Species Diversity, Ecology and Laccase Gene Diversity of Saprotrophic Fungi Across Different Plant Community Types

By

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Abstract

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This dissertation explores the diversity, ecology and functional diversity of saprotrophic fungal across different plant community types. Saprotrophic fungi are responsible for recycling the majority of carbon from dead organic matter and have the unique ability to break down and release nutrients that can then be readily available for other organisms. A number of different biotic and abiotic factors affect the structure of saprotrophic fungal communities. Here, we investigated the effects of nutrient addition, substrate availability, and dominant plant community type on the structure and diversity of saprotrophic fungal communities. In addition, we investigated the diversity and structure of functional diversity of saprotrophic fungal communities.

The first chapter explores saprotrophic fungal diversity found in leaf litter in a diverse lowland tropical forest. We investigated the affect of nutrient addition on the saprotrophic fungal community using a replicated factorial N, P, K, micronutrient fertilization experiment. We found that the control plots had the lowest alpha diversity compared to treatments that received fertilization. The long-term addition of nutrients increased species richness relative to controls and had an effect on taxonomic composition of the leaf litter fungal communities at lower taxonomic levels, but not at higher taxonomic levels.

The second chapter characterizes saprotrophic fungal communities on straw and wood substrates across forest and grassland plant community types and characterized how these communities changed over time as substrate quality changed. The goal was to determine if substrate, space, time or plant community were the major determinants of fungal saprotrophic community composition. We found that the wood substrates collected in the grassland and forest had the highest richness, estimated richness and phylogenetic diversity. Overall, there was a decrease in richness on the substrates in both plant communities over time, and plant community type appears to have a greater influence on saprotrophic fungal community structure than substrate type or substrate quality.
In the third chapter, we investigated the diversity of saprotrophic basidiomycete laccase genes in grassland and forest plant community types and on straw and wood substrates. Functional diversity was assessed by the functional gene encoding laccase. Laccases play an important role in soil organic matter turnover and are able to completely degrade lignin. We found that the wood substrates had significantly higher richness of estimated OTUs as compared to the straw substrates. Laccase gene diversity was compared to basidiomycete diversity. We also found that both the laccase genes and basidiomycete assemblages associated strongly with plant community type.
This dissertation is dedicated to my parents, who have continuously been supportive and encouraging of all of my endeavors, to my sister who has been an amazing friend, to my husband, Andy, for all of his love and support, and to my son, Jesse, who I encourage to follow his dreams.
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Table of Contents

Abstract ................................................. 1
Dedication ................................................. i
Acknowledgements ........................................ ii
Table of Contents ....................................... iii
Introduction .............................................. iv

Chapter 1: Nutrient enrichment increased species richness of leaf litter fungal communities in a tropical forest 1

Tables ..................................................... 15
Figures ..................................................... 17
Supplemental Appendix ................................ 23

Chapter 2: Saprotrophic fungal assemblages are influenced by plant community type more strongly than by substrate type or time 27

Tables ..................................................... 43
Figures ..................................................... 48
Supplemental Appendix ................................ 57

Chapter 3: Comparison of basidiomycete functional diversity to species diversity reveals both are influenced by plant community type 60

Tables ..................................................... 72
Figures ..................................................... 74
Supplemental Appendix ................................ 77
Introduction

Microbial communities play a major role in terrestrial ecosystem functioning, and fungi can compose up to 90% of the total decomposer biomass of such communities. Fungi are one of the most diverse groups of Eukarya and are an essential functional component of terrestrial ecosystems as decomposers, mutualists and pathogens. Saprotrophic fungi in particular are responsible for recycling the majority of carbon from dead organic matter and have the unique ability to break down and release nutrients that can then be readily available for other organisms. Fungi are the main decomposers of recalcitrant components of plant litter and lignified cellulose by producing lignin-modifying enzymes. Despite fungi’s important role in ecological and biogeochemical processes, little is known about the structure, richness and functional diversity of saprotrophic fungal communities.

Traditional approaches to studying soil fungal diversity included pure culture techniques and fruiting body surveys. However, there are many limitations to both of these techniques with respect to accurately assessing fungal diversity and community structure. Molecular tools and methods have allowed for a better understanding and characterization of fungal diversity and community structure. Recent method papers focused on molecular approaches have found that diversity of soil fungi (including saprotrophic, mycorrhizal and pathogenic fungi) is higher than expected (Lynch & Thorn 2006; Buee et al. 2009), biodiversity of soil fungi is greatest in natural sites with less anthropogenic disturbance (Midgley et al. 2007; Kennedy et al. 2006), a floristic gradient across grasslands does not affect soil fungal diversity (Brodie et al. 2003), there are differences in community composition of plantation ecosystems as compared to natural forest ecosystems (He et al. 2005), and saprotrophic fungi are most often found in the litter layers (O’Brien et al. 2005; Lindahl et al. 2007; Dickie et al. 2002) whereas mycorrhizal fungi are most often found in the deeper layers of the soil profile. Many of these studies focused on agricultural or managed sites rather than natural ecosystems. Massively parallel pyrosequencing, a new DNA sequencing technique, is one of the leading technologies replacing conventional Sanger sequencing. To date, there are only a few studies applying this technique to fungi. The sequence data generated from this technique will allow us to understand finer spatial scale dynamics of fungal diversity, community assembly rules, and diversity that was not possible previously.

A number of different biotic and abiotic factors affect the structure of saprotrophic fungal communities. Here, we investigated the effects of nutrient addition, substrate availability, and dominant plant community type on the structure and diversity of saprotrophic fungal communities. In addition, we investigated the diversity and structure of functional diversity of saprotrophic fungal communities using a 454 pyrosequencing approach.

Nutrient enrichment effects on saprotrophic fungal community structure is not well understood. Recent studies and reviews have investigated the effect of macronutrients, primarily nitrogen (N), on fungal communities and, such studies have demonstrated a decrease in fungal species diversity and shifts in microbial communities (Cox et al. 2010; Avis et al. 2003, 2008; Treseder 2004, 2008; Parrent et al. 2006). However, these studies primarily focused on ectomycorrhizal fungi. Previous studies of microbial communities have only shown significant responses to nutrient manipulation after longer periods (Rinnan, Michelsen, Bååth, & Jonasson, 2007), indicating the importance of long-term treatments.
Soils and leaf litter are highly heterogeneous environments providing numerous niches for fungi. Bruns (1995) first proposed the idea of niche differentiation as a driver of diversity in ectomycorrhizal communities, and other more recent studies have also noted niche partitioning as one hypothesis to explain the species-rich ectomycorrhizal communities (Dickie et al. 2002; Koide et al. 2007). Previous work using molecular approaches have independently studied the diversity of fungal communities in grasslands (Deacon et al. 2006; Kennedy et al. 2006; Jumpponen et al. 2010) and natural forest ecosystems (He et al. 2005; Jumpponen 2007; Buée et al. 2009); however, there are no studies comparing fungal diversity across both grassland and forest ecosystems in close proximity in order to determine if plant community type has an effect on fungal communities.

Fungi are able to decompose lignin and cellulose by releasing extracellular enzymes that degrade these polymers. White rot fungi in particular produce several types of enzymes, including lignin peroxidases, manganese peroxidases, and laccases. These enzymes allow the fungus to break down and utilize organic substrates as an energy and nutrient source (Osono, 2007). Lignin is a large, complex, aromatic polymer made up of phenylpropane-based monomers linked via a variety of bonds that bind cell-wall components together (Osono, 2007). Fungal laccases appear to be involved in a variety of physiological functions, including fruit body development, detoxification of phenolic compounds, pigment production, and antimicrobial activity (Levin, Forchiassin, & Ramos, 2002; Thurston, 1994). In addition, fungal laccases also appear to have roles in stress defense and fungal plant-pathogen/host interaction (Baldrian & Snajdr, 2006; Crowe & Olsson, 2001; Thurston, 1994). Laccases play an important role in soil organic matter (SOM) turnover and are able to completely degrade lignin (Eggert, Temp, & Eriksson, 1997).

While there have been few studies linking the relationship between saprotrophic diversity to functional diversity, such relationships are of great interest. Understanding soil enzyme functional diversity could significantly increase our understanding of the linkages between resource availability, microbial community structure and function, and ecosystem processes (Caldwell, 2005).

The aims of this study were to 1) determine the effect of increased nutrients on leaf litter fungal community structure in a lowland tropic forest; 2) compare community diversity and composition of saprotrophic fungal communities in different temperate plant community types within close proximity and on different substrates, and to investigate how these communities change over time; and 3) compare how the observed saprotrophic basidiomycete diversity relates to functional diversity, assessed by the functional gene encoding laccase.
Literature Cited


CHAPTER 1

Nutrient enrichment increased species richness of leaf litter fungal communities
in a tropical forest
Abstract

Microbial communities play a major role in terrestrial ecosystem functioning. In this study we explored leaf litter fungal diversity in a diverse lowland tropical forest in which a replicated factorial N, P, K, micronutrient fertilization experiment of 40 x 40 m plots had been on going for nine years. We extracted DNA from leaf litter samples and used fungal-specific amplification and a 454 pyrosequencing approach to sequence two loci, the nuclear ribosomal internal transcribed spacer (ITS) region and the nuclear ribosomal large subunit (LSU) D1 region. Using a 95% ITS sequence similarity threshold revealed 5,717 OTUs, and the number of unique ITS1 OTUs per sample ranged from 55 – 177. Ascomycota (including approximately 29 orders, 44 families, and 88 genera) are the dominant phylum among the leaf litter fungi (71% of the sequences; 2,844 OTUs), followed by Basidiomycota (including approximately 31 orders, 38 families and 52 genera) (26% of the sequences; 1,053 OTUs). The control plots had the lowest Shannon and Simpson diversity compared to treatments that received fertilization. The long-term addition of nutrients increased species richness relative to controls and had an effect on taxonomic composition of the leaf litter fungal communities at lower taxonomic levels, but not at higher taxonomic levels.
Introduction

Saprotrophic fungi, a major component of leaf litter fungi, are responsible for recycling the majority of carbon from dead organic matter and have the unique ability to break down and release nutrients that can then be readily available for other organisms. Although microbial communities play an important role in controlling the soil carbon balance, there is very little knowledge of the effects of climate change, including increased nutrient availability due to increased plant biomass and anthropogenic activities, on microbial communities and microbial activity. The objective of this study is to understand how leaf litter microbial communities in a lowland tropical forest respond to the long-term addition of macro- and micronutrients (N, P, K) and how the increase of these nutrients affects community composition.

Here, we investigate the species richness and diversity of leaf litter fungal communities in a diverse tropical lowland forest. Compared with soil and ectomycorrhizal fungal diversity studies, leaf litter fungal diversity and community studies have not been as extensive, especially in the tropics (but see Lodge 1997; and Lindahl et al. 2007; Blackwood et al. 2007; U’ren et al. 2010) for temperate studies).

Two loci were amplified in this study, the nuclear ribosomal internal transcribed spacer (ITS) region, used for DNA barcoding of fungi (Seifert 2008; Ryberg et al. 2008; Seifert 2009), and the nuclear ribosomal large subunit (LSU) D1 region. The ITS region appropriately delimits species, allowing for a finer-scale taxonomic resolution (Horton & Bruns 2001), while the LSU gene region is appropriate for phylogenetic inference (Kõljalg et al. 2005) and is appropriate for sequence alignment of fungi. We choose to combination of these two gene regions because they together they provide a near species-level resolution (ITS) and a phylogenetic insight into the leaf litter community (LSU).

Many recent studies and reviews have investigated the effect of macronutrients, primarily nitrogen (N), on fungal communities and, such studies have demonstrated a decrease in fungal species diversity and shifts in microbial communities (Cox et al. 2010; Avis et al. 2003, 2008; Treseder 2004, 2008; Parrent et al. 2006). However, these studies primarily focused on ectomycorrhizal fungi. In addition, many nutrient addition studies only span a few years. Previous studies of microbial communities have only shown significant responses to nutrient manipulation after longer periods (Rinnan et al. 2007), indicating the importance of long-term treatments.

