

Spatial analysis of ectomycorrhizal fungi reveals that root tip communities are structured by competitive interactions

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Abstract

Microbial ecology has made large advances over the last decade, mostly because of improvements in molecular analysis techniques that have enabled the detection and identification of progressively larger numbers of microbial species. However, determining the ecological patterns and processes taking place in communities of microbes remains a significant challenge. Are communities randomly assembled through dispersal and priority effects, or do species interact with each other leading to positive and negative associations? For mycorrhizal fungi, evidence is accumulating that stochastic and competitive interactions between species may both have a role in shaping community structure. Could the methodological approach, which is often incidence based, impact the outcomes detected? Here, we applied an incidence-based Terminal Restriction Fragment Length Polymorphism (T-RFLP) database approach to examine species diversity and ecological interactions within a community of ectomycorrhizal (ECM) fungi. Co-occurrence analysis revealed that the ECM community colonizing root tips was strongly structured by competitive interactions, or ecological processes generating a similar spatial pattern, rather than neutral processes. Analysis of β -diversity indicated that community structure was significantly more similar (spatially autocorrelated) at distances equal to or <3.41 m. The eight most frequently encountered species in the root tip community of ECM fungi displayed significant competitive interactions with at least one other species, showing that the incidence-based approach was capable of detecting this sort of ecological information.

Keywords: co-occurrence, ecological interactions, ectomycorrhiza, incidence data, microbial biodiversity, T-RFLP

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Introduction

Ectomycorrhizal (ECM) associations, involving thousands of species of woody plant hosts and symbiotic fungi (Brundrett 2009), have come to dominate forest ecosystems (Smith & Read 2008) since their

evolution from saprotrophic fungi (Hibbett *et al.* 2000) in or around the Early Cretaceous period 150–99 Mya (Berbee & Taylor 2010). Despite the importance of ECM symbioses for carbon cycling and nutrient dynamics (Courty *et al.* 2010), species invasions (Nuñez, *et al.* 2009; Dickie *et al.* 2010) and other vital ecological processes, there are still many basic questions about the ecology of ECM fungi that require answers. Significant advances in understanding and explaining the biotic and abiotic interactions affecting organisms as individuals, species and communities have been made by applying

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spatial analysis techniques (Tilman & Kareiva 1997). However, examination of spatial distribution and abundance, which is more easily achieved for ECM host plant species, poses a significant challenge for microbial symbionts (Ettema & Wardle 2002; Prosser *et al.* 2007). Detection and analysis of these spatial patterns remain key issues in modern microbial ecology.

Classical niche theory (Vandermeer 1972) predicts that species assemblages will be structured by the ability of individual species to persist in a given set of abiotic conditions, while successfully competing with other species for resources and space. Neutral theory (Hubbell 2001) predicts that assemblages will be structured by stochastic processes such as initial dispersal into an area, or founder effects (where the first species to colonize a resource remains there). Many communities are most likely structured by both of these theoretical models to a greater or lesser degree (Adler *et al.* 2007), and certainly in the case of bacterial communities both processes appear to take place with different levels of importance (e.g. Woodcock *et al.* 2007; Lauber *et al.* 2009; Dumbrell *et al.* 2010; Langenheder & Székely 2011). For obligate symbionts, like mycorrhizal fungi, we might expect a nestedness of interspecific interactions (Thompson 2005) in which host distribution is the strongest structuring factor at the landscape scale (Gilbert *et al.* 2007). However, at scales important to individual fungi (or perhaps individual colonization sites) where different species directly come into contact for access to a growth substrate, do we observe communities structured by ecological interactions, or do they appear to be randomly assembled as might be expected under neutral assembly models?

Evidence for stochastic community assembly exists for arbuscular mycorrhizal (AM) fungi (Lekberg *et al.* 2012), suggesting neutral structuring processes, whereas ericoid mycorrhizal (ERM) fungi appear to display facilitative interactions (Gorzelak *et al.* 2012). Studies of ECM fungi indicate that several different types of ecological interactions may be taking place. This research has mostly focused on site-scale (tens of metres) investigations of the spatial and temporal properties of below-ground ECM communities (Lilleskov *et al.* 2004; Koide *et al.* 2005a,b, 2007; Peay *et al.* 2007, 2010; Thiet & Boerner 2007; Scattolin *et al.* 2008; Pickles *et al.* 2010), although recently the extension of data to the landscape (Kennedy *et al.* 2011; Obase *et al.* 2011) and global (Tedersoo *et al.* 2012) scales have been reported. Meta-analysis of earlier ECM studies found that communities were probably patchy on a scale below 4 m (Lilleskov *et al.* 2004), although insufficient data were available at small scales to clarify this. Competitive interactions were detected in an ECM community (Koide *et al.* 2005a) between only the

two most abundant species. This suggested either that the rest of the community was randomly distributed or that there was not enough statistical power in the analysis to detect interactions between less abundant species. Greenhouse studies (Kennedy *et al.* 2009; Kennedy 2010) have indicated that priority effects are important structuring processes for ECM fungi, in which the first fungi to colonize a root system remains dominant. This implies a more stochastic structuring effect that is dependent on dispersal. While priority effects appear important in the initial colonization of seedlings, the rapid turnover of ECM below ground at small scales (Guidot *et al.* 2001, 2003; Zhou & Hogetsu 2002; Izzo *et al.* 2005) may mean more substrate to colonize, potentially reducing the importance of this strategy in a more mature forest setting. Strong evidence for competitive interactions between ECM fungi has been demonstrated for a variety of species associated with Scots pine (Pickles *et al.* 2010), where positive and negative interactions took place between different species within the fungal community, and species were distributed in patches of different sizes within the study area. Temporal dynamism was observed, with changes in both the relative abundance of species and the position and size of individual patches over time (Pickles *et al.* 2010).

