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Host Phylogenetic Relatedness and Soil Nutrients Shape Ectomycorrhizal Community Composition in Native and Exotic Pine Plantations

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Abstract: Exotic non-native *Pinus* species have been widely planted or become naturalized in many parts of the world. Pines rely on ectomycorrhizal (ECM) fungi mutualisms to overcome barriers to establishment, yet the degree to which host specificity and edaphic preferences influence ECM community composition remains poorly understood. In this study, we used high-throughput sequencing coupled with soil analyses to investigate the effect of host plant identity, spatial distance and edaphic factors on ECM community composition in young (30-year-old) native (*Pinus massoniana* Lamb.) and exotic (*Pinus elliottii* Engelm.) pine plantations in China. The ECM fungal communities comprised 43 species with the majority belonging to the Thelephoraceae and Russulaceae. Most species were found associated with both host trees while certain native ECM taxa (*Suillus*) showed host specificity to the native *P. massoniana*. ECM fungi that are known to occur exclusively with *Pinus* (e.g., *Rhizopogon*) were uncommon. We found no significant effect of host identity on ECM communities, i.e., phylogenetically related pines shared similar ECM fungal communities. Instead, ECM fungal community composition was strongly influenced by site-specific abiotic factors and dispersal. These findings reinforce the idea that taxonomic relatedness might be a factor promoting ECM colonization in exotic pines but that shifts in ECM communities may also be context-dependent.

Keywords: ectomycorrhizal fungi; host origin; host specificity; *Pinus massoniana*; *Pinus elliottii*

1. Introduction

Plantation forestry has been widely used to accelerate reforestation, offset greenhouse gas emissions, and relieve timber demands from native forests [1]. Fast-growing and high-yielding exotic tree species such as *Pinus* and *Eucalyptus* are intensively planted in monocultures and frequently introduced outside their natural range. However, a tree species planted outside its native range can have dramatic and long-lasting impacts on biogeochemical cycling (carbon, nitrogen), water and nutrient availability, and mycorrhizal communities [2,3]. Here, we focus on ectomycorrhizal (ECM) fungi, the ubiquitous symbiont of many forest trees, and *Pinus* (pine), the most widely planted plantation tree [4].

Pines are obligately dependent on symbioses with ECM fungi for nutrient uptake, growth and plant survival, and ECM are therefore important components of forest management. More than 200 species of ECM fungi representing 54 genera have been intentionally introduced in the roots of pine seedlings during the development of large-scale plantations [5]. This co-introduction has facilitated

a massive range expansion of the ECM–*Pinus* mutualism, a situation that is widely compared to co-invasion (or 'enemy escape' [4,6–9]). Exotic pines may also form novel symbioses with native ECM fungi or cosmopolitan mutualists [10]. Nevertheless, plants may be limited by the availability of compatible ECM fungal inoculum. Exotic pine ECM communities are remarkable for their low species richness (<50 taxa) in comparison to native ECM forests [11], and an abundance of ECM taxa that are almost exclusively associated with *Pinaceae* (e.g., *Suillus*, *Rhizopogon* [12,13]).

Such low-diversity limitations may reflect both neutral (dispersal) and niche processes (abiotic factors; e.g., [14–17]). Studies show that longer distances (>10 km) may limit the stochastic dispersal of fungal propagules from one location (native forest) to another (plantation) whereas deterministic traits such as dispersal via spores [14,18] versus mycorrhizal root tips and hyphal networks may operate at finer scales [19–22]. In addition, biotic (e.g., host nutrient demands, seed dispersal) and abiotic factors (e.g., soil chemistry) may facilitate ECM fungal species with physiological and ecological adaptations depending on the environmental context [23].

Recent evidence has demonstrated that phylogenetic distance between exotic and native hosts might explain their (dis)similarities in ECM community composition and richness [24,25]. For example, studies have shown that co-occurring exotic pine and native trees host similar ECM fungal communities and share the same dominant ECM fungal species [8,10,26–28]. Most ECM fungal species were also host generalists and, as a result, species-specific ECM taxa were rare [29,30]. Conversely, other studies show that hosts in different subgenera (e.g., hard pines vs. soft pines) better explained the covariation between exotic and native pines and their ECM communities [10,25]. Thus, a major challenge is to disentangle the effects of host phylogeny from environmental covariation on ECM-host interactions.

Here, we examined the influence of phylogenetic relatedness, distance, and environmental factors on ECM communities in plantations of exotic slash pine (*Pinus elliottii* Engelm.) adjacent to native *Pinus massoniana* Lamb. (masson pine) at two sites. Our study sites were selected to represent forests with inherent differences in soil N and P fertility. Both plantations were located in southern China. Plantations comprise ~69 million hectares of forest in China, or ~36% of the total forest area [31]. Masson pine is a slow-growing, dominant native species in native forests in subtropical China. Slash pine was introduced from the southeastern U.S. during reforestation efforts in the 1920s [32], and is now one of the most popular plantation timber species due to its high-quality timber, fast growth, and resistance to insect damage [33]. The standardized forestry practices (e.g., thinning) used across both study sites makes this a model system in which to examine the effects of native versus exotic pines on ECM communities. We used high-throughput sequencing analyses of ECM communities coupled with analyses of soil physicochemical properties to address two questions:

(1) Do closely related exotic and native host species support similar ECM communities?
(2) To what extent do neutral (dispersal) versus niche (abiotic factors) shape ECM assemblages in exotic and native pines?

2. Materials and Methods

2.1. Study Sites

The study was carried out at two forest areas of south China, Longli Forest Farm, Guizhou Province (LFF, 26°45′ N, 106°45′ E), and Hunan Botanic Garden, Hunan Province (HBG, 28°60′ N, 113°20′ E) (Figure S1). LFF has a subtropical humid climate characterized by abundant precipitation and mild temperatures; mean annual precipitation is 1077 mm and average temperatures is 15 °C. The soil is a well-drained sandy-loam yellow soil developed from quartz sandstone parent rock, classified as *Hapli-Udic Ferrosols* according to the Chinese Soil Taxonomic Classification System (CSTC [34]). In HBG, the climate is typical mid-range subtropical monsoonal with mean annual precipitation of 1422 mm and annual air temperature of 17.2 °C. The soil is a well-drained clay-loam red soil developed from slate parent rock, classified as *Alliti-Udic Ferrosols* by the CSTC. Both masson

pines and slash pine plantations were planted in 1988 as monocultures after clear-cutting of mixed evergreen broadleaved (Fagaceae)-conifer mixed forests (masson pines).

