

Phylogenetic similarity and structure of Agaricomycotina communities across a forested landscape

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Abstract

The Agaricomycotina are a phylogenetically diverse group of fungi that includes both saprotrophic and mycorrhizal species, and that form species – rich communities in forest ecosystems. Most species are infrequently observed, and this hampers assessment of the role that environmental heterogeneity plays in determining local community composition and in driving β -diversity. We used a combination of phenetic (TRFLP) and phylogenetic approaches [Unifrac and Net Relatedness Index (NRI)] to examine the compositional and phylogenetic similarity of Agaricomycotina communities in forest floor and surface soil of three widely distributed temperate upland forest ecosystems (one, xeric oak – dominated and two, mesic sugar maple dominated). Generally, forest floor and soil communities had similar phylogenetic diversity, but there was little overlap of species or evolutionary lineages between these two horizons. Forest floor communities were dominated by saprotrophic species, and were compositionally and phylogenetically similar in all three ecosystems. Mycorrhizal species represented 30% to 90% of soil community diversity, and these communities differed compositionally and phylogenetically between ecosystems. Estimates of NRI revealed significant phylogenetic clustering in both the forest floor and soil communities of only the xeric oak-dominated forest ecosystem, and may indicate that this ecosystem acts as a habitat filter. Our results suggest that environmental heterogeneity strongly influences the phylogenetic β -diversity of soil inhabiting Agaricomycotina communities, but has only a small influence on forest floor β -diversity. Moreover, our results suggest that the strength of community assembly processes, such as habitat filtering, may differ between temperate forest ecosystems.

Keywords: Agaricomycetes, Agaricomycotina, ectomycorrhizal, habitat filters, temperate forests, phylogenetic community analysis, saprotrophic

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Introduction

Beta diversity is the change in species composition over geographic space (Whittaker 1972). High beta diversity (low community similarity) may result from habitat heterogeneity when species differ in their abilities to colonize distinct habitats (Kerr *et al.* 2001), and habitat characteristics can therefore be seen as defining aspects of a species β – niche (Pickett & Bazzaz 1978). However,

high β -diversity may also be observed if dispersal limitation prevents species from reaching all suitable habitats, or if the nature of the species is such that comprehensive sampling is problematic. Microbial communities may be especially problematic, because high species richness and cryptic growth forms make comprehensive sampling a challenge (Hughes *et al.* 2001; O'Brien *et al.* 2005; Lynch & Thorn 2006). Fungi are an important component of terrestrial ecosystem biodiversity (Hawksworth 2001). Within the fungi, the Agaricomycotina (*sensu* Hibbett 2006; Agaricomycetes, Dacrymycetes and Tremellomycetes) represent about

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20% of all described fungal species (Hawksworth 2001). Saprotrophic Agaricomycotina are important agents of plant litter decomposition (Lynch & Thorn 2006), whereas ectomycorrhizal species are an important component of the mutualistic symbiotic community beneath *Pinus*, *Picea*, *Quercus*, *Populus* and *Betula* (Smith & Read 1997).

Relatively few studies have considered the role of habitat heterogeneity on fungal β -diversity, and these have focused on ectomycorrhizal species (Nantel & Neumann 1992; DeBellis *et al.* 2006). Plant community composition is considered a primary factor defining the β -niche of ectomycorrhizal fungi (Molina *et al.* 1992), because these symbiotic species often exhibit host-specificity (Nantel & Neumann 1992; Bruns *et al.* 2002; DeBellis *et al.* 2006). Edaphic factors also influence ectomycorrhizal species distributions (Nantel & Neumann 1992; Kranabetter *et al.* 2009). In comparison with ectomycorrhizal species, much less is understood about the factors affecting saprotrophic species distributions at both the local and landscape scales (O'Brien *et al.* 2005; Porter *et al.* 2008), although edaphic factors may be important here too (Tyler 1992). Saprotrophic Agaricomycotina also rely on plant-derived organic compounds for energy, and because the biochemical composition of litter varies between plant species, the landscape-scale distribution of plant species might also influence the distribution of these species (Tyler 1992; O'Brien *et al.* 2005; Porter *et al.* 2008).

At landscape scales (1000s to 100000s of ha), plant community assembly has a strong deterministic component, with local variation in physiographic and edaphic properties strongly affecting species distributions (Barnes *et al.* 1982; Laliberté *et al.* 2009). Because of this, landscapes can be conceptualized of as a mosaic of plant communities, each of reasonably predictable composition, and each potentially colonized by a subset of the regional Agaricomycotina species pool (Barnes *et al.* 1982; Villeneuve *et al.* 1989; Laliberté *et al.* 2009).

For communities such as the Agaricomycotina, which are characterized by high species richness and highly variable local species assemblages (Villeneuve *et al.* 1989; DeBellis *et al.* 2006; Peay *et al.* 2007), phylogenetic analyses may provide a better measure of community similarity than more traditional species-based ordination analyses (Lozupone & Knight 2005; Graham & Fine 2008). Moreover, the phylogenetic structure of communities might provide insight into dominant community assembly processes (Kembel & Hubbell 2006; Losos 2008). Yet, the degree to which the Agaricomycotina communities of forest ecosystems are phylogenetically distinct or exhibit significant phylogenetic structure is largely unknown. In this study, we asked three questions: (1) Do the floristic and edaphic characteristics of

temperate forest ecosystems significantly affect Agaricomycotina community composition? (2) Are the Agaricomycotina communities of temperate forest ecosystems phylogenetically distinct? (3) What types of phylogenetic structure characterize the Agaricomycotina communities of temperate forest ecosystems? We hypothesized that because of intrinsic floristic and edaphic differences, the Agaricomycotina communities of temperate forest ecosystems should be compositionally distinct. Moreover, we hypothesized that these fungal communities should also be phylogenetically distinct, i.e. that despite local differences in community composition, each different temperate forest ecosystem nevertheless recruits Agaricomycotina from a restricted range of evolutionary lineages. Finally, we hypothesized that the Agaricomycotina communities of distinct forest ecosystems should be phylogenetically clustered. To test these hypotheses, we examined the composition, phylogenetic similarity, and phylogenetic structure of Agaricomycotina communities across a temperate forested landscape, using replicated stands of three common upland forest ecosystem types.

