DIVERSITY AND ECOLOGY OF MYCORRHIZAL FUNGI ASSOCIATED WITH OAK SEEDLINGS IN THE APPALACHIAN MOUNTAINS

by

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(ABSTRACT)

Diversity of ectotrophic mycorrhizal (EM) fungi on out-planted seedlings of two oak species (*Quercus rubra* and *Q. prinus*) was estimated at two sites in mature mixed forests in the southern Appalachian mountains. Late – stage fungi were well represented. Total richness was 73 types, with 42 types having a frequency of only one. Thelephoroid / tomentelloid, russuloid, and cortinarioid groups were the richest. Dominant fungi included a putative *Tuber* sp. and *Craterellus* sp., and *Laccaria* cf *laccata*. Diversity was lower at a high elevation chestnut oak dominated site compared to a lower mesic cove – hardwood forest site. There was little evidence for fungal specificity to red oak versus white oak seedlings.

We also compared EM fungus distributions on root systems of oak seedlings from samples taken in mid-July and early-September. The majority of EM types occurred only in the mid- or late-summer samples respectively. Dramatic shifts in mycobiont dominance were observed in relation to sample date, including increases in *Cortinarius* spp. richness, decreases in Thelephoraceae richness, and the disappearance of *Amanita* spp. types in the late- compared to mid-summer samples. A multi-stage model of seasonal EM

dynamics is proposed, with implications for the niche expansion of associated phytobionts. In this model, generalistic mycobionts are most frequent and occur throughout the season. Other more specialized fungi show seasonal specificity.

Relationships between species and communities of EM fungi and environmental parameters such as ericoid shrub abundance and edaphic characteristics were also examined. High diversity of EM fungi limited resolution of community level relationships given our sample sizes. Intraspecific variation in EM fungi with regard to microsite characteristics was also undetectable. No association between ericoid shrub dominance (*Kalmia latifolia* and *Rhododendron maximum*) and EM fungi was observed. We present a listing of EM fungus types with associated ranges of edaphic parameters and ericoid shrub abundance.

The family Sebacinaceae is a basal hymenomycete lineage that includes members of the genera *Tremellodendron* and *Sebacina.* We present evidence suggesting the putative mycorrhizal status of two species of *Tremellodendron. Tremellodendron* appears to form both endophytic associations with achlorophyllous orchids and ectomycorrhizae with species of *Quercus, Pinus* and *Tilia cordata*.

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INTRODUCTION

Rhododendron maximum: background and seedling inhibition

Throughout the southern Appalachians Mountains, *Rhododendron maximum* L. (Ericaceae) (Rm) is a dominant subcanopy evergreen species on mesic north facing slopes, stream banks, and in coves. The Rm shrubs form dense thickets known as 'slicks', and achieve heights of 4-5 m. Mycorrhizal associations of Rm are primarily with ericoid mycoboints, which form intracellular coils in the cortical cells of Rm fine roots. Few extramatrical hyphae are associated with these dense intracellular coils, however. In addition, Dighton and Coleman (1992) reported ectomycorrhizal colonization of Rm, including *Cenococcum geophilum*, but were unable to quantify their occurrence. Largent and Sugihara (1980) also reported evidence of ectomycorrhizal colonization of *Rhododendron* spp. However, Smith et al. (1995) found that *Rhododendron macrophyllum* D. Don *ex* G. Don was rarely colonized by ectomycorrhizal fungi when grown in a greenhouse in soils from young Douglas fir forests in the Pacific Northwest, USA.

At the USDA Forest Service Southern Research Station Coweeta Hydrologic Laboratory (Coweeta) experimental forest, Rm occupies about 30% of the forest and has expanded more than 20% since 1975 (Dobbs, 1995). Historically, areas where Rm occurs are also known to be highly

productive for the generation of quality hardwood lumber. The perception of strong canopy tree seedling inhibition in Rm thickets, beginning in the 1940's (Minkler, 1941; Wahlenberg & Doolitle, 1950), has engendered considerable concern among foresters and plant ecologists. Wahlenberg and Doolitle (1950) stated that "the hardwoods are hopelessly inhibited within the *R. maximum* slicks in comparison to outside the slicks". More recently, Phillips and Murdy (Phillips & Murdy, 1985) found that hardwood regeneration and rates of succession were reduced in Rm thickets. Significantly lower seedling density in gaps with heavy Rm cover was reported by Clinton et al. (1994). Numerous authors have considered Rm to be a problem weed for hardwood production (e.g. Martinez, 1975), and control programs have been investigated (Hooper, 1969; Neary et al., 1984; Romancier, 1971; Wahlenberg & Doolitle, 1950). Yet the biological basis of the interaction between Rm and tree seedlings remains poorly understood. Therefore research into the mechanisms by which Rm thickets suppress the growth of canopy tree seedlings remains a high priority for forest biologists and foresters alike. One potential mechanism for this suppression is inhibition of mycorrhizal colonization by Rm thickets, either by competition for space and nutrients or production of inhibitory compounds by Rm.

Several other studies have examined the relationship between mycorrhizal fungi and allelopathy. Reduced ectomycorrhizal colonization and growth of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco)

seedlings in response to litter leachates *in vitro* was reported by Rose et al. (1983). Robinson (1972) found that "living" heather (*Calluna vulgaris* [L.] Hull; Ericaceae) produced a compound that reduced the growth of ectomycorrhizal fungi. Lyon and Sharpe (1996) found that hayscented fern (*Dennstaedtia punctilobula* [Michx.] Moore) reduced growth and mycorrhizal colonization of northern red oak (*Quercus rubra* L.) in mini-terracosms. Hansen and Dixon (1987) carried out a pot study with red oak and reported that inoculation with an ectomycorrhizal fungus (*Suillus luteus* [L.] Fr.) reduced the allelopathic effect of interrupted fern (*Osmunda claytoniana* L.) on seedling mortality in a pot study.

Similar interactions between ericaceous shrubs and tree seedlings have been the focus of research in the Pacific Northwest (Messier, 1993) and central Newfoundland (Yamasaki et al., 1998). In coastal British Columbia, salal (*Gaultheria shallon* Pursh), a member of the Ericaceae like Rm, inhibits the growth and survival of seedlings. Messier (1993) found no difference between levels of mycorrhizal colonization in sites with various densities of salal, and similar results for vegetation removal versus control treatments. In Messier's (1993) study containerized seedlings also were not colonized differentially over a range of salal planting densities. Messier (1993) attributed the seedling inhibition to competition for limited soil resources between the seedlings and the salal, and ruled out light as a factor because the seedlings were taller than the salal. In central Newfoundland, Yamasaki et al. (1998) found

that black spruce (*Picea mariana* Mill.) seedlings grown close to mountain laurel (*Kalmia angustifolia* L.) had significantly lower mycorrhizal colonization (66%) than seedlings grown far from *K. angustifolia* (96%). The reduced mycorrhizal colonization was attributed to allelopathic compounds produced by the *K. angustifolia*, based on the results of *in vitro* studies by Titus et al. (1995).

Results from previous work at Virginia Tech

Previous studies on seedling suppression in Rm thickets were conducted at Coweeta. Field plots were established at an elevation of 1000 m on a single north-facing slope. The site was dominated by mature northern red oak (*Quercus rubra*), and also included hickory (*Carya* spp.), red maple (*Acer rubrum* L.) yellow-poplar (*Liriodendron tulipifera* L.), sweet birch (*Betula alleghaniensis* L.), eastern hemlock (*Tsuga canadensis* L.), witch hazel (*Hamamelis virginiana* L.), and flowering dogwood (*Cornus florida* L.). Tree species composition was similar in blocks within and outside Rm thickets. The following sections summarize the findings of these studies.

Suppression of mycorrhizae

Inhibition of mycorrhizal synthesis in situ

Examination of root systems of seedlings collected from all experimental beds indicated that ectomycorrhizal synthesis is inhibited in the presence of Rm. mycorrhizal root tips were three times fewer (61.9% vs 18.8 %) on *Tsuga canadensis* seedlings and about 20% less (71.1% vs 54.2%) on *Q. rubra* seedlings, when in the presence of Rm, compared to that for seedlings outside of the Rm thicket after the first year of growth (Figure 1).

The difference in infection rate decreased in the second and third year which may have been due to increased mortality of plants without mycorrhizal synthesis (Walker, 1998; Walker et al., in press).

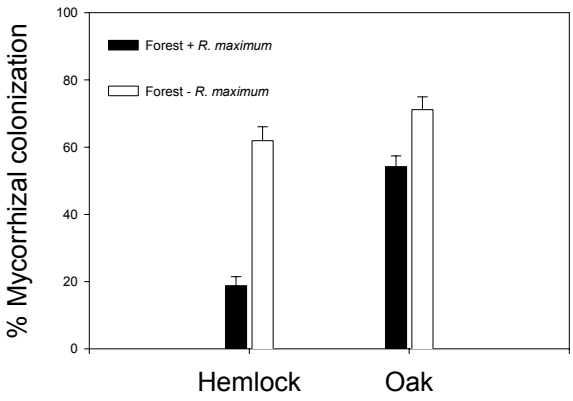

Figure 1. Inhibition of mycorrhizae on first year seedlings of two tree species planted in forest with and without R. maximum in the subcanopy. Both groups are statistically significantly different.

Ectomycorrhizal fungus availability:

The relative abundance and diversity of ectomycorrhizal sporocarps in the two forest types were equivalent (Walker & Miller, 2002). However, there were some differences in the distribution of certain ectomycorrhizal species between

forest types (Walker & Miller, 2002). Although the survey of mycorrhizal sporocarps indicated a rich and diverse community of ectomycorrhizal fungi in the rhizosphere under Rm thickets (Walker & Miller, 2002), seedlings were primarily colonized by a single generalistic mycobiont (*Cenococcum geophilum* Fr.) when grown under the thickets (Walker et al., 1999). The seedlings were either incompatible with the majority of the mycobionts under the Rm thickets or the process of mycorrhization was inhibited under the thickets (Walker & Miller, 2002).

Allelopathic inhibition of mycorrhizal growth

Three ectomycorrhizal fungi were inoculated onto plates (ten each) containing sterilized media including pieces of fresh Rm leaves or media without pieces of Rm leaves. The isolates of *Cenococcum geophilum* and *Piloderma bicolor* did not show reduced growth rates while *Pisolithus tinctorius* showed highly significant reduction in growth on the medium with Rm leaf pieces. This experiment was repeated with *Pisolithus tinctorius* using the same treatments but including controls of media containing pieces of leaves from four dominant canopy tree species. Inhibition by leaves of other canopy tree species was equal to or greater than that of Rm. Therefore toxicity of Rm leaves is not a likely explanation for differential mycorrhizal synthesis between forest with and without Rm (Nilsen et al., 1999; Walker et al., 1999). Litter from birch, red oak, and cherry inhibited growth of *P. tinctorius* over 50% more than

that of *R. maximum*. The radial growth of *P. tinctorius* was not inhibited by leachates from *R. maximum* litter or organic layer substrates.

Ericoid inhibition of ectomycorrhizae

Plates that were dual-inoculated with the ericoid mycorrhizal fungus *Hemenoscyphous ericae* and isolates of various ectomycorrhizal fungi showed no strong evidence of somatic incompatibility. There was no evidence for a zone of inhibition in any confrontation. Based on these experiments, there is no indication that ericoid mycorrhizae inhibit the growth of ectomycorrhizal fungi (Nilsen et al., 1999).

Molecular techniques in mycorrhizal ecology

Before the recent advent of DNA-based methods for typing and identification of symbiotic fungi from ectomycorrhizal root tips (Gardes & Bruns, 1993), studies investigating ectotrophic mycobiont diversity and ecology were relegated to the use of sporophore abundance and morphotyping. Both methods produced important information and meaningful conclusions. However, each had limitations which curtailed exploration of the "black box" enveloping the distributions and ecological interactions of strictly hypogeous and infrequently fruiting mycobiont thalli (Horton & Bruns, 2001). While molecular typing presents promising new options for the mycorrhizal researcher, we are only

beginning to develop the baseline data needed to evaluate the power and limitations of these methods.

Applicability of sporophore distributions to ecological questions has been discussed frequently in previous literature (Gardes & Bruns, 1996; Walker & Miller, 2002). The limitations revolve largely around the failure of sporophore records to accurately map the fungal thallus in its substrate, and lack of information regarding the specificity of the interaction between the fungi and the roots of a given tree species. While sporophore observations allow for reasonably large sampling regimes (depending strongly on identification skills), thalli of dominant fungi may likely be present in niches which are not conducive to production of sporophores. Furthermore fungi that do not fruit above ground are infrequently detected in sporophore collections and would not be included in most diversity estimates based on sporophores.

The main limitation of classical morphotyping of ectomycorrhizal roots is sample size, owing to the time consuming necessity of detailed microscopic observation. Additionally, morphologically similar ecological types may not be separated accurately, yet on the other hand the same species of fungus may have a different appearance on the roots of various tree species (Agerer, 1991), leading to potential fragmentation of ecological data.

The initial promise of molecular applications seems to alleviate many of the restrictions associated with sporophore based and

morphological approaches. Sample sizes can be much larger than with morphotyping alone, and typing can be very accurate (depending on the specific techniques employed). Both the fungus and the tree can be identified, and the data are in an association specific context, detailing host–fungus interactions. Overall diversity may be underestimated where fungi form few associations. However, highlighting the diversity of fungi actively associating with a given component of the plant community can be most valuable when evaluating the influence of the fungi on the plants.

Research Objectives

Because of the discrepancy between high inoculum potential (estimated by sporocarp distributions) and low colonization levels on seedlings in Rm thickets, we conducted research on the diversity and ecology of ectotrophic fungi colonizing the seedlings. This research was initially aimed at identifying and quantifying species of fungi colonizing seedlings in areas with varying levels of ericoid shrub presence in the southern Appalachian Mountains.

In chapter one, we examined how ectotrophic fungus assemblages differed in diversity and abundance between a high and low elevation site, and between a red and a white oak species as host seedlings. The inferences which can be drawn from the diversity of fungi associated with the seedlings in relation to previously noted ericoid shrub effects are also

discussed in the first chapter. The seasonal dynamics of ectotrophic fungus assemblages were explored in chapter two. In chapter three, we considered how EM fungal abundance related to a variety of edaphic parameters and ericoid shrub gradients. The identity of Sebacinaceae mycorrhizal isolates from the seedlings are clarified in chapter four, and a fungal genus assumed to be saprophytic is identified as mycorrhizal.

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Chapter One: Diversity of ectotrophic mycorrhizal fungi on oak seedlings in southern Appalachian Mountain mixed forests.

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Abstract

Diversity of ectotrophic mycobionts on out-planted seedlings of two oak species (*Quercus rubra* and *Q. prinus*) was estimated at two sites in mature mixed forests in the southern Appalachian mountains. The "bait" seedlings captured a high diversity of mycorrhizal ITS-types and late – stage fungi were well represented. Total richness was 73 types, with 42 types having a frequency of only one. Among Basidiomycetes, thelephoroid / tomentelloid, russuloid, and cortinarioid groups were the richest. The Ascomycete *Cenococcum geophilum* was ubiquitously present. Dominant fungi included a putative *Tuber* sp. (Ascomycete), and Basidiomycetes including a putative *Craterellus* sp., and *Laccaria* cf *laccata*. Diversity was lower at a drier high elevation oak forest site compared to a lower mesic cove – hardwood forest site. Fungal specificity for red oak versus white oak seedlings was suggested. The high degree of rarity in this system imposes limitations on the power of community analyses, and identifications based on ITS data in public databases were frequently tentative at the generic level, especially in groups needing systematic attention.

Keywords: Diversity, Molecular typing, Mycorrhiza, *Quercus*, seedling regeneration.

Introduction

Initially, the impetus for this study developed from observations on mycobiont diversity during previous research examining colonization parameters of oak seedlings in relation to *Rhododendron maximum* L. shrub presence (Walker et al., 1999). Macroscopic observations gave the impression that a limited set of dominant fungi were colonizing the seedlings, with most ectomycorrhizal tips (other than those colonized by *Cenococcum geophilum* Fr.) appearing slightly swollen and orange to orange-brown, some being variably ramified and with limited variation in type and presence of extramatrical hyphae (pers. obs.).

Testing this observation became important in the context of differences in the way sporophore distributions and mycorrhizal colonization levels of the oak seedlings related to *R. maximum* presence and absence. While sporophore distributions were similar (Walker & Miller, 2002), the seedlings were poorly colonized in the presence of thickets of *R. maximum* (Walker et al., 1999). The impact of *R. maximum* and the type of differential response observed could be explained if young oak seedlings were only colonized by a limited assemblage within the overall mycobiont community, as our observations suggested.

Questions basic to our understanding of mycorrhizal community development on seedlings remain unanswered. In general, the diversity of EM fungi increases during succession (Helm et al., 1996). In mature

forests, early colonizers remain present and late-stage fungi become more diverse. This is known as the multi-stage model of fungal succession (Visser, 1995). However, phenetic constraints on mycorrhizal development may lead to limited diversity of fungi on seedlings. Conversely, EM diversity on seedlings may be representative of overall EM diversity in a stand. These considerations are also important in regard to the utility of seedlings to bait fungi for estimating diversity.