In each site and forest type, two (HBG) or three (MA) 20 × 20 m plots were established at least 50 m apart; the small stand size in HBG limited the number of plots possible (Figure S1). Within each plot, five randomly selected mature pine trees (DBH 15–20 cm) separated by at least 4 m were selected as 'island' trees.

2.2. Sampling Methods

We sampled the ECM community in each site and tree species using a combination of sporocarp surveys (collected monthly since August 2015) and hyphal in-growth bags. In-growth bags were made of anti-static polyester fabric with 50 µm diameter pores and filled with sand (0.6~1.8 mm) that had been washed with 5% (*v*/*v*) HCl and 10% (*v*/*v*) H_2O_2, thoroughly rinsed with deionized water, and dried. Such bags allow for efficient, well-replicated community sampling of active ECM fungi hyphae growing in soil [35,36]. In July 2016, we planted three 5 × 5 cm mesh bags under each island tree. Bags were buried 45 cm from the base of the tree along one of the four cardinal directions, and 5 cm below the soil surface (A horizon). Additional bags containing sand were stored in the laboratory as negative controls. All bags were harvested in October 2016 and transported to the laboratory over ice. At harvest, soil cores (5 cm diameter × 10 cm deep) were collected next to each buried bag. Soil cores were homogenized and pooled by each 'island' tree, sieved to 2-mm to remove gravel and coarse organic matter, and used for soil physical and chemical analyses.

2.3. Soil Physicochemical Analysis

Soil pH was determined in a soil suspension with soil: water ratio of 1:2.5 (*w*/*v*) using a Delta 320 pH-meter (Melter-Toledo Instruments Co., Shanghai, China). Soil texture was determined by the hydrometer method for estimating particle size. Soil ammonium (NH_4-N), nitrate (NO_3-N), and phosphate (PO_4) were extracted with 2M KCl and measured using a continuous flow analyzer (SAN++, Skakar, Breda, Holland). Available potassium (K) was determined on ammonium acetate extraction by flame photometry (Model FP6410; Jingke Ltd. Co. Shanghai, China). Total N was measured using Semi-micro-Kjedahl digestion with a mixture of H_2SO_4, K_2SO_4, $CuSO_4$ and Se. Additional soil samples were digested with HNO_3: HCl (1:3), and analyzed for total P using a molybdenum colorimetric method and macro- or micronutrients (Ca, Mg, Fe, Cu, Pb, Zn, Mn) using atomic absorption spectrophotometry (Mode AA-7000; Shimadzu Corp. Nakagyo-ku, Kyoto, Japan). Soil samples for organic matter analysis were oven-dried at 105 °C, milled and sieved through 0.25 mm mesh and analyzed by wet combustion method (Walkley-Black procedure) with potassium dichromate.

2.4. Molecular Identification of Ectomycorrhizal Fungal Community

Fungal hyphae were obtained from the mesh bag following [36]. Briefly, each bag was emptied into a sterile 50 mL centrifuge tube, 10 mL of sterile deionized water was added and each tube was vortexed for 2 min. After a 5 min settling period, the top 2 mL of suspension was transferred to a new, sterile 2 mL centrifuge tube and the contents pelleted via centrifugation. Hyphal pellets were pooled by 'island tree', which yielded 55 samples from forest types across both the LFF and HBG sites. We also extracted DNA from 18 different ECM sporocarps that had been collected opportunistically from both sites. These extracts were only used to create a positive control (or mock ECM community) comprising equimolar aliquots of genomic DNA from each fungal species (Table S2). We did not include these fungi in analyses of masson and slash pine ECM communities because sporocarp sampling was infrequent, and local farmers collect edible ECM sporocarps (e.g., *Lactarius*) in pine plantations.

DNA from sample mesh bags and negative controls (bags stored in the laboratory) was extracted using Mobio PowerSoil kit (Hercules, CA, USA) following the manufacturer's instructions for maximum DNA yield. The ITS1 region of the fungal rDNA subunit was amplified by polymerase chain reaction (PCR) (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C

for 30 s and a final extension at 72 °C for 5 min) using primers ITS1F and ITS2 [37]. PCR reactions were performed in triplicate—20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using QuantiFluor™-ST (Promega, Fitchburg, WI, USA). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform according to the standard protocols at Majorbio Technic Group Laboratory, Shanghai, China.

Raw Illumina fastq files were demultiplexed and quality-filtered using QIIME v1.17 [38] with the following criteria: reads were truncated at any site receiving an average quality score of <20 over a 50-bp sliding window; truncated reads shorter than 50 bp were discarded; any read containing one or more ambiguous base calls was discarded; only sequences that overlapped by more than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded. The ITS1 sub-region was then extracted from each sequence using FungalITSextractor (http://emeren-cia.org/FungalITSextractor.html) [39]. Operational units (OTUs) were initially clustered with 97% similarity cutoff by USEARCH v7 [40] and followed by de novo chimera checking using the UNITE-QIIME reference (http://unite.ut.ee/repository.php) [41]. All global singletons were removed to avoid data artifacts [42,43]. Taxonomy was assigned using NCBI BLAST+ v2.2.29 [44] and a custom database compiled from sequences in UNITE v7 and vouchered mushrooms collected from the field sites. The mycorrhizal status of OTUs was tentatively assigned using the 'FUNGuild' algorithm [45] and checked manually [46].

After bioinformatic processing, we first calibrated our clustering threshold [47] and cut-off for low abundance OTUs [48]. To do so, we examined and compared the number and abundance of OTUs recovered after sequencing versus the original composition of the mock ECM community (Table S2). Using 97% clustering threshold resulted in the recovery of 22 species out of the 18 species. Adjusting the clustering threshold to 96.8% (de novo UCLUST) resulted in the recovery of the correct number of species (Table S2). Analyses also showed that four sequence reads (OTU read abundance $\geq$4) was the lowest cut-off that was not significantly different from a 10-sequence threshold ($p > 0.05$; pairwise Wilcoxon rank-sum tests). We used this value as a conservative threshold for taxon inclusion in the species richness analysis.

