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Research

Evidence of species interactions within an ectomycorrhizal fungal community

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Summary

• Ectomycorrhizal fungal communities can be structured by abiotic and biotic factors. Here, we present evidence for community structuring by species interactions.

• We sampled ectomycorrhizas and forest floor seven times during a 13-month period. The presence of various ectomycorrhizal fungal species was determined for each sample, and species co-occurrence analyses were performed.

• For both ectomycorrhizas and forest floor samples there was significantly less co-occurrence among species within the community than expected by chance, mostly because of negative associations involving *Cenococcum geophilum* or *Clavulina cinerea*. For some species pairs, there was significantly more co-occurrence than expected by chance. Both nitrogen and tannin additions to the forest floor altered some interactions among species.

• The causes of these nonrandom distributions are currently unknown. Future investigations on competition, antibiosis, parasitism and facilitation among ectomycorrhizal fungal species appear to be warranted.

Key words: antibiosis, *Cenococcum geophilum*, *Clavulina cinerea*, community structure, competition, competitive network, ectomycorrhizal fungi, nonrandom distribution.

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Introduction

Natural forests and forest plantations possess a relatively high diversity of ectomycorrhizal fungi (Bruns, 1995; Horton & Bruns, 2001; Taylor, 2002; and references therein). The species composition of ectomycorrhizal fungal communities can be strongly influenced by various soil properties, including parent material (Gehring *et al.*, 1998), soil stratification (Malajkzuk & Hingston, 1981; Dickie *et al.*, 2002; Landeweert *et al.*, 2003; Rosling *et al.*, 2003), organic matter content (Harvey *et al.*, 1987), litter quality (Goodman & Trofymow, 1998; Conn & Dighton, 2000), moisture content (O'Dell *et al.*, 1999) and fertility (Sagara, 1995; Lilleskov *et al.*, 2001). Variation in any of these soil properties therefore has the potential to contribute to ectomycorrhizal fungal species diversity. This kind of partitioning of the environment may be the result of the abiotic preferences of a species, which delineate its fundamental niche, as well as interactions among species that limit a species to its realized niche.

As noted above, we have previously shown that spatial differentiation among ectomycorrhizal fungal species occurs according to soil horizon (Dickie *et al.*, 2002). At the scale of soil horizons, therefore, the occurrences of the species will not be random. It follows, then, that the examination of species interactions capable of contributing to realized niches must occur at a scale that is smaller than the observed niches. In this contribution we tested for the existence of interactions among ectomycorrhizal fungal species by assessing the frequency of co-occurrence of species on individual, colonized roots, or within 0.25 ml samples of the forest floor. As far as we are aware, there have been no other systematic tests to determine whether distributions of

ectomycorrhizal fungal species deviate from random on a scale this fine.

Interactions among ectomycorrhizal fungal species probably occur frequently. Zhou & Hogetsu (2002), for example, showed that many species of ectomycorrhizal fungi coexist in mosaic fashion in small volumes of soil. Several studies (Gryta *et al.*, 1997; Fiore-Donno & Martin, 2001; Guidot *et al.*, 2001, 2003; Zhou *et al.*, 2002) indicated that genets of some species of ectomycorrhizal fungi, even those of mature, late-successional forests, are relatively small and turn over rapidly. These findings suggest that there is frequent opportunity for at least some ectomycorrhizal fungal species within a community to interact with others as their hyphae attempt to colonize either newly produced roots in order to acquire carbon, or recently vacated volumes of forest floor in order to capture water and nutrients. Moreover, many researchers have noted multiple species of ectomycorrhizal fungi colonizing a single root (for example Mamoun & Olivier, 1993a,b; Wu *et al.*, 1999), and there are some notable examples of association between specific pairs of fungi on colonized roots (Olsson *et al.*, 2000). Systematic investigation of such nonrandom distributions at the whole-community level, however, has not been made.

Interactions among mycorrhizal fungal species may be either positive or negative in nature. Compared with random distributions, more frequent than predicted co-occurrence of species might occur as a consequence of physiological complementation. For example, one species may secrete enzymes that allow it to be superior at acquiring nitrogen (N) from organic sources, while another may secrete enzymes allowing it to be superior at acquiring phosphorus (P). By living in close association, both species may be able to acquire the necessary N and P. More frequent than predicted co-occurrence of species might also occur as a consequence of parasitic interactions between fungi such as if one species obtains some of its reduced carbon from another species, or if root colonization by one species is facilitated by the presence of another. Less frequent than predicted co-occurrence of species (avoidance) might be the result of either antibiosis or a competitive hierarchy, either of which could result in the exclusion of one species by another.

Competitive exclusion may be exacerbated by nutrient addition, as suggested by the reduction in species richness with increasing N availability (Lilleskov *et al.*, 2001, 2002; Peter *et al.*, 2001). We therefore tested the hypothesis that the addition of nitrogen to the forest floor would reduce co-occurrence of species. Polyphenolic compounds, including tannins, which are abundant in many forest floors (Kuiters & Dennemann, 1987), may have beneficial effects on some ectomycorrhizal fungal species and detrimental effects on others (Baar *et al.*, 1994; Koide *et al.*, 1998), and this may also influence competitive exclusion. Therefore, we also determined whether the addition of tannin to the forest floor could significantly alter the co-occurrences of species.