Another aspect of our study involved a comparison of oak seedling mycobiont diversity at a high versus a low elevation site. Virtually nothing is known about how elevation related environmental parameters influence seedling associated fungus assemblages in the Appalachian Mountains. What is known about elevational changes in mycorrhizal fungus community composition in the Appalachian Mountains comes from sporophores and considered areas with limited host species overlap (Bills et al., 1986). Furthermore, because the two sites for this study are in close proximity to one another and share common host species, but differ markedly in edaphic and climatic characteristics, the presence of overlapping fungal taxa could provide an indication of low ecological specificity.

The objectives of this study were to: 1) provide an estimate of mycobiont diversity on oak seedlings in a fungus rich, mature, mixed forest in the southeastern Appalachian Mountains, 2) to assess the distribution of early and late stage fungi on the seedlings, 3) to determine

whether the diversity of mycobionts on the seedlings explains the previously noted effects of *R. maximum*, 4) to compare seedling mycobiont assemblages on a red versus a white oak species, and 5) to compare seedling mycobiont assemblages at a high versus a low elevation site in close relative proximity. We hypothesized that the diversity might be low based on the previously mentioned morphotype observations, and that late – stage and multi – stage fungi would be found to colonize the seedlings.

In addition to assessing mycobiont diversity and ecology on young oak seedlings, we were interested in adding to the growing information base on molecular applications in mycorrhizal ecology. Two important aspects of this will be: 1) to determine whether the diversity of mycobionts from a subset of the plant root community (i.e. oak seedlings) is in a range that would facilitate correlation with ecological gradients given the sample size recovered in this study, and 2) to determine if current ITS sequence repository databases are sufficient to provide meaningful identifications based on matching with sequenced sporophore vouchers.

Materials and Methods

Site description

The sites for this study were located within the Coweeta Hydrologic Laboratory (Coweeta), part of the NSF Long Term Ecological Research

Station network. Coweeta (35° 02' 29" N, 83° 27' 16" W) is located in the Blue Ridge Mountain Physiographic Province in the southwestern corner of North Carolina. Vegetation types and water availability at Coweeta vary with elevation from lower mesic cove – hardwood forests, mixed – oak at mid-elevations to "xeric" oak – pine forests at higher elevations (Day et al., 1988). Climatically classified as marine, humid; Coweeta experiences relatively high moisture levels and mild temperatures typify the area. Precipitation is distributed equally throughout the season, averaging 180 cm annually (Swank & Crossley, 1988). As a result, diversity of ectomycorrhizal fungi is quite high in this area (Walker & Miller, 2002).

The low elevation mesic site (LM site), with a northwestern aspect, was located upslope from Ball Creek at an elevation of approximately 690 m above sea level. Oak species at the LM site included *Quercus alba* L., *Q. falcata* Michx., *Q. coccinea* Muenchh., *Q. prinus* L., *Q. velutina* Lam., and *Q.rubra* L. (in order of dominance). Other EM host trees present included *Fagus* spp., *Betula* spp., *Tsuga canadensis* Carr, *Carya* spp., and *Pinus* spp.. The drier high elevation site (HD site) was located above Dryman's Fork at approximately 1530 m above sea level and had a north – northeasterly aspect. Oak species in order of dominance included *Q. prinus*, *Q. alba*, *Q. rubra*, and *Q. velutina* at the HD site. In addition, *Betula* spp., *Carya* spp., and *T. canadensis* were present at the HD site. The dominance of chestnut oak (*Q. prinus*) at the HD site is indicative of

rocky, shallow soils with less moisture retention capacity than at the red oak dominated LM site. Soil depths were measured at four locations in each plot and averaged. The average soil depth was significantly lower ($P<.001$) at the HD site (29 cm) than at the LM site (41 cm). Of the two sites it is therefore referred to as the drier site.

Bait seedling propagation

Seedlings of two oak species, *Quercus rubra* and *Q. prinus* (a red and a white oak, respectively), were germinated from acorns collected at Coweeta. The acorns were surface sterilized in 10% bleach solution for 10 minutes and then rinsed with tap water for five minutes prior to sowing in coarse Vermiculite in a greenhouse. *Pinus rigida* Ait. seeds, also collected at Coweeta, were surface sterilized in hydrogen peroxide for twenty minutes and germinated in sterilized sand. After germination, the seedlings were transplanted to nursery cells with coarse vermiculite. After two months of growth, the seedlings were fertilized weekly with quarter strength Hoagland's solution (Hoagland & Arnon, 1950). After four months of growth in the semi-opaque greenhouse, the seedlings were planted out at the field sites during the last week of June, 2000.

At each site (LM and HD) 60 1x2m plots were randomly located along four transects oriented cross – slope. At the LM site, four seedlings of each species (*Quercus rubra* and *Q. prinus*) were planted evenly spaced within each 1x2m plot. At the HD site *Q. rubra* and *Pinus rigida* were

planted, again with four seedlings per plot. There were too few *P. rigida* seedlings surviving to analyze after the first growing season. One randomly chosen seedling from each species/site set (*Q. rubra* at LM and HD; *Q. prinus* at LM) was harvested from each plot with surviving seedlings in mid-July and again in early September, 2001. Herbivores eliminated all seedlings from some plots. At the time of harvest, each seedling was carefully removed and bagged with the roots and surrounding soil as intact as possible. After transportation to the lab, the seedlings were stored at approximately 5°C until processed.

Mycobiont sampling

From the first harvest (July), approximately half of the surviving seedlings were systematically chosen for mycobiont sampling by using a seedling from every other plot along each transect. From the second harvest, all seedlings were sampled. The combined first and second harvest seedling sample totals were 78 *Quercus rubra* seedlings from the HD site, 90 *Q. rubra* seedlings from the LM site, and 86 *Q. prinus* seedlings from the LM site.

The soil was removed from the root system of the seedlings manually. Each root system was examined under a dissecting microscope and all mycorrhizal root tips (excluding those colonized by *Cenococcum geophilum*) were picked free of debris, removed with tweezers and stored frozen in 100µl 2x CTAB buffer. Those colonized by *C.*

geophilum were excluded because they were ubiquitously present and were quantified accurately by morphology in a previous study (Walker et al., 1999). All seedlings were processed within two weeks from the time of harvest.

DNA was extracted from each root tip using CTAB buffer with chloroform:isoamyl alcohol following standard procedures (Hibbett & Vilgalys, 1993). Following extraction, the nuclear 5.8S rRNA gene and the flanking internal transcribed spacer regions I and II were amplified by PCR with primers ITS1F and ITS4 (Gardes & Bruns, 1993; White et al., 1990). After purification of the PCR products with QIAquick PCR Purification Kits (QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355), sequencing reactions were run using the same primers and ABI PRISM® BigDye™ Terminators Cycle Sequencing Kits (Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404 USA). Final amplification products were cleaned and sequenced by the Virginia Bioinformatics Institute Core Lab Facility (Virginia Tech, Blacksburg, VA 24061-0477 USA) using an ABI automated sequencer. Sequences were assembled into sequence types that share 97% or greater similarity, and were manually edited. Unique ITS-types were compared with sporophore voucher sequences by blast searching against (GenBank) and private sequence databases (Jeri L. Parrent and Rytas Vilgalys, Department of Biology, Duke University) for identification.

Names for ITS-types are derived from the closest matching sporophore voucher sequence. The taxonomic specificity of the name reflects the authors' opinion based on the amount of sequence data available for the group, the apparent heterogeneity of the ITS regions in the group, and the level of match between the sample and voucher sequences. To help clarify the distinction between ITS-types and real taxa, type names are not presented in italics.

An additional two types were matched to taxa (*Phialophora finlandia* and a salal root associate) for which the ecological role needs further study, and were therefore excluded from the analyses. Both types occurred on a single root tip each at the LM site, and would not appreciably alter the results of this study. Their inclusion would only slightly increase the difference in diversity we present for the two sites.

Analytical methods

For site comparisons, the frequency for each ITS-type was defined as the number of plots from which the type was isolated from a *Quercus rubra* seedling (the only seedling species planted at both sites), regardless of harvest date. In comparisons between seedling species, frequency was recorded as the number of plots from which the type was isolated from a seedling of the given species at the LM site (the only site with both species), regardless of harvest date. Frequency for each type-site and type-species were entered as a matrix for Reciprocal Averaging ordination

and calculation of diversity measures using PC-ORD Multivariate Analysis of Ecological Data version 3.0 for windows (McCune & Medfford, 1997). Diversity measures comprised Richness (S, total number of types), Evenness (or equitability, E) of Pielou (1969), and Diversity (H) of Greig-Smith (Greig-Smith, 1983), based on Shannon and Weaver (1949). Ordination was performed without the "downweight rare species" option. Euclidean distance was used for the ordination (no option) and therefore was also used for coefficients of determination (R^2).

Results

Total diversity

All ITS-types are listed in Table 1, along with frequencies by site and seedling species, and are organized by frequency at each site. The species – area curve is climbing rapidly at the maximum area sampled (Figure 1). Total richness of putative EM types counting both sites and both seedling species was 73 ITS-types. Of these, 42 types were isolated from only one plot. The highest richness was in tomentelloid / thelephoroid types (15 types), followed by russuloid, and cortinarioid types (11 types each). The most frequent types were cf Tuber #01 (20 plots), Laccaria cf laccata, Cantharellaceae #01, Tomentella terrestris (eight plots each), Lactarius chrysorheus (seven plots), Hebeloma #01, Russula #02 (five plots each), Corticiaceae #01, Russula #04, Russula #06, and Tomentella #01 (four plots each) (Table 1).

Diversity by site (*Quercus rubra* only)

The *Quercus rubra* seedlings at the LM site had a richness of 43 types (S), with 0.91 evenness (E) and diversity (H) equal to 3.43. At the HD site, richness totaled 30 types (S), with E = 0.95 and H = 3.22. While 31 types were only collected at the LM site, only 14 types occurred only at the HD site. Of those types occurring only at the LM site, the richest were again cortinarioid, tomentelloid / thelephoroid (eight types each), and russuloid (six types). The most frequent types occurring only at the LM site included Hebeloma #01, Russula #02 (five plots each), Russula #04 (four plots), Tremellodendron #01, and cf Tuber #02 (three plots each). Frequent types found only at the HD site included cortinarioid, cf *Lactarius* spp., and tomentelloid / thelephoroid types (two types each) (Table 1).

Thirteen types occurred at both the HD and LM sites (considering only *Quercus rubra*). The most frequent types occurring at both sites were Tuber #01 (20 plots), Cantharellaceae #01, Laccaria cf laccata, Tomentella terrestris (eight plots each), Lactarius #01 (seven plots), Tomentella #01, Corticiaceae #01, and Russula #06 (four plots each). Most of the types frequent at both sites were more frequent at the LM site. Tomentelloid / thelephoroid (five types), cf *Russula* spp. (three types) and cf *Lactarius* spp. (two types) were the richest types occurring at both sites (Table 1).

Seedling species specificity (LM site only)

Quercus prinus seedlings had a richness of 34 types (R), evenness (S) of 0.95, and diversity (H) equal to 3.33. For *Q. rubra* seedlings, R = 43, S = .92, and H = 3.46. Of the 59 total types at the LM site, 16 types occurred only on *Quercus prinus* seedlings. However, none of these types had a frequency greater than one. Twenty five of the LM types occurred only on *Quercus rubra* seedlings. Of these, only Bolete #02, Cortinarius #10, Russula #05, Tomentella #01, and Tricholoma #01 (2 plots each) had a frequency greater than one. Eighteen types occurred on both seedling species. The most frequent types on both seedling species included Tuber #01 (Qr = 12, Qp = 5), Cantharellaceae #01 (Qr = 4, Qp = 4), Hebeloma #01 (Qr = 4, Qp = 1), Russula #04 (Qr = 4, Qp = 1) Lactarius #01 (Qr = 3, Qp = 1), Corticiaceae #01 (Qr = 2, Qp = 1), Russula #02 (Qr = 2, Qp = 3), Laccaria cf laccata (Qr = 2, Qp = 4), and Tuber #02 (Qr = 1, Qp = 2) (Table 1).

Ordination results

The RA ordination coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space (an index of the percent of variation in the distance matrix explained by the ordination axis) were axis 1 $R^2 = 0.150$, axis 2 $R^2 = 0.033$, and axis 3 $R^2 = -0.001$. The cumulative R^2 for the first two axes shown (Figure 2) equaled 0.183. Isolated groups of types related to

seedling species / site combinations are clearly delineated on the ordination (Figure 2). These groups represent types occurring only on *Quercus prinus* at the LM site (Qp / LM), types occurring only on *Q. rubra* at the LM site (Qr / LM), and types occurring only on *Q. rubra* at the HD site (Qr / HD). Types with overlapping seedling species affinities from the LM site are spread between the two groups on the left (Qp / LM and Qr / LM). Types occurring only on *Q. rubra* with overlapping site affinities are spread between the Qr / LM and Qr / HD groups. Those types floating in the center show no specificity to site type or seedling species and may be considered generalists (Figure 2).

Discussion

Seedlings and overall diversity

Little is known about the ability of tree seedlings to form mycorrhizal symbioses *in situ* in forests. Gibson and Deacon (1988) found that only older portions of birch sapling root systems were able to form EM with *Lactarius pubescens* (Fr. ex Krombh.) Fr., a late – stage fungus when in a glasshouse. While the use of bait seedlings has been putatively successful in estimating diversity when planted in field soils in laboratories, these studies are conducted under high light conditions. Theoretically, mycorrhizal colonization with shaded seedlings might be limited because seedlings living at low light levels in closed forests have limited and potentially negative carbon budgets.

In our study, seedlings were colonized by a high diversity of mycorrhizal fungi under a closed forest canopy. Horton and Bruns (2001) compiled richness estimates from 14 previous studies. Higher richness was found only in mature (90 year old) Douglas fir/western hemlock and red fir (350-400 year old) forests with 200 and 80 types respectively (Bidartondo et al., 2000; Luoma et al., 1997), even though samples were taken by coring (including the roots of mature trees of various species). Seedlings appear to be compatible with a similar diversity of EM fungi as represented by sporophores in this forest (figure one), which has important implications for the potential for seedlings to acquire carbohydrates from mature canopy trees. If seedling mycobiont diversity was low, the potential for carbon sharing would likely be reduced because fewer hyphal networks could be accessed.

The high diversity of mycorrhizal types which colonized the seedlings in this study also confirms the applicability of using seedlings to effectively document mycobiont diversity *in situ*. In fact, we documented similar total diversity in a single year using bait seedlings and molecular techniques in this study compared to what we found over a three year period in a nearby area relying solely on sporophores (Walker & Miller, 2002) (Figure 1). In a coniferous boreal forest with markedly lower overall diversity, Jonsson et al. (1999a) found that 72% of the ectomycorrhizal types found on mature roots (i.e. in soil cores) were also found on seedling root systems. These findings both support

the conclusion that seedlings in natural forest communities can be colonized by a wide assemblage of EM fungi. However, Baxter et al. (1999) found less than half as many EM types on oak seedlings in comparison to mature oaks in urban and rural oak forests.

Implications for Rhododendron – seedling interactions

Our initial impression of low morphotype diversity on oak seedlings from a previous study with similar conditions proved to be false. Previous studies reported reduced EM colonization levels (ramification index and percent colonization) and diversity in one hand (Walker et al., 1999), with apparently equitable sporophore distributions in the other (Walker & Miller, 2002) where *Rhododendron maximum* thickets were present compared to open forest. The high diversity found in this study indicates that reduced overall colonization levels of oak seedlings in *R. maximum* thickets is not merely due to association with a limited subset of the overall ectotrophic fungus community. It is therefore likely that some component of the environment in the shrub thickets is inhibitory to mycorrhizal colonization. Furthermore, inhibition of mycorrhization is not specific to a small subset of the mycorrhizal fungus community, and it is most likely the process of mycorrhization with seedlings which is inhibited in the thickets.

Notes on fungal community composition

Oak seedlings were associated with a high diversity of fungi typically considered as late – stage forest inhabitants. For example, *Albatrellus* spp., *Amanita* spp., boletes, *Cortinarius* spp., *Gautieria* spp., *Hydnellum* spp., Russulales taxa, *Tricholoma* spp., and truffles can for the most part be considered late – stage (Chu-Chou, 1979; Danielson, 1984; Dighton et al., 1986; Hintikka, 1988; Last et al., 1987). The hypothesis that oak seedlings might be predominantly associated with mixed – stage fungi was not supported. Many of the most frequent fungi were representative of the taxa thought of as ubiquitous and frequently encountered as sporophores, with the notable exception of hypogeous taxa.

Because it is so ubiquitous, *Cenococcum geophilum* was not treated in our analyses. It should be noted, however, that this was the most frequent mycobiont at both sites and on both seedling species. Excluding that, the dominant type was a truffle (Tuber #01) which would have gone unnoticed in most sporophore collections. Several tomentelloid types such as *Tomentella terrestris* and a corticioid type (Corticiaceae #01) were also among the most frequent. Types referred to the common epigeous sporophores *Laccaria laccata*, *Lactarius chrysorrheus*, and *Craterellus lutescens* had high frequency.