Materials and methods

Study sites

Our study was conducted in northwestern Lower Michigan, Lat. 44°48', Long. 85°48' (Fig. 1). This region has a temperate climate, with a mean annual temperature of 7.2 °C and a growing season of 100–150 days. Mean annual precipitation is 81 cm and precipitation is evenly distributed throughout the year (Albert *et al.* 1986). We sampled three replicate stands in each of three common and widely distributed upland forest ecosystems: black oak – white oak/*Vaccinium* (BOWO), sugar maple – red oak/*Maianthemum* (SMRO), and sugar maple-basswood/*Osmorhiza* (SMBW) ecosystems; they are named for the dominant overstory tree species and a characteristic ground flora species (Host

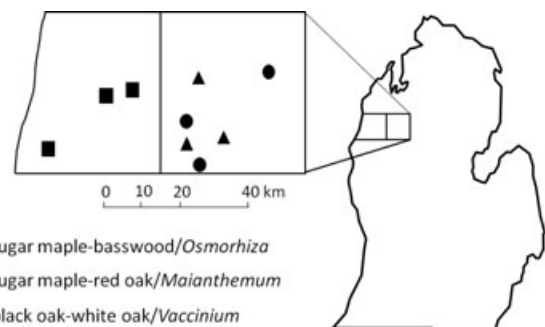


Fig. 1 A map of Lower Michigan showing the location of nine replicate stands in three upland forest ecosystems.

Table 1 Soil and vegetation characteristics of three upland forest ecosystems in northern Lower Michigan, USA

	Ecosystem type		
	Black oak/white oak	Sugar maple/red oak	Sugar maple/Basswood
	BOWO	SMRO	SMBW
Overstory species	<i>Quercus velutina</i> *	<i>Q. rubra</i>	<i>A. saccharum</i> *
	<i>Quercus alba</i>	<i>A. rubrum</i>	<i>Tilia americana</i>
	<i>Quercus rubra</i>	<i>P. grandidentata</i>	<i>P. grandidentata</i>
	<i>Acer rubrum</i>	<i>Fagus grandifolia</i>	<i>F. grandifolia</i>
	<i>Populus grandidentata</i>	<i>O. virginiana</i>	<i>Fagus americana</i>
		<i>Tilia americana</i>	<i>O. virginiana</i>
		<i>Acer saccharum</i> *	
Ground flora	<i>Vaccinium</i>	<i>Maianthemum</i>	<i>Ozmorhiza</i>
Litter Lignin (mg/g)†	340	300	260
Litter Cellulose (mg/g)†	440	440	500
Soil Type	Entic Haplorthod	Typic Haplorthod	Typic Haplorthod
pH‡	3.9	4.1	5.5
Organic C (g/kg)‡	440	390	550
Total N‡ (µg/N g ⁻¹)	1913	1835	3040
Net N mineralization‡ (µg N g ⁻¹ year ⁻¹)	313	382	426
Net Nitrification‡ (µg N g ⁻¹ year ⁻¹)	18	43	364

*Indicates canopy dominant.

†Litter characteristics from Blackwood *et al.* 2007.

‡All soil properties measured in 0–3.8 cm depth, from Zak *et al.* 1990.

et al. 1988; Zak *et al.* 1990; Table 1). Distance between replicate stands of an ecosystem type ranged from 0.5–35 km (average, 12.9 km); all stands are late successional, with overstory trees that are approximately 100 years old (Table 1).

Field sampling

Because ectomycorrhizal and saprotrophic species are often vertically stratified between forest floor and soil (Lindahl *et al.* 2007), we sampled these two horizons separately. Within each stand, 12 forest floor and 12 surface soil samples were collected at 5-m intervals along two parallel transects spanning a pre-existing 30-m × 10-m plot. At each sampling location, forest floor was collected from a 0.1 m² area and surface soil was collected to a depth of 5 cm with a 2.5 cm diameter soil corer. In the SMBW ecosystem, forest floor (O_i) abruptly gives way to the underlying mineral soil, whereas in the BOWO and SMRO ecosystems, a 3 to 5-mm thick O_e horizon forms on the surface of the mineral soil. This horizon, which is densely interpenetrated by fine roots, is continuous in the BOWO ecosystem and discontinuous in the SMRO ecosystem. We collected the O_e as part of the surface soil, not the forest floor. Forest floor and surface soil samples collected in each plot were combined to produce one composite

forest floor and one composite surface soil sample for each replicate stand; these samples were stored on ice prior to DNA extraction.

Sample preparation and DNA extraction

We used a soil-washing technique prior to DNA extraction in order to increase the probability of sampling DNA from actively growing hyphae, rather than potentially dormant basidiospores (Bååth 1988; Lynch & Thorn 2006). Briefly, soil samples were homogenized by hand and passed through a 2-mm sieve to remove roots and coarse fragments. Ten grams of sieved, homogenized soil was shaken with 100 mL of 0.1 M sodium pyrophosphate for 1 h, and the slurry washed through sieves of 0.25 mm and 0.053 mm mesh with ~ 4 L of deionized water (Lynch & Thorn 2006). DNA was extracted from the 0.053–0.25 mm fraction using PowerMax™ Soil kits (Mo Bio) as per manufacturer's instructions. Forest floor samples were chopped (Hamilton Beach R 10 speed blender) to facilitate homogenization and subsequently treated as described for soils, except that 2.5 g of material was washed and used for DNA extraction. Duplicate DNA extractions were performed on each sample in order to assess the effectiveness of the homogenization procedure; DNA was stored at –80 °C.

Polymerase chain reaction

The entire ribosomal rDNA internal transcribed spacer region and approximately 400 bases of the large-subunit rDNA was selectively amplified from DNA extracts using primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3', Gardes & Bruns 1993) and LR21 (5'-ACT TCA AGC GTT TCC CTT T-3', Hopple & Vilgalys 1994). ITS1F is considered a general fungal primer and was designed to minimize co-amplification of non-fungal DNA, while LR21 preferentially amplifies basidiomycete DNA. We used the same primers for both cloning and Terminal Restriction Fragment Length Polymorphism (TRFLP), with the ITS1F primer labelled with 6-FAM for TRFLP. Each PCR reaction cocktail contained 50–200 ng of soil DNA, 200 nM dNTPs, 1X PCR buffer including 1.5 mM MgCl₂ (Roche), 0.5 µM of each primer and 50 µg of BSA. After an initial denaturation step of 3 min at 94 °C, 35 cycles (TRFLP) or 25 cycles (clone libraries) of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s were carried out using Stratagene PCR cyclers (La Jolla, CA). A final extension step of 72 °C for 15 min was used to minimize the production of pseudo-restriction products (Egert & Friedrich 2003).