In this study we use a factorial N, P, K fertilization of 40 x 40 m plots, plus an additional micronutrient treatment (i.e. B, Ca, Cu, Fe, Mg, Mn, Mo, S, Zn), compared with control plots that did not receive fertilization, to evaluate possible nutrient effects on leaf litter fungal communities in a diverse lowland tropical forest. The plots have received nutrient additions since 1998 and were sampled in 2007. Previous work by Kaspari et al. 2008 investigated nutrient control of litter production and decomposition in these plots and documented that fertilization enhanced leaf litter element concentrations by up to 7% on +N plots, 27% on +P
plots and 34% on +K plots. Interestingly, cellulose decomposition increased by 49% on +P and 30% +K plots, and leaf litter decomposition was 30% faster on +P plots. The goals of our current study was to determine the effect of increased nutrients on leaf litter fungal community structure and with the factoral design we expected to determine which nutrients or nutrient combinations where the strongest drivers of fungal community structure in these tropic leaf litter habitats.

Materials and Methods

Study site
Leaf litter samples were collected from the Smithsonian Tropical Research Institute’s Gigante Fertilization Experiment (9°06′31″ N, 79°50′37″ W) in the Barro Colorado Nature Monument (BCNM) on the Gigante peninsula, Republic of Panama. Thirty-six 40 x 40 m plots were established in 1998 along a macronutrient (NPK) gradient on the Gigante peninsula within the macronutrient fertilization plot. Four replicate leaf litter samples were collected from the 9 treatments (nitrogen (N), phosphorus (P), potassium (K), NP, NK, PK, NPK, micronutrients (M) and control plots) (n=36). The control plots did not receive any fertilization. The plots are approximately 30-40 m apart and span over 27 ha. The forest is categorized as a low land primary rainforest (older than > 300 years) (Leigh 1999). The soils are characterized as oxisol soils low in available P and K (Yavitt & Wieder 1988; Cavelier 1992). Fertilizers were applied four times a year during the wet season (beginning in June 1998) to reach the following total doses for each year’s four applications: 125 kg N ha⁻¹ year⁻¹ (as coated urea [(NH₂)₂CO₃]), 50 kg N ha⁻¹ year⁻¹ (as triple superphosphate [Ca(H₂PO₄)₂.H₂O]), and 50 kg K ha⁻¹ year⁻¹ (as KCl). Similar doses are used in forestry (Binkley 1986) and have also been used in tropical montane studies (100-150 kg N, 50-65 kg P, 50 kg K, (e.g. Tanner et al. 1992, Vitouset et al. 1995). An additional four ‘+M’ plots were dosed with a micronutrient fertilizer (Scott’s S.T.E.M) consisting of HB₂O₂, CuSO₄, FeSO₄, MnSO₄, ZnSO₄, and (NH₄)₆Mo₇O₂₄ at 25 kg ha⁻¹ year⁻¹ plus dolomitic limestone CaMg(CO₃)₂ (36.8 kg year⁻¹) at 230 kg ha⁻¹ year⁻¹(Kaspari et al. 2008).

Sampling
Four replicate samples were collected in September 2007 from the 8 treatments (nitrogen (N), phosphorus (P), potassium (K), NP, NK, PK, NPK, micronutrients (M) and control plots) (n=36). The control plots did not receive any fertilization. Sampling from each plot consisted of a 10 x 10 cm quadrant of leaf litter located in the center of the plot. Leaf litter was collected down to mineral soil and then sifted for 30 s through a 1 cm screen. Samples were processed within 12 hours or stored at 3°C for 24 hours and then processed. Sampling was in collaboration with Dr. Mike Kaspari and Dr. Brad Stevenson at the University of Oklahoma.

DNA extraction
Total DNA was isolated from 0.5-1.0 g (wet wt.) from each homogenized litter sample using the MOBIO Power Soil DNA Extraction Kit (MOBIO Laboratories, Carlsbad, CA, USA) according to manufacturer’s protocols. DNA was extracted from two replicate sub-samples, pooled for each sample, and then stored at -20°C until needed.
PCR Amplification

Both the ITS region (ITS1F/ITS4) (Gardes & Bruns 1993; White et al. 1990) and the LSU region (LROR_F (Amend et al. 2010)/LR5-F (Tedersoo et al. 2008)) were PCR amplified (n=36) with 8 bp barcodes. The forward primers included the “A” pyrosequencing adaptor along with the 8 bp multiplex tag and forward gene (5′-A + 8 bp multiplex tag + forward gene primer-3′), and the reverse primers included the “B” pyrosequencing adaptor along with the reverse gene primer (5′-B + reverse gene primer-3′) for the ITS and LSU loci regions respectively (Amend et al. 2010). Each PCR consisted of 1.2 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, 0.5 μM of each primer and H₂O to a final concentration of 25 μL. Thermocycling in an Eppendorf Mastercycler Gradient thermocycler was carried out under the following conditions: initial denature for 10 min at 95°C; 34 cycles of 1 min at 95°C, 1 min at 51°C (ITS) or 54°C (LSU), and 1 min at 72°C; and final extension for 7 min at 72°C. PCR products were purified with the QIAGEN QIAquick 96 PCR Purification Kit (QIAGEN, Valencia, CA) following the manufacturer’s instructions. Purified PCR products were quantified with the Invitrogen Qubit Fluorometer (Invitrogen, Carlsbad, CA) and pooled in equimolar concentrations by locus and then sequenced on separate ¼ plate (ITS) and ¼ plate (LSU) of a 454 Titanium sequencing run (454 Life Sciences/Roche Applied Biosystems, NJ, USA). Samples were sequenced at the Duke Institute for Genome Sciences and Policy.

Sequence processing and analyses

Sequencing half of a plate with two loci, (1/4 of a plate) ITS and (1/4 of a plate) LSU, resulted in 157 364 ITS sequences and 31 023 LSU sequences total. Reads were trimmed using the Qiime software (Caporaso et al. 2010), with a minimum of 200 bp and maximum of 650 bp for both ITS and LSU and were filtered with the following default parameters in Qiime: 0 number of allowable Ns, homopolymers maximum length 6, and an average Q score of at least 25. All Qiime commands were executed using MacQIIME version 1.2.1-20110224a. The ITS1 region was verified and extracted using the ITS1 extractor for fungal ITS sequences (Nilsson et al. 2010), and the D1 region was verified and extracted using a modified version of V-Xtractor ((Hartmann et al. 2010); Supplementary X). Within the program Qiime, operational taxonomic units (OTUs), a representation of species (ITS) or higher taxonomic groups (LSU), were determined at 90%-99% sequence similarity using the UCLUST clustering method (Edgar 2010), and plotted against number of OTUs defined by each cut-off level to determine where sequence error level became problematic. For ITS OTUs we selected 95% and for LSU OTUs we selected XX%. Representative OTUs (the longest sequence in each cluster) were deposited in GenBank (Accession number XX-YY for ITS, and WWW-ZZZ for LSU) and were Blasted against the Fungal Internal Transcribed Spacers (ITS) rDNA Sequence Database and the Fungal Large Subunit (LSU) Database curated by the Fungal Metagenomic Project (http://www.borealfungi.uaf.edu/) (Lee Taylor, James Long, Shawn Houston). Both databases are compiled from GenBank, AFTOL and TreeBASE. The BLAST (version 2.2.18) output was imported into MEGAN 4.3 (Huson et al. 2007) using default parameters. Non-fungal taxa and BLAST hits that resulted in no matches were removed from the analysis. Alpha and beta diversity analyses were conducted on the
confirmed fungal taxa (minus singletons) in Qiime. Total study rarefaction was also calculated using EstimateS 8.0 (Colwell 2009).

Alpha diversity indices, including Shannon-Wiener diversity (H’), Simpson, Phylogenetic Diversity (PD) (Faith 1992; Faith & Baker 2006), and Fisher’s alpha for each sample were computed in Qiime. A Mantel test was computed to test for spatial autocorrelation. The Mantel test, ANOVA, and ANOSIM were computed in the R programming environment (R Core Development Team, RDC 2005). The LSU D1 extracted sequences (all non-fungal taxa and singletons removed) were aligned using the PyNAST (a python implementation of the NAST alignment algorithm) in Qiime using LSU sequences from (James et al. 2006) as the core alignment template (Amend et al. 2010). FastTree (Price et al. 2009) was used as the tree building method in Qiime. For community ecological analyses, singletons were removed (Tedersoo et al. 2010), and only samples with 950 reads per treatment for the ITS1 region and samples with at least 250 LSU reads (D1) were included. Non-metric Multidimensional Scaling (NMDS) ordinations were used to represent the dissimilarities in community composition among samples using the Vegan package (Dixon 2003) in R. Community phylogenetic dissimilarity was calculated using the unweighted UniFrac metric in Qiime.

Results

454 Pyrosequencing Results
A total of 117,331 ITS sequences (median length 523 bases, range 200 – 650 bp) and 23,730 LSU sequences (median length 533 bases, range 200 – 630 bp) passed the default Qiime quality control steps. 40,033 sequences were removed due to reads that were too short or contained ambiguous bases, low mean quality score or long homopolymer runs. A total of 23,730 LSU sequences (median length 533 bases, range 200 – 630 bp) passed the default Qiime quality control steps. 7,293 sequences were removed due to reads that were too short or contained ambiguous bases, low mean quality score or long homopolymer runs.

The entire ITS amplicon, the entire LSU amplicon, and the extracted ITS1 spacer and extracted D1 regions were assigned to Operational Taxonomic Units (OTUs) based on 90.0-99.0% sequence similarity and plotted against the number of OTUs that were determined (Figure 1, Figure 2s). These graphs illustrate that the number of OTUs for the unextracted sequences are higher than the component extracted variable region at “higher” similarity thresholds. In addition, the number of OTUs for all regions is initially linear but increases sharply as bin size for OTU similarity is narrowed. The sharp upturn in OTU numbers seen at the narrowest bin sizes is likely a result of sequence error, chimeras, or possibly intraspecific variation. For this reason, OTUs were conservatively determined at 95% sequence similarity.

Using a 95% ITS sequence similarity threshold revealed 5,717 OTUs, 90% sequence similarity revealed 4,599 OTUs, 97% sequence similarity revealed 6,547 OTUs, and 99% sequence similarity revealed 11,565 OTUs (Figure 1). Figure 1 is a comparison of ITS OTU richness determined at different sequence similarities with the full ITS gene region, the ITS1
region extracted, full ITS region with singletons removed and the ITS1 extracted region with singletons removed. ITS1 extracted with singletons removed is the most conservative when determining the number of OTUs. For the purpose of this study, OTUs were conservatively determined at 95% sequence similarity based on the accumulation of OTUs at different sequence similarities (Jumpponen & Jones 2009; Jumpponen et al. 2010; Kunin et al. 2009) (see note in discussion).

Using a 95% sequence similarity threshold revealed 1 618 OTUs for the LSU D1 extracted gene region, 90% sequence similarity revealed 861 OTUs, 97% sequence similarity revealed 2 078 OTUs and 99% sequence similarity revealed 3 529 OTUs (Supplemental Figure 1).

A species accumulation curve of all the ITS OTUs (95%) in the study is close to leveling off, indicating the leaf litter community was sufficiently sampled (Figure 2). Rarefaction curves of the control, M, N, P, and K treatments approach leveling off around 1 500 sequences indicating the sequencing effort obtained a large proportion of the leaf litter diversity among the different treatments (Figure 3).

Alpha diversity indices of the total study area (ITS1) by treatment are presented in Table 1. No significant difference was observed among Observed Species [based on a one-way ANOVA] among all nutrient treatments (ANOVA: F_{9,26}=1.33 \ p=0.27) or with just control, M, K, P, N treatments (ANOVA: F_{4,14}=1.51 \ p=0.25). The control plots have the lowest Shannon and Simpson diversity compared to treatments that received fertilization. The control plots also had the fewest number of observed species, followed by the M and NPK plots. PK plots had the highest Shannon and Simpson diversity indices, while NP plots had the total highest number of observed species, although the Chao 1 species estimates predicts the N plot to have the highest number of species. The control plots also had the lowest number of Observed Species and PD in the LSU dataset.

