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Distinctive fungal and bacterial communities are associated with mats formed by ectomycorrhizal fungi

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ABSTRACT

The distinct rhizomorphic mats formed by ectomycorrhizal *Piloderma* fungi are common features of the organic soil horizons of coniferous forests of the Pacific Northwest. These mats have been found to cover 25–40% of the forest floor in some Douglas-fir stands, and are associated with physical and biochemical properties that distinguish them from the surrounding non-mat soils. In this study, we examined the fungal and bacterial communities associated with *Piloderma* mat and non-mat soils. Each mat and non-mat area was repeatedly sampled at four times throughout the year. Characterization of the mat activity and community was achieved using a combination of N-acetylglucosaminidase (NAGase) enzyme assays, and molecular analysis of fungal and bacterial communities using T-RFLP profiles, clone libraries, and quantitative PCR. *Piloderma* mats had consistently greater NAGase activity across all dates, although the magnitude of the difference varied by season. Furthermore, we found distinct fungal and bacterial communities associated with the *Piloderma* mats, yet the size of the microbial populations differed little between the mat and non-mat soils. Significant temporal variation was seen in the NAGase activity and in the sizes of the fungal and bacterial populations, but the community composition remained stable through time. Our results demonstrate the presence of two distinct microbial communities occupying the forest floor of Douglas-fir stands, whose populations and activities fluctuate seasonally but with little change in composition, which appears to be related to the physiochemical nature of mat and non-mat habitats.

1. Introduction

The coniferous forests of the Pacific Northwest (USA) are often thought of as N limited because most of the N is tied up in complex organic forms that are unavailable for direct plant uptake (Chapin et al., 2002). Through their production of extracellular enzymes, fungi are especially adept at breaking down complex organic substrates with low N content that are ubiquitous in coniferous forest ecosystems (Dix and Webster, 1995). Although saprotrophic fungi play a primary role in the degradation of woody substrates, nearly all types of fungi participate in nutrient cycling within Pacific Northwest forest ecosystems (Molina et al., 2001).

Tree species that form ectomycorrhizal (ECM) associations are dominant in Pacific Northwest coniferous forests creating one of the largest assemblages of ECM hosts in the world (Trappe et al., 2009). ECM fungi play a critical role in these forest ecosystems through their ability to provide host trees with enhanced nutrient uptake in exchange for photosynthate C (Allen et al., 2003; Smith and Read, 2008). A subset of ECM fungi that form dense hyphal mats (Agerer, 2001) are prominent features in some Douglas-fir forests, covering 25–40% of the forest floor in some stands (Cromack et al., 1979; Griffiths et al., 1996; Phillips, 2009). Early work on ECM mats in Douglas-fir forests focused on characterizing mats formed by *Hysterangium* and *Gautieria* species (Cromack et al., 1979; Griffiths et al., 1991). At that time, identification of the mat-forming fungi was limited to morphotype and sporocarp identification, potentially leading to misidentification or underestimated diversity. Not surprisingly, the findings of a recent molecular-based survey on the phlyogenetic diversity of ECM mat-forming fungi found much greater diversity than previously described (Dunham et al., 2007). Furthermore, Dunham et al. (2007) found mats formed by *Piloderma* species to be dominant, accounting for nearly 50% of the ECM mats sampled in old- and second-growth stands. These findings correlate with other studies that also found the rhizomorphic structures formed by *Piloderma* to be prominent in Douglas-fir forests (Smith et al., 2000; Phillips, 2009).

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ECM mats formed by *Piloderma* fungi occur in the organic horizon, and may perform an ecologically significant role in accessing the organically bound N from the organic horizon and providing it to the host plant. Rhizomorphic ECM mats, including those formed by *Piloderma*, have enhanced enzymatic capabilities compared to the non-mat organic horizon (Kluber et al., 2010). Most notably, the activity of N-acetylglucosaminidase (NAGase), an enzyme involved in the degradation of chitin, was 1.7 times greater in mat than non-mat soils. Additionally, Phillips (2009) determined that *Piloderma* mats had greater respiration rates than non-mat soil, accounting for over 30% of rhizosphere (root plus ECM fungi) respiration.

Bacterial associations with mycorrhizal fungi have long been documented (Oswald and Ferchau, 1968), and range from "helper bacteria" (Garbaye, 1994) to mycophagous bacteria (Leveau and Preston, 2008), although many of the mycorrhizal-associated microbes most likely lie between these extremes, as reported by Bending et al. (2002). Culture-dependent methods have shown that microorganisms of the ectomycorrhizosphere have distinct functional capabilities compared to organisms from the bulk soil (Timonen et al., 1998; Heinonsalo et al., 2001; Calvaruso et al., 2007). Using molecular techniques, Warmink et al. (2009) examined the bacteria associated with ephemeral hyphal mats from under fungal fruiting bodies and found decreased overall bacterial diversity and increased *Pseudomonas* diversity compared to the bulk soil. Furthermore, they found that the bacteria associated with the ECM fungi *Lactarius hepaticus* and *Scleroderma citrina* were similar for samples collected a year apart, suggesting that the bacterial communities may be specific to ECM phylotypes. However, others have found that bacterial community composition appeared unrelated to ECM presence (Burke et al., 2006) or the taxonomic identity of the ECM root tip (Burke et al., 2008).