Cloning

Triplicate PCR products from each horizon of the three replicate stands in each ecosystem ($n = 3$) were combined and purified (UltraClean PCR Clean-up, Mo Bio) prior to cloning into pCR[®]2.1-TOPO[®] using the TOPO TA Cloning kit (Invitrogen). Thirty-two clones for each soil horizon in each stand were selected to create six 96-clone libraries, one for each horizon ($n = 2$) of each ecosystem ($n = 3$). Clones were grown overnight in Luria broth supplemented with 10% glycerol, and sent to the University of Georgia for bidirectional sequencing with M13F and M13R primers. Sequence quality was assessed by visual inspection, and full length contiguous sequences for each clone were constructed in Geneious 3.7.0 (Biomatters Ltd.).

Terminal restriction fragment length polymorphism

For TRFLP, approximately 500 ng of purified PCR product (see above) was digested overnight with 10 U of *Hae*III (Promega) at 37 °C. Digests were desalted with Microcon YM-30 centrifugal filters (Millipore) prior to genotyping. All samples were mixed with ROX 1000 size marker, and genotyping was performed at the University of Michigan's Core Sequencing Facility using an ABI 3730XL DNA Sequencer with a 96 capillary array. Terminal restriction fragment lengths were determined

relative to the ROX 1000 size standard with Genemarker 1.51 (SoftGenetics). Each digest was run four times, (duplicates on two separate runs) and all TRF greater than 50 fluorescence units that occurred in at least both duplicate electropherograms of each run were scored in a presence-absence matrix. Initial sensitivity analyses demonstrated that the number of TRF peaks observed in each sample was essentially independent of total signal intensity after a threshold intensity of 20 000 fluorescence units was reached; samples were reanalyzed if they did not meet this minimum intensity. Terminal restriction fragment length profiles obtained from each sample were highly reproducible, and we estimated measurement precision as ± 0.5 bp for TRF up to 1000 bp, and ± 1.0 bp for TRF 1000–1200 bp.

Operational taxonomic units

There is no ideal single-gene approach to defining either fungal species or the phylogeny of the Agaricomycotina (Taylor *et al.* 2000; Hibbett 2006; Nilsson *et al.* 2008). Generally, the rDNA ITS has a higher degree of resolution at the subgeneric level than more conserved regions such as rDNA 28S (Nilsson *et al.* 2008). However, alignment problems beyond the generic or family level due to the variability of the non-coding ITS1 and ITS2 spacers make this region of little use in phylogenetic community analysis. Some recent studies have defined fungal operational taxonomic units (OTUs) in terms of rDNA 28S sequence similarity, using 99% sequence similarity as an arbitrary cutoff (Lynch & Thorn 2006; Porter *et al.* 2008). While pragmatic, this approach is also conservative and may unintentionally lump together closely related species (Lynch & Thorn 2006). In this study, we used a two-step procedure to define OTUs in order to capitalize on the information provided by both ITS and rDNA 28S regions recovered with the ITS1F-LR21 primer set. First, we created a rDNA 28S alignment of all sequences using Clustal W (Thompson *et al.* 1997) within Geneious (Drummond *et al.* 2008). This alignment was used to generate a similarity matrix in MEGA 4.0 (Tamura *et al.* 2007), and subsequently to cluster sequences into groups based on $\geq 99\%$ similarity using the furthest neighbour algorithm in DOTUR (Schloss & Handelsman 2005). Subsequently, for each of the rDNA 28S groups that included more than one sequence, we repeated this procedure using full length (ITS + 28S) alignments and defined OTUs at $\geq 97\%$ similarity. Sequences representing each OTU have been deposited in GenBank (GU328501-GU328639). Non-parametric estimates of species richness (Chao I and Jackknife) were also calculated by DOTUR (Fig. S1 Supporting information).

OTU identification and chimera checking

BLAST searches were performed for all OTUs to retrieve the top-matching sequence from GenBank. To check for chimeric sequences, we repeated the BLAST searches using first the ITS sequence and then the 28S fragment. When the two searches returned dramatically different results, we considered this as possible evidence of a chimera and the sequence was excluded from subsequent analyses. After examination of preliminary phylogenetic trees with the reference sequences, only OTUs which placed clearly in the Agaricomycota were retained for analysis.

Phylogenetics

The rDNA 28S gene fragments (~450bp) from each OTU were aligned using Clustal W (Thompson *et al.* 1997) in Geneious (Drummond *et al.* 2008), and the alignment was manually edited. The hypervariable D1 region was excluded from the alignment. ModelTest (Posada & Crandall 1998) was used to compare evolutionary models, and a bootstrapped neighbour-joining tree created using MEGA 4.0. Ascomycete sequences were used to root the tree.

Gradient analysis

We used Detrended Correspondence Analysis (DCA) to assess the similarity of the Agaricomycotina communities in each horizon across the landscape, and Canonical Correspondence Analysis (CCA, Ter Braak 1986) to determine the significance of the species – environment correlation. Species data consisted of a *HaeIII* TRFLP presence-absence matrix, and we assumed that each unique TRF peak represented a single species. This assumption is known to not be strictly true (Edwards & Turco 2005; Avis *et al.* 2006; Dickie & FitzJohn 2007) but it is unavoidable in the absence of extensive *a priori* knowledge of these communities. Environmental parameters included in CCA were: relative abundance of overstorey species, soil pH, soil N mineralization rate (N_{min}), total organic carbon (TOC), and forest floor cellulose and lignin contents (Zak *et al.* 1990; Blackwood *et al.* 2007). Significance of community-environment correlations was tested with a Monte Carlo test, using 999 unrestricted permutations. In both DCA and CCA, forest floor and soil fingerprints were examined together, and singleton TRFs were not removed, although their influence on the ordination was minimized using the 'downweight rare species' option in CANOCO (Biometris, Wageningen). TRFLP fingerprints were also used to assess the influence of distance between sites on fungal community similarity. Finger-

print similarity was estimated as Sorensen's index, (1 = identical, 0 = no overlap) and this was regressed against distance (km) between study sites using linear regression. Regressions for litter and soil horizons were performed separately using SAS 8.1.