The richest epigeous groups were also fairly representative of the local sporophore records, with the typically specious groups such as

Cortinarius spp. and *Russula* spp. being well represented. Considering both hypogeous and epigeous forms, such high richness of Russulaceae taxa and tomentelloid / thelephoroid types is typical of 16 previous studies in coniferous forests synthesized by Horton and Bruns (Horton & Bruns, 2001). Tomentelloid / thelephoroid fungi are clearly important EM associates in the southeastern Appalachian mountain mixed forests as well, being represented by frequent ubiquitous types and relatively high richness at both sites.

This is obviously a rich community with high equitability and diversity. Since only oak seedlings were used, additional fungi specific to other potential host taxa (e.g. *Betula* spp. and *Pinus* spp.) should be expected in the area. Many fungi were recovered only from a single root tip in this study, and the species-area curve is ascending steeply across the range of the area examined. Because of this high diversity, our findings with regard to tree seedling species and site specificity must be interpreted cautiously. Extrapolation to other sites is clearly not possible. Similar cautions were presented in Stendell et al. (1999), Jonsson et al. (1999a), and Jonsson et al. (1999b).

Diversity by site

Richness and diversity of mycorrhizal root tip ITS-types were both higher at the low elevation mesic site (LM), while evenness was higher at the drier high elevation site(HD). Nearly one third of the types at the LM

site were also found at the HD site. At the HD site approximately half of the types found also occurred at the LM site. Species common to both sites were typically more frequent at the LM site. Most groups occurring only at the LM site were also found at the HD site, but had much lower richness. For example, richness of tomentelloid / thelephoroid types at the LM site was four times higher than at the HD site, with eight versus two types respectively. Notably, no Amanita types were found at the HD site.

These observations indicate that at the LM site the mycorrhizal fungus assemblage was richer and dominant species were more frequent than at the HD site, possibly in response to differences in elevation and associated changes in soil water availability and edaphic characteristics. In addition, host tree diversity was lower at the HD site in comparison to the LM site, which should be expected to affect EM fungus composition strongly (Nantel & Neumann, 1992). Diversity and dominance of hypogeous taxa did not increase with elevation. Hypogeous fungi avoid atmospheric aspects of harsher sites at higher elevations where greater exposure and less moisture occur. Multiple sites at high and low elevations in the Appalachian Mountains would be necessary to confirm the generality of these patterns and to more fully test relationships between the environment and EM fungal communities.

Seedling species specificity

Due to the high diversity and evenness at the LM site, little can be said about actual tree seedling species specificity by mycorrhizal fungi for *Quercus rubra* versus *Q. prinus* (a red and white oak respectively) given our sample sizes. However, it is interesting to note that the dominant types found at both the LM and the HD site also occurred on both seedling species at the LM site. Sixteen additional types were added to the total richness estimate for the LM site due to the inclusion of *Q. prinus*.

Mycobiont community analyses

Analyses of mycorrhizal fungus community relationships to environmental gradients in this system lack power. In comparison to informative types, the high number of types occurring only once obscures relationships between species variability and ordination space. The ordination performed for this study captures minimal variation in species composition even at the site level. Variability in species composition at the microsite level that could be correlated with gradients within a site will have to be estimated based on a large number of sites with similar overall characteristics. Results meaningful at the fine ecological scale within which ectotrophic mycorrhizal fungi seem to operate are not possible based on our results. Current methods of direct amplification and sequencing are probably too limited to generate the

sample sizes needed to assess community level relationships in this system.

Notes on identifications

It should be noted that identification of mycobiont species based on matching ITS-types to voucher sporophore sequences is not strictly possible because of lack of heterogeneity between species in some groups. Furthermore, ITS-types can be vouchered just like a sporophore and identifications can be reevaluated as additional sequences become publicly available.

In this study, only two types were identically matched to available Genbank sequences, along with five matched to private sequence databases. Numerous sporophores of known taxa will still have to be sequenced for identification purposes in this ecosystem. The need for sporophore sequencing would be compounded for larger studies. Reliability of identification to the generic level based solely on ITS sequences is variable in relation to the amount of systematic attention the group has received and the evolutionary history of the group. There appears to be a great deal of undocumented diversity in tomentelloid / thelephoroid types, and one type (Thelephoraceae #08) matches a *Tomentella* sp. (563/609; 92%) and a *Thelephora* sp. (561/608; 92%) equally. This is clearly a group where increased sampling effort and traditional systematic study would benefit mycorrhizal ecologists.

Members of the Tuberales seem poorly identified, as indicated by the closest match for the cf Tirmania #01 type. *Tirmania nivea* has not been collected in North America or in temperate forests to our knowledge, and the genus is thought of as strictly a desert taxon. However, *Terfezia gigantea* Imai is known from Pennsylvania, North Carolina and Tennessee (Trappe & Sundberg 1977), so Terfezia #01 may be matched to genus. The closest match to Tuber #01, *Tuber borchii*, seems quite similar in appearance to *T. schearii* which we have collected near the LM site. Both species also have similar sized reticulated spores. Whether *T. schearii* is a better match for the Tuber #01 type needs to be investigated further.

Summary

The ectomycorrhizal fungus assemblage on oak seedlings in mature mixed forests in the southeastern Appalachian mountains is highly diverse with a high proportion of infrequently collected and rare species. Planted seedlings were colonized by a broad range of EM fungi typically characterized as late – or mixed – stage fungi. These results support the multi – stage theory of ectotrophic mycorrhizal fungus succession in well developed EM forests. Mycobiont diversity in this study is reflective of general patterns from previous studies, but is among the highest measured. Diversity was lower at a drier high elevation oak forest site compared to a lower mesic cove – hardwood

forest site. Fungal specificity for red oak versus white oak seedlings was suggested, but not strongly.

High diversity of beneficial tree associated fungi and high numbers of rare species have important implications for conservation of biodiversity. The southeastern Appalachians is apparently a hot spot for ectomycorrhizal diversity, and conservation efforts should include large areas spread through all habitat types in order to capture as many rare species as possible.

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Table 1: Frequency of mycorrhizal ITS-types. LM, low elevation mesic site; HD, drier high elevation site; Qr, *Quercus rubra* seedling; Qp, *Q. prinus* seedling. *Types not included in diversity estimates and ordination.

	Frequency					
Type	**LM**	**HD**	**Qr**	**Qp**	**Closest matching sporophore voucher**	**Code**
Hebeloma #01	5	0	4	1	dfmo0659 *Hebeloma* sp. 624/624 (100%)	HEBELOMA
Russula #02	5	0	2	4	*Russula decolorans* 435/459 (94%)	RUSSUL02
Russula #04	4	0	4	1	*Russula pascua* 345/361 (95%)	RUSSUL04
Tremellodendron #01	3	0	2	1	*Tremellodendron pallidum* 457/509 (89%)	TREMELL1
cf Tuber #02	3	0	1	2	*Tuber borchii* 426/466 (91%)	TUBER2
Albatrellus #01	2	0	1	1	*Albatrellus flettii* 169/171 (98%)	ALBATREL
Bolete #02	2	0	2	0	*Chalciporus piperatus* (408)	BOLETE2
Cortinarius #03	2	0	1	1	*Cortinarius traganus* 454/478 (94%)	CORTIN03
Cortinarius #04	2	0	1	1	*Cortinarius traganus* 511/558 (91%)	CORTIN04
Lactarius #05	2	0	1	1	*Lactarius quietus* 529/569 (92%)	LACTAR5
Russula #05	2	0	2	0	dfmo2008 *Russula* species O 590/593 (99%)	RUSSUL05
Thelephoraceae #01	2	0	1	1	*Thelephora americana* 548/604 (90%)	THELEPH1
Tricholoma #01	2	0	2	0	*Tricholoma pardinum* 523/569 (91%)	TRICHOL1
Amanita cf gemmata	1	0	0	1	*Amanita gemmata* 627/628 (99%)	AMANITA1
Amanita #02	1	0	1	1	*Amanita muscaria* 503/527 (95%)	AMANITA2
Amanita DFMO1078	1	0	1	0	*Amanita* 637/637 (100%)	AMANITA3
Athelia cf neuhoffii	1	0	1	0	*Athelia neuhoffii* 177/178 (99%)	ATHELIA
Bolete #03	1	0	1	0	*Boletus mirabilis* (s=266)	BOLETE3
Cortinarius #01	1	0	1	0	*Cortinarius traganus*	CORTIN01
Cortinarius #05	1	0	0	1	*Cortinarius traganus* 339/368 (92%)	CORTIN05
Cortinarius #06	1	0	1	0	*Cortinarius umbilicatus* 212/241 (87%)	CORTIN06
Cortinarius #07	1	0	1	0	*Cortinarius* sp. 299/311 (96%)	CORTIN07
Cortinarius #09	1	0	1	0	*Cortinarius* sp. 559/589 (94%)	CORTIN09

Table 1 continued

	Frequency					
Type	**LM**	**HD**	**Qr**	**Qp**	**Closest matching sporophore voucher**	**Code**
Cortinarius #11	1	0	1	0	*Cortinarius leucopus* 367/393 (93%)	CORTIN11
Entolomataceae #01	1	0	1	0	*Entoloma nitidum*	ENTOLOMA
Gautieria #01	1	0	1	0	*Gautieria* sp. SLTahoe2264CA 166/167 (99%)	GAUTIER1
Gautieria #02	1	0	1	0	*Gautieria monticola* 152/160 (95%)	GAUTIER2
Hydnellum #01	1	0	0	1	*Hydnellum diabolus* 218/225 (96%	HYDNELLU
Lactarius #02	1	0	1	0	dfmo1034 *Lactarius volemus* 576/592 (97%)	LACTAR2
Russula #01	1	0	0	1	*Russula postiana* 470/510 (92%)	RUSSUL01
Russula #03	1	0	0	1	*Russula rosacea* 305/335 (91%)	RUSSUL03
Russula #08	1	0	0	1	dfmo1104 *Russula* 480/480 (100%)	RUSSUL08
Peziza #01	1	0	0	1	dfmo1344 *Peziza* 557/564 (98%)	TIRMANI2
Thelephoraceae #02	1	0	0	1	*Thelephora penicillata* 575/630 (91%)	THELEPH2
Thelephoraceae #04	1	0	0	1	*Tomentella cinerascens* 584/626 (93%)	TOMENT02
Thelephoraceae #05	1	0	0	1	*Tomentella galzinii* 463/516 (89%	TOMENT06
Thelephoraceae #06	1	0	1	0	*Tomentella cinerascens* 359/389 (92%)	TOMENT07
Thelephoraceae #08	1	0	1	0	*Tomentella* sp. 563/609 (92%)	TOMENT09
Thelephoraceae #09	1	0	0	1	*Tomentella* sp. 587/635 (92%)	TOMENT10
Tomentella #02	1	0	0	1	*Tomentella* sp. 632/646 (97%)	TOMENT11
Tremellodendron #03	1	0	0	1	*Tremellodendron pallidum* 287/304 (94%)	TREMELL3
Tricholoma #02	1	0	1	0	*Tricholoma mutabile* 343/376 (91%)	TRICHOL2
Tricholoma #03	1	0	1	0	*Tricholoma muricatum* 504/526 (95%)	TRICHOL3
cf Tuber #01	14	6	12	5	*Tuber borchii* (S=656) maculatum (s=656)	TUBER1
Cantharellaceae	7	1	4	4	*Craterellus lutescens* 287/305 (94%)	CRATER1
Laccaria cf laccata	5	3	2	4	dfmo0370 *Laccaria laccata* 431/433 (99%)	LACCARIA
Tomentella terrestris	5	3	3	2	*Tomentella terrestris* (906)	TOM_TERR
Lactarius chrysorheus	3	4	3	1	*Lactarius chrysoreus* 652/652 (100%)	LACTAR1

Table 1 continued:

	Frequency					
Type	**LM**	**HD**	**Qr**	**Qp**	**Closest matching sporophore voucher**	**Code**
Corticiaceae #01	3	1	2	1	Uncultured cf. *Piloderma* 382/417 (91%)	PILODERM
Russula #06	2	2	1	1	*Russula emetica* 178/184 (96%)	RUSSUL06
Tomentella #01	2	2	1	0	*Tomentella galzinii* 561/583 (96%)	TOMENT05
Cortinarius #10	2	1	3	0	*Cortinarius* sp. O14 473/502 (94%)	CORTIN10
Russula #11	1	2	1	0	*Russula raoultii* 420/441 (95%)	RUSSUL11
Thelephoraceae #03	1	2	0	1	*Tomentella fusco-cinerea* (509/568; 89%)	TOMENT01
Tomentella #03	1	2	0	1	*Tomentella subclavigera* 602/614 (98%)	TOMENT03
Clavariaceae #01	1	1	1	0	*Clavulina cinerea* 598/641 (93%)	CLAVULIN
Lactarius #03	1	1	1	0	*Lactarius deliciosus* 181/187 (96%)	LACTAR3
Russula #09	1	1	1	0	*Russula pascua* 467/480 (97%)	RUSSUL09
Tomentellopsis zygodesmoides	1	1	0	1	*Tomentellopsis zygodesmoides* 531/531 (100%)	TOM_ZYGO
***Phialophora**	1	0	1	0	*Phialophora finlandia*	
***Salal Associate**	1	0	0	1	Salal root associated fungus 309/342 (90%)	
Bolete #01	0	3	0	0	*Xerocomus pruinatus* 179/182 (98%)	BOLETE1
Russula #07	0	2	0	0	*Russula integra* 565/609 (92%)	RUSSUL07
Amphinema #01	0	1	0	0	*Amphinema* sp. 170/174 (97%)	AMPHINEM
Boletus auriporus	0	1	0	0	dfmo4639 *Boletus auriporus* 601/601 (100%)	BOLETE4
Cortinarius #02	0	1	0	0	*Cortinarius traganus* (524/554; 94%)	CORTIN02
Cortinarius #08	0	1	0	0	*Cortinarius* sp. 555/577 (96%)	CORTIN08
Lactarius #04	0	1	0	0	*Lactarius deliciosus* 173/177 (97%)	LACTAR4
Lactarius #06	0	1	0	0	*Lactarius utilis* 376/391 (96%)	LACTAR6
Russula #10	0	1	0	0	*Russula puellula* 526/560 (93%)	RUSSUL10
cf Terfezia #01	0	1	0	0	*Terfezia claveryi* 301/326 (92%)	TERFEZIA
cf Tirmania #01	0	1	0	0	*Tirmania nivea* 109/119 (91%)	TIRMANI1
Tomentella cf sublilacina	0	1	0	0	*Tomentella sublilacina* 522/526 (99%)	TOMENT04

	Frequency					
Type	**LM**	**HD**	**Qr**	**Qp**	**Closest matching sporophore voucher**	**Code**
Thelephoraceae #07	0	1	0	0	*Tomentella* sp. 435/485 (89%)	TOMENT08
Tremellodendron #02	0	1	0	0	*Tremellodendron pallidum*	TREMELL2

FIG. 1. Species versus area curve – average number of species versus number of plots for all ITS-types, based on sub-sampling with 500 repetitions.

