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Hyperdiversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains

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Abstract

Diversity of ectotrophic mycobionts on outplanted seedlings of two oak species (*Quercus rubra* and *Quercus prinus*) was estimated at two sites in mature mixed forests in the southern Appalachian Mountains by sequencing nuclear *5.8S rRNA* genes and the flanking internal transcribed spacer regions I and II (ITS). The seedlings captured a high diversity of mycorrhizal ITS-types and late-stage fungi were well represented. Total richness was 75 types, with 42 types having a frequency of only one. The first and second order jackknife estimates were 116 and 143 types, respectively. Among Basidiomycetes, tomentelloid/thelephoroid, russuloid, and cortinarioid groups were the richest. The ascomycete *Cenococcum geophilum* was ubiquitously present. Dominant fungi included a putative *Tuber* sp. (Ascomycetes), 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 low elevation mesic cove — hardwood forest site. Fungal specificity for red oak vs. white oak seedlings was unresolved. The high degree of rarity in this system imposes limitations on the power of community analyses at finer scales. The high mycobiont diversity highlights the potential for seedlings to acquire carbon from mycelial networks and confirms the utility of using outplanted seedlings to estimate ectomycorrhizal diversity.

Keywords: ectomycorrhizal diversity, molecular identification, *Quercus* spp., *Rhododendron* spp., richness, seedling regeneration

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Introduction

Trees of the family Fagaceae, which includes *Quercus* spp., are obligately dependent on ectomycorrhizal (EM) fungi under natural conditions in temperate forests (Smith & Read 1997). EM fungi provide plants with essential nutrients such as nitrogen, phosphorus, and water (Smith & Read 1997). Furthermore, EM colonization is thought to be critical in the regeneration of canopy trees (Amaranthus & Perry 1987), and under certain circumstances EM fungi may even potentially supply carbohydrates to seedlings (Simard *et al*. 1997).

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Understanding diversity is an important prerequisite for conservation efforts and framing of ecological studies. With regards to oak seedlings in the southeastern Appalachian Mountains, enumerating diversity of EM fungi colonizing roots is of particular interest because limited EM colonization has been observed in conjunction with strong suppression of seedling regeneration in dense thickets of *Rhododendron maximum* L. 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). This investigation of the diversity of fungi colonizing oak seedlings will facilitate estimation of the sample sizes needed in experimental designs targeting the relationship between mycorrhizal fungi and seedling suppression. Furthermore, the sequences provided herein can be utilized as markers for further investigation of fungi indicated to be dominant in this system.

In addition, questions basic to our understanding of mycorrhizal community development on seedlings remain unanswered. The early developmental stage of seedlings may impose phenetic constraints on mycorrhizal development, leading 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.

The objectives of this study were: (i) to characterize mycobiont diversity on oak seedlings in a fungus-rich, mature, mixed forest in the southeastern Appalachian Mountains; (ii) to compare seedling mycobiont assemblages at a high vs. a low elevation site in close relative proximity; and (iii) to compare seedling mycobiont assemblages on a red vs. a white oak species.

Materials and methods

Site description

The sites for this study were located within the Coweeta Hydrologic Laboratory (Coweeta), part of the National Science Foundation Long Term Ecological Research (LTER) 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 through mixed oak at mid-elevations to 'xeric' oak hypiens pine forests at higher elevations (Day *et al*. 1988). Climatically classified as marine humid, Coweeta experiences relatively high moisture levels and mild temperatures. Precipitation is distributed equally throughout the season, averaging 180 cm annually (Swank & Crossley 1988). As a result, diversity of EM fungi is quite high in this area (Walker & Miller 2002). Historical disturbances to the area include logging (*c*. 1900) and the Chestnut blight which extirpated *Castanea dentata* (Marsh.) from the overstory in the 1930s. The second-growth forest sites for this study have remained undisturbed since the inception of the LTER in 1934.

The low elevation mesic site (LM site), with a northwestern aspect, was located upslope from Ball Creek at an elevation of approximately 720 m above sea level (a.s.l.). Oak species at the LM site included (in order of dominance) *Quercus alba* L., *Quercus falcata* Michx., *Quercus coccinea* Muenchh., *Quercus prinus* L., *Quercus velutina* Lam., and *Quercus rubra* L. Other EM host trees present included *Fagus* spp., *Betula* spp., *Tsuga canadensis* Carr, *Carya* spp., and *Pinus rigida*. The drier high elevation site (HD site) was located above Dryman's Fork at approximately 1170 m a.s.l. 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 which were then averaged. The average soil depth was significantly lower ($P < 0.001$) at the HD site (29 cm) than at the LM site (41 cm). Of the two sites, the HD site is therefore referred to as the drier site.