DNA sequences in this study have been deposited in Sequence Read Archive of the NCBI database under accession number SRP113701.

2.5. Statistical Analyses

All statistical analyses were conducted in R v3.3.2 (R Foundation for Statistical Computing; available at http://www.R-project.org) with indicated packages.

Prior to all tests, read abundances were normalized using the 'metagenomeSeq' package [49], since rarefaction may reduce the sensitivity of analyses [50,51]. Soil properties were standardized using scale transformation (function 'scale' in R). Data sets were then checked for multi-collinearity among variables using the variation inflation factor (VIF) function of the 'FMSB' package, and highly correlated variables removed (variation inflation factor scores >5: C, K, Cu, Fe, % sand and clay).

We calculated ECM species richness (S), evenness (Shannon–Wiener index), estimated species richness (Chao1 richness), and species accumulation curves (Mao Tau) for each sample. Differences in species richness and evenness between host tree species and sites were analyzed using two-way analysis of variance (ANOVA) with post-hoc Tukey Honestly Significant Difference (HSD) tests for significant variables. Indicator species analysis was used to identify OTUs unique to each host species and site ('indicspecies' [52]). This analysis was based on presence-absence of each ECM fungal OTU in each 'focal' tree. The indicator values were group-equalized and their statistical significance was tested by a randomization procedure with 999 permutations. Heat maps and network-based analyses (Cytoscape 3.7.0 [53]) were used to visualize the number of ECM species that were cosmopolitan

(present in all forest types), specific to a host species or site (i.e., only detected in one forest type) or broadly dispersed (found in two or three sites).

Next, an ECM community distance matrix (Bray–Curtis dissimilarity, OTU abundances) and environmental matrix (Euclidean dissimilarity, spatial distance and soil properties) were constructed. We tested for differences in ECM community and soil properties between sampling sites and host species using permutational multivariate analysis of variance (PERMANOVA; adonis function in vegan, 999 permutations), and multivariate homogeneity of group dispersions (betadisper function in vegan). Patterns of ECM community dissimilarity were visualized using non-metric multidimensional scaling (NMDS). Differences in community structure were compared visually using centroids and 95% confidence intervals associated with a χ^2-distribution around the standard error of the centroid. To disentangle the relative importance of soil factors controlling ECM community composition, we identified and fitted significant environmental vectors to the NMDS (envfit function in vegan; 999 permutations). Mantel tests were used to directly compare geographic distances with community composition differences (mantel.rtest in ade4 package) and to determine differences in spatial scaling between sites (mgram in ecodist package). These analyses were run using both abundance-based and presence–absence ECM community data. Both analyses showed similar results (data not shown), and only the abundance-based data are presented.

3. Results

Our initial dataset included a total of 2,921,761 raw sequence reads. After quality filtering, there were 1,992,789 sequences for downstream analysis, with an average of 37,060 (±12,664 standard error (SE)) reads per sample. These sequences clustered to 403 OTUs. Eighty-percent of the sequences (1,593,614) were identified as putative ECM fungal species.

The ECM community comprised a broad range of phylogenetic lineages. A total of 43 ECM fungal species were assigned to 16 genera, many of which were native to China. The most abundant taxa were *Russula* and *Tomentella* with 10 OTUs each (Table 1; Figure 1). The majority of ECM species were either cosmopolitan (14%) and found in all sites with both tree species (e.g., *Cenococcum*) or broadly dispersed across sites and/or species (63%; e.g., *Tylospora*, *Clavulina*; Table 1; Figures 1 and 2). In contrast, *Suillus* OTU_963 and *Lactarius* OTU_984 were specific to masson pine in both LFF and HBG while ECM specific to slash pine were site dependent, e.g., *Lactifluus* in LFF and *Chloridium* OTU_349 in HBG. Further, fewer fungal taxa were indicator species in masson pine than slash pine (IndVal; Table 1). Analyses showed that ECM fungi of other native pines (e.g., *P. yunnanensis*) were indicator species of masson pine. In contrast, a variety of ECM were indicator species in slash pine, including taxa that were best matched to pines in North America (*Clavulina*) and Eastern Europe (*Pseudotomentella*; Table 1). We also found significant differences in ECM community between sites (Figures 1 and 2), whereby 44% of ECM fungal species were specific to HBG or LFF. Of the 43 ECM species identified in the study, 16 were recovered only in LFF, most of which were species of *Pseudotomentella* (2 species), *Tomentella* (6 species), or *Russula* (5 species). Conversely, only three ECM were restricted to HBG: *Laccaria* OTU_1219, *Chloridium* OTU_349, and *Russula* OTU_1000.