A number of studies have documented seasonal trends in soil microbial communities and activities in a variety of ecosystems (Allison and Treseder, 2008; Björk et al., 2008; Cruz-Martinez et al., 2009), including the coniferous forests of the Pacific Northwest (Brant et al., 2006; Moore-Kucera and Dick, 2008). Studies specifically examining ECM fungi have found that their community structure and enzymatic and metabolic capabilities exhibit considerable temporal variation (Buée et al., 2005; Courty et al., 2007, 2008).

The mycosphere has been referred to as a "nutritional hotspot" for soil bacteria (Nazir et al., 2010) and the presence of ECM fungi has been shown to have an influence on microbial community structure (Warmink and van Elsas, 2008; Warmink et al., 2009) and function (Calvaruso et al., 2007). To gain a greater understanding of the relationship ECM fungi have with soil fungi and bacteria, it is necessary to determine whether the communities associated with ECM fungi are consistent across space and time. Because of their abundance and persistence, *Piloderma* mats offer a unique opportunity to examine microbial communities and activities associated with ECM fungi on a temporal scale. Furthermore, this system allows us to test whether 1) microbial activity responds as a function of the size of microbial populations, or 2) changes in microbial activity reflect changes in microbial community composition. We tested these hypotheses, by analyzing the size and structure of fungal and bacterial communities, and NAGase activity in *Piloderma* mat and non-mat soils across four seasons and investigated the synchronicity of the response of microbial activity, composition, and population size to seasonal changes in environmental factors.

2. Materials and methods

2.1. Site description and sample collection

This study was conducted at the H.J. Andrews Experimental Forest (HJA) located in the western Cascade Mountains of Oregon (44°13′25′N, 122°15′30′W). The mean annual temperature is 8.7 °C and annual precipitation is ~2300 mm. Despite the relatively high annual precipitation, the region is classified as having a xeric climate with the majority of precipitation occurring between October and April. Soils at the HJA generally are weakly developed and andic in origin.

Five old-growth (350+ years) Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) stands ranging in elevation from 451 to 1192 m were selected for this study based on the previously high number of detected ECM mats formed by *Piloderma* fungi (Dunham et al., 2007). During the summer of 2006, two *Piloderma* mats and two non-mat areas were located and flagged at each of the five sites. All mats were identified by extracting, amplifying, and sequencing DNA from ECM rhizomorphs and root tips as described in Dunham et al. (2007). According to their best GenBank match, all mats used in this study have been tentatively identified as *Piloderma fallax*; however, *Piloderma* taxonomy is known to be unsettled and the sequences found to be the best GenBank matches fall within two different clades of the phylogenetic tree constructed in Dunham et al. (2007) (see Supplementary material Table 1). Both clades contained the sequences previously described as *P. fallax*, but only one contained a *P. fallax* fruiting body, thus the authors suggested that the clade without a representative fruiting body be simply refered by the genus. For the sake of simplicity, and to prevent perpetuation of poor taxonomy, we will simply refer to the mats as *Piloderma* in this paper. Samples were taken at four times throughout the year: 10 September 2006, 5 November 2006, 5 May 2007, and 23 June 2007. These four dates correspond to summer, when soils were hot and dry; fall, when soils were cool and wet; early spring, around snowmelt when soils were cold and wet; and late spring when soils were warm and moist. To provide a context for the environmental conditions surrounding each sampling time, average daily air temperature and precipitation data from the HJA primary meteorological station was provided from 15 July 2006 to 15 July 2007 (Fig. 1). Additionally, soil moisture content and temperature at the time of sampling were measured (Fig. 2a). At each sampling time, three (2 cm diameter) cores were taken from each previously identified mat and non-mat area then composited into a single sample, resulting in two mat and two non-mat samples per site per sampling date. The core size was intentionally small to decrease the possibility that repeated sampling would negatively impact the mats over the course of the study. Because these *Piloderma* mats colonize the organic soil horizon and our focus was on comparing mat and non-mat soil communities and activities, only the organic horizon (3–6 cm deep, depending on location) was removed for analysis for each sample type. All samples were transported on ice and sieved using a 4 mm mesh

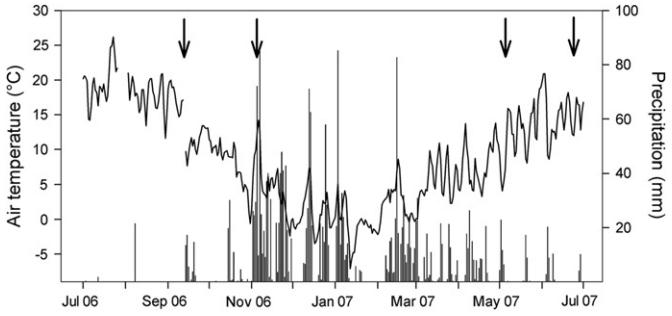

Fig. 1. Time series of air temperature (black line) and precipitation (black bars) from the H.J. Andrews headquarters weather station (430 m above sea level) from July 2006 through July 2007. Black arrows indicate our four sampling times. Data from Daly and McKee (2009).