Conceptual advances in ECM fungal ecology require a better understanding of the processes that structure field communities in simple and relatively benign environments. By focusing on low-diversity, environmentally homogeneous communities, we may be able to discern at a basic level the relative importance and structuring effects of neutral/stochastic processes (Hubbell 2001) vs. niche/deterministic processes (Vandermeer 1972) on ECM fungi. Testable hypotheses can then be formed about how host and resource heterogeneity and increased species diversity may alter the basic structure of ECM fungal communities. The spatial analyses of ECM fungi mentioned previously indicated that the patch size of most species, and hence the distances over which they are likely to interact, was less than the major sampling scale used in the relevant study (Lilleskov *et al.* 2004; Koide *et al.* 2005a,b; Pickles *et al.* 2010). This is a common issue in ecological studies of micro-organisms that can be overcome with rigorous spatially explicit sampling of communities at scales relevant to the target organisms (Tilman & Kareiva 1997; Prosser *et al.* 2007). Additionally, the scale at which species turnover (β -diversity) is spatially autocorrelated can then be determined and used to guide future field studies.

Incidence-based approaches to community analysis may, by their nature, provide less resolution on species interactions in a community than relative abundance

approaches. However, they are often better at detecting rare species in a community and are commonly used in studies of microbial biodiversity (Furman 2009). The loss of resolution may be avoided by choosing a sample size appropriate to the organism in question, which is difficult for most micro-organisms but possible in the case of ECM fungi (which bridge the scales between micro- and macro-organisms) by focusing on their colonization of host root tips. By taking an incidence-based approach to examining a community of ECM fungi that was previously shown to display competitive interactions using abundance data (Pickles *et al.* 2010), do we lose the signal of interspecific competition? In the context of this study, we defined interactions between species based on their presence or absence as ECM root tips in O-horizon soil cores, where all extracted tips were combined for a given sample.

Here, we applied database Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis to the detection of spatial patterns in ECM fungal community composition, to determine the ecological processes structuring the community, to examine the specific interactions taking place between species below ground and to determine the scale at which species turnover occurs. The aims of our study were to use species incidence data from a relatively homogeneous community: (i) to determine whether the distributions of ECM fungi supported communities structured by positive or negative interactions, or random assembly; (ii) to examine the spatial patchiness of species distributions on a 1-m scale; (iii) to determine the scale at which species turnover in an ECM community is spatially autocorrelated (i.e. the scale of intracommunity patchiness). We hypothesized that, when using incidence data, stochastic/neutral processes would appear to be more important than deterministic/niche processes in structuring ECM root tip communities.

Materials and methods

Study site

The study site was an even-aged 120-year-old Scots pine (*Pinus sylvestris* L.) plantation in Culbin Forest, Morayshire, Scotland (57°38'08" N, 03°42'07" W). The site was flat with no obvious environmental gradients. The soil profile consisted of an organic horizon [bryophyte/litter (L; c. 0–2 cm), fermentation (F; c. 2–4 cm) and humic (H; c. 4–12 cm)] above deep aeolian sand deposits (Gauld 1981). Dominant bryophytes present were *Rhytidiadelphus triquetrus* (Hedw.) Warnst. and *Hylocomium splendens* (Hedw.) B.S.G. Vascular plants were absent. The same site has been used in previous studies (Genney *et al.* 2006; Pickles *et al.* 2010).

Development of an ECM T-RFLP database

A database of diagnostic terminal restriction fragments (TRFs) from vouchered sporocarps and ectomycorrhizas was created (Table S1, Supporting information). This database contained 117 entries distributed as shown in Table S2 (Supporting information). The majority (84%) of the entries were collected in Culbin Forest from sporocarps (58% of entries) under *P. sylvestris*, *P. nigra* ssp. *laricio*, or a mixture of the two with occasional *B. pubescens*, or from ectomycorrhizas (26% of entries) collected in the 20 × 20 m study plot (Genney *et al.* 2006; Pickles *et al.* 2010; H. Izumi unpublished). TRF sizes were determined for each database entry by performing T-RFLP with both *Hinf*I and *Taq*I restriction endonucleases as described in Genney *et al.* (2006). Briefly, DNA from each was amplified using the fluorescently labelled primers ITS1F (6-FAM) and ITS4 (HEX) (White *et al.* 1990; Gardes & Bruns 1993) in a 50 µL reaction mix that contained 1-µL DNA template, 1× buffer [16 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween-20]; 2.0 mM MgCl₂; 250 µM dNTPs (Bioline Ltd, London, UK); 20 pmol of each primer, 1 µL BSA and 2.5 U BIOTAQ polymerase (Bioline Ltd). The PCR conditions comprised an initial denaturation step at 95 °C for 5 min followed by 29 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension step of 72 °C for 5 min on a PTC-220 DYADTM Thermal Cycler (MJ Research Inc., Waltham, MA, USA). PCR products were purified using a magnetic bead ChargeSwitch PCR clean-up kit (Invitrogen, Paisley, UK) prior to T-RFLP analysis. TRF separation was performed on an ABI PRISMTM 3130xl genetic analyser (Applied Biosystems, Warrington, UK) using POP 4 and a 50-cm column with a 15 s injection time at 1.5 kV for 40 min at 60 °C. A GS-500 ROX size standard (Applied Biosystems) was included in every sample run to facilitate accurate sizing of each detected TRF. The four unique TRFs for each sporocarp/species (i.e. two for each restriction enzyme) were imported into the Fragmatch program (Saari *et al.* 2007) to produce a reference database for the detection of individual ECM species in subsequent experiments.