After non-fungal taxa and sequences with no BLAST hits were removed, 4 941 ITS1 OTUs remained. Singletons were also removed (Tedersoo et al. 2010), leaving 3 248 OTUs remaining for the treatment analyses. Samples containing 950 sequences reads (and rarified to 950 sequence reads were included in the treatment analyses. Fifty-three percent of unique OTUs were present in only one sample and only 1.3% of OTUs were present in half or more of the samples. For the LSU gene region, the D1 region was extracted and non-fungal taxa were removed resulting in 1 577 OTUs. Singletons were also removed, resulting in 904 OTUs and indicating 43% of the cleaned up dataset represented singletons. Samples including 250 sequence reads were included in the treatment analyses.

**Taxonomic diversity analysis**

Based on BLAST analyses of ITS1 sequences followed by MEGAN analyses, *Ascomycota* (including approximately 29 orders, 44 families, and 88 genera) are the dominant phylum among the leaf litter fungi (71% of the sequences; 2 844 OTUs), followed by *Basidiomycota* (26% of the sequences; 1 053 OTUs) (including approximately 31 orders, 38 families, and 52 genera), *Glomeromycota* (2% of the sequences; 95 OTUs), *Chytridiomycota* (1% of the sequences; 33 OTUs), *Neocallimastigomycota* (<1%; 5 OTUs), *Blastocladiomycota* (<1%; 6 OTUs), and subphyla *Mucoromycotina* (<1%; 17 OTUs) (Figure 4) (following (Hibbett et al.
Two ‘basal’ Fungi genera (Hibbett et al. 2007), *Basidiobolus* (5 OTUs) and *Olpidium* (1 OTU) were also represented. The percentages represent the presence of unique OTUs and are not based on abundances of sequence reads. Of the 1.3% of ITS1 OTUs present in half or more of the samples (n ≥18), all were Ascomycetes, with the exception of one Basidiomycete (genus: *Mycena*) OTU.

A similar pattern was observed with the LSU gene region OTUs: *Ascomycota* (72%), *Basidiomycota* (24%), *Glomeromycota* (<1%) and *Chytridiomycota* (3.6%) (data not shown). The majority of ITS1 OTUs at the class taxonomic level are *Sordariomycetes* (30% of the sequences; 984 OTUs), followed by *Agaricomycetes* (24% of the sequences; 784 OTUs), *Dothideomycetes* (14% of the sequences; 455 OTUs), *Eurotiomycetes* (9% of the sequences; 290 OTUs), and *Lecanoromycetes* (3% of the sequences; 94 OTUs) (Supplemental Figure 2).

The majority of ITS1 OTUs at the class taxonomic level are *Sordariomycetes* (30% of the sequences; 984 OTUs), followed by *Agaricomycetes* (24% of the sequences; 784 OTUs), *Dothideomycetes* (14% of the sequences; 455 OTUs), *Eurotiomycetes* (9% of the sequences; 290 OTUs), and *Lecanoromycetes* (3%) of the sequences. The distribution of higher taxonomic levels is very similar across the treatments (control, M, N, P, K, NP, PK, and NPK) and only those represented by 3% of the OTUs or less were apparently variable. For example at the phylum level, *Chytridiomycota* (1% of the overall sample) were not represented in the PK, NP, NK or NPK treatments. At the class level there is also similar distribution of ITS1 OTUs between the treatments, although the PK, NK, and NPK have fewer representatives across different classes (e.g. no *Orbiliomycetes*, *Tremellomycetes* or *Glomeromycetes*). At the order level, no *Xylariales*, *Polyporales*, *Auriculariales*, and *Tremellales*, and at the family level, no *Marasmiaceae*, and *Agaricaceae* were detected. There is a slight increase in *Sordariomycetes* and a decrease in *Agaricomycetes* in these plots compared to the control plots. *Chytridiomycetes* are only present in M, P and K. *Saccharomycetes* are only found in N and NP plots. There is a shift in microbial communities across different nutrient treatments at lower taxonomic levels. At the genera level, the dominant taxa varied across the nutrient treatments (Supplemental Figure 3).

**Treatment analysis**

The number of unique ITS1 OTUs per sample, (presence/absence) ranged from 55 – 177. A comparison of the single factor treatments (N, P,K,M, C) with non-metric multidimensional scaling (NMDS), using the Jaccard index, shows the P treatments tightly clustered, N and K are loosely clustered, and micronutrient and the controls are highly dispersed (Figure 5). In the NMDS plot of all the treatments combined, the P sites also clump closely together (Figure 6). There is a general similarity (or clumping) of plots that have received fertilization (primarily the K, P, NP, PK, and NPK sites). A PCoA plot provided a similar ordination diagram as the NMDS plots (data not shown). A Mantel test was run to test for correlations between the species data distance matrix (LSU) and the geographic distances of the sites. Both the Jaccard and unweighted Unifrac measures of associations were not significant, indicating species composition are not related to geographic distances (Table 2). Analysis of similarities (ANOSIM) method using phylogenetic composition dissimilarity distance matrix using unweighted UniFrac was not significant (R=0.07, p=0.153) indicating there is not a
significant difference between the treatments. However, an unweighted UniFrac beta significance test, with OTUs grouped by treatment, yielded the following sample pair-wise comparisons statistically significantly different (Bonferroni corrected): control-N; control-P; K-N; K-NK; M-N; N-P (representing 21% of the comparisons). This test indicates N treatment plots are statistically significant from the control, K, P, and M treatment plots.

Discussion

This study revealed high species diversity of leaf litter fungi in the tropics. The diversity reported here is comparable, if not higher, to recent pyrosequencing fungal studies (including a temperate soil study, an alpine soil study and a global indoor air study, respectively (Buée et al. 2009; Lentendu et al. 2011; Amend et al. 2010). Interestingly, recent soil studies have demonstrated a dominance of Basidiomycota taxa (Buée et al. 2009; Jumpponen, Jones & Blair 2010). Here we report a dominance of Ascomycota, followed by Basidiomycota among leaf litter fungi. High-throughput 454 pyrosequencing has allowed for greater sampling depth and has provided a greater insight into fungal richness and diversity across the study site, compared with a previous T-RFLP study (Kaspari et al. 2010).

The addition of nutrients had an effect on the taxonomic composition of the leaf litter fungal communities at lower taxonomic levels (i.e. family, genus and species), but not at higher taxonomic levels (i.e. phylum, class and order). There was no significant difference among observed species (ITS1 OTUs at 95% sequence similarity) based on a one-way ANOVA across the different nutrient treatments. However, it does appear that the addition of nutrients had an effect on the richness and diversity of the leaf litter fungal communities on the plots receiving fertilization compared with the control plots. Control plots for both the ITS and LSU datasets had the lowest observed species (OTUs) and estimated species richness (Chao 1), along with the lowest phylogenetic diversity (PD) for the LSU data set compared to the other plots that received nutrients. The interaction of nutrients on the NPK plots resulted in low diversity (as defined by alpha diversity indices – Shannon, Simpson, Chao1, and PD).

Overall, both the ITS and LSU gene regions had similar results. The use of two separate gene regions for the analysis provides a complimentary look at the data. OTUs were determined conservatively at 95% sequence similarity (Figure 1) as a function of sequence similarity and in an effort to avoid overestimating richness, which is inherent with 454 pyrosequencing technologies compared with traditional sequencing methods (Huse et al. 2007; Quince et al. 2009; Kunin et al. 2009). The ITS dataset was re-analyzed with both the ITS and ITS1 regions clustered at 97%, which is typically used for OTU delimitation (O’Brien et al. 2005; Tedersoo et al. 2010; Amend et al. 2010). Similar patterns resulted (data not shown) in the community analysis, and the major difference was the number of OTUs (Figure 1). Only OTUs occurrence (presence/absence of unique and not abundances of sequences) was included in community ecological analyses paper due to the fact that 454 reads are not a good proxy for relative abundance when comparing between species (Amend, Seifert & Bruns 2010).
As has been previously mentioned, the results of ecological community analyses and species reporting is only as good as the databases that we can compare our sequences with (Kõljalg et al. 2005; Buée et al. 2009; Hibbett et al. 2011; Rolf Henrik Nilsson et al. 2009). Overall, this study provides insights into the taxonomic composition of leaf litter fungi in the tropics and the potential effects of nutrient addition.
Literature Cited


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Table 1. Alpha diversity indices of treatments (ITS OTUs). Each treatment is averaged across the four replicates. No significant difference was observed among Observed Species based on an ANOVA.
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**Table 2.** Results of the Mantel test comparing community dissimilarity to physical distance based on 9999 permutations using LSU gene region.
Figure 1. Comparison of ITS OTUs determined at different sequence similarities. Black – Full ITS, Blue – ITS 1, Grey – ITS with singletons removed, and Light Blue – ITS 1 with singletons removed. ITS 1 extracted and with singletons removed is the most conservative when determining the number of OTUs. For the purpose of this study, OTUs were conservatively determined at 95% sequence similarity. Arrow shows chosen value for OTU determination.
Figure 2. Species accumulation curve of total study area. ITS1 OTUs clustered conservatively at 95% sequence similarity. The curve is reaching an asymptote, indicating the community was sufficiently sampled.
**Figure 3.** Rarefaction curves of observed ITS1 OTUs per treatment. The control plots - C (red) plots have fewer OTUs compared with the plots that received fertilization treatments. Potassium - K (blue), Nitrogen - N (green) and Phosphorus - P (purple) plots have similar number of OTUs while Micronutrients - M (orange) plots have fewer OTUs.
Figure 4. Percentages of the phyla represented across the entire study site of leaf litter fungi. Ascomycota represent the majority of ITS1 OTUs. (OTU percentages based on occurrence and not sequence abundance-based.)
Figure 5. NMDS plot using the Jaccard index of ITS1 sequences normalized to 950 sequences per treatment (N,P,K,M and control treatments). The numbers following the treatment represent the replicate of each treatment. (Stress 11.78)
Figure 6. NMDS plot of ITS1 sequences normalized to 950 sequences per treatment. All treatments included. The numbers following the treatment represent the replicate of each treatment. (Stress 12.600)
**Supplemental Figure 1.** Comparison of LSU OTUs determined at different sequence similarities. Blue – Full LSU, Grey – LSU D1 region extracted.
Supplemental Figure 2. Distribution of total leaf litter fungal ITS1 OTUs (95%) at the class taxonomic level. (OTU percentages based on occurrence and not sequence abundance-based.)
Supplemental Figure 3. Comparison of ITS1 OTU (95%) distribution across class (A), order (B) and family (C). X axis: all ITS1, Control, Micronutrient, N, P, K, PK, NP, NK, NPK.
CHAPTER 2

Saprotrophic fungal assemblages are influenced by plant community type more strongly than by substrate type or time.
Abstract

In this study we characterized saprotrophic fungal assemblages on straw and wood substrates across forest and grassland plant community types and characterized how these assemblages changed over time as substrate quality changed. The goal was to determine if substrate, space, time or plant community were the major determinants of fungal saprotrophic community composition. Straw and wood baits (n=160) were buried and retrieved at two different time points to select for saprotrophic fungi that were active in this system. We extracted DNA from the substrates and soil samples and used fungal-specific amplification and a 454 pyrosequencing approach to sequence the nuclear ribosomal large subunit (LSU) D1 region. Using a 95% LSU sequence similarity threshold revealed 508 Operational Taxonomic Units (OTUs), and the number of unique LSU D1 OTUs per sample ranged from 13 to 89. *Ascomycota* was the dominant phylum among the saprotrophic fungi colonizing the straw (74%) and wood substrates (74%), followed by *Basidiomycota* (20% and 23%, respectively). Wood substrates collected in the grassland and forest at the first time point had the highest richness, estimated richness and phylogenetic diversity. Results from cloning and sequencing of the nuclear ribosomal internal transcribed spacer (ITS) and LSU regions and TRFLP of the ITS region were compared with the pyrosequencing results. Here, we investigated possible factors affecting saprotrophic fungal community structure, such as dominant plant community type, resource type (represented by different substrates) and resource quality (represented by different time points). Overall, there was a decrease in richness on the substrates in both plant communities over time, and plant community type appears to have a greater influence on saprotrophic fungal community structure than substrate type or substrate quality.
Introduction

Fungi can compose up to 90% of the total decomposer biomass in some ecosystems (Kjoller & Struwe 1982). Saprotrophic fungi in particular are responsible for recycling the majority of carbon from dead organic matter and have the unique ability to break down and release nutrients that can then be readily available for other organisms. Basidiomycete fungi are capable of decomposing recalcitrant components of plant litter and lignified cellulose by producing lignin-modifying enzymes. Despite fungi’s important role in ecological and biogeochemical processes, little is known about the structure and richness of saprotrophic fungal communities.