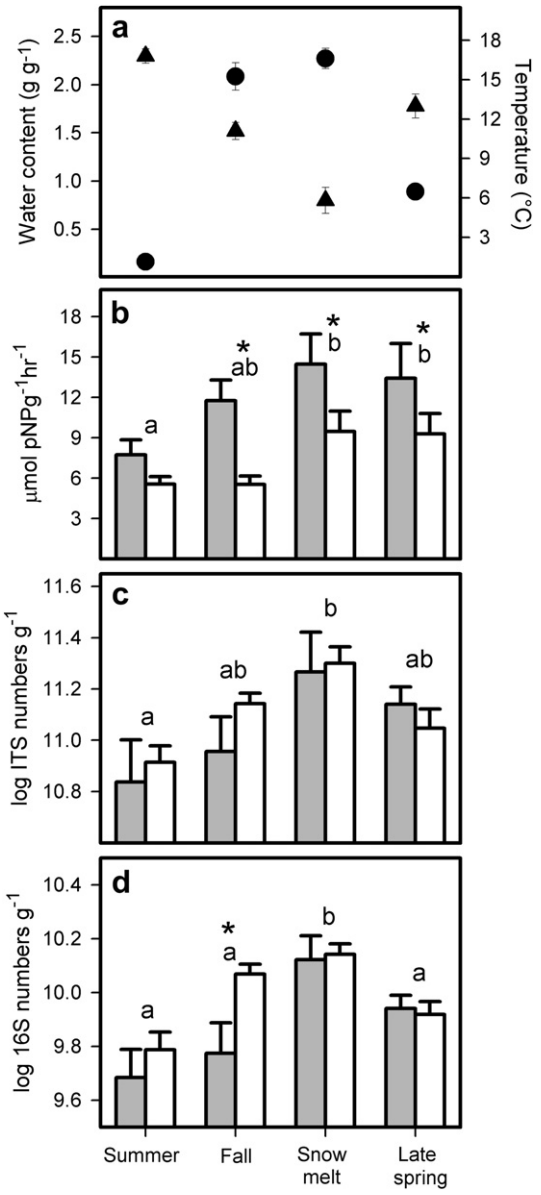

Fig. 2. Means and standard errors for soil water content and temperature (a), NAGase activity (b), fungal ITS numbers (c), and bacterial 16S rRNA numbers (d) at each of the four sampling times. In panel a, soil water content (circles) and soil temperature (triangles) are presented to demonstrate seasonal variation in soil conditions. In panels b–d, *Piloderma* mat (gray bars) and non-mat (white bars) data are shown for each season. Letters denote statistical differences between seasons and asterisks indicate statistical differences between mat and non-mat samples within a given season.

screen to exclude rocks and large pieces of organic matter; ECM rhizomorphs were rubbed through the sieve with the soil. Soils for water content and enzymatic analysis were stored at 4 °C until analysis (up to 2 days), and soils used for molecular analysis were frozen at −20 °C.

2.2. Enzyme analysis

A standard *p*-nitrophenol assay with a pH 5.5 sodium acetate buffer was used to measure N-acetyl-β-D-glucosaminidase activity (Parham and Deng, 2000). NAGase activity was selected as the sole enzyme assay for this study because elevated levels of NAGase have previously been reported in rhizomorphic mats, including those formed by *Piloderma*, whereas other enzymes involved in C, N, and P cycling did not differ significantly from the organic non-mat soils (Kluber et al., 2010).

2.3. DNA extraction and amplification

DNA was extracted from 0.5 g of each soil sample using a MOBio PowerSoil™ DNA isolation kit (MOBio Laboratories, Carlsbad, CA) according to the manufacturer's protocol, except that the bead beating tubes were shaken for 45 s on a FastPrep instrument (Bio 101, Carlsbad, CA) to lyse cells. All extracts were quantified using a NanoDrop ND-1000 UV-visible spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to 25 ng μl^{-1}. Diluted DNA aliquots were stored at −80 °C awaiting downstream analysis.

Amplification of DNA was carried out in 50-µl PCR reactions with 100 ng of template DNA and 5′-6-FAM (6-carboxyfluorescein) fluorescently labeled forward primers. The fungal ITS spacer region was amplified using ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primers and the thermocycling procedure from Manter and Vivanco (2007). Bacterial 16S rRNA genes were amplified with Eub8F (Edwards et al., 1989) and Eub907R (Muyzer et al., 1995) using the thermocycling protocol from Hackl et al. (2004). Duplicate PCR reactions were pooled for each sample prior to cleaning with a Qiaquick™ PCR Purification kit (Qiagen Inc., Valencia, CA) and quantified.

2.4. Community fragment analysis and clone library construction

Fungal ITS PCR products were restricted with HinfI and bacterial 16S PCR products were digested using MspI and AluI restriction enzymes using the manufacturer's protocol (Promega Corp., Madison, WI). Restricted samples were submitted to the Oregon State University Center for Genome Research and Biocomputing for purification using Illustra SephadexTM columns (GE Healthcare, Piscataway, NJ) and analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Unrestricted fungal ITS amplicons were also submitted for analysis using the same procedure to produce length heterogeneity (LH) profiles. GeneMapper v4.0 software (Applied Biosystems, Foster City, CA) was used to analyze LH and terminal restriction fragments (T-RF) following methods described by Avis and Feldheim (2005). Peak area data were exported for each dataset and normalized by total fluorescence of each individual sample.