For 113 of the 117 entries in the database, an ITS sequence was also generated and deposited in UNITE (sporocarps only) (Abarenkov *et al.* 2010) and GenBank (see Table S1, Supporting information for accession codes). Sequencing reactions were performed with the primers ITS1F and ITS4 using the BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI PRISMTM 3130xl genetic analyser (Applied Biosystems). DNA sequences were manually checked and edited where necessary using the SEQUENCHER software package (version 3.0; Gene Codes Corporation, MI,

USA) and matched against NCBI and UNITE (Abarenkov *et al.* 2010) database sequences using the BLASTn algorithm.

The plausibility of the TRF profile generated for each species was checked by subjecting the ITS sequence for that species to *in silico* fragment generation using REMA (Szubert *et al.* 2007). Entries having implausible TRF profiles were discarded. In around 30 cases, TRFs were generated from multiple fruit body (FB) collections of the same species or from both FB and ECM collections. Where duplicate collections of the same species produced identical TRFs, only one was entered into the database. In 15 cases where one or more TRFs were different, all the variants were entered in the database, and the Fragmatch output was adjusted to record only the presence or absence of the species in a sample, irrespective of how many of the TRF profile variants were present. In the case of *Laccaria laccata* and *Laccaria proxima*, the TRFs did not distinguish between the spp., so these were entered in the database as *Laccaria* sp.

Spatial sampling

A 441-point grid with 1-m spacing between sample points was overlain on the 20 × 20 m study area, and soil cores (5 cm diam × 5 cm deep) were extracted at every grid point. Because many of the grid points had been sampled previously (Pickles *et al.* 2010), cores were taken 10 cm NE of each point. All 441 cores were collected on the same day, and the depth of the organic horizon was measured. The mineral horizon was discarded from each sample, and the organic horizons were stored at <3 °C, for a maximum of 3 weeks, until further processing.

Root tip extraction

Owing to the large number of samples involved in this study, a new time-based procedure was used in which six minutes were allocated to root tip extraction per core. Ectomycorrhizal root tips were extracted from the surface layer first, working down into the organic horizon, following soaking of cores in deionized water. Extensive familiarity with the soil profile and ECM species present at the field site (Genney *et al.* 2006; Pickles 2007; Pickles *et al.* 2010) enabled unbiased selection of root tips with this method, thus avoiding preferential selection of more easily observed morphotypes. For each core, the total number of ECM tips extracted was recorded (mean = 34.36, SEM = 0.918) prior to being washed, bulked and stored in a single 1.5-ml Eppendorf tube at −20 °C.

DNA extraction, PCR and T-RFLP

Each bulked ECM root tip sample (Dickie & FitzJohn 2007) was homogenized using a micro pestle and a Pellet Pestle® Motor (Koren, Anachem Ltd, Luton, UK), and DNA was extracted from each homogenate using the DNeasy Plant Mini Kit (Qiagen Ltd, Crawley, UK). The internal transcribed spacer (ITS) region was amplified for each DNA sample using fluorescently labelled primers as described in the database development section above. T-RFLP profiles were analysed with a second-order least squares size calling method for peaks between 25 and 500 bp and with minimum peak amplitude of 50 using GENEMAPPER V3.7 software (Applied Biosystems). This generated four sets of data for each individual soil core (ITS1F and ITS4 peaks for both *HinfI* and *TaqI*), and the peak height information for each of these was analysed using FragMatch (Saari *et al.* 2007).

Data analysis

The reference database described above was used to identify the presence of ECM fungal species in the T-RFLP profiles generated from each homogenized bulk root tip sample. Species were determined as being present if all TRFs were detected within an error limit of ±1.5 bp. Following the identification of all species within each sample, the information was converted into a binary table, and the relative and absolute frequency was calculated for all species detected.

EstimateS 8.0 (Colwell 2009) was used to calculate estimates of α -diversity, using the incidence-based Chao2 richness estimator (Chao *et al.* 2005), Simpson diversity and evenness. In each case, estimations were based on 500 Monte Carlo simulations of the data without replacement.

Species turnover (β -diversity) was used to examine spatial patterns of change in community composition. Mantel correlogram analysis was performed in R (R Development Core Team 2011), using the VEGAN package (Oksanen *et al.* 2011), to test for spatial autocorrelation in ECM fungal community composition across the field site. Presence/absence data for each sample was converted into a similarity matrix using the Jaccard index, and standardized to meet the statistical requirement of second-order stationarity. The similarity matrix was then compared with a geographic distance predictor matrix of Euclidean distances between samples, which was partitioned into discrete distance classes for the purpose of the correlogram analysis. Mantel statistics (r_M) were calculated for each distance class and tested for significance using 5000 permutations and Holm's correction for multiple testing. In this study, positive values of r_M represent positive autocorrelation.