Bait seedling propagation

Seedlings of two oak species, *Q. 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 min 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 20 min and germinated in sterilized sand. After germination, the seedlings were transplanted to nursery cells with coarse vermiculite. After 2 months of growth, the seedlings were fertilized weekly with quarter strength Hoagland's solution (Hoagland & Arnon 1950). After 4 months of growth in the greenhouse, the seedlings were planted out at the field sites during the last week of June, 2000. Greenhouse EM contaminants, especially members of the Thelephoraceae, are frequently observed when seedlings are grown in greenhouses. However, EM tree species are rarely grown in the greenhouse we utilized. To test for possible contaminants, 20 seedlings of each oak species were destructively sampled and screened for mycorrhizal colonization at the time of planting. The root systems of the screened seedlings were examined under a dissecting microscope and multiple root tip sections were examined at high magnification under a compound microscope. No evidence of mycorrhizal colonization was observed.

At each site (LM and HD), 60 1×2 m plots were randomly located along four transects orientated cross—slope. The plots were distributed over an area of approximately 18 000 m^2 at each site. At the LM site, four seedlings of each species (*Q. rubra* and *Q. prinus*) were planted evenly spaced within each 1×2 m plot. At the HD site *Q. rubra* and *P. rigida* were planted, again with four seedlings per plot. There were too few *P. rigida* seedlings surviving to analyse 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 sampled plot with surviving seedlings in mid-July and again in early September, 2001. Herbivores eliminated all seedlings from two plots at each site. 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 *c*. 5 °C until processed.

Mycobiont sampling

From the first harvest (July), half of the plots were systematically chosen for mycobiont sampling by using a seedling from every other plot along each transect (30 plots at each site). For the second harvest, all plots with surviving seedlings were sampled (58 plots at each site). The first harvest seedling sample totals were 30 *Q. rubra* seedlings from the HD site, 30 *Q. rubra* seedlings from the LM site, and 30 *Q. prinus* seedlings from the LM site. From the second harvest seedling, sample totals were 58 *Q. rubra* seedlings from the HD site, 58 *Q. rubra* seedlings from the LM site, and 53 *Q. prinus* seedlings from the LM site. There were 284 and 309 root tip samples (593 total) from the first and second harvests, respectively. We purposely over-sampled root tips which showed signs of potential mycorrhization but were not well developed to maximize the recovery of EM fungi from the seedlings. Multiple (three to four) polymerase chain reaction (PCR) attempts were made at various template concentrations for all un-amplified samples. Of the 593 root tips sampled, 291 were successfully sequenced (49%).

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 100 µL 2× CTAB buffer. Those colonized by *C. geophilum* were not sequenced because they were quantified accurately by morphology. All seedlings were processed within 2 weeks from the time of harvest. The remainder of the root system was cleaned, oven-dried at 80 °C, and weighed. Root biomass was compared between sites for each harvest using *t*-tests in SIGMASTAT (version 3.0, SPSS).

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 (White *et al*. 1990; Gardes & Bruns 1993). After purification of the PCR products with QIAquick PCR Purification Kits (QIAGEN), sequencing reactions were run using the same primers and ABI PRISM BigDye Terminators Cycle Sequencing Kits (Applied Biosystems). Final amplification products were cleaned and sequenced by the Virginia Bioinformatics Institute Core Laboratory Facility (Virginia Tech) using an ABI automated sequencer. Sequences were manually edited and then assembled into sequence types that share 97% or greater similarity. Unique ITS-types were compared with sporophore voucher sequences by BLAST searching GenBank and private sequence databases (Jeri L. Parrent and Rytas Vilgalys, Department of Biology, Duke University) for identification.

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 *Q. rubra* seedling (the only seedling species planted at both sites), regardless of harvest date. The site comparisons were thus based on 88 *Q. rubra* seedlings per site from a total of 58 plots at each site. 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. The *Q. rubra* seedlings from the five plots with no surviving *Q. prinus* seedlings at the time of the second harvest were excluded from the seedling species comparisons. The seedling species comparisons are therefore based on 83 *Q. rubra* seedlings and 83 *Q. prinus* seedlings from 53 plots.