Molecular approaches and methods have allowed for a better understanding and characterization of fungal diversity and community structure compared with culturing and fruiting body surveys. Recent method papers focused on molecular approaches have found that diversity of soil fungi is higher than expected (Lynch & Thorn 2006; Buee et al. 2009), biodiversity of soil fungi is greatest in natural sites with less anthropogenic disturbance (Midgley et al. 2007; Kennedy et al. 2006), a floristic gradient across grasslands does not affect soil fungal diversity (Brodie et al. 2003), there are differences in community composition of plantation ecosystems as compared to natural forest ecosystems (He et al. 2005), and saprotrophic fungi are most often found in the litter layers (O’Brien et al. 2005; Lindahl et al. 2007; Dickie et al. 2002) whereas mycorrhizal fungi are most often found in the deeper layers of the soil profile. Many of these studies focused on agricultural or managed sites rather than natural ecosystems. In this study, we used bait bags as a method to capture the active decomposer fungal community found in soil.

Soils are highly heterogeneous environments providing numerous niches for fungi. Bruns (1995) first proposed the idea of niche differentiation as a driver of diversity in ectomycorrhizal communities, and other more recent studies have also noted niche partitioning as one hypothesis to explain the species-rich ectomycorrhizal assemblages (Dickie et al. 2002; Koide et al. 2007). Previous work using molecular approaches have independently studied the diversity of fungal assemblages in grasslands (Deacon et al. 2006; Kennedy et al. 2006; Jumpponen et al. 2010) and natural forest ecosystems (He et al. 2005; Jumpponen 2007; Buée et al. 2009); however, there are no studies comparing fungal diversity across both grassland and forest ecosystems in close proximity in order to determine if plant community type has an effect on fungal assemblages.

Swift and colleagues (1979) (in Dighton 2007) proposed a general model of fungal succession of uncolonized substrates, with “sugar” fungi as the first to colonize and progressing to basidiomycetes which have a greater enzymatic capability to break down more recalcitrant plant debris, such as lignin and cellulose. In addition, Frankland (1998) described fungal succession on leaf litter in relation to the rate of loss of cellulose and lignin and the decrease in C:N ratio. A three stage model of litter decomposition was proposed recently (Snajdr et al. 2011) with an initial fast decomposition stage of readily available substrates followed by cellulose decomposition and then later lignin. Along with litter, wood is also an important component of available substrates in forests. There is evidence that with wood-decay
basidiomycetes, fungal competition is the most common type of interaction and often results in a hierarchy of combative ability (Boddy 2000; Holmer & Stenlid 1993; Holmer & Stenlid 1997). A recent study reported an increase in richness of active fungi (based on extracted RNA) with decomposition stage in Norway spruce logs (Rajala et al. 2011).

In this study, we used baits (wood blocks and mesh bags filled with straw) to select for active saprotrophic fungi that are capable of digesting cellulose, as opposed to mycorrhizal or pathogenic fungi, which are also present in the soils. Soil fungal communities, as judged by sequencing, are hyperdiverse, but the roles and activity of that diversity are unclear. Total DNA extractions from environmental samples mainly detect living organisms; however, DNA can also represent dead or dormant organisms (Rajala et al. 2011). Therefore, with this approach we are sampling only the active saprotrophic fungi. Soil samples from the plots were also collected as a way to compare the active fungal communities with the total diversity present in the surrounding soils.

The goals of this study were to compare species richness and diversity of saprotrophic fungal communities in different plant community types within close proximity and on different substrates, and to investigate how these communities change over time. More specifically, we were interested in testing the impact of substrate, space, time and plant community type on the active saprotrophic community.

Materials and Methods

Study site and experimental design
This study was conducted along Bolinas ridge on Mount Tamalpais in the Marin Municipal Water District watershed, in Marin County, California, USA (N37° 55.35', W122° 38.11'). Four 10 x 10 m blocks, each with 10 randomly assigned 2 x 2 m plots were established (Figure 1). Two blocks were in the coastal grassland, characterized as northern coastal grassland and dominated by Festuca and Danthonia, along the forest edge, and two blocks were in the adjacent forest dominated by Pseudotsuga menziesii with an understory dominated by Notholithocarpus densiflorus and Quercus agrifolia. One grassland block was paired with a forest block. The distance between the two paired blocks was roughly 30 meters. A second set of the paired blocks (one grassland block and one forest block 30 meters apart) was established over 300 meters away.

The region is characterized as a Mediterranean climate with a seasonal summer drought and average annual precipitation of 1250 mm (Dunne & Parker 1999) (see Kennedy et al. 2003 for further site characteristics). Sterile bait bags (5 x 5 cm mesh bags filled with 0.07 g of sterile wheat straw with a nylon string attached) and small sterilized conifer wood blocks (5 x 5 cm, average 4 g), also with a nylon string attached (Figure 1), were placed in the grassland and forested plots, with two bait bags per plot (n=80) and two wood blocks per plot (n=80), each at a depth of <10 cm. Ten bait bags and ten wood blocks were removed from each block at two different time points. In February 2007, after six months, one-half of the bait bags (n=40) and one-half of the wood blocks (n=40) were removed and collected from each plot. The
remaining bait bags (n=40) and wood blocks (n=40) were removed and collected in February 2008 after being buried for 18 months. In all cases, the bait bags and the wood blocks were collected near the end of the rainy season. The samples were placed on ice and processed immediately upon returning to the lab. A substrate analysis (performed by University of California, Department of Agriculture and Natural Resources Lab) compared the similarity of cellulose, lignin, carbon (C), nitrogen (N), phosphorus (P), and potassium (K) contents among the prepared substrates (in this case, the straw and wood blocks) as compared to the natural substrates (i.e. conifer needles, oak leaves and native grass) collected in the blocks (Supplemental Table 1). Percentage of mass loss of the buried substrates over 6 and 18 months was measured by dry weight and reported in Supplemental Table 2. Substrates were placed in coin envelopes and dried in a 37°C oven for two days. Soil moisture, pH, N, P, K, and C:N ratios were measured in every block and are reported in Supplemental Table 3. Soil cores of 5 x 5 x 5 cm were collected from 5 randomly assigned plots within each block (n=20) in early April 2009. The soil samples were only included in the pyrosequencing dataset.

**Culturing**
At both time points (February 2007 and February 2008) the straw and wood substrates were cultured for basidiomycete fungi. The substrates were washed under running DI water, surface sterilized for 15 seconds in 30% hydrogen peroxide, and then rinsed in sterile water. Four 2 x 2 mm pieces of straw or wood from each plot and from both time points (n=160) were plated on 2% Malt Agar 20 ml plates with (per liter of distilled water) 50 mg of Chloramphenicol, 50 mg of Streptomycin, 50 mg of Carbenicillin and 1 mg of Benomyl (to deter Ascomycete growth) (Thorn et al. 1996). DNA was extracted from a small amount of visible hyphae on the plates that colonized the straw and wood blocks using a modified Qiagen Kit protocol (Valencia, CA) and the internal transcribed spacer region (ITS) was sequenced (method described below). Sequences were queried in GenBank (NCBI) using BLASTn for identification.

**DNA extraction**
The straw and wood substrates were pulverized for 30 seconds with a 3 mm glass bead beater (Biospec) and suspended in 1000 µl CTAB/PVPP buffer (2% CTAB, 1% PVPP, 0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA). After a 60 minute incubation at 65°C, DNA was extracted from the straw and wood substrate samples (n=160) using a chloroform:isoamyl alcohol extraction method (Kennedy et al. 2003), and extracts were purified using the Qiagen DNeasy Tissue Kit (Valencia, CA).

**TRFLP**
The ITS region was amplified with fluorescently-labeled primers, ITS1F and ITSF4B (Gardes & Bruns 1993), from each DNA sample from both time points (n=160). The 5' end of primer ITS1F was linked with the fluorescent dye 6-FAM (Integrated DNA Technologies, Coralville, IA). Each PCR consisted of PCR buffer (containing 50 mM KCl, 10 mM Tris, 2.5 mM MgCl₂, and 0.1 mg/ml gelatin) (Gardes & Bruns 1996), 0.2 mM dNTPs, 0.25 µM of each primer, 0.05 U of AmpliTaq (PerkinElmer, Waltham, MA), and 2 µl of sample DNA in a total volume of 20 µl. Thermocycling in an Eppendorf Mastercycler Gradient thermocycler was carried out under the following conditions: initial denature for 1 min at 94°C; 28 cycles of 1
min at 94°C, 1 min at 51°C, and 3 min at 72°C; and final extension for 8 min at 72°C. Up to three replicate PCRs were pooled and visualized on a 1.5% agarose gel. Successful amplifications of 200 nanograms were then digested using either HaeIII or Hinf1 restriction enzyme following the manufacturer’s protocol (New England Biolabs Inc., Beverly, MA). The resulting DNA fragments were separated on an ABI 3100 Genetic Analyzer with GeneScan (Applied Biosystems, Foster City, CA) and analyzed with GeneMapper software (Applied Biosystems, Foster City, CA). Non-metric Multidimensional Scaling (NMDS) ordinations were used to represent the dissimilarities in community composition among samples using the PRIMER 5 (Clarke & Gorley 2001) software.

Cloning
The primer pair ITS1F and LR3 (Vilgalys & Hester 1990) was used to PCR amplify one large region containing the rDNA ITS and a partial region of the LSU region. The LSU region is phylogenetically conserved enough to allow for alignment across the Ascomycota and Basidiomycota and provide higher-level taxonomic assignment. The ITS region is used to identify finer-scale taxonomic assignment and is widely used as a validated DNA barcode marker for the identification of fungal species (Seifert 2008). Samples were cloned with the TOPO cloning kit (Invitrogen, Carlsbad, CA). Positive clones were screened for correct sized inserts using plasmid primers T3/T7 and the following PCR conditions: initial denature for 10 min at 94ºC; 95ºC for 2 min, 50ºC for 45 sec, and 72ºC for 1 min 30 sec; 29 cycles of 95ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 1 min 30 sec; and final extension for 7 min at 72ºC. PCR products were cleaned with ExoSAP-IT using the manufacturer’s instructions (USB, Cleveland, OH) and sequenced in one direction using ITS1F or LR3 primer with BigDye version 3.1 chemistry (Applied Biosystems, Foster City, CA). Sequences were determined with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were edited and clustered into Operational Taxonomic Units (OTUs) using Sequencher 4.2.2. (Gene Codes, Ann Arbor, MI) and queried in GenBank (NCBI) using BLASTn for identification. Species accumulation curves, along with richness and diversity estimates, were calculated using EstimateS 8.2 (Colwell 2004).

454 Pyrosequencing
PCR Amplification – A subset of the samples were chosen for pyrosequencing due to a limit in the number of barcodes available at the time. Thirty-two samples from the first time point (16 straw and 16 wood substrate samples) with equal representation from the grassland and forest plant communities were sequenced. An equal number of samples from the second time point were also sequenced from the same plots. Nineteen soil samples (9 grassland and 10 forest samples) were also sequenced, resulting in a total of 83 samples. The LSU region (LROR_F (Amend et al. 2010)/LR5-F (Tedersoo et al. 2008)) were PCR amplified with 10 bp MID barcodes. Each sample (n=83) was PCR amplified with a unique barcode. The forward primers included the “A” pyrosequencing adaptor along with the 10 bp multiplex tag and forward gene (5’-A + 10 bp multiplex tag + forward gene primer-3’), and the reverse primers included the “B” pyrosequencing adaptor along with the reverse gene primer (5’-B + reverse gene primer-3’) for the LSU loci. Each PCR consisted of 1.2 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, 0.5 µM of each primer, and H₂O to a final concentration of 25 µL.
Thermocycling in an Eppendorf Mastercycler Gradient thermocycler was carried out under the following conditions: initial denature for 10 min at 95°C; 34 cycles of 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C; and final extension for 7 min at 72°C. PCR products were purified with the Qiagen QIAquick 96 PCR Purification Kit (QIAGEN, Valencia, CA) following the manufacturer’s instructions. Purified PCR products were quantified with the Invitrogen Qubit Fluorometer (Invitrogen, Carlsbad, CA) and pooled in equimolar concentrations by locus, gel purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and then sequenced on 1/8 of a plate on a 454 Titanium sequencing run (454 Life Sciences/Roche Applied Biosystems, NJ, USA). Samples were sequenced at the Duke Institute for Genome Sciences and Policy.