Interactions within the community of ECM fungi were assessed using the co-occurrence application of EcoSim (Gotelli & Entsminger 2009). Two co-occurrence indices were used, each of which compares observed data against randomized null models to examine a different aspect of association. C-score analysis (Stone & Roberts 1990) averages the number of 'checkerboard units' between all pairs of species in the data set. In a competitively structured community, the observed C-score (C_{obs}) is significantly greater than that generated by the randomized null models. Lack of significance suggests that species co-occur randomly, and a significantly smaller C_{obs} than the null models indicates aggregations of species. Unique species combination analysis (Pielou & Pielou 1968) compares observed species co-occurrence in the data set with that obtained by fully randomized null models. A competitively structured community possesses fewer unique species combinations than are expected by the null models. Each of these co-occurrence analyses also provides a measure called the standardized effect size (SES), which indicates the number (and direction) of standard deviations that the observed data differs from that expected by the null models. SES of ≥ 2 or ≤ -2 is generally considered ecologically significant (Gotelli & Entsminger 2009). In all analyses, rows (species) were always 'fixed', meaning that the number of occurrences of each species in a null model was the same as in the observed data. Columns (soil cores) were constrained in three different ways: (i) 'equiprobable', where observed differences in species richness were removed from null models (i.e. assuming each core was equally suitable for each species), (ii) 'fixed', where observed differences in species richness were maintained in the null models, (iii) 'weighted', where the number of root tips in a soil core affected the relative probability of a species occurring there in each null model. In all cases, observed data were compared against 10 000 null models generated from Monte Carlo randomizations using the sequential swap algorithm. Species present in fewer than three soil cores were removed from the data set as they were unlikely to provide much meaningful information, which resulted in a data set of 26 species in 405 soil cores. Species of interest were sequentially removed from the data set to examine the effects on observed interactions within the community. These species were selected based on their frequency of occurrence in the data set or based on previous work indicating a potential contribution to species interactions within this (Pickles *et al.* 2010) or other (Koide *et al.* 2005a) ECM communities. If a significant result was obtained using the C-score index, the pairwise data for each species combination were examined to determine which species pairs made a significant contribution to community

interactions. The largest 5% of pairwise C-score values were considered to represent significant interactions as per Arrington *et al.* (2005).

Joint-count statistics were calculated for each of the twelve most frequently encountered species using the Rook's adjacency definition in ROOK-CASE (Sawada 1999). *P*-values were generated from 10 000 Monte Carlo randomizations of the frequency data for each species. This technique provided a simple method for detecting clustering within species distributions in a binary data set.

For all analyses, unless otherwise stated, the Benjamini and Hochberg false discovery rate (FDR) correction (Verhoeven *et al.* 2005) was applied to *P*-values to account for multiple testing while minimizing error.

Results

Community structure and diversity

In the ECM fungal root tip community detected with T-RFLP, 43 ECM fungal species were recorded (Fig. 1), with twenty found in three or fewer soil cores. Estimated α -diversity using the Chao2 richness estimator was 65.69 species, with a Simpson diversity index of 9.42, and evenness of 0.712.

Mantel correlograms

The number of species detected was found to increase with the number of tips per core ($r^2 = 0.325$, $P < 0.001$), with only one species found in those cores with fewer than 20 tips. To eliminate this relationship from the data set, all cores with <20 or >100 root tips (75 in total) were removed. Following this manipulation, a weak relationship between root tip number and species richness remained, although it only accounted for 7.7% of the variation ($r^2 = 0.077$, $P < 0.001$). Data were subsequently converted to a Jaccard similarity matrix, and Mantel statistics (r_M) were obtained for nine distance classes (Fig. 2). These revealed weak but significant positive spatial autocorrelation in β -diversity at mean distances of 1.80–3.41 m (Fig. 2). No significant negative autocorrelation was detected, although a significant downward trend in r_M was observed with increasing distance ($r^2 = 0.821$, $P < 0.001$).

Co-occurrence analysis

C-score revealed significant interactions taking place within the community regardless of the null model or grid size used (Table 1). Analysis using the number of unique species pairs also revealed significant interac-