Phylogenetic similarity, diversity and structure

The phylogenetic similarity of the Agaricomycotina communities was assessed using Unifrac (Lozupone & Knight 2005). UniFrac estimates the distance between communities as the fraction of the branch length of the phylogenetic tree that leads to descendants from either one environment or another, but not both; we used this distance matrix to cluster environments using Jackknifed UPGMA. Phylocom-3.40 (Webb 2000) was used to calculate Faith's index of phylogenetic diversity (PD, Faith 1992) and the Net Relatedness Index (NRI). PD is defined as the minimum branch length spanning any given set of species on a phylogenetic tree. The phylogenetic structure of each community was estimated with the Net Relatedness Index (NRI, Webb 2000). NRI is defined as $[-(MPD - MPD_{null})/SD(MPD_{null})]$, where MPD is the mean pairwise phylogenetic distance between species in a community, MPD_{null} is the mean MPD for 1000 random communities, and $SD(MPD_{null})$ is the standard deviation. The significance of phylogenetic pattern was determined relative to 999 randomly assembled communities created under Phylocom's null model 2 (Webb *et al.* 2002) whereby species richness was maintained in each sample, and species were drawn randomly from the list of all species present in all samples. Because the significance of phylogenetic structure can be sensitive to tree topology (Swenson 2009), we manually edited the basic input tree to collapse all unsupported nodes and repeated the analysis with the resulting polytomy tree.

Results

Gradient analysis

Overall, we recorded 171 unique 5' *HaeIII* terminal restriction fragments (TRF) ranging in size from 73 ± 0.5 to 1062 ± 1 base pairs; 66 of these were unique to the forest floor, 86 were unique to surface soil and 19 were recovered from both horizons. Most TRF were recovered from a single stand, and ~90% were recovered from three stands or less (Supporting information, Fig. S2). Sorensen similarity values between sites ranged from 0.08 to 0.74 in the forest floor and from 0.0 to 0.46 in the soil, and similarity was independent of distance between sites (soil, $P = 0.68$; forest floor $P = 0.85$).

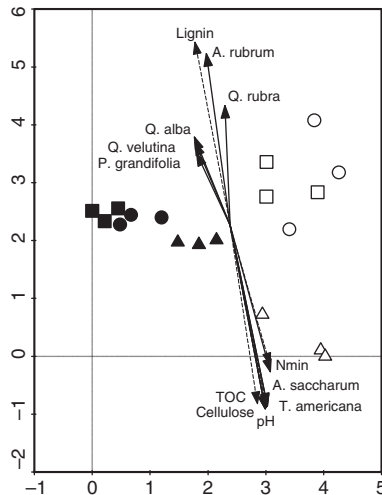


Fig. 2 Detrended Correspondence Analysis (DCA) biplot displaying the similarity of nine upland hardwood forest stands in terms of fungal community composition. Soil (open symbols) and forest floor (closed symbols) are presented separately. The nine points represent three replicate stands in three contrasting upland forest types; squares: black oak/white oak; circles: sugar maple – red oak; triangles: sugar maple - basswood. Soil properties (dashed vectors), and overstory plant abundances (solid vectors) are plotted as passive vectors. Axes scale, standard deviation. The first correspondence axis explains 20.6% of the variance, and the second axis 8.8%. Both axes are highly significant ($P = 0.002$).

Detrended Correspondence Analysis separated forest floor from soil TRFLP profiles along a primary axis with a gradient length of 4.3 SD that accounted for 12.7% of the overall variance (Fig. 2). A second axis with a gradient length of 4.1 SD, accounted for an additional 7.9% of the variance. This second axis provided little extra separation within the forest floor communities, but tended to separate the oak soil horizon fungal communities from the sugar maple-basswood soil horizon communities. The primary CCA axis accounted for 12.0% of the variance and was significantly correlated to soil horizon ($r^2 = 0.98$, $P = 0.01$). The second canonical axis was strongly correlated to *Quercus* and *Acer* distribution, net N mineralization rate, and forest floor lignin and cellulose contents. This second axis accounted for a further 6.6% of the variance and was also significant ($P = 0.02$).

Phylogenetic diversity

We obtained non-chimeric rDNA ITS-28S sequences from 529 clones, and initial BLAST and phylogenetic analyses indicated that the majority of these were fungal and that 461 placed in the Agaricomycotina. Clustering of these sequences based on 99% rDNA 28S similarity produced 106 groups, 44 of which included

multiple sequences. Full length rDNA ITS-28S alignments within each of these groups showed sequence similarity levels of 87–100% (average 96.03%), and based on 97% full length similarity, we recovered 139 OTU with rDNA 28S similarities of 99 – 100% (Table S1, Supplementary material). Based on the rDNA 28S alignment, Chao I and Jackknife estimates of species richness ranged from 170 to 295, and the curves for the overall library had positive slopes, suggesting that further sampling would likely have recovered more OTUs (Fig. S1, Supporting information).

The phylogenetic dataset included 141 rDNA 28S sequences (including two Ascomycete outgroup tax) aligned in 468 positions. Forty ambiguous sites were excluded from the analysis, and of the remaining 428, 192 were variable and 236 constant. ModelTest indicated that a Kimura 2-parameter model with a gamma function provided an efficient fit to the data, and we used this to create a bootstrapped neighbour-joining tree (Supporting information, Fig. S3). Generally, terminal groups in the phylogeny were moderately to well supported, but deeper nodes were not (Supporting information, Fig. S3). Despite this, the overall topology of the tree accorded reasonably well with recent analyses of the Agaricomycota (e.g. Hibbett 2006). Both Agaricomycetes and Tremellomycetes were recovered (Supporting information Fig. S3) and the OTUs represented species from at least 15 orders of Agaricomycotina (Table 2). Agaricomycetes represented 64 – 96% of the OTUs recovered from each forest ecosystem, and Agaricomycete OTU richness was higher in soil than forest floor (Table 2). Tremellomycetes represented 6 – 36% of OTUs within each forest ecosystem, and Tremellomycete OTU richness was higher in the forest floor (Table 2).

Within the Agaricomycetes, Agaricales were dominant and were significantly more diverse in soil than forest floor (Table 2). The diversity of Russulales and Boletales also tended to be greater in soil than forest floor (Table 2). In contrast, Cantharellales were exclusively recovered from forest floor (Table 2). In both forest floor and soil, phylogenetic diversity was lowest in BOWO ecosystem and highest in SMBW ecosystem (Table 3). Although we recovered more OTUs from the soil than the forest floor, species in the forest floor tended to encompass a greater phylogenetic diversity both at the landscape scale and within each of the three forest ecosystems (Table 3).

