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REGULAR ARTICLE

Differences in ectomycorrhizal community assembly between native and exotic pines are reflected in their enzymatic functional capacities

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Abstract

Background and Aims Introducing exotic tree species for afforestation out of their natural range may alter the local ectomycorrhizal (ECM) fungal communities. The potential functional consequences shaped by exotic trees with recruited local ECM fungi rather than native trees remain unclear. This study examined (a) whether the composition and extracellular enzyme function of ECM fungal communities differed between native masson pine (Pinus massoniana) and exotic slash pine (Pinus elliottii) during seedling establishment; and (b) how differences in enzyme functioning were linked to the growth pattern of the host plants.

Methods Native (masson) and exotic (slash) pine seedlings were planted into soil cores collected from each study site. At three months growth, root tips were collected from seedlings and assayed for ECM fungal community composition using high-throughput sequencing, and functioning using single root tip assays for enzymes associated with N, P and C acquisition.

Results ECM fungi on masson pines showed higher activities of nitrogen- (N-acetylglucosaminidase, 280–300%), phosphorus- (acid phosphatase, 105–152%), and cellulose (β-glucosidase, 204–235%; cellobiohydrolase, 142–255%) compound degrading enzymes compared to those on slash pines. Those differences was attributed to the host-specific performance of certain ECM fungal taxa, such as *Rhizopogon* spp. Information theory model selection showed that plant nutrient status in masson pines was correlated with the enzymatic contribution of *Rhizopogon spp.,* whereas slash pines depended on a diverse enzyme palette from multiple ECM fungal taxa.

Conclusions Host identity strongly influenced ECM fungal community composition and extracellular enzymatic functions of specific ECM fungal taxa, which could feedback to host establishment and nutrient cycling processes of restored ecosystem. Therefore, the origin of afforestation tree species should be an important factor when selecting tree species for restoration of degraded lands.

Keywords Community structure and function · Ectomycorrhizal fungi · Extracellular enzyme · Exotic pine · *Rhizopogon* · *Russula*

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Introduction

Exotic tree species have been widely introduced for afforestation due to their rapid growth rate, high

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productivity and local pathogen resistance (Richardson 1998). When a tree is planted outside its natural range, both local abiotic factors (e.g. soil physiochemical properties) and biotic factors (e.g. fitness with local community) can determine the success of establishment (Dodet and Collet 2012). Additionally, recent studies have highlighted the important contribution that belowground communities such as mycorrhizal symbionts may play in host plant health and adaptation to novel environmental conditions (Pringle et al. 2009). Therefore, understanding the relative effects of soil mycorrhizal communities on exotic trees establishment and persistence and their interactions in shaping those complex ecosystems are critical to sustainable forest management.

As one of the main belowground components, ectomycorrhizal (ECM) fungi form symbioses with host plants and play an essential role in uptake of nutrients (e.g. nitrogen (N) and phosphorus (P)) and water from soil to support plant growth in forest ecosystems (Smith and Read 2008). ECM fungi produce a diversity of extracellular oxidative and hydrolytic enzymes that mobilize and release smaller organic molecules from soil organic matter (SOM) (Courty et al. 2005). While ECM fungal communities are functionally similar in their roles as mycorrhizae, individual species can substantially differ in exoenzyme activity profiles (Courty et al. 2005; Maillard et al. 2018; Nicolás et al. 2019). For example, rhizomorph-forming ECM fungal species (e.g. species of *Cortinarius*, *Suillus, Rhizopogon*) have been found to secrete high levels of N-compound and cellulose degrading enzyme (e.g. N-acetylglucosaminidase, β-glucuronidase), and hence are typically abundant in nutrient limited soils (Leski et al. 2010; Lilleskov et al. 2011). Conversely, fungal taxa with short/contact hyphal morphology, such as *Russula* and *Tomentella* species, are generally found to secrete high levels of lignin-degrading enzyme (e.g. phenol oxidase (laccase)) and tend to access and assimilate inorganic nutrients more readily (Lilleskov et al. 2011; Jones et al. 2012; Ning et al. 2018; van der Linde et al. 2018). As exoenzymatic activity profiles of ECM fungal communities depend on their host plant nutrient status (reviewed in Hawkins et al. 2015), ECM fungal species with specific enzyme activities are selected by hosts to fulfill their nutrient requirements (Kipfer et al. 2012; Walker et al. 2014). Further, ECM fungi may respond to shortages in host carbon (C) allocation by enhancing the activity of enzymes to obtain labile carbohydrate from soil organic matter (Courty et al. 2010a; Hupperts et al. 2017; Akroume et al. 2019). These 'saprotrophic' capacities vary among ECM fungal species and depend on the quality of surrounding N resources, e.g. inorganic N level versus different organic N types (Maillard et al. 2018; Akroume et al. 2019; Nicolás et al. 2019). It follows that if the introduction of exotic tree species alters the ECM fungal community composition, the physiological functioning of their constituent ECM fungal species could feedback to impact nutrient cycling patterns in restored forest ecosystems.

Compatibility between ECM fungi and the host is also important for the successful establishment of seedlings. Recent studies have found that exotic ECM hosts may expediently adopt local fungal communities (Ishida et al. 2007; Tedersoo et al. 2007; Kohout et al. 2011; Trocha et al. 2012; Bahram et al. 2013), or rely on strictly co-evolved ECM fungal partners (Dickie et al. 2010; Hayward et al. 2015). The latter pattern was further explained by tree-fungal host specificity systems (e.g. pine-suilloid fungi in Liao et al. 2016), in which the host-specific fungal partner could confer sufficient advantage to the ECM symbiont (host plant) and thus overcome the cost of mutualism in low niche-requirement condition (e.g. seedling establishment) (Bever et al. 2009; Kiers et al. 2011; Peay 2016; Hortal et al. 2017). Although broad investigations show that ECM fungal communities respond strongly to edaphic and climatic variables, patterns of specificity are inferred by evidence at the plant species level (e.g. Rusca et al. 2006; Nguyen et al. 2016). Even at the plant intraspecific level, certain evolved traits, such as mutualistic benefits and preferential associations, are heritable features that permit the plant host to select ECM fungal partners (e.g. Hoeksema et al. 2012), and thus determine local adaptation processes and specialized ecological functions (e.g. drought tolerance, Gehring et al. 2017). Therefore, if a ECM fungal community is comprised of species that are highly restricted to a specific host and have highly specialized ecosystem function (e.g. enzyme activities), a shift in plant host could results in profound changes in the structure and function of niche-realized ECM fungal communities, and in turn impact plant nutrient uptake, forest structure and productivity. Conversely, if the native ECM fungal community can persist in the exotic tree restoration site with a high level of functional trait similarity (redundancy) or phenotypic plasticity, it may minimize potential changes in ecosystem structure and function (Konopka 2009; Rineau and Courty 2011).