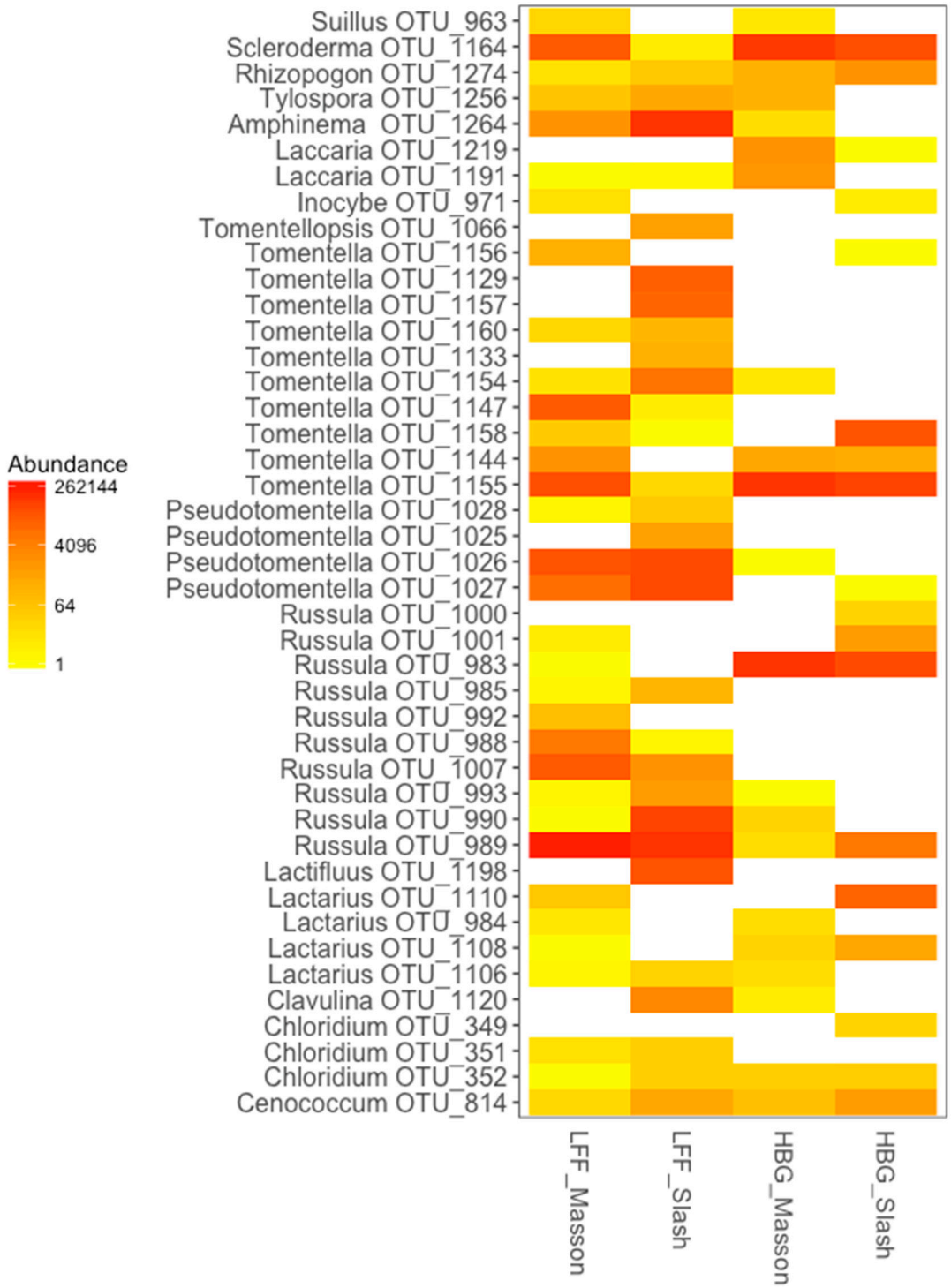

Figure 1. Ectomycorrhizal fungal operational units (OTUs) showing sequence read abundance across sites for each pine host. LFF—Longli Forest Farm; HBG—Hunan Botanic Garden.

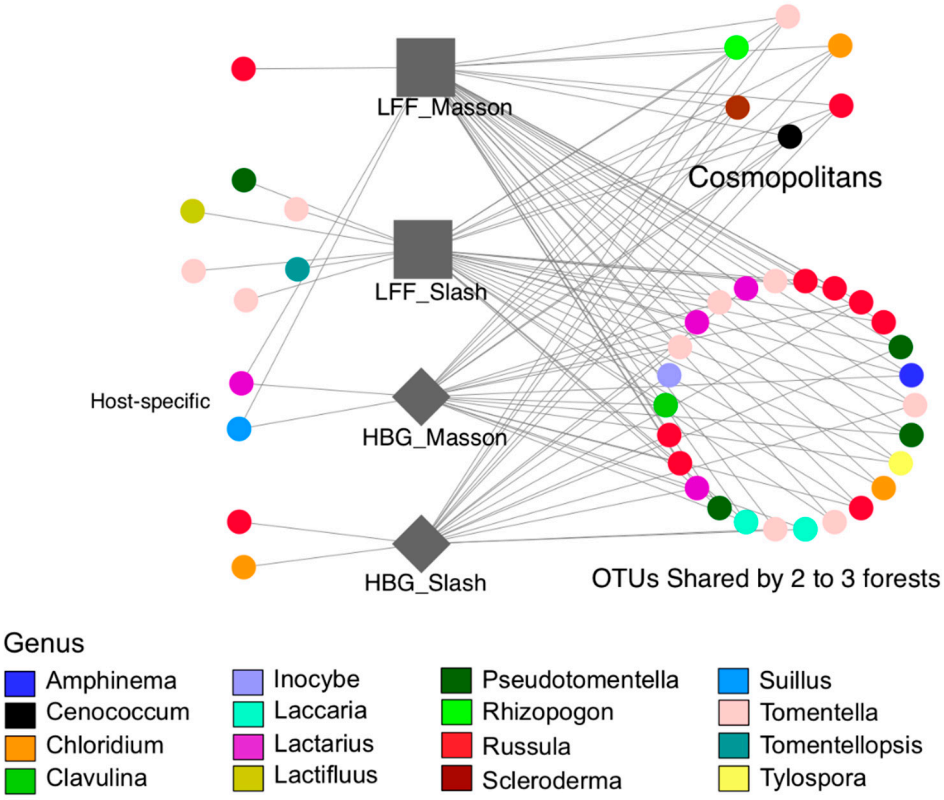

Figure 2. A network map showing the interactions of the OTUs among all the samples from different hosts at each site. Each point represents one independent fungal OTU (43 OTUs in total). OTUs in the left column were unique to one forest type, while those in the right belonged to multiple forest types. LFF—Longli Forest Farm; HBG—Hunan Botanic Garden.

Rarefaction curves showed that the number of ECM fungal species was higher in LFF than HBG. However, species richness did not reach saturation, suggesting that additional ECM species could be added with further sampling (Figure 3). *Host* × *Site* interactions influenced both observed and estimated ECM fungal richness (Table 2). Species richness was significantly higher in slash than masson pine at LFF whereas there was no significant difference in richness between pine species at HBG. Species evenness was significantly higher in slash than masson pines (Table 2).

Table 1. Indicator species analyses. The table shows the number of ectomycorrhizal (ECM) fungal molecular taxa (OTU) significantly associated with each habitat type, their indicator species value [52] and associated probability. Based on species hypotheses, the distribution area and possible host species was assigned by closest similar representative sequence data in UNITE. MA-masson pine, SL-slash pine.