Materials and Methods

Study site

The study was performed in an approximately 65-yr-old red pine (*Pinus resinosa* Ait.) plantation located in State College, Centre County, PA, USA. The site has a well-developed O-horizon comprising litter, a fermentation (F) layer and a thin humified (H) layer, which overlies the mineral soil (Morrison sandy loam), consisting of a thin eluviated A-horizon over a well-defined sandy B-horizon. There is little or no understory vegetation in most of the stand. Where understory vegetation exists, it consists of saplings of red pine and hardwoods such as *Acer rubrum* and *Quercus alba*, and few *Vaccinium* spp. shrubs. Samples were collected only from areas without understory vegetation.

Study 1, ectomycorrhizal hyphae in F-layer samples

We used a cylindrical soil sampler (30 cm long and 2 cm inside diameter) featuring one open side to obtain samples of F-layer. These were collected from 80, randomly located 0.25 m^2 plots on 18 July, 16 September and 16 October 2002, and on 16 January, 5 April, 26 June and 22 August 2003. On each date 60 samples were taken randomly from among the 80 plots. There were, therefore, a total of 420 samples taken altogether. Because samples were taken from the same plot on more than one sampling date, care was exercised to remove each sample from a previously unsampled place within the plot. A single, approximately isodiametric, 1.0 ml sample of F-layer was collected from each core taken from the forest floor. The samples were never taken from the outer surface of the core but, instead, were taken from its interior so as to avoid any material carried over from the previous core. Samples were placed into microfuge tubes in the field and were frozen (−20°C) on the day of collection and left frozen prior to DNA extraction.

Study 2, ectomycorrhizal fungi on colonized roots

Ectomycorrhizal roots were collected from cubes of F-layer sampled within a few centimeters of the cores taken in Study 1, within the same plots and on the same dates. On the first four dates 40 samples of F-layer were cut out of the forest floor using a small knife. On the subsequent three dates 60 samples were taken. The ground surface of each sample was approximately 5 × 5 cm square. The samples were taken back to the laboratory where all viable ectomycorrhizas were removed from each F-layer sample and placed onto a moist paper towel. Three ectomycorrhizas (for the first six dates) or four ectomycorrhizas (for the last date) were randomly chosen from among all ectomycorrhizas of each sample and individually dried over silica gel in microfuge tubes. They were then stored at −20°C until they were extracted. A total of 1080 ectomycorrhizas were thus processed.

Study 3, effects of nitrogen and tannin additions on hyphae in the F-layer

Forty locations were randomly selected within the plantation and a 0.25 m^2 plot was established at each location for each of the three treatments: N addition, tannin addition and control (no additions). The three plots at each location were spaced approximately 2 m apart from each other to avoid treatment contamination. Nitrogen was added in the form of dry, type 120 R, 4-month, controlled-release urea (Exxon Mobil, Houston, TX, USA). The concentration added was 500 kg N ha^{-1} yr^{-1} to each plot in June 2002 and April 2003. Tannin was applied in June, July, August, September and October 2002, and April, May and June 2003. Tannin additions to individual plots consisted of 2.48 g dry extract of Quebracho (*Schinopis* sp.; LH Lincoln & Sons Coudersport, PA, USA), equal to 1.45 g tannic acid equivalents, mixed in 125 ml water. Samples from the F-layer were taken from each plot on 20 June 2003 with the 2 cm diameter soil sampler (above), using care to avoid cross-contamination from one plot to another as before. Samples were placed into sealed microfuge tubes in the field and, upon return to the laboratory, stored at −20°C.

Molecular methods, all studies

The DNA extraction of all samples (both F-layer and ectomycorrhizas) was performed within 4 wk of collection in order to minimize DNA degradation. For F-layer samples, all ectomycorrhizas were removed under a dissecting microscope before extraction to determine the presence of the ectomycorrhizal fungi as hyphae rather than as ectomycorrhizas. A 0.25-ml subsample of root-free F-layer was used for DNA extraction, which was performed using a commercially available soil DNA extraction kit (Ultra Clean Soil DNA Isolation Kit; Mo Bio Laboratories, Solana Beech, CA, USA). The standard Mo Bio method for soil DNA extraction using a vortex shaker with a Mo Bio adapter for holding 12 microfuge tubes was slightly modified to obtain a higher yield of inhibitor-free DNA, as given in Dickie *et al.* (2002). Ectomycorrhizas were placed individually into extraction tubes of a commercially available DNA extraction kit (Ultra Clean Microbe DNA Isolation Kit; Mo Bio Laboratories) from which the garnet beads had been removed. In their place we added 3 2.4 mm zirconia beads (Biospec Products, Bartlesville, OK, USA). The ectomycorrhizas were shaken dry with the beads for 10 min with the vortex shaker on the highest setting, which pulverized the ectomycorrhizas. Then, 300 µl bead solution, 50 µl IRS solution and 50 µl MD1 solution (MoBio) were added and the mixture was shaken for a further 15 min. The remaining extraction procedure was the same as for F-layer samples.

Genomic DNA thus extracted was amplified using a 'Hot Start' DNA polymerase (Tempase, GeneChoice, Frederick, MD, USA) to avoid nonspecific amplification (Roux, 1995). The total PCR reaction volume was 50 µl and the final concentrations of the reaction mix were as follows: 1× ammonium-based buffer (PGC Scientifics Corp., Frederick, MD, USA), 3 mM $MgCl_2$, 0.8 µg $µl^{-1}$ bovine serum albumin, 0.4 mM dNTPs (PGC Scientific), 30 pmol internal transcribed spacer (ITS)-1F (Gardes & Bruns, 1993) with 5′ VIC fluorescent label (Operon, Alameda, CA, USA), 30 pmol ITS4 (Gardes & Bruns, 1993) with 5′-NED fluorescent label (Operon), 2.5 units Tempase DNA polymerase (PGC Scientifics), and 5 µl 1× genomic DNA (as extracted above). Bovine serum albumin was used to reduce polymerase chain reaction (PCR) inhibition by substances coextracted from soils with the DNA (Kreader, 1996; Watson & Blackwell, 2000). The PCR program for the MWG Primus 96 plus thermocycler (MWG AG, Ebersberg, Germany) began with an enzyme activation step at 95°C for 15 min, followed by four cycles of 95°C for 40 s, 49°C for 30 s and 72°C for 48 s, 31 cycles of 94°C for 40 s, 49°C for 30 s and 72°C for 48 s, and a final 7 min extension period at 72°C. The thermocycler lid was held at 102°C for the entire reaction. The PCR products were visualized following electrophoresis in 2% agarose gel and ethidium bromide staining. The PCR products were purified using a commercially available kit (UltraClean DNA Purification Kit; Mo Bio Laboratories) before restriction digestion.