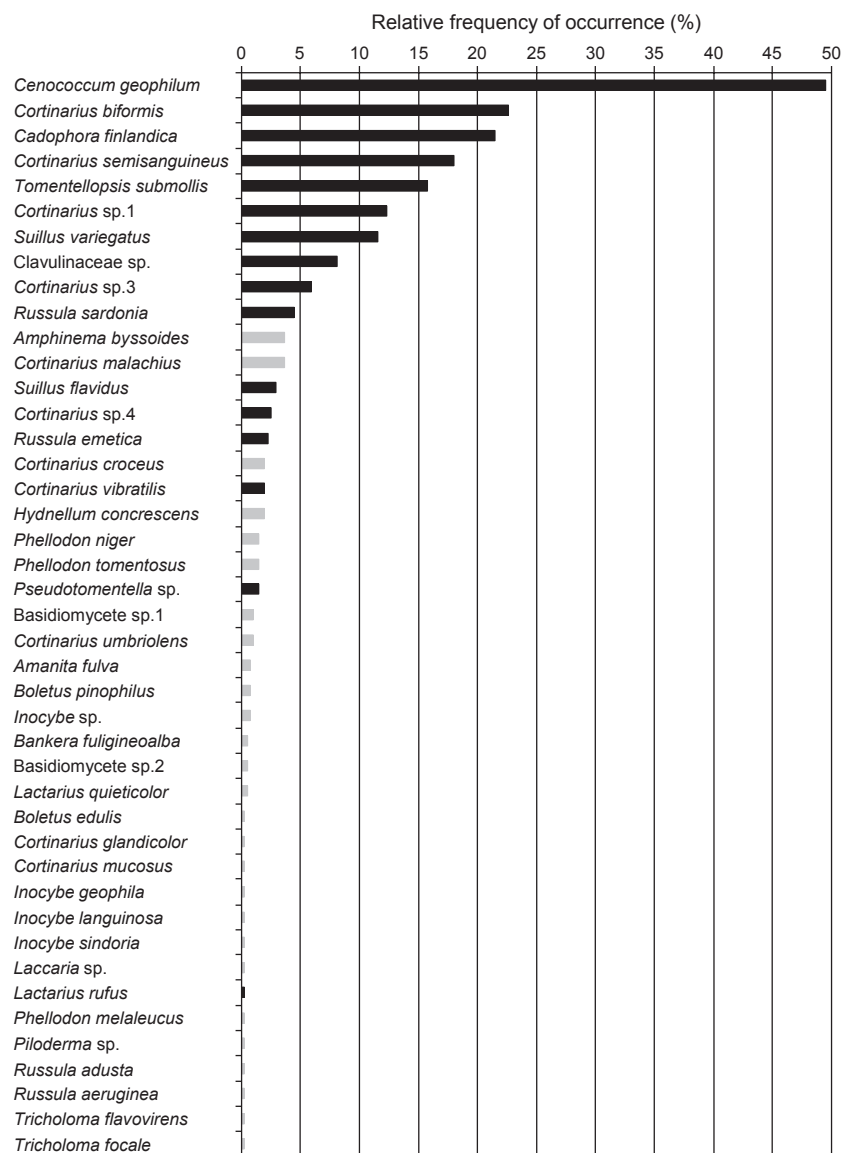


Fig. 1 Relative frequency of occurrence of ectomycorrhizal fungal species detected by T-RFLP. Black bars are those species previously detected by morphotyping (Pickles *et al.* 2010), and grey bars are species detected using T-RFLP only.

tions (Table 2). Standardized effect size was strongest when sample constraints were equiprobable or weighted by root tip number. Treating each core as equally likely to be colonized (equiprobable) was the most conservative model; however, weighting by root tip number may be the most ecologically relevant approach as it reflects the available substrate for fungal colonization. SES remained ecologically and statistically significant after increasing the size of the sampling grid, which shows that the results are robust and species co-occur less often than expected. Using all community data, eight species showed significant interactions (C-scores in the largest 5% of pairwise interactions) with at least one other species (Table 3); this dropped

to five and four species, respectively, when the grid size spacing increased to 2 m and >3.41 m.

Joint-count statistics

Joint-count statistics were obtained for the twelve most frequently encountered species across the site at the 1-m sampling scale (Table 4). *Cadophora finlandica*, *Cortinarius semisanguineus*, *Cortinarius sp.3* and *Russula sardonia* all displayed significant patches and gaps in their distribution. *Cortinarius sp.1* showed significant aggregation into patches, while *Suillus variegatus* and *Clavulinaceae sp.* both displayed significant gaps in their distributions.

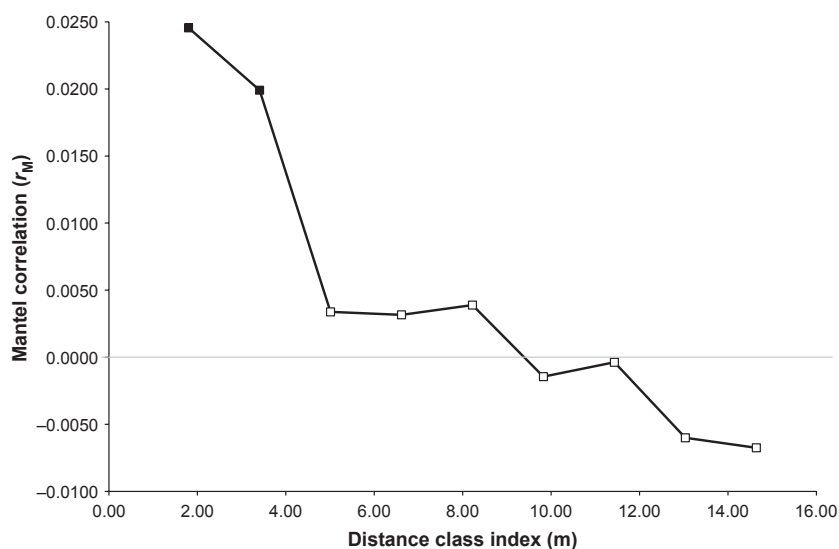


Fig. 2 Mantel correlogram of spatial autocorrelation in β -diversity. Positive Mantel statistics (r_M) represent positive autocorrelation. Solid symbols represent significant values of r_M based on 5000 Monte Carlo randomizations of the data and Holm's correction for multiple testing.

Table 1 Co-occurrence analysis of ectomycorrhizal fungal community incidence data using C-score

Data	Constraints*	Samples†	Species	Obs C-score	Exp C-score‡	P-value	SES
Whole community (1-m grid)	Fixed–Equiprobable	405	26	597.59	555.75	0.000	4.76
	Fixed–Fixed				579.24	0.000	6.17
	Fixed–Weighted				555.70	0.000	4.91
Community minus <i>C. geophilum</i>	Fixed–Equiprobable	344	25	432.18	401.79	0.000	5.29
	Fixed–Fixed				419.94	0.000	5.67
	Fixed–Weighted				370.58	0.000	8.35
<i>Cortinarius</i> spp. only	Fixed–Equiprobable	217	9	644.42	476.41	0.000	8.06
	Fixed–Fixed				627.21	0.000	2.55
	Fixed–Weighted				423.64	0.000	9.51
2-m sampling grid	Fixed–Equiprobable	108	16	98.21	82.98	0.000	3.92
>3.41-m sampling grid	Fixed–Equiprobable	39	13	22.08	18.08	0.000	2.91

*Row–Column.

†All samples with 1 or more species of frequency 3 +.

‡Estimated C-score from 10 000 Monte Carlo randomizations in which all species were equally likely to appear in any sample.