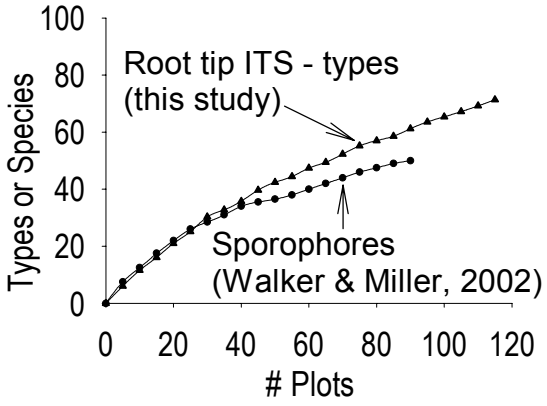
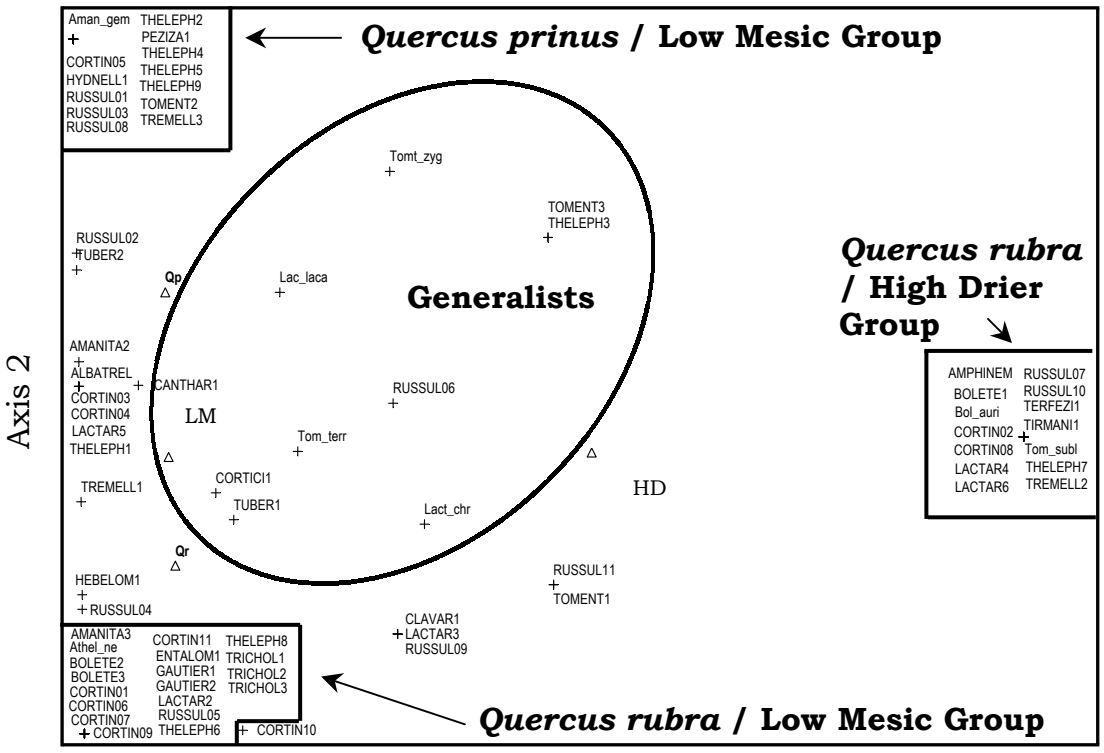


FIG 2. Reciprocal averaging ordination of ITS-types by site and bait seedling species. Triangles: LM, low elevation mesic site; HD, drier high elevation site; Qr, *Quercus rubra* seedlings; and Qp, *Q. prinus* seedlings; Crosses: EM fungi, fungus codes given in Table one. Groups of types which occur uniquely in one site / species combination are designated, along with generalists which overlap in site and seedling species occurrence.



Chapter Two: Seasonal dynamics of ectotrophic fungus assemblages on oak seedlings in the southeastern Appalachian Mountains.

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Abstract

The potential for seasonal dynamics in ectotrophic mycorrhizal (EM) fungus assemblages has important implications for the ecology of both the host trees and the fungal associates. We compared EM fungus distributions on root systems of out-planted oak seedlings at two sites in mixed southeastern Appalachian Mountain forests at the Coweeta Hydrologic Laboratory in North Carolina, from samples taken in mid-July and early-September. Species level EM fungus type specificity, and identification in some cases, was enabled by direct sequencing of the mycobionts from the seedling roots. Seventy three EM fungus ITS-types were documented, most of which occurred only in the mid- or late-summer samples respectively. Generalistic dominants were found fairly equally at both sites and on both sample dates. Dramatic shifts in mycobiont dominance were observed in relation to sample date, including increases in *Cortinarius* spp. richness, decreases in Thelephoraceae richness, and the disappearance of *Amanita* spp. types in the late- compared to mid-summer samples. Patterns similar to sporophore assessments, systematic affinity of shifting EM types, and the generalistic nature of non-seasonal types all lend support to our assessment of dramatic seasonal variability in EM associations in this system. A multi-stage model of seasonal EM dynamics is proposed, with implications for the niche expansion of associated phytobionts.

Introduction

Temporal dynamics of mycorrhizal fungus assemblages have been studied more intensively in arbuscular mycorrhizal (AM) associations than in ectotrophic mycorrhizal associations (EM). For AM types, there are data on seasonal patterns of root colonization on a non-type specific basis (Allen et al., 1998; Gavito & Varela, 1993), and seasonal sporulation data for given species (Gemma et al., 1989; Rich & Schenck, 1981). However, temporal patterns associated with EM types are known only for higher scale changes such as during stand development, and are based largely on sporophore distributions and morphotype analyses (typing based on macro- and microscopic characteristics). Seasonality is strong and well documented for sporophore production in many EM fungus species, for example see Miller (1977). Still, virtually nothing is known about type specific aspects of seasonal dynamics for either AM or EM fungi on plant roots. For AM fungi, type specific dynamics within and between years is cited as being little studied by Abbott and Gazey (1994), who also noted difficulties associated with typing and identification. Dahlberg and Stenlid (1995) noted the dearth of information on this topic regarding EM fungi, and strongly promoted the need for better understanding of EM temporal dynamics. A single prior study reporting limited observations on the seasonal dynamics of EM associations is a rare example which will be discussed later (van der Heijden & Vosatka,

1999). Historically, it was believed that EM fungi store carbohydrates assimilated by the phytobiont in the first portion of the growing season when the mycorrhizae are abundant, and then shift resources to sporophore production later in the year. However, methodological limitations restricted our ability to assess these patterns until recently.

Dependence on morphotyping EM root tips imposed limitations on type specificity and the potential for mycobiont identification which precluded the type of study described herein. In this study, we compared EM fungus assemblages from mid- and late- season samples on oak seedlings in a mature mixed forest in the southeastern Appalachian Mountains. EM fungus type frequency data were derived from direct amplification and sequencing of the ITS region of the mycobiont from EM root tips on out-planted seedlings. We hypothesized that there would be differences in the mid- and late-season assemblages of EM fungi on the seedlings. The relationship between seasonal patterns of EM sporophore production and patterns for EM types found on the seedlings will also be compared.

Information on the seasonal dynamics of the EM fungi is important in the plant specific context that carbohydrate expenditures to EM fungi occur during the time period when the EM are active on the roots. Furthermore, this is the time period during which nutrients and water are available to the plant from the fungus. Thus seasonal dynamics of major resource pools are strongly tied to seasonal dynamics of EM

colonization and persistence. Seasonal plasticity in EM fungus associations may translate into ecological flexibility for the phytobiont. These aspects along with implications for future research on EM fungus communities will be discussed.

Materials and Methods

Site description

The sites for this study were located within the Coweeta Hydrologic Laboratory (Coweeta), part of the NSF Long Term Ecological Research Station network. Coweeta (35° 02' 29" N, 83° 27' 16" W) is located in the Blue Ridge Mountain Physiographic Province in the southwestern corner of North Carolina. Climatically classified as marine, humid; Coweeta experiences relatively high moisture levels and mild temperatures typify the area. Precipitation is distributed equally throughout the season, averaging 180 cm annually (Swank & Crossley, 1988). As a result, diversity of ectomycorrhizal fungi is quite high in this area (Walker & Miller, 2002).

The low elevation mesic site (LM site), with a northwestern aspect, was located upslope from Ball Creek at an elevation of approximately 690 m above sea level. The drier high elevation site (HD site) was located above Dryman's Fork at approximately 1530 m above sea level and had a north – northeasterly aspect.

Bait seedling propagation

Seedlings of two oak species, *Quercus rubra* and *Q. prinus* (a red and a white oak, respectively), were germinated from acorns collected at Coweeta. The acorns were surface sterilized in 10% bleach solution for 10 minutes and then rinsed with tap water for five minutes prior to sowing in coarse Vermiculite in a greenhouse. *Pinus rigida* Ait. seeds, also collected at Coweeta, were surface sterilized in hydrogen peroxide for twenty minutes and germinated in sterilized sand. After germination, the seedlings were transplanted to nursery cells with coarse vermiculite. After two months of growth, the seedlings were fertilized weekly with quarter strength Hoagland's solution (Hoagland & Arnon, 1950). After four months of growth in the semi-opaque greenhouse, the seedlings were planted out at the field sites during the last week of June, 2000.

At each site (LM and HD) 60 1x2 m plots were randomly located along four transects oriented cross – slope. At the LM site, four seedlings of each species (*Quercus rubra* and *Q. prinus*) were planted evenly spaced within each 1x2m plot. At the HD site *Q. rubra* and *Pinus rigida* were planted, again with four seedlings per plot. There were too few *P. rigida* seedlings surviving to analyze after the first growing season. One randomly chosen seedling from each species/site set (*Q. rubra* at LM and HD; *Q. prinus* at LM) was harvested from each plot with surviving seedlings in mid-July and again in early September, 2001. Herbivores eliminated all seedlings from some plots. At the time of harvest, each

seedling was carefully removed and bagged with the roots and surrounding soil as intact as possible. After transportation to the lab, the seedlings were stored at approximately 5°C until processed.

Mycobiont sampling

From the first harvest (July), approximately half of the surviving seedlings were systematically chosen (by using a seedling from every other plot along each transect) for mycobiont sampling. From the second harvest, all seedlings were sampled. The first harvest seedling sample totals were 20 *Quercus rubra* seedlings from the HD site, 31 *Q. rubra* seedlings from the LM site, and 33 *Q. prinus* seedlings from the LM site. From the second harvest seedling sample totals were 58 *Quercus rubra* seedlings from the HD site, 59 *Q. rubra* seedlings from the LM site, and 53 *Q. prinus* seedlings from the LM site. There were 284 and 309 root tip samples from the first and second harvests respectively.

The soil was removed from the root system of the seedlings manually. Each root system was examined under a dissecting microscope and all mycorrhizal root tips (excluding those colonized by *Cenococcum geophilum* Fr.) were picked free of debris, removed with tweezers, and stored frozen in 100µl 2x CTAB buffer. Those colonized by *C. geophilum* were excluded because they were ubiquitously present and were quantified reasonably accurately by morphology in a previous study

(Walker et al., 1999). All seedlings were processed within two weeks from the time of harvest.

DNA was extracted from each root tip using CTAB buffer with chloroform:isoamyl alcohol following standard procedures (Hibbett & Vilgalys, 1993). Following extraction, the nuclear 5.8S rRNA gene and the flanking internal transcribed spacer regions I and II were amplified by PCR with primers ITS1F and ITS4 (Gardes & Bruns, 1993; White et al., 1990). After purification of the PCR products with QIAquick PCR Purification Kits (QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355), sequencing reactions were run using the same primers and ABI PRISM® BigDye™ Terminators Cycle Sequencing Kits (Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404 USA). Final amplification products were cleaned and sequenced by the Virginia Bioinformatics Institute Core Lab Facility (Virginia Tech, Blacksburg, VA 24061-0477 USA) using an ABI automated sequencer. Sequences were assembled into sequence types that share 97% or greater similarity, and were manually edited. Unique ITS-types were compared with sporophore voucher sequences by blast searching against (GenBank) and private sequence databases (Jeri L. Parrent and Rytas Vilgalys, Department of Biology, Duke University) for identification.

Names for ITS-types are derived from the closest matching sporophore voucher sequence. The taxonomic specificity of the name reflects the authors' opinion based on the amount of sequence data

available for the group, the apparent heterogeneity of the ITS regions in the group, and the level of match between the sample and voucher sequences. Two types were matched to taxa (Phialophora finlandia and a salal root associate) for which the ecological role needs further study, and were therefore excluded from the analyses. Both types occurred on a single root tip each at the LM site, and their inclusion would not appreciably alter the results of this study.

Analytical methods

The site – frequency for each ITS-type was defined as the number of plots from which the type was isolated from a seedling regardless of harvest date. Seasonal – frequency was recorded as the number of plots from which the type was isolated from on that sample date regardless of site. Frequency for each type – site and type – sample date were entered as a matrix for Reciprocal Averaging ordination (RA) using PC-ORD Multivariate Analysis of Ecological Data version 3.0 for windows (McCune & Medfford, 1997). Ordination was performed without the "downweight rare species" option. Euclidean distance was used for the ordination (no option) and therefore was also used for coefficients of determination (R^2). The ordination was rotated +40 degrees.

Results

A total of 73 EM fungus ITS-types were documented. Thirty one types were recovered only from the first harvest in mid-July (Table 1), 25

types were recovered only in early-September (Table 2), and 17 types were recovered from both harvests (Table 3). The RA ordination coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space (an index of the percent of variation in the distance matrix explained by the axis) were axis 1 $R^2 = 0.558$, axis 2 $R^2 = 0.237$, and axis 3 $R^2 = -0.001$. The cumulative R^2 for the first two axes shown (Figure 1) equaled 0.795. Grouping of ITS-types is evident at the site level and within sites at the sample date level. Fungi lacking site affinity also typically lacked seasonality (Figure 1).

Discussion

There is strong support for seasonal shifts in the EM assemblage on oak seedlings in this system. Most of the EM fungus ITS-types were found in only one of the two seedling harvests, and the composition of the EM fungus assemblage appears to be quite different between the two harvests. Shifts in dominance were related to seasonal patterns of sporophore production and systematic affinities of the shifting types.

Seasonal patterns of sporophore production are well known for many EM fungus species. Virtually nothing is known, however, regarding the relationship between sporophore production and root colonization and turnover events. While our data likely resolved to species level for most types, unfortunately, identities are not known for many of the types

here and little is known from sporophore records about the fruiting patterns of many of the hypogeous taxa. Nonetheless, the dramatic increase in *Cortinarius* types and the absence of *Amanita* types in the late summer (Table 1-3) is remarkably consistent with generally known seasonal sporophore production patterns. *Cortinarius* types were split between seasons with two types forming mycorrhizae on our seedlings only in the mid-summer, versus nine types which were only represented in the autumn. Species specific identifications of our ITS-types would help clarify these patterns, however mid-summer and fall sporulating *Cortinarius* spp. are well known.

It is apparent that seasonal shifts in EM fungi follow systematic affinities for some groups at least. Three Amanita types were present in the mid-summer samples from the LM site, while there were none in the late summer harvest (Figure 1). *Gautieria* types were only present in the mid summer (Tables 1-3). No Bolete types occurred only in the autumn samples (Tables 1-3). Thelephoroid and tomentelloid types (listed as Thelephoraceae if unresolved at the generic level) were three times richer in the mid-summer samples, with nine types, than in the late summer when there were only three types (Figure 1, Tables 1-3). For *Cortinarius* types this pattern was reversed, as described above. The types that were recovered in both the mid-summer and late summer harvests were very frequently recovered at both sites also (Figure 1).

The only recent study which suggested seasonal patterns of EM fungi was done in *Salix repens* L. stands of Canadian dune ecosystems by van der Heijden and Vosatka (1999). Based on observations of morphotype samples from large numbers of root tips at 16 sites, they hypothesized that relative abundance and nutrient contributions of different morphotypes might change throughout the season. The observed shifts were largely similar among replicate samples within sampling dates, which occurred in October, 1994, August, 1995, and April, 1996. However, van der Heijden and Vosatka (1999) did not emphasize these results because of the potential for spatial variation within field sites, and because they were more interested in interactions between AM and EM fungi on the non-specific *S. repens* hosts. Furthermore, data are presented only for a handful of dominant types, only fifteen types were documented (in spite of 78 sporphore species being present), and typing by morphological characteristics are accepted by the authors as likely being variable to the generic level. In a separate publication, van der Heijden et al. (1999) acknowledge that their *Cortinarius* type may comprise up to 30 species. Of particular interest, however, is their observation that *Cortinarius* only formed abundant mycorrhizae in the autumn. This corroboration between the fine scale results in our study and broader ranging coarser scale results from Canadian *S. repens* stands strongly suggests that seasonal dynamics in

EM communities are real, and occur in at least two disparate ecosystems.

It is acknowledged that there is a high proportion of rare species in our data, and there will remain the potential that rare types exaggerate the apparent seasonal differences in EM fungus assemblages. However, our data show fairly dramatic changes in EM fungus composition which parallels the systematic grouping of the fungi (discussed above). Dominant types, or those with the highest frequency, were typically found at both sites and in both mid- and late-summer. Stendell et al. (1999) sampled EM fungus types (by soil cores) in May in consecutive years and found apparent changes in the assemblage, even though samples from consecutive years were frequently only 25 cm apart. However, in contrast to this study, several dominant types from year one were not represented at all in year two, and dramatic shifts in dominant groups were not reported (Stendell et al., 1999). In fact, based on our results, the inter-annual variation they present could have been explained by fine scale temporal dynamics in addition to high spatial variability. Furthermore, the high degree of spatial variation reported by many authors (Horton & Bruns, 2001) may be exaggerated by seasonal temporal dynamics.

Our results suggest that EM fungus associations are much more dynamic than previously considered. Mid-summer (or earlier) may represent a generalized peak in EM abundance and diversity (based on

our frequency), however the early associations seem to be largely replaced by alternate fungi later in the season. Blasius et al. (1990) found that gross EM root tip abundance varied throughout the growing season and the seasonality varied during separate years. In 1985, peak abundance occurred in May and October, with August being the least abundant time period. In 1986, however, abundance bottomed out in September and October, with peaks in August and November. Furthermore, it was thought that changes in weather patterns influenced the yearly variation in abundance cycles. These data are not incongruous with the idea of alternate EM fungus assemblages for the early and late portions of the season.

A multi-stage model of seasonal succession by EM fungi on tree roots is proposed here. We hypothesize that certain generalist EM fungi associate with roots throughout the season. Other more specialized fungi are more temporally as well as more spatially variable. The potential for well defined early and late season groups of EM fungi associated with roots will require further research, as is the case for other aspects of the model. This model compliments the larger scale temporal dynamics models for EM fungi over successional periods of time, i.e. the multi-stage model of succession *sensu* Visser (1995).