To construct clone libraries, 20 µl of each 25 ng μl^{-1} DNA extract from each sample type were pooled within each site, resulting in a single mat and non-mat sample per site. The pooled DNA samples were then amplified using the fungal ITS and bacterial 16S rRNA primers and protocols as described for the fragment analysis except that unlabeled primers were used. The resulting PCR product was cleaned, quantified, and diluted to 10 ng μl^{-1}. Equal volumes of diluted PCR product were pooled across sites to create mat and non-mat samples containing sequences from all sites and sampling times. Four clone libraries (mat fungi, mat bacteria, non-mat fungi, and non-mat bacteria) were constructed from the final pooled PCR product using the TOPO TA® Cloning kit (Invitrogen, Carlsbad, CA) for sequencing by employing the manufacturer's protocol. Ligation products were shipped on ice to the Genome Center at Washington University (St. Louis, MO) where the ligation product was transformed into competent cells, and 96 colonies from each clone library were selected and sequenced using M13 forward and reverse primers. Priming from the plasmid, rather than the target DNA, was chosen to increase our ability to obtain complete sequence reads and improve our chances for matching sequences with LH and T-RF peaks seen in community profiles.

Sequences were screened for quality, trimmed, and contigs of forward and reverse reads were assembled using Geneious Pro (Drummond et al., 2009). Clone libraries were screened for chimeric sequences with Chimera_check (Maidak et al., 2000) and Bellerophon (Huber et al., 2004) programs. Fungal and bacterial clone libraries were grouped into Operational Taxonomic Units (OTUs) of 97% sequence similarity using Geneious Pro. The 97% sequence similarity cutoff was chosen because it has previously been used to approximate species diversity in both fungi (Taylor et al., 2008) and bacteria (Schloss and Handelsman, 2005). Fungal sequence identities were determined by comparison to the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and UNITE (http://unite.ut.ee/) databases, and the bacterial sequences were identified using the Ribosomal Database Project Classifier (Wang et al., 2007). Sequences from the clone libraries presented in this manuscript are available in GenBank under accession numbers HM488453–HM488737. In silico digests of full-length sequences from the clone libraries were carried out in Geneious Pro using the HinfI for fungal sequences and MspI and AluI for bacterial sequences.

2.5. Quantitative PCR

Relative population size of soil fungal and bacterial communities was determined with quantitative PCR. This method provides an index of microbial biomass by measuring the abundance of fungal and bacterial rRNA gene numbers. Bacterial 16S rRNA gene and fungal ITS numbers were measured following the method of Fierer et al. (2005) as modified by Boyle et al. (2008) using general fungal (5.8S and ITS1f) and bacterial (Eub338 and Eub518) primers. DNA template from each soil sample was diluted to 5 ng μl^{-1}, and 2 μl were then amplified in triplicate on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using the Brilliant II qPCR Master Mix with ROX (Stratagene, Jolla, CA). Amplification conditions, clone standards, and quality control measures were as described in Boyle et al. (2008).

2.6. Statistical analyses

Prior to statistical analysis, data from within-site mat and non-mat pseudoreplicates were averaged, resulting in a single mat and non-mat data point for each site at each date. Repeated measures ANOVAs were used to test whether mat presence or season had a significant effect on the NAGase activity, fungal population size, or bacterial population size. Variables were log transformed to meet normality assumptions as needed, and analysis was carried out using a PROC MIXED model in SAS 9.2 (SAS Institute, Inc., Cary, NC) with treatment and date as fixed effects and site (block) as a random effect. AICC, similar to AIC but corrected for small sample size, was used to determine the best covariance structure for each data set, and the SLICES option was used to test for the effect of mat presence within each date.

The structure of the fungal and bacterial communities was investigated with non-metric multidimensional scaling (NMS) (McCune and Grace, 2002) using PC-ORD v5 (MjM Software Design, Gleneden Beach, OR). Two data matrices were constructed: the fungal community matrix containing the combined LH and HinfI fragment profiles, and the bacterial community matrix containing the MspI and AluI T-RF profiles. Ordinations were constructed in autopilot mode using the "slow and thorough" setting with the Sørensen distance measure. PC-ORD was used also to run indicator analysis on the fungal and bacterial communities to determine which fragments were most influential in determining treatment differences (McCune and Grace, 2002). Indicator values (IV) were calculated for each fragment, and ranged from 0 (no indication) to 100 (perfect indication); therefore, a fragment with a high IV is more likely to be associated with a given sample type. Multivariate analysis of variance (MANOVA) PROC GLM code in SAS 9.2 tested the effects of sample type and season on NMS axes scores while blocking for site. This method was chosen to examine clustering of samples on the NMS ordination over the more commonly used multi-response permutation procedures (MRPP) because MANOVA is able to account for more complicated study designs, thus allowing us to test for the effects of mat presence or season while accounting for site-to-site variation. MANOVA has been used previously to test for treatment differences using the axes scores resulting from other ordination methods (Brenner, 2000; Kourtev et al., 2002; Yi et al., 2009) and has been shown to be a robust, quantitative method for examining treatment and seasonal patterns in community ordinations. EstimateS (Colwell and Coddington, 1994) was used to create rarefaction curves for the community fragment profiles and clone libraries.

3. Results

3.1. NAGase activity

On average, the *Piloderma* mat activity was 1.4 times greater than non-mat activity ($p < 0.01$), and both sample types showed significant variation in NAGase activity across seasons ($p < 0.01$). We found that during the summer sampling there was no significant difference between the NAGase activity of *Piloderma* mat and non-mat soils, whereas mats had greater activity than non-mats at all other times (Fig. 2b), with the fall sampling date showing the greatest difference between the two sample types.

