2010

The Fungal Connection: Characterizing the Ectomycorrhizal Community and Belowground Response to Restoration Treatments in Northern Mississippi.

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The Fungal Connection: Characterizing the Ectomycorrhizal Community and Belowground Response to Restoration Treatments in Northern Mississippi.

A Thesis

Presented for the
Master of Science
Degree
The University of Mississippi

Ashley “Anjel” Craig
December 2010

ABSTRACT

Ectomycorrhizal (ECM) fungi are symbionts on the roots of woody plant species throughout the world. These fungi provide plants with nutrients and are important drivers of ecosystem processes. ECM fungi vary in their effect on host plants and host-specificity, making them important considerations in restoration projects seeking to restore target tree species. Restoration strategies such as burning and thinning may have strong impacts on ECM fungi, and given the that ECM are important in structuring aboveground communities and maintaining certain dominant plant taxa, knowledge of ECM fungal response is needed to ensure restoration efforts succeed. Using molecular methods, this research aimed to identify the ECM fungal community in a restoration project in northern Mississippi, comparing the belowground fungal community on plant roots between replicated control and treatment plots. We also measured abiotic factors that may structure the ECM fungal community, including litter depth, canopy openness, burn regimen, and soil compaction. Results indicate that the ECM fungal community is very diverse with 175 operational taxon units recovered from sequence data, 106 OTUs only found once. The fungal species had high site fidelity, with site being the factor explaining the most variation in community structure. Taxa in the family Russulaceae represented the most abundant fungi found on roots, followed by Thelephoraceae. The abiotic factors measured accounted for only 10% of the variation in community structure, indicating that other unmeasured variables may account for the remaining variation in ECM community distribution. Spatial autocorrelation was found at one of the six plots, indicating similar ECM fungal species composition at scales greater than in the other 5

sites. This plot also had the greatest canopy openness and oak regeneration, suggesting that this greater spatial autocorrelation could be related to oak seedling facilitation. The restoration treatments did not have a strong impact on fungal community structure except in the Tallahatchie plots, where there was a strong difference between treatment and control plots. This study was the first assessment of belowground ECM fungal diversity in Mississippi, and will serve as a starting point for further investigation into shifts in the fungal community as a result of restoration.

DEDICATION

This work is dedicated to my husband, Paul “Puppy” Tate, whose support, encouragement and cooking helped keep my soul and stomach fed through the duration of this degree.

ACKNOWLEDGMENTS

I would like to firstly thank my adviser, Dr. Jason Hoeksema, for his patience, benevolence and encouragement through this process in helping me to be his first graduate student to graduate. I would also like to thank Dr. Tiffany Bensen for her "relentless eye for detail" that helped me produce many good drafts for this thesis and grants. My thanks also go out to my labmates, Bridget Piculell and Kris Hennig, who suffered through many hours of practice presentations and reading drafts. I also want to thank the members of my committee, Dr. Tamar Goulet and Dr. Steve Brewer, both of whom taught me a great deal about ecology, being a grad student, and life in general. I would also like to thank the other USDA fellows with whom I shared many hours, a cross-continent internship, and the pain and rewarding completion of this degree: Erynn Maynard, Jason Ryndock, and Anthony Rietl. In addition I want to thank the members of the British Columbia Ministry of Forests & Range, the Arkansas Game and Fish Commission service, the Mississippi Department of Wildlife, Fisheries and Parks, and the USDA Forest Service for enriching my degree with their expertise and time spent teaching me about restoration. Finally I would like to thank the USDA for providing me the opportunity to pursue my degree and for funding my living expenses throughout.

TABLE OF CONTENTS

LIST OF TABLES

FIGURE PAGE

LIST OF FIGURES

INTRODUCTION

Restoration ecology is the applied science of restoring degraded, damaged or destroyed environments in an effort to revive ecosystem integrity and processes (SER 2004). Methods and policies developed to help restore ecosystems are expected to find intensive application in the 21st century as more research is geared towards repairing anthropogenically induced environmental damage. Due to the fluidity of natural systems, the ultimate goal of restoration ecology is not simply to return an ecosystem to a prior state of biological history, but to restore natural key processes, functions and biodiversity (Primack 2002). A growing body of research on how to best implement restoration treatments and gauge success of restorative efforts suggests that a holistic approach is essential to monitoring total ecosystem response to restoration (Naveh 1994, Cabin 2007, Giai and Boerner 2007). A holistic approach includes all ecological components and considers constituents that may not be readily perceived such as fungi and bacteria. Such an approach assures that a full assessment of ecosystem health can be achieved. Furthermore, failing to take into account all effects from ecological restoration, including belowground and microscopic ones, can often lead to pitfalls and disappointing results in attempts to restore environments (Perry *et al.* 1989, Pickett and Parker 1994, Cairns and Heckman 1996). In the research reported here, I used molecular methods to identify the important belowground symbionts of trees: ectomycorrhizal fungi associated with tree hosts located in experimental treated restoration and unmanipulated plots in a research site in northern Mississippi.

An important component often overlooked in ecosystems is the contribution of

mycorrhizal fungi to several key ecosystem processes. Mycorrhizal fungi live symbiotically on plant roots beneath the soil and are prolific throughout the world in most major biomes and plant communities (Smith and Read 2008). In many temperate forests, the dominant type of mycorrhizal fungi is ectomycorrhizal (ECM) fungi, which grow in sheathing mycelial networks surrounding the roots of many different tree species, including those of economically important *Quercus* and *Pinus* species (Smith and Read 2008). Mycorrhizae have been shown to play key roles in maintaining soil structure (Perry *et al.* 1989), plant successional process and assemblages (van der Heijden *et al.* 1998b, Koide and Dickie 2002), and nutrient cycling (Treseder and Allen 2000, Treseder 2004, Smith and Read 2008) as well as directly affecting plant growth (Karst *et al.* 2008). Furthermore, different mycorrhizal taxa at varying successional stages contribute differently to these ecological processes and range from beneficial to parasitic for their plant hosts (Molina *et al.* 1992, Johhnson *et al.* 1997, Bruns *et al.* 2002b). Therefore, due to their instrumental importance in structuring forest ecosystems and controlling ecological processes, mycorrhizae are an important consideration in any study seeking to restore the environment.

Frequent fires ignited naturally or by Native Americans historically regulated much of the processes and successional patterns in ecosystems throughout North America and especially in the southeastern United States. As a result of the removal of fire, there has been extensive overgrowth and expansion of lowland fire-intolerant species moving into upland, fire-maintained forests, shading out the native oaks (Brewer 2001). Today there remains less than 1 % of the former estimated 13-11 million acres of open oak

woodlands in the Eastern US (Nowacki and Abrams 2008). These ecosystems are in danger of disappearing completely due to the accumulated leaf litter of lowland species, which prevents fire from carrying into the understory and prohibits understory bunch grasses and younger oaks from gaining any foothold in the environment (Nowacki and Abrams 2008). With the loss of this plant community, we stand to lose not only important habitat for quail and other shrub birds, but essential environmental heterogeneity that maintains biodiversity throughout the southeastern United States. Fire disturbances acts as an environmental filter, selecting and maintaining plant species that have fire-adapted traits in areas of high fire frequency (Verdu and Pausas 2007). The ECM fungal species with fire adapted traits potentially evolved with open oak woodland plant assemblages as both these belowground and aboveground communities were regulated by fire. The ECM fungal community belowground which would have been maintained by fire may be an important component to restoring these endangered systems, specifically in maintaining the dominance of obligately mycorrhizal oaks and facilitating oak seedlings (Dickie *et al.* 2002). After the removal of this important disturbance regime from the system, both aboveground and belowground communities may have shifted towards those dominated by more weedy, fire-intolerant species, and the mycorrhizal community may have become less specific towards *Quercus* hosts. Reintroducing fire cycles to the ecosystem would be predicted to restore historical fungal communities that evolved in association with fire-mediated open oak woodlands, creating a belowground- aboveground feedback between ECM fungi and their tree hosts that will encourage the maintenance and persistence of these ecosystems (Heneghan *et al.* 2008).