The implication of seasonal dynamics in EM fungus communities impacts how we think of nutrient flux between plants and fungi involved in EM associations. Based on the frequency of ITS-types in our study,

seasonal carbohydrate drain by EM fungi may peak in the in mid-summer or earlier, but should be extended throughout the season by a progression of fungi. Shifting EM fungi throughout the summer should provide the phytobiont with an adaptable assemblage with greater plasticity with regard to associated changes in environmental conditions over the course of the growing period, potentially enhancing nutrient and water uptake for the plant. van der Heijden and Vosatka (1999) also considered that seasonal shifting of AM and EM fungus types, and among EM types, by *Salix repens* contributes to the ecological plasticity of the phytobiont. Thus it appears likely that the diversity of the root – soil interface, *sensu* Pirozynski (1981), which is mediated by EM fungi in this system, is enhanced by the seasonal dynamics of the fungi.

Implications for future studies

A great number of studies have been conducted comparing EM fungus distributions based on a single sample time per year (e.g. Gehring et al., 1998). Generalizations based on these sampling regimes must be restricted to the appropriate portion of the season, and future studies should consider the potential for seasonal dynamics. A great deal remains to be understood about seasonal dynamics within this system and about the applicability of these results to other systems, although similar patterns have now been found in both a dune ecosystem by van der Heijden and Vosatka (1999) and two southern Appalachian Mountain

ecotypes in this study. Seasonality is also promoted as a candidate niche dimension in studies assessing the autecology of EM fungus species.

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Table 1: EM fungus ITS-types for mid-July samples from out-planted oak seedlings in a mature mixed forest in the southeastern Appalachian Mountains.

Type Name	Code	Frequency
Amanita #02	AMANITA2	1
Amanita cf gemmata	Aman_gem	1
Amanita DFMO1078	AMANITA3	1
Athelia cf neuhoffii	Athel_ne	1
Bolete #03	BOLETE3	1
Boletus auriporus	Bol_auri	1
cf Tirmania #01	TIRMANI1	1
Corticiaceae #01	CORTICI1	4
Cortinarius #01	CORTIN01	1
Cortinarius #02	CORTIN02	1
Entolomataceae #01	ENTALOM1	1
Gautieria #01	GAUTIER1	1
Gautieria #02	GAUTIER2	1
Hydnellum #01	HYDNELL1	1
Lactarius #02	LACTAR2	1
Russula #01	RUSSUL01	1
Russula #03	RUSSUL03	1
Russula #05	RUSSUL05	2

Thelephoraceae #01	THELEPH1	2
Thelephoraceae #02	THELEPH2	1
Thelephoraceae #03	THELEPH3	3
Thelephoraceae #04	THELEPH4	1
Thelephoraceae #05	THELEPH5	1
Thelephoraceae #06	THELEPH6	1
Thelephoraceae #09	THELEPH9	1
Tomentella #03	TOMENT3	3
Tomentella cf sublilacina	Tom_subl	1
Tremellodendron #01	TREMELL1	3
Tremellodendron #02	TREMELL2	1
Tricholoma #01	TRICHOL1	2
Tricholoma #02	TRICHOL2	1

Table 2: EM fungus ITS-types for early-September samples from out-planted oak seedlings in a mature mixed forest in the southeastern Appalachian Mountains.

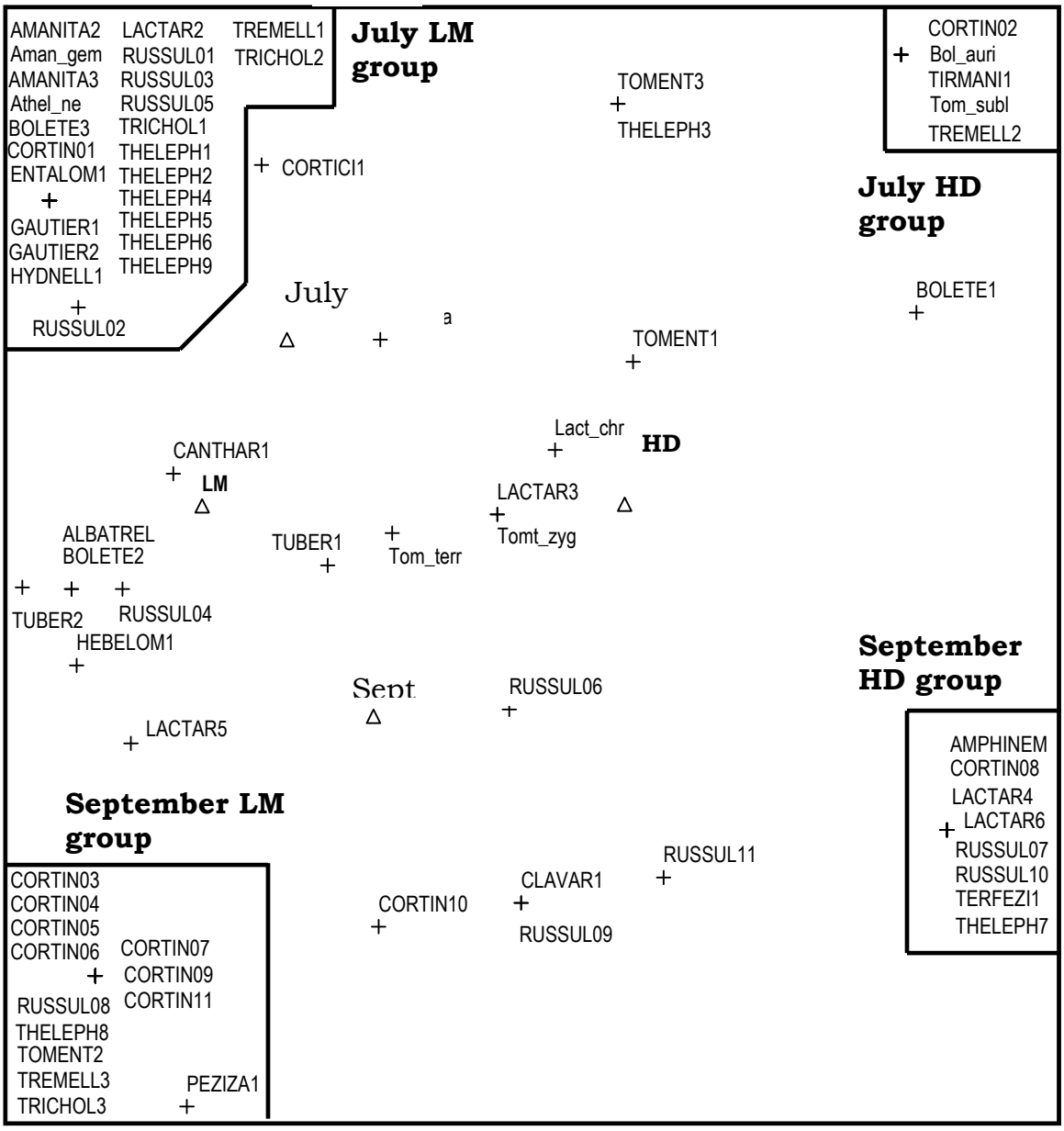
Type Name	Code	Frequency
Amphinema #01	AMPHINEM	1
cf Terfezia #01	TERFEZI1	1
Clavariaceae #01	CLAVAR1	2
Cortinarius #03	CORTIN03	2
Cortinarius #04	CORTIN04	2
Cortinarius #05	CORTIN05	1
Cortinarius #06	CORTIN06	1
Cortinarius #07	CORTIN07	1
Cortinarius #08	CORTIN08	1
Cortinarius #09	CORTIN09	1
Cortinarius #10	CORTIN10	3
Cortinarius #11	CORTIN11	1
Lactarius #04	LACTAR4	1
Lactarius #06	LACTAR6	1
Peziza #01	PEZIZA1	2
Russula #07	RUSSUL07	2
Russula #08	RUSSUL08	1
Russula #09	RUSSUL09	2

Russula #10	RUSSUL10	1
Russula #11	RUSSUL11	3
Thelephoraceae #07	THELEPH7	1
Thelephoraceae #08	THELEPH8	1
Tomentella #02	TOMENT2	1
Tremellodendron #03	TREMELL3	1
Tricholoma #03	TRICHOL3	1

Table 3: EM fungus ITS-types recovered on both sample dates (mid-July and early-September) from out-planted oak seedlings in a mature mixed forest in the southeastern Appalachian Mountains.

		July	Sept
Type Name	Code	Frequency	Frequency
Albatrellus #01	ALBATREL	1	1
Bolete #01	BOLETE1	2	1
Bolete #02	BOLETE2	1	1
Cantharellaceae #01	CANTHAR1	5	3
cf Tuber #01	TUBER1	11	12
cf Tuber #02	TUBER2	1	1
Hebeloma #01	HEBELOM1	2	3
Laccaria cf laccata	Lac_laca	6	2
Lactarius #03	LACTAR3	1	1
Lactarius #05	LACTAR5	1	2
Lactarius chrysorheus	Lact_chr	4	3
Russula #02	RUSSUL02	5	1
Russula #04	RUSSUL04	3	3
Russula #06	RUSSUL06	1	3
Tomentella #01	TOMENT1	2	1
Tomentella terrestris	Tom_terr	4	4
Tomentellopsis zygodesmoides	Tomt_zyg	1	1

Figure 1: Reciprocal averaging ordination of EM fungus ITS-types from oak seedlings out-planted in two mature mixed forest sites in the southern Appalachian Mountains. Types (crosses) are named by code (see Table 1-3) and separated by site (triangles, LM and HD) and by sample date (triangles, July and Sept). The ordination was rotated so that differences between sites are oriented horizontally, and differences between sample dates are oriented approximately vertically. Grouping is delineated (boxes) within sites (Low Mesic, LM; High Drier, HD) by season (July, September).



Chapter Three: Autecology and community structure of Em fungi on oak seedlings in the southeastern Appalachian Mountains – Edaphic characteristics and ericoid shrub abundance.

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Abstract

Little is known about the relationships between species and communities of EM fungi and environmental parameters such as ericoid shrub abundance and edaphic characteristics. Previous studies only provided information on sporophore distributions and gross EM morphotypes. We derived species specific information on frequency and abundance of EM fungi on oak seedlings by sequencing EM fungal rDNA directly from root tips. The community level and autecological relationships between EM fungi and a wide range of edaphic characteristics and the abundance of ericoid subcanopy shrubs were analyzed. While sequencing methods overcame the traditional limitations of inaccurate typing and under-representation of infrequent and hypogeous sporophore producers, community level relationships were still obscured. Relationships between abundance of individual species of EM fungi and microsite characteristics was also undetectable in light of the aforementioned diversity. Many EM fungi were amplified from only a single root tip. The dominant types, for which recovery was more frequent, did not appear to be specialists, and occurred over a broad range of most measured parameters. No association between ericoid shrub abundance (*Kalmia latifolia* and *Rhododendron maximum*) and EM fungi was observed. Differences in community composition and autecological relationships between EM fungi and ericoid shrub abundance were not detected.

Nonetheless, we present a listing of EM fungus types with associated ranges of edaphic parameters and ericoid shrub abundance. These parameters could have practical significance once identifications of the EM fungi are better known.

Introduction

Structure in communities of EM fungi in the Appalachian Mountains has been reported on two levels by Nantel and Neumann (1992). They found that host tree composition of a stand was the primary determinant of EM fungus composition, while within stands EM distributions varied in relation to edaphic characteristics (Nantel & Neumann, 1992). However, these results are derived from a single study which was based on sporophore distributions, and therefore do not incorporate either hypogeous species or those which produce sporophores infrequently. Furthermore, Walker and Miller (2002) were unable to detect any strong evidence of correlations between edaphic parameters and EM fungus sporophore distributions in the southeastern Appalachian Mountains. Thus, the parameters controlling the fine scale distributions and frequencies of EM fungi remain an open question.

In other systems, both sporophore sampling and morphotyping of EM root tips has been applied to assess relationships between EM fungus distributions and environmental parameters. A study by van der Heijden et al. (1999) compared soil chemistry with EM fungus

distributions in *Salix repens* L. stands in Canadian dune ecosystems. They found that at the species level only pH and available phosphorus explained a significant proportion of the variation in EM fungus sporophore distributions, while at the generic level, pH and moisture were significantly explanatory (van der Heijden et al., 1999). However, results based on morphotypes, which were probably identified only to the generic level, were variable depending on the sample date (van der Heijden et al., 1999).

In the southeastern Appalachian Mountains, seedlings experience high rates of mortality under *Rhododendron maximum* L. (Ericaceae) thickets. While this effect was known at least as early as 1941 (Minkler, 1941), recent spread of the thickets and the potential for induced changes in forest composition has engendered considerable concern (Lei et al., 2002; Nilsen et al., 1999; Walker et al., 1999). In the current study, we were interested in determining whether ericoid shrubs (*R. maximum* and *Kalmia latifolia* L.) affected the composition of EM fungi associated with roots of oak seedlings (*Quercus rubra* L. and *Q. prinus* L.). In addition, edaphic parameters were measured and compared with EM fungus distributions. Species specific identification of EM fungi from the seedling roots was facilitated by direct sequencing of the mycobiont rDNA from the EM root tissue.

Materials and Methods

Site description

The two sites for this study were located within the Coweeta Hydrologic Laboratory (Coweeta), part of the NSF Long Term Ecological Research Station network. Coweeta (35° 02' 29" N, 83° 27' 16" W) is located in the Blue Ridge Mountain Physiographic Province in the southwestern corner of North Carolina. Vegetation types and water availability at Coweeta vary with elevation from lower mesic cove – hardwood forests, mixed – oak at mid-elevations to "xeric" oak – pine forests at higher elevations (Day et al., 1988). Climatically classified as marine, humid; Coweeta experiences relatively high moisture levels and mild temperatures typify the area. Precipitation is distributed equally throughout the season, averaging 180 cm annually (Swank & Crossley, 1988). As a result, diversity of ectomycorrhizal fungi is quite high in this area (Walker & Miller, 2002).

The low elevation mesic site (LM site), with a northwestern aspect, was located upslope from Ball Creek at an elevation of approximately 690 m above sea level. Oak species at the LM site included *Quercus alba* L., *Q. falcata* Michx., *Q. coccinea* Muenchh., *Q. prinus* L., *Q. velutina* Lam., and *Q.rubra* L. (in order of dominance). Other EM host trees present included *Fagus* spp., *Betula* spp., *Tsuga canadensis* Carr, *Carya* spp., and *Pinus* spp.. The drier high elevation site (HD site) was located above

Dryman's Fork at approximately 1530 m above sea level and had a north – northeasterly aspect. Oak species in order of dominance included *Q. prinus*, *Q. alba*, *Q. rubra*, and *Q. velutina* at the HD site. In addition, *Betula* spp., *Carya* spp., and *T. canadensis* were present at the HD site.

Bait seedling propagation

Seedlings of two oak species, *Quercus rubra* and *Q. prinus* (a red and a white oak, respectively), were germinated from acorns collected at Coweeta. The acorns were surface sterilized in 10% bleach solution for 10 minutes and then rinsed with tap water for five minutes prior to sowing in coarse Vermiculite in a greenhouse. *Pinus rigida* Ait. seeds, also collected at Coweeta, were surface sterilized in hydrogen peroxide for twenty minutes and germinated in sterilized sand. After germination, the seedlings were transplanted to nursery cells with coarse vermiculite. The seedlings were fertilized weekly with quarter strength Hoagland's solution (Hoagland & Arnon, 1950) beginning after two months of growth. After four months of growth in the semi-opaque greenhouse, the seedlings were planted out at the field sites during the last week of June, 2000.

At each site (LM and HD) 60 1x2m plots were randomly located along four transects oriented cross – slope. At the LM site, four seedlings of each species (*Quercus rubra* and *Q. prinus*) were planted evenly spaced within each 1x2m plot. At the HD site *Q. rubra* and *Pinus rigida* were planted, again with four seedlings per plot. There were too few *P. rigida*

seedlings surviving to analyze after the first growing season. One randomly chosen seedling from each species/site set (*Q. rubra* at LM and HD; *Q. prinus* at LM) was harvested from each plot with surviving seedlings in mid-July and again in early September, 2001. Herbivores eliminated all seedlings from some plots. At the time of harvest, each seedling was carefully removed and bagged with the roots and surrounding soil as intact as possible. After transportation to the lab, the seedlings were stored at approximately 5°C until processed.

Mycobiont sampling

From the first harvest (July), approximately half of the surviving seedlings were systematically chosen for mycobiont sampling by using a seedling from every other plot along each transect. From the second harvest, all seedlings were sampled. The combined first and second harvest seedling sample totals were 78 *Quercus rubra* seedlings from the HD site, 90 *Q. rubra* seedlings from the LM site, and 86 *Q. prinus* seedlings from the LM site.

The soil was removed from the seedling root systems manually. Each root system was examined under a dissecting microscope and all mycorrhizal root tips (excluding those colonized by *Cenococcum geophilum* Fr.) were picked free of debris, removed with tweezers, and stored frozen in 100µl 2x CTAB buffer. Those colonized by *C. geophilum* were excluded because they were ubiquitously present and were

quantified reasonably accurately by morphology in a previous study (Walker et al., 1999). All seedlings were processed within two weeks from the time of harvest.