Site	Species Name	OTU No.	Indicate Value	*p*-Value	Closest BLAST Match in Genbank	Closest UNITE Species Hypothesis Match	Distribution [1]	Host of *Pinus*
Longli Forest Farm, LFF								
MA	*Russula virescens*	OTU_988	0.683	0.003	KU552087 (100)	SH179772.07FU	China	not indicated
	Russula violeipes	OTU_1007	0.619	0.012	LT201954 (100)	SH191296.07FU	Yunnan (China)	*P. yunnanensis*
	Russula.sp1	OTU_992	0.447	0.05	LT602950 (97)	SH218430.07FU	Yunnan (China)	*P. yunnanensis*
SL	*Amphiema.sp*	OTU_1264	0.961	0.001	LC176645 (100)	SH193510.07FU	China	*P. massoniana*
	Tomentella stuposa	OTU_1154	0.856	0.001	UDB024437 (100)	SH529807.07FU	Laos	not indicated
	Clavulina corralloides	OTU_1120	0.816	0.001	KF359593 (94)	SH220215.07FU	North America	*P. banksiana*
	Russula sp.2	OTU_990	0.774	0.001	AB636419 (93)	SH218466.07FU	Korea	*P. koraiensis*
	Russula sp.3	OTU_993	0.682	0.001	AB211253 (95)	SH186553.07FU	Japan	*P. densiflora*
	Tomentella sp.1	OTU_1157	0.632	0.001	UDB018462 (97)	SH010050.07FU	China	not indicated
	Tomentella sp.2	OTU_1129	0.632	0.002	UDB018462 (99)	SH010050.07FU	China	not indicated
	Pseudotomentella sp1	OTU_1027	0.628	0.011	AB839386 (100)	SH223400.07FU	East Europe	*P. sylvestris*
	Pseudotomentella sp2	OTU_1025	0.577	0.011	AB587791 (100)	SH189639.07FU	China	*P. massoniana*
	Tomentella.sp.3	OTU_1133	0.516	0.011	AB587791 (100)	SH189639.07FU	Japan, Korea	*P. thunbergii*
Site	*Russula sp.4*	OTU_989	0.941	0.001	AB211253 (99)	SH186553.07FU	Japan	not indicated
	Pseudotomentella sp.3	OTU_1026	0.913	0.001	AB839386 (100)	SH223400.07FU	East Europe	*P. sylvestris*
Hunan Botanic Garden, HBG								
MA	*Russula sp.5*	OTU_983	0.785	0.001	AB839393 (100)	SH201481.07FU	South China	*P. massoniana*
	Laccaria amethystina	OTU_1219	0.548	0.015	KF692988 (100)	SH220964.07FU	China, Japan	*P. densiflora*
	Laccaria aurantia	OTU_1191	0.547	0.03	KU685645 (99)	SH179274.07FU	Yunnan (China)	*P. yunnanensis*
SL	*Russula sp.6*	OTU_1000	0.577	0.001	UDB032527 (100)	SH189355.07FU	Laos	not indicated
	Tomentella.sp.4	OTU_1158	0.577	0.044	AB587783 (100)	SH189355.07FU	a.w.	multiple
	Russula sp.7	OTU_1001	0.576	0.006	KP866130 (99)	SH199912.07FU	Hunan (China)	*P. elliottii*
Site	*Tomentella sp.5*	OTU_1155	0.891	0.003	JX556209 (100)	SH189353.07FU	South China	not indicated
	Scleroderma sp.1	OTU_1164	0.772	0.006	KP866131 (100)	SH189277.07FU	Hunan (China)	*P. elliottii*

[1] a.w.-reprehensive sequences have wild distribution all over the world.

Table 2. Single-sample based observed operational taxonomic unit (OTU) richness, Chao1, and Shannon-Wiener indices for ECM fungal communities from all forest types across the sites. Different letters indicate significant differences among forest types based on Tukey Honestly Significant Difference (HSD) post-hoc test ($p < 0.05$).

	Analysis of Variance (ANOVA) Test			**Mean (Standard Error) of Index**			
				Longli Forest Farm (LFF)		**Hunan Botanic Garden (HBG)**	
				Masson Pine (n = 15)	**Slash Pine (n = 15)**	**Masson Pine (n = 10)**	**Slash Pine (n = 15)**
Observed Richness		*F*	*P*	7.07 (0.62) b	10.33 (0.8) a	7.50 (1) ab	6.27 (0.53) b
	Host	2.11	0.152				
	Site	7.95	0.007				
	Interaction	9.31	0.004				
Chao1		*F*	*P*	8.13 (0.88) ab	11.32 (0.8) a	8.85 (1.6) ab	6.77 (0.71) b
	Host	0.43	0.513				
	Site	5.36	0.025				
	Interaction	7.51	0.008				
Shannon		*F*	*P*	0.47 (0.09) b	0.72 (0.1) a	0.30 (0.09) b	0.58 (0.12) a
	Host	5.49	0.023				
	Site	2.93	0.174				
	Interaction	0.02	0.899				