Each purified PCR reaction was digested with two restriction enzymes. We incubated 5 µl of the PCR product (approx. 10 ng of DNA) with either 2 units *Hin*f1 or 4 units *Hae*III (New England Biolabs, Beverly, MA, USA) using the specified buffers at the specified concentrations, in a total of 10 µl at 37°C for 4 h. Digestion products were diluted with 80 µl H_2O, then stored at 4°C.

To reveal the terminal restriction fragment length polymorphism (T-RFLP), 1 µl of digestion product was resuspended in 10 µl formamide (ABI, Foster City, CA, USA) and 0.125 µl X-Rhodamine MapMarker 1000 standard, denatured at 95°C for 5 min, then immediately cooled on ice. Capillary gel electrophoresis was performed with an ABI 3100 genetic analyser using Performance Optimized Polymer 4 in 36 cm capillaries (Applied Biosystems, Foster City, CA, USA). The samples were injected for 22 s at 15 kV, and separated for 45 min at 40°C. Terminal restriction fragment lengths were determined by GENESCAN analysis software version 3.7 (Applied Biosystems). These lengths were compared with those in our database comprising ectomycorrhizal fungi collected from our research site over several years as sporocarps or ectomycorrhizas. The database used in this study is different from the database published in Dickie *et al.* (2002). That database was derived from T-RFLP patterns produced on a slab gel sequencer. The current database was produced using a capillary sequencer and a different DNA size standard and is considered to be more accurate than the previous one. We considered a species present if at least three of its four restriction fragment lengths (VIC *Hin*f1, NED *Hin*f1, VIC *Hae*III,

and NED *Hae*III) were seen in the sample T-RFLP fingerprint at less than 3 bp error. If a species were present, all four of its terminal restriction fragments must have been present, but on occasion the DNA concentrations were low enough that a particularly small peak corresponding to one or another fragment would get lost in the 'noise' of the sequencer output. In the vast majority of cases the fingerprints were matched to within 1.5 bp error, but a few terminal fragments produced two peaks within 2 bp of each other. Therefore, an allowable error of 3 bp was necessary. When no restriction site existed for one of the enzymes, the PCR product length was recorded as the restriction fragment length for that enzyme. A description of the Excel (Microsoft Corporation) program used to match unknown T-RFLP patterns to those in the database is given in Dickie *et al.* (2002).

Statistical analyses

A total of 385 of the 420 possible F-layer samples in Study 1, 1026 of the 1080 possible ectomycorrhizas in Study 2 and 107 of the 120 possible F-layer samples in Study 3 contained identifiable DNA from ectomycorrhizal fungal species. The results of each study were analysed separately. The presence or absence of the various ectomycorrhizal fungal species was recorded for each sample and these data were then arranged into matrices of rows (species) and columns (samples). The checkerboard scores, or C-scores (Stone & Roberts, 1990), were calculated using the co-occurrence module of the ECOSIM programs (Gotelli & Entsminger, 2001). As used in this fashion, the C-score is a measure of co-occurrence of species within a sample among all possible species pairs within the community. The ECOSIM program calculates the C-score for the community and then compares it to a distribution of C-scores calculated from a user-defined number of simulated matrices assuming random distributions of species. For ectomycorrhizas and F-layer hyphae, the simulated matrices were generated within ECOSIM maintaining the same row totals in the simulated matrices as in the observed matrix and allowing all columns (samples) to be equally likely to be represented. An observed C-score that is significantly larger than the C-score calculated for a community with randomly distributed species, for example, indicates that within samples there is significantly less co-occurrence (more avoidance) among the species than expected.

The degrees of association between all pairs of species were determined by calculating the Spearman rank correlation coefficients (Zar, 1999) with the StatsDirect programs (StatsDirect Ltd, 2002). Correlations were considered significant when $P = 0.05$. We calculated the correlation coefficients for all pairs of species that had frequencies of three or more because correlations based on two or fewer occurrences were judged to have little meaning.

In Study 1, *Cenococcum geophilum* and *Clavulina cinerea* were the most abundant species and they were negatively associated with each other at the sample level (see the Results section). To test whether this pattern could have been produced by patchiness at the plot level, C-scores were also calculated for their distributions among plots. We determined the actual number of 0.25 m^2 plots (not samples) containing both species, only *Cenococcum*, only *Clavulina*, or neither species. The data were arranged into a matrix of two rows (the two species) and columns (plots). By comparing observed with expected C-scores for distributions of the two species, we were able to determine whether the degree of co-occurrence within plots was significantly different from random at the plot level rather than the sample level.

