.

Data quality control and statistical analyses

Both data quality control and statistical analyses were performed primarily in the software package MacQIIME (Caporaso *et al.* 2010). Raw reads (sequences) files (.fna) and base quality score (quality of each base calls) files (.qual) were imported into MacQIIME where primers and multiplex barcode sequences were removed, followed by annotation of these sequences with the associated sample metadata. The minimum quality score parameter was set to 25. Only sequences that were between 675-790bp in length were kept (sequences of 711bp were expected based on the primer). Because of a newer implementation of the way each base was added and read (called Flow B), the denoising algorithm in MacQIIME could not be used, and at the moment there is no replacement for that algorithm. As a suitable substitute, a graph of OTU number vs. % similarity between the sequences (from 90-100%) was created (Fig. 1) to visually determine the inflection point of the exponential curve. All sequences that lie to the right of the inflection point are considered to have been created by errors where the OTUs are false. Using this data, a 97.5% similarity was chosen as the cutoff point for OTU delimitation. OTUs were picked using the default UCLUST method using the Greengenes core alignment dataset as a guide. Taxonomic data was assigned to each OTU using the RDP Classifier. Chimeras were removed using ChimeraSlayer. Singletons (OTUs that occur only once) were removed, as were OTUs that occurred in the negative control. OTUs that did not match to bacterial 16S rRNA genes, and those that matched to plant chloroplasts (Streptophyta) were removed. Samples that failed to sequence or those that produced too few sequences relative to the other samples (sequencing failures) were removed. Representative sequences from each OTU were selected and aligned using the PyNAST algorithm, followed by construction of a phylogenetic tree using the FastTree method. The resulting tree was used for phylogeny-based community analyses in later steps.

All statistical analyses were performed in MacQIIME, except for the rarefaction curves of samples (Fig. 3C) which was performed in EstimateS 8.2 (Colwell 2009). OTU abundances and alpha-diversity metrics (Shannon H', Simpson's Diversity, and Faith's Phylogenetic Diversity = PD, Faith 1992) were measured using default parameters. To perform beta-diversity analyses on found OTUs, all samples were rarified to 148 sequences and samples below this threshold were automatically discarded. Two approaches were taken to analyse this dataset. The first was to filter out samples of uncolonized roots and soil from the dataset. Bacterial community comparisons based on fungal genus, fungal species, year, and site were analyzed using all EMF samples (results in Table 1). The second approach was to analyze only the most common EMF species that were found 5 or more times (results in Table 2).

To answer the questions posed in the introduction, overall beta-diversity analyses were performed using Principle Coordinate Analysis (PCoA), *adonis* (a nonphylogenetic based test very similar to a PERMANOVA), and the UniFrac Significance test. The UniFrac Significance method randomly permutes the environmental variable on a phylogenetic tree where significance is determined if the UniFrac value of initial tree is greater than the expected UniFrac values of the randomly distributed trees. A significant p-value from the UniFrac Significance test means that one community is phylogenetically different from another. Since *adonis* is based on F-statistics, R^2 values are also reported. Both the *adonis* and UniFrac tests were based on 1000 randomizations. All statistical tests were performed using both incidence (presence/absence) and abundance data. PCoA and UniFrac methods used UniFrac-based distance matrices whereas the other analyses relied on Soronsen (presence/absence) and Jaccard (abundance) distances. All p-

values were corrected for multiple comparisons using Bonferroni corrections where multiple comparisons were performed.

Results

Data statistics and quality control

The ½ plate run of 94 barcoded samples produced a total of 252,018 sequences with the average length of 709bp. After the thorough data filtering process, 73,633 sequences (29%) were discarded and 178,385 (71%) sequences were retained. Rarefaction analysis from the retained sequences showed that in general, sampling of individual samples had not reached saturation, even with Chao1 estimation (Fig. 2A & B). However, the combined data showed that the overall sampling was near saturation with the observed and estimated richness curves converging (Fig. 2C).

Bacterial diversity of the whole dataset

A total of 12,896 bacterial OTUs and 28 fungal OTUs were found in the whole dataset containing EMF root-tips, soil surrounding EMF roots, and uncolonized roots (average 137 bacterial OTUs per sample). Figure 3 shows that the proportions of OTUs belonging to different bacterial Classes and that the types of sample are visually similar. However, the UniFrac Significance test showed that the underlying communities were significantly different among soil, colonized root-tips, and uncolonized root-tips. The figure also shows a community cluster analysis of these three environments, indicating that the bacterial communities inside the EMF root-tips and soil surrounding the colonized root-tips were more similar than the communities found in uncolonized root-tips (UniFrac $p \leq 0.003$). However, variation within the samples were not well explained by these three factors ($R^2 = 0.03$).

Since my questions of interest pertain to only the bacteria inside of EMF root-tips, the soil and uncolonized root-tips were excluded from further reported analyses. However, analyses with soil and uncolonized root-tips included did not significantly affect the results of downstream analyses (data not shown). After rarefaction, the dataset contains 66 EM samples and 3746 OTUs (average 57 OTUs/sample). The rarefied, ranked-abundance graph in Figure 4 shows the 10 most common bacterial OTUs in this dataset. The three most common OTUs belong to the Burkholderiales, followed by members of the Rhizobiales and Rhodospirillales. *Burkholderia sordidicola* was the most abundant OTU.

Unweighted UniFrac PCoA suggested that the bacterial communities were clumped by fungal species (Fig. 5). Statistical comparison of these communities showed that all four factors (fungal genus, fungal species, site, year) were significant, although only the factors “fungal genus” and “fungal species” had strong explanatory power (Table 1). The genus level explained 22-26% of the variation and the species level explained 43-46% of the variation in this dataset. Conversely, “site” and “year” only explained at most 5% of the variation. Results from the phylogenetic based UniFrac Significance test supported the *adonis* test.

Bacterial diversity within major EMF species

To have sufficient power to answer Question 1 of community differences between fungal species, I examined the associations between microbial communities and the five most common

fungus species. These fungi were *Rhizopogon salebrosus* (found in 13 samples), *Russula cerolens* (12 samples), *Tricholoma imbricatum* (9 samples), *Tomentella sublilacina* (7 samples), and *Clavulina* sp. (7 samples). This dataset contains 48 samples and 2092 OTUs (average of 44 OTUs per sample). The relative proportions of bacterial OTUs found in the EMF root-tips of these common fungal species are summarized in Figure 6. Summed across the five fungal species, the most highly represented group was the Burkholderiales, followed by the Rhizobiales, Acidobacteriales, Rhodospirillales, and Actinomycetales (Fig. 6).

The rarefacted ranked abundance curve of the 10 most commonly represented bacterial OTUs are shown in Figure 7. There were no major differences between this ranked abundance curve and the one for all EMF root-tips (Fig 4). The top four bacterial OTUs remained the same, although there were some changes in rank among the less common OTUs.

Community comparisons between the samples showed a clearer clustering of the species in a PCoA ordination (Fig. 8). Some fungal species (same colored dots) tend to cluster more closely indicating more similar community compositions, whereas others tend to have more spread indicating less similar community compositions. Similar to the dataset above with all EMF samples, the statistical comparisons in this dataset showed that all factors (fungal genus, fungal species, site, year) were significant. However, only the factors “fungal species” was able to explain the variation of up to 16% (Table 2). It should be pointed out that the factors “fungal genus” and “fungal species” are the same, since there was no replication of species within genus, thus the factor “fungal genus” was removed from the table. Results from a UniFrac Significance test showed that *Clavulina* sp., *Russula cerolens*, *Tomentella sublilacina*, and *Tricholoma imbricatum* had significant bacterial communities from each other ($p \leq 0.006$), whereas *Rhizopogon salebrosus* did not (Table 3). This indicates that there was no specificity of bacteria that associates with *R. salebrosus*.

Discussion

The overall results suggest that even within the root-tips of ectomycorrhizal fungi, the bacterial diversity is relatively high with an average of 150 OTUs per sample. This is an underestimate since the sampling was not saturated for any single sample. However, in comparison with other environments, such as soils, the number of OTUs found here is still relatively small. The environment within the root-tips are probably more selective for certain types of bacteria when compared to the soil environment, which has a lot more homogeneity and thus is able to support a greater number of bacteria.

The similarity between colonized root-tips and soil surrounding these root-tips vs. uncolonized root-tips was not surprising since the soils surrounding the root-tips were directly influenced by roots and fungal hyphae surrounding roots and *vice versa*. The lack of fungi from uncolonized roots may explain the community similarity between colonized roots and soil, and the results suggest that fungi colonizing root-tips can influence the bacterial community.

All together, the results from the statistical analyses and ordination (Fig. 8 and Tables 1-3) showed that fungal genus was strongly correlated with the bacterial communities within the root-tips. Although sites and year were also significant, they only explained a small portion of the

variance. Only fungal species explained the most variance. These results answered Question 1: root-tips colonized by different fungal species harbor different bacterial communities. This result contrasts with the study by Burke *et al* (2006) where the authors found no associations between fungal species and bacterial communities. The difference is probably due to techniques. The study by Burke *et al* (2006) used t-RFLP and Sanger sequencing of the bands, which underestimates diversity and does not allow for a full analysis of these communities.

The finding that members of the Burkholderiales and Rhizobiales are the major associates of EMF root-tips in this study corroborates published studies that also found these bacteria in association with EMF root-tips (Bending *et al* 2002, Izumi *et al* 2007, 2008, Burke *et al* 2008). It also answers Question 2: there are specific groups of bacteria associated with the ectomycorrhizal fungi in this study.

Members of the Burkholderiales, particularly *Burkholderia* have emerged as common, and potentially important, associates of plant, animal, and, especially, fungi. Several species of *Burkholderia* have been described directly from fungi such as *Burkholderia fungorum* from *Phanerochaete chrysosporium* and *Burkholderia sordidicola* from *Phanaerochaete sordida* (Coenye *et al* 2001, Lim 2003). A few studies have identified *Burkholderia* as endosymbionts of fungi such as *Burkholderia endofungorum* from *Rhizopus microsporus* (Partida-Martinez *et al* 2007a) or *Candidatus Glomeribacter gigasporarum* (basically a *Burkholderia*) from the spores of several genera of arbuscular mycorrhizal fungi and the hyphae of *Mortierella elongate* (Bianciotto 2003, Sato 2010). Other *Burkholderia* species seem to be associated with plants and soil, such as *B. sediminicola*, and *B. bryophila* and *B. megapolitana* associated with mosses (Vandamme *et al* 2007, Lim *et al* 2008). These substrates may not have been without fungi as there are usually numerous fungal hyphae traversing the soil adjacent to, and in or on the tissue of, mosses (U'ren *et al* 2010).

Burkholderia sordidicola was the most abundant OTU in the dataset. It has a 99% sequence similarity to the type strain of the species described from Korea on the surface of the fungus *Phanaerochaete sordida* (Lim 2003). It was also found by two independent studies to be associating with the EMF root-tips of *Pinus sylvestris* in Europe (Izumi *et al* 2006, Timonen & Hurek 2006), with the hyphae of a *Lyophyllum* sp. (Warmink & Van Elsas 2009), and the lichen *Cladonia rangiferina* (Cardinale 2006). These repeated isolations suggest that this taxon has a strong predilection towards fungi. The next two most common OTUs, *Leptothrix* sp. and *Burkholderia sediminicola*, have not been previously found in association with fungi, but their relative abundance here suggests that they play a major role in the Bishop Pine-EMF system

The next most abundant group of bacterial associates of EMF root-tips were the Rhizobiales. Although reports of their presence with fungi are not as common as for *Burkholderia*, their presence in environments where fungi are also present combined with their ability to fix nitrogen has stirred interest. This group of bacteria were reported to be associated with pine ectomycorrhizae (Izumi *et al.* 2007, Tanaka & Nara 2009), and lichens (Hodkinson & Lutzoni 2010, Bates *et al.* 2011). Outside of fungal associations, these bacteria have been found widespread in the soil as “free-living” organisms (Ludwig 1984, Estrada-de Los Santos *et al* 2001, Chapter 3 of this dissertation). However, they are most widely known for their symbiosis

with leguminous plants where they nodulate the roots and perform nitrogen fixation within the symbiotic nodules.

Nitrogen fixation of bacteria living with fungi has been the topic of interest by mycorrhizal ecologists. Studies using acetylene reduction assays have been used to measure fixed nitrogen from EMF root-tips (Li 1992, Paul 2007). Despite the evidence for increased nitrogen fixation rates, the amount of total nitrogen contributed to plant hosts from fixation is small. Although my study did not test for nitrogen fixation within EMF root-tips, the presence of members of the Rhizobiales suggests via taxon association that this might happen. As shown in Fig 7, many of the top 10 OTUs were members of the Rhizobiales with potential nitrogen fixing abilities. And even though the top three OTUs belonged to the *Burkholderiales*, other members of the genus *Burkholderia* have been shown to fix nitrogen when associated with plants (Elliott *et al* 2007) and thus their nitrogen fixing potentials in fungi cannot yet be ruled out.