Sequence processing and analyses – Sequencing 1/8 of a plate resulted in a total of 123,117 LSU sequences. Reads were trimmed using the Qiime software, which allows for analysis of high-throughput community sequencing data (Caporaso et al. 2010), with a minimum of 200 bp (and maximum 650 bp), and were filtered with the following default parameters in Qiime: 0 number of allowable Ns; homopolymers maximum length 6; and an average Q score of at least 25. All Qiime commands were executed using MacQIIME version 1.2.1-20110224a. The D1 region was verified and extracted using a modified version of V-Xtractor for the LSU region (Hartmann et al. 2010). The extraction of the D1 LSU region removes the conserved region, therefore allowing for sensitive OTU clustering, and it detects sequences that pass quality scores, but are not LSU sequences. This step also decrease the chance of chimeras in the dataset. Within the program Qiime, OTUs were determined at 90%-99% sequence similarity using the UCLUST (Edgar 2010) clustering method. Representative OTUs (the longest sequence in each cluster) were deposited in GenBank (Accession number XX-YY for LSU) and were Blasted against the Fungal Large Subunit Database curated by the Fungal Metagenomic Project (http://www.borealfungi.uaf.edu/) (Lee Taylor, James Long, Shawn Houston) which is compiled from GenBank, AFTOL and TreeBASE. The BLAST (version 2.2.18) output was imported into MEGAN 4.3 (Huson et al. 2007) using default parameters. Non-fungal taxa and BLAST hits that resulted in no matches were removed from the analysis. Alpha and beta diversity analyses were conducted in Qiime. Total study rarefaction was also calculated using EstimateS 8.0 (Colwell 2009).

Alpha diversity indices, including Shannon-Wiener, Simpson, Phylogenetic Diversity (PD) (Faith 1992; Faith & Baker 2006), and Fisher’s alpha were computed on rarified samples in Qiime and based on a similar cutoff of 95% for OTUs. A Mantel test was computed to test for spatial autocorrelation. The Mantel test and ANOSIM were computed in the R programming environment (R Core Development Team, RDC 2005). The LSU D1 extracted sequences with non-fungal taxa and singletons removed were aligned using the PyNAST (a python implementation of the NAST alignment algorithm) in Qiime using LSU sequences (from James et al. 2006) as the core alignment template (James et al. 2006). FastTree (Price et al. 2009) was used as the tree building method in Qiime. For community ecological analyses, non-fungal taxa and singletons were removed (Tedersoo et al. 2010), and only samples with at least 600 LSU D1 reads were included. NMDS ordinations were calculated using the Vegan package (Dixon 2003) in R. Community phylogenetic dissimilarity was calculated using the unweighted UniFrac metric in Qiime.
Results

Culturing
Culturing resulted in 11 Basidiomycota species (Table 1) found on 11 plates. Of the 160 plates, 55 plates resulted in a sequence with a closest Blast match. The rest of the plates were either contaminated early or did not have hyphal growth. Twenty-four of the sequences had Ascomycota as the closest Blast match, and 20 sequences had a Mucorales as the closest Blast match.

TRFLP
The TRFLP analysis was focused only on basidiomycete fungi. From the 160 substrate samples, the total number of phylotypes for basidiomycete fungi with HaeIII was close to 300. The mean number of phylotypes, per substrate sample, ranged from 2.6 to 29.5 (Table 2). Results indicate there is a greater diversity of basidiomycete fungi on the straw substrate in the grassland than in the forest. Over time, basidiomycete diversity decreased on the substrates in the different plant community types (Table 1). NMDS was used to ordinate the basidiomycete community data by different substrates, plant community type, and time. The NMDS analysis suggested that the basidiomycete communities could be clearly separated by time (Figure 2).

Cloning and sequencing
TRFLP is a DNA fingerprinting method that is commonly used for profiling microbial communities. However, there are some limitations to this molecular technique (Avis et al. 2006). In order to analyze the total community diversity, a broader approach was taken with the clone libraries in order to address the question of how the taxonomic composition of fungal assemblages change across substrates, plant community type, and time. The PCR amplicons from each substrate per plant community type were pooled for a total of 16 clone libraries (There were 8 clone libraries for each gene region. The eight clone libraries consisted of samples pooled from: 1. Straw from Grassland TP1; 2. Straw from Forest TP1; 3. Straw from Grassland TP2; 4. Straw from Forest TP2; 5. Wood from Grassland TP1; 6. Wood from Forest TP1; 7. Wood from Grassland TP2; 8. Wood from Grassland TP2). A total of 1120 clones were sequenced. Analysis of the sequence data resulted in taxa from 12 Basidiomycota classes and 5 Ascomycota orders. OTUs were determined at 97% sequence similarity for both the ITS and LSU gene regions which has been demonstrated to correspond well with taxonomic species groups (Vellinga et al. 2003; Smith et al. 2007).

The species accumulation and the Chao 1 Estimator curves remain non-asymptotic, indicating that the clone pool sampling did not capture the total diversity (data not shown). 264 ITS OTUs were recovered (with 97% sequence similarity). The LSU clone library data indicates that the Sordariomycetes are the most dominant taxa in both the forest and grassland with both time points combined, followed by the Agaricales in the forest and the Dothideomycetes in the grassland (Table 3). Overall, Ascomycota dominated both time points (62% and 70%, respectively) followed by Basidiomycota (27% and 29%, respectively) (Figure 3). Ordination of the samples using NMDS showed that assemblages associated with plant community type more strongly than time or substrate (data not shown).
**454 Pyrosequencing Results**

A total of 100,263 LSU sequences (average length 343 bases, range 200 – 559 bp) passed the default Qiime quality control steps. 22,854 sequences were removed due to reads that were too short or contained ambiguous bases, low mean quality score or long homopolymer runs. Thirty-two barcodes of the 83 resulted in very low or 0 sequence reads.

The data was parsed into two datasets. One dataset included the substrates (straw and wood), and the second dataset included both the soil samples and the substrates. The entire LSU amplicon and LSU D1 extracted region were assigned to OTUs based on 90-99% sequence similarity and plotted against the number of OTUs that were determined (Figure 4). Figure 4 is a comparison of LSU OTU richness determined at different sequence similarities with the full LSU gene region and the LSU D1 extracted region. This graph illustrates that the number of OTUs for the unextracted sequences are higher than the component extracted variable region at “higher” similarity thresholds. In addition, the number of OTUs for all regions is initially linear but increases sharply as bin size for OTU similarity is narrowed. The sharp upturn in OTU numbers seen at the narrowest bin sizes is likely a result of sequence error, chimeras, or possibly intraspecific variation. For this reason, OTUs were conservatively determined at 95% sequence similarity.

For the substrate (straw and wood) and soil samples combined, a 95% LSU sequence similarity threshold revealed 2,182 OTUs and for the substrate-only samples, a 95% LSU sequence similarity threshold revealed 863 OTUs. After non-fungal taxa, sequences with no BLAST hits and singletons were removed, 1,174 OTUs (substrates and soil samples) and 508 OTUs (substrate only samples) at 95% sequence similarity remained.

A species accumulation curve of all the LSU OTUs (95%) in the study reached an asymptote for the substrate communities, indicating the substrate communities were sufficiently sampled. However, the soil community accumulation curve is non-asymptotic. Rarefaction curves of the substrates approach leveling off around 200 sequences indicating the sequencing effort obtained a large proportion of the diversity of the substrates (Figure 5 and Figure 6).

Alpha diversity indices of the total study area (LSU D1) by treatment are presented in Table 4. The soil alpha diversity indices were calculated from a rarified dataset containing both the soil and substrate samples (which is important for the PD). The PD for the straw substrate in this dataset is 3.40 and the wood is 5.28 (not included in the table). The rest of the treatment alpha diversity indices were calculated from the substrate dataset. The grassland samples have a greater number of observed species, estimated species (Chao 1), and PD as compared to the forest samples, while the wood samples have a greater number of observed OTUs, estimated OTUs (Chao 1), and PD as compared with the straw samples. However, the soil samples had greater alpha diversity indices as compared to both the straw and wood samples. Also, the diversity indices for each time point were fairly similar to each other, although the first time point samples have a slightly higher richness as compared to the second time point samples.
Based on BLAST analyses followed by MEGAN analyses, *Ascomycota* is the dominant phylum among the fungi found in the grassland and forest plant community types and is the dominant phylum on both the straw and wood substrates. In the grassland, *Ascomycota* represents 82% of the sequences (209 OTUs), and *Basidiomycota* represents 17% of the sequences (43 OTUs). Within the forest samples, *Ascomycota* represents 64% of the sequences (180 OTUs), and *Basidiomycota* represents 34% of the sequences (96 OTUs). *Agaricales, Sebacinales, Polyporales, Auriculariales, and Corticiales* have a greater number of OTUs in the forest than grassland. Both the straw and wood substrates are dominated by *Ascomycota* (74% and 74%, respectively), followed by *Basidiomycota* (20% and 23%, respectively). There is a slightly greater phylogenetic diversity on the wood substrates than on the straw substrates; however, the two substrates appear similar across higher taxonomic levels. The percentages here represent the presence of unique OTUs and are not based on abundances of sequence reads. Samples from both time points have similar percentages of *Ascomycota* and *Basidiomycota* OTUs (74.4% and 20.6%, respectively, for the first time point; 74.8% and 22.4%, respectively, for the second time point). However, samples from the first time point have a much greater phylogenetic diversity than samples from the second time point. Samples from the first time point have 462 *Ascomycota* OTUs as compared to 169 *Ascomycota* OTUs for samples at the second time point, and 128 *Basidiomycota* OTUs as compared to 50 *Basidiomycota* OTUs at the second time point. In almost all cases, the genera present in samples at the second time point were also present in samples at the first time point. Dominant *Basidiomycota* genera present in samples at the second time point are *Aleurodiscus, Sebacina, Trechispora, Ramariopsis*, and *Mycena*.

The number of unique LSU OTUs, at 95% (presence/absence), ranged from 13 to 89 per sample. NMDS method, using the Jaccard index, shows the assemblages associated strongly with plant community type type (forest or grassland) (Figure 7). A similar ordination was observed with the unweighted UniFrac metric (data not shown). The soil samples group together and by plant community type. A PCoA plot of paired samples (straw and wood substrates from the same plot, including both time points) showed the paired samples most often are grouped together, indicating there is not a large shift in taxa from the first time point to the second time point. In addition, the wood substrates clearly grouped by plant community type type (data not shown). Analysis of similarities (ANOSIM) using the Jaccard dissimilarity distance matrix indicated there is a significant difference between the ecosystem type (R=0.11, p=0.005). A UniFrac significance test (100 Monte Carlo randomizations), with OTUs grouped by plot type (substrate, ecosystem, and time) indicated that there is a significant difference between wood substrates in the forest compared with wood substrates in the grassland, as well as a significant difference between wood substrates in the forest at the first time point as compared to the second time point. Table 5 shows all pair-wise comparisons. Mantel tests were run to test for correlations between the species data distance matrix (LSU) and the geographic distances of all the blocks. The Jaccard measure of association was significant (p < 0.05) for both datasets (substrate only and substrate plus soil samples), indicating there is a relationship between species composition and geographic distance. However, there was no significance between the two sets of paired blocks, indicating the ecosystem is driving the correlation, as opposed to stochastic spatial patterning, between the species data matrix and the geographic distance.
Discussion

In this study, saprotrophic fungal diversity on two different substrates (straw and wood) from two ecosystem types, collected at two different time points, was analyzed using ITS and LSU rDNA. A high-throughput pyrosequencing approach was used, along with TRFLP and cloning and sequencing. Here we report a dominance of *Ascomycota*, followed by *Basidiomycota*, in the straw and wood substrates and in the grassland and forest plant community types. Overall, plant community type and time appear to have a greater effect on fungal communities than substrate (straw and wood).