Discussion

Construction of a reference database of TRFs from locally obtained specimens of ECM fungi proved to be an extremely effective tool for ecological investigation of the ECM root tip community. Community composition as detected in this rigorously sampled, spatially explicit study was similar to that observed in previous studies, with a few species making up the majority, followed by a long tail of rare species (Horton & Bruns 2001; Taylor 2002). All species present in $\geq 4\%$ of soil cores, and fourteen of the fifteen most commonly encountered TRFs, matched species or members of a species complex, recorded during the previous morphotyping/sequenc-

ing study at this site (Pickles *et al.* 2010). Co-occurrence analysis determined that the major structuring effect on the community was competition, or processes generating the same pattern as competition, with the eight most frequently encountered species all implicated in competitive interactions with at least one other species.

Factors affecting community structure

Spatial autocorrelation. Mantel correlograms revealed a tendency for the species composition of cores to be slightly but significantly more similar when sampled ≤ 3.41 m apart. This observation that β -diversity was

Table 2 Co-occurrence analysis of ectomycorrhizal fungal community incidence data using number of unique species combinations

Data	Constraints*	Samples†	Species	Obs Uniques	Exp Uniques‡	P-value	SES
Whole community (1-m grid)	Fixed–Equiprobable	405	26	150	181.27	0.000	–6.81
	Fixed–Fixed				167.88	0.000	–4.25
	Fixed–Weighted				181.15	0.000	–6.95
Community minus <i>C. geophilum</i>	Fixed–Equiprobable	344	25	121	155.17	0.000	–8.04
	Fixed–Fixed				134.96	0.000	–3.43
	Fixed–Weighted				152.85	0.000	–7.21
<i>Cortinarius</i> spp. only	Fixed–Equiprobable	217	9	30	44.62	0.000	–5.86
	Fixed–Fixed				33.99	0.043	–2.15
	Fixed–Weighted				43.08	0.000	–5.87
2-m sampling grid	Fixed–Equiprobable	108	16	55	61.33	0.010	–2.51
>3.41-m sampling grid	Fixed–Equiprobable	39	13	23	28.20	0.002	–3.12

*Row–Column.

†All samples with 1 or more species of frequency 3 +.

‡Estimated number of unique species combinations from 10 000 Monte Carlo randomizations in which all species were equally likely to appear in any sample.

Table 3 Significant competitive interactions within the whole fungal community as detected by C-score analysis

Species	No. of significant interactions	Interspecific interactions
<i>C. geophilum</i>	7	<i>C. finlandica</i> *, <i>Clavulinaceae</i> sp., <i>C. biformis</i> *†, <i>C. semisanguineus</i> *†, <i>Cortinarius</i> sp.1, <i>S. variegatus</i> *†, <i>T. submollis</i>
<i>C. finlandica</i>	6	<i>C. geophilum</i> *, <i>C. biformis</i> , <i>C. semisanguineus</i> , <i>Cortinarius</i> sp.1, <i>S. variegatus</i> , <i>T. submollis</i>
<i>C. biformis</i>	6	<i>C. geophilum</i> *, <i>C. finlandica</i> , <i>C. semisanguineus</i> *†, <i>Cortinarius</i> sp.1, <i>S. variegatus</i> *, <i>T. submollis</i>
<i>C. semisanguineus</i>	5	<i>C. geophilum</i> *, <i>C. finlandica</i> , <i>C. biformis</i> *†, <i>S. variegatus</i> , <i>T. submollis</i>
<i>T. submollis</i>	5	<i>C. geophilum</i> , <i>C. finlandica</i> , <i>C. biformis</i> , <i>C. semisanguineus</i> , <i>Cortinarius</i> sp.1
<i>Cortinarius</i> sp.1	4	<i>C. geophilum</i> , <i>C. finlandica</i> , <i>C. biformis</i> , <i>T. submollis</i>
<i>S. variegatus</i>	4	<i>C. geophilum</i> *†, <i>C. finlandica</i> , <i>C. biformis</i> *, <i>C. semisanguineus</i>
<i>Clavulinaceae</i> sp.	1	<i>C. geophilum</i>

Significance determined as a C-score value in the 95th percentile of all pairwise combinations (as per Arrington *et al.* 2005).

*95th percentile using 2-m grid.

†95th percentile using >3.41-m grid.

significantly spatially autocorrelated at scales below approximately 3.5 m, closely corresponds to that observed previously at the same site for abundance data

(Pickles *et al.* 2010) and to that observed in a meta-analysis of earlier work (Lilleskov *et al.* 2004). This finding further confirmed that ECM communities (or at least, those few for which such an investigation has been carried out) appear spatially aggregated at scales below 3–4 m. This is probably related to the patch size of individual species or genets, and/or the fine roots of their hosts. Future studies concerned with detecting the full range of species present in a community may therefore be best served by sampling with a separation distance below approximately 3.41 m, whereas those concerned with avoiding small-scale community pseudo-replication should sample with a separation distance >3.41 m.

Species interactions. Spatial autocorrelation in a data set generally has the effect of making the detection of significance more likely (Legendre & Legendre 1998). As most ecological processes are spatially autocorrelated to some extent, rather than throwing out data following the detection of a given level of spatial dependency, it is advisable to analyse the entire data set while considering the potential mechanisms causing the dependency (Legendre 1993; Legendre *et al.* 2002). For example, C-score has often been applied to the study of bird communities on islands, such as finches of the West Indies (Gotelli & Abele 1982), which are assumed to be independent of each other. Given the dispersal ability of birds, the relative proximity of these islands, and their shared evolutionary history of colonization, a small level of spatial dependence must exist. Fortunately, the analysis programme allows for the observed patterns to be compared against those generated by tens of thousands of null models, vastly increasing the ability to detect real effects in the data (Gotelli & Entsminger 2009). For our investigation of species interactions

Table 4 Joint-Count statistics for assessing the clustering of ectomycorrhizal fungal species* based on incidence data