Two common techniques often used in combination to restore fire-tolerant plant communities to historical assemblages are prescribed burning and thinning of overstory and/or midstory trees. Nearly a century of fire suppression policy has necessitated the use of prescribed burning to restore environments to previous historical assemblages and promote the stability and maintenance of fire dominated ecosystems (Neary *et al.* 1999, Fernandes and Botelho 2003). Indeed, prescribed burning represents the majority of restoration work being implemented throughout many parts of the world. Thinning is another common restoration technique that employs selective removal of invasive or unwanted plant species in order to encourage a particular plant assemblage. Thinning also opens up the canopy to allow regeneration of fire adapted species that would otherwise not re-establish due to the overgrowth of non-fire adapted species. In the research proposed here, burning and thinning techniques are being used to attempt restoration of a northern Mississippi mixed upland forest to a historical open oak woodlands ecosystem that proliferated in this area during the 1800's, prior to the advent of fire suppression polices (Brewer 2001). Burning treatments are designed to encourage the growth of fire-tolerant understory and canopy plant species, including grasses and oak (*Quercus*) species. The thinning treatments are targeted for the removal of invading fire intolerant lowland species, especially sweet gum (*Liquidambar styraciflua*).

Fire changes ECM fungal species composition to varying degrees (Buchholz and Gallagher 1982, Visser 1995, Baar *et al.* 1999, Jonsson *et al.* 1999b, Stendell *et al.* 1999, Grogan *et al.* 2000). ECM fungi occupy mostly the organic layers of the soil (Smith and Read 2008) which makes them susceptible to fire due to the elevated temperatures near

the surface during the burn. The initial response of the community depends on the severity of the burn (Neary *et al.* 1999), with low intensity burns tending to show little difference in species composition post burn (Jonsson *et al.* 1999b, Bastias *et al.* 2006). In contrast, high intensity blazes may kill off the entire soil biota and initiate successional changes in ECM community structure (Visser 1995, Torres and Honrubia 1997, Horton *et al.* 1998). Fungi have lower heat tolerance than other soil biota (Ahlgren and Ahlgren 1965, Vazquez *et al.* 1993, Neary *et al.* 1999) further increasing the propensity of community assemblage shifts post fire.

When the ECM fungal community does change in response to high-intensity fire, the initial response is often an increase in the dominance of r-selected, fast growing, rapidly reproducing and aggressively colonizing species found in the soil sporebank (Baar *et al.* 1999, Stendell *et al.* 1999, Bruns *et al.* 2002a), presumably because their spores survived the fire. In the case of low intensity fires, the inoculum source for seedlings tends to come from vegetative surviving mycelia growing from the roots of surviving trees (Jonsson *et al.* 1999a, Stendell *et al.* 1999). Succession in post-fire environments eventually restores the original species composition of ECM fungi; however, some species may take years and even decades to appear (Visser 1995). Since the fires employed in this study are slow-moving, patchy, moderate-intensity fires, it was predicted that the ECM fungal community in the burn areas would likely be moderately affected, but would quickly recover with mycelium growing from remaining tree roots serving as inoculum.

Extreme thinning treatments, such as clear-cutting, have also been shown to

modify the composition of ECM communities. The removal of trees tends to skew the community towards fungi that reproduce via spores rather than vegetatively, increasing the soil temperature, and changing other aspects of the microhabitiat (Jones *et al.* 2003). Previous research has concluded that extra-radical expansion of vegetative mycelia is the major source of inoculum for ECM fungi in mature forest ecosystems (Deacon and Fleming 1992, Amaranthus and Perry 1994). Thus removing living roots, from which fungi may colonize, changes the species composition to favor fungi that colonize via spores. This suggests the vital importance of allowing "island trees" which serve as inoculum sources for trees targeted by the restoration treatments (Kranabetter *et al.* 1999, Luoma *et al.* 2006). An increase in the degree of spatial aggregation of the ECM fungal community could be expected when substantial thinning causes significant vegetative ECM fungal colorization to take place, although this prediction has not been tested as far as I am aware.

To date, most research known to follow the successional progress of ECM communities after thinning includes severe thinning or clear-cutting (Visser 1995, Jones *et al.* 2003). Clear-cutting is not an ideal analogue for understanding effects of restoration thinning treatments or natural disturbances on ECM fungi, since the latter are typically much less extreme than clear-cuts. Few studies exist that report ECM fungal responses to less severe forms of thinning and more research is needed on the combined effects of thinning and fire as restoration treatments on ECM fungi. The few such studies that have been conducted have indicated the intensity of prescribed burns to be among the most indicative factors in how the ECM community will be affected (Smith *et al.* 2004,

Smith *et al.* 2005).

The importance of ECM to the maintenance of aboveground structure and ecosystem functioning necessitates that the impacts of controlled burns and thinning on belowground components be fully monitored in restoration projects. If we are to thoroughly understand the consequences and effectiveness of these efforts, it is important to incorporate belowground components into both research designs and monitoring. The research proposed here sought to identify ECM fungal community structure in forest restoration treatments at study sites in a northern Mississippi upland hardwood dominated forest. Specifically, I compared ECM fungal community structure on the roots of trees in plots subject to two different treatments: treatment and control, where treatment plots were subjected to a combination of burning and anthropogenic thinning or natural thinning by wind damage from a tornado. No studies or survey of the ECM fungal community have been conducted in this area, and knowledge of ECM fungal communities in hardwood forests of the southeastern United States is generally sparse. This research will contribute to a growing body of data on restoration ecology and will also help to characterize the species composition of an important group of organisms in a dominant habitat in the Southeast.

This project considered the following questions and tested the proceeding hypotheses:

Question 1*: Does ECM community composition and diversity differ between treatment and control plots or among sites?*

Hypothesis 1: The ECM community composition will differ between treatment and

unmanipulated plots with treatment plots containing a less diverse assemblage of more tolerant species to the abiotic conditions and control plots comprising of a more diverse ECM fungal community.

Question 2*: Which abiotic factors most influence ECM community composition?*

Hypothesis 2: Abiotic factors of light, soil texture, litter depth, and burn regime explain a significant amount of variation in the species composition of ECM.

Question 3: *Does spatial proximity explain any variation in the ECM community structure?*

Hypothesis 3a: There will be no spatial autocorrelation at the 10 m spatial scale at which we sampled between cores because most previous studies have shown spatial autocorrelation in ECM fungal communities at smaller scales (Lilleskov *et al.* 2004).

Hypothesis 3b: Spatial autocorrelation in the ECM fungal community will be highest in treatment plots, which are predicted to have low canopy cover and to retain large living oak "legacies" to serve as sources for rapid vegetative expansion of ECM fungi.

METHODS

Study site

Two of the three study sites were located at Strawberry Plains Audubon center, a 1000 hectare wildlife sanctuary located in Marshall County, Mississippi. The study area is characterized by gently rolling hills, 10 to 50 meters in elevation from ridge to hollow (Surrette *et al.* 2008). The forest species assemblages that dominate this area include mostly second growth stands of oaks such as *Quercus velutina*, *Q. marilandica*, *Q. rubra*, Q. *stellata, and Q. rubra* in the upland areas, with *Liquidambar styraciflua*, *Acer rubrum*, *Quercus alba*, and *Nyssa sylvatica* commonly occurring in the floodplain regions. The soil at this site is characterized as Providence Cahaba with a loess silt texture (Brewer 2001, Surrette *et al.* 2008). The third study site is located in the Little Tallahatchie Experimental Forest, in Holly Springs National forest, Lafayette County, Mississippi and consists of a mixed upland forest with similar composition to the first site, however with a larger population of *Pinus echinata* Miller [shortleaf pine], and *Pinus taeda* L. [loblolly pine]. The soil at this site is designated as Smithdale sandy loams and Lucy loamy sands on the slopes, with some Lexington silt loams on the ridges, and are lighter sandier than Strawberry Plains soils. (J.S. Brewer pers. comm.). Table 1 summarizes the characteristics and locations of the three study site.

Table 1. Site locations and characteristics.

Site	Abbreviation	GPS location	Elevation	Treatment notes	Soil Type
Front Strawberry	FSC: Control FST: treatment	34°49'59.161"N 89°28'31.967"W	137.12 m	Treatment site was burned September 2004, early October of 2006, and July 1, 2008 and at site 2 on July 1, 2008	Providence cahaba with a loamy silt texture, underlain by loess
Back Strawberry	BSC: Control BST: Treatment	34°49'51.19"N 89°27'17.70"W	143.26 m	Treatment was burned July 1, 2008.	Providence cahaba with a sandier texture than Front Strawberry sites, underlain by loess
Tallahatchie	TC: Control TT: Treatment	34°30'8.93"N 89°26'3.04"W for TC 34°30'25.20"N 89°26'37.4"W for TT	121.9 m for TC 131 m for TT	TT site was hit by a tornado in Feb. 2008 and burned March of 2005. Both plots had been burned during the 1980s.	Primarily Lucy loamy sand on slopes; Lexington silt loam on ridges.