*Plant community effects on fungal assemblages*

Plant community type, as compared to substrate or time, appears to have the greatest influence on structuring fungal assemblages. Ordination of the samples using NMDS, for the cloning and pyrosequencing data, indicated that assemblages associated with plant community type (forest or grassland) more strongly than time or substrate. *Ascomycota* are the dominant phylum among the fungi found in the grassland and forest plant community types. The grassland samples have a greater number of observed OTUs, estimated OTUs (Chao 1), and PD as compared to the forest samples. The difference in composition of the saprotrophic assemblages between the grassland and forest plant community types may be driven by the substrate typically present and dominant in each site (i.e. the dominant plant debris is woody and higher in lignin content in the forested areas). Alternatively, the physical differences between the plant community types (e.g. pH, soil moisture, temperature, and shade), and the stochastic spatial patterning of active saprobes might have a greater influence on saprotrophic assemblages. We anticipated the soil saprotrophic basidiomycete assemblages would be more uniform in plant communities dominated by woody plants than in those dominated by grasses due to the presence of dominant wood decomposer fungi in the forest plant community type. However, the forest samples have a greater number of *Basidiomycota* OTUs as compared to the grassland samples. One possible explanation for the greater number of basidiomycete genera represented in the forest is due to the higher available substrate diversity as compared to the grassland.

*Temporal effects on fungal assemblages*

Substrates were buried for a total of 6 months or 18 months. Over time, substrate quality changed as indicated by mass loss (82% mass loss in straw substrates and 17% mass loss in wood substrates overall). The NMDS ordination of the TRFLP data suggests that the basidiomycete communities are clearly separated by time (Figure 2). Over time, basidiomycete diversity decreases both on the substrates and in both plant community types. The pyrosequencing data also indicated that the first time point samples appear to have slightly higher alpha diversity indices as compared to the second time point samples. The samples from the first time point have a much greater phylogenetic diversity as compared to the samples from the second time point. The samples from the first time point have 462 *Ascomycota* OTUs as compared to 169 *Ascomycota* OTUs in samples at the second time point, and 128 *Basidiomycota* OTUs as compared to 50 *Basidiomycota* OTUs in samples at the second time point. In almost all cases, the genera present in samples at the second time point were also present in samples at the first time point. Also, there was a decrease in
richness on the substrates in both plant community types over time. This may indicate that a select group of the first colonizers to arrive at the resource remain there over time and outcompete many of the other species as the resource quality changes over time. The patterns observed here complement the ideas of succession presented by Swift et al. (1979) and Frankland (1998), such that there is a greater species diversity and richness colonizing the substrates and then decreasing over time. Although this study was not designed to study all the stages of succession, we were able to characterize how the assemblages changed over a period of time and how the assemblages changed as the resource quality changes over time.

**Substrate effects on fungal assemblages**
Overall, there was not a strong substrate preference. TRFLP, cloning and pyrosequencing had different results regarding species richness on the different substrates. TRFLP results indicate there is a greater diversity of basidiomycete fungi on the straw substrate. Cloning and sequencing results indicated a slightly higher (fungal or basidiomycete?) richness on the straw substrates (132 OTUs) as compared to the wood substrates (122 OTUs). The pyrosequencing data highlighted that wood samples have a greater number of unique OTUs, estimated OTUs (Chao 1), and PD as compared to the straw samples. As expected, the soil samples had much greater alpha diversity indices as compared to both the straw and wood substrates. *Ascomycota* are the dominant phylum on both the straw and wood substrates. The fungal assemblages on the wood substrates were influenced strongly by plant community type. Paired samples (straw and wood substrates from the same plot) most often grouped together in the NMDS.

**Different molecular approaches**
The use of three separate molecular approaches provided a complimentary look at the data. TRFLP remains a valid approach to find patterns in a study with large number of samples. High-throughput 454 pyrosequencing allowed for greater sampling depth as compared to TRFLP and cloning and sequencing. The use of two separate gene regions for the analysis also provided a complimentary look at the data.

**Pyrosequencing OTU determination**
OTUs were determined conservatively at 95% sequence similarity (Figure 4) as a function of sequence similarity and in an effort to avoid overestimating richness, which is inherent with 454 pyrosequencing technologies as compared to traditional sequencing methods (Huse et al. 2007; Quince et al. 2009; Kunin et al. 2009). The dataset was re-analyzed with the LSU region and LSU D1 region clustered at 97%, which is typically used for OTU delimitation (O’Brien et al. 2005; Tedersoo et al. 2010; Amend et al. 2010). Similar patterns resulted in the community analysis, and the major difference was the number of OTUs (Figure 4). Only OTU occurrence (presence/absence of unique and not abundances of sequences) was included in community ecological analyses due to the fact that 454 pyrosequencing reads are not a good proxy for relative abundance when comparing between species (Amend, Seifert & Bruns 2010). Overall, this study provides insights into the richness and diversity of saprotrophic fungi in a temperate environment and highlights the effect of plant community type on saprotrophic fungal community structure.
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http://aem.asm.org/cgi/content/abstract/71/9/5544.


Table 1. List of basidiomycetes cultured from buried straw and wood substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ecosystem</th>
<th>Time Point</th>
<th>Order</th>
<th>Genus</th>
</tr>
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<tbody>
<tr>
<td>Straw</td>
<td>Forest</td>
<td>1</td>
<td>Tremellales</td>
<td>Trichosporon</td>
</tr>
<tr>
<td>Straw</td>
<td>Forest</td>
<td>1</td>
<td>Agaricales</td>
<td>Mycena</td>
</tr>
<tr>
<td>Straw</td>
<td>Forest</td>
<td>2</td>
<td>Agaricales</td>
<td>Armillaria</td>
</tr>
<tr>
<td>Straw</td>
<td>Forest</td>
<td>2</td>
<td>Agaricales</td>
<td>Galerina</td>
</tr>
<tr>
<td>Straw</td>
<td>Forest</td>
<td>2</td>
<td>Russulales</td>
<td>Hericium</td>
</tr>
<tr>
<td>Straw</td>
<td>Grassland</td>
<td>2</td>
<td>Pucciniomycotina</td>
<td></td>
</tr>
<tr>
<td>Straw</td>
<td>Grassland</td>
<td>2</td>
<td>Hymenochaetales</td>
<td>Schizopora</td>
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<td>Wood</td>
<td>Grassland</td>
<td>2</td>
<td>Agaricales</td>
<td>Clitopilus/Omphalina</td>
</tr>
<tr>
<td>Wood</td>
<td>Grassland</td>
<td>2</td>
<td>Agaricales</td>
<td>Clitopilus/Omphalina</td>
</tr>
<tr>
<td>Wood</td>
<td>Grassland</td>
<td>2</td>
<td>Corticiales</td>
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<tr>
<td>Treatment</td>
<td># of Phylotypes</td>
<td>Treatment</td>
<td># of Phylotypes</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>----------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>1st time point:</td>
<td></td>
<td>2nd time point:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forest Block</td>
<td></td>
<td>Forest Block</td>
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<tr>
<td>Straw substrate</td>
<td>14.2</td>
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<td>Mean</td>
<td>4.2</td>
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<td>Grassland Block</td>
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<td>Grassland Block</td>
<td></td>
<td></td>
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<tr>
<td>Wood substrate</td>
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<td>Wood substrate</td>
<td>3.7</td>
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<tr>
<td>Mean</td>
<td>22.2</td>
<td>Mean</td>
<td>3.2</td>
<td></td>
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</tbody>
</table>

**Table 2.** Mean TRFLP basidiomycete specific ITS phylotypes per treatment for *HaeIII* restriction enzyme with the primer pair ITS1F/4B (basidiomycete specific).
Table 3. Taxonomic composition of saprotrophic fungi Operational Taxonomic Units (OTUs) were determined at 97% sequence similarity for the LSU gene region from cloning and sequencing. 2 indicates samples harvested at the second time point (after 18 months).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>OTUs</th>
<th>OTUs(_{est})</th>
<th>Shannon</th>
<th>Simpson</th>
<th>PD</th>
<th>Fisher's alpha</th>
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<tbody>
<tr>
<td>Straw in forest 1st TP</td>
<td>38.60</td>
<td>56.38</td>
<td>3.08</td>
<td>0.76</td>
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<tr>
<td>Straw in forest 2nd TP</td>
<td>21.40</td>
<td>33.60</td>
<td>1.85</td>
<td>0.48</td>
<td>2.70</td>
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<td>30.58</td>
<td>2.25</td>
<td>0.66</td>
<td>2.58</td>
<td>4.18</td>
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<tr>
<td>Straw in grassland 2nd TP</td>
<td>18.00</td>
<td>23.00</td>
<td>1.34</td>
<td>0.33</td>
<td>3.02</td>
<td>3.50</td>
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<tr>
<td>Wood in forest 1st TP</td>
<td>45.88</td>
<td>100.61</td>
<td>3.46</td>
<td>0.81</td>
<td>5.12</td>
<td>11.76</td>
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<tr>
<td>Wood in forest 2nd TP</td>
<td>46.90</td>
<td>67.84</td>
<td>3.52</td>
<td>0.82</td>
<td>4.50</td>
<td>12.00</td>
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<td>Wood in grassland 1st TP</td>
<td>59.55</td>
<td>101.88</td>
<td>4.18</td>
<td>0.89</td>
<td>5.66</td>
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<tr>
<td>Wood in grassland 2nd TP</td>
<td>48.84</td>
<td>88.39</td>
<td>3.80</td>
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<td>5.45</td>
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<td>Grassland total</td>
<td>43.79</td>
<td>75.03</td>
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<td>3.40</td>
<td>11.44</td>
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<td>Forest total</td>
<td>39.52</td>
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<td>3.07</td>
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<tr>
<td>Straw total</td>
<td>29.34</td>
<td>46.25</td>
<td>2.46</td>
<td>0.64</td>
<td>2.55</td>
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<td>Wood total</td>
<td>49.36</td>
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<td>Soil total</td>
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<td>0.77</td>
<td>3.21</td>
<td>10.38</td>
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**Table 4.** Summary of alpha diversity indices for the LSU D1 gene region. The totals were calculated from the substrate-only rarified pyrosequencing dataset (except for the soil alpha diversity indices; they were calculated from a dataset containing both the soil and substrate samples). All samples were rarified. Estimated OTU richness based on Chao 1 estimator.
Table 5. Results of the UniFrac significance test (100 monte carlo randomizations) with OTUs grouped by plot type (substrate/ecosystem and time). * Indicates significant difference between samples ($p<0.01$). Labels: S – straw; W – wood; F – forest; G – grassland; 1 – 1st time point; 2 – 2nd time point.

<table>
<thead>
<tr>
<th></th>
<th>SF1</th>
<th>SF2</th>
<th>SG1</th>
<th>SG2</th>
<th>WF1</th>
<th>WF2</th>
<th>WG1</th>
<th>WG2</th>
</tr>
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<tbody>
<tr>
<td>SF1</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
<td>SG1</td>
<td>0.01*</td>
<td>0.57</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
<td>SG2</td>
<td>0.35</td>
<td>0.88</td>
<td>0.97</td>
<td>--</td>
<td>--</td>
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<td>WF1</td>
<td>$p&lt;0.01*$</td>
<td>0.12</td>
<td>0.10</td>
<td>0.91</td>
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<tr>
<td>WF2</td>
<td>$p&lt;0.01*$</td>
<td>0.46</td>
<td>0.25</td>
<td>0.97</td>
<td>$p&lt;0.01*$</td>
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<tr>
<td>WG1</td>
<td>$p&lt;0.01*$</td>
<td>0.01*</td>
<td>0.22</td>
<td>0.44</td>
<td>$p&lt;0.01*$</td>
<td>$p&lt;0.01*$</td>
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<tr>
<td>WG2</td>
<td>$p&lt;0.01*$</td>
<td>0.29</td>
<td>0.16</td>
<td>0.60</td>
<td>$p&lt;0.01*$</td>
<td>$p&lt;0.01*$</td>
<td>$p&lt;0.01*$</td>
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</tr>
</tbody>
</table>
Figure 1. Schematic of the experimental design including a picture of the straw and wood baits.
**Figure 2.** NMDS of TRFLP basidiomycete specific ITS phylotypes. Green – first time point; Blue – second time point.
Figure 3. Rank abundance curves of OTUs determined at 97% for the LSU gene region based on cloning and sequencing. A. Ecosystem comparison; B. Substrate comparison; C. Time point comparison.
B.