Results

Study 1, ectomycorrhizal hyphae in F-layer samples

A total of 28 species of ectomycorrhizal fungi were identified from the 420 F-layer samples (Table 1). By far the most frequently occurring species was *C. geophilum*, which was followed by *C. cinerea*, the unknown species Jori 10, etc. (we use the 'Jori' designation followed by a serial number for ectomycorrhizal fungi identified only from repeated observation on ectomycorrhizas but not from known sporocarps).

Table 1 The frequencies of occurrence of all species of ectomycorrhizal fungi detected as hyphae in Study 1

	Frequency
Cenococcum geophilum (CEGE)	255
Clavulina cinerea (CLCI)	128
Jori 10 (J10)	68
Russula white 1 (RUW1)	47
Scleroderma citrinum (SCCI)	35
Jori 1 (J1)	31
Ramaria concolor (RACO)	31
Lactarius oculatus (LOC)	28
Tylopilus felleus (TYFE)	28
Jori 4 (J4)	26
Jori 5 (J5)	24
Suillus intermedius (SUIN)	22
Amanita brunnescens (AMBR)	18
Amanita cf. *vaginata* (AMVA)	15
Gyroporus castaneus (GYCA)	10
Jori 12 (J12)	7
Tricholoma virgatum (TRVI)	7
Amanita rubescens (AMRU)	4
Lactarius 4 (L4)	4
Suillus 1 (SU1)	4
Thelephora terrestris (THTE)	3
Boletus cf. *illudens* (BOIL)	2
Cortinarius b (COB)	2
Amanita virosa (AMVI)	1
Cortinarius 3 (CO3)	1
Jori 13 (J13)	1
Russula 3 (RU3)	1
Russula pseudolepida (RUPS)	1

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Table 2 The frequency distribution of the number of species of ectomycorrhizal fungi detected as hyphae per 0.25-ml F-layer sample in Study 1

Number of species per 0.25-ml F-layer sample	Number of samples
1	164
2	109
3	63
4	26
5	14
6	6
7	2
8	1

Many F-layer samples contained a single ectomycorrhizal fungus species, but a great many also contained two or more (up to eight) species (Table 2).

We calculated C-scores using all samples for which there were at least two species (see Table 2). The observed C-score (487.3) was significantly greater than the expected C-score (mean 432.1) based on random distributions (Table 3), indicating that there was significantly less co-occurrence (more avoidance) among species within the community than expected.

Among the large, significant, negative Spearman rank correlation coefficients were those between *Russula* white 1 and *Clavulina*, *Clavulina* and *Cenococcum*, and Jori 10 and *Clavulina* (Table 4). *Cenococcum* and *Clavulina* also participated in a number of other negative associations with various other species (Table 4). We therefore recalculated the C-score in various ways (Table 3). When *C. geophilum* was removed from the analysis the observed C-score (404.1) was still significantly larger than the expected C-score (mean 373.4). However, when *C. geophilum* and *C. cinerea* were removed, the observed C-score (315.1) was not significantly different from expected (mean 308.6), indicating that there was neither more nor less avoidance among species than expected based on a random distribution of the remaining species.

We also determined the distributions of *C. geophilum* and *C. cinerea*, the two most abundant species, with respect to plot (not sample). During the 13-month period of sampling, *Cenococcum* occurred without *Clavulina* in 21 plots, *Clavulina* appeared without *Cenococcum* in seven plots, the two species occurred together in 48 plots and neither occurred in two plots. The C-score for the observed distribution (147.0) was not significantly different from the C-score for randomly distributed *Cenococcum* and *Clavulina* (mean 146.5), indicating that with respect to plot there was neither more nor less avoidance between the two species than expected based on a random distributions. Thus, there did not appear to be any evidence for the existence of separate patches of the two species at the plot scale.

The largest Spearman correlation coefficient was positive and occurred between *Thelephora terrestris* and *Lactarius* 4 (Table 4). Other large, significant positive coefficients included those between *Amanita* cf. *vaginata* and *Russula* white 1, *Amanita rubescens* and *Tricholoma virgatum*, *A.* cf. *vaginata* and *Scleroderma citrinum*, Jori 5 and *Clavulina*, and between *T. terrestris* and *Amanita brunnescens*.

Cenococcum and *Clavulina* interacted with the other species in different ways. For example, Jori 10 was positively correlated with *Cenococcum* but negatively correlated with *Clavulina*, while Jori 1 and Jori 5 were negatively correlated with *Cenococcum* but positively correlated with *Clavulina* (Table 4).

Study 2, ectomycorrhizal fungi on colonized roots

A total of 25 species of ectomycorrhizal fungi were identified from the 1080 ectomycorrhizas (Table 5). By far the most frequently occurring species was *C. geophilum*, which was followed by *C. cinerea*, *Lactarius oculatus*, *Tylopilus felleus*, etc. We identified a single ectomycorrhizal fungus species on the vast majority of ectomycorrhizas, but approximately 10% harbored more than one species (Table 6). We calculated C-scores using all ectomycorrhizas for which there were at least 2 species (see Table 6). The observed C-score (245.8) was significantly larger than the expected C-score (mean 172.2), $P < 0.0001$. This indicated that there was significantly less co-occurrence (more avoidance) among species within the community than expected.