In southern China, a large portion of climax vegetation of evergreen broadleaved forests has been destroyed due to anthropogenic activities (i.e. clear-cut logging, firewood collection) in the 1950s (Wang et al. 2007). Reforestation and afforestation campaigns were then launched to prevent land degradation and the original evergreen broadleaved forests were converted into secondary conifer forests or replaced with plantations. Masson pine (Pinus massoniana Lamb.) is the pioneer native tree species that dominates subtropical areas of southern China with a total coverage of 10 million ha (SFA 2014). Additionally, nearly 2 million ha of exotic tree species, such as slash pine (Pinus elliottii Engelm.), was introduced for afforestation due to its high productivity and resistance to local pests (Chen et al. 1995). Previous studies have shown that slash pine plantations exhibit distinct patterns of biomass and soil nutrient stocks compared to masson pine plantations (Tian et al. 2004; Ma et al. 2014; Wang et al. 2015). Slash and masson pine plantations also have distinct ECM fungal community compositions across young-aged plantations (Ning et al. 2019). Some seedling inoculum experiments have found that slash pine had relative low compatibility with indigenous ECM fungal strains (Chen et al. 2006), but the consequences of this limited compatibility in naturalized slash pine in plantations is unclear. Understanding the extent to which exotic slash pine forms symbioses with local ECM fungal communities and whether the ECM fungal species recruited by exotic slash pine are functionally similar to masson pine is critical for regional restoration and afforestation efforts.

In this study, we conducted a greenhouse bioassay experiment by planting native masson pine and exotic slash pine seedlings into soils collected from three forest sites: semi-natural masson pine forests, masson pine plantations and slash pine plantations. We used high-throughput sequencing to identify ECM fungal community composition on seedling roots, and assayed the potential activity of ECM root tips to produce extracellular enzymes used in C, N, and P acquisition and cycling. In addition, host growth responses to ECM fungal communities were determined using shoot/root biomass and tissue C, N, and P concentrations. These results were used to address three questions: a) Are there shifts in ECM fungal community structure between native masson pine and exotic slash pine? b) Are there differences in ECM enzymatic functioning between native/exotic pine seedlings? c) Are there changes in native/exotic pine seedling biomass and nutrient content relate to ECM extracellular enzyme activities?

Materials and methods

Study site description and seedling inoculation

Soil samples for this study were collected from three forests in Longli Forest Farm (26°22′~26°45′ N, 106°45′~107°11′E, ~1200 m above sea level), Guizhou Province, China. Based on ecological contexts, the three forest sites were: (1) semi-naturally regenerated masson pine (*P. massoniana*) forest (hereafter abbreviated as Masson-N), which spontaneously regenerated after a forest fire in 1994 and was wind-sown by surviving maternal masson pine trees; the site has not been managed or disturbed since the fire; (2) established masson pine plantation (Masson-PL) and (3) slash pine (*P. elliottii*) plantation (Slash-PL). Both plantations were established on clear-cut areas of former masson pine dominated forests in the 1990s. These sites differ significantly in soil N and P fertility in that the Masson-N site showed significantly higher level of total N (1.4 ± 0.1 g kg^{-1}) and available P (4.7 ± 0.3 mg kg^{-1}) than soils in the other two pine plantation sites (N, 0.8–1.2 g kg^{-1}; available P, 3.2–3.7 mg kg^{-1}) (Table S1). At each site, three 20 × 20 m subplots (at least 100 m apart) were set up, and three mature pine trees (DBH 15-20 cm) at random locations (>5 m apart) in each subplot were set as 'focal' trees for soil sampling.

In March 2016, four soil cores from each focal tree (10 cm diam, ~12 cm deep, 45 cm apart from base of trunk in a random cardinal direction) were sampled using a pre-sterilized steel soil core, sealed in plastic bags with as little disturbance as possible, and transported on ice to the growth facilities. Pre-sterilized PVC pots (top diameter 10.5 cm, bottom diameter 9 cm, height 13.5 cm, and volume ~750 ml) were used as the containers for all the prepared soil samples.

The soil cores were kept intact and undisturbed to maintain the residual live fine roots and associated mycelia since several studies have indicated that the residual mycorrhizal root tips can act as important inoculum resources for 'late stage' ECM fungal species, such as *Russula* (Avis and Charvat 2005), and remain physiologically-active for several months after collection (reviewed in Cairney 2012). After soil cores were placed into pots, the soil was sown with either native

masson pine or exotic slash pine seeds collected during the previous year by Longli Forest Farm staff. Prior to sowing, all seeds were surface-sterilized in 1% bleach solution and cold-stratified at 4 C for 3 weeks and then evenly sown in each pot. Posts were thinned after germination to keep 8 seedlings per pot. A total of 108 planted-pots (4 cores × 3 trees × 3 plots × 3 sites) were placed in greenhouse with a randomized block design. To confirm whether the greenhouse experiments were influenced by potential ruderal fungal spore contamination, eighteen pots of autoclaved soil (121 C for 3 h) were used as a control (2 plant species × 3 sites × 3 replicates; autoclaved at) and initiated and seeded with aforementioned masson or slash pine seeds. Growth conditions were natural day-length (12-13 h) at a greenhouse located at Central South University of Forestry and Technology (28°06′N, 113°02′E), Changsha, Hunan, China. Soil moisture was maintained around 50–80% by an automatic watering system to meet seedling growth requirements.

Seedling harvesting and root tip sampling

In July 2016 (3 months after seed germination), three pine seedlings per pot were harvested following an equilateral triangle distribution. Roots were gently rinsed with tap water and morphologically scanned (number of root tips and surface area of <2 mm fine roots) using the computer program $W_{IN}R_{HIZO}$ (Regent Instrument, Quebec, Canada). To quantify ECM colonization, six lateral roots (about 10 cm) from the seedlings per pot were randomly selected and examined under a stereomicroscope. After that, we cut the roots into 2-3 cm lengths in a Pyrex dish containing distilled water, and mixed thoroughly. From the pool of roots, six root fragments were randomly selected and the first 5 root tips encountered on each fragment were determined to be mycorrhiza or not. In total, 30 healthy ECM root tips were selected from each pot and pooled for DNA extraction and subsequent Illumina sequencing analysis. Seedlings were then separated into two parts (shoot, root), dried for 48 h at 60 C and weighed. For each pot, dry biomass was pooled for roots or shoots, ground to a fine power, and root and shoot levels of C, N and P were analyzed. (for more details see supplementary Method S1).