Frequency for each type-site and type-species (seedling species) were entered as matrices for reciprocal averaging (RA) ordination and calculation of diversity measures using PC-ORD MULTIVARIATE ANALYSIS OF ECOLOGICAL DATA version 3.0 for Windows (McCune & Medfford 1997). Ordination techniques including RA are used to describe multidimensional data such as species composition on a reduced number of axes while retaining as much of the original information as possible. Similar samples are positioned close to one another in RA ordination space, thus grouping is evident when the samples appear clustered on the ordination diagram. 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 & Weaver (1949). For the ordination, frequency by site includes both seedling species; frequency by seedling species includes only LM samples. 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 first and second order jackknife estimates were also calculated using PC-ORD from a matrix of fungus-type abundance (number of root tips colonized) by plot.

Notes on identifications

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. The ITS-types are deposited at GenBank and identifications can be re-evaluated as additional sequences become publicly available.

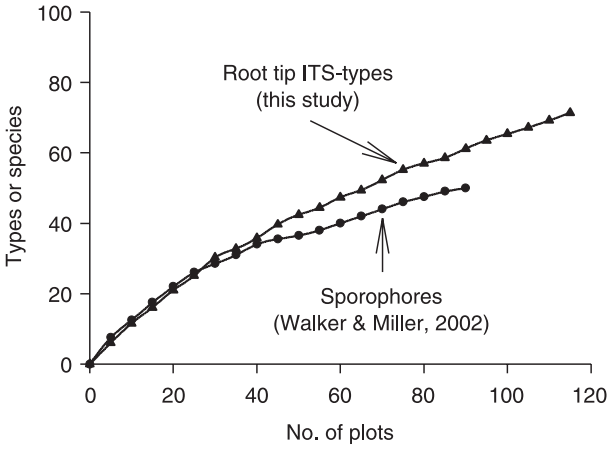

Fig. 1 Species vs. area curve–average number of species vs. number of plots for all fungal ITS-types recovered from oak seedling roots, based on subsampling with 500 repetitions. The sporophore accumulation curve is presented for comparison, and is based on 2 × 2 m plots located in a similar sized area nearby the study site.

Results

Total diversity

All ITS-types are listed in Table 1 with frequencies by site and seedling species. The species–area curve is climbing rapidly at the maximum area sampled (Fig. 1). Total richness of putative EM types counting both sites and both seedling species was 75 ITS-types (Table 1). Of these, 42 types were isolated from only one plot. The first and second order jackknife estimates were 116 and 143 types, respectively. The highest richness was in russuloid (*Russula* spp. & *Lactarius* spp.; 17 types) and tomentelloid/thelephoroid types (15 types), followed by cortinarioid types (10 types; Table 1). The most frequent types were *Cenococcum geophilum* (116 plots), cf. *Tuber* #01 (20 plots), *Laccaria* cf. *laccata*, Cantharellaceae #01, *Tomentella* cf. *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).

*Diversity by site (*Quercus rubra *only)*

The *Quercus rubra* seedlings at the LM site had a richness of 43 types (*S*), with 0.70 evenness (*E*) and diversity (*H*) equal to 2.6. At the HD site, richness totalled 30 types (*S*), with $E = 0.63$ and $H = 2.14$. While 29 types were only collected at the LM site, only 17 types occurred only at the HD site (Table 1). Of those types occurring only at the LM site, the richest were cortinarioid (six types), russuloid (five types), tomentelloid/thelephoroid (three types) and *Tricholoma* spp. (three types; Table 1). The most frequent types occurring only at the LM site included *Hebeloma* #01 and *Russula* #04 (four plots each; Table 1). The richest types found only at the HD site were the tomentelloid/thelephoroid (five types) and russuloid (four types; Table 1). Bolete #01 was the most frequent type occurring only at the HD site (three plots; Table 1).

Fourteen types occurred at both the HD and LM sites (again considering only *Q. rubra*). The most frequent types occurring at both sites were *Cenococcum geophilum* (116 plots), cf. *Tuber* #01 (17 plots), *Lactarius chrysorheus*, *Tomentella* cf. *terrestris* (six plots each), Cantharellaceae #01, and *Laccaria* cf. *laccata* (five plots each; Table 1).