Bacterial nitrogen fixation in fungal tissue is one of the most obvious of the many potential interactions between fungi and bacteria, but there are many other potentially interesting interactions to be studied that could lead to deeper understanding of fungal-bacterial symbioses. For example, it was long thought that the fungus *Rhizopus microsporus* produced toxins that kill rice seedlings post-infection. However, Partida-Martinez *et al* (2005, 2007a) found that the endohyphal symbionts *Burkholderia endofungorum* and *Burkholderia rhizoxinica* were the actual toxin producers. Furthermore, fungi removed of these bacteria lost the ability to produce asexual spores and can only be cured by reintroduction of the symbiont (Partida-Martinez *et al* 2007b). The *Burkholderia* discovered here has not been studied in depth and future isolation and closer examination would be worthwhile.

The results presented here showed that fungal species have a significant effect on the microbial community within EMF root-tips. Within these EMF root-tips, the two dominant groups of bacteria were the Burkholderiales and the Rhizobiales. This suggested a potential for nitrogen fixation within the root-tips, although direct evidence is missing at this point. In future works, nitrogen fixing abilities of these microbes should be tested through expression and acetylene reduction assays. If they do fix nitrogen, additional future work should track the fixed nitrogen to determine if it travels to the fungi and eventually to the plant tissue. Other symbiotic interactions may easily be found because so little is known about fungal-bacterial interactions. It is a field wide open for study.

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Table 1. Significance p-values for the *adonis* and UniFrac tests when comparing the overall bacterial community based on all EMF root-tip samples. R^2 values based on F-statistics are also reported for the *adonis* test. These values indicate the explained variation within a comparison. uw = unweighted data based on presence/absence, w = weighted data based on read abundance.

factor	<i>adonis</i> (uw)	<i>adonis</i> R^2 (uw)	<i>adonis</i> (w)	<i>adonis</i> R^2 (w)	UniFrac (uw)	UniFrac (w)
fungus genus	0.001	0.22	0.001	0.26	0.001	0.001
fungus species	0.001	0.43	0.001	0.46	0.001	0.001
site	0.001	0.03	0.001	0.04	0.001	0.001
year	0.001	0.02	0.001	0.02	0.001	0.001

Table 2. Significance p-values for the *adonis* and UniFrac tests when comparing the bacterial community based on the 5 major EMF species dataset. R^2 values based on F-statistics are also reported for the *adonis* test. uw = unweighted data based on presence/absence, w = weighted data based on read abundance. Note that the factor “fungus genus” is not shown in this table because every species belonged to a different genus, thus the results were the same for these two factors.

factor	<i>adonis</i> (uw)	<i>adonis</i> R^2 (uw)	<i>adonis</i> (w)	<i>adonis</i> R^2 (w)	UniFrac (uw)	UniFrac (w)
fungus species	0.001	0.12	0.001	0.16	0.001	0.001
site	0.002	0.03	0.003	0.04	0.001	0.001
year	0.001	0.12	0.001	0.16	0.001	0.001

Table 3. Significance p-values (corrected for multiple comparisons) for the UniFrac test when comparing the communities within each fungal species vs. all other species (pair-wise comparisons). uw = unweighted data based on presence/absence, w = weighted data based on read abundance.

Factor (fungus species)	UniFrac (uw)	UniFrac (w)
<i>Clavulina</i> sp.	0.001	0.001
<i>Rhizopogon salebrosus</i>	0.06	0.075
<i>Russula cerolens</i>	0.008	0.006
<i>Tomentella subllilacina</i>	0.001	0.001
<i>Tricholoma imbricatum</i>	0.001	0.001

Figure 1. An exponential curve that resulted from graphing the number of OTUs vs. % sequence similarity. The inflection point of this curve is between 97-99% and thus a 97.5% sequence similarity value (arrow) was used for OTU delimitation in this study (see Materials and Methods).

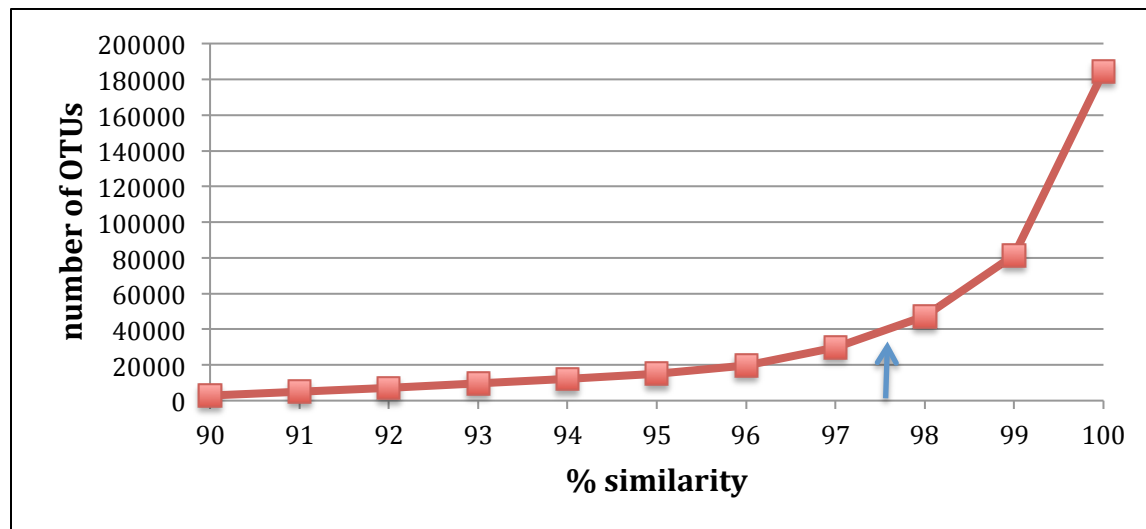


Figure 2. Rarefaction curves of the data, post quality control. A. Observed OTUs of individual sequences per sample. B. Chao1 estimates of OTUs per individual sequence. C. Observed and Chao1 estimate of all OTUs per sample together.

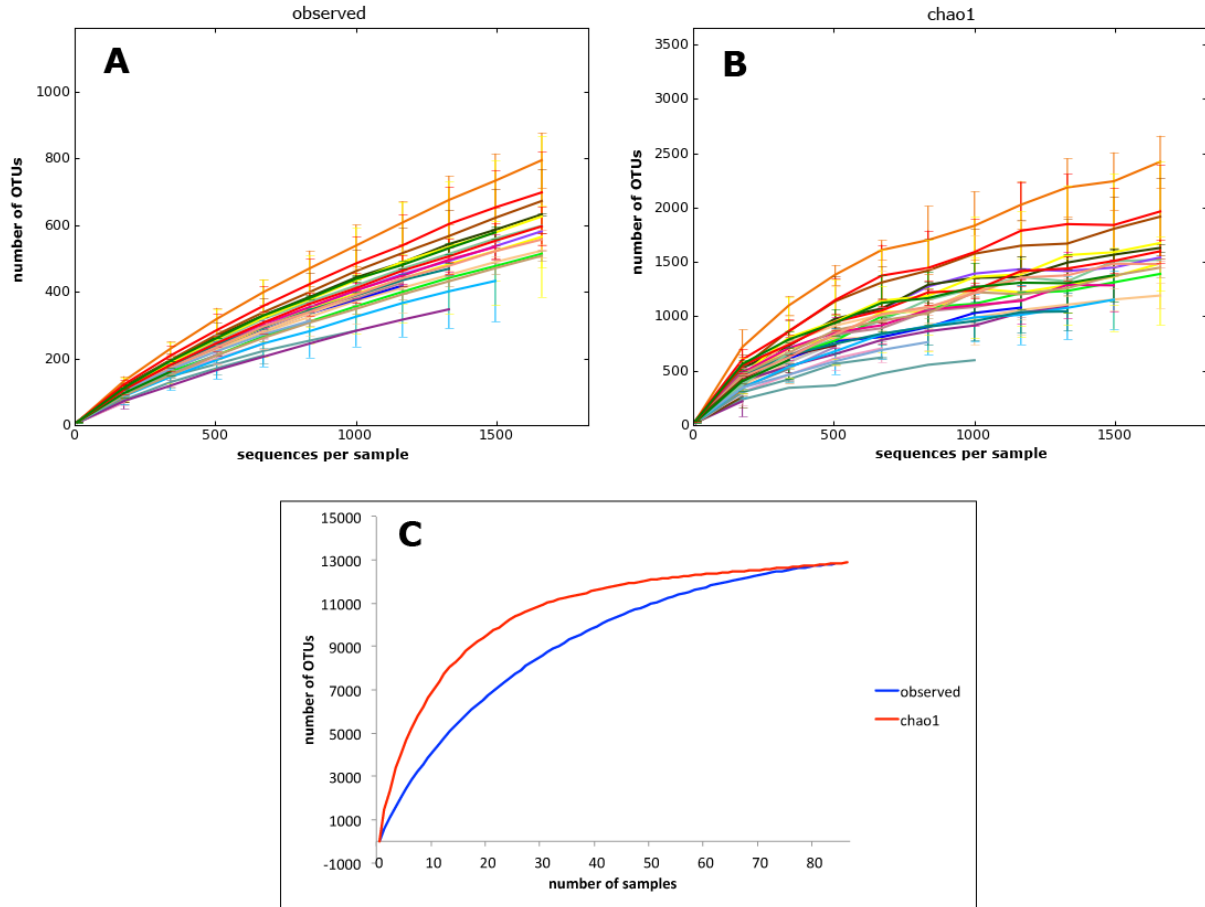


Figure 3. Relative proportions of bacterial OTUs belonging to different classes. Data for the graph came from unrarified samples. The tree at the bottom of the figure shows a hierarchical grouping of the samples based on rarified beta diversity statistics. The letters above the bars indicate whether communities of bacteria within the three environmental types (soil surrounding the EMF root-tips, EMF root-tips, and uncolonized roots) are more different than expected by chance (UniFrac Significance test, $p < 0.05$).

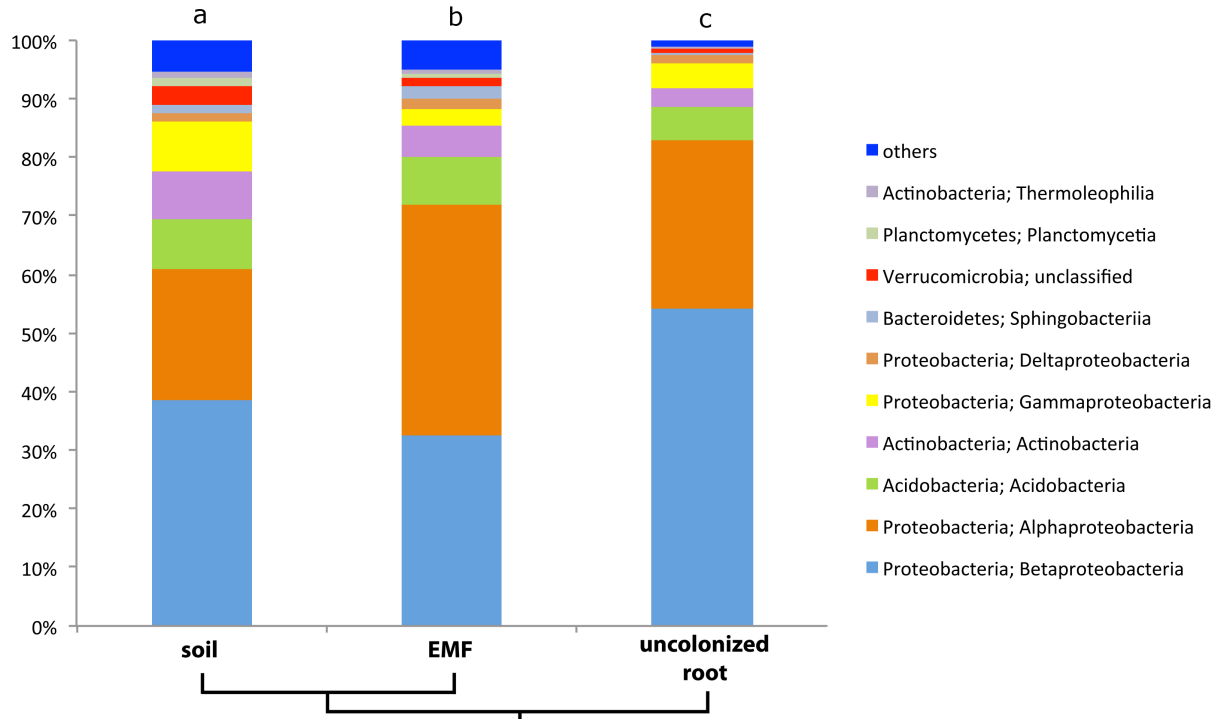


Figure 4. Rarified ranked abundance graph showing the 10 most commonly represented OTUs from all EMF root-tips. Those with potential nitrogen fixing ability are marked with an asterisk (*) based on taxonomic similarity to the species in literature review. The genera *Burkholderia* and *Leptothrix* belong to the Order Burkholderiales, the genera *Bradyrhizobium*, *Agrobacterium*, and *Rhizobium* belong to the order Rhizobiales, and *Azospirillum* belongs to the order Rhodospiralles.