Community phylogenetic similarity

There was little overlap in community membership between forest floor and soil, with only two OTUs recovered from both. Moreover, as for TRFLP, most

Table 2. Comparison of the Agaricomycotina communities of three upland forest ecosystems. Data is expressed as proportion of community OTU richness

Order	Forest floor			Soil			Comment
	BOWO	SMRO	SMBW	BOWO	SMRO	SMBW	
Agaricomycetes	0.74	0.64	0.66	0.96	0.96	0.94	
Agaricales	0.39	0.39	0.28	0.52	0.67	0.44	Greater richness in soil
Cantharellales	0.17	0.11	0.19	0.00	0.00	0.00	Forest floor only
Other*	0.09	0.04	0.03	0.00	0.07	0.06	
Russulales	0.04	0.04	0.03	0.24	0.07	0.09	Greater richness in soil
Stereales	0.04	0.00	0.00	0.00	0.00	0.00	
Atheliales	0.00	0.00	0.00	0.12	0.00	0.00	BOWO soil only
Boletales	0.00	0.00	0.03	0.08	0.00	0.15	
Geastrales	0.00	0.00	0.00	0.00	0.00	0.03	
Phallales	0.00	0.00	0.03	0.00	0.00	0.00	
Polyporales	0.00	0.00	0.00	0.00	0.00	0.03	
Sebacinales	0.00	0.00	0.03	0.00	0.00	0.12	SMBW only
Thelephorales	0.00	0.00	0.03	0.00	0.07	0.00	
Trechisporales	0.00	0.07	0.00	0.00	0.07	0.06	
Tremellomycetes	0.26	0.36	0.34	0.00	0.04	0.06	
Filobasidiales	0.17	0.18	0.28	0.00	0.04	0.03	Greater richness in forest floor
Tremellales	0.04	0.14	0.06	0.00	0.00	0.03	Greater richness in forest floor
Cystofilobasidiales	0.04	0.04	0.00	0.00	0.00	0.00	Greater richness in forest floor

*Other includes OTUs that placed in poorly represented groups such as Corticiales and Gomphales.

OTUs showed a very limited distribution across the landscape, with ~97% of all OTU recovered from three or less stands (Fig. S2 Supporting information). Only three OTUs were recovered from all three ecosystems and all of these were recovered from the forest floor. OTUs that placed within known ectomycorrhizal clades were recovered almost exclusively from the soil horizon. Putative ectomycorrhizal OTUs dominated the soil in the BOWO ecosystem, in which they comprised 92% of all OTUs; however, these organisms were less prominent in SMRO and SMBW ecosystems (30% and 25% respectively, Fig. S4 Supporting information). No ectomycorrhizal OTU was recovered from all three ecosystems, although some showed high fidelity to a particular forest type (e.g. *Piloderma* sp. and some *Russula* spp. in BOWO soils).

UniFrac unambiguously distinguished soil and forest floor communities (UniFrac $P < 0.01$). Within the forest floor, UniFrac revealed a higher level of similarity between BOWO and SMRO fungal communities than between these and SMBW (Fig. 3). Despite this, the UniFrac metric indicated no significant difference between these three communities in terms of Agaricomycotina lineages (BOWO vs. SMRO, $P = 0.26$; BOWO vs. SMBW, $P = 0.20$; SMRO vs. SMBW $P = 0.26$). Within the soil horizon, UniFrac revealed a higher level of similarity between SMBW and SMRO fungal communities than between these and BOWO (Fig. 3). Moreover, within the soil horizon the UniFrac metric

indicated significant differences between the BOWO fungal community and those of SMRO and SMBW (BOWO vs. SMRO, $P = 0.03$; BOWO vs. SMBW, $P = 0.02$). There was also a marginally significant difference between the SMRO soil and SMBW soil communities ($P = 0.08$).

Community phylogenetic structure

Forest floor and soil horizon Agaricomycotina communities had similar phylogenetic diversity when pooled across the three ecosystems, and moreover both communities had positive NRI values, suggesting phylogenetic clustering; however this was not statistically significant (Table 3).

Both NRI and PD values were highly variable at the stand scale (300 m²) and no clear trends were apparent (Fig. 4). Twelve of the 18 communities had positive NRI values; although for only three of these, two BOWO soil horizon and one BOWO forest floor, did these values significantly exceed null expectations. The remaining six communities had negative NRI values, suggesting phylogenetic overdispersion, but this was only significant in one SMBW forest floor community.

We estimated the effect of spatial scale by pooling replicate stand data. At this scale (900 m²) the Agaricomycotina soil horizon community of the BOWO ecosystem exhibited significant phylogenetic clustering, while the forest floor community of SMBW was

Table 3. Phylogenetic diversity and structure in the Agaricomycotina communities associated with three upland forest ecosystems in northern Lower Michigan, USA

Ecosystem	Horizon	N*	P.D.†	NRI	Obs > Sim‡	P§	Pattern
Agaricomycotina							
ALL	Forest floor	72	0.569	0.5608	699	n.s.	Random
ALL	Soil	75	0.566	1.2100	892	0.11	Random
Agaricomycotina							
BOWO	Forest floor	23	0.231	0.8582	813	n.s.	Random
SMRO	Forest floor	28	0.278	-0.0629	479	n.s.	Random
SMBW	Forest floor	32	0.353	-1.1688	120	0.12	Random
BOWO	Soil	25	0.182	2.4011	988	0.01	Clustered
SMRO	Soil	27	0.304	-0.5923	289	n.s.	Random
SMBW	Soil	34	0.344	0.1311	544	n.s.	Random
Agaricomycotina (polytomy)							
BOWO	Forest floor	23	0.205	1.0359	856	n.s.	Random
SMRO	Forest floor	28	0.265	-0.3169	362	n.s.	Random
SMBW	Forest floor	32	0.329	-1.0367	142	0.14	Random
BOWO	Soil	25	0.157	2.4724	993	0.006	Clustered
SMRO	Soil	27	0.267	-0.0179	488	n.s.	Random
SMBW	Soil	34	0.307	0.5834	724	n.s.	Random
Agaricomycetes							
BOWO	Forest floor	17	0.150	1.9247	969	0.03	Clustered
SMRO	Forest floor	18	0.173	0.8493	795	n.s.	Random
SMBW	Forest floor	21	0.252	-0.0310	469	n.s.	Random
BOWO	Soil	25	0.182	2.4657	991	0.01	Clustered
SMRO	Soil	26	0.287	-0.5648	292	n.s.	Random
SMBW	Soil	32	0.301	0.4881	698	n.s.	Random
Agaricomycetes (polytomy)							
BOWO	Forest floor	17	0.124	1.9421	975	0.02	Clustered
SMRO	Forest floor	18	0.155	0.5466	688	n.s.	Random
SMBW	Forest floor	21	0.227	-0.1463	441	n.s.	Random
BOWO	Soil	25	0.157	2.4060	988	0.01	Clustered
SMRO	Soil	26	0.251	0.0911	529	n.s.	Random
SMBW	Soil	32	0.265	0.9456	819	n.s.	Random

*N, number of OTU.