![Rank abundance curve - Wood substrate](image)

![Rank abundance curve - Straw substrate](image)
Figure 4. Comparison of LSU OTUs determined at different sequence similarities. Black – LSU substrate and soil; Blue – LSU D1 extracted, substrate and soil; Grey – LSU D1 substrate only. Arrow indicates OTUs sequence similarity chosen to represent OTUs.
Figure 5. Rarefaction curves of substrates. Red – grass; orange – wood; blue – soil.
Figure 6. Rarefaction curves of substrates (soil, straw or wood) by ecosystem type (grassland or forest). Red – forest soil; Green – grassland soil; yellow – wood substrates in grassland; purple – wood substrates in forest; blue – straw substrates in forest; orange – straw substrates in grassland.
Figure 7. NMDS of straw, wood and soil substrates using the Jaccard index (stress 16.90). The blue circles highlight the soil samples. F – Forest soils; G – Grassland soils. The green circle highlights the grassland samples and the brown circle highlights the forest samples. Labels: 1st letter: W – wood; G – straw; 2nd letter: F – forest; G – grassland; 1 – first time point; 2 – second time point.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cellulose</th>
<th>Lignin (with ash)</th>
<th>Hemi-cellulose</th>
<th>C (total)</th>
<th>N (total)</th>
<th>P (total)</th>
<th>K (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straw</td>
<td>41.1</td>
<td>8.7</td>
<td>30.8</td>
<td>42.2</td>
<td>0.40</td>
<td>0.02</td>
<td>2.11</td>
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<tr>
<td>Native grass</td>
<td>32.2</td>
<td>4.3</td>
<td>27.9</td>
<td>42.2</td>
<td>0.81</td>
<td>0.13</td>
<td>1.43</td>
</tr>
<tr>
<td>Conifer needles</td>
<td>11.7</td>
<td>10.9</td>
<td>14.1</td>
<td>48.9</td>
<td>2.15</td>
<td>0.26</td>
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<tr>
<td>Oak leaves</td>
<td>27.3</td>
<td>15.4</td>
<td>14.6</td>
<td>14.6</td>
<td>1.05</td>
<td>0.06</td>
<td>0.30</td>
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<tr>
<td>Wood blocks</td>
<td>49.3</td>
<td>24.7</td>
<td>*</td>
<td>47.2</td>
<td>0.10</td>
<td>&lt;0.010</td>
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**Supplemental Table 1.** Substrate analysis compared substrates with natural substrates found on the plots. * indicates hemicellulose was not analyzed from the wood blocks.
### Supplemental Table 2
Percentage of mass loss of buried substrates. Straw substrates in the Forest ecosystem had the highest percent mass loss.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ecosystem</th>
<th>Time point</th>
<th>% mass loss</th>
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<tbody>
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<td>Straw</td>
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<td>1</td>
<td>76%</td>
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<td>74%</td>
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<td>13.7%</td>
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<tr>
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<td>21.27</td>
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<td>15.1%</td>
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</tbody>
</table>

**Supplemental Table 3.** Soil analysis from all four blocks. OM – Organic Matter; ENR – Estimated Nitrogen Release; VH – Very High; H – High; M – Medium; L – Low; VL – Very Low.
CHAPTER 3

Comparison of basidiomycete functional diversity to species diversity reveals both are influenced by plant community type
Abstract

In this study, the diversity of basidiomycete laccase genes was assessed in grassland and forest plant community types and on straw and wood substrates. Straw and wood baits (n=160) were buried and retrieved at two different time points to select for saprotrophic fungi. We extracted DNA from the substrates and used fungal-specific amplification and a 454 pyrosequencing approach to sequence the basidiomycete laccase gene (Cu1F/Cu2R) and the nuclear ribosomal large subunit (LSU) D1 region. The wood substrates had significantly higher richness of estimated OTUs (Chao 1) (p ≤ 0.05) as compared to the straw substrates. Laccase gene diversity was compared to basidiomycete diversity. NMDS ordination shows that both the laccase genes and basidiomycete assemblages associated strongly with plant community type.
Introduction

Fungi are able to decompose lignin and cellulose by releasing extracellular enzymes that degrade these polymers. White rot fungi in particular produce several types of enzymes, including lignin peroxidases, manganese peroxidases, and laccases. These enzymes allow the fungus to break down and utilize organic substrates as an energy and nutrient source (Osono 2007). Lignin is a large, complex, aromatic polymer made up of phenylpropane-based monomers linked via a variety of bonds that bind cell-wall components together (Osono 2007). Lignin provides strength and support to plant cells, bonds cellulose fibers, and makes the limited nitrogen in wood less available. Lignin can make up 20 – 35% of wood, while cellulose can make up 40 – 50% of wood, and hemicellulose can make up 25 – 40% of wood (Pointing et al. 2003).

In comparison to wood decay fungi, there has been less attention focused on litter-decomposers, which are also capable of producing extracellular enzymes, such as laccases and manganese peroxidases. However, litter-decomposers vary in their ability to decompose lignin in leaf litter. Basidiomycete genera such as Clitocybe, Collybia, Marasmius, and Mycena have been studied for their bleaching activity and enzyme production (Osono 2007). Bleaching of leaf surfaces and humus is correlated with ligninolytic activity of fungi in that lignin content has been found to be lower in both bleached leaf surfaces and humus as compared to non-bleached surfaces.

Laccase is a phenol-oxidizing enzyme that catalyzes the reduction of oxygen to water (Thurston 1994; Baldrian & Snajdr 2006). Fungal laccases appear to be involved in a variety of physiological functions, including fruit body development, detoxification of phenolic compounds, pigment production, and antimicrobial activity (Levin et al. 2002; Thurston 1994). In addition, fungal laccases also appear to have roles in stress defense and fungal plant-pathogen/host interaction (Thurston 1994; Crowe & Olsson 2001; Baldrian & Snajdr 2006). Laccases play an important role in soil organic matter (SOM) turnover and are able to completely degrade lignin (Eggert et al. 1997).

Laccases are a multigene family, and some fungi, such as Coprinopsis cinera, have as many as 17 different laccase genes (Kilaru et al. 2006). Laccases have been found in almost all wood decay fungi; however, Phanerochaete chrysosporium, a well studied white rot basidiomycete, does not produce laccases, and likely breaks down lignin using a variety of peroxidases (Martinez et al. 2004; Larrondo et al. 2003; Hoegger et al. 2006).

While there have been few studies linking the relationship between saprotrophic diversity to functional diversity, such relationships are of great interest. Understanding soil enzyme functional diversity could significantly increase our understanding of the linkages between resource availability, microbial community structure and function, and ecosystem processes (Caldwell 2005). Previous studies using molecular approaches have looked at the diversity and distribution of laccase genes from basidiomycetes, which are involved in lignin degradation. Luis et al. (2004) first described the Cu1F/Cu2R basidiomycete specific laccase primer pair and identified a number of laccase genes from mycelial cultures and fruit-bodies.
They also demonstrated that saprotrophic fungi have a greater diversity of laccase genes as compared to mycorrhizal fungi. In a follow-up study, they found that soil fungi with laccase genes occupied different niches and showed a vertical distribution in the soil profile with the greatest number of laccase genes found in the upper horizons (Luis et al. 2005a). In nitrogen amended soils, suppression of laccase gene expression was observed (Edwards et al. 2011). Christ et al (2011) reported that laccase-producing fungi differed between soil and stone compartments.

The aim of this study was to compare the functional diversity of saprotrophic fungi on straw and wood substrates across forest and grassland plant community type and compare laccase diversity with the saprotrophic fungal taxonomic diversity. Functional diversity was assessed by the functional gene encoding laccase and saprotrophic fungal diversity was assessed by the nuclear ribosomal large subunit (LSU) D1 region.

**Materials and Methods**

**Study site and experimental design**

This study was conducted along Bolinas ridge on Mount Tamalpais in the Marin Municipal Water District watershed, in Marin County, California, USA (N37° 55.35', W122° 38.11'). Four 10 x 10 m blocks, each with 10 randomly assigned 2 x 2 m plots were established (Chapter 2, Figure 1). Two blocks were in the coastal grassland, characterized as northern coastal grassland and dominated by Festuca and Danthonia, along the forest edge, and two blocks were in the adjacent forest dominated by Pseudotsuga menziesii with an understorey dominated by Notolithocarpus densiflorus and Quercus agrifolia. One grassland block was paired with a forest block. The distance between the two paired blocks was roughly 30 meters. A second set of the paired blocks (one grassland block and one forest block 30 meters apart) was established over 300 meters apart.

The region is characterized as a Mediterranean climate with a seasonal summer drought and average annual precipitation of 1250 mm (Dunne & Parker 1999) (see Kennedy et al. 2003 for further site characteristics). Sterile bait bags (5 x 5 cm mesh bags filled with 0.07 g of sterile wheat straw with a nylon string attached) and small sterilized conifer wood blocks (5 x 5 cm, average 4 g), also with a nylon string attached (Figure 1), were placed in the grassland and forested plots, with two bait bags per plot (n=80) and two wood blocks per plot (n=80), each at a depth of <10 cm. Ten bait bags and ten wood blocks were removed from each block at two different time points. In February 2007, after six months, one-half of the bait bags (n=40) and one-half of the wood blocks (n=40) were removed and collected from each plot. The remaining bait bags (n=40) and wood blocks (n=40) were removed and collected in February 2008 after being buried for 18 months. In all cases, the bait bags and the wood blocks were collected near the end of the rainy season. The samples were placed on ice and processed immediately upon returning to the lab. This method of using baits selects for active saprotrophic fungi that are capable of digesting cellulose, as opposed to mycorrhizal or pathogenic fungi, which are also present in the soils. A substrate analysis (performed by University of California, Department of Agriculture and Natural Resources Lab) compared
the similarity of cellulose, lignin, carbon (C), nitrogen (N), phosphorus (P), and potassium (K) contents among the prepared substrates (in this case, the straw and wood blocks) as compared to the natural substrates (i.e. conifer needles, oak leaves, and native grass) collected in the blocks (Chapter 2, Supplemental Table 1). Percentage of mass loss of the buried substrates over 6 and 18 months was measured by dry weight and reported in Chapter 2, Supplemental Table 2. Substrates were placed in coin envelopes and dried in a 37°C oven for two days. Soil moisture, pH, N, P, K, and C:N ratios were measured in every block and are reported in Chapter 2, Supplemental Table 3. Soil cores of 5 x 5 x 5 cm were collected from 5 randomly assigned plots within each block (n=20) in early April 2009.

DNA extraction
The straw and wood substrates were pulverized for 30 seconds with a 3 mm glass bead beater (Biospec) and suspended in 1000 µl CTAB/PVPP buffer (2% CTAB, 1% PVPP, 0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA). After a 60 minute incubation at 65°C, DNA was extracted from the straw and wood substrate samples (n=160) using a chloroform:isoamyl alcohol extraction method (Kennedy et al. 2003), and extracts were purified using the Qiagen DNeasy Tissue Kit (Valencia, CA).