There were no differences in root biomass between sites for either harvest one (LM = 960 mg, SE = 56 mg; HD = 925 mg, SE = 50 mg; $P = 0.64$) or harvest two (LM = 1021 mg, SE = 55 mg; HD = 1124 mg, SE = 71 mg; $P = 0.25$).

Seedling species specificity (LM site only)

Quercus prinus seedlings had a richness of 37 types (*S*), evenness (*E*) of 0.65, and diversity (*H*) equal to 2.36. For *Q. rubra* seedlings, $S = 38$, $E = 0.68$, and $H = 2.48$. Of the 57 total types (excluding those plots without a *Q. prinus* seedling) at the LM site, 19 types occurred only on *Q. prinus* seedlings. However, none of these types had a frequency greater than one. Twenty of the LM types occurred only on *Q. rubra* seedlings. Of these, only Bolete #02 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 *Cenococcum geophilum* (Qr = 53, Qp = 53), *Tuber* #01 (Qr = 11, Qp = 5), Cantharellaceae #01 (Qr = 3, Qp = 4), *Laccaria* cf. *laccata* (Qr = 2, Qp = 4), *Russula* #02 (Qr = 2, Qp = 4), *Tomentella* cf. *terrestris* (Qr = 3, Qp = 2), *Hebeloma* #01 (Qr = 4, Qp = 1), and *Russula* #04 (Qr = 3, Qp = 1; 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 (Fig. 2) equalled 0.183. Isolated groups of types related to seedling species/site combinations are clearly delineated on the ordination (Fig. 2). These groups represent types occurring only on *Q. 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

Table 1 Frequency (number of plots) of fungal ITS-types recovered from oak seedling roots