Species†	BW joins‡	BW <i>P</i> -value	BB joins§	BB <i>P</i> -value	WW joins¶	WW <i>P</i> -value
<i>Ce. geophilum</i>	396	0.072	258	0.111	186	0.065
<i>Co. biformis</i>	272	0.286	528	0.388	40	0.243
<i>Ca. finlandia</i>	220	0.000	564	0.000	56	0.000
<i>Co. semisanguineus</i>	213	0.011	597	0.008	30	0.049
<i>T. submollis</i>	200	0.126	618	0.181	22	0.126
<i>Cortinarius</i> sp.1	152	0.013	668	0.141	20	0.002
<i>S. variegatus</i>	147	0.021	679	0.020	14	0.071
Clavulinaceae sp.	110	0.092	725	0.034	5	0.500
<i>Cortinarius</i> sp.3	78	0.016	756	0.039	6	0.024
<i>Co. malachius</i>	58	0.183	782	0.249	0	0.395
<i>R. sardonias</i>	55	0.001	780	0.002	5	0.007
<i>A. byssoides</i>	52	0.122	785	0.309	3	0.059

Values in bold were significant following FDR correction for multiple testing.

*Analysis performed for 12 most frequently encountered species.

†Ca = Cadophora, Ce = Cenococcum, Co = Cortinarius.

‡Number of 0 & 1 adjacencies.

§Number of 0 & 0 adjacencies.

¶Number of 1 & 1 adjacencies.

using C-score, we decided to examine our data at 1-m, 2-m and >3.41-m intervals. This allowed comparison between the full data set and restricted sets that progressively removed any potential effect of slight spatial autocorrelation in species composition. Even after dramatically reducing the available data set from 405 to 39 samples, C-score and unique species analyses indicated that the ECM fungal root tip community displayed significant competitive structure. Because the SESs were so large, and the associated *P*-values remained highly significant even after reducing the data set, we are confident that analysing the entire data set is justified. The detection of interspecific interactions was not solely dependent on the most frequently encountered species, unlike the only previous ECM study to use the C-score technique (Koide *et al.* 2005a). This result also differed from a recent C-score analysis of ERM communities, which implied that community structure was determined by facilitative processes rather than competitive ones (Gorzela *et al.* 2012).

In this study, many different combinations of species interacted across the study area. Several of the species involved, such as *C. semisanguineus* and other *Cortinarius* spp., have been shown to interact negatively with each other during an earlier study at the same site using abundance data (Pickles *et al.* 2010). Species of the genus *Cortinarius* made up a significant proportion of the ECM fungal community at the Culbin site and are a common component of boreal and temperate ECM communities (Garnica *et al.* 2005). There are several possible ecological explanations for the consistently observed dissociation between *C. semisanguineus* and all other *Cortinarius* species. Biotic interactions, particularly

competition (for nutrients, new host roots, etc.) or even antibiosis (the production of a compound that suppresses the growth of another), may be taking place between these species. The growth form, or exploration type (Agerer 2001) of one of these two groups may also make the establishment of the other extremely difficult. Given that *C. semisanguineus* produces thick, medium length external hyphae, whereas all of the other *Cortinarius* spp. morphotypes produce copious short range or very few medium ranged hyphae, it would seem likely that it is *C. semisanguineus* that may suppress the establishment of other *Cortinarius* species. Another explanation may be found in resource partitioning, which might act to separate these species if *C. semisanguineus* has substantially different nutritional requirements from the other members of the *Cortinarius* spp., and those nutrients are distributed patchily and dissociated with each other. Because the abiotic properties of the site were not studied here, it is not possible to confirm or reject this hypothesis. Finally, it is possible that the observed distributions of ECM fungi are simply the result of the respective species being the first to colonize those particular root tips as they became available, thus excluding the other species from the substrate (e.g. Kennedy *et al.* 2009; Lekberg *et al.* 2012). This would be more in line with neutral theory (Hubbell 2001; Adler *et al.* 2007) than the previous types of interaction which support classical niche theory. However, given that those species mentioned above have consistently shown negative associations, regardless of (i) the type of data collected, (ii) the analysis applied or (iii) fine root turnover, some form of competition, antibiosis or resource partitioning appears the more likely expla-

nation. For species such as *Cenococcum geophilum*, *Cadophora finlandica*, *Suillus variegatus* and *Clavulinaceae* sp., which have previously been seen to display both positive and negative pairwise interactions (Pickles *et al.* 2010), the founder effect may well be an appropriate explanation. Although genets were not specifically studied here, it is possible that the patchy distributions of several species that were observed in each year of this study represent individual genets of the different species. Other studies have shown that species such as *Cortinarius rotundisporus* Cleland & Cheel (Sawyer *et al.* 1999), *Hebeloma cylindrosporum* Romagn. (Guidot *et al.* 2001) and *Rhizopogon vinicolor* A. H. Sm. (Beiler *et al.* 2010) can form several large genets at a single site.

The presence-/absence-based TRF approach used in this study was usefully applied to identify many interspecific interactions, despite that it cannot provide the same level of detail as an abundance or biomass approach. Still it is particularly important to note that interactions between species can be identified using high-throughput techniques and binary data if the correct analytical approaches are used. In this case, biologically meaningful results were maintained by analysing binary data from samples containing an appropriate number of both species and colonization sites, thus preventing the signal of interaction from being lost.