Restoration treatments

The goal of restoration in mixed upland forests of northern Mississippi is to restore the historical open oak or oak/shortleaf pine woodland habitat from a dense, closed-canopy hardwood forest that has resulted from fire suppression practices of the

past century (Brewer and Menzel 2009). The species that have been targeted for restoration include several oak species that historical records suggest were dominant in oak woodlands of the 1800s: *Quercus velutina*, *Q. stellata*, *Q. marilandica*, and *Q. falcata*. Each of these species is a known ectomycorrhizal host.

At each of the three study sites, we established treatment and control plots measuring 70 x 75 meters: two sites at the Strawberry Plains Audubon Center ("Front Strawberry" and "Back Strawberry"), and one site located in the Little Tallahatchie Experimental Forest, in Holly Springs National forest ("Tallahatchie"). Treatment plots received thinning and burning treatments, with varying burn regimens. The burning at the Audubon sites includes four recent fires: September 2004 (Front Strawberry only), April 2005 (Front Strawberry only), October 2006 (Front Strawberry only) and July 2008 (both sites). The spring fire in 2005 burned the entire Front Strawberry treatment plot while the other three fires were patchier and only affected areas near the edges of the plots. The thinning treatments in the Front Strawberry site have been ongoing since 2005, and have targeted specific species for removal; most notably *Liquidambar styraciflua* which historically has been relegated to flood plains, but due to fire suppression has extended its range into upland forests (Brewer 2001, Surrette *et al.* 2008, Brewer and Menzel 2009). Thinning at the Back Strawberry site was initiated in 2007. Thinning at the Audubon sites consists mostly of mechanical removal via girdling along with chemical treatments of 8% Triclopyr (an herbicide) to undesired trees. "Thinning" in the Tallahatchie site was accomplished naturally from a tornado in February 2008 with canopy coverage thinning to around 30% of the original canopy. The Tallahatchie treatment plot was burned in

March of 2005, while the whole experimental forest area had previous been burned at various different intervals during the 1980s.

Sample collection and processing

In May, 2009, within each 70 x 75 meter plot, 36 root cores 15 cm deep and 3 cm in diameter were collected. A systematic grid sampling design was utilized collecting cores every 10 meters throughout the plots in order to maximize sampling of ECM diversity while minimizing potential spatial autocorrelation between samples, which has been found to occur typically among samples of ECM communities less than 2 m apart (Lilleskov *et al.* 2004). Soil cores were kept on ice in coolers until they were returned to the laboratory, where samples were then refrigerated at 4 degrees Celsius until processing. Roots in each core were washed carefully over a 2 mm sieve, and 10 individual ECM root tips were randomly selected for removal with the aid of a dissecting microscope. Each root tip was classified initially according to a crude morphotype (e.g., fuzzy brown, smooth yellow, grainy white) and then placed in individually labeled tubes for immediate DNA extraction.

Abiotic variables were measured for each of the soil core samples in the field, including soil density, litter depth, canopy cover and burning regimen. A penetrometer (i.e., soil compaction tester, Dickey-John, Inc.) was used to estimate the density of the soil at 7.5 and 15 cm depths. Litter depth was assessed using a measuring stick and gauging the height of the litter layer from top to mineral soil. To estimate canopy cover, canopy photos were taken with a Nikon Coolpix 990 digital camera fit with a fisheye

lens, and then analyzed using Gap Light Analyzer software (version 2, Frazer *et al.* 1999) to produce estimates of % canopy openness and total light penetration. Burn history at each soil core (burned or not) was assessed by searching for burn evidence (scorched tree trunks, blackened large coarse woody debris).

Molecular identification of ECM fungi

DNA was extracted from fresh root tip samples using the Sigma Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO), as follows: 10 µl of the Sigma Extraction Buffer was added to each root tip, each sample was heated at 65 °C for 10 minutes and 95 °C for 10 minutes in a thermocycler, and then 30 µl of the Sigma Neutralization Solution was added to each sample. PCR was performed using the fungal-specific primers ITS1-F and ITS4 (Gardes *et al.* 1991). These primers amplify the internal transcribed spacer (ITS) region between the small subunit (SSU) and large subunit (LSU) ribosomal genes of the fungal nucleus, which is effective for distinguishing fungi at the species level due to the rapid evolution of this area of the genome. Each 8 µl PCR reaction contained 0.4 µl (10 µM stock concentrations) of each primer, 2.7 µl of sterile PCR-grade water, 4 µl of Sigma Extract-N-Amp PCR Reaction mix, and 0.5 µl of DNA extract. Thermocycling for PCR used the following conditions: 93°C for 3 minutes followed by 35 cycles of 1 minute at 93°C, 55 seconds at 53°C, and 35 seconds with +5 seconds per cycle at 72°C, followed by 10 minutes at 72°C. The PCR products were checked for amplification on a 1 % agarose gel with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). Excess primer and unincorporated nucleotides were removed using ExoSAP-IT

(USB Corporation, Cleveland, Ohio, USA), with the following protocol: 1 ul of ExoSAP-IT and 4 µl of PCR-grade water were combined with 5 µl of PCR product, and each sample was heated to 37°C for 45 minutes, 80°C for 15 minutes and 4°C for 5 minutes. Sequencing was performed using the ITS1 primer and the ABI Big Dye Terminator Sequencing Kit (v3.1). Each Big Dye reaction contained 1 µl Big Dye Reaction Pre-Mix, 1.5 µl Big Dye 5 X sequencing buffer, 0.5 µl of the primer (10 µM stock concentration), 6 µl of sterile PCR-grade water, and 1 µl of the cleaned PCR product. Thermocycling conditions were 96°C for 1 minute followed by 35 cycles of 30 seconds at 95°C, 20 seconds at 50°C, and 60°C for 4 minutes. Reactions were then dried and mailed overnight to the DNA Lab in the School of Life Science at Arizona State University, in Tempe, Arizona, where sequencing reactions were purified and read on a capillary genetic analyzer.

Sequences were imported into Codoncode Aligner software (version 1.6.3; CodonCode Corporation) where sequence ends were trimmed and sequences with fewer than 200 bases were removed from the dataset. In addition, sequences with > 6% ambiguous bases (defined as bases with Phred-Phrap quality scores of less than 15) were not used for further analysis. Sequences passing these initial screening criteria were then assembled into operational taxonomic units (OTUs) with the CAP3 software package (Huang and Madan 1999) running on the University of Alaska, Fairbanks (UAF) Life Science Informatics computing cluster, using default parameters with the exception of the following changes: h = 60 (max. % overhang length), m = 6 (match score factor), p = 97 (overlap % identity cutoff), y = 6 (clipping range). This analysis sorted the sequences

into contigs (OTUs appearing more than once) and singlets (OTUs only appearing once). After this analysis, singlets with >3% ambiguous bases were removed.

A merged file containing the filtered singlets and consensus sequences for the contigs was submitted for BLAST comparisons with GenBank using the BLASTALL utility on the UAF Life Science Informatics computing cluster. Database hits with Hit Overlap of less than 150 bases were not used. OTUs sequences were also compared with matches from the UNITE database (Kessy *et al.* 2010) as well as our in-house database generated from known ectomycorrhizal mushroom samples collected in northern Mississippi. Top hits from these database queries were used to assign likely taxonomic identity based on the degree of matching, with hits matching at 99% or greater identity assigned to matching species. Sequences with 95-98% similarity were assigned to genus level resolution, designated with a number based on the order with which they were determined (e.g. *Lactarius* 1). OTU matches at the 90-94% identity level were assigned family level resolution, with a number denoting the order with which they were assigned (e.g. Russulaceae 1). All queries found to be <90% or matching non-ectomycorrhizal species were excluded from the final analyses.