One seedling from each pot was harvested for enzyme assay. First, all root tips were scanned by $W_{IN}R_{HIZO}$ and surface area (SA) calculated; the diameter of fine root ranges was set from 0 to 0.5 mm. After that, seven root tips per seedling were collected for extracellular enzyme analyses. Under a dissecting microscope, the total root system was divided into 7 equal sections and one root tip was chosen from each section. Morphological types were distinguished by branching pattern, color, texture, presence of rhizomorphs, and other morphological features of the mantle and emanating hyphae. The seven root tips from each seedling were selected to ensure representation of all morphotypes. Typically, there were three or four morphological groups on each seedling. For enzyme assays, microplate wells were first filled with morphotypes that had only one representative and then with those that has more than two representatives. In addition, each set of seedling root tips included at least one non-ECM root tips as control since several studies have reported that non-ECM root tips may yield exoenzymatic activities (Walker et al. 2014). Individual root tips of each morphotype were then loaded into a well (one root tip per well) in a 96-well plate (BRAND plates®, Wertheim, Germany) for immediate enzyme assay.

Potential extracellular enzyme activities of ectomycorrhizae

Potential activities of hemicellulases (**GU**; β-glucuronidase (EC 3.2.1.31), β-xylosidase (**X**; EC 3.2.1.37)), cellulases (**CEL**; cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (**G**; EC 3.2.1.21)), enzymes degrading chitin (**NAG**; N-acetylglucosaminidase (EC 3.2.1.14)), P- and N- containing organic compounds (**AP**; acid phosphatase (EC 3.1.3.2) and leucine aminopeptidase (**LEU**; EC 3.4.11.1), respectively), and an oxidative enzyme, laccase (**LAC**; EC 1.10.3.2) were assessed following the methods of Pritsch et al. (2011). Once assays were completed, the root tips were frozen at −80 °C for DNA analysis (see details in supplementary Method S2) to confirm the identities of the mycobionts.

Molecular identification of the ectomycorrhizal community

DNA of pooled root tips from each pot was extracted using Mobio PowerSoil kit (Hercules, CA, USA) following manufacturer's instructions. The ITS1 region of the fungal rDNA subunit was amplified by PCR using ITS1F-ITS2 primer set (Smith and Peay 2014) and sequenced (2 × 300 bp) on the Illumina Miseq platform. Paired-end reads were assembled and quality filtered

bioinformatically (see details in supplementary Method S3) to generate the final species table. Sequences of sample data are deposited in the NCBI sequence read archive with accession number SRP140037.

Statistical analysis

Data sets were square root (enzyme potential) or log-transformed (tissue nutrients) before analyses to meet the assumptions of normality. Significant level of each test was considered at $\alpha = 0.05$.

For ECM fungal community composition, we used a normalization method for the read abundance data of fungal communities using the 'metagenomeSeq' package (Paulson et al. 2013) of R 3.3.2 software (R project for Statistic Computing; http://www.R-project.org) since several reports revealed that rarefying methods could cause a loss of sensitivity due to elimination of a portion of available data (McMurdie and Holmes 2014). The effects of *Site* (Masson-N, Masson-PL, Slash-PL) and *Host* (masson or slash) as well as interactions on ECM fungal community composition were compared using program Primer v7 with PERMANOVA+ (PRIMERE Ltd., Lutton, Ivybridge, UK) on Bray-Curtis dissimilarity matrices. We also included *Plot* identity nested in site and 'focal' *Tree* identity nested in plot as random factors in the mixed model. Analysis of community structure was visualized using non-metric multidimensional scaling (NMDS) in 'vegan' package in R (Oksanen et al. 2017).

A number of ECM fungal taxa were encountered with sufficient replication (ECM root tips present in at least three pot samples per *Site* × *Host* combination) to examine the relationship between the relative abundance and relative potential enzyme activity of ECM fungal communities. The exploration types of ECM fungal taxa were classified as 'long−/medium−/short-distance' using presence and length of emanating hyphae because ECM exploration types often show consistent responses to soil nutrient status (Lilleskov et al. 2011). The main groups of ECM fungal taxa and their exploration strategies were: *Rhizopogon* (long-distance), Atheliaceae (long-distance), *Russula* (contact/short-distance), Thelephoraceae (short/medium-distance).

The relative abundance (RA) data of ECM fungi was calculated from the Illumina sequencing reads. Given that various patterns for correspondence between high-throughput sequences and quantified amount of DNA (Amend et al. 2010; Taylor et al. 2016) have been reported, we used a series of 'mock' communities as a proxy. The mock communities were composed of different initial DNA concentration of dominant ECM fungal species collected from this experiment to verify if gene copy numbers can accurately estimate fungal abundances. The read abundance of the dominant ECM fungal species was significantly correlated with their initial DNA concentrations in 'mock' communities ($R^2 = 0.37$, $P = 0.002$) (see supplementary Table S2 and Fig. S1). Thus, the total activity of the ECM fungal community on each seedling was calculated as:

$$\sum(\text{activity} \times \text{RA} \times \text{colonization} \times \text{SA})_{ECMgroup} \quad (1)$$

In which the activity was the mean level of enzyme activity of that ECM fungal group in each *Site* × *Host* treatment. The relative contribution of each ECM fungal group to community enzyme activity in each pot was calculated as [activity of ECM fungal group/ total activity of ECM fungal community per seedling]. The effect of *Site* and *Host* on mean/total enzyme activities of ECM fungal communities was similarly analyzed as ECM fungal community data based on Euclidean distance with PERMANOVA and NMDS analyses. We then compared the *Host* effect (native vs. exotic) on mean and total levels of ECM enzymatic activities in each seedling (based on eq. [1]) using t-tests.

To test the effects of various enzymatic activities on each host seedling biomass and nutrient status, we used an information theory model selection in the 'MuMIN' package of R (Bartoń 2013). The variable models were ranked from the lowest AIC_c scores and further compared using model averaging from the inclusion enzyme activities. The beta coefficients, standard errors, significance, and relative importance values were based on top number models within AIC_c score < 4.