	Frequency						
Type	LM	HD	Qr	Qp	Closest sporophore match	Accession no.	Code
Albatrellus #01	1	0	1	1	*Albatrellus flettii* (98% 506 bp)	AY656960	ALBATREL
Amanita #02	1	0	1	1	*Amanita muscaria* (95% 551 bp)	AY656923	AMANITA2
Amanita #03	0	0	0	1	*Amanita* sp. dfmo1078 (100% 660 bp)	AY656916	AMANITA3
Amanita cf. *gemmata*	0	0	0	1	*Amanita gemmata* (99% 667 bp)	AY656924	Aman_gem
Amphinema #01	0	1	0	0	*Athelia neuhoffii* (96% 500 bp)	AY656917	AMPHINEM
Athelia cf. *neuhoffii*	1	0	0	0	*Athelia neuhoffii* (99% 612 bp)	AY656918	Athel_ne
Bolete #01	0	3	0	0	*Xerocomus pruinatus* (98% 669 bp)	AY656925	BOLETE1
Bolete #02	2	0	2	0	*Boletus erythropus* (93% 782 bp)	AY656926	BOLETE2
Bolete #03	1	0	0	0	*Boletus mirabilis* (90% 413 bp)	AY656922	BOLETE3
Boletus auriporus	0	1	0	0	*Boletus auriporus* dfmo4639 (100% 609 bp)	AY656919	Bol_auri
Cantharellaceae	4	1	3	4	*Craterellus lutescens* (94% 862 bp)	AY656927	CANTHAR1
Cenococcum geophilum	58	58	53	53	Morhpotyped		Cenococcum
cf. *Terfezia* #01	0	1	0	0	*Terfezia claveryi* (92% 600 bp)	AY656921	TERFEZI1
cf. *Tirmania* #01	0	1	0	0	*Tirmania nivea* (91% 624 bp)	AY656920	TIRMANI1
cf. *Tuber* #01	11	6	11	5	*Tuber borchii* (95% 619 bp)	AY656958	TUBER1
cf. *Tuber* #02	1	0	1	2	*Tuber borchii* (91% 527 bp)	AY656959	TUBER2
Clavariaceae #01	1	1	1	0	*Clavulina cinerea* (93% 629 bp)	AY730686	CLAVAR1
Corticiaceae #01	2	1	2	1	*Piloderma fallax* (92% 598 bp)	AY656928	CORTICI1
Cortinarius #02	0	1	0	0	*Cortinarius traganus* (94% 575 bp)	AY656964	CORTIN02
Cortinarius #03	1	0	1	1	*Cortinarius paleaceus* (96% 474 bp)	AY656929	CORTIN03
Cortinarius #04	1	0	1	1	*Cortinarius traganus* (91% 578 bp)	AY656930	CORTIN04
Cortinarius #05	0	0	0	1	*Cortinarius saturninus* (94% 347 bp)	AY656965	CORTIN05
Cortinarius #06	1	0	1	0	*Cortinarius paleaceus* (89% 344 bp)	AY656966	CORTIN06
Cortinarius #07	1	0	1	0	*Cortinarius paleaceus* (96% 317 bp)	AY656967	CORTIN07
Cortinarius #08	0	1	0	0	*Cortinarius atrocoerulaeus* (96% 570 bp)	AY656968	CORTIN08
Cortinarius #09	1	0	1	0	*Cortinarius cedriolens* (94% 590 bp)	AY656969	CORTIN09
Cortinarius #10	2	1	1	0	*Cortinarius atrocoerulaeus* (93% 570 bp)	AY656961	CORTIN10
Cortinarius #11	1	0	1	0	*Cortinarius duracinus* (93% 438 bp)	AY656931	CORTIN11
Entolomataceae #01	1	0	1	0	*Entoloma nitidum* (91% 620 bp)	AY656932	ENTOLOM1
Gautieria #01	1	0	0	0	*Gautieria* sp. SLTahoe2264CA (99% 443 bp)	AY656933	GAUTIER1
Gautieria #02	1	0	1	0	*Gautieria monticola* (95% 328 bp)	AY656970	GAUTIER2
Hebeloma #01	4	0	4	1	*Hebeloma* sp. dfmo0659* (100% 637 bp)	AY730685	HEBELOM1
Hydnellum #01	0	0	0	1	*Hydnellum diabolus* (96% 574 bp)	AY656934	HYDNELL1
Laccaria cf. *laccata*	2	3	2	4	*Laccaria laccata* dfmo0370 (99% 694 bp)	AY656938	LACCARIA
Lachnum #1	0	0	0	1	*Lachnum pygmaeum* (97% 527 bp)	AY656935	LACHNUM1
Lactarius #02	1	0	1	0	*Lactarius volemus* dfmo1034 (97% 613 bp)	AY656936	LACTAR2
Lactarius #03	1	1	1	0	*Lactarius deliciosus* (96% 620 bp)	AY656971	LACTAR3
Lactarius #04	0	1	0	0	*Lactarius deliciosus* (97% 610 bp)	AY656972	LACTAR4
Lactarius #05	1	0	1	1	*Lactarius quietus* (92% 125 bp)	AY656973	LACTAR5
Lactarius #06	0	1	0	0	*Lactarius utilis* (96% 680 bp)	AY656974	LACTAR6
Lactarius chrysorheus	2	4	1	1	*Lactarius chrysoreus* (100% 659 bp)	AY656937	Lact_chr
Peziza #01	0	0	0	1	*Peziza* dfmo1344 (98% 583 bp)	AY656939	PEZIZA1
Phialophora #1	1	0	0	0	*Phialophora finlandia* (94% 556 bp)	AY656940	PHIALO1
Russula #01	0	0	0	1	*Russula postiana* (92% 696 bp)	AY656941	RUSSUL01
Russula #02	2	0	2	4	*Russula decolorans* (94% 637 bp)	AY656942	RUSSUL02
Russula #03	0	0	0	1	*Russula rosacea* (91% 446 bp)	AY656943	RUSSUL03
Russula #04	4	0	3	1	*Russula pascua* (95% 603 bp)	AY656944	RUSSUL04
Russula #05	2	0	1	0	*Russula* sp. dfmo2008 (99% 608 bp)	AY656945	RUSSUL05
Russula #06	1	2	1	1	*Russula emetica* (96% 360 bp)	AY656962	RUSSUL06
Russula #07	0	2	0	0	*Russula integra* (92% 688 bp)	AY656975	RUSSUL07
Russula #08	0	0	0	1	*Russula* sp. dfmo1104 (100% 461 bp)	AY656946	RUSSUL08
Russula #09	1	1	1	0	*Russula pascua* (97% 696 bp)	AY656978	RUSSUL09
Russula #10	0	1	0	0	*Russula puellula* (93% 670 bp)	AY656976	RUSSUL10
Russula #11	1	2	1	0	*Russula raoultii* (95% 440 bp)	AY656977	RUSSUL11
Sebacinaceae #01	2	0	2	1	*Tremellodendron pallidum* (89% 544 bp)	AY656955	TREMELL1