3.2. Fungal and bacterial communities

NMS ordinations of fungal and bacterial fragment profiles showed significant grouping of the mat communities (Fig. 3). MANOVAs on the NMS axes scores for each ordination confirmed that fungal and bacterial communities differed between *Piloderma* mat and non-mat samples ($p < 0.01$ for each ordination). Furthermore, there were significantly different fungal and bacterial communities among sites ($p < 0.01$ for each ordination). In contrast, neither the fungal ($p = 0.12$) nor the bacterial ($p = 0.82$) community exhibited significant temporal variation. To examine the fungal community structure without the influence of the mat-former, we removed the LH and T-RF fragments associated with *Piloderma* from the species matrix, repeated the analysis, and found statistical significance unchanged (sample type $p < 0.01$, site $p < 0.01$, season $p = 0.13$).

The microbial communities represented by the fragment profiles were likely representative of the total soil community because the rarefaction curves constructed from these data reach an asymptote (Fig. 4). Although T-RFLP and LH-PCR allow us to examine differences in overall community structure, these techniques provide no information on the identities of the community members. For this reason, clone libraries were constructed to identify dominant members of the fungal and bacterial communities and to potentially determine which organisms were associated with indicator fragments from the community analysis.

A total of 384 clones were sequenced for the four clone libraries. After chimeras and sequences of poor quality were excluded, 149 fungal (78 mat and 71 non-mat) and 138 bacterial (67 mat and 71 non-mat) sequences remained. At a 97% similarity level the fungal ITS sequences grouped into 57 OTUs representing >12 orders (Fig. 5a). Atheliales sequences dominate the ITS clone library from mats and are much more abundant in the mats than the non-mats; not surprisingly, the majority of the Atheliales sequences are from

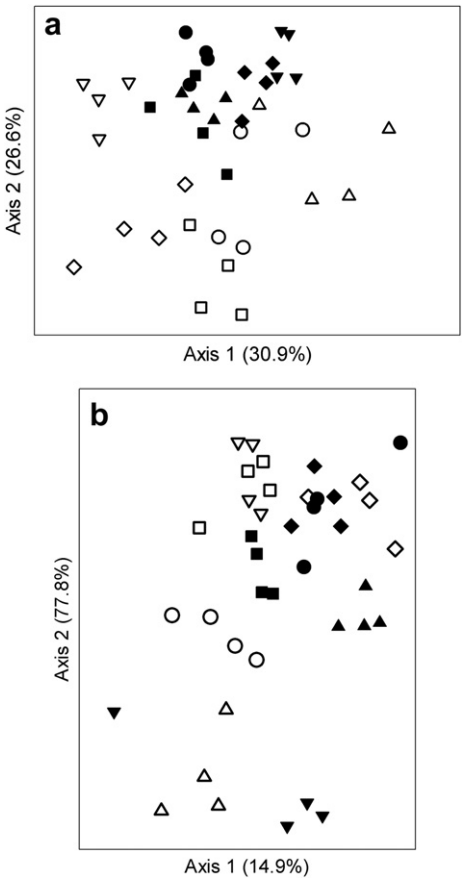

Fig. 3. Composition of fungal and bacterial communities in *Piloderma* mat (solid symbols) and non-mat (open symbols) samples over the four seasons. The five sites are represented by different symbols to demonstrate the spatial and temporal variation. Panel a shows the first two axes of a 3D NMS ordination of the fungal LH-PCR and HinfI T-RFLP profiles (stress = 13.99) and panel b shows the 2D NMS ordination of the bacterial AluI and MspI T-RFLP profiles (stress = 12.24).