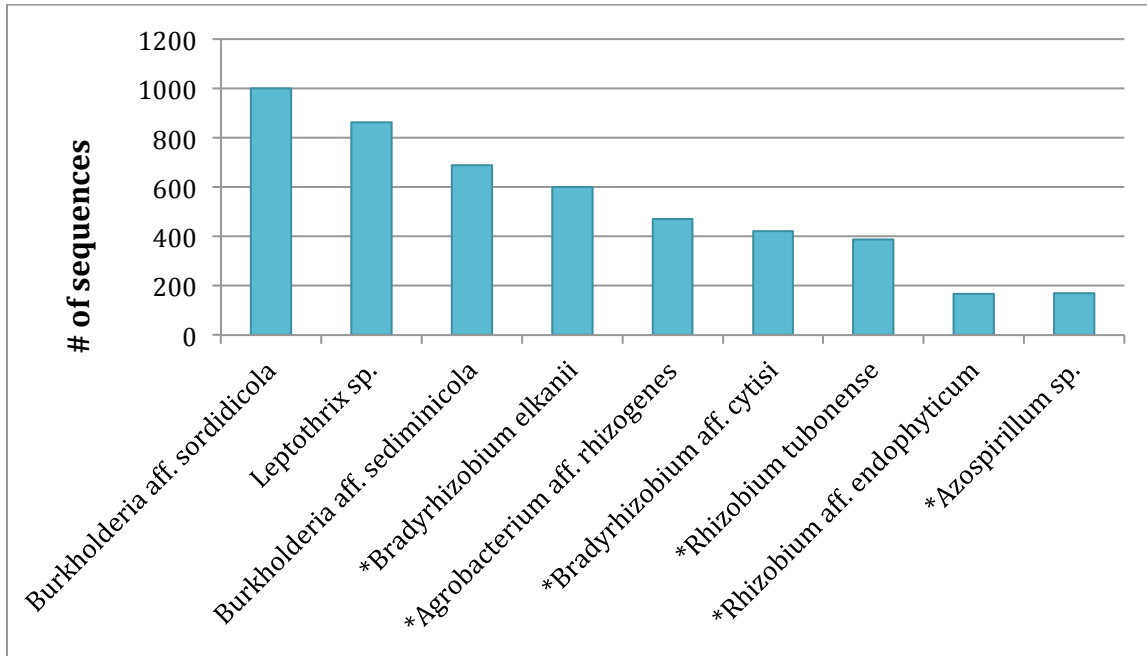


Figure 5. Unweighted UniFrac PCoA showing general clumping of bacterial community composition based on fungal species. This ordination uses data from all EMF root-tips. Each color represents a different fungal species and each dot represents a different sample.

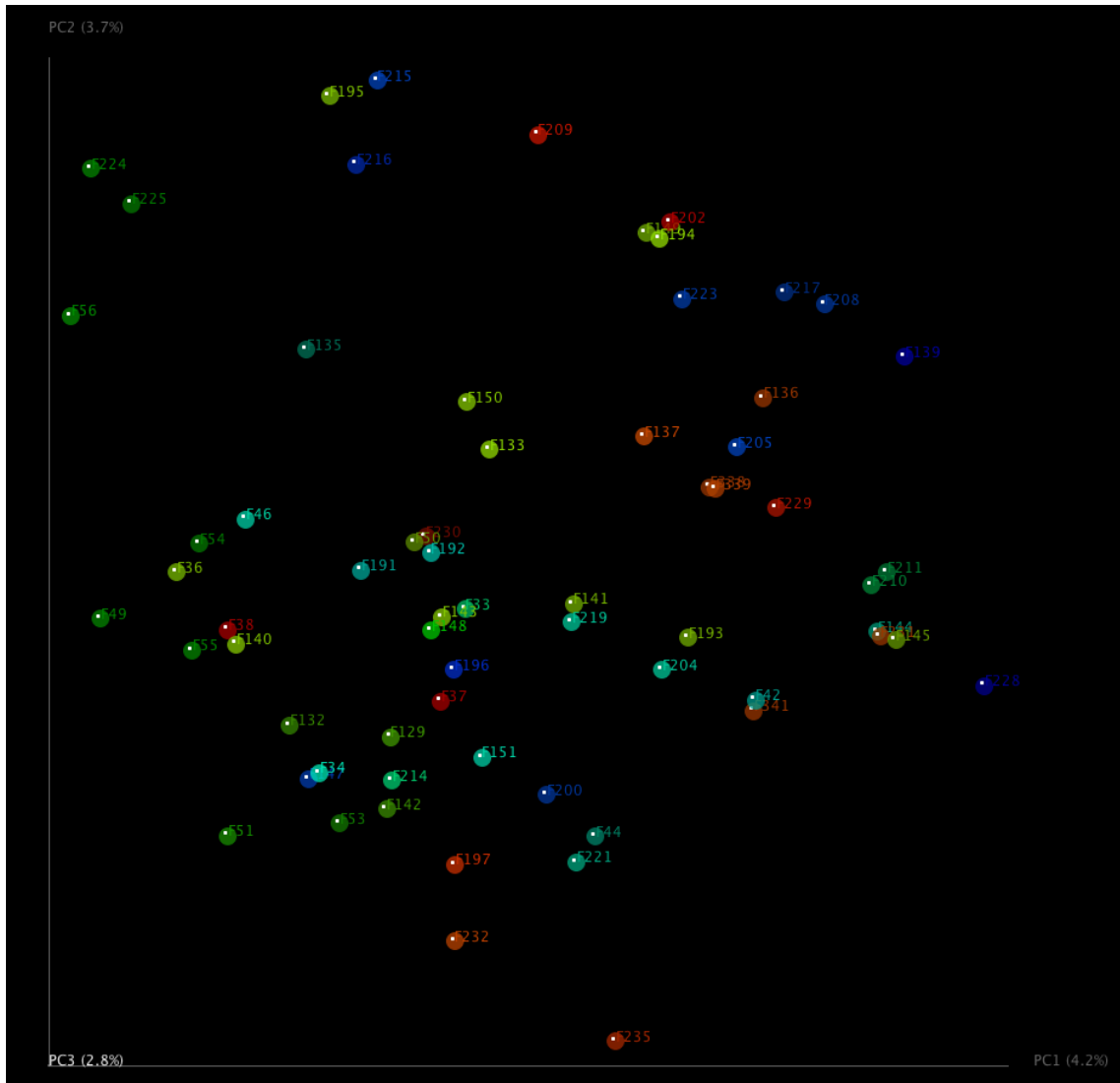


Figure 6. Relative proportions of bacterial OTUs belonging to different bacterial orders in the five most commonly encountered fungal species. All fungal species except for *Rhizopogon salebrosus* contained statistically different bacterial communities based on the UniFrac Significance test.

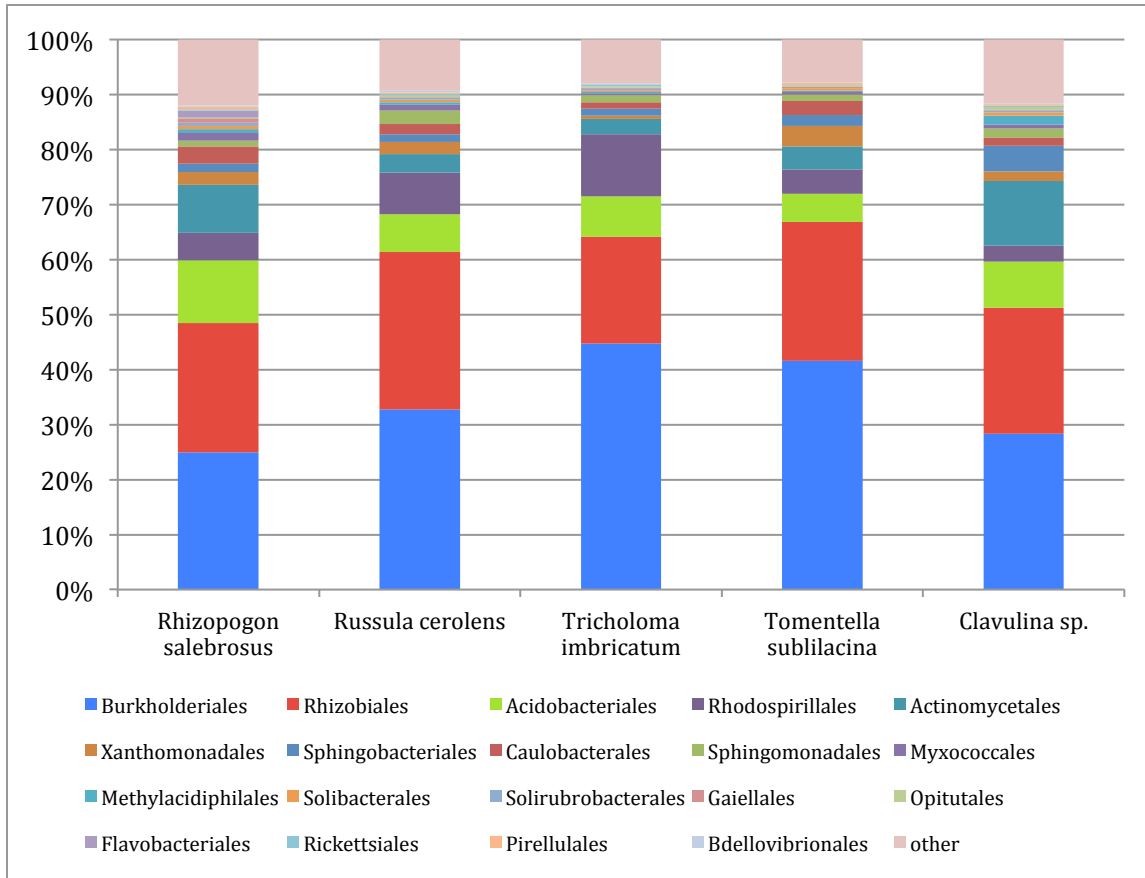


Figure 7. Ranked abundance curves showing the 10 most commonly represented OTUs from the five most common EMF fungi. Those with potential nitrogen fixing ability are marked with an asterisk (*) based on taxonomic similarity to the species in literature review. The genera *Burkholderia* and *Leptothrix* belong to the Order Burkholderiales, the genera *Bradyrhizobium*, *Agrobacterium*, and *Rhizobium* belong to the order Rhizobiales, *Actinospica* belongs to the order Actinomycetales, and *Azospirillum* belongs to the order Rhodospiralles.