Table 1 *Continued*

Type	Frequency				Closest sporophore match	Accession no.	Code
	LM	HD	Qr	Qp			
Sebacinaceae #02	0	1	0	0	*Tremellodendron pallidum* (94% 563 bp)	AY277943	TREMELL2
Sebacinaceae #03	0	0	0	1	*Tremellodendron pallidum* (94% 355 bp)	AY656985	TREMELL3
Thelephoraceae #01	1	0	0	1	*Thelephora americana* (90% 612 bp)	AY656947	THELEPH1
Thelephoraceae #02	0	0	0	1	*Thelephora penicillata* (91% 640 bp)	AY656979	THELEPH2
Thelephoraceae #03	0	2	0	1	*Tomentella fusco-cinerea* (89% 649 bp)	AY656948	THELEPH3
Thelephoraceae #04	0	0	0	1	*Tomentella cinerascens* (93% 561 bp)	AY656980	THELEPH4
Thelephoraceae #05	0	0	0	1	*Tomentella galzinii* (89% 535 bp)	AY656949	THELEPH5
Thelephoraceae #06	1	0	1	0	*Tomentella cinerascens* (92% 433 bp)	AY656950	THELEPH6
Thelephoraceae #07	0	1	0	0	*Tomentella subclavigera* (88% 604 bp)	AY656981	THELEPH7
Thelephoraceae #08	1	0	1	0	*Tomentella terrestris* (92% 635 bp)	AY656982	THELEPH8
Thelephoraceae #09	0	0	0	1	*Tomentella terrestris* (92% 636 bp)	AY656983	THELEPH9
Tomentella #01	1	2	1	0	*Tomentella galzinii* (96% 630 bp)	AY656951	TOMENT1
Tomentella #02	0	0	0	1	*Tomentella coerulea* (96% 644 bp)	AY656984	TOMENT2
Tomentella #03	0	2	0	1	*Tomentella subclavigera* (98% 630 bp)	AY656963	TOMENT3
Tomentella cf. *sublilacina*	0	1	0	0	*Tomentella sublilacina* (99% 569 bp)	AY656953	Tom_subl
Tomentella cf. *terrestris*	3	3	3	2	*Tomentella terrestris* (97% 448 bp)	AY656954	Tom_terr
Tomentellopsis zygodesmoides	0	1	0	1	*Tomentellopsis zygodesmoides* (100% 637 bp)	AY656952	TOM_ZYGO
Tricholoma #01	2	0	2	0	*Tricholoma pardinum* (91% 645 bp)	AY656986	TRICHOL1
Tricholoma #02	1	0	1	0	*Tricholoma mutabile* (91% 430 bp)	AY656987	TRICHOL2
Tricholoma #03	1	0	1	0	*Tricholoma fulvum* (95% 519 bp)	AY656988	TRICHOL3

LM, low elevation mesic site; HD, drier high elevation site; Qr, *Quercus rubra* seedling; Qp, *Q. prinus* seedling. Frequency by site includes *Q. rubra* seedlings only; frequency by seedling species includes only LM samples. The closest matching sporophore sequence from GenBank is listed along with the percentage match and the length of the sequence read. Representative sequences for each type are deposited at GenBank under the Accession nos listed. The code name is the type designation used for Fig. 2.
*dfmo no. — Duke Forest Mycological Observatory sporophore voucher reference number.