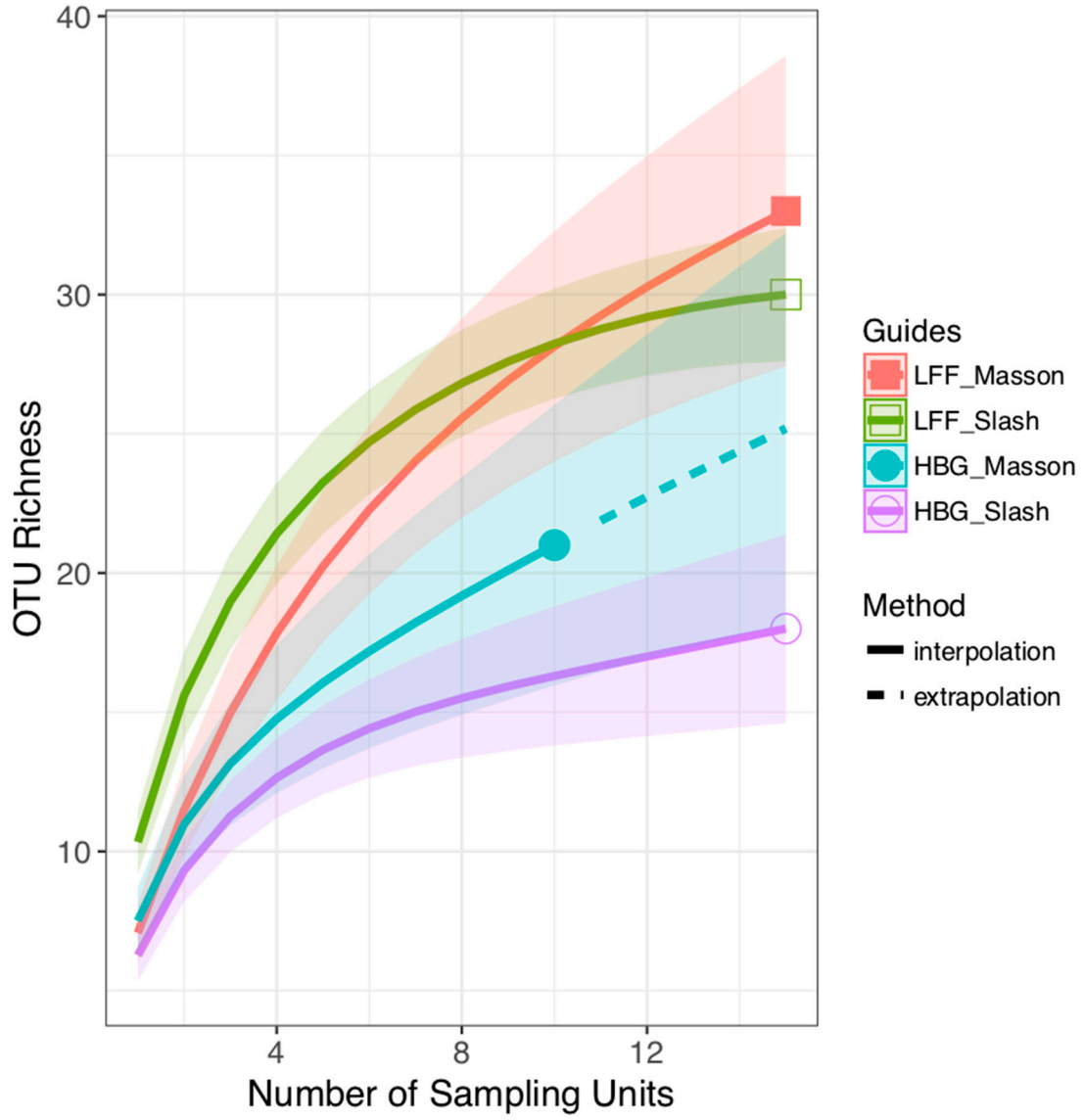

Figure 3. Sample-size-based species accumulation rarefaction (solid line segment) and extrapolation (dotted line segments) sampling curves with 95% confidence intervals (shaded areas) for estimated total ectomycorrhizal OTU richness across different host/sites. LFF—Longli Forest Farm; HBG—Hunan Botanic Garden.

NMDS ordination coupled with PERMANOVA analyses (Table 3) showed that ECM communities differed significantly between *Site* and *Host* (Figure 4). This distribution was nonrandom (PERMDISP; Table 3), and best explained by site (R^2 = 0.151, p = 0.001) rather than host plant (R^2 = 0.057, p < 0.02; Table 3). NMDS also showed strong influence of local soil factors on ECM community. Although soil factors varied significantly between *Site* and *Host* (Table 3; see supplementary Table S1), the largest effects were detected between sites (R^2 = 0.929, p = 0.001). In particular, levels of soil N (including N as NO_3), P, and K were higher in HBG than LFF whereas macronutrients (Ca, Mg, Mn) and silt levels were higher in LFF than HBG (Table S1). Within each site cluster, HBG ECM communities were more homogeneous than those in LFF (PERMDISP; Table 3).

Table 3. PERMANOVA analyses performed to test the differences in ECM fungal community and soil properties among forest types. Post hoc PERMDISP pair-wise analysis tests were applied to data from all forest types across the sites. Values represent the site mean with standard error in parentheses. Different letters indicate significant differences among forest types based on Tukey HSD post-hoc test ($p < 0.05$).

		PERMANOVA Test			PERMDISP Pair-Wise Test			
					Longli Forest Farm (LFF)		Hunan Botanic Garden (HBG)	
					Masson Pine	Slash Pine	Masson Pine	Slash Pine
ECM fungal community		*F*	*P*	R^2				
	Host	3.89	0.002	0.057	0.42 (0.05) c	0.57 (0.03) b	0.51 (0.03) ab	0.64 (0.01) a
	Site	10.26	0.001	0.151				
	Interaction	2.94	0.004	0.043				
Soil nutrient status		*F*	*P*	R^2				
	Host	9.66	0.007	0.012	0.04 (0.01)	0.05 (0.01)	0.08 (0.02)	0.04 (0.01)
	Site	828.02	0.001	0.929				
	Interaction	2.5	0.101	0.003				