DNA was extracted from each root tip using CTAB buffer with chloroform:isoamyl alcohol following standard procedures (Hibbett & Vilgalys, 1993). Following extraction, the nuclear 5.8S rRNA gene and the flanking internal transcribed spacer regions I and II were amplified by PCR with primers ITS1F and ITS4 (Gardes & Bruns, 1993; White et al., 1990). After purification of the PCR products with QIAquick PCR Purification Kits (QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355), sequencing reactions were run using the same primers and ABI PRISM® BigDye™ Terminators Cycle Sequencing Kits (Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404 USA). Final amplification products were cleaned and sequenced by the Virginia Bioinformatics Institute Core Lab Facility (Virginia Tech, Blacksburg, VA 24061-0477 USA) using an ABI automated sequencer. Sequences were assembled into sequence types that share 97% or greater similarity, and were manually edited. Unique ITS-types were compared with sporophore voucher sequences by blast searching against (GenBank) and private sequence databases (Jeri L. Parrent and Rytas Vilgalys, Department of Biology, Duke University) for identification.

Names for ITS-types are derived from the closest matching sporophore voucher sequence. The taxonomic specificity of the name

reflects the authors' opinion based on the amount of sequence data available for the group, the apparent heterogeneity of the ITS regions in the group, and the level of match between the sample and voucher sequences. To help clarify the distinction between ITS-types and real taxa, type names are not presented in italics. An additional two types were matched to taxa (Phialophora finlandia and a salal root associate) for which the ecological role needs further study, and were therefore excluded from the analyses. Both types occurred on a single root tip each at the LM site, and their inclusion would not appreciably alter the results of this study.

Resource and subcanopy ericoid shrub measurements

Litter, humus and soil samples were collected in the late summer of 2000 from four locations in each plot and composited for soil analyses. Separate samples in each location were taken of the litter, humus, and soil layers respectively. Litter was defined as the undecomposed leaf material on the forest floor, subtended by the humus layer, or partially decomposed leaf and organic material. The soil layer was defined as the mineral soil below the humus layer, and was sampled to a depth of 10 cm in each location. Depth of each layer was recorded for all four locations in each plot at the time of sampling and mean depth was recorded. After compositing, carbon and nitrogen content of the litter and humus samples were analyzed using an Elementar CNS Analyzer

(Elementar Americas, Trenton, NJ). Soil pH and concentration of cations (Ca, Mg, P, K, Mn, Zn, Fe, Al, Cu, and B) were determined by the Soil Testing Laboratory (Virginia Tech, Blacksburg, VA) using inductively coupled mass spectroscopy. Basal area of all *Rhododendron maximum* and *Kalmia latifolia* stems within a 2.5 m radius of the center of each plot was summed (cumulative basal area).

Analytical methods

The frequency for each ITS-type was defined as the number of plots from which the type was isolated from a *Quercus* spp. seedling. Abundance was tabulated for each ITS-type as the number of mycorrhizal root tips of the type amplified in a given plot, regardless of seedling species. Abundance of each EM fungus type – plot combination were entered as a matrix for ordination using PC-ORD Multivariate Analysis of Ecological Data version 3.0 for windows (McCune & Medfford, 1997). Canonical Correspondence Analysis (CCA) ordinations were performed without the "downweight rare species" option. A second matrix containing resource and ericoid shrub parameters by plot was used to constrain the CCA. Because no relationships were detected in the ordinations, they are not presented.

Results

Ordination (CCA) of the gross abundance of EM ITS-types per plot captured only a very small amount (<5%) of the variation in species

composition. Ordination within sites and within site – sample date combinations were similarly weak. When limited to EM fungus types with frequency greater than five, the ordinations still captured minimal variation in species composition. Because of the low variation explained by the first three ordination axis, relationships between species composition and variation in ericoid shrub presence could not be evaluated. Ordinations limited to those plots with greater than zero *R. maximum* cumulative basal area also captured minimal variation. Similarly, edaphic characteristics could not be correlated with EM fungus species composition. When constrained with the resource parameters and ericoid shrub presence, no species specific relationships were observed in the ordinations. Limiting the analysis to the generic level did not improve the ordinations. The range of several pertinent parameters are listed for each EM fungus type in Tables one and two, including soil pH, litter depth, litter carbon content, litter nitrogen content, humus depth, humus carbon content, humus nitrogen content soil depth, soil carbon content, soil nitrogen content, potassium, phosphorus, and calcium concentrations, and cumulative basal area of both *Rhododendron maximum* and *Kalmia latifolia*.

Discussion

No relationships between EM fungus assemblages and edaphic characteristics or ericoid shrub abundance were detected in this study.

Most EM fungus types were recovered from a single root tip only. Increased sample sizes and additional sites may partially ameliorate the limitations imposed by such high diversity and occurrence of rare species.

Previous work also failed to detect differences in EM fungus assemblages in areas with or without dense *R. maximum* thickets based on sporphore collections (Walker & Miller, 2002). However, the proportion of EM root tips colonized on seedlings has been shown to be lower in locations with dense *R. maximum* thickets compared to areas without thickets (Walker et al., 1999). If the EM fungus assemblages are not different on seedlings in areas with higher versus lower *R. maximum* abundance, then colonization of seedlings by a limited set of EM fungi cannot explain the reduced colonization level observed in the thickets by Walker and Miller (2002).

Because EM fungi frequently appear to be resident *in situ* for periods considerably longer than a year (reviewed in Horton & Bruns, 2001), the limited amount of time the seedlings were growing at the field site (up to two growing seasons) should not be considered a major limitation in this study. The seedlings were used to bait resident EM fungi, thereby estimating the distribution of preexistent fungal thalli in the mycorhizosphere. However, in the *R. maximum* context, reduced carbohydrate supplies may limit the ability to form mycorrhizae as *R. maximum* induced mortality sets in (typically 2-3 years). This may in turn

partially explain why we observed differences between gross EM abundance from previous work (Walker et al., 1999) and species specific EM abundance here.

The range of edaphic parameters given in Tables one and two will have practical implications once species specific identities are known for the majority of the EM fungus ITS-types listed. Those interested in managing EM mycobionts on outplanted seedlings by inoculation will then be able to consult a baseline estimate of several niche dimensions for the listed taxa. Furthermore, the taxa listed might be expected to perform well in areas with similar characteristics.

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Table 1: EM fungus ITS-types on oak seedlings and edaphic characteristics under which they were found. Asterisk denotes a single observation.

Type	pH	Litter Depth (mm)	Litter %C	Litter %N	Humus Depth (mm)	Humus %C	Humus %N
Albatrellus #01	4.8 - 4.9	46 - 77	41 - 46	1.00 - 1.20	17 - 23	24 - 31	0.95 - 1.15
Amanita #02*	5.0	47	47	1.17	13	26	0.91
Amanita cf gemmata*	4.8	55	46	1.17	16	14	0.49
Amanita DFMO1078*	5.0	73	43	0.91	13 - 13	22	0.8
Amphinema #01*	4.8	63	48	1.44	41	48	1.66
Athelia cf neuhoffii	4.9 - 5.0	42 - 73	43 - 44	0.91 - 1.23	13 - 17	20 - 22	0.78 - 0.8
Bolete #01	4.7 - 4.8	48 - 87	47 - 48	1.22 - 1.54	31 - 117	42 - 49	1.52 - 1.78
Bolete #02	4.8 - 5.0	47 - 55	46 - 47	1.17	13 - 16	14 - 26	0.49 - 0.91
Bolete #03*	4.9	42	44	1.23	17	20	0.78
Boletus auriporus*	4.6	51	46 - 46	1.27	18	34	1.18
Cantharellaceae #01	4.8 - 5	32 - 73	37 - 50	0.91 - 1.73	10 - 22	10 - 35	0.41 - 1.16
cf Terfezia #01*	4.6	56	50	1.54	67	49	1.70
cf Tirmania #01*	4.9	36	49	1.34	31	25	0.97
cf Tuber #01	4.3 - 5.3	32 - 93	43 - 51	0.91 - 1.72	7 - 66	15 - 49	0.52 - 1.72
cf Tuber #02	5.0 - 5.1	57 - 63	45	0.94 - 1.28	14 - 15	16 - 19	0.63 - 0.65

Table 1 continued.

Type	pH	Litter Depth (mm)	Litter %C	Litter %N	Humus Depth (mm)	Humus %C	Humus %N
Clavariaceae #01	4.4 - 5.0	26 - 63	44 - 50	0.87 - 1.76	7 - 41	12 - 47	0.47 - 1.54
Corticiaceae #01	4.7 - 5.0	33 - 63	44 - 50	0.87 - 1.76	7 - 28	21 - 28	0.84 - 0.93
Cortinarius #01*	4.8	60	45	1.03	21	23	0.70
Cortinarius #02*	4.6	42	50	1.53	36	38	1.50
Cortinarius #03	4.8 - 5.0	45 - 46	42	1.00 - 1.05	10 - 16	10 - 22	0.41 - 0.59
Cortinarius #04	4.9 - 5.0	45 - 68	42 - 47	1.00 - 1.20	16 - 17	10 - 30	0.41 - 1.05
Cortinarius #05*	5.0	50	39	0.10	23	11	0.40
Cortinarius #06*	5.1	66	44	1.25	5	20	0.65
Cortinarius #07*	4.9	40	47	1.91	16	25	0.98
Cortinarius #08*	4.6	49	50	1.31	90	51	1.37
Cortinarius #09*	4.8	57	46	1.40	14	32	1.07
Cortinarius #10	4.7 - 5.0	42 - 52	44 - 47	1.02 - 1.58	12 - 30	20 - 34	0.78 - 1.46
Cortinarius #11*	4.8	60	45	1.03	21	23	0.70
Entolomataceae #01*	5.0	72	48	1.13	41	39	1.18
Gautieria #01*	4.9	42	44 - 44	1.23	17	20	0.78
Gautieria #02*	4.7	55	44	1.29	13	26	1.03
Hebeloma #01	4.9 - 5.0	47 - 112	42 - 47	1.02 - 1.20	12 - 23	23 - 31	0.78 - 1.15

Table 1 continued.

Type	pH	Litter Depth (mm)	Litter %C	Litter %N	Humus Depth (mm)	Humus %C	Humus %N
Hydnellum #01*	4.9	42	44	1.23	17	20	0.78
Laccaria cf laccata	4.3 - 5.0	33 - 73	39 - 50	0.91 - 1.76	13 - 55	11 - 48	0.4 - 1.64
Lactarius #02*	4.9	64	44	1.16	22	21	0.72
Lactarius #03	4.8 - 4.9	44 - 57	44 - 48	0.94 - 1.53	17 - 23	21 - 47	0.71 - 1.73
Lactarius #04*	4.6	45	49	1.52	52 - 52	40	1.47
Lactarius #05	4.8 - 5.1	55 - 75	41 - 46	1.12 - 1.17	15 - 16	14 - 20	0.49 - 0.82
Lactarius #06*	4.7	65	49	1.35	48	48	1.47
Lactarius cf chrysorheus	4.7 - 5.1	33 - 87	44 - 51	1.03 - 1.64	13 - 117	20 - 48	0.70 - 1.57
Peziza #01*	5.1	57	45	1.28	15	16	0.63
Russula #01*	4.9	32	47	1.26	20	35	1.16
Russula #02	4.8 - 5.0	42 - 95	39 - 46	0.96 - 1.40	14 - 25	20 - 33	0.49 - 1.23
Russula #03*	4.8	93	45	1.21	25	33	1.13
Russula #04	4.8 - 5.3	60 - 98	45 - 45	0.94 - 1.23	11 - 21	19 - 32	0.65 - 1.05
Russula #05	5.0 - 5.0	49 - 54	41 - 44	0.91 - 1.02	11 - 21	21 - 30	0.72 - 0.74
Russula #06	4.6 - 5.0	38 - 74	42 - 48	1.00 - 1.27	15 - 60	9 - 47	0.35 - 1.51
Russula #07	4.6 - 4.6	42 - 45	49 - 50	1.53	36 - 52	38 - 40	1.47 - 1.50
Russula #08	4.9	63 - 63	40	1.05	21	19	0.59

Table 1 continued.

Type	pH	Litter Depth (mm)	Litter %C	Litter %N	Humus Depth (mm)	Humus %C	Humus %N
Russula #09	4.7 - 4.7	64 - 67	42 - 50	1.00 - 1.42	34 - 45	33 - 49	1.20 - 1.74
Russula #10*	4.8	58	51	1.72	34	44	1.71
Russula #11	4.6 - 5.0	49 - 50	44 - 50	1.06 - 1.73	7 - 90	21 - 51	0.84 - 1.37
Thelephoraceae #01	4.8 - 5.0	54 - 57	44 - 46	0.91 - 1.4	11 - 14	30 - 32	0.72 - 1.07
Thelephoraceae #02*	4.9	71	45	1.09	30	29	0.99
Thelephoraceae #03	4.7 - 4.9	42 - 67	39 - 48	0.93 - 1.27	21 - 61	23 - 45	0.49 - 1.50
Thelephoraceae #04*	4.9	64	44	1.16	22	21	0.72
Thelephoraceae #05*	4.9	40	47	0.91	16	25	0.98
Thelephoraceae #06*	5.0	73	43	0.91	13	22	0.80
Thelephoraceae #07*	4.8	42	46	1.27	13	36	1.33
Thelephoraceae #08*	4.9	43	45	1.26	19	37	1.16
Thelephoraceae #09*	4.9	40	43	1.19	11	15	0.52
Tomentella #01	4.7 - 5.0	33 - 49	41 - 50	1.02 - 1.76	21 - 30	21 - 34	0.74 - 1.46
Tomentella #02*	5.1	66	44	1.25	5	20	0.65
Tomentella #03	4.4 - 4.9	26 - 87	44 - 50	1.22 - 1.32	17 - 117	32 - 48	1.23 - 1.57
Tomentella cf sublilacina*	4.7	92 - 92	49	0.10	127	49	1.16
Tomentella terrestris	4.6 - 5.0	42 - 73	41 - 51	0.91 - 1.54	13 - 67	17 - 49	0.63 - 1.70

Table 1 continued.

Type	pH	Litter Depth (mm)	Litter %C	Litter %N	Humus Depth (mm)	Humus %C	Humus %N
Tomentellopsis zygodesmoides	4.6 - 4.8	56 - 62	47 - 50	0.87 - 1.54	21 - 67	27 - 49	0.88 - 1.70
Tremellodendron #01	4.8 - 5.1	46 - 95	41 - 46	1 - 1.23	14 - 17	24 - 32	0.95 - 1.12
Tremellodendron #02*	4.6	42	50	1.53	36	38	1.50
Tremellodendron #03*	4.9	112	42	1.15	14	23	0.83
Tricholoma #01	5.0 - 5.3	38 - 72	46 - 48	1.13 - 1.3	27 - 41	21 - 39	0.79 - 1.18
Tricholoma #02*	4.8	55	46	1.17	16	14	0.49
Tricholoma #03*	4.9	69	48	1.26	37	44	1.40

Table 2: EM fungus ITS-types on oak seedlings with additional edaphic characteristics and ericoid shrub abundance. BA, cumulative basal area within a 2.5m radius; Kl, *Kalmia latifolia*; Rm, *Rhododendron maximum*. Asterisk denotes a single observation.

Type	Soil Depth (cm)	Soil %C	Soil %N	K (ppm)	P (ppm)	Ca (ppm)	Kl BA (m^2)	Rm BA (m^2)
Albatrellus #01	20 - 31	1.9 - 2.1	0.09 - 0.10	13 - 16	0.00 - 0.04	41 - 43	0.012 - 0.043	0.000 - 0.006
Amanita #02*	47	3.7	0.17	22	0.00	57	0.014	0.000
Amanita cf gemmata*	40	2.5	0.14	22	0.00	46	0.008	0.000
Amanita DFMO1078*	43	2.1	0.10	18	0.00	35	0.020	0.000
Amphinema #01*	35	4.2	0.19	33	0.00	112	0.000	0.000
Athelia cf neuhoffii	43 - 50	2.1 - 2.7	0.10 - 0.13	14 - 18	0.00	35 - 42	0.010 - 0.020	0.000
Bolete #01	14 - 40	2.0 - 6.2	0.09 - 0.29	22 - 33	0.00 - 0.96	39 - 42	0.000	0.000 - 0.041
Bolete #02	40 - 47	2.5 - 3.7	0.14 - 0.17	22 - 22	0.00	46 - 57	0.008 - 0.014	0.000
Bolete #03*	50	2.7	0.13	14	0.00	42	0.0102	0.000
Boletus auriporus*	35	3.5	0.17	28	0.00	43	0.001	0.000
Cantharellaceae #01	31 - 50	2.1 - 3.6	0.1 - 0.17	15 - 37	0.00 - 0.03	35 - 58	0.000 - 0.048	0.000 - 0.025
cf Terfezia #01*	35	3.5	0.15	32	0.00	39	0.008	0.000
cf Tirmania #01*	16	3.0	0.15	38	0.00	52	0.000	0.000
cf Tuber #01	15 - 50	1.7 - 9.5	0.08 - 0.37	15 - 60	0.00 - 1.84	32 - 86	0.000 - 0.048	0.000 - 0.044
cf Tuber #02	37 - 48	1.8 - 3.0	0.07 - 0.15	15 - 22	0.20 - 0.70	56 - 64	0.002 - 0.044	0.000

Table 2 continued.