Result

ECM fungal community composition

We examined 126 mixed root tip samples from pine seedlings in this study. High throughput sequencing recovered 62 putative ECM fungal OTUs assigned to 27 genera (Fig. 1a). The most common genera were *Rhizopogon* (Rhizopogonaceae; 54.2%), *Tomentella* and *Thelephora* (Thelephoraceae; 25.1%), *Amphinema* and *Tylospora* (Atheliaceae; 16.8%), and *Russula* (Russulaceae; 6.3%). We found negligible read

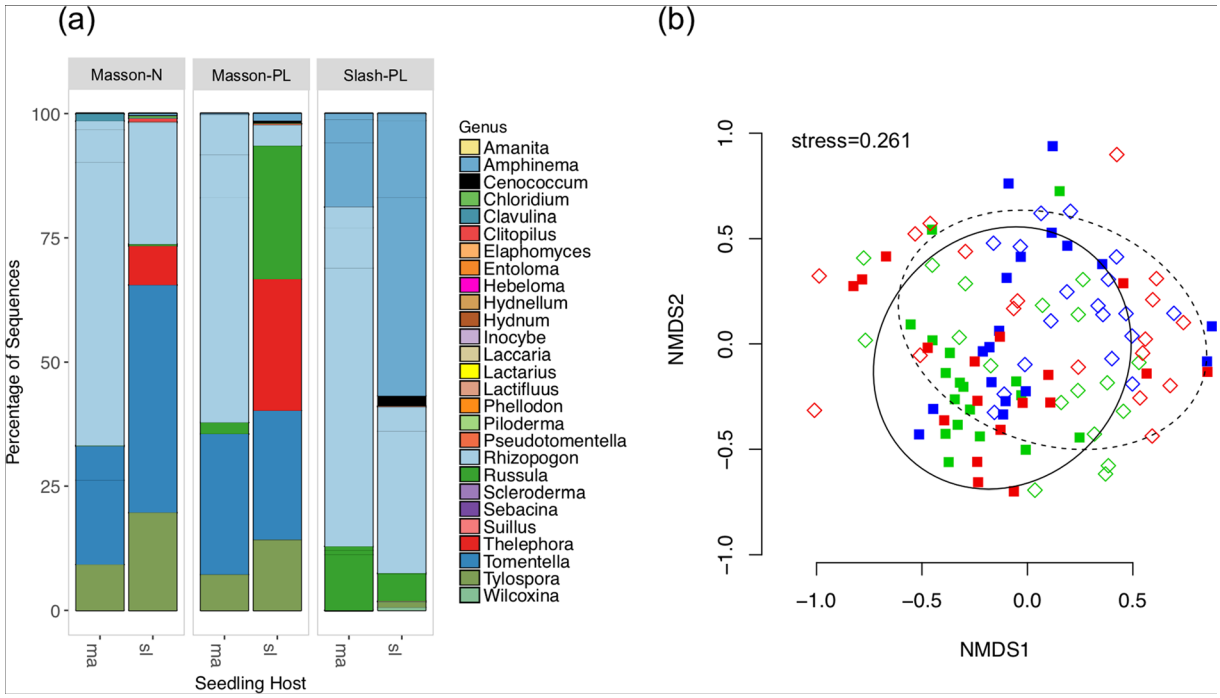

Fig. 1 **a)** Relative abundances of ectomycorrhizal fungal genera and b) NMDS plot based on Bray-Curtis distance of ectomycorrhizal fungal community colonizing pine seedlings in a reciprocal transplant experiment with respect to combination of *Sites* (Masson-N, red; Masson-PL, green; Slash-PL, blue) and *Hosts* (masson, closed squares; slash, open diamonds). 95% confidence ellipses were projected on the ordination plot to visualize multivariate dispersion in community composition by hosts (masson, solid; slash, dash)

abundances of some ECM fungal species, as well as *Thelephora terrestris,* (OTU_1214), which generally occurred as a contaminant colonizer in most nursery systems (e.g. Velmala et al. 2014), in 18 sterilized control pots (Fig. S2). The mean recovered ECM fungal richness was 6.8 ± 0.4 (mean ± s.e.) for masson pine seedlings and 8.8 ± 0.5 for slash pine seedlings.

In Masson-N soils, the ECM fungal community of masson pine seedlings was dominated by *Rhizopogon* (65.3%) whereas *Tomentella* (45.8%) was the most abundant species on slash pine seedlings. Similar patterns were also found between the two hosts species planted in Masson-PL forest soils. In Slash-PL forest soil, the ECM fungal community of masson pine was dominated by *Rhizopogon* (68.3%), whereas the community of slash pine was dominated by *Amphinema* (56.8%).

Analysis by PERMANOVA and NMDS provided evidence for distinct communities of ECM fungi in seedling roots based on *Site*, as well as significant differences between *Hosts* (Fig. 1b; Table 1). There were also significant spatial effects (tree location; $p = 0.001$ for *Tree* identity nested in *Plot* identity) on recruited ECM fungal community structures of seedlings.

Exoenzyme activities of ectomycorrhizae

A total of 690 root tips from the 108 seedlings harvested for enzyme analysis were submitted for enzyme assay. Of these, four hundred and thirty two yielded successful PCR amplification and 311 were identified to genus or species level. Additional replicates from each seedling were identified by combining morphotyping and molecular identification. Twenty of the 37 detected OTUs were ECM fungal species; the most abundant ECM fungal species were *Rhizopogon* OTU_1298, *Rhizopogon* OTU_1463, *Tomentella* OTU_1227, *Tomentella* OTU_1219, *Tylospora* OTU_1494, *Amphinem*a OTU_1288, and several *Russula* spp. Based on Species Hypotheses in the UNITE database (https://unite.ut.ee), all these taxa were classified as local (Asian/China) species except for *Tylospora* OTU_1494, which was classified as a cosmopolitan species (Table S3).