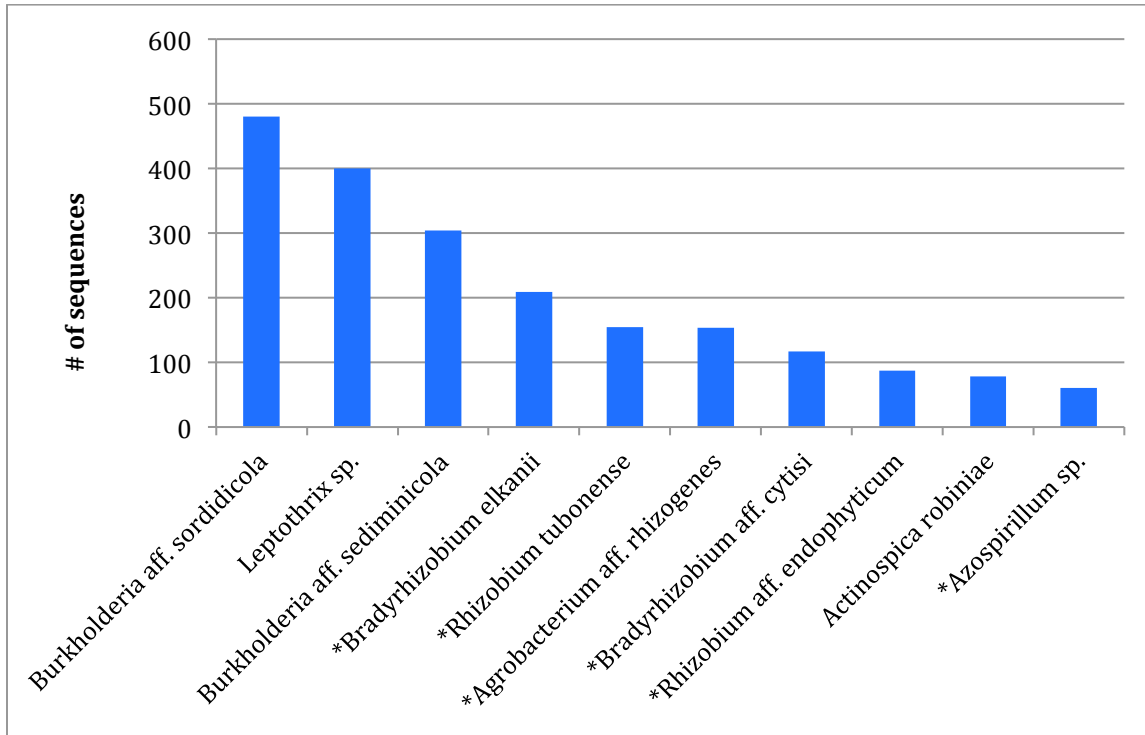
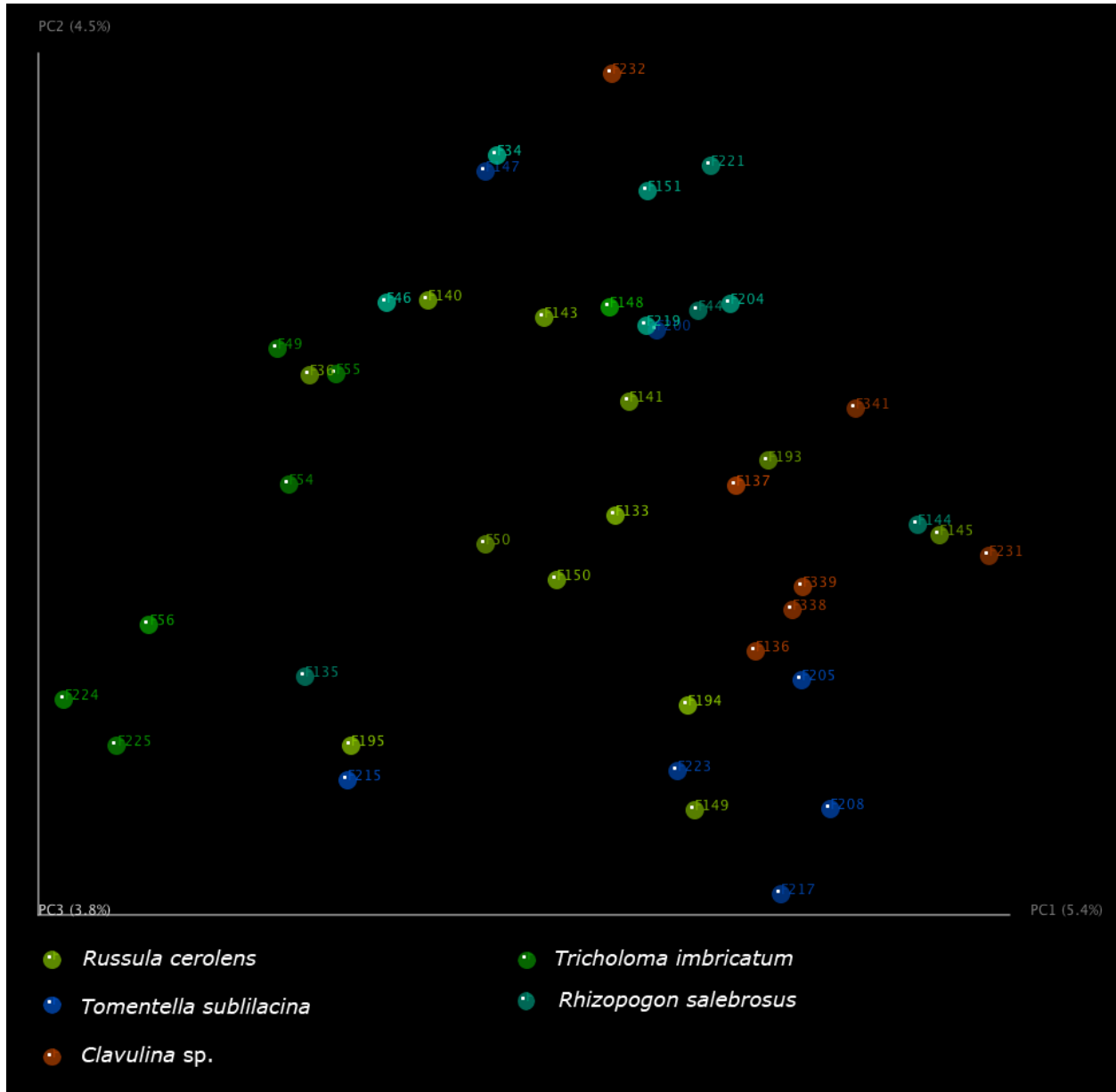


Figure 8. Unweighted UniFrac PCoA showing general clumping of bacterial community composition based on fungal species. This ordination uses data from only the 5 major EMF species. Each color represents a different fungal species and each dot represents a different sample.



CHAPTER 3

Bacterial communities associated with saprobic fungal mats with the potential for antagonistic and mutualistic symbioses

Abstract

Fungal mats can be very common in the litter layer of many forests and are thought to harbor communities of bacteria that differ from litter not colonized by fungal mats. This chapter addresses three basic questions relating to mat and non-mat forest litter and the three host fungal species (*Leucopaxillus gentianeus*, *Leucopaxillus albissimus*, and *Phaeoclavulina curta*) that make these mats. Specifically, (1) are bacterial communities within fungal mats different from non-mat? (2) Do the two fungal species of the genus *Leucopaxillus* contain bacterial communities that are more similar to each other than to the community within the more distantly related *Phaeoclavulina*? And, (3) are certain groups of bacteria found exclusively in fungal mats when compared to non-mat litter? To address these questions, 454 pyrosequencing was used as the platform to sequence part of the bacterial 16S rDNA region. Analyses of 44,000 sequences showed that the bacterial community compositions differed between mat and non-mat litter, but fungal species had marginal effects on community structure. *Pseudonocardia* aff. *alaniniphilia*, the second most common OTU, was significantly associated with fungal mat samples. *Pseudonocardia* species are well-known antibiotic producers and have been previously isolated from the bodies leaf-cutting ants where they are involved in a complex multi-partner symbiosis. This study provides a model from which experiments could be performed to test for symbiotic associations between *Pseudonocardia* and the mat forming fungi.

Introduction

There has not been a more exciting time to study fungal-bacterial interactions. Recent estimates suggested that there may be up to 6 million species of fungi (Blackwell 2011, D. Lee Taylor pers comm.) and with the countless bacteria that have escaped estimation, there appears to be a vast number of potential interactions between these two diverse groups. Despite the open possibilities, the field of fungal-bacterial association is a still small one, consisting of a handful of researchers worldwide and a few sporadically published articles. With so many organisms and so few questions being asked, the potential for novel discovery is wide open.

With such a huge number of possible fungal-bacterial interactions, high throughput molecular methods can be valuable for peering into this vast unexplored space. In this chapter, I used 454 pyrosequencing, a high throughput DNA sequencing technology, to study the bacteria that live in association with fungal mats in forest litter. The system was chosen because of the initial impression I received while collecting mushrooms in the genus *Leucopaxillus*. These mushrooms are impressively large and atypical entities in that they are able to withstand decay for months on the forest floor until they are eaten away by fly larvae and collapse from within. Even more interesting than the mushrooms themselves are the underground, dense networks of hyphae that may extend many meters (Arora 1986, personal observation). These networks are called fungal or mycelial mats. Could the ability of the mushrooms to withstand decay be due to the symbiotic relationships between bacteria and fungi in their mats? This question led to my interest to study the microbes within these fungal mats.

Fungal mats are prevalent in forest ecosystems and can make up 10-20% of the forest organic matter (Griffiths *et al* 1996). The definitions of what makes up a fungal mat are numerous (see Trappe *et al.* 2012 for definitions from various researchers), but these mats are probably best defined as “forest soils and litter layers that are characterized by a dense profusion of rhizomorphs...form[ing] distinct morphological entities that are easily differentiated from adjacent noncolonized soil [and litter]” (Griffiths *et al* 1990). Hereafter, they will be called fungal mats or simply mats. Studies of fungal mats in the Pacific Northwest found that they alter the physical and chemical properties of the substrates that they inhabit (Griffiths *et al* 1991, 1994, Trappe 2012). Some notable differences in chemical properties and processes such as pH, denitrification, mineralization, and nitrogen fixation rates have been recorded in fungal mats when compared with non-mat soils (Griffiths *et al* 1991), leading to the hypothesis that such environmental differences could change local microbial composition.

The hypothesis that fungal mats could alter microbial composition of litter led Hesse (2012) to a descriptive study using next generation sequencing of the bacteria that live within the fungal mats of two ectomycorrhizal fungi in the genera *Piloderma* and *Ramaria*. The general conclusions of this study were that there were different microbial compositions between the types of mats when compared to uncolonized soils and the bacterial families Sphingobacteriaceae, Burkholderiaceae, Microbacteriaceae, and Acetobacteraceae were associated with *Piloderma* mats, but no families showed distinct associations with *Ramaria* mats. Unfortunately, the author only carried out the analysis at the bacterial Family level, which was useful for a general understanding of microbial composition, but it was not useful to detect species that may participate in symbioses.

While mycorrhizal species do not have the ability to break down complex plant polysaccharides such as cellulose and lignin, saprobic fungi are masters at such work. These fungi invade a new substrate and immediately stake claim to that substrate by growing around all the material available, forming a dense fungal mat in the organic horizon. It had been suggested that fungal hyphae often leak nutrients that attract bacteria, which may in turn behave positively or negatively towards the fungal host (reviewed by Leveau & Preston 2008, Scheublin *et al* 2010). I suspected that with such prevalence of hyphae in the mat environment, certain groups of bacteria would be found living with the fungi, growing with them either mutualistically, parasitically, or somewhere within continuum of symbiosis.

In this chapter, I present data that would not only complement the Hesse (2012) ectomycorrhizal mat study, but extend beyond it. This was achieved by studying a different system of fungal mats (saprobic here vs. mycorrhizal mats), including deeper and more detailed analyses of bacterial species to detect potential symbiotic partners. Three saprobic mat-forming fungi were selected for this study because of their relatively common occurrence in California: *Leucopaxillus albissimus*, *Leucopaxillus gentianeus*, and *Phaeoclavulina curta* (*Ramaria myceliosa*). Three hypotheses to be tested were: 1) the communities within fungal mats are different from non-mat, 2) the communities of bacteria in closely related fungal species (*Leucopaxillus*) are more similar to each other than to those in an unrelated fungal species (*Phaeoclavulina*), and 3) there are certain groups of bacteria that are preferentially associated with fungal mats.

Materials and methods

Site description

The samples were collected from three field sites. Site 1 was in the watershed of San Francisco Bay (SFW, 37.498397° -122.355608°). Sites 2 (38.043889° -122.804094°) and 3 (38.046683° -122.804248°) were in Point Reyes National Seashore (PtR). The two sites in PtR were 0.31 km from each other, and 72 km from Site 1. Site 1 was underneath the canopy of a monodominant stand of Monterey Cypress (*Cupressus macrocarpa*) and the fungus of interest was *Leucopaxillus gentianeus* (Fig. 1A). Site 2 was underneath the canopy of a stand of Blue Gum Eucalyptus (*Eucalyptus globulus*) and the fungus of interest was *Leucopaxillus albissimus* (Fig. 1B). Site 3 was underneath the canopy of a massive Coast Live Oak (*Quercus agrifolia*) and the fungus of interest was *Ramaria myceliosa* (*Phaeoclavulina curta*, Fig. 1C). The litter in which the fungi grow is composed of nearly 100% of leaves and twigs from the associated trees.

Sampling

For a summary of the sampling scheme, refer to Table 1. Sites 1 and 2 each had two plots, and site 3 had only 1 plot. Plot selection was determined by finding fruiting bodies of the fungus of interest. Plot selection was relatively easy for Sites 1 and 2, but only one fruiting body could be located for Site 3; hence only one plot for that site. Within a site, the plots were about 30m apart.

Within each plot, six litter samples were taken. Three of those samples came from within a single mat where each sample was about 10m from each other, right underneath mushroom(s). The other three came from non-mat litter that were 0.5m away from the edge of a mat. A mat edge

was easily distinguished as an abrupt ending to mycelial colonization, much like the edge of a fungal colony growing in a petri plate. The mat and non-mat samples were not paired. Each sample was the approximately 100 x 70 x 20 mm deep. Care was taken so that for every sample, only leaf litter of similar depth was collected without any of the soil beneath the litter. This was usually not difficult because the leaf litter could be as thick as 100mm in certain plots. Thirty samples were collected, all within an 8-day period starting Dec. 2, 2011.