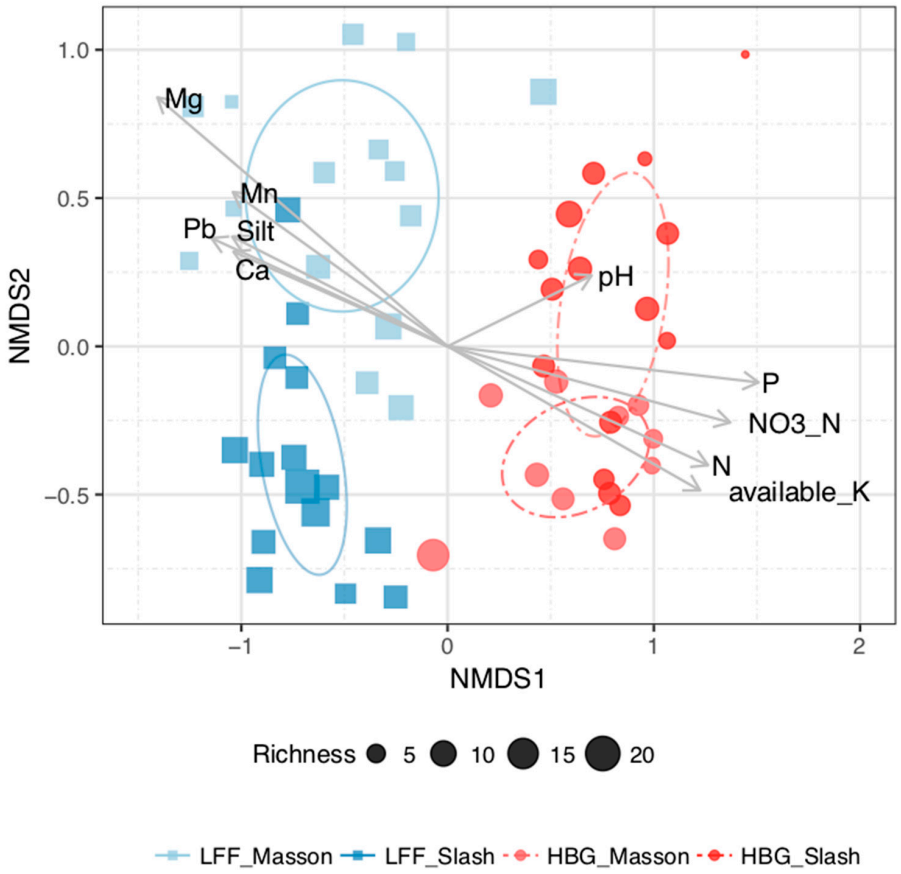

Figure 4. Non-metric multidimensional scaling (NMDS) of ectomycorrhizal fungal community composition. Point size reflects the richness of each sample. Significant abiotic variables ($p < 0.05$) were fitted as vectors onto the NMDS graph. LFF—Longli Forest Farm; HBG—Hunan Botanic Garden.

ECM communities scaled very differently between sites. In LFF, ECM communities were more similar than expected by chance within ~10 m (Figure 5). In HBG, ECM communities were also more similar than expected at ~10 m and 350 m but more dissimilar than expected at spatial scales ranging from ~80 m to 400 m (Figure 5). Within each site, however, host effects on ECM community and soil property scaling were not correlated (LFF Mantel test: $r = -0.0675$, $p = 0.63$; HBG Mantel test: $r = 0.0196$, $p = 0.73$, respectively).

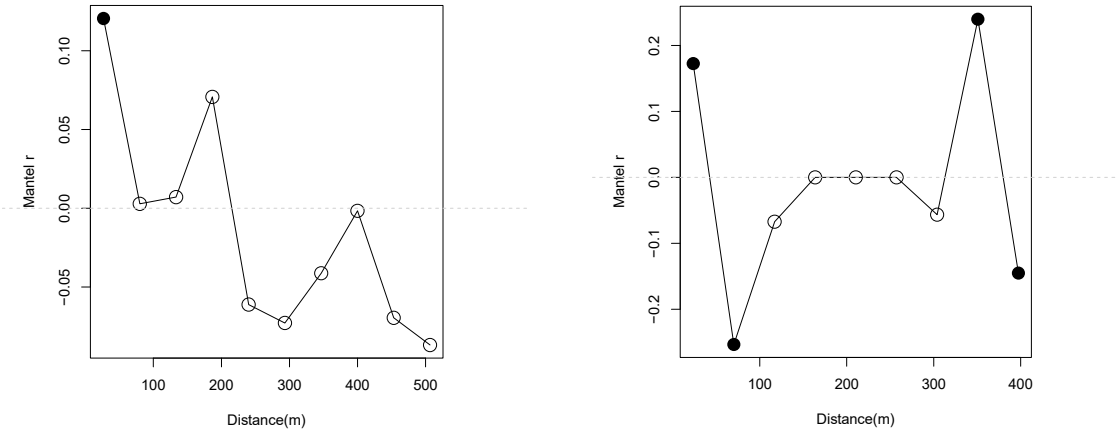

Figure 5. Mantel correlogram showing the strength of the correlation between spatial proximity and ectomycorrhizae for samples within different distance species composition classes in (**a**) Longli Forest Farm and (**b**) Hunan Botanic Garden. Points are plotted at the midpoint of each distance class. Solid circles represent significant correlations ($p < 0.05$), and open circles show correlations that are not significantly different from zero ($p > 0.05$).

4. Discussion

In this study, we systematically investigated the effect of host plant identity, distance and edaphic factors on ECM community composition in native and exotic pine plantations using hyphal ingrowth bags and high throughput sequencing. Although the hyphal ingrowth bag approach has its limitations (see [54]), we found no significant effect of plant identity on ECM communities, i.e., phylogenetically related exotic and native pines shared similar ECM communities (Q1). In the absence of a phylogenetic effect, we found that ECM community composition was influenced by site-specific abiotic factors and dispersal (Q2).

The few differences in ECM community between slash and masson pine (both subgenus *Pinus*) reinforces the idea that taxonomic relatedness might be a factor influencing ECM fungal colonization in exotic pines. Plant–ECM interactions are also believed to be conserved at the family level [55]. Analogous results have been noted in previous studies of ECM in native and exotic pines [8,10,24,26]. However, other studies have found that ECM host specialization contributes significantly to the composition of ECM communities [25,56]. Although the reason(s) for these opposing responses are not clear, they may reflect differences in the evolutionary co-adaptation between ECM fungi and their host [25,30], or differences in host range [57].

We found that exotic slash pine readily developed ECM with native fungi. Overall, the ECM community in exotic pine plantations was composed of a subset of the ECM fungi usually associated with native pines in their native ranges. Other studies have similarly shown a restricted suite of ECM species in exotic pine species (e.g., [12]). However, masson and slash pines exhibited very little systematic differences in ECM community composition and relative abundance of ECM species. Most of the recorded ECM fungi are considered cosmopolitan with the result that out of 43 ECM fungal taxa identified, 40 were associated with non-native slash pine (vs 35 in masson). Compositionally, members of the Thelephoraceae (e.g., *Tomentella*) emerged as the most species-rich, frequent and abundant genera on both pine species and sites. These taxa typically dominate ECM communities in many systems [58], form ECM with a wide range of plant host species, and exhibit broad ecological profiles [59,60]. Along with *Inocybe* and *Laccaria*, these taxa also form relatively long-lived spore banks that have the capacity to rapidly colonize roots of new plants [16]. This is important because it indicates that in plantations there is an ECM community that can immediately assist with reforestation.