An ordination of mean level and total level of all eight enzyme activities of the dominant ECM fungal taxa illustrated a clear separation of enzymatic functions associated with different *Hosts* (Fig. 2a and b; Table 1). *Site* effect on mean level of ECM root tip enzyme

Table 1 PERMANOVA of ectomycorrhizal fungal community composition (Bray-Curtis distance) and root tip mean/total enzyme activities (Euclidean distance)

Factor.	df	Sum sq	Mean sq	Pseudo-F	P-value[1]
ECM fungal composition					
Host	1	23,674	23,674	5.678	0.014 *
Site	2	19,380	9690	1.771	0.018 *
Host x Site	2	5005	2502	0.6	0.859
Plot (Site)	6	32,828	5471	0.943	0.579
Tree (Plot(Site))	18	1.0441E+05	5801	1.635	0.001**
Host x Plot (Site)	6	25,019	4170	1.147	0.207
Host x Tree (Plot(Site))	18	65,443	3636	1.025	0.38
Residual	54	1.9156E+05	3547		
ECM mean enzyme activities per seedling					
Host	1	31,049	31,049	34.224	0.001 **
Site	2	8222	4111	1.659	0.224
Host x Site	2	1953	976	1.076	0.375
Plot (Site)	6	14,871	2479	1.628	0.143
Tree (Plot(Site))	18	27,404	1522	1.597	0.044 *
Host x Plot (Site)	6	5443	907	0.886	0.508
Host x Tree (Plot(Site))	18	18,430	1024	1.074	0.368
Residual	54	51,495	954		
ECM total enzyme activities per seedling					
Host	1	24,367	24,367	5.58	0.034 *
Site	2	40,713	20,356	4.23	0.009 **
Host x Site	2	14,526	7263	1.663	0.277
Plot (Site)	6	28,872	4812	1.664	0.713
Tree (Plot(Site))	18	1.3042E+05	7245	2.083	0.004 **
Host x Plot (Site)	6	26,203	4367	1.776	0.103
Host x Tree (Plot(Site))	18	44,260	2459	0.707	0.828
Residual	54	1.8787E+05	3479		

[1] *p < 0.05; **p < 0.01; ***p < 0.001

activities was marginally insignificant but significant on total level of all ECM root tips on each seedling. Similar to fungal community structure, tree location also showed significant effects on both mean and total level of ECM enzyme activities ($p = 0.044$ for mean level and $p = 0.004$ for total level).

Within the same forest soil, the mean enzyme activity of root tips for all ECM fungal groups differed significantly according to their host identities. Generally, ECM fungi associated with native masson pine seedlings had significantly higher levels of N- (NAG) (280–300%), P- (AP) (105–152%), and cellulose (G 204–235%, CEL 142–255%) degrading enzyme activities than those associated with exotic slash pine (Fig. 3a). These enzymatic functional differences also contributed to the disparity in total enzyme activity in masson and slash pine seedlings inoculated from Masson-N and Masson-PL soils, but overlapped in Slash-PL (Fig. 3b). No significant difference of enzyme activities was found on non-ECM root tips between two hosts across the forest sites (Fig. S3).

Rhizopogon species associated with masson pine seedlings had relatively higher LEU, NAG, and G activities and total enzymatic contributions (40~75%) than those associated with slash pine seedlings (averaged 19–39% contributions) (Fig. 4a). Significant variations in the enzyme activities of Atheliaceae was found among *Site* × *Host* treatments as *Tylospora* (in Masson-N and Masson-PL) and *Amphinema* (in Slash-PL) showed disparate dominance and contribution to total enzyme activities (4~38% for masson pine and 26–47% for slash pine)

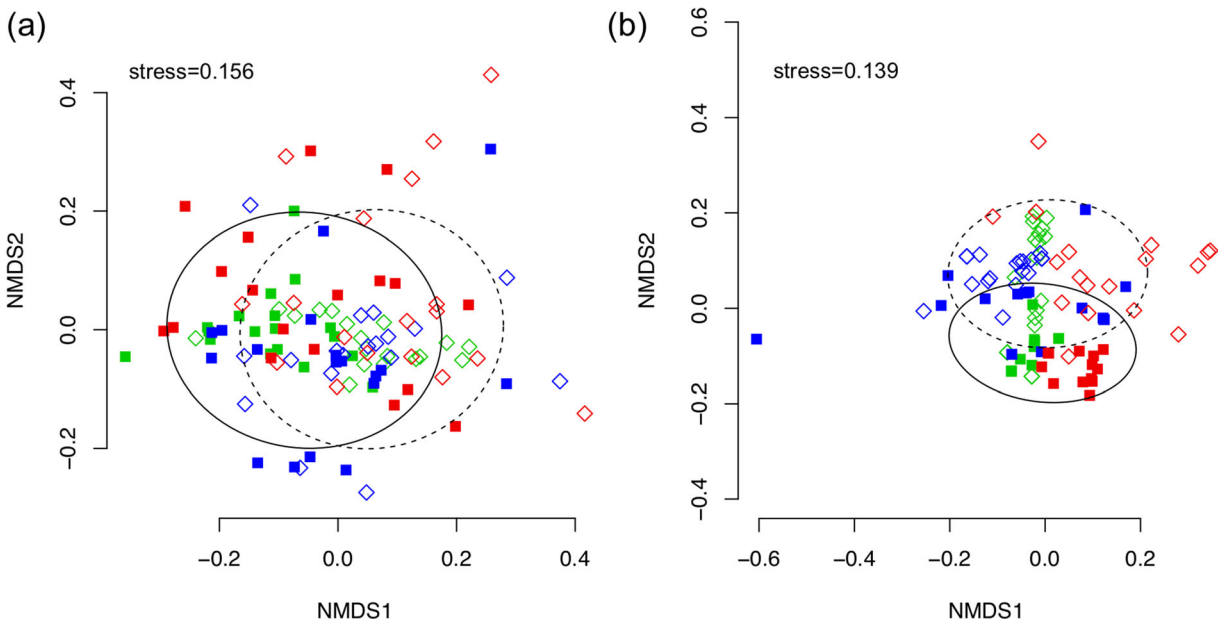

Fig. 2 NMDS plot illustrating differences in a) mean and b) total enzyme activities of ectomycorrhizal root tips from masson pine (closed squares) and slash pine (open diamonds) seedlings among forest types (colored) across *Sites* (Masson-N, red; Masson-PL, green; Slash-PL, blue). Data points represent individual seedlings and lines show 95% confidence ellipses for each host, with solid for masson pine and dashed for slash pine

(Fig. 4d). The enzyme activities of *Russula* and Thelephoraceae were dependent on species identity or associated host and site (Fig. 4b, c). For example, *Russula* spp. recovered on slash pine seedlings from Masson-PL and Slash-PL sites presented relatively high activities of hydrolytic enzymes (> mean levels of all ECM root tips) and contributed 10–30% to the total enzyme activities.