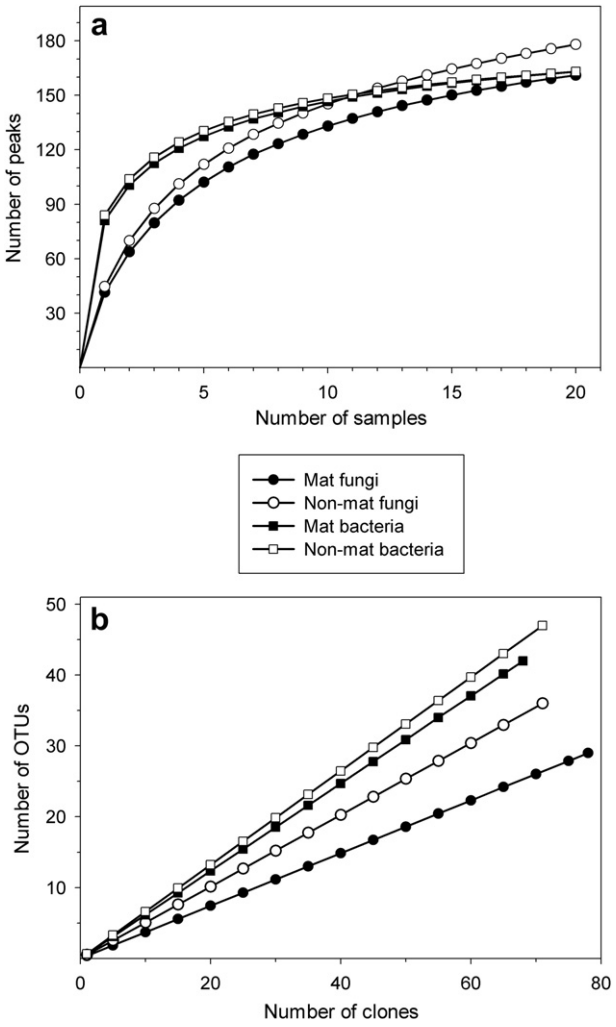

Fig. 4. Rarefaction curves for fungal and bacterial communities. Panel a shows the curves constructed with community fragment profiles; the curves in panel b were constructed using clone library data. In both panels, *Piloderma* mats are represented with solid symbols and non-mats with open symbols; fungal communities are shown by circles and bacterial communities by squares.

the genus *Piloderma*. The non-mat clone library is more evenly distributed among taxa, and tends to have more Sebacinales, Russulales, and Agaricales than the mat library. The mat and non-mat ITS libraries had roughly equal abundance of *Cenococcum geophilum* sequences and percentage of Ascomycota and Basidiomycota.

Bacterial 16S rRNA gene sequences grouped into 77 OTUs representing 10 bacterial classes (Fig. 5b). α-Proteobacteria and Acidobacteria clones were abundant in both the mat and non-mat 16S rRNA gene libraries, although the total number of Acidobacteria clones was greater in the non-mat library. γ-Proteobacteria was the third most abundant class in the mat library, yet had only a few representatives in the non-mat library. The rarefaction curves constructed from clone library data (Fig. 4) show fewer fungal and bacterial OTUs encountered in the mat samples than the non-mat samples but, differences were not statistically significant, as the 95% confidence intervals overlap (data not shown to improve figure clarity). Furthermore, because these rarefaction curves do not reach an asymptote, the clone libraries do not represent the total diversity of the microbial communities and caution is needed when comparing sample diversity with these data.

Indicator analysis on the fungal fragment profiles revealed three significant indicator fragments for the mat samples and five for the non-mat samples. Not surprisingly, the three mat indicator fragments (IVs = 64, 80, and 84) matched *Piloderma* sequences from the clones and phylotype samples. Of the four non-mat indicator fragments, two could not be matched to clone libraries, however the remaining two (IV's = 77 and 60) matched Ascomycota sequences representing the Myxotrichaceae.

Overall, the bacterial indicator values were not as high as the fungal indicators. Nonetheless, three significant bacterial indicator fragments for the bacterial mat community matched clone sequences representing α-Proteobacteria (IV = 58), β-Proteobacteria (IV = 57), and Actinobacteria (IV = 56). Five bacterial fragments were found to be significant non-mat indicators; however, only three matched sequences from the clone library, including Sphingobacteria (IV = 60), Acidobacteria and Actinobacteria (IV = 59) and γ-Proteobacteria and β-Proteobacteria (IV = 58). It should be noted that the sequences associated with each indicator fragment represent only a subset of sequences from the listed taxon, and other sequences from each taxon may be represented by additional fragments. Thus it is possible for sequences from the same taxon to be indicators for *Piloderma* mats while others are indicators for non-mat soils.

3.3. Quantitative PCR

The fungal population size, measured by ITS numbers, was not significantly different between the *Piloderma* mat and non-mat soils ($p = 0.47$) although there was significant seasonal variation

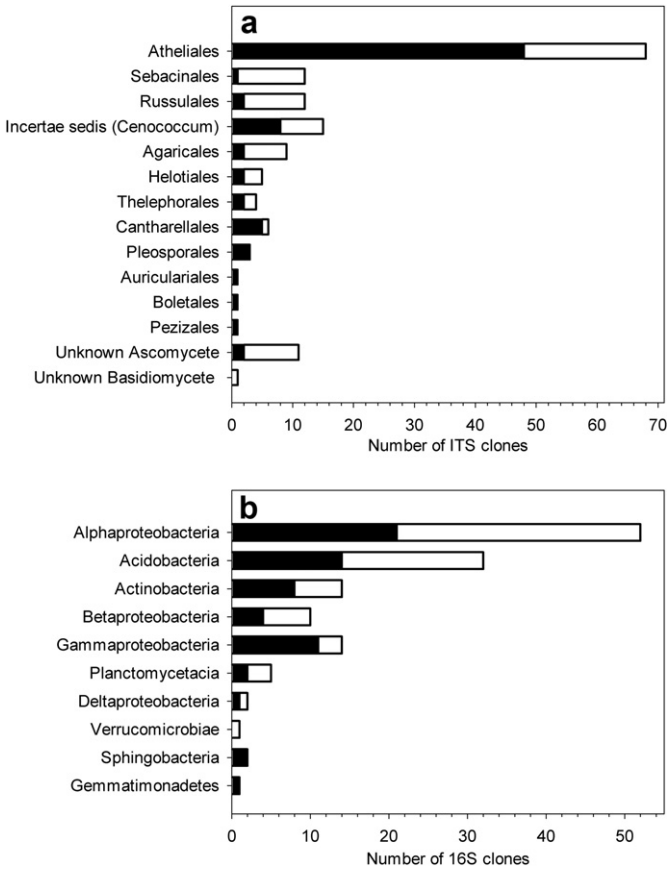

Fig. 5. Abundance of taxa present in *Piloderma* mat (black) and non-mat (white) clone libraries. Fungal orders are shown in panel a, and bacterial classes are shown in panel b.