Data analysis

Question 1: *Does ECM community composition and diversity differ between treatment and control plots or among sites?*

Distance Based Redundancy Analysis graphic output was used to visualize the similarities among soil cores in species space and to discern patterns. PermANOVA, a

non-parametric analysis of variance technique that is robust against non-normality associated with ecological species abundance data (Anderson 2001) was used to test the influences of treatment and site on multivariate fungal community structure, with site and treatment as fixed-effect predictor variables in the model. Multivariate homogeneity of dispersion among treatments and sites was verified using the PERMDISP procedure (see results). Indicator species analysis was further used to determine which species most contributed to significant effects in the PermANOVA. These analyses were performed using the PCOrd (McCune and Mefford 2006) and PRIMER6 (Clarke and Gorley 2006) software packages.

Diversity and richness for each plot and site were estimated with EstimateS software (Colwell 2009). EstimateS was further used to generate sample based rarefaction species accumulation curves for each plot. Univariate ANOVA (in SAS Proc GLM) was then used to test the influences of treatment and site on plot-level estimated fungal species richness and diversity, with site and treatment as fixed-effect predictor variables in the model.

Question 2: *Which abiotic factors most influence ECM community composition?* Distance Based Linear Modeling (Legendre and Anderson 1999) was used to test which measured abiotic environmental factors, including canopy cover, soil texture, litter depth, and burn history most influenced ECM fungal community structure and relative abundances of particular fungal taxa. This statistical method permits using multivariate species relative abundance data as a response to multiple quantitative predictor variables. Site and Treatment were also included as factors in these analyses, to explore the degree

to which community structure varied among sites and treatments due to factors besides our measured abiotic variables. Model selection based on Akaike's Information Criterion corrected for small sample seizes (AICc) was conducted on all possible multifactor models to assess which predictor variables best explained the multivariate community data. This statistical analysis was conducted in the PRIMER6 software package (Clarke and Gorley 2006).

Question 3: *Does spatial proximity explain any variation in the ECM community structure?*

A statistical approach to measure spatial autocorrelation was used, with cores as the sample units, at the site and plot levels (Legendre and Fortin 1989). Specifically, a Mantel test was conducted to test for a correlation between two distance matrices, a species distance matrix (consisting of Sorensen distances generated from species relative abundance data), and a physical distance matrix (consisting of Euclidean physical distances among cores generated from X-Y coordinates). Monte Carlo permutation was used to generate *p*-values for the significance of spatial autocorrelation in structuring the community data. This procedure was performed using PCOrd software (McCune and Mefford 2006). When significant spatial autocorrelation was found in a plot or site, the scale of spatial autocorrelation was explored by calculating a Mantel correlogram, consisting of normalized Mantel correlation coefficients for multiple distance classes of soil cores calculated using the procedure described by Legendre & Fortin (1989).

RESULTS

Overall Patterns in Community Composition

A total of 486 ECM fungal sequences were generated from the 216 root cores, after low quality and non-mycorrhizal sequences were discarded. The fungal community was highly diverse, with 69 operational taxon units (OTUs) occurring more than once, and 106 singlet OTUs (i.e. OTUs occurring only once) across all sites. The number of OTUs recovered in each plot indicated the Front Strawberry plots to have the highest species richness with 83 OTUs recovered from cores in this site compared to 48 from Back Strawberry plots and 72 from the Tallahatchie plots. Front Strawberry also contained 40 of the 69 total contig OTUs (species appearing more than once) from the study samples, while Back Strawberry plots contained only 26 and Tallahatchie plots contained 31 contigs. Sample based rarefaction species accumulation curves generated from each of the sites and plots did not achieve an asymptote, indicating that our sampling effort did not reach species saturation. The most abundant species included taxa from the Cantharellaceae, Thelephoraceae, Russulaceae, and Sebacinaceae families (see Fig. 1). Richness and diversity estimators (including Chao1, Chao2, Jack-knife, incidence based coverage estimates (ICE), and Shannon diversity did not differ significantly by site or treatment (see Table 2). The maximum number of OTUs as calculated by Chao 1 diversity estimates included 356 for Front Strawberry, 240 for Back Strawberry and 230 for Tallahatchie.

Table 2: ANOVA table results for five diversity measures used between treatment and site.

	Treatment				Site			
Diversity measure	df	MS	F value	*p*	df	MS	F value	*p*
ICE	1	486.9004	2.21	0.28	2	91.4367	0.41	0.71
Chao 1	1	10.5073	0.49	0.56	2	47.5395	2.23	0.30
Chao 2	1	75.1188	2.83	0.23	2	65.9313	2.48	0.29
Jack knife	1	13.4401	0.32	0.63	2	67.2460	1.62	0.38
Shannon Diversity	1	0.01306	3.06	0.22	2	0.0701	16.42	0.06

The most abundant OTUs (greater than 6% of the total species community) made up 67% of the total samples and included OTUs from the families Sebacinaceae, Thelephoraceae and Russulaceae. OTUs in the family Russulaceae alone accounted for 45% of the total OTUs for all plots (see Fig 1).

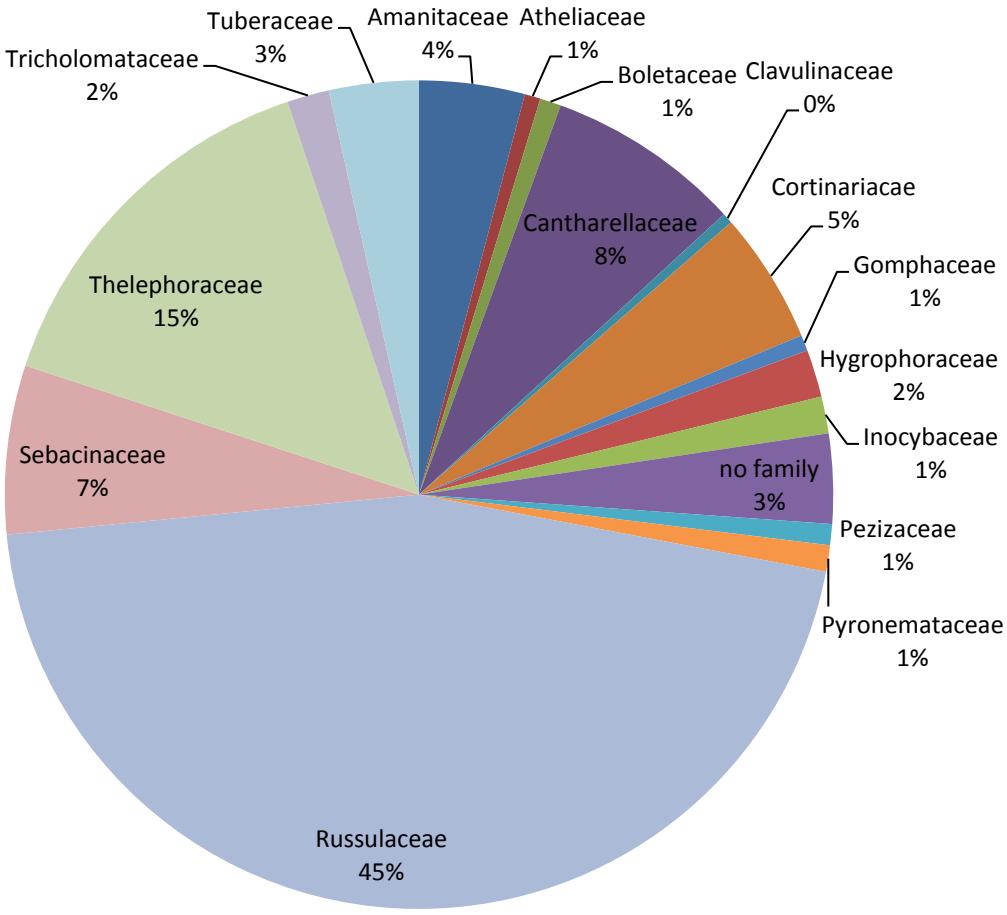

Fig. 1. Percent frequency of all operational taxon units sorted by family level across all plots.

At the upper taxonomic levels of family and genus levels, the fungal communities showed similar distribution in abundance across all three sites; however, several taxa at the species level occurred in only 1 plot and or were specific to a site. Russulaceae and Thelephoraceae consistently made up the largest portion of the belowground community;

however, the next most abundant taxa differed between the Strawberry sites and the Tallahatchie sites (see Fig 2).