The effect of the potential exoenzyme activities on seedling nutrient acquisition

Seedling biomass accumulation and tissue nutrient levels were differentially influenced by ECM exoenzyme activity (Fig. 5; Table S5-S10; see Table S4 for seedling biomass and nutrient content data). Overall, either mean or total level of cellulose (G/CEL) and hemicellulose (X/GU) degrading enzymes showed a positive relation to root biomass, N or P content of plant tissue of two hosts. Higher levels of total LEU activities in both host species was also associated with higher tissue C content.

On the other hand, the nutrient status of the two host species showed distinct correlations with the relative enzymatic contribution from certain ECM fungal taxa (Fig. 5;Table S7-S10). For masson pine, only the relative contribution of LEU from *Rhizopogon* spp. positively correlated with plant C content (MAE = 17.53 ± 7.52, $p = 0.022$, RVI = 0.86). In contrast, the nutrient status of slash pine seedlings varied with the enzyme activities provided by multiple ECM fungal groups. For example, the relative contribution of X from *Rhizopogon* (MAE = 387.6 ± 115.5, $p < 0.001$, RVI = 1), NAG from *Russula* (MAE = 82.27 ± 32.37, $p = 0.013$, RVI = 1), and AP (MAE = 210.64 ± 94.05, $p = 0.027$, RVI = 0.71) and CEL (MAE = 85.04 ± 40.21, $p = 0.038$, RVI = 0.69) from Thelephoraceae were all positively correlated with plant tissue C content.

Discussion

The recovery of disparate ECM fungal communities on congeneric native/exotic pine seedlings grown in the same forest soils is consistent with previous studies (Rusca et al. 2006; Kohout et al. 2011; Nguyen et al. 2016). Our study further documents that these differences are associated with changes in enzyme profiles of certain co-occurring ECM fungal taxa in response to host pine associations. For example, although both of the pine hosts in our study were colonized by the same *Rhizopogon* species, these species consistently displayed higher N-

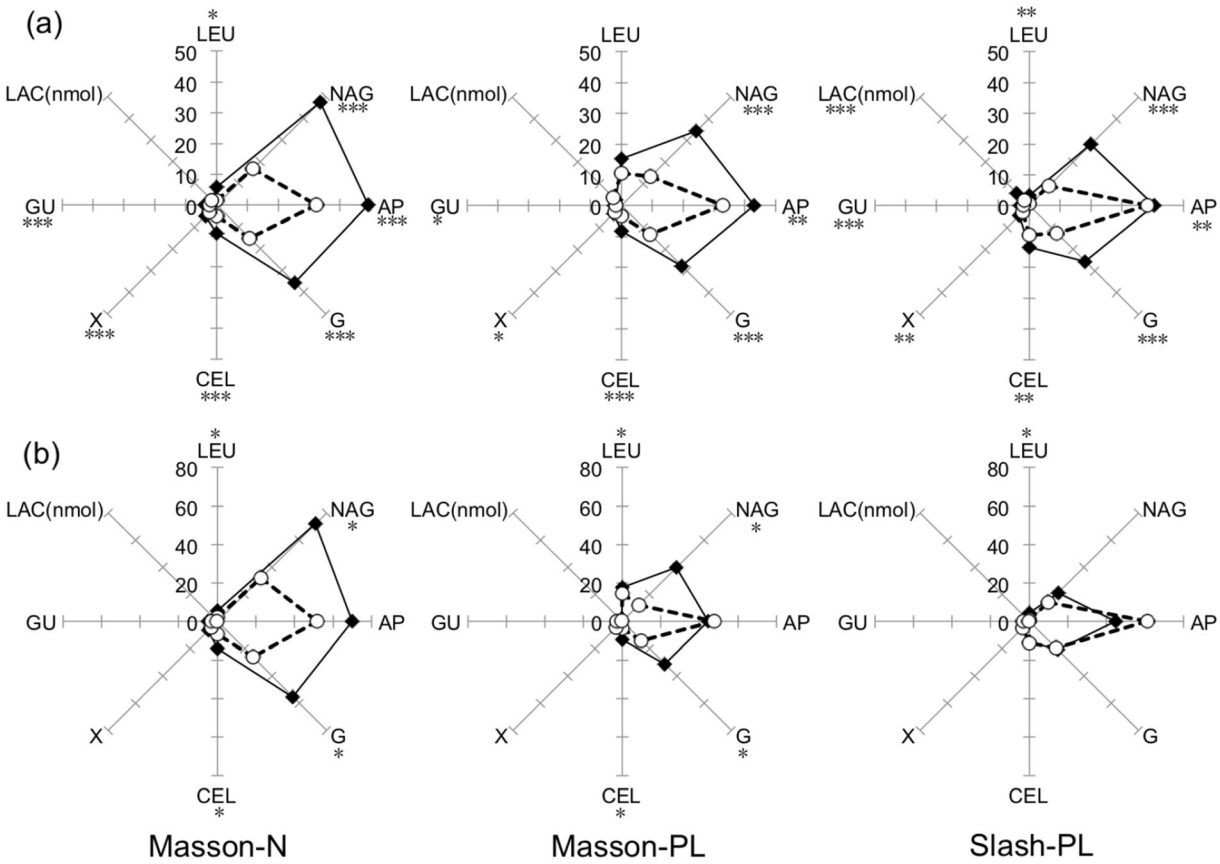

Fig. 3 a) Mean and b) total levels of potential enzymatic activity (pmol mm^{-2} min^{-1}/LAC: nmol mm^{-2} min^{-1}) profiles of ectomycorrhizal fungal root tips on seedlings sampled among *Sites*. Masson pine- solid line, slash pine – dash line. Abbreviations for enzymes: LEU, leucine aminopeptidase; NAG, N-acetyl-glucosaminidase; AP, acid phosphatase; G, β-glucosidase; CEL, cellobiohydrolase; X, xylosidase; GU, glucuronidase; LAC, laccase. Asterisks indicate t-tests with significance levels at $p < 0.05$*, $p < 0.01$**, $p < 0.001$***

and P-related enzymatic activities with native masson pine than with slash pine. This result suggests that tree species and their associated ECM fungi are co-adapted to their local environment and can thus maximize nutrient acquisition and SOM utilization strategies. It is also possible that the relatively higher enzyme activities in masson pine-*Rhizopogon* symbioses was due to a 'compatibility pattern' developed over their co-evolutionary history so that the gene expression may be interlinked with the realized plant-mycobiont associations (Plett et al. 2015; Liao et al. 2016; Policelli et al. 2019). In contrast, the slash pine-*Rhizopogon* associations encountered in our study were novel symbioses that likely developed due to a lack of suitable/compatible ECM fungi in the species pool and/or the developmental stage of the seedlings used in the study.