The Spearman rank correlation coefficients indicated that significant negative interactions primarily involved *Cenococcum*, *Clavulina*, *L. oculatus* and Jori 4 (Table 7). The coefficient of greatest magnitude occurred between *Cenococcum* and *Clavulina*. There were also significant positive interactions between *A.* cf. *vaginata* and *Russula* white 1, *L. oculatus* and *C. geophilum*, Jori 5 and *Clavulina*, Jori 4 and *Clavulina*, and between Jori 14 and *Cenococcum* (Table 7). By contrast with

Table 3 Relationship between observed and expected C-scores for ectomycorrhizal fungal species detected as hyphae in Study 1

Species composition	Observed < or > expected C-score	*P*	Interpretation
All species	>	< 0.0001	Greater than expected avoidance
All species less *Cenococcum*	>	< 0.0001	Greater than expected avoidance
All species less *Cenococcum* and *Clavulina*	nsd	0.085	Avoidance nsd from expected

C-scores (a measure of species avoidance) were calculated for species with frequencies of three or greater and present in F-layer samples containing two or more species (see Table 2). The expected values were based on 1000 random simulations with fixed row sums and equiprobable column sums. nsd, Not significantly different.

Table 4 Matrix of Spearman rank correlation coefficients for ectomycorrhizal fungal species detected as hyphae in Study 1

	CEGE	CLCI	J10	RUW1	SCCI	J1	RACO	LOC	TYFE	J4	J5	SUIIN	AMBR	AMVA	GYCA	J12	TRVI	AMRU	L4	SU1
CLCI	***−0.363***																			
J10	***0.144***	***−0.278***																		
RUW1	0.044	***−0.400***	−0.030																	
SCCI	***−0.207***	0.100	−0.125	0.111																
J1	***−0.165***	***0.165***	−0.061	−0.075	−0.018															
RACO	0.026	***−0.179***	***0.197***	***0.184***	−0.092	0.062														
LOC	0.123	***−0.163***	0.042	−0.080	***−0.150***	−0.107	−0.107													
TYFE	−0.032	0.013	−0.097	0.010	−0.041	0.003	***0.160***	−0.055												
J4	−0.024	−0.020	***−0.143***	−0.010	−0.110	***0.176***	0.055	−0.090	−0.012											
J5	***−0.229***	***0.329***	−0.034	***−0.176***	−0.102	***0.236***	−0.015	−0.082	−0.089	0.098										
SUIIN	0.046	−0.114	−0.074	−0.087	−0.002	−0.086	0.002	0.073	0.016	0.025	−0.064									
AMBR	***−0.218***	−0.131	−0.077	−0.031	−0.048	−0.101	0.010	−0.032	0.078	−0.032	−0.087	***0.181***								
AMVA	***−0.140***	***−0.189***	−0.056	***0.400***	***0.349***	−0.005	0.046	−0.099	−0.049	−0.043	−0.094	−0.026	−0.067							
GYCA	−0.021	−0.094	***0.194***	−0.002	0.034	0.037	−0.025	−0.079	−0.017	−0.012	0.064	−0.071	0.038	0.114						
J12	−0.029	−0.099	−0.060	−0.027	−0.074	0.076	0.001	0.014	−0.069	0.014	0.020	−0.059	−0.045	−0.049	0.085					
TRVI	−0.083	0.111	−0.060	−0.091	−0.074	***0.150***	0.001	0.014	0.009	0.094	0.020	0.118	0.065	0.054	−0.039	−0.033				
AMRU	−0.113	−0.045	−0.013	0.016	−0.056	0.043	0.043	0.056	0.050	−0.050	−0.047	0.072	0.110	−0.037	−0.030	−0.025	***0.363***			
L4	−0.042	−0.114	***0.136***	−0.069	0.041	0.043	−0.055	−0.050	−0.052	−0.050	−0.047	−0.044	***0.255***	0.098	***0.297***	0.169	−0.025	−0.018		
SU1	0.100	−0.114	−0.013	0.016	−0.056	0.043	0.043	−0.050	***0.152***	0.056	−0.047	−0.044	0.110	−0.037	−0.030	−0.025	−0.025	−0.018	−0.018	
THTE	−0.077	−0.019	0.010	−0.059	−0.048	−0.047	−0.047	−0.043	−0.045	−0.043	−0.041	−0.038	***0.303***	−0.032	***0.163***	−0.021	−0.021	−0.016	***0.571***	−0.016

Species were those with frequencies of three or greater and present in F-layer samples containing two or more species (see Table 2). n = 221. Bold italic values indicate significant slopes ($P < 0.05$). Species abbreviations are given in Table 1.

Table 5 The frequencies of occurrence of all species of ectomycorrhizal fungi detected as ectomycorrhizas in Study 2

	Frequency
Cenococcum geophilum (CEGE)	309
Clavulina cinerea (CLCI)	214
Lactarius oculatus (LOC)	183
Tylopilus felleus (TYFE)	146
Russula white 1 (RUW1)	92
Jori 4 (J4)	59
Amanita brunnescens (AMBR)	30
Jori 5 (J5)	14
Jori 2 (J2)	11
Jori 14 (J14)	9
Amanita cf. *vaginata* (AMVA)	9
Thelephora terrestris (THTE)	7
Suillus intermedius (SUIN)	6
Scleroderma citrinum (SCCI)	5
Cortinarius b (COB)	3
Jori 1 (J1)	2
Amanita rubescens (AMRU)	2
Boletus projectellus (BOPR)	2
Jori 17 (J17)	2
Jori 20 (J20)	1
Jori 21 (J21)	1
Jori 23 (J23)	1
Jori 24 (J24)	1
Jori 10 (J10)	1
Jori 25 (J25)	1

Study 1, however, removal of *Cenococcum* and *Clavulina* from the calculation still resulted in a significantly larger C-score than expected, probably owing to the high frequency of insignificant but nonetheless negative correlations among various species pairs (Table 7).