Type	Soil Depth (cm)	Soil %C	Soil %N	K (ppm)	P (ppm)	Ca (ppm)	Kl BA (m^2)	Rm BA (m^2)
Clavariaceae #01	15 - 50	2.4 - 5.9	0.13 - 0.26	16 - 47	0.00 - 3.56	41 - 56	0.000 - 0.028	0.000 - 0.033
Corticiaceae #01	18 - 50	2.6 - 3.6	0.13 - 0.18	16 - 39	0.00 - 3.56	41 - 56	0.000 - 0.0274	0.000 - 0.033
Cortinarius #01*	20	3.2	0.15	25	0.00	37	0.048	0.027
Cortinarius #02*	42	3.0	0.13	40	0.52	51	0.015	0.000
Cortinarius #03	36 - 47	2.6 - 3.3	0.13 - 0.17	24 - 27	0.00	44 - 47	0.028 - 0.064	0.000 - 0.048
Cortinarius #04	39 - 47	2.6	0.13	24 - 25	0.00	45 - 47	0.000 - 0.028	0.000
Cortinarius #05*	48	3.4	0.17	24	0.00	56	0.0234 - 0.0234	0.000
Cortinarius #06*	38	2.8	0.13	20	0.00	109	0.0166 - 0.0166	0.000
Cortinarius #07*	42	2.7	0.13	18	0.00	44	0.000	0.000
Cortinarius #08*	32	1.5	0.08	20	0.00	40	0.000	0.000
Cortinarius #09*	39	3.2	0.15	20	0.00	45	0.021 - 0.021	0.000
Cortinarius #10	21 - 50	2.1 - 2.7	0.11 - 0.13	14 - 29	0.00 - 0.57	42 - 86	0.000 - 0.035	0.000
Cortinarius #11*	20	3.2	0.15	25	0.00	37	0.048	0.027
Entolomataceae #01*	45	2.6	0.11	17	0.00	54	0.000	0.0505 - 0.0505
Gautieria #01*	50	2.7	0.13	14	0.00	42	0.010	0.000
Gautieria #02*	32	2.0	0.1	14	0.00	38	0.000	0.000
Hebeloma #01	31 - 50	1.9 - 3.7	0.09 - 0.17	15 - 25	0 - 0.57	41 - 86	0.000 - 0.0431	0.000 - 0.006

Table 2 continued.

Type	Soil Depth (cm)	Soil %C	Soil %N	K (ppm)	P (ppm)	Ca (ppm)	Kl BA (m^2)	Rm BA (m^2)
Hydnellum #01*	50	2.7	0.13	14	0.00	42	0.010	0.000
Laccaria cf laccata	16 - 48	1.7 - 9.5	0.09 - 0.37	18 - 60	0 - 1.84	32 - 80	0.000 - 0.048	0.000 - 0.033
Lactarius #02*	31	3.6	0.17	22	0.00	58	0.033	0.000
Lactarius #03	16 - 50	1.8 - 2.7	0.09 - 0.13	13 - 25	0.00	40 - 43	0.011 - 0.019	0.000 - 0.019
Lactarius #04*	50	3.7	0.17	39	0.00	88	0.003	0.000
Lactarius #05	34 - 40	1.9 - 2.5	0.10 - 0.14	16 - 22	0 - 0.01	40 - 46	0.000 - 0.008	0.000
Lactarius #06*	28	4.2	0.18	30	0.00	44	0.022	0.000
Lactarius cf chrysorheus	16 - 50	2.4 - 5.1	0.12 - 0.19	14 - 43	0 - 0.86	37 - 73	0.000 - 0.048	0.000 - 0.041
Peziza #01*	48	1.8	0.07	15	0.70	56	0.044	0.000
Russula #01*	50	2.2	0.10	18	0.00	40	0.048	0.001
Russula #02	37 - 50	1.9 - 3.2	0.09 - 0.15	14 - 29	0.00	32 - 64	0.005 - 0.068	0.000 - 0.090
Russula #03*	37	2.2	0.10	20	0.00	40	0.0236	0.044
Russula #04	20 - 49	2.5 - 3.2	0.12 - 0.15	20 - 28	0.00 - 0.51	37 - 104	0.002 - 0.048	0.000 - 0.090
Russula #05	39 - 50	2.0 - 3.0	0.09 - 0.14	15 - 23	0.00	72 - 86	0.001 - 0.016	0.000 - 0.024
Russula #06	31 - 47	1.7 - 8	0.09 - 0.34	14 - 42	0.00	38 - 52	0.000 - 0.030	0.000 - 0.016
Russula #07	42 - 50	3.0 - 3.7	0.13 - 0.17	39 - 40	0.00 - 0.52	51 - 88	0.003 - 0.015	0.000
Russula #08	43	2.8	0.13	36	0.00	48	0.030	0.000

Table 2 continued.

Type	Soil Depth (cm)	Soil %C	Soil %N	K (ppm)	P (ppm)	Ca (ppm)	Kl BA (m^2)	Rm BA (m^2)
Russula #09	37 - 50	3.2 - 4.1	0.16 - 0.17	31 - 42	0.00	47 - 56	0.000 - 0.008	0.000 - 0.026
Russula #10*	15	3.6	0.17	28	0.88	75	0.000	0.000
Russula #11	32 - 39	1.5 - 2.7	0.08 - 0.14	16 - 37	0.00	40 - 57	0.000 - 0.027	0.000
Thelephoraceae #01	39 - 39	2.0 - 3.2	0.09 - 0.15	15 - 20	0.00	45 - 72	0.016 - 0.021	0.000
Thelephoraceae #02*	48	3.6	0.18	27	0.00	43	0.001 - 0.001	0.100
Thelephoraceae #03	31 - 42	1.7 - 3.1	0.09 - 0.14	20 - 42	0.00	39 - 64	0.000 - 0.068	0.000 - 0.041
Thelephoraceae #04*	31	3.6	0.17	22	0.00	58	0.033	0.000
Thelephoraceae #05*	42	2.7	0.13	18	0.00	44	0.000	0.000
Thelephoraceae #06*	43	2.1	0.10	18	0.00	35	0.020	0.000
Thelephoraceae #07*	32	2.6	0.12	37	0.65	69	0.003	0.000
Thelephoraceae #08*	50	3.2	0.15	17	0.00	40	0.005	0.022
Thelephoraceae #09*	45	2.4	0.12	15	0.03	37	0.011	0.025
Tomentella #01	21 - 50	2.1 - 3.2	0.12 - 0.16	23 - 36	0.00 - 0.42	44 - 86	0.000 - 0.001	0.000 - 0.033
Tomentella #02*	38	2.8	0.13	20	0.00	109	0.017 - 0.017	0.000
Tomentella #03	15 - 40	1.9 - 5.9	0.09 - 0.26	29 - 47	0.00 - 2.67	32 - 47	0.000 - 0.048	0.000 - 0.041
Tomentella cf sublilacina*	47	4.1	0.17	24	0.00	44	0.014	0.000
Tomentella terrestris	20 - 48	2.1 - 3.6	0.10 - 0.18	13 - 42	0.00 - 0.04	35 - 58	0.000 - 0.040	0.000 - 0.100

Table 2 continued.

Type	Soil Depth (cm)	Soil %C	Soil %N	K (ppm)	P (ppm)	Ca (ppm)	Kl BA (m^2)	Rm BA (m^2)
Tomentellopsis zygodesmoides	35 - 50	3.5 - 3.6	0.15 - 0.18	26 - 32	0.00	39 - 43	0.008 - 0.012	0.000
Tremellodendron #01	20 - 49	2.1 - 2.8	0.10 - 0.14	13 - 21	0.00 - 0.04	41 - 44	0.005 - 0.012	0.000 - 0.090
Tremellodendron #02*	42	3.0	0.13	40	0.52	51	0.015	0.000
Tremellodendron #03*	46	1.9	0.10	15	0.56	47	0.002	0.000
Tricholoma #01	29 - 45	2 - 2.6	0.1 - 0.11	17 - 21	0.00	53 - 54	0.000 - 0.011	0.000 - 0.050
Tricholoma #02*	40	2.5	0.14	22	0.00	46	0.008	0.000
Tricholoma #03*	45	2.3	0.10	27	0.00	38	0.039	0.009

Chapter Four: Molecular phylogenetic evidence for the mycorrhizal status of *Tremellodendron* (Sebacinaceae)

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Abstract

The family Sebacinaceae is a basal hymenomycete lineage that includes members of the genera *Tremellodendron* and *Sebacina.* Results from i*n vitro* synthesis experiments and from field studies have shown that several *Sebacina* species are important members of the ectomycorrhizal community in some forests. The mycorrhizal status of the closely related genus *Tremellodendron* has not yet been explored. We present evidence suggesting the putative mycorrhizal status of two species of *Tremellodendron. Tremellodendron* appears to form both endophytic associations with achlorophyllous orchids and ectomycorrhizae with species of *Quercus*, *Pinus*, and *Tilia cordata*.
Keywords: Auriculariales, Sebacinaceae, *Sebacina*, *Tremellodendron*, ectomycorrhizal fungi, orchid mycorrhiza.

Introduction

The family Sebacinaceae (Auriculariales, Basidiomycota) is a basal hymenomycete lineage encompassing the genera *Sebacina*, *Tremelloscypha*, *Craterocolla*, *Efibulobasidium* and *Tremellodendron* (Wells & Oberwinkler, 1982). Although exact placement of Sebacinaceae in the larger Basidiomycete phylogeny remains unresolved, molecular phylogenetic evidence suggests that it is a well-supported clade (Weiß & Oberwinkler, 2001). Species in the Sebacinaceae have septate hyphae

with dolipore septa that lack clamp connections (Wells & Oberwinkler, 1982), and typically form terricolous basidiocarps, a unique character distinguishing them from other Heterobasidiomycete fungi (Weiß & Oberwinkler, 2001). Basidiocarp morphology in this group includes resupinate forms found in the genus *Sebacina*, pulvinate forms of members in the genus *Craterocolla*, and coralloid forms such as those found in *Tremellodendron*. One common species found in Eastern USA is *T. pallidum* (Schwein) Burt., which is shown in Figure 1.

All the species of Sebacinaceae were historically thought to be saprophytic in habit. This was shown not to be the case when Warcup and Talbot (1967) examined *Rhizoctonia* orchid symbionts and found that teliomorphs of several *Rhizoctonia* isolates possessed characters consistent with *Sebacina vermifera sensu* Warcup & Talbot (1967). The mycorrhizal status of *S. vermifera* was later confirmed using *in vitro* synthesis experiments with *Melaleuca uncinata* R. Br. ex Aiton F. (Myrtaceae) and other host species (Warcup 1988). Although this provided the first direct evidence that a *Sebacina* species may form mycorrhizal associations, it remained unclear how broad the geographic or host range of *Sebacina* might be.

More recently, community studies using molecular methods to identify mycorrhizal fungi from root tips have found that species in the Sebacinaceae commonly form mycorrhizal associations in Australian and European forests, suggesting that species in this family may be

ubiquitous in ectotrophic forests (Glen et al., 2002; Selosse et al., 2002a; Urban et al., 2003). Molecular evidence from these studies clearly demonstrates that there are multiple *Sebacina* species that are mycorrhizal in habit, which raises the question as to whether or not other genera in the Sebacinaceae, previously assumed to be saprophytic such as *Tremellodendron* (Fergus, 1960), are also mycorrhizal.

We present results from field studies conducted in two separate regions of the southeastern United States where *Sebacina* and *Tremellodendron* species were frequently identified from mycorrhizal root tips. The implications of these results for revising the ecological role of *Tremellodendron* species is discussed, along with results from recent studies by other authors in regard to the role and diversity of mycorrhizal *Sebacina* species. Finally, certain aspects of the origin of EM associations will be revisited.

Materials and Methods

Sampling

Field studies were conducted in two locations in the southeastern USA; Coweeta Hydrologic Laboratory, Macon County, NC, and Duke Forest, Orange Co., NC. Root tips were collected from oak seedlings out-planted on two sites at Coweeta, and from soil cores taken from *Pinus taeda* L. stands in Duke Forest. Root tips were either cleaned manually under a dissecting scope or by gently washing roots in water. Root tips to

be sequenced were chosen randomly and mycorrhizal colonization was determined by visually confirming the presence of a mantle. All roots were processed within two weeks from the time of harvest and each individual, colonized root tip was stored in 2X CTAB until DNA extraction could be performed.

DNA extraction, amplification, and Sequencing

DNA extraction was performed according to the protocol of Hibbett and Vilgalys (1993). For Coweeta DNA samples, the internal transcribed spacer (ITS) and 5.8S region of ribosomal DNA was amplified by PCR with primers ITS1F and ITS4 (Gardes & Bruns, 1993; White et al., 1990). For Duke Forest DNA samples, this same region was amplified using ITS1F in combination with a newly designed universal basidiomycete – specific primer, ITS 4NA (primer sequence: 5'-CTTTTCATCTTTCCCTCACGG-3'). Amplified DNA was sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), and visualized on an ABI3700 automated sequencer (Applied Biosystems). Sequences were queried against both the Genbank database (NCBI) and a large database of EMF ITS sequences compiled by the Vilgalys laboratory from collections made in eastern USA forests. Sequences were assembled into sequence types that share 97% or greater similarity, and were manually edited.

Phylogenetic Analysis

Our sequences were combined with additional sequences of the Sebacinaceae from Genbank. *Pseudohydnum gelatinosum* (Scop. ex Fr.) P. Karst. was used as the outgroup species. ITS sequences were aligned into a matrix of 649 base pairs in length with 278 parsimony informative characters. The most parsimonious phylogenies were found using 1000 heuristic searches with the software package PAUP 4.0b.10 (Swofford, 2002). Gaps were treated as missing, and multistate taxa were interpreted as uncertainty. Starting trees were obtained by random addition; tree-bisection-reconnection was used with the MulTrees option. Statistical support was estimated using 1000 bootstrap replicates.

Results

Results of the phylogenetic analysis using ITS and 5.8S sequence data are shown in Figure 2. Phylogenetic analysis resolves two well-supported clades (98% and 100% bootstrap support) in the Sebacinaceae. One clade ("clade one") contains only three *Sebacina* species, *S. umbrina* Rogers, *S. calcea* (Pers.) Bres., and *S. grisea* (Pers. ex Fr.) Bres.. The second, larger clade ("clade two") contains all additional samples including all of the sequences from root tip samples.

A large diversity of Sebacinaceae ITS sequence types from root tips were obtained from a broad range of host types and geographic locations. However, clustering by these factors was not evident. This suggests that

Sebacinaceae mycorrhizal species are not host specific and have a broad geographic distribution.

Within clade two (Fig. 2), *Efibulobasidium* and *Tremellodendron* are nested within the clade containing two *Sebacina* species and root tips assigned to the Sebacinaceae. These data suggest that *Sebacina* is not a monophyletic group, a result consistent with other phylogenetic analyses of the Sebacinaceae using large subunit sequence data (Weiß and Oberwinkler 2001). Furthermore, the two *Tremellodendron* sequences generated from basidiocarps, *T. pallidum* and *T. schweinitzii* (Peck) GF Atk., which share 89.4% sequence identity, do not cluster with one another, and therefore *Tremellodendron* is probably not a monophyletic genus.

Our North American *Tremellodendron schweinitzii* sequence is 99% identical with an ITS sequence type isolated from a *Neottia nidus avis* (L.) L.C.M. Rich root tip from France (Sebacinaceae GB10 in Fig. 2). *Tremellodendron pallidum* is nested within a clade of ITS sequence types obtained from orchid and ectomycorrhizal root tips. These results are suggestive of a mycorrhizal habit by *T. pallidum* and *T. schweinitzii*.

Discussion

Our results add to the burgeoning support for the ectomycorrhizal status of Sebacinaceae species, and identify members of the genera *Sebacina* and *Tremellodendron* as putative mycorrhizal symbionts. *In*

vitro synthesis experiments with *S. vermifera* have shown that *Sebacina* species are capable of forming ectomycorrhizal associations with *Eucalyptus obliqua* L'Herit. (Warcup, 1988). Other EMF community studies employing molecular methods for EMF identification have found that Sebacinaceae species are dominant EMF community members in Australian *Eucalyptus marginata* Donn ex Sm. forests (Glen et al., 2002), and are also prominent mycorrhizal associates of various hardwood species (Selosse et al., 2002a). However, this study is the first to provide evidence that *Tremellodendron* species are also likely to be mycorrhizal. In the study by Glen et al. (2002) reference was made to a high match (96% sequence identity) between one EMF ITS-type and *T. pallidum*. However, they did not include *T. pallidum* in their phylogenetic analysis and tentatively assign their sequence types to the genus *Sebacina*. Other recent phylogenetic studies of the Sebacinaceae have also omitted *Tremellodendron* species from their analyses (Selosse et al., 2002b; Weiß & Oberwinkler, 2001). Urban et al. (2003) presented phylogenetic evidence depicting the presence of mycorrhizal samples in a "*Sebacina*/*Tremellodendron* complex", but only *Sebacina incrustans* (Fr.) Tul. is clearly identified as a mycorrhizal associate.