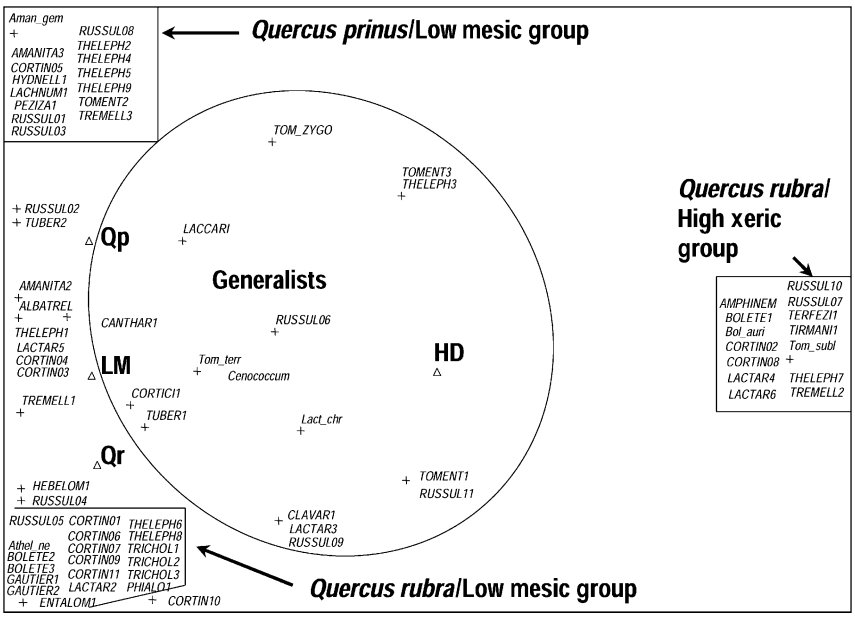

Fig. 2 Reciprocal averaging ordination of EM fungus ITS-types. Frequencies by site and bait seedling species determine the horizontal and vertical distribution (respectively) of the types in the ordination space. Triangles: LM, low elevation mesic site; HD, drier high elevation site; Qr, *Quercus rubra* seedlings; and Qp, *Quercus prinus* seedlings. Crosses: fungi recovered from oak seedling roots, fungus codes given in Table 1. Frequency by site includes both seedling species; frequency by seedling species includes only LM samples. Groups of types which occur uniquely in one site/species combination are designated, along with generalists which overlap in site and seedling species occurrence.

and Qr/HD groups. Those types floating in the centre show no specificity to site type or seedling species and may be considered generalists (Fig. 2).

Discussion

Seedlings and overall diversity

In our study, seedlings planted under a forest canopy were colonized by a high diversity of mycorrhizal fungi. Among 14 previous studies compiled by Horton & Bruns (2001), higher richness was found only in mature Douglas fir/western hemlock (90 years) and red fir (350–400 years) forests with 200 and 80 types, respectively (Luoma *et al*. 1997; Bidartondo *et al*. 2000). However, these studies used soil cores that included the roots of mature trees of various species.

Until recently, the ability of tree seedlings to form mycorrhizal symbioses *in situ* in forests was relatively unknown. Gibson & Deacon (1988) found that only older portions of birch sapling root systems were able to form EM with *Lactarius pubescens*, a late-stage fungus, when in a glasshouse. However, in a field study, Fleming (1983) found that late-stage fungi were able to colonize birch seedlings when planted under mature trees. In our study, 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, *Tricholoma* spp., and Tuberales can be for the most part considered late-stage (Chu-Chou 1979; Danielson 1984; Dighton *et al*. 1986; Hintikka 1988). The hypothesis that oak seedlings might be predominantly associated with early- and mixed-stage fungi was not supported.

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. However, in a coniferous boreal forest, Jonsson *et al*. (1999a) found that 72% of the ectomycorrhizal types recovered from mature roots (i.e. in soil cores) were also found on seedling root systems. In the study of Kennedy *et al*. (2003), naturally occurring *Lithocarpus densiflora* seedlings (less than 1 m tall and with stem diameter less than 1 cm) shared 17 of 56 EM fungi with overstory *Pseudotsuga menzesii* trees. Similar total diversity compared to that found over a three year period in a nearby area relying solely on sporophores (Walker & Miller 2002) was documented in a single year using bait seedlings and molecular techniques in this study (Fig. 1). Our findings support the conclusion that seedlings in natural forest communities can be colonized by a wide assemblage of EM fungi. The high diversity of mycorrhizal types colonizing seedlings in this study also confirms the applicability of using seedlings to effectively document mycobiont diversity *in situ*.

High mycobiont diversity has important implications for the potential for seedlings to acquire carbohydrates from mature canopy trees. Net transfer of fixed carbon to shaded seedlings through common mycelial networks was demonstrated by Simard *et al*. (1997). However, according to Robinson & Fitter (1999), considerable clarification remains necessary as to the amount and significance of carbon translocated to seedlings directly via EM hyphae. Nonetheless, unidirectional carbon transfer to shaded seedlings may be especially important in temperate forests where seedlings generally persist under a closed canopy until gap formation enables colonization of canopy space (Runkle 1982; Canham 1988; Clinton *et al*. 1994). If seedling mycobiont diversity was low in this system, the potential for carbon sharing would likely be reduced because fewer hyphal networks could be accessed.