Study 3, effects of nitrogen and tannin additions on hyphae in the F-layer

There were 11, 20 and 22 ectomycorrhizal fungal species recorded in control, N and tannin treatments, respectively. Several F-layer samples contained a single ectomycorrhizal fungus species, but a great many also contained two, three, four and up to eight species (Table 8). We calculated C-scores using all samples for which there were at least two species (see Table 8). In the control plots, the observed C-score (12.6) was significantly greater than the expected C-score (mean 10.4) based on random distributions (Table 9), indicating that there was significantly less co-occurrence (more avoidance) among species within the community than expected. For both the tannin and N plots, the observed C-scores (15.2 and 19.7, respectively) were not significantly different from the expected C-scores (means of 15.6 and 18.7, respectively) based on random distributions, indicating that species associations were essentially those of a randomly organized community.

The Spearman rank correlations showed that, irrespective of treatment, a significant negative correlation occurred between *C. cinerea* and *C. geophilum* (Table 10). Tannin eliminated the significant negative association between Jori 10 and *Clavulina*, N eliminated the significant positive interaction between Jori 5 and *Clavulina*, and tannin treatment caused a significant positive association between *S. citrinum* and *Clavulina*. Further comparisons were not possible because not all species were found in high enough frequency in all treatments to be included in the analyses.

Table 6 The frequency distribution of the number of species of ectomycorrhizal fungi detected per ectomycorrhiza in Study 2

Number of species per ectomycorrhiza	Number of ectomycorrhizas
1	900
2	95
3	7
4	1

Discussion

We cannot be absolutely sure that the presence of spores in the F-layer samples did not lead us to suppose that hyphae were

Table 7 Matrix of Spearman rank correlation coefficients for ectomycorrhizal fungal species detected as ectomycorrhizas in Study 2

	CEGE	CLCI	LOC	TYFE	RUW1	J4	J5	AMVA	J2
CLCI	***−0.596***								
LOC	***0.400***	***−0.392***							
TYFE	−0.063	−0.120	0.062						
RUW1	−0.048	***−0.462***	−0.062	−0.144					
J4	***−0.354***	***0.358***	***−0.376***	−0.161	−0.184				
J5	***−0.334***	***0.413***	***−0.205***	−0.116	***−0.212***	***−0.297***			
AMVA	***−0.227***	−0.200	−0.139	−0.079	***0.547***	−0.161	−0.116		
J2	0.180	−0.146	0.111	−0.041	−0.075	−0.126	−0.060	−0.041	
J14	***0.290***	***−0.234***	−0.116	−0.066	−0.120	***−0.202***	−0.097	−0.066	−0.034

Species were those with frequencies of 3 or greater and colonizing ectomycorrhizas containing 2 or more species (see Table 6). $n = 96$. Bold italic values indicate significant slopes ($P < 0.05$). Species abbreviations are given in Table 5.

Table 8 The frequencies of occurrence of all species of ectomycorrhizal fungi detected as hyphae in 0.25-ml F-layer samples in the control, tannin and nitrogen plots of Study 3

Number of species in 0.25-ml F-layer samples	Frequency in control plots	Frequency in tannin plots	Frequency in N plots
1	16	15	7
2	10	11	10
3	7	4	9
4	0	2	2
5	1	1	2
6	0	2	1
7	0	1	0
8	0	1	0

present when they were not. However, we did sample from the middle of the F-layer, which was often several centimeters below the surface of the forest floor. The concentration of spores there is not likely to be as high as on the surface of the forest floor, where the spores of all known ectomycorrhizal fungal species are known to be shed, with the exception of *Russula* white 1, which sheds spores below the litter layer. Moreover, many of the species we detected as hyphae either do not make sporocarps (*C. geophilum*), have not yet made sporocarps that we have observed over the course of several seasons (all the Jori fungi), or exist relatively frequently as hyphae but only occur rarely as sporocarps (*Gyroporus castaneus*). The most frequently occurring species as ectomycorrhizas were also among the most frequently occurring species as hyphae.

Our studies revealed that the distribution of the frequencies of the fungal species in this community was typical, with a few abundant species and many species of significantly lower abundance (Kårén & Nylund, 1996; Gehring *et al.*, 1998; Jonsson *et al.*, 1999; Mahmood *et al.*, 1999; Stendell *et al.*, 1999; Grogan *et al.*, 2000; Taylor, 2002). *Cenococcum geophilum* was the most frequently encountered species, both as hyphae in the F-layer and as a root colonist. It is a cosmopolitan species – one of the most frequently observed species in nature (LoBuglio, 1999) – and is frequently one of few dominant ectomycorrhizal fungal species in other communities (Kårén & Nylund, 1996; Goodman & Trofymow, 1998; Kranabetter & Wylie, 1998; Baxter *et al.*, 1999; Hagerman *et al.*, 1999; Byrd *et al.*, 2000; Dahlberg, 2001).

For the F-layer samples of Study 1, the ectomycorrhizas of Study 2, and the control F-layer samples of Study 3, the ectomycorrhizal fungal community appeared to be structured, in part, by strong amensal interactions such as competition or antibiosis because the overall C-scores were significantly larger (there was less species co-occurrence or more species avoidance within a sample) than expected. When we removed *C. geophilum* and *C. cinerea* from the C-score calculation for the community in Study 1 (the study with the most samples containing multiple species of ectomycorrhizal fungi), the degree of co-occurrence among species within samples was not significantly different from what would be expected if the species were distributed randomly among samples. Thus, *Cenococcum* and *Clavulina* contributed strongly to the overall avoidance among species within the community. These were also the species most frequently observed having negative Spearman rank correlations with other species.