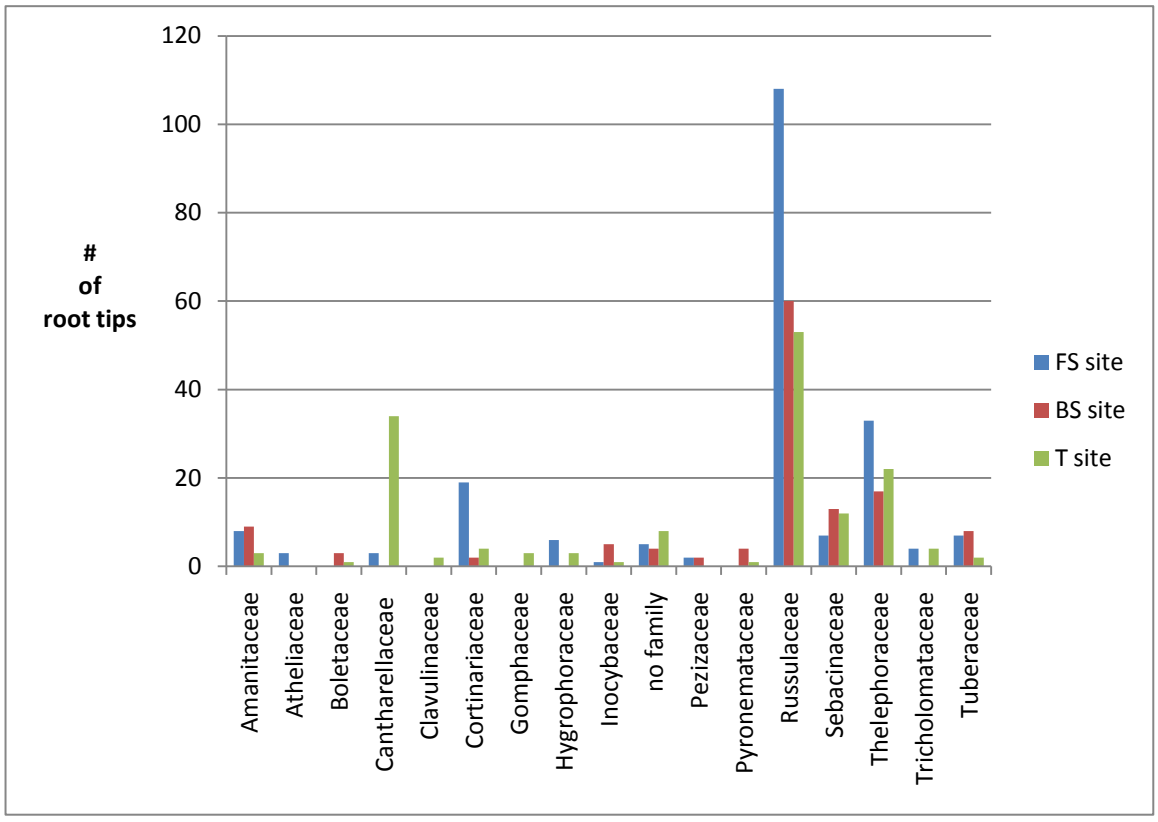

Fig. 2. Number of root tip sequences of fungal OTUs at the family level compared between each of the 3 sites.

Question 1: *Does ECM community composition and diversity differ between treatment and control plots or among sites?*

ECM fungal community composition differed significantly among sites, (df = 2, Pseudo-F = 2.28, and $p = 0.001$), but not between treatments (df = 1, Pseudo F = 1.455 $p = 0.145$) or the site by treatment interaction (df = 2, Pseudo F = 1.3297, $p = 0.143$). In order to ensure this analysis was robust, multivariate dispersion was checked between sites and found to be non-significant (df = 2, F = 0.749 $p = 0.51$). The fungal community structure showed high site fidelity throughout all three of the sites, with certain taxa occurring at either only one site, or both of the Strawberry Plains sites. Indicator Species Analysis elicited some of the species responsible for the site-specific pattern found. Operational taxon units (OTUs) in the family Russulaceae were found in each of the 3 sites, while Cantharellaceae and *Cenococcum geophilum* species were only found at the Tallahatchie sites. One particular OTU, *Craterellus cornucopioides*, was found exclusively in one of the plots in abundance (21 root tips). Amanitaceae species were found exclusively in the Strawberry sites, both control and treatment plots. Furthermore the two most abundant taxa, both Russulaceae species, were found in both the Strawberry sites, but not in the Tallahatchie sites (see Fig 3).

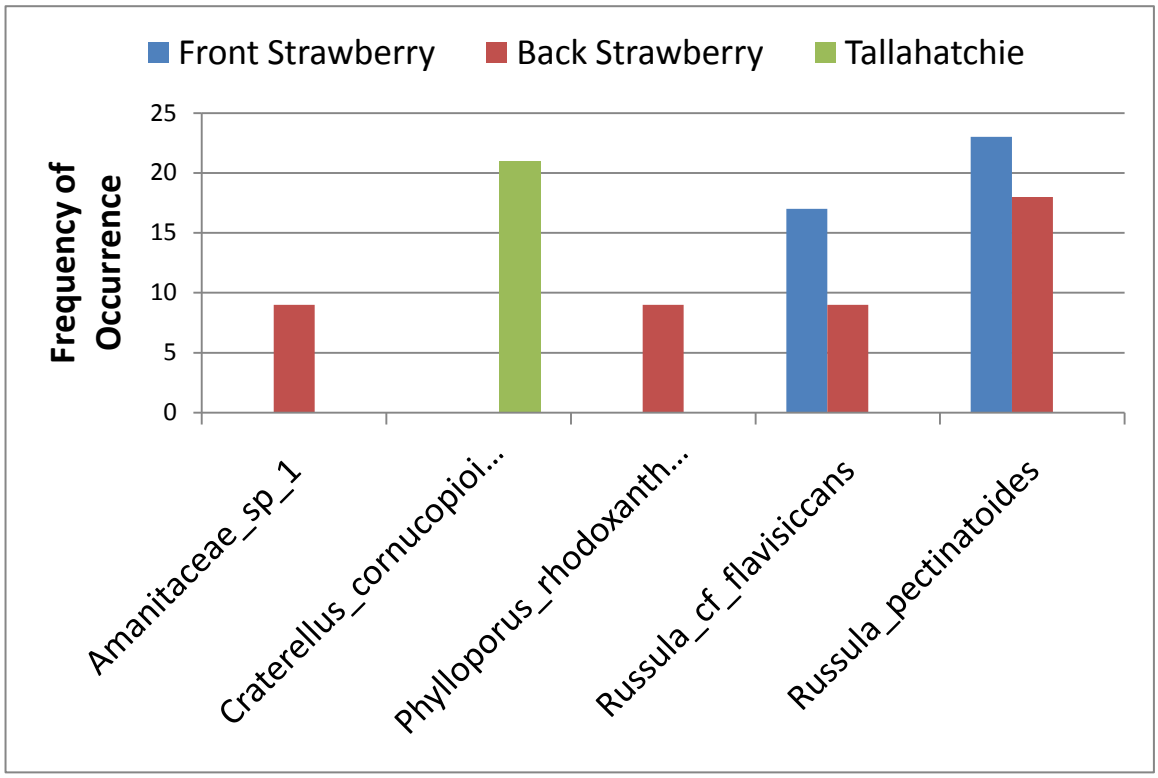

Fig. 3. The three taxa on the left were found to be significantly perfect indicators of the unique fungal community at each of the site, according to Indicator Species Analysis. The two most abundant taxa in the dataset, shown on the right, were not found at the Tallahatchie site.

Question 2: *Which abiotic factors most influence ECM community composition?*

Distance-Based Linear Modeling (DBLM) was employed to relate measured abiotic data to community composition data. Site was found to be the factor that accounted for the most variation in community structure according to this analysis, with the Tallahatchie site (df = 1 Pseudo F = 3.2662 p = 0.001) appearing in all best AICc models along with soil density up to 10 centimeters (df = 1 Pseudo F = 1.566 p = 0.028). Burn history was found to be a significant factor accounting for variation in community structure (df = 1 Pseudo F = 2.5397 p = 0.001) but was not included as a top five factor in the AICc models. The other two sites as categorical variables also accounted for a

significant amount of the variation however were secondary to the Tallahatchie site in structuring the community according to the AICc analysis (see Table 3). In a graphical Distance Based Redundancy analysis generated from the Distance Based Linear modeling results, the Tallahatchie site vector was parallel to the horizontal axis, whereas soil density up to 10 centimeters depth was the next most important measured factor and was correlated with the second ordination axis (Fig 4). Despite inclusion of various abiotic factors, the most important explanatory variables were the Site factors, since site factors appeared in all the AICc-best models. A model with two factors, Site and Soil compaction at 10 centimeters exhibited the lowest AICc score (see Table 3). Overall, less than 10% of variation in community composition was explained by most models.