Samples were stored on ice and processed within 24 hours. In the lab, each sample was homogenized by hand. pH was measured by macerating each sample in an equal volume of dH₂O. DNA was extracted from 1g of each sample using a MO BIO PowerSoil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA). Timing of bead beating was modified such that each 1g sample was divided in half. One half of the sample received 5 minutes of beating and the other half received 10 minutes of beating, each at maximum speed on a Fisher Vortex Genie 2 (Thermo Fisher Inc.). The reason for the dual beating time was to obtain DNA from groups of bacteria that vary in extraction efficiency (DeSantis *et al.* 2005). DNA extracts for each sample were pooled and stored at -20°C.

Molecular methods

The V1-V2 region of the 16S rRNA gene was amplified using the universal primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3'; Lane 1991) and 357r (5'-CTGCTGCCTYCCGTA-3'). The reverse primer was attached to 454 adapters and 8bp multiplex barcodes: A-adaptor + MID barcode + primer (357r) and used in combination with B-adaptor + primer (8f). Each PCR reaction contains 1.25 units of HotStarTaq polymerase (Qiagen), PCR buffer (containing 50 mM KCL, 10 mM Tris, 2.5 mM MgCl₂, and 0.1 mg/ml gelatin), 0.2 mM dNTPs, 25µg BSA, 2 mM MgCl₂, 0.3 µM of each primer and H₂O to a final concentration of 25 µL. Thermocycle conditions are: initial denature for 15 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 55[58 and 61]°C, 1 min at 72°C, and final extension for 10 min at 72°C. Each PCR reaction was produced in triplicate using the three annealing temperatures (55, 58, 61°C) to minimize PCR bias. The three PCR products were pooled and magnetically cleaned using an Agencourt AMPure XP kit (Beckman Coulter, Inc.) according to manufacturer's instructions. Purified PCR products were quantified using an Invitrogen Qubit Fluorometer (Invitrogen, Carlsbad, CA). The barcoded samples were then pooled in equimolar concentration. The pooled sample was cleaned once again through sodium borate agarose gel purification, during which extraneous bands of unexpected sizes were excised to prevent biased amplification. Samples were sent to the University of Illinois Keck Center for sequencing on ¼ of a plate with the Roche GS FLX+ system with 2.8 software, Flow B.

Data quality control and statistical analyses

Both data quality control and statistical analyses were performed primarily in the software package MacQIIME (Caporaso *et al.* 2010) unless noted otherwise. Raw sequence reads (.fna) and base quality score files (.qual) were imported into MacQIIME where primers and multiplex barcode sequences were removed, followed by annotation with sample metadata. The minimum quality score parameter was set to 25. Only sequences that were between 200-375bp in length were kept (sequences of 350bp were expected from these primers). Because of a newer implementation of the way each base was obtained (called Flow B), the denoising algorithm in MacQIIME could not be used, and at the moment there is no replacement for such an algorithm.

In its place, a graph of OTU number vs. % sequence similarity (from 90-100%) was created (Fig. 2) to visually determine the inflection point of the exponential curve. Percent similarity to the right of the inflection point is considered to be sequencing error where erroneous OTUs are created. Using this data, a 98% similarity was chosen as the cutoff point for OTU delimitation. OTUs were picked using the default UCLUST method using the Greengenes core alignment dataset as a guide. Taxonomic data was assigned to each OTU using the RDP Classifier. Chimeras were removed using ChimeraSlayer. Singletons (OTUs that occur only once) were removed, as were OTUs that occurred in the negative control. OTUs that could not be assigned to bacterial 16S, and those that matched to plant chloroplasts (Streptophyta) were removed. Samples that failed to sequence or those that produce too few number of sequences relative to other samples were removed. Representative sequences from each OTU were selected and aligned using the PyNAST algorithm, followed by construction of a phylogenetic tree using the FastTree method. The resulting tree was used for phylogenetic based community analyses (UniFrac) in later steps.

All analyses were performed in MacQIIME, except for the rarefaction of samples (Fig. 3C) which was performed in EstimateS 8.2 (Colwell 2009). OTU abundances and alpha-diversity metrics (Shannon H', Simpson's Diversity, and Faith's Phylogenetic Diversity = PD, Faith 1992) were measured using default parameters. To perform beta-diversity analyses, all samples were rarefied to 132 sequences and samples below this threshold were automatically discarded. Two approaches were taken to analyse this dataset. The first was to analyse community effects based on mat vs. nonmat litter, site, pH, and fungal species using all bacterial OTUs (Table 3). The second approach was to parse out bacterial OTUs at three taxonomic levels: Phylum, Order, and Genus (Table 4). At the Phylum level, all phyla found in the dataset were subjected to comparisons (see below). At the Order level, only orders that were represented by more than one OTU were compared. At the Genus level, only the genera that were present in at least three samples were compared.

Overall beta-diversity analyses include Principle Coordinate Analysis (PCoA), *adonis* (a nonphylogenetic based test very similar to a PERMANOVA where significance is determined by permutation of the dataset compared to the observed dataset), and UniFrac Significance (a test that randomly permutes the environmental variable on a phylogenetic tree and significance is determined if the UniFrac value of the observed tree is greater than the expected UniFrac values of the randomly permuted trees). A significant p-value from the UniFrac Significance test means that one community is phylogenetically different from another. Since *adonis* is based on F-statistics, R^2 values are also reported. Both the *adonis* and UniFrac Significance tests were based on 1000 randomizations. All statistical tests were performed using both incidence (presence/absence) and abundance data from the number of sequence reads. PCoA and UniFrac methods used UniFrac-based distance matrices whereas the other analyses relied on Sorosen (presence/absence) and Jaccard (abundance) distances. When required, p-values were corrected for multiple comparisons using Bonferroni correction.

Results

Data statistics and quality control

The ¼ plate run produced a total of 83,495 sequences with an average length of 336bp. After the thorough data filtering process, 39,324 sequences (47%) were discarded, resulting in a set of 44,080 sequences. The discarded sequences consisted of 28,222 sequences (33.8%) that did not pass quality control criteria, 6673 sequences (8%) that belonged to singleton OTUs, 1979 sequences (2.4%) that were found in the negative control, and 2450 sequences (2.9%) that were confirmed to be plant chloroplast in origin. Rarefaction of the retained 44,080 sequences showed that in general, the sequences have not reached saturation (Fig. 3). Chao1 estimates show that all but four samples were saturated (Fig. 3B). The accumulation curve for all samples together was not saturated (Fig. 3C).

Alpha-diversity

The total number of OTUs from the whole dataset was 5014. The non-mat samples contained 4387 OTUs and the mat samples contained 1658 OTUs. The major phyla of Bacteria were represented in all samples: Proteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria, and Firmicutes. Figure 4 compares proportions of OTU that belong to different bacterial Classes within mat and non-mat samples. The proportions of each Class varied depending on the type of sample. The last two bars in the figure show the overall diversity in mat and non-mat samples. Only the Bacilli seemed to have been overly represented in mat samples (5%) and almost absent in the non-mat samples (0.07%). In addition, the Actinobacteria appear to have been more abundant in mat than non-mat samples, but the Alphaproteobacteria and Sphingobacteria were more abundant in non-mat samples.

Rarefaction (to 132 sequences) did not have major effects on the most abundant taxa at the OTU level. The two most abundant OTUs kept their ranks after rarefaction. A few other OTUs swapped places with their neighbors, but not to a notable degree (data not shown). In the rarified ranked-abundance graph of the 16 most common OTUs shown in Fig. 5, *Ensifer* (*Sinorhizobium*) aff. *medicae* was the most abundant OTU, followed by *Pseudonocardia* aff. *alaniniphila*. Some OTUs appeared in both mat and non-mat samples, whereas other OTUs seemed to appear preferentially in either mat or non-mat samples (Fig. 5).

The three diversity indices showed that there is a broad range of diversity among the samples (Table 2). Shannon's diversity index ranged from 3.79-9.52, whereas Simpsons Diversity index ranged from 0.79-0.99, compared to Faith's PD that ranged from 4.43-69.33. When the samples were grouped into mat and non-mat for comparison, Shannon's ($p = 0.001$) and Faith's PD ($p = 0.003$) showed that the diversity in mat samples were significantly lower than non-mat. However, Simpson's index was not significantly different between mat and non-mat samples.

Beta-diversity

Figure 6 visually shows that the community structure is strongly partitioned by litter habitat (mat [red dots] and non-mat [blue dots]). Of the four environmental parameters examined (site, fungal species, pH, and mat/non-mat litter) only mat/non-mat litter showed clear separation between communities. The other parameters also showed general groupings but there is considerable overlap between samples (data not shown). When compared to unweighted UniFrac data (Fig. 6A), weighted data (Fig. 6B) showed more spread in the mat-samples. In 3-dimensional space (not shown here), the non-mat samples have less spread in the z coordinate and generally clump well together.

In this experimental design, site and tree species covaried with each other. As such, the language below will only refer to "site". Fungal species also covaried with site and tree species, although in this case, fungal species only applies to mat samples. Because the data were analyzed by two tests, weighted or not, significant p-values will be reported based on the least significant value of all tests (e.g. $p \leq 0.04$).

Overall, beta-diversity comparisons using the nonphylogenetic test *adonis* showed comparable results for weighted and unweighted data (Table 3). Only pH and mat/non-mat litter had significant effects on the bacterial community. Both of these environmental factors also had respectable R^2 values, especially when the weighted data was considered. In both cases, pH had greater explanatory power than mat/non-mat litter. Surprisingly, bacterial communities from the three sites were not significantly different from each other. Similarly, the communities among fungal species were not significantly different from each other. The UniFrac Significance test did not agree with these results.

Phylogenetic UniFrac tests using unweighted and weighted data showed similar results to each other, and have more resolution than the taxon-based *adonis* comparisons because it employs phylogenetic data (Table 3). All four unweighted parameters, site ($p \leq 0.001$), fungal species ($p \leq 0.003$), pH ($p \leq 0.005$), mat/non-mat litter ($p \leq 0.001$) had statistically significant effects on bacterial community composition. However, the weighted data showed that while site, pH, and mat/non-mat litter were significant ($p \leq 0.006$), fungal species was not always. In particular, the bacterial community from *L. gentianeus* was not significantly different from that of *L. albissimus*, but they were significantly different from *Phaeoclavulina curta* ($p \leq 0.003$).

Bacterial taxon differences between mat vs. non-mat litter and between fungal species

All five bacterial phyla were subjected to the *adonis* test in order to determine if any one of the phyla were significantly present in either mat or non-mat litter. Table 4 shows these results. All four of the major phyla differed in relative abundance between mat and non-mat litter more than expected by chance, except when Bacteroidetes data were weighted. However, the differences had large explanatory power only for the Firmicutes ($R^2 = 0.32$). At the ordinal level, relative abundances of Acidobacteria Group 6, Actinomycetales, Sphingobacteriales, Bacillales, Clostridiales, and Caulobacterales differed between mat and non-mat litter more than expected by chance. Explanatory power of the model was greatest for Bacillales ($R^2 = 0.33$). All of the orders were more strongly associated with mat litter, except for Acidobacteria Group 6 which was associated more strongly with non-mat litter. At the level of genera, relative abundance of *Asanoa*, *Bacillus*, *Micobacterium*, *Paenibacillus*, *Pseudonocardia*, *Streptomyces*, and *Terrimonas* differed between mat and non-mat litter more than expected by chance, where the greatest explanatory power was with *Paenibacillus* ($R^2 = 0.22$). As a general trend, these genera were generally more strongly associated with mat litter, except for *Terrimonas* which occurred almost exclusively in non-mat litter.

The most highly represented OTUs were examined to further understand their importance in mat and non-mat litter (Figure 5). However, because only one OTU was examined at a time at this taxonomic level, the tests for community comparisons could be used. *Ensifer* aff. *medicae* was the most abundant OTU, and it frequently occurred in both mat and non-mat litter. A similar

pattern held for *Rhizobium. Pseudonocardia* aff. *alaniniphila*, the second most abundant OTU, occurred in high abundance on almost all mat samples (131 sequences) but only occurred sporadically in several non-mat samples (6 sequences). Two other *Pseudonocardia* OTUs (*Pseudonocardia* sp. and *Pseudonocardia* aff. *yunnanensis*) showed a similar pattern; they mostly occurred with mat litter and rarely with non-mat litter. These three OTUs belong to a species complex within the genus *Pseudonocardia*. Two OTUs of *Streptomyces* showed a similar pattern, but were not shown in Figure 5 because they were below the cut-off threshold of at least 20 sequences. As a general trend, the less abundant OTUs did not show obvious biases toward either mat or non-mat litter. The few OTUs that appeared only in mat samples were not consistently present. There were four such taxa: *Chryseobacterium* sp., *Rhodococcus* sp., *Leifsonia* sp., and *Microbacterium oxydans*, and even though they were relatively abundant, each was present from only one mat sample.