Implications for rhododendron — seedling interactions

Previous studies reported reduced EM colonization levels (ramification index and percent colonization) and diversity shifts (increased abundance of *Cenococcum geophilum*) where *Rhododendron maximum* thickets were present compared to open forest (Walker *et al*. 1999). However, sporophore distributions were similar in the open forest compared to the thickets (Walker & Miller 2002). One hypothesis was that the seedlings were associated with a limited set of fungi which were affected by the presence of *R. maximum*. The high diversity found in this study indicates that reduced overall colonization levels of oak seedlings in *R. maximum* thickets is not because of an affect limited to a 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. Because inhibition of mycorrhization is not specific to a small subset of the mycorrhizal fungus community, it is most likely the process of mycorrhization with seedlings that is inhibited in the thickets. Furthermore, in temperate forest ecosystems, ectomycorrhizae are found primarily near the soil surface and in accumulated litter (Harley 1940; Meyer 1973). Because a dense rhododendron root mat forms at the soil surface, the density of mycorrhizae of mature trees may be reduced because of spatial competition. If this is the case, it is likely that the seedling root systems are spatially isolated from mycorrhizae formed by mature trees in the rhododendron thickets, thus explaining the reduced colonization of the seedlings in the thickets.

Notes on fungal community composition

Most types were referred to commonly accepted ectomycorrhizal groups. Exceptions include the Sebacinaceae types and the *Phialophora* #1 and *Lachnum* #1 types. The identity and ectomycorrhizal role of Sebacinaceae types are presented

in Walker & Parrent (2004). We do not know of any reports indicating a mycorrhizal habit for *Lachnum* spp. The *Lachnum* #1 type is included in our analyses, however, additional research is needed to confirm the ecological role of this type. *Phialophora finlandia* is capable of a variety of interactions including dark septate endophytes (Jumpponen & Trappe 1998), ectomycorrhizae and ectendomycorrhizae (Wilcox & Wang 1987), and ericoid mycorrhizae (Monreal *et al*. 1999).

C. geophilum was the most frequent mycobiont at both sites and on both seedling species. Excluding *C. geophilum*, the dominant type was a truffle (Tuber #01) which would have gone unnoticed in most sporophore collections. Several tomentelloid types such as *Tomentella* cf. *terrestris* and a corticioid type (Corticiaceae #01) were also among the most frequent. Types referred to the common epigeous sporophores *Laccaria laccata*, *Lactarius chrysorheus*, and *Craterellus lutescens* had high frequency.

The richest epigeous groups were representative of 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 the family Russulaceae and tomentelloid/thelephoroid types is typical of 16 previous studies in coniferous forests synthesized by Horton & Bruns (2001). Tomentelloid/thelephoroid fungi are clearly important EM associates in the mixed forests of the southeastern Appalachian Mountains 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. As 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), and Jonsson *et al*. (1999a and 1999b).

Diversity by site

Richness, evenness and diversity of mycorrhizal root tip ITS-types were all higher at the low elevation mesic site (LM) compared to the drier high elevation site (HD). Nearly twice as many types occurred only at the LM site than were found only at the HD site. At the HD site, approximately half of the types found also occurred at the LM site. Of species common to both sites, frequencies tended to be higher at the LM site. Although richness was higher at the LM site, the major groups including russuloid, tomentelloid/thelephoroid, and cortinarioid were all present at both sites. Notably, no *Amanita* spp. or *Tricholoma* spp. types were recovered at the HD site.

These observations indicate that at the LM site the mycorrhizal fungus assemblage was richer and the 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 strongly affect EM fungus composition (Nantel & Neumann 1992). Although hypogeous fungi avoid atmospheric aspects of harsher sites at higher elevations where greater exposure and less moisture occur, diversity and dominance of hypogeous taxa did not appear to increase with elevation in this system. Because root biomass was similar between sites, lower diversity does not appear to be related to smaller root systems at the HD site. 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

Because of 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* vs. *Quercus 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. Nineteen additional types were detected at the LM site because of 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 larger number of sites with similar overall characteristics. Interpretations 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 hyperdiverse system. For a quantitative analysis of spatial structure in a variety of ectomycorrhizal fungus communities and discussion of optimal sampling designs, see Lilleskov *et al*. (2004).

Summary

The EM 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 including those typically characterized as late- or mixed-stage fungi. 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 vs. white oak seedlings was unresolved. The high diversity of associated mycorrhizal fungi highlights the potential for seedlings to acquire carbon from mycelial networks and confirms the utility of using outplanted seedlings to estimate ectomycorrhizal diversity.

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

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