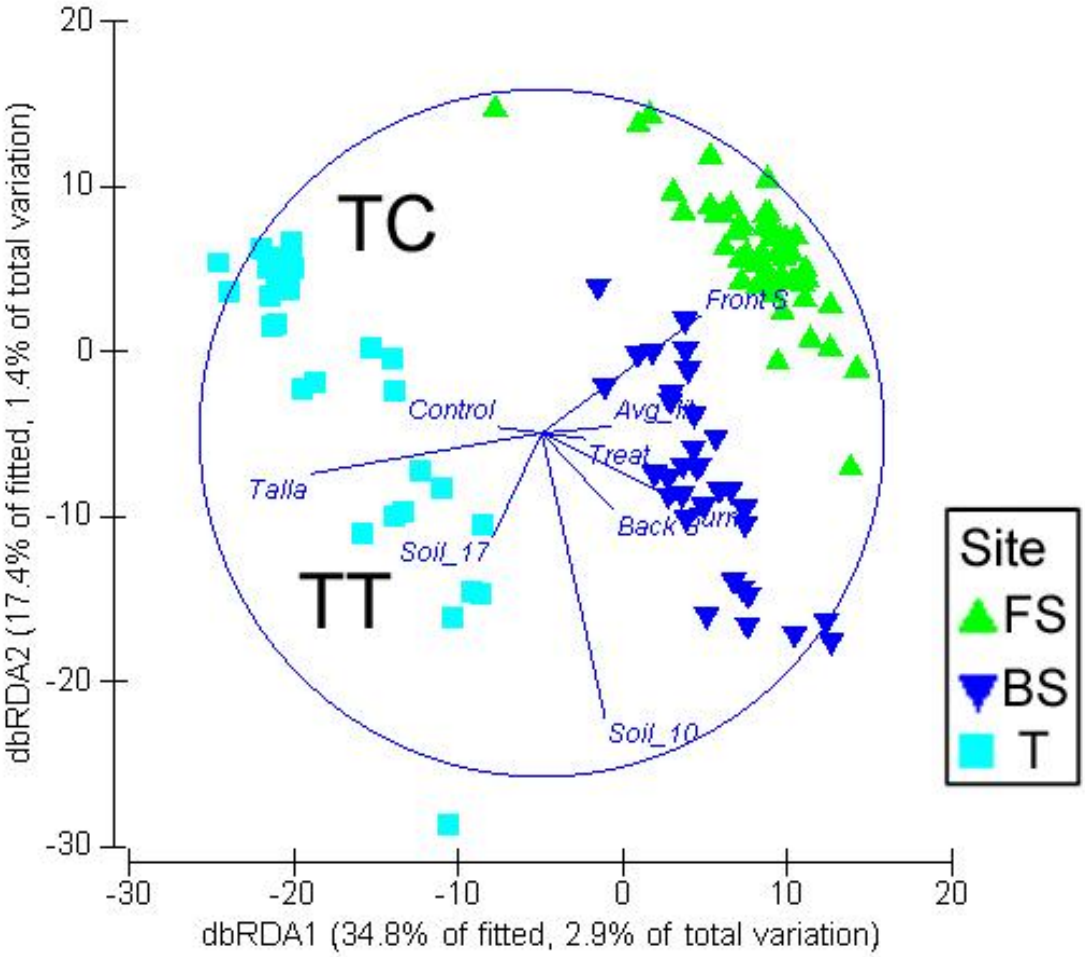

Fig. 4. Distance based redundancy analysis ordination graph, overlayed with abiotic factors included in the analysis, generated in PRIMER6 software.

Table 3: AICc scores of ten best models from Distance-based Linear Modeling analysis, with lowest AICc score indicating best fit. The following variables are soil compaction at 10 centimeters (soil_10), soil compaction at 17 (soil_17), burn regime (burn), Tallahatchie site categorical variable (Talla), Back strawberry site categorical variable (Back S), Front Strawberry categorical variable (Front S), treatment (treat), canopy openness, average leaf litter depth (avg_lit).

AICc	r^2	Number of variables	Variables in model
1025.3	2.67×10^{-2}	1	Talla
1025.7	3.98×10^{-2}	2	Soil_10, Talla
1026	3.80×10^{-2}	2	Front_S, Back_S
1026	3.80×10^{-2}	2	Front_S, Talla
1026	3.80×10^{-2}	2	Back_S, Talla
1026	3.80×10^{-2}	3	Front_S, Back_S, Talla
1026	3.78×10^{-2}	2	Soil_17, Talla
1026	2.09×10^{-2}	1	Burn
1026.1	3.73×10^{-2}	2	Burn, Talla
1026.3	1.83×10^{-2}	1	Front_S

Question 3: *Do spatial patterns explain any variation in the ECM community structure?*

One plot was found to have significant spatial autocorrelation of fungal community composition, i.e. a correlation between spatial proximity and species composition similarity among cores: Tallahatchie Treatment (TT). This result indicates similar species composition among cores at distances of greater than 10 meters at this plot, whereas for the other 5 plots, communities were completely dissimilar at those spatial scales. A distance class correlogram showed that the spatial autocorrelation was highest at the 10-15 meter scale for the Tallahatchie Treatment plot (Fig. 5).

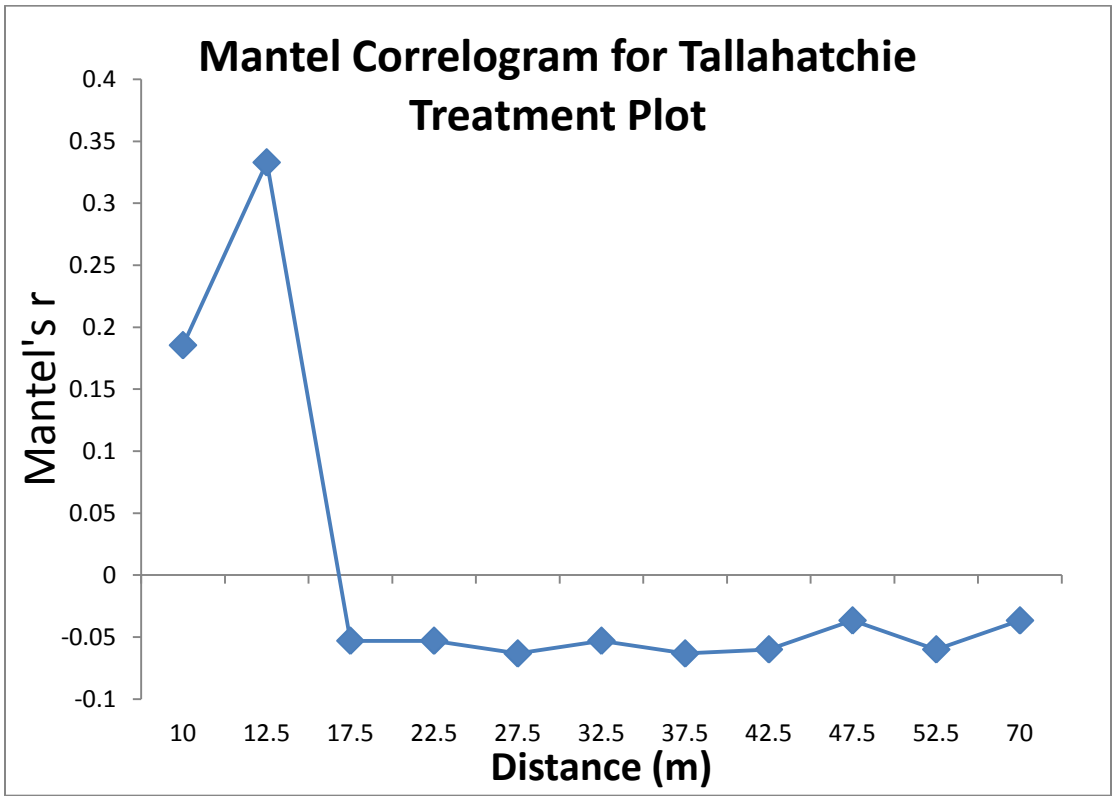

Fig. 5. Correlogram indicating spatial autocorrelation among pairs of cores in different distance classes in the Tallahatchie Treatment plot.