Discussion

The dataset that remained after quality control filtration was still robust enough to bring the Chao estimates in most samples to saturation (Fig 3B). However when all samples were considered together (Fig. 3C) the curve was not saturated. This suggests that a one-quarter 454 pyrosequencing plate was not enough to saturate sampling of forest litter bacteria. This was not unexpected as a plethora of microbial ecology studies have found that soil microbial communities are very diverse (review by Marona *et al.* 2011 and references therein).

As predicted by Hypothesis 1, communities within mat litter were different than those in non-mat litter. Overall, the microbial diversity of mat litter was lower than non-mat. Shannon's Index and Faith's PD both showed that diversity was significantly lower in mat versus non-mat litter. However, Simpson's diversity index showed the same trend but was not significant. The lower diversity in mat litter was expected for two reasons. First, established fungi likely prevent invaders (both bacteria and other fungi) from entering the mats based on priority effects where the first organism to colonize the environment takes hold and keep out the others. Second, the hydrophobic nature of the mat litter is likely to help deter invaders (Trappe *et al* 2012). Isolation of the microbes followed by lab competition assays will be necessary to answer these two hypotheses.

The bacterial community composition of mats and non-mat litter also differed more than expected by chance. However, community diversity was also influenced by pH and, and the effect of pH was not independent of the presence/absence of fungal mat. The pH in mats (5.5) was significantly lower than in non-mat litter (5.9), with an average difference of 0.4. Cromack *et al* (1979) and Griffiths *et al* (1991) showed that mats of ectomycorrhizal fungi produce oxalates and other organic acids that lower the pH level when compared to non-mat soils. It is reasonable to speculate the saprobic fungal mats studied here also contain organic acids that lower the pH, and thus influence the bacterial community. The effect of pH on bacterial communities was first demonstrated by Fierer & Jackson (2006) and more recently in the fungal mat study of Hesse (2012). The results reported here support these findings.

Presence of a fungal mat had different effects on different bacterial taxonomic groupings (see Table 4). Overall, these significant effects seemed to have come from the bias presence of many

of the taxa in mats vs. non-mat litter. The general mycophilic nature of the mat-dwelling bacteria is also supported by other studies between fungi and bacteria. For example, the genera *Bacillus*, *Paenibacillus*, *Pseudonocardia* and *Streptomyces* have been reported in the literature as having been found from either within or on fungal hyphae (Knutson & Hutchins 1980, Li *et al* 1992, Bertaux *et al* 2003, Izumi *et al* 2006, Toljander *et al* 2006, Sharma *et al* 2008, Sen *et al* 2009). Surprisingly, *Burkholderia* and the genera within the Rhizobiales were not significantly associated with fungal mats in this study, despite the many studies that have reported their occurrence and often times intimate associations with fungi (Bianciotto & Bonfante 2002, Izumi *et al* 2008, Partida-Martinez & Hertweck 2005, Coenye *et al* 2001, Lim 2003, Tanaka *et al* 2009). Only a few taxa with significant effects were found outside of the mat such as those within the Phylum Acidobacteria and the genus *Terrimonas*.

Surprisingly bacterial communities at the different sites, covarying with different host trees, did not differ more than expected by chance based on both UniFrac and *adonis* with weighted and unweighted data. This result contrasts with numerous studies that found that site, even in millimeter scale, significantly affected microbial communities (for example Kunin 2008, Martiny 2011). These studies, however, were able to determine that chemical gradients within the environment contributed to the differences observed. In this study, site and host tree are covariates and together they did not have an effect on the bacterial community. The fact that mat/non-mat litter had a significant effect with well-explained variance points to the strong selection pressure within fungal mats. It also implies that decomposing litter isn't a habitat preferred by bacteria because of the high competition with fungi.

There was mixed support for Hypothesis 2, that similar fungal species share similar bacteria communities. The non-phylogenetic test *adonis* did not show any significant effects whereas the phylogenetic test UniFrac, when weighted by read number, showed that bacterial communities were similar between the two *Leucopaxillus* species, but both these communities differed from that of *Phaeoclavulina curta*. This result agreed with the results by Hesse (2012), which found that different fungal species support different bacterial communities. However, our study was not ideal because fungal species covaried with site and host tree. To dissect the confounding effects of sites and host tree from fungal species, future studies should focus on sites with *Cupressus macrocarpa* because all three fungal species will grow in the leaf litter of this plant.

The different types of tests (taxon based vs. phylogenetic based and unweighted vs. weighted data) gave varied results. Taxon based methods such as *adonis* do not account for the different degrees of similarity between sequences and thus fail to use informative data about relatedness. Phylogenetic based methods on the other hand, take account of relatedness and produce what is generally considered more complete results (Lozupone *et al* 2006). The current software implementation of the *adonis* test is performed on a combined dataset of all samples together, and thus any differences within a set of samples could not be observed. Conversely, the UniFrac test allows for pairwise comparisons (followed by correction for the multiple comparisons) and thus has greater sensitivity. Despite the limitations of taxon based tests, they are especially useful when comparing data at lower taxonomic levels because phylogenetic tests fail to detect community differences due to phylogenetic relatedness. For example, phylogenetic tests will fail to find community differences between samples if all of organisms were very similar. Because very similar organisms will group together phylogenetically, shuffling of the branches will not

produce trees that are different from the original tree, thus giving a non-significant result. In such cases, taxon based tests are more sensitive.

Even though interpretation of weighted data must be made very carefully due to the many biases of PCR (Amend *et al* 2010, Sipos 2010 and the references therein), the use of weighted data can give certain signals that could be missed otherwise as in the case of fungal species comparisons discussed above. As an example from this study, *Pseudonocardia* aff. *alaniniphila* was the most highly represented taxon and it occurs in both mat and non-mat samples. However, when weighted data was considered, the majority of the occurrence (=sequences) were found in the mat samples. Had occurrence not been taken into account, this taxon would not have been picked out as having a potentially important role within fungal mats. Therefore, it is important to consider both taxon based and phylogenetic based tests using both weighted and unweighted metrics.

Specific OTUs and their potential effects on the mat environment

The most abundant OTU in the samples was *Ensifer* aff. *medicae*. This OTU belongs to the Rhizobiales and taxonomic association indicates that it might fix nitrogen, and perhaps contribute nutrients to the nitrogen-poor leaf litter environment. However, this OTU was similarly associated with both mat and non-mat litter in both occurrence and abundance. Similarly, four of the 16 most abundant OTUs had the potential ability to fix nitrogen (Fig. 5B), but they too were these enriched within the mat. It is interesting to note that Griffiths *et al* (1991) found that nitrogen fixation rates did increase (from ~2-9 times) in mat samples of two different truffle-forming fungi in the genera *Hysterangium* and *Gautieria*. Perhaps *Ensifer* aff. *medicae* and other abundantly represented potential nitrogen fixers live in both mat and non-mat litter, but nitrogen fixation rate would be preferentially increased in mat samples. This may be due to the protective environment of the fungal mat where oxygen level is lower, allowing for nitrogen fixation to occur. Another explanation would be that these are actually active within the mat, and are not active in the soil.

In contrast to the potential N fixers, *Pseudonocardia* spp. were significantly enriched in mat samples. *Pseudonocardia* aff. *alaniniphila* was the second most abundant OTU in the study and was found primarily in mat samples (131 sequences) but only sporadically in non-mat samples (6 sequences). Phylogenetic analysis (not shown) showed this OTU nested within a group of *Pseudonocardia* species in the *aniniphila/yunnanensis* complex, described from China. *Pseudonocardia aniniphila* was isolated from soil and was able to resist two types of antibiotic, rifampicin and neomycin, both derived from other Actinobacteria. Two other *Pseudonocardia* OTUs that appeared on the top 16 OTU list (Fig. 5B) also belong to this species complex, and along with one more OTU identified as “Actinomycetales sp.”, were found almost exclusively in mat litter.

Other reports of *Pseudonocardia* lend support to the idea that they may be symbiotically associated with fungal mats. This genus is important in associations with animals, from marine sponges to insects, but particularly leaf-cutting ants (Mueller *et al* 2010). In the leaf-cutting ant model, the *Pseudonocardia* grows on the ants and provides antibiotics to ward away fungal weeds of cultivated fungal gardens (Cafaro *et al* 2011). A similar association could be occurring in saprobic fungal mats (without the ants), with *Pseudonocardia* producing antibiotics that

protect fungal mats by warding off pest fungi and bacteria. After all, the ants' fungal gardens are just mats of saprobic fungi growing in leaf litter (Fig. 7).

Hesse (2012) specifically mentioned that there was no increase or decrease in the family Streptomycetaceae (closely related to the Pseudonocardiaceae) in the mats. Since *Streptomyces* species are able to utilize oxalate as a sole carbon compound, and oxalates are present in notable amounts in fungal mats, it was thought that *Streptomyces* are drawn to mats because of this compound (Cromack *et al* 1979, Knutson *et al* 1980, Hesse 2012). In this study, the members of genus *Streptomyces* were significantly biased towards fungal mats (Table 4), but they were not present in as high numbers as *Pseudonocardia*. Hesse (2012) made no mention of *Pseudonocardia* in his work.

The fact that mushrooms of *L. albissimus* resist decay has not gone unnoticed and had been described in field guides. In David Arora's lyrical *Mushrooms Demystified*, he suggested that to identify the mushroom, "hide it in your housemate's closet. . .if unchanged after one month, it is *definitely L. albissimus*" (Arora 1986). The mushrooms of *L. gentianeus* also can last for weeks in the field, but often fall apart due to mycophagy by fungus gnat larvae (personal observation). Could the ability of *Leucopaxillus* species to withstand decay be related to their association with antibiotic producing bacteria? Isolation of host fungi, potential pest fungi, and *Pseudonocardia* followed by bioassays would be very interesting to answer this question. Together, these findings support Hypothesis 3, that certain groups of bacteria (in this case *Pseudonocardia* aff. *alaniniphila* and its relatives) are associated with fungal mats.

Of the three hypotheses that were made, two were well supported, and one was marginally supported. 1) The bacterial communities of fungal mats were indeed different than those of non-mat litter, 2) the communities in mats of related fungal species were more similar to each other, at least when their phylogenetic relatedness was taken into account, and 3) there was a group of bacteria that was found specifically in association with fungal mat litter. Further, this work provides a basis for further experimental dissection of the association between *Pseudonocardia* species and fungal mats. Finally, the rich opportunities for bacterial-fungal interactions in the environment are likely to provide many additional examples of important, but overlooked symbioses.

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Table 1. Summary of the sampling scheme.

site	plot	mat sample	non-mat sample	Associated leaf litter
1 San Francisco watershed	1	<i>Leucopaxillus gentianeus</i> 1-A	<i>Leucopaxillus gentianeus</i> 1-a	<i>Cupressus macrocarpa</i>
		<i>Leucopaxillus gentianeus</i> 1-B	<i>Leucopaxillus gentianeus</i> 1-b	<i>Cupressus macrocarpa</i>
		<i>Leucopaxillus gentianeus</i> 1-C	<i>Leucopaxillus gentianeus</i> 1-c	<i>Cupressus macrocarpa</i>
	2	<i>Leucopaxillus gentianeus</i> 2-A	<i>Leucopaxillus gentianeus</i> 2-a	<i>Cupressus macrocarpa</i>
		<i>Leucopaxillus gentianeus</i> 2-B	<i>Leucopaxillus gentianeus</i> 2-b	<i>Cupressus macrocarpa</i>
		<i>Leucopaxillus gentianeus</i> 2-C	<i>Leucopaxillus gentianeus</i> 2-c	<i>Cupressus macrocarpa</i>
2 Point Reyes	3	<i>Leucopaxillus albissimus</i> 3-A	<i>Leucopaxillus albissimus</i> 3-a	<i>Eucalyptus globulus</i>
		<i>Leucopaxillus albissimus</i> 3-B	<i>Leucopaxillus albissimus</i> 3-b	<i>Eucalyptus globulus</i>
		<i>Leucopaxillus albissimus</i> 3-C	<i>Leucopaxillus albissimus</i> 3-c	<i>Eucalyptus globulus</i>
	4	<i>Leucopaxillus albissimus</i> 4-A	<i>Leucopaxillus albissimus</i> 4-a	<i>Eucalyptus globulus</i>
		<i>Leucopaxillus albissimus</i> 4-B	<i>Leucopaxillus albissimus</i> 4-b	<i>Eucalyptus globulus</i>
		<i>Leucopaxillus albissimus</i> 4-C	<i>Leucopaxillus albissimus</i> 4-c	<i>Eucalyptus globulus</i>
3 Point Reyes	5	<i>Phaeoclavulina curta</i> 5-A	<i>Phaeoclavulina curta</i> 5-a	<i>Quercus agrifolia</i>
		<i>Phaeoclavulina curta</i> 5-B	<i>Phaeoclavulina curta</i> 5-b	<i>Quercus agrifolia</i>
		<i>Phaeoclavulina curta</i> 5-C	<i>Phaeoclavulina curta</i> 5-c	<i>Quercus agrifolia</i>

Table 2. Three diversity indices calculated for mat and non-mat samples. The p-value indicates comparison of the diversity metrics, as calculated using a two-tailed Student's T-test.