($p < 0.01$; Fig. 2c). Similarly, the bacterial population size, measured by 16S rRNA gene numbers, did not differ between mat and non-mat soils when compared across seasons ($p = 0.07$), although bacterial populations were significantly greater in the non-mat soils at the fall sampling ($p < 0.01$). There were significant seasonal fluctuations in bacterial population size ($p < 0.01$; Fig. 2d). Both the fungal and bacterial populations showed similar seasonal trends with lower gene numbers in the summer when soils were hot and dry, and greater gene numbers in the early spring when soils were cool and moist.

4. Discussion

ECM mats have previously been associated with elevated microbial activity compared to their non-mat counterparts (Griffiths and Caldwell, 1992; Kluber et al., 2010). Consistent with earlier work, our study found greater NAGase activity in the *Piloderma* mats than in the non-mat soils (Fig. 2b). The matter of "who" is responsible for producing the NAGase activity remains a question, however. A number of ECM fungi express NAGase activity (Courty et al., 2010) and *P. fallax* has been found to possess the genetic potential to produce these chitinolytic enzymes as well (Lindahl and Taylor, 2004). It has been hypothesized that ECM fungi may degrade chitin as a means of obtaining N (Lindahl and Taylor, 2004) or allow ECM fungi to potentially prey on saprotrophic fungi as a means of obtaining N as described by Buée et al. (2007). Although it is tempting to conclude that the enhanced NAGase activity of *Piloderma* mats is a product of the mat-forming fungi, mats harbor a diversity of microorganisms that could potentially produce NAGase to access the chitin-rich fungal hyphae of the mat-former. Accordingly, N-acetylglucosamine has been found to be a preferred substrate of mycorrhizosphere bacteria (Timonen et al., 1998), and a variety of organisms likely contribute to the enhanced NAGase activity of ECM mats.

4.1. Fungal communities

Piloderma mats harbored a fungal community that was significantly different from the community occupying the non-mat soils, and no significant community shifts were observed across seasons (Fig. 3a). From the NMS ordination, it was apparent that the non-mat fungal communities tended to group with other samples from the same site with little temporal variation. It is not unexpected that the fungal communities from *Piloderma* mats group due to of the abundance of *Piloderma* in these samples; however, the significance of the mat grouping did not change when fragments associated with *Piloderma* were removed. This finding indicates that the difference between the fungal communities goes beyond the dominance of the mat-forming fungi.

Atheliales sequences were abundant in the mat clone library and, although the majority of these sequences were *Piloderma*, we also found another non-mycorrhizal genus within the Atheliales, *Leptosporomyces* (K.H. Larsson, personal communication) to be associated with the mats. The second most abundant sequence in the mat clone library was from *C. geophilum*, an ECM fungus commonly found on a wide range of hosts. Beyond these were a number of orders with only a few representative sequences. The clone library rarefaction curve (Fig. 4) tended to be lower for mats, indicating less species-level diversity; however, there were a greater number of orders found in the mat clone library. Conversely, although the non-mat community was not as diverse across orders, the number of species represented by the orders was greater than in the mat soils. Fungi that form mycorrhizal associations with the ericoid plants, common in the understory of this ecosystem, seemed to play a role in structuring the non-mat community. The Myxotrichaceae sequences that were associated with the non-mat indicator peaks belong to the genus *Oidiodendron*, which forms ericoid mycorrhizas (Dalpé, 1989). Furthermore, *Sebacina*, an orchid and ectomycorrhizal fungus (Warcup, 1988; Weiss and Oberwinkler, 2001) that has been found in the fine roots of salal (*Gaultheria shallon* Pursh)(Berch et al., 2002), a common understory plant at these sites, was found in greater abundance in the non-mat clone library. The non-mat library also contains clones from common ECM genera, such as *Cortinarius*, *Russula*, and *Piloderma*, in addition to a number of sequences from the Leotiomycetes.

Piloderma mats are one of the most visually striking ECM mats due to their tendency to heavily colonize the soil with thick, cord-like rhizomorphs. Using direct count methods, Ingham et al. (1991) found that ECM mats had considerably greater hyphal length per g soil compared to non-mat soils and that rhizomorphic structures accounted for up to 50% of the dry weight of ECM mat soils. Despite previous findings and the strong visual delineation between *Piloderma* mats and adjacent non-mat soil, we did not find significantly larger fungal populations in mats (Fig. 2c). Perhaps the total biomass of the mat-forming rhizomorphs was underrepresented with our method of quantifying fungal populations using ITS numbers, which represent only the active, or nucleated, biomass. Despite this, the results from our fragment analysis and clone libraries demonstrated that *Piloderma* sequences were dominant in the mat samples, indicating that much of the *Piloderma* biomass was detected with our methods. In this case, one may argue that although the non-mat soils do not contain rhizomorphs, they are certainly not devoid of fungi. Indeed, a variety of saprotrophic and

ECM (Dickie et al., 2002; Robinson et al., 2005; Genney et al., 2006) fungi are known to colonize the organic horizon soils. Perhaps the microscopic hyphae from these fungi are indeed equal in abundance to the fungal biomass in mats, yet not visible to the naked eye.