Patchiness of species distributions. Joint-count statistics revealed clustering into patches and gaps for several species. The limitation with the Joint-count method in this study was that it only detected clustering at scales ≥ 1 m, while several of these species have previously been observed to cluster into smaller-scale patches (Pickles *et al.* 2010). However, the results of this analysis do allow several conclusions to be drawn about the distribution of ECM fungi across the field site. *Cadophora finlandica*, *Cortinarius semisanguineus*, *Cortinarius* sp.3 and *Russula sardonia* all displayed spatial aggregations and absences of root tips at the ≥ 1 m scale, meaning that their patches and gaps were both heterogeneous on the 20×20 m scale. *Cortinarius* sp.1 only displayed aggregation into patches, meaning that any gaps in its distribution occurred at scales < 1 m (indicating a more even distribution across the site in addition to significant clusters). *Suillus variegatus* and *Clavulinaceae* sp. only displayed aggregation into gaps, so any clusters in their distribution occurred at scales < 1 m (indicating an even distribution across the site coupled with significant areas in which they were absent). Other species did not appear to have clusters or gaps at the scale of analysis used in this study.

Ecological processes. By exploring interactions within a site chosen for its homogeneity (even-aged stand of a

single host species, no herbaceous plant layer or apparent environmental gradients), using a comprehensive sampling approach at an explicit spatial scale, this study was able to draw conclusions about major structuring forces in simple ECM fungal root tip communities. Deterministic rather than stochastic processes appear to be driving community structure, much like in other mutualistic systems (Palmer *et al.* 2003). Similar to communities of annual plants (Levine & Rees 2002), tropical forests (Wright 2002) and fish (Munday *et al.* 2001), competition appears to be an important structuring factor in this simplified system. The most probable driver here was access to growth substrate (competition for host root tips). However, other factors may have been responsible for the observed pattern such as interspecific variation in preference for patchy soil nutrients, interactions with host genotypes or strong and persistent priority effects, the last of which seems least likely. Studying how a relatively simple ECM fungal community is structured helps to generate hypotheses about more complex communities, based on the existing ecological literature, because many of these structuring processes have been well studied in other organisms (Huston 1994; Gaston 2003). As soil heterogeneity increases, the patchiness of individual fungal species is also expected to increase because of niche partitioning. As disturbance increases, neutral structuring processes like dispersal are expected to become more important. Host age and community structure are also likely to have varying and complicated impacts on the structure of ECM communities.

Methodological considerations

Studies of inter- and intraspecific interactions within ECM fungal communities continue to provide important insights into the ecology of this vital symbiosis (Kennedy 2010). T-RFLP is an example of a high-throughput technique that has been successfully used in several recent ecological studies of ECM fungi (Koide *et al.* 2005a,b; Genney *et al.* 2006; Dickie & FitzJohn 2007; Dickie *et al.* 2010). One of the main benefits of the approach is that large numbers of samples can be processed at a relatively low cost in a short period of time (Anderson & Cairney 2004), an important consideration for spatial investigation of ECM communities (Genney *et al.* 2006; Pickles *et al.* 2009, 2010). In addition, when used in conjunction with an extensive reference database, its resolution is well suited to the studies of ECM fungal root tips (Dickie & FitzJohn 2007; Saari *et al.* 2007). Thus, T-RFLP remains a very viable option for investigation of ECM community ecology, particularly in lower diversity systems and/or small samples for which the newer and currently popular pyrosequencing

technique (e.g. Caporaso *et al.* 2011) may be inappropriate. Morphotyping/molecular approaches are still valid for studies of interactions between dominant ECM species in a community given that (i) abundance/biomass data are more effective at revealing interactions than presence/absence data; (ii) studies of ecological interactions are generally not concerned with extremely rare species in such a system because of the difficulty in assessing potential ecological relevance. The TRF with accompanying database approach makes it easier to quickly distinguish between closely related species than the morphotyping/molecular approach. Analysis of species richness data using Chao2 estimators shows that the detection of species within the community using this method was successful. If the true number of species present on the site was in fact closer to the α -diversity estimate, this might simply be due to missing some of the rarest species on the site, or may in part be attributed to the T-RFLP database being incomplete, rather than a sampling issue. So for studies of ECM ecology, a morphotyping/sequencing approach is still justified when investigating the interactions between the most common or dominant species.

Conclusions

Spatial and co-occurrence analyses provide evidence that, in a relatively homogeneous environment, ECM fungal root tip communities are competitively structured or structured by processes that generate the same pattern as competition. The hypothesis that analysis of incidence data would suggest a community structured by neutral rather than deterministic processes was rejected. The combination of a high-throughput molecular technique with sampling at a scale that was both appropriate to the organism's biology and suitable for the detection of relevant spatial patterns was probably a key factor. It seems that the relatively large patch size of ECM structures enables the detection of the interactions between species, as compared to smaller microbial species that are either subject to neutral structuring processes or studied at scales that do not allow for successful identification of interspecific interactions. Recent advances in pyrosequencing (which is now capable of generating sequences >500 bp) show great promise for ECM fungal community studies; however, using this technique for quantitative analysis may lead to relative abundance errors of several orders of magnitude (Amend *et al.* 2010), and it can be susceptible to high error rates (Kunin *et al.* 2010). For now, studies such as the one presented here show that it is possible to derive ecologically relevant information on species interactions and other ecological processes from incidence data generated by high-throughput techniques. Future studies may readily scale up ECM ecology from

the plot to the landscape scale using the 3–4 m separation distance between samples to account for spatial autocorrelation in community β -diversity. Such work would be a big step in determining whether ECM fungi behave like other communities of organisms across geographical scales.

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Data accessibility

DNA sequence/TRF database: See Table S1 (Supporting information) for all GenBank and UNITE accessions.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Table S1. ECM T-RFLP database.

Table S2. Summary of collection information for Table S1.

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