454 Pyrosequencing
PCR Amplification – A subset of the samples were chosen for pyrosequencing due to a limit in the number of barcodes available at the time. Thirty-two samples from the first time point (16 straw and 16 wood substrate samples) with equal representation from the grassland and forest plant community types were sequenced. An equal number of samples from the second time point were also sequenced from the same plots. Nineteen soil samples (9 grassland and 10 forest samples) were also sequenced, resulting in a total of 83 samples. The laccase gene region was PCR amplified with the basidiomycete specific laccase degenerate primer pair Cu1F [5’-CAT(C) TGG CAT(C) GGN TT(C) TTT(C) CA-3’] and Cu2R [5’-G G(A)CT GTG GTA CCA GAA NGT NCC-3’] (P Luis et al. 2004) with 10 bp MID barcodes. Each sample (n=83) was PCR amplified with a unique barcode. The forward primers included the “A” pyrosequencing adaptor along with the 10 bp multiplex tag and forward gene (5’-A + 10 bp multiplex tag + forward gene primer-3’), and the reverse primers included the “B” pyrosequencing adaptor along with the reverse gene primer (5’-B + reverse gene primer-3’) for the laccase gene region. Each PCR consisted of 1.2 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, 0.5 µM of each primer, and H₂O to a final concentration of 25 µL. Thermocycling in an Eppendorf Mastercycler Gradient thermocycler was carried out under the following conditions: initial denature for 10 min at 95°C; 34 cycles of 1 min at 95°C, 1 min at 51°C, and 1 min at 72°C; and final extension for 7 min at 72°C. PCR products were purified with the Qiaqen QIAquick 96 PCR Purification Kit (QIAGEN, Valencia, CA) following the manufacturer’s instructions. Purified PCR products were quantified with the Invitrogen Qubit Fluorometer (Invitrogen, Carlsbad, CA) and pooled in equimolar concentrations by locus, and then sequenced on 1/8 of a plate on a 454 Titanium sequencing run (454 Life Sciences/Roche Applied Biosystems, NJ, USA). Samples were sequenced at the Duke Institute for Genome Sciences and Policy.
Sequence processing and analyses – Sequencing 1/8 of a plate resulted in a total of 79,786 laccase gene sequences. Reads were trimmed using the Qiime software, which allows for analysis of high-throughput community sequencing data (Caporaso et al. 2010), with a minimum of 120 bp (and maximum 240 bp), and were filtered with the following default parameters in Qiime: 0 number of allowable Ns; homopolymers maximum length 6; and an average Q score of at least 25. All Qiime commands were executed using MacQIIME version 1.2.1-20110224a. Within the program Qiime, laccase OFUs (Operational Functional Units) were determined at 77% sequence similarity (Blackwood et al. 2007) and the LSU (D1) OTUs were determined at 95% sequence similarity (Chapter 2) using the UCLUST (Edgar 2010) clustering method. OFU determination at 77% was based on an approach by Blackwood et al. (2007) where they determined that closely related fungal laccase sequences consistently clustered together at 77% sequence similarity. Singletons (OFUs represented by a single sequence) and doubletons (OFUs represented by only two sequences) were removed from the laccase dataset (Tedersoo et al. 2010), and only samples with 300 laccase gene sequence reads were included in analyses. Representative laccase OTUs (the longest sequence in each cluster) were deposited in GenBank (Accession number XX-YY). The basidiomycete LSU OTUs were Blasted against NCBI GenBank using Blastn. 147 laccase or laccase-like multicopper oxidase (LMCO) sequences with an associated taxon id were downloaded from GenBank and aligned with MAFFT (version 6) (Katoh et al. 2009). This served as the core alignment. Representative laccase OTUs (the longest sequence in each cluster) from this study were aligned with the core alignment. The resulting multiple alignment was optimized visually using the online MAFFT tool, Jalview, and laccase OFUs that did not properly align or were too short were deleted and not included in further analyses. The coding regions were deduced after alignment with known laccases (and primarily with *Trametes versicolor*, Accession No. L78077) and introns were deleted for the phylogenetic analysis (Luis et al. 2004). Nucleotide sequences were translated to amino acids to confirm that OTUs determined at 77% sequence similarity did not encode identical amino acid sequences. A neighbor-joining (NJ) tree was conducted using the MAFFT online tools and a maximum likelihood tree was conducted using PhyML 3.0 (Guindon & Gascuel 2003) on the University of Oslo Biportal. Three ascomycete laccase sequences were used as the out-group to root the trees (*Colletotrichum lagenarium* (GenGank Accession NO. AB055709), *Gaeumannomyces graminis* (GenBank Accession No. AF243855) and *Botryotinia fuckeliana* (GenBank Accession No. AF243855). Alpha diversity indices were computed on rarified samples in Qiime. ANOVAs were computed in the R programming environment (R Core Development Team, RDC 2005), and NMDS ordinations were calculated using the Vegan package (Dixon 2003) in R.

The LSU D1 extracted sequences, non-fungal taxa and singletons removed, and the processed sequences were aligned using the PyNAST (a python implementation of the NAST alignment algorithm) in Qiime using LSU sequences (from James et al. 2006) as the core alignment template (James et al. 2006). FastTree (Price et al. 2009) was used as the tree building method in Qiime.
Results

A total of 34,021 laccase sequences (average length 192 bases, range 120 – 240 bp) passed the default Qiime quality control steps. 45,765 sequences were removed due to reads that were too short or contained ambiguous bases, low mean quality score or long homopolymer runs. Thirty-five of the 83 barcodes resulted in very low or 0 sequence reads and the associated samples were dropped.

A species accumulation curve of the observed laccase gene OFUs (77%) is close to leveling-off, and the estimated (Chao 1) laccase gene OFUs reached an asymptote for the substrate communities and soil communities, indicating these communities were sufficiently sampled (Figure 1).

The laccase data was parsed into two datasets. One dataset included the substrates (straw and wood), and the second dataset included both the soil samples and the substrate samples. For the substrate (straw and wood) and soil samples combined, a 77% laccase gene sequence similarity threshold revealed 1,612 OFUs. After singletons and doubletons were removed, 523 laccase gene OTUs remained. 123 OFUs were specific only to the soil and not found on the substrates.

Observed and estimated richness (Chao 1) of the total study area (laccase gene) by plot type are presented, along with basidiomycete richness (portion of potentially laccase-producing fungi), in Table 1. The wood samples had significantly higher richness of estimated OFUs (Chao 1) \((p \leq 0.05)\) and observed species as compared to the straw substrates. The wood samples in the forest had the highest richness of the substrate combinations. However, the soil samples had significantly greater richness \((p \leq 0.05)\) for both observed and estimated OFUs as compared to both the straw and wood samples. The forest soil samples had the highest richness. Also, the diversity indices for each time point were fairly similar to each other.

The number of unique laccase gene OFUs, at 77% (presence/absence) for the substrate data, ranged from 3 to 59 per sample (up to 80 in the soil samples). The number of unique basidiomycete LSU OTUs, at 95% (presence/absence) for the substrate data, ranged from 1 to 23 per sample (up to 61 in the soil samples). Different laccase gene OFU distribution patterns were found on the substrates and soil samples. The 10 most dominant (present in greater than 10 plots) basidiomycetes are presented in Table 2. NMDS plots, using the Jaccard index, show that both the laccase genes and basidiomycete assemblages associated strongly with plant community type (forest or grassland) (Figure 2 and 3, respectively). A PCoA plot of the laccase gene OFUs shows that the samples in the forest had greater variability as compared to laccase OFUs from samples in the grassland, and shows that the laccase OFUs on wood had greater variability as compared to OFUs on both the straw substrate and soil substrate. The soil laccase samples clumped closely together, indicating the assemblages were very similar, as compared to both the straw and wood substrates (data not shown). A PCoA plot of the basidiomycete assemblages also shows a slight separation by time point. There was a greater
variability of the basidiomycete OTUs from the first time point, as compared with basidiomycete OTUs from the second time point.

A maximum likelihood tree of the fungal laccases from this study along with the laccase sequences from GenBank is presented in Supplemental Figure 1. Laccase sequence clades that were genus or family specific were Boletaceae, Psathyrella, Coprinus, Trametes, Mycena, Russula, and Ramaria, and the largest genus specific clades were Mycena, Russula, and Ramaria. Overall, there was limited congruence between laccase gene diversity and fungal diversity.

Discussion

In this study, saprotrophic fungal functional diversity was analyzed using a pyrosequencing approach of the laccase gene as a functional marker. Functional diversity of saprotrophic fungi was also compared to saprotrophic fungal diversity using LSU rDNA. The use of two different genetic markers provided two distinct representations of saprotrophic fungi and provided a complementary view of saprotrophic fungal diversity and function.

Chapter 2 described the total richness and diversity of saprotrophic fungi on two different substrates (straw and wood) from two plant community types (forest and grassland), collected at two different time points. In this study, the same samples were analyzed; however, only basidiomycete fungi were included in the fungal diversity analysis in order to better compare the potentially laccase-producing fungi with the basidiomycete specific functional marker (laccase). In addition, soil samples were analyzed in a second dataset with the substrates in order to compare the diversity colonizing the substrates with the diversity in the surrounding soil.

Overall, both the laccase OFUs and basidiomycete assemblages associated strongly with plant community type (forest or grassland). The wood samples had significantly higher richness of estimated laccase OFUs (Chao 1) \( p \leq 0.05 \) as compared to the laccase OFUs on straw substrates. The laccase OTUs and basidiomycete assemblage from the soil samples also grouped strongly by plant community type, and within plant community type the soil samples grouped closely together.

This is the first known attempt of pyrosequencing the laccase gene with basidiomycete specific degenerate laccase primers. Laccase sequences were confirmed by aligning with a core alignment of laccase sequences downloaded from GenBank. The nucleotide sequences were translated to amino acids to confirm that OFUs determined at 77% sequence similarity did not encode identical amino acid sequences. OFU determination at 77% was based on an approach by Blackwood et al. (2007) where they determined that closely related fungal laccase sequences consistently clustered together at 77% sequence similarity.
Since laccases are involved in a number of processes (both intra- and extracellular) including lignin and polyphenol degradation, nutrient acquisition and developmental processes (Luis et al. 2004; Hoegger et al. 2006), the study here is not specific to just functional diversity described as lignin degradation and SOM cycling. In addition, most fungal taxa have multiple laccase genes, and therefore, there is a limited congruence between laccase gene diversity and fungal diversity. However, Luis et al. (2004) found clades of laccase sequences in their NJ phylogenetic tree that were congruent with fungal diversity at the genera or family level. However, they were not able to always place amplified laccase sequences of unknown origins to specific taxa. This was observed in this study as well. Another possible limitations of using laccase genes as a functional marker is that there is still a need to link genetic potential to expression in the environment (Luis et al. 2005b; Theuerl & François Buscot 2010).

Despite the limitations of using laccase genes as a functional marker, laccases are key players in lignin and polyphenol degradation, and within soil and decaying substrates it is anticipated that the basidiomycete laccases are primarily involved in lignin degradation (Blackwood et al. 2007). Understanding the link between taxonomic diversity and functional diversity remains an important question and goal towards a greater understanding of ecosystem processes and function.
Literature Cited


Table 1. Summary of alpha diversity indices for the laccase gene region and basidiomycete fungi (LSU D1). All samples were rarified to equal sample size, 600 and 300 sequences for LSU and Laccase respectively. Estimated OTU richness based on Chao 1 estimator. The wood samples (in forest?) had significantly higher richness of estimated laccase OTUs (Chao 1) (p ≤ 0.05) and observed OTU richness as compared to the straw substrates.

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<th>Plot</th>
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<th>LSU Est. OTUs</th>
<th>Laccase gene Obs. OTUs</th>
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**Table 2.** List of the 10 most dominant (present in greater than 10 plots) basidiomycete fungi across all the plots.
Figure 1. Rarefaction curves of observed laccase gene OTUs (77% similarity) by substrate type. Estimated (Chao1) laccase gene OTU curves reach an asymptote. Blue – soil; orange – wood; red – grass.
Figure 2. NMDS of the laccase gene OTU distribution by ecosystem type using the Jaccard index. The green circle highlights the grassland samples and the brown circle highlights the forest samples.
Figure 3. NMDS of basidiomycete fungi (LSU D1) using the Jaccard index. The green circle highlights the grassland samples and the brown circle highlights the forest samples.
Supplemental Figure 1. Maximum likelihood tree of the laccase gene OTUs and laccase sequences from GenBank. The blue clades represent sequences that are genus specific.