4.2. Bacterial communities

Trends seen in the bacterial community were similar to those of the fungi, with significant differences between mat and non-mat soils, and among sites, but showing little temporal variation (Fig. 3b). It should be noted, however, that the bacterial communities had a greater tendency to be site specific, even within the *Piloderma* mats, which differs from the findings in the fungal community, where the mats had similar community structure regardless of site. Because of the greater site-to-site variation, bacterial indicator values were generally weaker than for the fungal community. Although α-Proteobacteria, β-Proteobacteria, and Actinobacteria sequences were associated with indicator fragments for the mats, other sequences from these classes were associated with indicator fragments for the non-mat community as well. Consequently no bacterial class stood out as a primary indicator for mats and unfortunately no indicator fragments definitively matched sequences at lower taxa levels. Somewhat unexpectedly, our study did not find greater numbers of actinomycetes, or other known chitin degraders, in the mat clone library; however, indicator species analysis showed that T-RFLP peaks from a subset of Actinobacteria sequences commonly were associated with the mats. The most notable difference between the bacterial clone libraries was the increased abundance of γ-Proteobacteria in the mat library. Members of the γ-Proteobacteria, such as *Pseudomonas*, have been previously associated with ECM fungi (Frey-Klett et al., 2007; Warmink et al., 2009). However, because we were unable to identify many of the γ-Proteobacteria sequences below the class level, we will not speculate about their role within ECM mats. The overall structures of the mat and non-mat clone libraries were similar to other studies of forest soils where α-Proteobacteria and Acidobacteria were abundant (Fierer et al., 2005; Lauber et al., 2008; Yarwood et al., 2010). Interestingly, the majority of α-Proteobacteria sequences were from the Rhizobiales, comprising 22% of the mat and 29% of the non-mat clone libraries. Several potentially N_2-fixing families within Rhizobiales were present in the clone libraries, and Bradyrhizobiaceae sequences were dominant. These bacteria have previously been reported from clone libraries of Douglas-fir forest mineral soils (Yarwood et al., 2010), although potential N_2-fixing plant hosts are not common in old-growth Douglas-fir forests. Several studies have associated Rhizobiales with ECM fungi (Izumi et al., 2006; Burke et al., 2008; Kretzer et al., 2009), which may account for their presence in this fungal-dominated forest ecosystem.

4.3. Temporal variation of communities and activities

The temporal variation in NAGase activity (Fig. 2b) is not surprising, as others have described seasonal variation in enzyme activities for bulk soil (Boerner et al., 2005) and ECM roots (Courty et al., 2007, 2010). It is likely that the low water content in summer was limiting microbial activity (Skopp et al., 1990) leading to the lowest levels of NAGase activity. With the fall moisture, NAGase activity in mat soils increased dramatically whereas the non-mat soils showed a slower response, despite increased fungal and bacterial populations (Fig. 2a–c). This indicates that the two communities had differing functional response to the flush of nutrient availability associated with the soils "wetting up" (Fierer and Schimel, 2002). A number of studies have shown temporal variation in soil microbial communities in this ecosystem (Brant et al., 2006; Moore-Kucera and Dick, 2008) and others (Burke et al., 2009; Cruz-Martinez et al., 2009), but no such trend was detected in our study. Although our ordinations showed slight changes in community composition among the four sampling dates for each site, there was no significant effect of season on the community structure, and the effects of sample type and site largely overshadowed any seasonal effect. Microbial populations, on the other hand, did show significant temporal changes, with both the fungal and bacterial populations following the same trend as the NAGase with lower populations in the late summer and the highest populations in the early spring during snowmelt in the early spring (Fig. 2b–d).

4.4. Conclusions

Correlations between soil pH and microbial community structure have drawn increased attention recently: Significant changes in bacterial, and to a lesser degree fungal, communities have been seen in soils varying by 4 or more pH units (Lauber et al., 2009; Rousk et al., 2010). It is unlikely, however, that pH had a strong influence on the structure of the communities described herein as Rousk et al. (2010) saw only minor changes in the fungal communities across a broad pH gradient and rhizomorphic mats, including *Piloderma*, differ from non-mat soils by only 0.31 pH units (Kluber et al., 2010). The mycosphere has been referred to as a "nutritional hotspot" for soil bacteria (Nazir et al., 2010), and our findings further support the notion that the ectomycorrhizosphere exhibits selective pressures on soil microbes (Calvaruso et al., 2007). By studying *Piloderma* mats, we were able to examine the structure of microbial communities associated with the ectomycorrhizosphere at multiple times and demonstrate that the dense rhizomorphic mats formed by *Piloderma* fungi are associated with distinct and fungal and bacterial communities. Although the mat and non-mat communities differ functionally, future work is necessary to determine 1) the nature of the interactions between ECM fungi and their associated microbial community, and 2) if the microbes contribute to enhanced nutrient uptake by the mat-forming fungi.

In examining ECM mat and non-mat soils types across seasons, our results paint a picture of two distinct microbial communities whose populations and activities wax and wane through the seasons yet their composition does not change. This indicates that, in this system, only the size and activity of the microbial populations responded to seasonal changes in environmental factors. The structure of the communities, on the other hand, seem to be in response to their physiochemical micro-habitats thus unique communities form in the presence of the ectomycorrhizosphere and among sites.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.soilbio.2011.01.022.

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