Several lines of evidence support the notion that *Tremellodendron* species are mycorrhizal. First, *T. pallidum* and *T. schweinitzii* are located in clade two of the Sebacinaceae (Figure 2), which contains *S. vermifera*, a species known to be mycorrhizal (Warcup, 1988), and all of the

mycorrhizal sequences included in this study. Second, the high degree of sequence similarity shared between *Tremellodendron* samples and several mycorrhizal root tip sequence types further supports the mycorrhizal status of *Tremellodendron*. Third, though the Sebacinaceae includes genera likely to be wood decomposers such as *Craterocolla* (Weiß & Oberwinkler, 2001), the strictly terricolous fruiting habit of *Tremellodendron* makes it unlikely for *Tremellodendron* to be a wood decomposer. The terricolous fruiting and clavarioid basidiocarp morphology is also reminiscent of other well know EMF genera such as *Clavulina* and *Thelephora*.

Based on our analyses, *Tremellodendron* species putatively form ectomycorrhizal associations with members of the genera *Eucalyptus* (Sebacinaceae GB2), *Quercus* (Coweeta), *Pinus* (DFMO and Sebacinaceae GB4), and *Tilia cordata* Miller (Sebacinaceae GB1, Sebacinaceae GB9). In addition, endophytes of the orchid *Neottia nidus-avis* are tentatively identified as *Tremellodendron* species (e.g. Sebacinaceae GB10). The range of mycorrhizal forms reported for Sebacinaceae fungi is striking. Orchid mycorrhizal taxa overlap with ectomycorrhizal taxa (Selosse et al., 2002b; Warcup, 1988). Remarkably, Sebacinaceae species have also been reported to form arbuscular mycorrhizae with *Phyllanthis calycinus* Labil (Warcup, 1988) and ericoid mycorrhizae with *Gaultheria shallon* Pursh (Berch et al., 2002). This seems to be the least specific group of mycorrhizal fungi ever reported.

These new findings add yet another Basidiomycete lineage to the list of mycorrhizal forming fungi previously reported (Bruns et al., 1998; Hibbett et al., 2000). Further *in vitro* synthesis experiments should be conducted to verify the ectomycorrhizal status of *T. pallidum* and *T. schweinitzii*. The role of other *Tremellodendron* species, and members of the genera *Efibulobasidium* and *Tremelloscypha*, should also be reexamined.

The Sebacinaceae is notable for its phylogenetic placement, basal to the Thelephorales, Russulales, Boletales, and the Agaricales (Weiß & Oberwinkler, 2001). The diverse range of mycorrhizal types formed by species in the Sebacinaceae and the basal position of this family relative to other major mycorrhizal lineages suggest that either the ancestor to the Basidiomycetes were mycorrhizal in habit, or the mycorrhizal condition is an evolutionarily labile trait (Hibbett et al., 2000). In either case, reexamination of the ecological role of species presumed to be saprophytic will undoubtedly lead to the discovery of additional mycorrhizal lineages.

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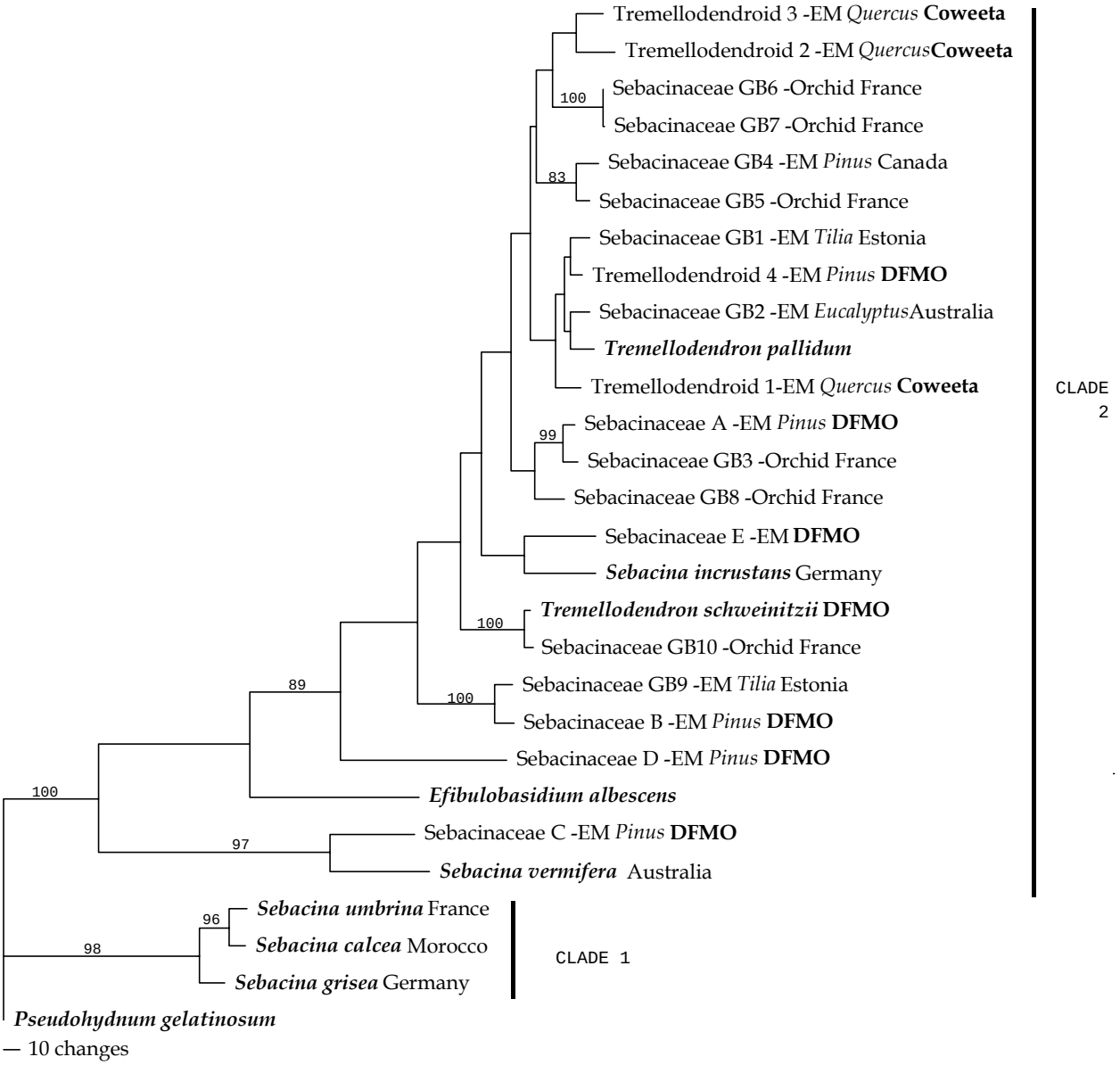
Table 1. Accession numbers and authors of reference sequences. [1]GenBank accession numbers. [2]DFMO, Duke Forest Mycological Observatory; Coweeta, Coweeta Hydrologic Laboratory.

Taxon	Accession[1]	Source[2]
Efibulobasidium albescens	AF384860	Lim, S.-R. and Berbee, M.L.
Pseudohydnum gelatinosum	AF384861	Lim, S.-R. and Berbee, M.L.
Sebacina calcea	AJ427408	Gonzalez, V.
Sebacina grisea	AJ427410	Gonzalez, V.
Sebacina umbrina	AJ427409	Gonzalez, V.
Sebacina vermifera	AF202728	Taylor, D.L., Bruns, T.D. and Hodges, S.A.
Sebacina incrustans	AY143340	Urban, A., Weiß, M. and Bauer, R.
Tremellodendron pallidum	AF384862	Lim, S. -R. and Berbee, M.L.
Tremellodendron schweinitzii		DFMO
Mycorrhizal Sebacinaceae A		DFMO
Mycorrhizal Sebacinaceae B		DFMO
Mycorrhizal Sebacinaceae C		DFMO
Mycorrhizal Sebacinaceae D		DFMO
Mycorrhizal Sebacinaceae E		DFMO
Mycorrhizal Sebacinaceae GB1	AJ534907	Tedersoo, L., Hallenberg, N., Larsson, K.H. and Koljalg, U.
Mycorrhizal Sebacinaceae GB2	AY093437	Glen et al. 2002
Mycorrhizal Sebacinaceae GB3	AF440653	Selosse et al. 2002b
Mycorrhizal Sebacinaceae GB4	AY192164	Bois, G. Piche, Y., and Khasa, D.P.

Mycorrhizal Sebacinaceae GB5	AF440651	Selosse et al. 2002b
Mycorrhizal Sebacinaceae GB6	AF440660	Selosse et al. 2002b
Mycorrhizal Sebacinaceae GB7	AF440658	Selosse et al. 2002b
Mycorrhizal Sebacinaceae GB8	AF440644	Selosse et al. 2002b
Mycorrhizal Sebacinaceae GB9	AJ534910	Tedersoo, L., Hallenberg, N., Larsson, K.H. and Koljalg, U.
Mycorrhizal Sebacinaceae GB10	AF440655	Selosse et al. 2002b
Mycorrhizal Tremellodendroid 1	AY277944	Coweeta
Mycorrhizal Tremellodendroid 2	AY277943	Coweeta
Mycorrhizal Tremellodendroid 3	AY277945	Coweeta
Mycorrhizal Tremellodendroid 4		DFMO

Figure 1. *Tremellodendron pallidum* (Schwein) Burt. Photograph by Orson K. Miller, Jr.

Figure 2. Phylogenetic relationships of the Sebacinaceae inferred from ITS and 5.8S regions of the nuclear rDNA using parsimony analysis. The topology was rooted with *Pseudohydnum gelatinosum*. Bootstrap support is shown above branches for nodes with >80% support. Two well-supported clades are designated. Basidiocarps are presented in bold italics. EM, sequences from ectomycorrhizal root tips; orchid, isolates from roots of the achlorophyllous orchid, *Neotia nidus-avis* (Orchidaceae); Coweeta, samples collected from Coweeta Hydrologic Laboratory; DFMO, samples collected from Duke Forest; GB, previously published sequences from GenBank (accession numbers are listed in Table 1). Host taxa and site of origin are indicated when available.



Conclusions

The ectomycorrhizal fungus assemblage on oak seedlings in mature mixed forests in the southeastern Appalachian mountains is highly diverse with a high proportion of infrequently collected and rare species. Seedlings were colonized by a broad range of EM fungi typically characterized as late – or mixed – stage fungi. These results support the multi – stage theory of ectotrophic mycorrhizal fungus succession in well developed EM forests. Mycobiont diversity in this study is reflective of general patterns from previous studies, but is among the highest measured. Diversity was lower at a high elevation oak forest site compared to a lower mesic cove – hardwood forest site. Fungal specificity for red oak versus white oak seedlings was suggested, but not strongly. High diversity of beneficial tree associated fungi and high numbers of rare species have important implications for conservation of biodiversity.

The southeastern Appalachians is apparently a hot spot for ectomycorrhizal diversity, and conservation efforts should include large areas spread through all habitat types in order to capture as many rare species as possible. An amendment to the baseline ectotrophic fungus check list (Walker & Miller, 2002) is provided for the Coweeta Hydrologic Laboratory USDA Long Term Ecological Research Station.

Our results suggest that EM fungus associations are much more dynamic than previously considered. Mid-summer (or earlier) may

represent a generalized peak in EM abundance and diversity (based on our frequency), however the early associations seem to be largely replaced by alternate fungi later in the season.

We propose a multi-stage model of seasonal succession by EM fungi on tree roots. We hypothesize that certain generalist EM fungi associate with roots throughout the season. Other more specialized fungi are more temporally as well as more spatially variable. The potential for well defined early and late season groups of EM fungi associated with roots will require further research, as is the case for other aspects of the model. This model compliments the larger scale temporal dynamics models for EM fungi over successional periods of time, i.e. the multi-stage model of succession.

The implication of seasonal dynamics in EM fungus communities impacts how we think of nutrient flux between plants and fungi involved in EM associations. Based on the frequency of ITS-types in our study, seasonal carbohydrate drain by EM fungi may peak in the in mid-summer or earlier, but should be extended throughout the season by a progression of fungi. Shifting EM fungi throughout the summer should provide the phytobiont with an adaptable assemblage with greater plasticity with regard to associated changes in environmental conditions over the course of the growing period, potentially enhancing nutrient and water uptake for the plant. Thus it appears likely that the diversity of the

root – soil interface, which is mediated by EM fungi in this system, is enhanced by the seasonal dynamics of the fungi.

Implications for future studies

A great number of studies have been conducted comparing EM fungus distributions based on a single sample time per year. Generalizations based on these sampling regimes should be restricted to the appropriate portion of the season, and future studies should consider the potential for seasonal dynamics. A great deal remains to be understood about seasonal dynamics within this system and about the applicability of these results to other systems. Seasonality is also promoted as a candidate niche dimension in studies assessing the autecology of EM fungus species.

No relationships between EM fungus assemblages and edaphic characteristics or ericoid shrub abundance were detected in this study. Most EM fungus types were recovered from a single root tip only. Increased sample sizes and additional sites may partially ameliorate the limitations imposed by such high diversity and occurrence of rare species. However, it is becoming increasingly apparent that ericoid shrubs do not have a strong influence on EM fungus distributions.

Previous work also failed to detect differences in EM fungus assemblages in areas with or without dense *R. maximum* thickets based on sporphore collections. However, the proportion of EM root tips

colonized on seedlings has been shown to be lower in locations with dense *R. maximum* thickets compared to areas without thickets. If the EM fungus assemblages are not different on seedlings in areas with higher versus lower *R. maximum* abundance, then colonization of seedlings by a limited set of EM fungi cannot explain the reduced colonization level observed in the thickets.

The range of edaphic parameters given in Tables one and two of Chapter Three will have practical implications once species specific identities are known for the majority of the EM fungus ITS-types listed. Those interested in managing EM mycobionts on outplanted seedlings by inoculation will then be able to consult a baseline estimate of several niche dimensions for the listed taxa. Furthermore, the taxa listed might be expected to perform well in areas with similar characteristics.

Based on our analyses, *Tremellodendron* species putatively form ectomycorrhizal associations with members of the genera *Eucalyptus*, *Quercus*, *Pinus*, and *Tilia cordata*. In addition, endophytes of the orchid *Neottia nidus-avis* are tentatively identified as *Tremellodendron* species. The range of mycorrhizal forms reported for Sebacinaceae fungi is striking. Orchid mycorrhizal taxa overlap with ectomycorrhizal taxa. Remarkably, Sebacinaceae species have also been reported to form arbuscular mycorrhizae and ericoid mycorrhizae. This seems to be the least specific group of mycorrhizal fungi ever reported.

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Vita

John F. Walker was born in Cleveland Ohio on March 3rd, 1966. He attended Shaker Heights High School where he graduated in 1984. After graduating from high school he spent a period of several years working at a variety of occupations and traveling throughout the United States. During this time he discovered a deep appreciation for the natural environment and a strong desire to study biology. In 1989 while living in Oregon, he attended a fascinating seminar by Dr. Chris Maser on mycorrhizae, and decided to pursue a scholastic career in botany at Portland State University to learn more about that topic. In 1991 while at Portland State, John was awarded a Dean's Scholarship, and in 1994 he received a Bachelor of Science with high honors in Biology, with a Botany concentration.

While at Portland State John heard of the exciting and highly regarded work of Dr. Orson Miller, Jr. from a guest speaker in a course on mushrooms. After attending Dr. Miller's mushroom course at the University of Montana Flathead Lake Biological Station in 1995, where he cultured fungi for Pfizer Pharmaceutical Corporation, he began the Masters of Science program in Biology with Dr. Miller. While at Virginia Polytechnic and State University John has worked as a Research Assistant and as a Graduate Teaching Assistant has taught labs in general biology, microbiology, ecology, and team taught the Introductory

Mycology course with Rebecca Abler. He was awarded a grant from Sigma XI, The Scientific Society, in 1997. Also in 1997 he received a scholarship to attend a summer course at the University of Virginia, Mountain Lake Biological Station where he was involved in a project investigating the molecular phylogeny of the Russulales with Dr. Rytas Vilgalys. On June 7, 1997 he was married to Lynette Ann Jedermann.

John has given numerous presentations at Mycological Society of America and Mid Atlantic States Mycology Conferences, and has spoken to a diverse range of local interest groups. He assisted with the preparation of USDA-NRI grant renewal #9502486, wrote an ASPIRES grant awarded from Virginia Tech, and also solicited funding from the Graduate Research and Development program at Virginia Tech. John has published his previous results in two publications, and coauthored an additional four publications.