DISCUSSION

Overall patterns of ECM fungal community composition

Results from this study indicate that the ECM fungi found in our mixed hardwood forest sites in northern Mississippi are very diverse, a pattern repeatedly confirmed in other ectomycorrhizal community surveys of belowground diversity (see reviews by Horton and Bruns 2001, Smith and Read 2008). Other studies that have surveyed ECM belowground diversity in oak forests or forests containing oak species have found an impressive amount of diversity, generating species area curves that never asymptote, indicating an even greater diversity than sampled (Avis *et al.* 2003, Walker *et al.* 2005, Morris *et al.* 2008a, Walker *et al.* 2008). The ecological significance of such high diversity on root tip communities is a question still debated today among mycorrhizal ecologists, though theories pertaining to the maintenance of ecosystem stability and micro-niche partitioning have been proposed to explain the hyper diversity of these cryptic communities (Bruns 1995).

With the caveat that diversity has rarely been completely sampled (in my study or other studies), in comparison with most other studies of ectomycorrhizal communities on the roots of oak species, the community found in this mixed-oak system seems to be more diverse. Avis *et al.* (2003) recovered a total of 72 OTUs from an mixed oak savannah habitat in Illinois, Lindahl (2002) found 72 OTUs in a *Quercus agrifolia* grassland in California, Valentine *et al.* (2004) found 39 OTUs in a woodland dominated by *Quercus garryana* in southern Oregon, Morris *et al.* (2008b) found 140 OTUs on the roots of co-occuring *Quercus douglasii* and evergreen *Quercus wislizeni* trees in the foothills of

the Sierra Nevada by and Walker *et al.* (2005) found 75 ITS types in a *Quercus rubra* and *Quercus prinus* dominated wood land in the Appalachians. Outside the continental US, 140 ectomycorrhizal OTUs were also found on the root tips of *Q. ilex* trees in Corsica (France) by Richard *et al.* (2005) and 41 taxa of both EM and non-EM species found on the roots of *Q. crassifolia* in a tropical montane cloud forest in southern Mexico (Morris *et al.* 2008a) . Smith *et al.* (2007) found a comparable species richness of ECM on root tips to the diversity reported in this study, with 161 species found on the roots of *Q. douglasii* xeric woodland. With a total of 175 OTU, 106 being found only once, this study echoes previous work indicating that ECM diversity found plant roots in oak systems to be very diverse. The greater diversity found in this study could be reflective of the multi-host species assemblages found in this ecosystem, as pines, birch, sweet gum and other species were also found on the study sites.

Host specificity to particular plant taxa in ectomycorrhizal fungi is highly variable, with some fungal taxa having a broad hoast range and other being restricted to a particular host plant genera or species (Molina *et al.* 1992). Certain taxa in the same fungal family can be very host specific while others can have very broad host ranges. Molina *et al.* (1992) provides an extensive listing of ectomycorrhizal fungal taxa and host specificity and is still to date one of the best resources on host specificity in mycorrhizal taxa. According to this review, none of the taxa we encountered in this study have been reported to be narrowly host specific. Among intermediate host specific taxa, i.e. those that are host specific at the family level (i.e Pinaceae), two *Rhizopogon* OTUs were found exclusively in the Tallahatchie site. *Rhizopogon* is known to be specific to Pinaceae, and

pines were found in much greater abundance at this Tallahatchie site. Many of the sequences were only identified to the genus or family level, leaving the possibility of still more known host specific taxa in the ECM community. Many of the taxa that were resolved to the genus and species level have previously been found to have broad host ranges, including *Russula cyanoxantha* and *Thelophora* species. Some of the most numerous taxa in this study have not been reported to have either broad or narrow host ranges (*Russula cf. flavisiccans* and *Craterellus cornucopioides*). Conversely, it may be that host specificity in this system is an exception rather than the rule, and previous work suggests that ECM fungal assemblages often have more regional or landscape fidelity (local adaptation) than strict host associations due to the large diversity found in ECM communities lending less pressure to develop host/fungal specialization (Karst *et al.* 2008). Further work is needed to determine the host specificity of ECM fungal taxa in this diverse system and what role these plant/fungal relationships can impart on structuring the forest community pre-and post restoration.

Taxa in the family Russulaceae dominated the ectomycorrhizal community, with nearly 50% of the OTUs found on roots belonging to this family. Species in this family are found prolifically throughout the world in association with a wide range of tree species. According to a review by Dahlberg (2001) taxa in the Russulaes order dominated the root tip communities in 4 different studies in the Canada, 2 in Sweden, and 8 in the US, and noted that Russulaceae, Thelephoraceae and Sulloid fungi were the most abundant mycorrhizal taxa on the roots of tree in the western US and tended to be the second most abundant in boreal systems (see table 1 in Dalhberg 2001). The pattern of

Russulaceae dominance seems to be even further widespread than this review reports with forests in the Neotropics, Australia and Japan, as well as in the sites of this study dominated by this family of ECM fungi. Tedersoo *et al.* (2008) reported *Lactarius eucalyptii* to be the second most frequent taxa on the roots of trees in a Tasmanian wet sclerophyll forest. In a tropical montane cloud forest in Mexico, taxa in Russulaceae once again represented the most abundant ECM fungi found on the roots, comprising close to 16% of the 44 taxa reported in this study (Morris *et al.* 2008a). Russulaceae was also reported to be the most abundant taxa found in other studies of oak forests in the eastern US (Avis *et al.* 2003, Walker *et al.* 2005). This cosmopolitan family represented taxa that dominated the root tip communities of *Pinus muricata* in California (Gardes and Bruns 1996), *Castanopsis cuspidata* forest in Japan (Murakami 1987), and were the most abundant basidomycete taxa on roots in a boreal western balsam fir–paper birch forest in Northern Quebec (DeBellis *et al.* 2006). It is likely that as the number of studies of belowground ECM fungal diversity increase with the decreased cost of molecular reagents and processing, taxa in the family Russulaceae will continue to dominate the root tip communities in many forests throughout the world. With increased capacity to molecularly identify ECM taxa, this taxon could possibly rival the dominance of the ubiquitous species *Cenococcum geophilum*, which had been previous reported to be the most abundant taxa in other root tip surveys due to its characteristic morphotype that made it very easy to detect (Horton and Bruns 2001).

Variation in ECM fungal community composition among sites and the influence of abiotic factors

While there was no significant difference in diversity between plots, the Front Strawberry sites contained a greater number of OTUs. This site had lower canopy openness and will require more thinning of weedy non-fire dominant species by future restoration grant cohorts and the continued efforts of the Brewer lab. The higher number of OTUs at the Front Strawberry sites could be on account of the higher soil moisture and more benign edaphic environment at these sites as a result of the early stages of the restoration project when samples for this project were collected. The ECM fungal community was found to have high site fidelity indicating that soil factors, land-use history and other unmeasured factors had a strong influence on ECM occurrence. The measured factors found to have the largest impact on structuring fungal community were burn history and soil compaction up to 10 cm depth (not 17 cm depth), indicating that soil chemistry and microclimate of the edaphic environment exerts pressure on selecting which fungal taxa will occur in a certain area. While Site did explain the most variation in fungal community structure, all the measured abiotic factors combined only explained 10 % of variation according to the distance-based linear modeling analyses. These results suggest that other un-measured biotic and abiotic factors, such as relative abundance of roots of different host species, need to be measured to further understand variation in the belowground ECM fungal structure.

Another possibility that may relate to the strong site affinity found in this study is the life history and dispersal patterns of the different fungal taxa. For instance at the

Front and Back Strawberry sites, taxa in the genus *Amanita* were found, where they were not found in the Tallahatchie sites. *Amanita* is considered to be a late-stage ECM fungal genus that colonizes from plant roots after it is established, and often will not occur in young recently-disturbed plant assemblages (Bruns *et al.* 2002). The Front Strawberry sites had experienced less disturbance than the Tallahatchie treatment, potentially permitting a more favorable habitat for the occurrence of *Amanita*.