†Faith's index of phylogenetic diversity.

‡Number of times that NRI values of the natural community exceeded those of 999 random simulated communities generated under Phylocom null model 2.

§P-value for a one-sided test.

overdispersed, although this was not significant (Table 3). We also estimated the effects of taxonomic scale by restricting the analysis to the Agaricomycetes within the pooled 900 m² communities; significant clustering was still observed in the BOWO soil community, but in addition the BOWO forest floor Agaricomycete community exhibited significant phylogenetic clustering (Table 3). In contrast, with Tremellomycetes removed from the analysis, the SMBW community no longer exhibited overdispersion (Table 3). Finally, we examined the robustness of these results to topological inaccuracy in the phylogenetic tree; all unsupported branches were collapsed, and NRIs recalculated for Agaricomycotina and Agaricomycetes at the 900 m² scale using the resultant poorly resolved polytomy.

Incomplete basal resolution had no effect on the phylogenetic patterns observed (Table 3).

Discussion

Do the floristic and edaphic characteristics of temperate forest ecosystems significantly affect Agaricomycotina community composition?

Generally, there was a greater similarity between the TRFLP community fingerprints of replicate stands of each forest ecosystem type than there was between fingerprints of differing ecosystem types (Fig. 2). Furthermore, constrained (CCA) analysis revealed significant correlations between fingerprint similarity and

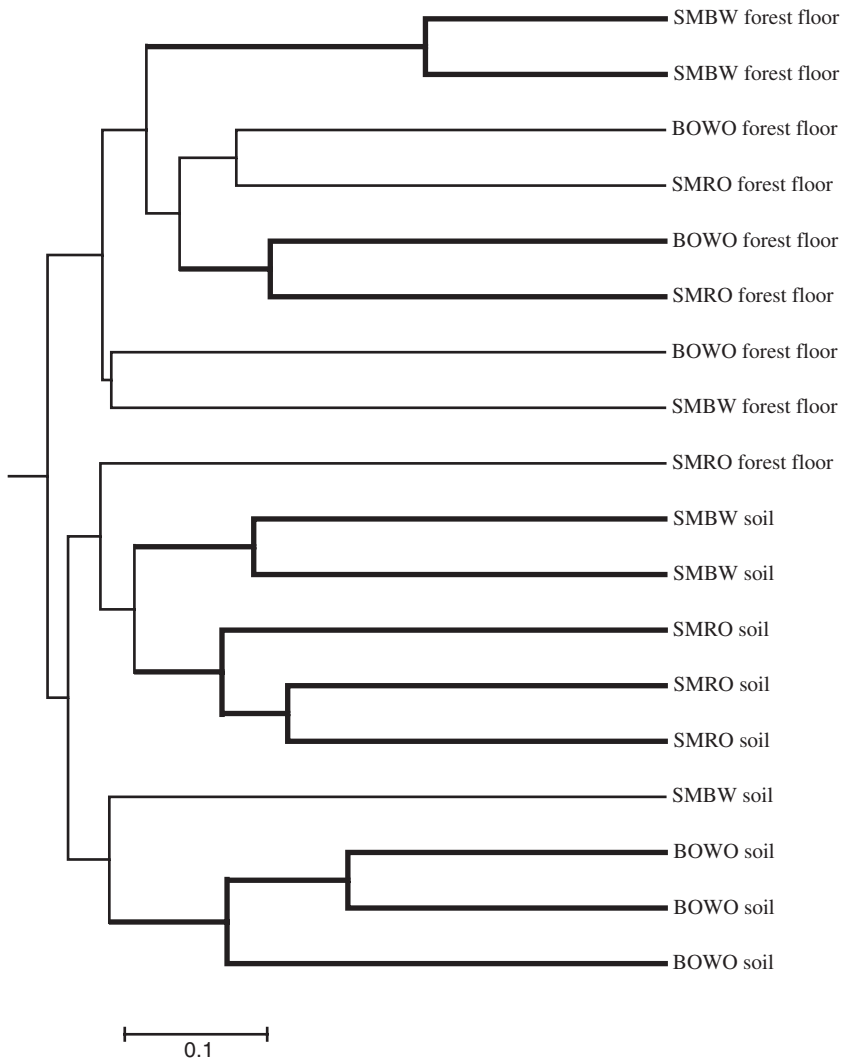


Fig. 3 Phylogenetic similarity of the basidiomycete communities from forest floor and soil horizons of three replicate stands in three contrasting upland hardwood forest ecosystems on UPGMA clustering of the Unifrac distance matrix. BOWO: black oak/white oak; SMRO: sugar maple – red oak; SMBW: sugar maple – basswood. Bold branches and nodes are supported by >70% of Jackknife replicates.

environmental factors such as soil horizon and the floristic and edaphic properties of the three upland forest ecosystems. As such, fingerprinting suggested that heterogeneity of the forested landscape increases β -diversity, because some degree of niche differentiation affects the distributions of Agaricomycotina species. These results are consistent with previous gradient analyses of the ectomycorrhizal community – environment relationship, despite differences in methodology (Nantel & Neumann 1992; DeBellis *et al.* 2006). Yet, the relationship between community composition and forest ecosystem characteristics accounted for only a small proportion (<10%) of the variance in species distributions. Conceivably, methodological limitations of the TRFLP approach might mask the true strength of the relationships between species distributions and forest ecosystem characteristics (Dickie & FitzJohn 2007). In this study, we employed TRFLP in what has been termed a ‘peak – profiling TRFLP’ approach (Dickie & FitzJohn 2007). The effec-

tiveness of peak-profiling depends on primer selectivity and on the assumption that each unique TRF peak represents a distinct species (Dickie & FitzJohn 2007). The ITS1F-LR21 primer pair is not selective for Agaricomycotina; yet our clone library results indicate that only ~13% of sequences amplified with ITS1F-LR21 are from fungal groups outside the Agaricomycotina. The assumption that each TRF represents a distinct species is also known not to be true (Edwards & Turco 2005; Avis *et al.* 2006). *In silico* digests of the 139 OTU recovered in this study suggest that *Hae*III digests were ~90% effective in resolving rDNA ITS-28S genotypes defined at the 97% similarity level (Edwards, unpublished data). Moreover the pattern of TRF distribution between forest stands parallel that of ITS1F-LR21 OTU distribution (Fig. S2 Supporting information). Therefore, we believe that the TRFLP data presented here are a reasonable approximation of species distributions across this landscape. Given this, the comparatively low