One might ordinarily expect highly abundant species to be positively correlated simply by virtue of their great abundance; they would all be present in nearly all samples. However, while *Cenococcum* and *Clavulina* were the two most frequently observed species, within samples their hyphae were actually significantly negatively correlated with each other. One possible explanation for this might be the existence of amensal interactions of some sort at the scale of the 0.25 ml samples. Another possible explanation might be nonoverlapping distributions of the two species at a larger scale. In Study 1 we noted the 0.25 m^2 plots from which the individual samples were taken. We were therefore able to assess whether *Cenococcum* and *Clavulina* had exclusive distributions at the plot level. According to our analysis of co-occurrence at this larger, plot scale, there was neither more nor less avoidance between the two species than expected based on random distributions. Thus, *Cenococcum* and *Clavulina* did not occupy distinct patches of the plantation at the plot scale. We conclude therefore that the negative interaction between hyphae of *Cenococcum* and *Clavulina* occurred at the 0.25 ml sample level.

Within samples, negative correlations between two species may be caused by the exclusion of one species by another, which has been shown frequently in studies where the persistence of inoculated ectomycorrhizal fungi is followed subsequent to seedling outplanting. Some inoculated species do not persist for long but are displaced by other species present

Table 9 Relationship between observed and expected C-scores for ectomycorrhizal fungal species detected as hyphae in Study 3

Treatment	Observed < or > expected C-score	*P*	Interpretation
Control	>	< 0.05	Greater than expected avoidance
Tannin	nsd	0.38	Avoidance nsd from expected
Nitrogen	nsd	0.25	Avoidance nsd from expected

C-scores (a measure of species avoidance) were calculated for species with frequencies of three or greater and present in F-layer samples containing two or more species (see Table 8). The expected values were based on 1000 random simulations with fixed row sums and equiprobable column sums. nsd, Not significantly different.

Table 10 Matrix of Spearman rank correlation coefficients for control, tannin and nitrogen plots for ectomycorrhizal fungal species detected as ectomycorrhizas in Study 3

	CEGE	CLCI	J10	J5	RACO	RUW1	SCCI			
(a) Control plots										
CLCI	***−0.685***									
J10	0.278	***−0.549***								
J5	−0.378	***0.491***	−0.074							
RACO	0.299	−0.436	***0.553***	−0.214						
RUW1	0.299	−0.436	−0.074	−0.214	0.190					
SCCI	−0.378	0.182	−0.387	−0.214	−0.214	−0.214				
J4	−0.040	0.182	−0.387	−0.214	−0.214	0.190	−0.214			
	CEGE	CLCI	J10	GYCA	SCCI	RACO	LOC	J4		
(b) Tannin plots										
CLCI	***−0.821***									
J10	−0.169	0.160								
GYCA	0.094	0.020	***0.466***							
SCCI	−0.327	***0.437***	0.043	0.241						
RACO	0.153	−0.069	***0.516***	***0.770***	0.083					
LOC	0.061	0.013	−0.363	−0.243	−0.187	−0.187				
J4	0.331	−0.271	−0.097	0.054	0.156	0.156	−0.158			
J23–	***−0.478***	0.297	***0.435***	0.054	0.156	−0.187	−0.158	0.158		
	CEGE	CLCI	SCCI	J5	SUIN	J17	RUW1	TYFE	J4	SCAE
(c) Nitrogen plots										
CLCI	***−0.505***									
SCCI	0.058	−0.300								
J5	0.038	0.088	0.088							
SUIN	0.118	−0.234	0.019	−0.154						
J17	0.038	−0.198	0.088	−0.130	0.180					
RUW1	0.245	−0.300	0.133	−0.198	0.019	0.374				
TYFE	−0.256	−0.042	−0.225	***−0.390***	−0.247	0.093	−0.225			
J4	−0.101	0.019	0.019	−0.154	−0.182	0.513	0.019	0.181		
SCAE	0.185	−0.267	***0.891***	0.129	0.062	0.129	−0.036	−0.136	0.062	
J23	0.118	0.272	0.019	***0.513***	−0.182	−0.154	−0.234	−0.247	−0.182	0.062

Species were those with frequencies of 3 or greater and present in F-layer samples containing 2 or more species (see Table 8). $n = 26$. Bold italic values indicate significant slopes ($P < 0.05$). Species abbreviations are given in Table 5.

in the outplanted soil, while other species are quite competitive and persist for years (Lamb, 1979; Fleming, 1985; McAfee & Fortin, 1986; Villeneuve *et al.*, 1991; Selosse *et al.*, 1998; Wu *et al.*, 1999). Persistence may be related to the ability of a species to exclude others from colonizing roots (Fleming, 1985; Mamoun & Olivier, 1993a,b; Olivier & Mamoun, 1994). In some cases, this could involve the production of chemical inhibitory substances. In the early 1960s research was performed at Penn State University on an isolate of 'strain A' of *Cenococcum graniforme* (= *C. geophilum*) originally from the collection of Dr E. Hacskaylo (Krywolap, 1964). This isolate produced antifungal compounds that were soluble in methanol and active against 'several filamentous fungi'. This antifungal activity, then, is one possible reason for the frequent negative interaction between *Cenococcum* and other mycorrhizal fungal species.