Burning was found to explain less of the variation in the ECM belowground community structure than originally predicted; however, this could be due to the early stage of this project as well as the patchy distribution of the burn in reference to where the samples for this project were taken. The burns that historically occurred in our plots were low creeping fires carried by perennial grasses (Brewer 2001) and previous studies on the response of fungal communities to less intense fire disturbance have shown minimal change in species richness or community composition post burn (Jonsson *et al.* 1999). Burns of greater intensity would have likely initiated a much more noticeable shift in the fungal community structure, but such a fire would have been a rare event throughout this landscape. Given that the mycorrhizal community can influence succession in plant communities through both direct and indirect mechanisms (Perry *et al.* 1989, van der Heijden *et al.* 1998, Twieg *et al.* 2007), the corresponding fungal community that influenced aboveground dynamics in these open oak ecosystems could have also been maintained by these fires. While these burns might not cause immediate drastic changes, we have no belowground reference state with which to compare the ECM community sampled in our sites. Discerning what sort of community may be the

soil counterpart to these ecosystems will require further research into host specific interactions as the burning regime of 3-5 year cycles is returned to the landscape. There is still much to learn about how microbial communities respond to fire and even further what sort of historical assemblages these communities might have sustained in areas of high fire frequency (Neary *et al.* 1999, Hart *et al.* 2005). Better understanding of the dynamics of fire, ECM response and consequential succession will be invaluable in helping to restore and maintain these ecosystems, reclaiming the belowground community that will maintain the above ground structure through appropriate fire cycles and burn intensities (Heneghan *et al.* 2008).

Canopy openness also accounted for much less variation than was originally predicted. While canopy openness can be correlated to thinning, it seemed not to serve as an adequate latent variable to discern thinning effects at the level or replication in this project. Thinning tree densities has been shown to have an effect on fungal community structure (Jones *et al.* 2003) and opening up the canopy can be correlated to the restoration thinning. Most of the plots had <20 % canopy openness with the exception of the Tallahatchie plot, which was struck by a tornado in 2008, and had 30% canopy openness when I estimated it in Summer 2009. The thinning work at the Strawberry Plains sites is still ongoing and tentative goals are to establish greater canopy openness in this area; however, natural disturbance such as the tornado damage through the Tallahatchie plots are seemingly effective in restoring historical canopy openness to the landscape (Surrette *et al.* 2008). It seems likely that greater replication of plots with the higher canopy openness of the Tallahatchie plot would reveal a more noticeable

distinction between the fungal communities of forest plots with open versus closed canopies.

Variation in spatial autocorrelation of ECM fungal communities

Perhaps the most surprising finding of this study was discerning spatial autocorrelation at a relatively large spatial scale (between 10 and 15 meters) in the Tallahatchie Treatment plot. Spatial autocorrelation in ECM fungal communities at this large scale using similar analyses has never been previously reported, and according to a review by Lilleskov (2002), spatial autocorrelation in ECM fungal community structure typically tends to break down between 3-5 meters. The ecological significance of this finding suggests an interesting topic for further research. ECM fungi form extensive mycelia networks through the soil (Simard and Durall 2004) and *Quercus* species have been shown to use these networks to facilitate congeneric seedlings (Dickie *et al.* 2002). If the oaks in historical landscapes maintained by fire were at greater distances apart from congeneric neighbors, perhaps their fungal linkages could have also been shared at greater spatial scales similar to that found in the TT plot. Spatial analysis of belowground fungal community structure is a fruitful topic for research that has the potential to elucidate a more holistic concept of what the reference conditions for these ecosystems might have been both above- and belowground.

Conclusions

Restoration ecology often employs the use of reference sites that consist of relatively pristine environments or plant assemblages representing the ideal or desired ecosystems for which the success restoration treatments will be gauged (Primack 2002).

In this case, no defined reference site exists in which one can examine the fungal community and define the target for restoration. Rather, I have compared ECM fungal communities between un-manipulated plots, representing modern forest structure, versus plots undergoing restoration treatments, which are expected to be more similar in structure to historical forests (Surrette *et al.* 2008, Brewer and Menzel 2009). Although I expect the ECM fungal community to continue to change as restoration continues in these habitats, these data can be used as a baseline for future studies in this area. From these data I have created a reference of ECM diversity with which future studies may track the progression of the community shifts as a result of further restoration work, and contribute to the first inventory of ECM diversity for this area.

As technology to identify the soil biota continues to progress, consideration into the impacts of belowground components will be a more accessible and necessary goal for the future of restoration ecology. Employing a strategy that seeks to restore the complete integrity of the ecosystem will facilitate more achievable restoration goals and total ecosystem management. Seeking a desired state of a restored ecosystem that is chemically, physically and biologically similar to the landscapes prior to fire suppression will require a fundamental understanding of the belowground constituents which helped to regulate and maintain these conditions (Heneghan *et al.* 2008). While the belowground "reference site" is often not available for comparison in restoration studies that consider the belowground community, broad opportunities exist for further research into soil ecology and restoration seeking to elucidate what belowground biotic assemblages comprise a healthy, stable, and complete ecosystem. Integrating theories and

models of climate change will be necessary to establish what landscapes will be able to persist into the 21st century in the face of changing hydrological, temperature, and carbon/nitrogen regimes. Understanding the dynamics of global transport and belowground community facilitation in a rapidly changing and less predictable environment will be necessary to combat the invasions of exotic species (Schwartz *et al.* 2006), while a better understanding of plant/soil interactions can increase the potential for agroecology and sustainable food production (Coleman *et al.* 2002, Plenchette *et al.* 2005). Knowledge of the interplay between the aboveground and belowground dynamics will be needed to achieve a desired trajectory that encompasses biological, physical and chemical integrity of ecosystem services and function, and furthermore provide a more complete understanding of our environment to better protect the future of our species' continued existence on our limited terrestrial habitat.

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VITA

Born on Leap-a-year day, February 29, 1984 in Slidell, Louisiana, Ashley Jane Craig grew up most of her early childhood in a small bayou town on the north shore of Lake Ponchtrain known as Lacombe, only 45 minutes from New Orleans. At 11 years old her family moved to a gated community just outside of Picayune, MS where she lived out the rest of her childhood into her early teens, swimming in the lake that made up the backyard of her family's home, riding bikes, and fishing with her younger brother, Alex. Ashley attended high school for her freshmen and sophomore year at Pearl River Central High School where her mother taught as a drama teacher.

At 16, Ashley was accepted into the Mississippi School for Math and Science, a state sponsored boarding school where she lived and graduated in 2002. It was at MSMS that Ashley first started calling herself Anjel, and continues to refer to herself as such today. While still attending high school at MSMS, Anjel met her would- be husband, Paul "Puppy" Tate at a live action role playing game in September 2001. They continued to date throughout high school, and moved in together upon her attending the University of Southern Mississippi for her Bachelor's degree.

After spending a year abroad in Victoria British Columbia in 2004, surviving Hurricane Katrina in 2005, attending a summer biology course in Belize in 2006, and finally completing her Honor's thesis with Dr. Kevin Kuehn and being awarded a McNair Scholarship in 2007, Anjel graduated with her Bachelor's of Science in December 2007. Anjel was then awarded a fellowship with a USDA Restoration Ecology Training Grant

to attend the University of Mississippi under the direction of Dr. Jason Hoeksema for her Master's degree. This grant funded her travel to a six week internship in British Columbia to learn about restoration from a very successful large scale project ongoing in the Canadian Rockies, in addition to several conferences including the Ecological Society of America Meeting in 2009 and 2010, the 2010 Gulf Coast Research Laboratory Symposium in Ocean Springs, MS, and the 2010 Western Mycorrhizal Conference in Bishop, CA.

In April of 2010, she was accepted as an IGERT fellow to pursue her PhD under the direction of Dr. Cathrine Gehring at the Northern Arizona University. That following month, on May 22, 2010, she married Paul Tate, and then 3 weeks later departed on another internship to Northern Thailand with an NSF team characterizing fungal diversity. Upon returning to Mississippi, she continued working toward completion of her Master's degree (expected in December, 2010), and is currently pursuing her PhD at Northern Arizona University in Flagstaff, AZ.