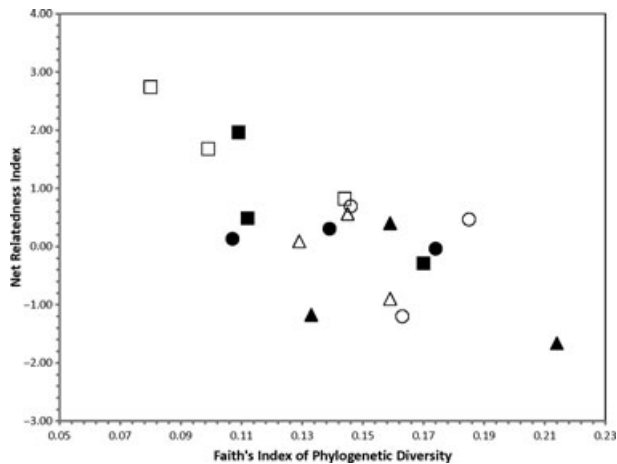


Fig. 4 Relationship between phylogenetic diversity and net relatedness index in the soil and forest floor Agaricomycotina communities of three temperate upland forests. Soil communities; open symbols. Forest floor communities; closed symbols. BOWO, squares; SMRO, circles, SMBW, triangles.

explanatory value of ecosystem characteristics was more likely due to the high proportion of singletons in the data, even though their influence was down-weighted in our analysis. However, a recent study in which singletons were excluded from gradient analysis assigned a similarly low explanatory value to forest type (DeBellis *et al.* 2006). Forest characteristics clearly define some portion of the β -niche of many Agaricomycotina, although the distribution of most species is too patchy for strong ecological inference.

Are the Agaricomycotina communities of temperate forest ecosystems phylogenetically distinct?

In addition to being species rich, fungal communities are often phylogenetically diverse. In these ecosystems, 461 rDNA clones recovered 139 species from >50 genera in at least 15 orders of Agaricomycotina. In comparison, using similar approaches to cloning and species definitions, Porter *et al.* (2008) recovered species from 17 orders in a study of hemlock-dominated forests and Lynch & Thorn (2006) found species from 11–12 orders in a study of grass and agricultural lands. Despite a combination of broad taxonomic range, limited representation at finer taxonomic scales (i.e. within families and genera) and limited resolution and support for phylogenetically defined clades (Supporting information Fig. S3), our results revealed significant differences between the forest floor and soil communities, and moreover, between the soil communities (Fig. 3).

UniFrac measures the distance between communities as the fraction of the total branch length of a phylogenetic

tree that is unique to each environment; the assumption is that if membership of a community requires lineage-specific adaptations, then that community should share less branch length with other communities than if membership to all communities was open (Lozupone & Knight 2005; Cavender-Bares *et al.* 2009). The significant UniFrac differences that we observed between forest floor and soil communities parallel the TRFLP results and reflect differential distribution of Agaricomycotina lineages at various taxonomic levels. Tremellomycetes generally were preferentially recovered from the forest floor, whereas within the Agaricomycetes, *Sistotrema* (Cantharellales), *Mycena* (Agaricales), and *Clitocybe* (Agaricales) were exclusive to forest floor. In contrast, Sebaciniales, *Cortinarius* (Agaricales), *Russula* (Russulales), *Thelephora* (Thelephorales), *Piloderma* (Atheliales) *Trechispora* (Trechisporales), *Collybia/Gymnopus* (Agaricales) and the Clavariaceae (Agaricales) were exclusive to the soil horizon. To some extent, this stratification reflects a life strategy change from saprotrophic in the forest floor to mycorrhizal in the fine root rich soil horizon. However, the extent to which mycorrhizal species dominate community diversity in temperate soil horizons can differ considerably, and in this study ranged from >90% to <40% (Supplementary material Fig. 4). Preferential distributions of saprotrophic lineages such as the Clavariaceae also drive the clear phylogenetic differences between forest floor and soil communities. Although we found a significant difference between the forest floor and soil communities, we found no significant phylogenetic differences between the forest floor communities of the three ecosystems, although they did appear to differ slightly in phylogenetic diversity (Table 2, Fig. 4). Moreover, forest floor communities showed the greatest degree of species overlap (Fig. 2). Our results suggest that differences in forest floor biochemistry between temperate hardwood ecosystems (Table 1) exert a comparatively small influence on saprotrophic Agaricomycotina community composition and β -diversity.

In comparison with the forest floor communities, we observed greater differences between the Agaricomycotina communities of the soil horizon. Specifically, the community of BOWO, which was dominated by mycorrhizal species, differed significantly from the communities of both SMRO and SMBW. This difference is clearly driven by the phylogenetic differences between an ectomycorrhizal *Cortinarius* – *Russula* dominated community and less mycorrhizal dominated communities. We also observed a marginally significant phylogenetic difference between SMRO and SMBW, and this appears to reflect the differential distribution of ectomycorrhizal *Quercus* associated species and of putatively endomycorrhizal *Sebacina* species.

Might temperate forest ecosystems act as habitat filters?

Despite evidence of species- and lineage-level sorting between the three ecosystems, generally the Agaricomycotina communities exhibited weak phylogenetic structure. Generally, local species assemblages can be seen as subsets of a larger species pool (Kraft *et al.* 2007). In comparison with this larger pool, the species in local assemblages may be more closely related than expected by chance (clustered), less closely related than expected by chance (overdispersed), or exhibit no phylogenetic structure (random). Interpretation of these patterns in terms of ecological process requires knowledge of the evolutionary history of life strategy/ecophysiological traits (Webb *et al.* 2002; Cavender-Bares *et al.* 2004; Kraft *et al.* 2007). For example, if different ecosystems favour different traits, and if these traits (β -niche traits, *sensu* Silvertown *et al.* 2006) evolve conservatively, then communities should exhibit little phylogenetic similarity and strong phylogenetic clustering (Lozupone & Knight 2005; Cavender-Bares *et al.* 2006; Kraft *et al.* 2007). Conversely, if ecological traits generally are conserved and habitat filtering is less important than limiting similarity, the communities of distinct ecosystems may have considerable phylogenetic overlap and even be phylogenetically overdispersed. However, if β -niche traits are convergent, strong habitat filtering should also lead to phylogenetic overlap and phylogenetic overdispersion, while strong limiting similarity leads to phylogenetically random or even possibly clustered patterns (Cavender-Bares *et al.* 2004; Kraft *et al.* 2007).