ECM fungal generalists (e.g. Atheliaceae, Thelephoraceae, Russulaceae) found in all three sites were readily available in slash pine plantations and appeared to maintain substantial enzyme functions. Specifically, in Slash-PL forest soil the activity of the N- acquisition enzyme (NAG) produced by *Amphinema* and *Russula* on slash pine seedlings enhanced the functional complementary of the whole root system and provided a level of functional redundancy between the two hosts. These findings reinforce the idea that a diverse ECM fungal community exhibits sufficient complementarity in enzyme activities to maintain the nutrient acquisition potential in a regenerating seedling (Kipfer et al. 2012; Velmala et al. 2014). However, this functional-based host filtering in an introduced non-native species may facilitate the recruitment of non-native host-preferred or even non-native ECM fungal symbionts (Bahram et al. 2013; Policelli et al. 2019), with a potential loss of ECM fungal taxa specific to native hosts (Dickie et al. 2010; Hayward et al. 2015). However, these results should be interpreted

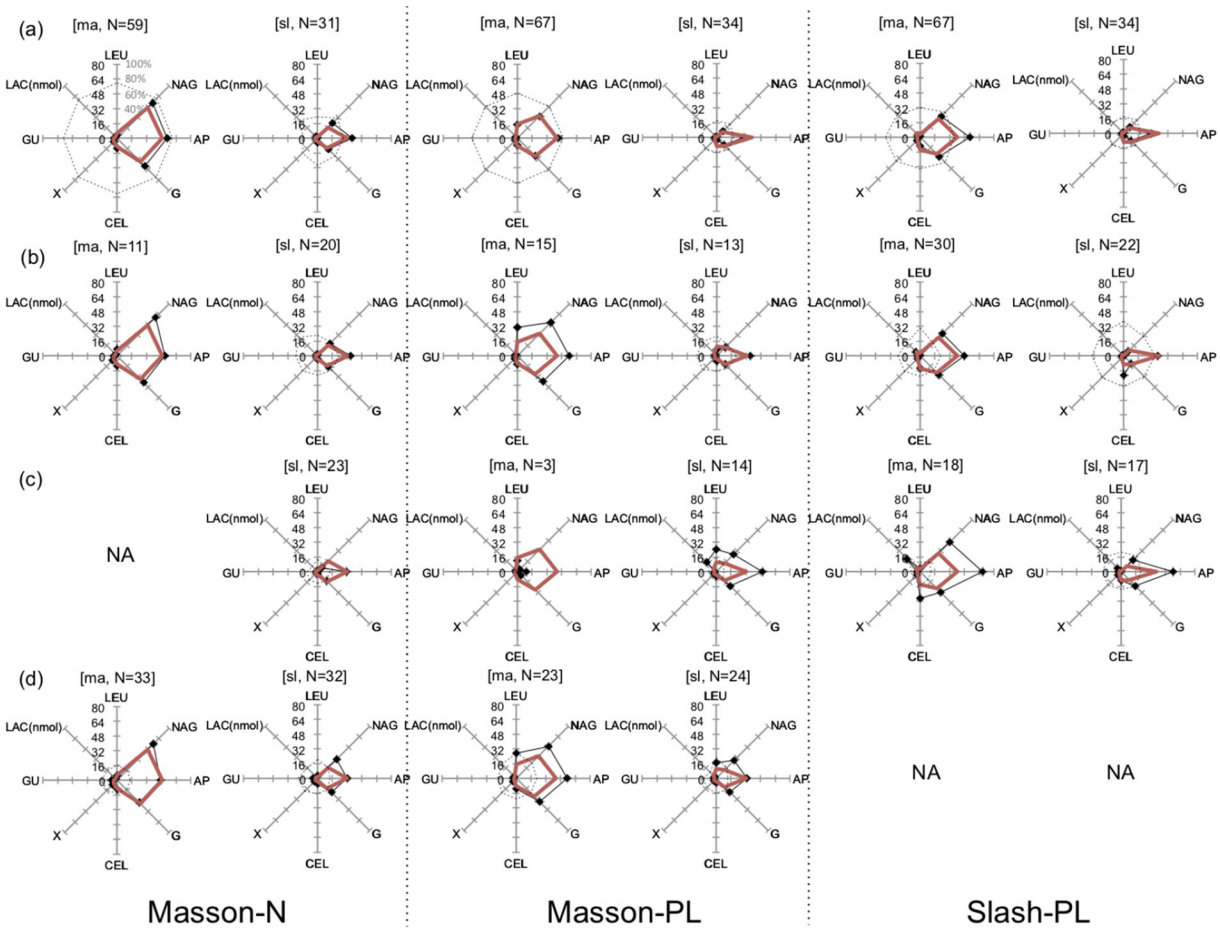

Fig. 4 Mean levels of potential enzymatic activity (pmol mm^{-2} min^{-1}/LAC: nmol mm^{-2} min^{-1}) profiles of a) *Rhizopogon* spp. b) Atheliaceae c) *Russula* spp. and d) Thelephoraceae; solid line- potential enzymatic activity, dash line – relative contribution to overall community activity. Red line in each panel indicates the mean community level of enzyme activity. Abbreviations for enzymes: LEU, leucine aminopeptidase; NAG, N-acetyl-glucosaminidase; AP, acid phosphatase; G, β-glucosidase; CEL, cellobiohydrolase; X, xylosidase; GU, glucuronidase; LAC, laccase

with caution because the experiment was undertaken in greenhouse containers for only one growing season. Spatial heterogeneity of fungal inocula (as tested in statistical models) (Pickles et al. 2010; Livne-Luzon et al. 2017), intraspecific competition of ECM fungal taxa (Kennedy et al. 2009, 2011), and temporal changes in plant and fungal phenology (e.g. Courty et al. 2010b; Hupperts et al. 2017) may be additional factors contributing to the observed ECM fungal communities. Further work on this topic is needed as a shift to ECM fungal community composition primarily comprised of generalist taxa with large scale plantings of non-native tree plantations would be a serious concern for maintaining the local biodiversity of ECM fungi and a mycorrhizal-induced community successional trajectory (Johnson et al. 2012).