Exclusion of species may also occur as a consequence of competition. Because N addition has been shown to reduce species richness (Lilleskov *et al.*, 2001, 2002; Peter *et al.*, 2001), we hypothesized that competitive exclusion should have been greater with N addition and therefore that N addition should have reduced the overall level of co-occurrence. It was found that N addition increased species richness, and greater than expected overall C-scores (significantly more species avoidance than expected) only occurred in the control plots. The reason for increased species richness and less species avoidance than expected with N addition may have been caused by the nature of competition. In some cases, competitive exclusion may be related to the capacity of a species to reduce the availability of a limiting nutrient to such a low level that other species cannot survive (Tillman *et al.*, 1981) and so, not surprisingly, relative competitive abilities may depend on resource availability (McAfee & Fortin, 1986; Mamoun & Olivier, 1993a,b). Based on this, the addition of N could promote greater co-occurrence and, in fact, the addition of N did eliminate the significant degree of overall species avoidance within the community, as indicated by the C-scores. However,

we cannot conclude much from that result because the list of species for which we had enough data to calculate C-scores in the control and N communities were slightly different. Moreover, the addition of N did not eliminate the significant negative correlation between *Cenococcum* and *Clavulina*. Although we have no data that suggest that competition for N was involved in the negative correlation between *Cenococcum* and *Clavulina*, this does not eliminate the possibility that competition for other resources, including water, P and other minerals, caused the observed avoidance.

Competitive exclusion may be related to more than just the ability to reduce the concentration of a limiting resource. It may also be related to tolerance to various conditions in the forest floor. Koide *et al.* (1998), for example, showed that compared with several other ectomycorrhizal fungal species, *Cenococcum* had a high tolerance to pine needle extracts, which contain a high concentration of tannin. One might then have predicted that the addition of tannin in Study 3 would allow *Cenococcum* to be more competitive against other species and thus demonstrate more negative correlations with other species, but this was not the case. As previously shown for the N addition, the addition of tannin did eliminate the significant degree of overall species avoidance within the community as indicated by the C-scores. However, we again cannot conclude much from that result because the list of species for which we had enough data to calculate c-scores in the control and tannin communities were slightly different.

Either antibiosis or superior competitive ability could allow an ectomycorrhizal fungal species to invade forest floor particles previously occupied by other species, and to persist in its new holdings. In the absence of too-frequent disturbance one might predict that the species with the greatest number of negative interactions would become very abundant in ectomycorrhizal fungal communities. It was found that *Cenococcum* had the greatest number of significant negative interactions and was the most abundant species in this community (Table 1). *Cenococcum* is also frequently seen overgrowing the surface of other ectomycorrhizas (Brand, 1992). The complete exclusion of other species by *Cenococcum*, or by any single species, could be prevented by either fluctuation in the environment (Lamb, 1979), by seasonal root production (Fleming, 1985) or by the presence of competitive networks, the situation in which no single species is competitively superior to all other species (Bruns, 1995).

The species interactions we observed within samples are reminiscent of competitive networks. While both *Cenococcum* and *Clavulina* contributed to the high degree of species avoidance in this community, they interacted with other species in different ways. For example, in Study 1 Jori 10 was positively correlated with *Cenococcum* but negatively correlated with *Clavulina*, while Jori 1 and Jori 5 were both negatively correlated with *Cenococcum* but positively correlated with *Clavulina*. In Study 2, *Lactarius oculatus* was positively correlated with *Cenococcum* but negatively correlated with *Clavulina*, while Jori 4 and Jori 5 were negatively correlated with *Cenococcum* but positively correlated with *Clavulina*.

In addition to negative interactions among ectomycorrhizal fungal species, positive interactions, such as revealed by our analyses, may also structure communities. Significant associations among species could occur when resources are not limiting so that competition would not have negative consequences. In that case, species with similar requirements would tend to occupy similar sites. Other types of interactions could also lead to significant association. For example, Fleming (1985), Shaw *et al.* (1995) and Mamoun & Olivier (1993a,b) have observed that the presence of one ectomycorrhizal fungal species may facilitate colonization by another. There are also a number of notable examples of associations between sporocarps of different species such as between *Boletus parasiticus* and *S. citrinum*, *Asterophora parasitica* and *Russula* or *Lactarius*, and between *Claudopus parasiticus* and *Cantharellus cibarius* (Bessette *et al.*, 1997). Associations among suilloid fungi and members of the Gomphidiaceae occur within ectomycorrhizal roots, as described by Agerer (1990, 1991) and Olsson *et al.* (2000). The nature of these interactions is unknown although there is some indication that members of the Gomphidiaceae are parasitic on the host plant or on the suilloid fungi (Olsson *et al.*, 2000). We do not know whether associations between sporocarps of different species or between fungi colonizing the same root are reflected in associations between the hyphae of different species in the soil.

Our database of DNA fingerprints undoubtedly limited us in a number of ways. First, it does not contain saprotrophic fungal species. It is quite possible that nonrandom interactions occur between ectomycorrhizal fungi and saprotrophic fungi or among saprotrophic fungi, but we were unable to detect them. Moreover, the database does not contain all possible ectomycorrhizal fungi and therefore some interactions among ectomycorrhizal fungi may not have been revealed. However, this may not be a particularly large shortcoming. This ectomycorrhizal fungal community clearly contains many rare species and, while we undoubtedly have not yet included many of them in the database, we are probably aware of most of the common species. Reason suggests that interactions among common species are more likely to be important than interactions among rare species in structuring a community.

As far as we are aware, this is the first systematic study to provide evidence for interactions among ectomycorrhizal fungal species using data from both ectomycorrhizas and hyphae in small samples of soil. The existence of nonrandom distributions at the level of the colonized root as well as in small soil samples suggests that interactions among species occurring at a very small scale can structure an ectomycorrhizal fungal community, perhaps resulting in the limitation of a species' ecological space from the fundamental niche to a realized niche. For now the causes of these interactions remain unknown. Future investigations on competition, antibiosis, parasitism and facilitation among ectomycorrhizal fungal species appear to be warranted.

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