We observed significant phylogenetic structure only in the Agaricomycete – dominated forest floor and soil horizon communities of the BOWO ecosystem, in which phylogenetic clustering indicated that species in this community were more closely related than expected by chance (Table 3). While phylogenetic studies of microorganisms remain rare (Vamosi *et al.* 2009), bacterial communities have been found to be phylogenetically clustered in a range of environments, and this has been interpreted as evidence of habitat filtering, even though trait information is lacking (Horner-Devine & Bohannan 2006; Bryant *et al.* 2008). Detailed trait information similar to that available for plants (Ackerly 2003) is lacking for fungi, and in the absence of such data, conclusions based on phylogenetic structure alone must be considered speculative. Mutualism is often a conserved generic trait and is believed to have multiple independent origins within the Agaricomycotina (Hibbett *et al.* 2000). Mutualism is clearly an important trait when considering successful colonization of the fine-root rich soil horizon in the BOWO ecosystem, in which mycorrhizal species accounted for >90% of the community

(Table 1., Supplementary material), and evolutionary conservation of this life strategy within speciose genera, such as *Russula* and *Cortinarius*, largely explains the phylogenetic clustering of this community and suggests that this environment may act as a habitat filter. However, phylogenetic clustering was also observed in the Agaricomycete community colonizing the forest floor of the BOWO ecosystem; species from the saprotrophic *Mycena* and *Clitocybe* and the predominantly saprotrophic *Sistotrema* genera were found in this environment. Therefore, traits such as the ability to maintain growth during periods of low moisture availability may be more generally important to colonization of this ecosystem, and the degree to which such traits show evolutionary conservatism requires further study.

In contrast to the BOWO ecosystem, the Agaricomycotina communities of the two maple – dominated forest ecosystems were more phylogenetically diverse and exhibited no significant phylogenetic structure. Conceivably, this might reflect a high degree of convergent trait evolution and a community assembly process dominated by competitive interactions (Webb *et al.* 2002). More recently Kembel & Hubbell (2006) and Kraft *et al.* (2007) have proposed that random phylogenetic patterns may also result when density dependent and environmental filtering processes are balanced or weak, or when neutral processes dominate community assembly. Dispersal limitation and high local variability in spore rain have been shown to affect fungal community composition (Vasiliauskas *et al.* 2005; Peay *et al.* 2007) and may lead to a lack of phylogenetic signal, especially if species have similar ecological traits or if trait evolution is random or convergent.

Lack of phylogenetic signal may also reflect methodological limitations (Swenson *et al.*, 2009). In this study, neither spatial nor taxonomic scale exerted strong influence on the results, but both should probably be examined in more detail in future work. Although Unifrac found only a marginally significant difference in the SMRO and SMBW fungal communities, close inspection of the data reveals that, as described above, preferential and restricted distribution of some lineages (e.g. Cortinariaceae and Sebaciniales) characterize the soil communities of these two ecosystems. Therefore, future family or genus level studies may yet reveal evidence of phylogenetic structure. Sampling intensity also needs to be addressed in future fungal community phylogenetic studies. Based on our estimates of species richness, we may have recovered between 36 to 62% of species from these sites, although these numbers have to be considered cautiously as they do not account for ribosomal copy number variation or PCR bias. It is likely that further sampling would reveal more species, and moreover that sampling efficiency was lower in the more

diverse SMBW ecosystem. Finally, it is plausible that altering the scale of the reference community may lead to greater evidence of phylogenetic structure in the SMRO and SMBW ecosystems (Swenson *et al.*, 2006; Cavender-Bares *et al.* 2006). Here, the reference community was defined solely by the samples, and within this context, the BOWO communities appear phylogenetically clustered. A more extensive reference community which integrated species information from other northern temperate forest ecosystems might also reveal phylogenetic structure in the SMRO and SMBW ecosystems.

Conclusions

Our results demonstrate that the distributions of Agaricomycotina species across a heterogeneous forested landscape are sensitive to the floristic and edaphic characteristics of distinct forest ecosystems. Habitat heterogeneity is therefore an important factor when considering fungal β -diversity. Moreover, our results provide a first indication of landscape-scale phylogenetic sorting, whereby the variation in community composition between ecosystems reflects the preferential distributions of distinct evolutionary lineages within the Agaricomycotina. Phylogenetic sorting was especially clear between the forest floor and soil communities, where it was in part the result of an ecological split between saprotrophic and mycorrhizal communities. Phylogenetic sorting was also observed between communities colonizing the soil horizon of the three ecosystems, and appeared to reflect differences in host plant specialization between mycorrhizal groups such as *Cortinari* and *Sebacina*. There was no evidence of phylogenetic sorting in the forest floor communities. Habitat heterogeneity therefore appears to be an important factor when considering fungal 'phylo'- β -diversity (*sensu* Graham & Fine 2008).

Although we found evidence for phylogenetic sorting between forest floor and soil, and between soils, significant phylogenetic clustering was only observed in one of the three ecosystems that we examined, and generally phylogenetic structure was inversely correlated with phylogenetic diversity. As such, our results suggest that within at least some lineages in the Agaricomycotina, phylogenetic relatedness may be an indication of ecological similarity, at least at the β -niche scale. Moreover our results suggest that some forest ecosystems may act as habitat filters. Further progress in understanding the determinants of Agaricomycotina β -diversity and community assembly process requires greater understanding of the correlation between ecological similarity and phylogenetic relatedness (Losos 2008).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 rDNA ITS-28S genotypes from upland temperate forest ecosystems

Fig. S1 Collectors curves. Top: Chao 1; Bottom: Jackknife. ITS – derived curves are indicated by solid symbols, and 28S curves by open symbols.

Fig. S2 Comparison of the distribution of TRFP and clone library Operational Taxonomic Units across nine temperate upland forest stands. Top: forest floor communities; Bottom: soil communities.

Fig. S3 Phylogenetic placement (Neighbour-joining analysis with Kimura 2-parameter model of partial rDNA 28S sequences) of 139 Agaricomycotina operational taxonomic units (OTU) recovered from rDNA random clone libraries of the forest floor and soil of three contrasting upland forest ecosystems. Source of OTU (forest floor or soil) is indicated, and best BLAST match is listed in parentheses. *Morchella esculenta* and *Morchella elata* were used as the outgroup.

Fig. S4 Functional guild breakdown of basidiomycete rDNA ITS-28S operational taxonomic units in three temperate upland forest ecosystems in upper lower Michigan, USA. Data are expressed as percentage of OTU in the clone libraries representing each system. BOWO, black oak – white oak/*Vaccinium*, SMRO, sugar maple- red oak/*Maianthemum*, SMBW, Sugar maple basswood/*Osmorhiza*. Solid black, saprotrophs; hashed, ectomycorrhizal; grey, unclassified.

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