	Sample	Shannon	Simpson	Faith
mat	F347	4.10	0.80	6.26
	F348	6.05	0.95	15.10
	F349	7.85	0.99	23.71
	F350	7.42	0.99	19.98
	F351	7.16	0.98	22.20
	F358	4.78	0.92	7.49
	F359	5.46	0.97	8.92
	F360	3.79	0.86	4.43
	F361	6.16	0.96	14.24
	F362	6.40	0.97	16.02
	F368	4.91	0.92	6.96
	F371	6.53	0.97	15.06
	F372	6.22	0.94	14.88
non-mat	F352	6.82	0.90	32.74
	F353	7.10	0.99	19.69
	F354	7.48	0.99	19.52
	F355	9.48	0.99	69.33
	F356	4.85	0.79	14.45
	F357	8.67	0.99	41.02
	F364	6.92	0.98	17.62
	F365	7.91	0.99	23.44
	F366	8.32	1.00	29.48
	F367	6.18	0.98	10.31
	F369	9.52	1.00	61.00
	F370	8.00	0.98	27.30
	F373	7.51	0.99	20.89
F374	8.94	1.00	43.67	
mat average		5.91	0.94	13.48
non-mat average		7.69	0.97	30.75
p-value		0.001	0.176	0.003

Table 3. Significance values from the *adonis* and UniFrac tests when comparing the overall bacterial community among samples that differed in particular environmental factors. R² values based on F-statistics are also reported for the *adonis* test. Significant p-values are highlighted in yellow. uw = unweighted data based on presence/absence, w = weighted data based on read abundance, varies = both significant and nonsignificant p-values were found. *Fungal species were based only on mat litter.

		<i>adonis</i> (uw)	<i>adonis</i> R ² (uw)	<i>adonis</i> (w)	<i>adonis</i> R ² (w)	UniFrac (uw)	UniFrac (w)
Environmental factor	mat/non-mat	0.001	0.10	0.001	0.21	0.001	0.001
	site	0.305	0.08	0.817	0.05	≤ 0.001	≤ 0.005
	pH	0.001	0.21	0.007	0.28	≤ 0.005	≤ 0.006
	fungal species*	0.627	0.16	0.357	0.17	≤ 0.003	varies

Table 4. Significance values for the *adonis* test comparing mat vs. non-mat litter at the Phylum, Order, and Genus levels. The R^2 values derived from F-statistics are also reported. p-values less than 0.05 are highlighted in yellow. uw = unweighted data based on presence/absence, w = weighted data based on read abundance.

	Order	<i>adonis</i> (uw)	<i>adonis</i> R^2 (uw)	<i>adonis</i> (w)	<i>adonis</i> R^2 (w)
Phylum	Actinobacteria	0.003	0.07	0.001	0.07
	Bacteroidetes	0.045	0.05	0.136	0.04
	Firmicutes	0.001	0.32	0.001	0.30
	Proteobacteria	0.020	0.05	0.098	0.05
	others	0.117	0.06	0.141	0.05
Orders within Acidobacteria	Acidimicrobiales	0.817	0.02	0.901	0.02
	Acidobacteria Group 1	0.748	0.02	0.711	0.02
	Acidobacteria Group 4	0.051	0.08	0.064	0.08
	Acidobacteria Group 6	0.005	0.14	0.006	0.14
	Acidobacteria Group 7	0.220	0.07	0.216	0.07
	Acidobacteria Group 16	0.899	0.02	0.849	0.02
Orders within Actinobacteria	Acidimicrobiales	0.258	0.05	0.133	0.05
	Actinomycetales	0.002	0.07	0.002	0.07
Orders within Bacteroidetes	Flavobacteriales	0.173	0.06	0.167	0.06
	Sphingobacteriales	0.037	0.05	0.125	0.05
Orders within Firmicutes	Bacillales	0.001	0.33	0.001	0.30
	Clostridiales	0.017	0.14	0.010	0.14
Orders within Proteobacteria	Burkholderiales	0.161	0.05	0.133	0.05
	Caulobacteriales	0.005	0.11	0.004	0.11
	Myxococcales	0.232	0.04	0.213	0.04
	Rhizobiales	0.104	0.05	0.162	0.05
	Rhodospirillales	0.885	0.02	0.908	0.02
	Legionellales	0.114	0.09	0.083	0.09
	Solirubrobacteriales	0.490	0.03	0.477	0.03
	Sphingomonadales	0.378	0.04	0.454	0.04
	Xanthomonadales	0.194	0.05	0.166	0.05
Genus	Aeromicrobium	0.707	0.02	0.786	0.02
	Asanoa	0.039	0.13	0.043	0.13
	Bacillus	0.020	0.17	0.021	0.16
	Bradyrhizobium	0.538	0.03	0.469	0.03
	Chryseobacterium	0.156	0.07	0.166	0.07
	Devosia	0.639	0.03	0.645	0.03
	Ensifer	0.038	0.32	0.036	0.29
	Ferruginibacter	0.117	0.06	0.093	0.06
	Gemmatimonas	0.101	0.08	0.117	0.08
	Hyphomicrobium	0.074	0.10	0.066	0.11
	Marmoricola	0.190	0.06	0.211	0.05
	Mesorhizobium	0.601	0.02	0.024	0.60
	Methylibium	0.172	0.10	0.157	0.07
	Microbacterium	0.015	0.13	0.019	0.13
	Mucilaginibacter	0.653	0.03	0.485	0.03
	Mycobacterium	0.223	0.06	0.231	0.06
	Novosphingobium	0.582	0.03	0.703	0.02
	Paenibacillus	0.010	0.22	0.011	0.20
	Pedobacter	0.749	0.02	0.729	0.02
	Phenylobacterium	0.380	0.04	0.313	0.04

	Pseudonocardia	0.023	0.10	0.008	0.12
	Rhizobium	0.178	0.06	0.204	0.06
	Solirubrobacter	0.153	0.07	0.104	0.07
	Sorangium	0.102	0.09	0.089	0.09
	Sphingobium	0.910	0.01	0.911	0.01
	Sphingopyxis	0.293	0.06	0.321	0.05
	Steroidobacter	0.216	0.06	0.202	0.06
	Streptomyces	0.001	0.19	0.004	0.19
	Terrimonas	0.007	0.15	0.010	0.15

Figure 1. Sporocarp and mat of the three species of fungi in this study from field collections. A. *Leucopaxillus gentianeus*. B. *Leucopaxillus albissimus*. C. *Phaeoclavulina curta*. Each of the three panels are divided into top and bottom where the top shows the fruiting structure, and the bottom shows the fungal mat. Photos were taken by the author.

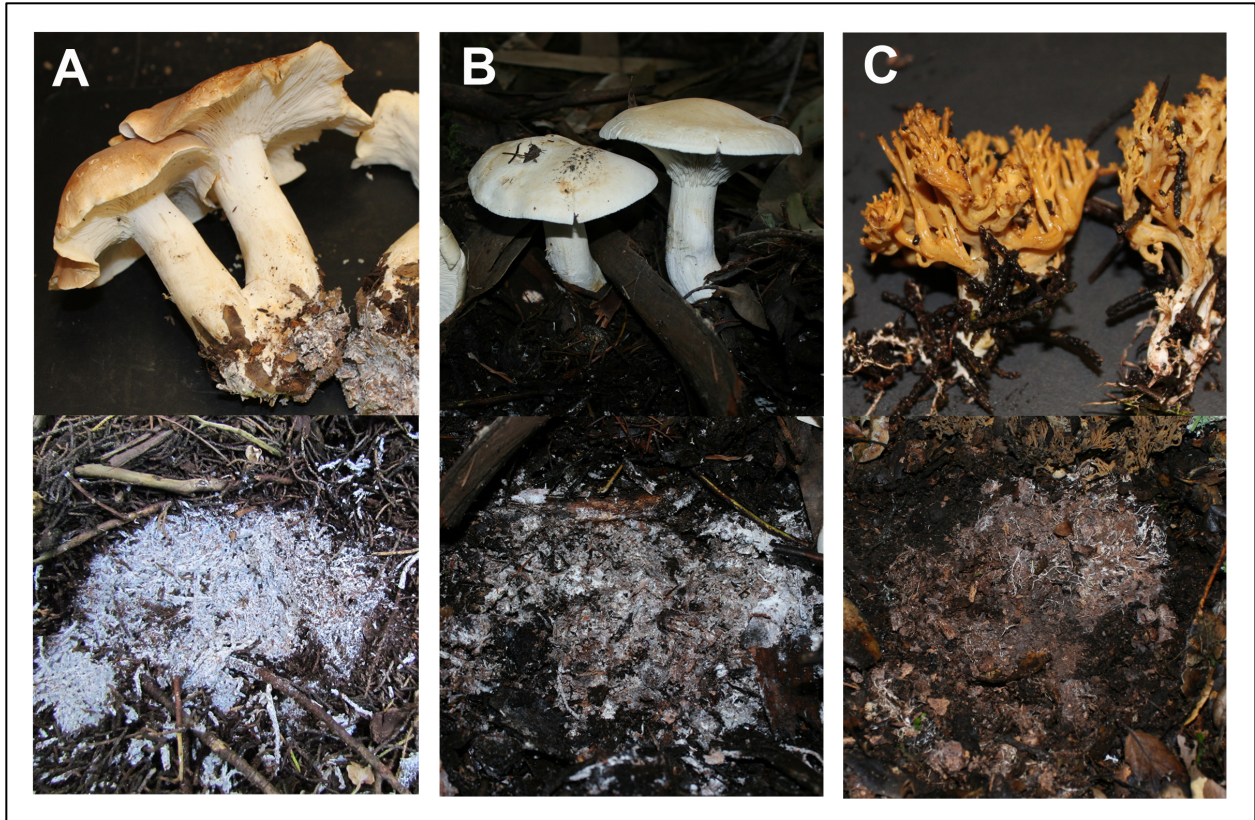


Figure 2. An exponential curve that resulted from graphing the number of OTUs vs. sequence % similarity. The inflection point of this curve is between 97-99%. The arrow shows the point on the curve (98%) that was chosen for OTU delimitation in this study.

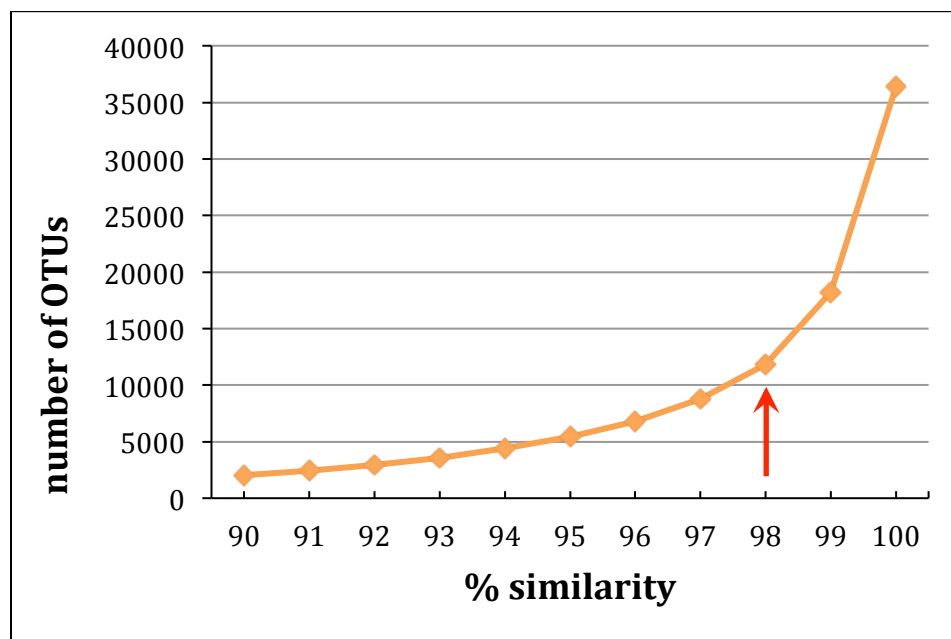


Figure 3. Rarefaction curves of the filtered data. A. Observed OTUs of individual samples. B. Chao1 estimates of individual samples. C. Observed and Chao1 estimate of all samples together.