Only a small number of ECM species displayed host specificity. Inspection of ECM communities shows that certain ECM fungi were specific to masson or slash pine. *Clavulina corralloides* (OTU_1120)

and *Pseudotomentella* (OTU_1025) were only found with slash pine. Sequence similarity shows that they are best matched with species of known North American (*Clavulina*) and Eastern Europe origin (*Pseudotomentella*). These species were likely co-introduced with slash pine. *Suillus* OTU_963- masson pine was an example of strict host specificity in both sites. Based on sequence similarity, this species is part of a distinct Asian *Suillus* clade [61], and its high masson pine- ECM specificity is consistent with patterns reported in other *Suillus*- pine associations [62]. The low abundance of *Rhizopogon* and *Suillus* in both pine species, however, was unexpected. These ECM genera are considered pine-specific [63] and abundant in pine roots in other ECM surveys (e.g., [20]). Yet, neither taxa were indicator species in masson or slash pine. These conflicting results may be due to the fact that *Rhizopogon* and *Suillus* are early successional taxa, and ECM fungal communities in 30 year-old plantation trees are not reflective of seedling ECM communities [64]. That *Rhizopogon* was equally abundant in both pine species and sites, however, supports the wide ecological amplitude of this taxon [65] and suggests that *Rhizopogon* has dispersed to these sites in the past and formed soil reservoirs of resistant spores that remain dormant in the soil for long periods [66].

We found that edaphic factors best explained the differences in ECM community composition between sites, not host specialization. Initially, we had expected ECM communities primarily to be structured by host plant identity. However, they were structured by differences in soil pH and nutrient levels between sites. Specifically, ECM communities in HBG were shaped by soil pH and macronutrient (N, P, K) levels whereas those in LFF were influenced by silt, Mn and base cations (Mg, Ca). These differences suggest that ECM fungi are limited by niche requirements (or abiotic tolerances) and that different ECM fungi may be available to plants depending on soil chemistry. This strong edaphic segregation is similar to previous large-scale ECM studies [14,67–69]. In addition, the correlation between soil N and P (and pH) and ECM community composition in HBG is consistent with previous work in forest systems showing a strong role for soil fertility (primarily N) in driving ECM community composition and richness (e.g., [70–74]).

More interestingly, the ECM community composition in LFF was correlated with soil Ca, Mg and Mn, and silt. In addition, many ECM species were detected in LFF but not HBG (e.g., *Tomentella*, *Russula*), which suggests that there are underlying physiological differences in ECM species that influence ECM community composition. Soil Mn levels are known to influence ECM fungi differentially [75]. Calcium and Mg are important plant macronutrients but have not been found to influence ECM community composition or favor certain ECM species in other studies [76]. The specific mechanism(s) driving this response is beyond the scope of this study. However, there are several possible processes that could generate this pattern: Soil Mn, Ca, and Mg availability is linked to soil pH, so that the response of ECM communities to these cations signals the effects of soil pH [77]. Plant morphology also varies with soil fertility meaning that major differences in root traits (root length, fine root density) could indirectly alter the ECM community [78]. Alternatively, the abiotic correlates of LFF and HBG ECM communities may simply reflect the idiosyncrasies in soil types that developed from two different parent materials.

We found consistent and strong spatial structuring of ECM populations in both study sites. Within each site, the highest spatial autocorrelation occurred at small scales (<5 m). This result lends support to dispersal limitation in our sites [79], and is in general agreement with the degree of spatial patterning found in temperate forests [21,30,66,80]. As a result, these ECM communities likely comprise individuals that are closely related or have similar environmental requirements. Against this backdrop, we found that ECM fungal taxa co-occurred less often than expected by chance (segregation) at ~80 and 400 m in HBG; a similar result was noted by Yamamoto et al. [81].

One possibility is that microhabitat differentiation inside HBG may have created a mosaic of environments that provided conditions for ECM fungi with varying environmental requirements [82]. Alternatively, there may be competition between ECM species [83] or between ECM and endophytic fungi for resources, such as host C [80]. These patterns may also be attributed to differences in ECM phenology or hyphal exploration networks. For example, ECM fungi with medium or long-distance

exploratory hyphae (e.g., *Tomentella*, *Tylospora*) might compete over larger distances so that there is open space for colonization by second and subsequent ECM species with contact- or short-range hyphae (*Russula*). Distinguishing between these possibilities is beyond the scope of this study but merits further investigation.

5. Conclusions

Our data sets provide important insights for separating the impact of host phylogenetic relatedness from abiotic and spatial factors on ECM community composition. Our results revealed that phylogenetic relatedness between pine trees resulted in a similar ECM community composition in both sites, and slash and masson pine were not limited by the availability of compatible ECM fungal inoculum. Instead, both dispersal and soil factors were stronger drivers of differences in ECM community composition between sites, meaning that ECM fungal communities in exotic pines are variable and context-dependent. In managed forests, improved productivity is reliant on belowground resource availability, uptake, and use efficiency, all of which are driven by ECM. Given the importance of soil and spatial factors in our study, there is now a need to better understand and align soil conditions with ECM fungi across a range of spatial scales, and identify how differences in ECM community functioning might feed back to influence host tree growth and productivity.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/3/263/s1, Figure S1: Map of sampling regime and sampling locations across the continental China. Circles represent individual sampling locations mapped on Google Map images. (A) Individual sampling plots are shown on a map of south China. (B) Individual plots 20 × 20 m within a sampling site are shown in Longli Forest Farm, Guizhou. (C) Individual plots 20 × 20 m within a sampling site are shown in Hunan Botanic Garden, Hunan. lat, latitude; lon, longitude, Table S1: Soil physico-chemical characteristics among different habitats, Table S2: Comparison of sequence abundances and operational taxomomic unit (OTU) counts in a mock community of 18 Basidiomycota species collected in local area.

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