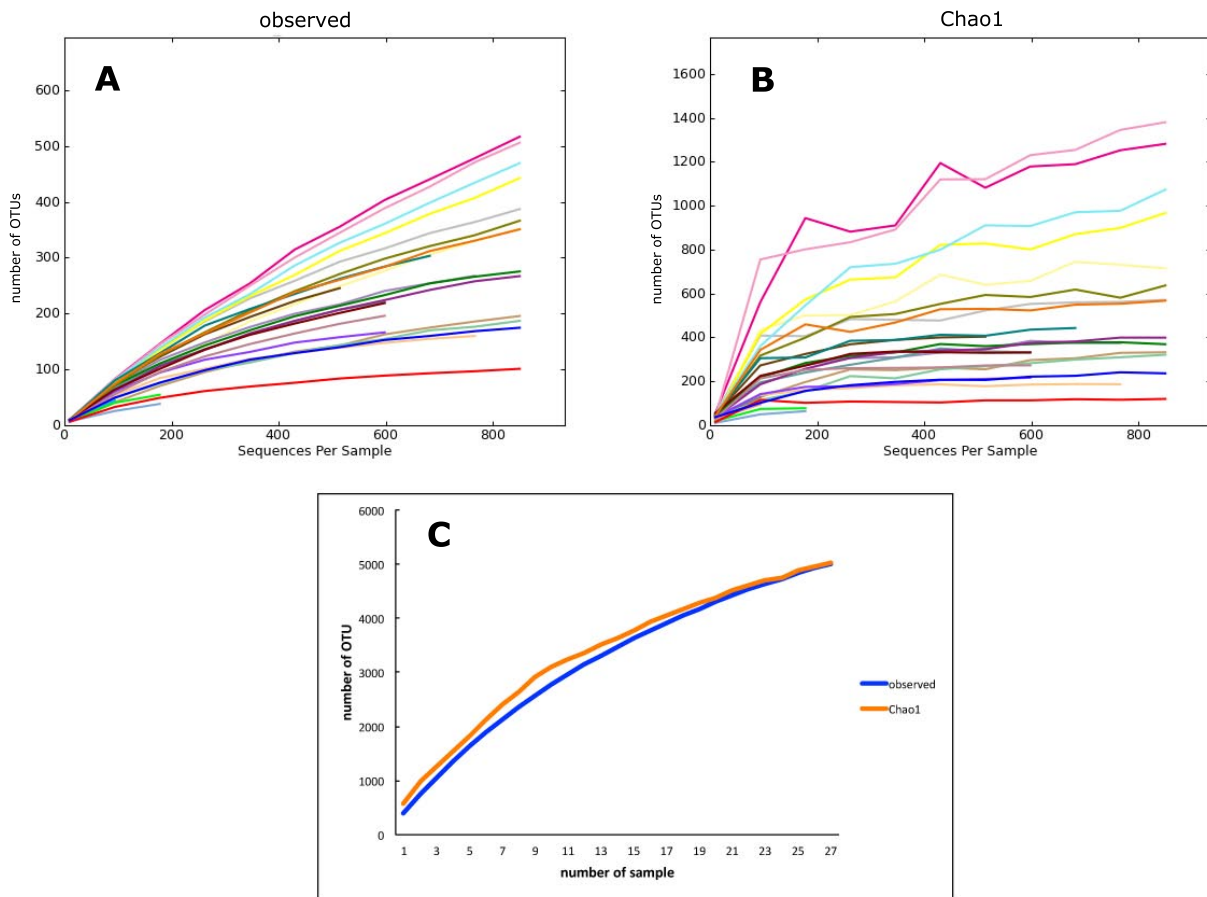


Figure 4. Relative proportions of bacteria OTUs at the Class level in mat and non-mat samples. Data for this graph came from unrarified samples. The last two bars are composites of all mats and non-mat samples.

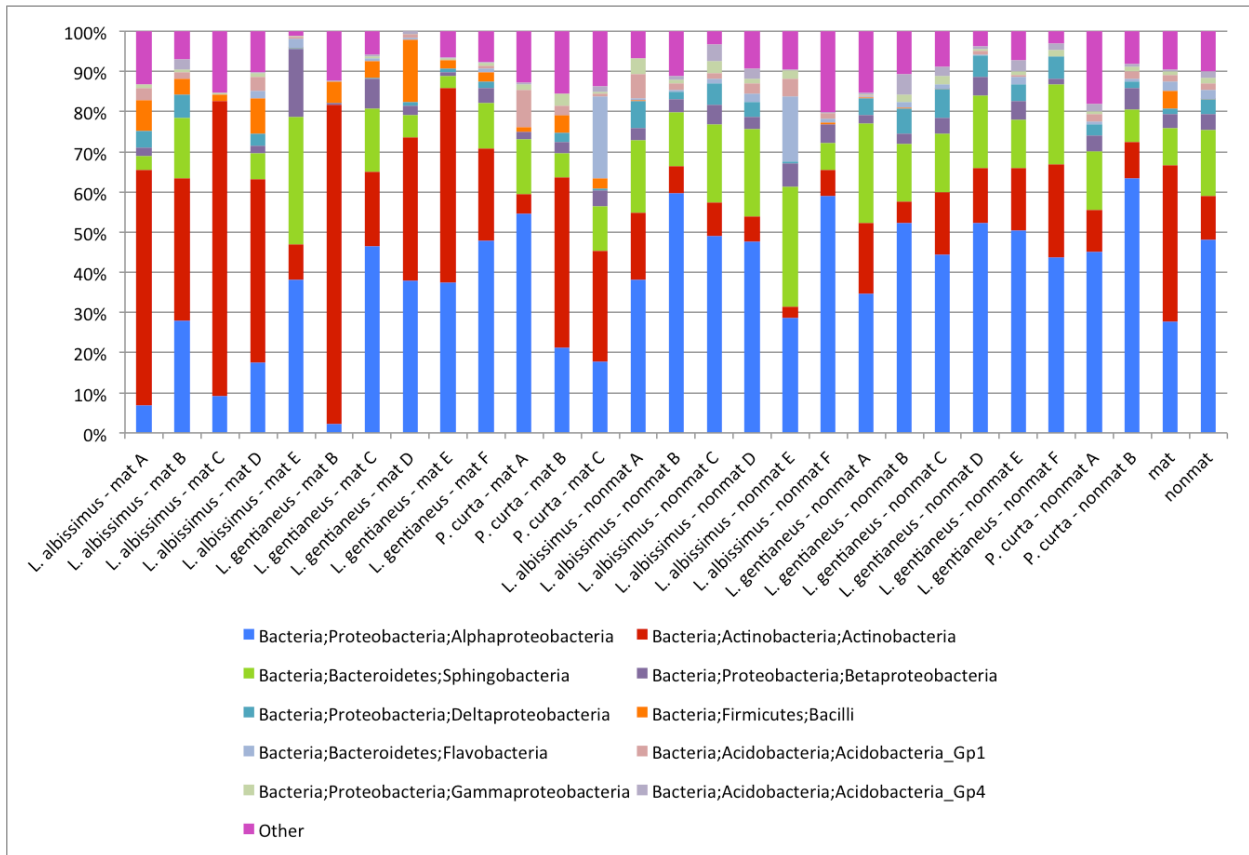


Figure 5. Ranked abundance graph showing the most common OTUs from rarified samples represented by ≥ 20 sequences. OTUs with the potential to fix nitrogen are marked with an asterisk (*) based on affinity to genera that have many diazotrophic species based on experimental evidence. Many of the other genera not marked by an asterisk also contain strains that (may be) are diazotrophs.

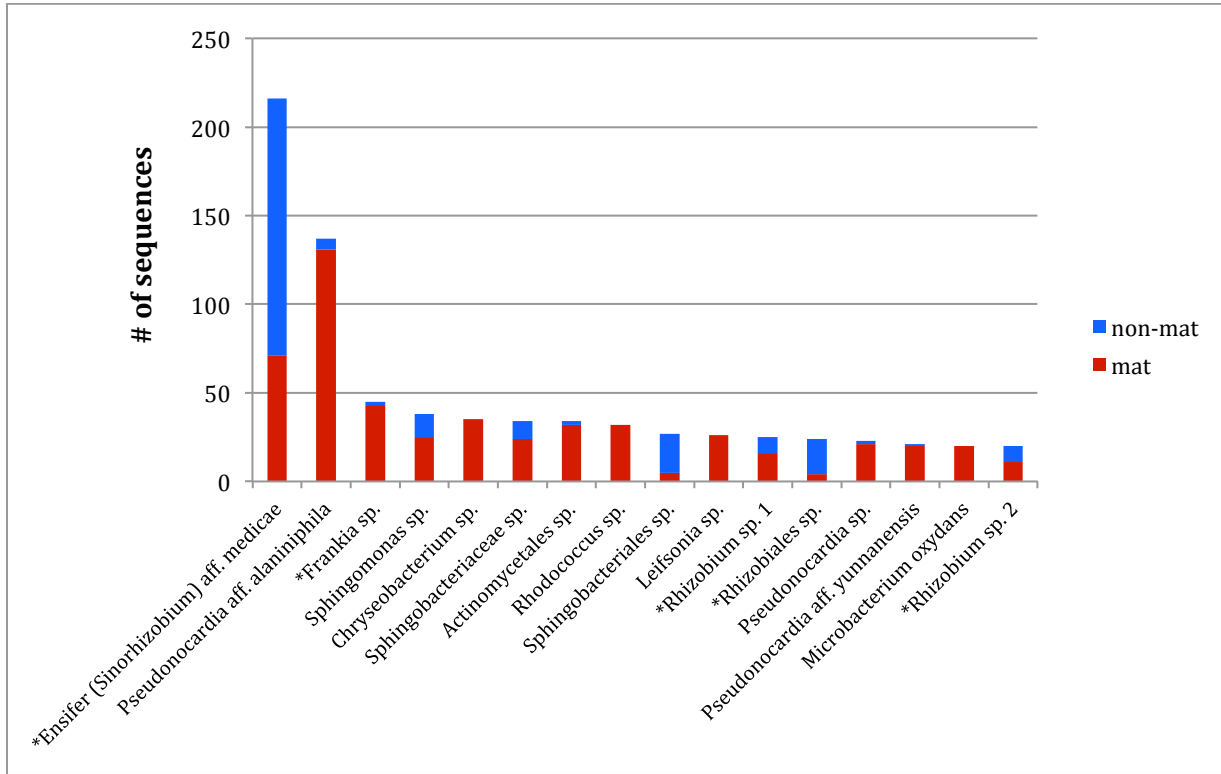


Figure 6. UniFrac PCA showing mat (red dots) and non-mat (blue dots) samples in 3-dimensional space. A. Unweighted UniFrac showing very little overlap. B. Weighted UniFrac showing a few overlapping samples.

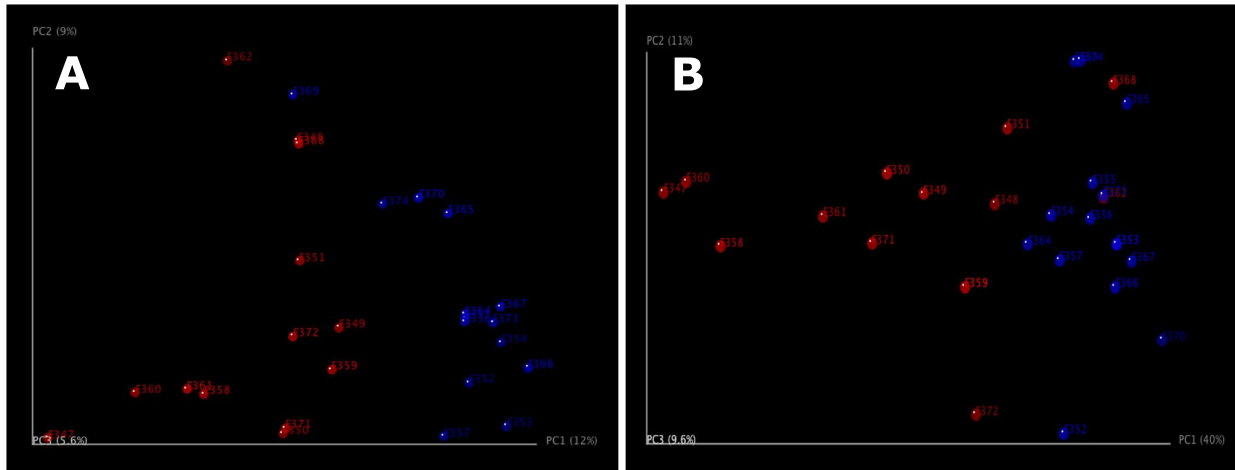


Figure 7. Fungal gardens of the leaf cutting ant *Atta texana*. The fungal gardens shown here can be compared directly to the fungal mats of *Leucopaxillus* and *Ramaria* species in this study. Photos were taken by the author.