The consistent differences in ECM fungal communities and extracellular enzymatic function between seedlings of the two host species may reflect a trade-off between biomass accumulation and the production of metabolically expensive enzymes (e.g. Moeller and Peay 2016). There is evidence that fungi with very extensive mycelial growth tend to supply less N to their hosts (Corrêa et al. 2012). This may explain the significant fluctuations in enzymatic activities we observed in host-ECM fungi associations. For masson pine seedlings, the high colonization rate of *Rhizopogon* spp. and their consistently higher N-acquisition enzyme activities suggest that the requirement of N for both host seedling and the mycobiont was maintained through oxidizing SOM by ECM fungi. This mechanism was

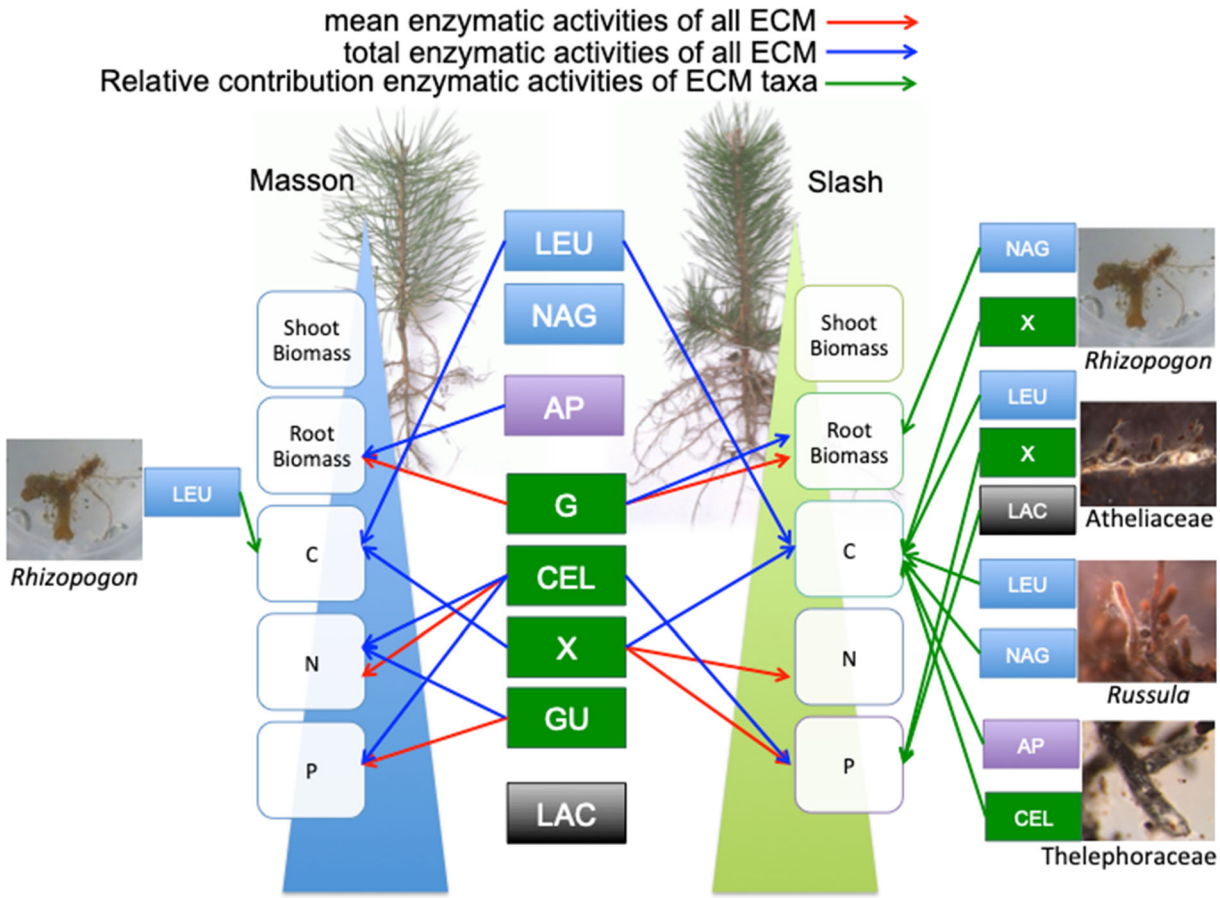

Fig. 5 Positive correlations between seedling growth and ectomycorrhizal fungal community and taxa level enzymatic activities based on information theory model. The effect indices of the models are presented in supplementary Table S5-S10. Abbreviations for enzymes: LEU, leucine aminopeptidase; NAG, N-acetyl-glucosaminidase; AP, acid phosphatase; G, β-glucosidase; CEL, cellobiohydrolase; X, xylosidase; GU, glucuronidase; LAC, laccase

further supported by a net up-regulation in enzymatic C acquisition (e.g. β-glucosidase) by *Rhizopogon,* which can be interpreted in the context of the 'saprotrophy model' wherein ECM fungi acquire labile carbohydrates when plant C allocation is low (Talbot et al. 2008; Courty et al. 2010a; Hupperts et al. 2017). In contrast, enzyme activity in the ECM fungal community on exotic slash pines appeared to be consistent with the 'nutrient acquisition model' (Talbot et al. 2008; Hupperts et al. 2017). In this symbiosis, the host slash pine accumulated more aboveground biomass than the other host-fungal combinations and could conceivably allocate more C to various ECM fungal taxa for extracellular enzyme production.

There is now substantial evidence that introducing exotic pines could alter the local ecosystems, which is mediated and facilitated by their belowground mutualists, particularly ECM fungi (Nuñez et al. 2017). Our results address two sets of dynamics related to the ECM fungal communities colonized on native pine versus exotic pine. The first focuses on dynamics associated with reducing potential enzyme activities for SOM degradation and the release of available nutrients. These changes could shift towards less SOM decomposition and faster nutrient depletion rate (e.g. Tian et al. 2004). Further, based on the 'preferential substrate utilization' hypothesis (see Lilleskov et al. 2011), the change in litter/fine root debris by an exotic host per se may provide new niches for functionally related microbes to colonize. This effect, combined with the observations that the input of litter from exotic trees can alter other soil microbial communities (Chen et al. 2011), may lead to distinct nutrient cycling pattern in exotic tree-based afforestation ecosystems.

The second focuses on ECM fungal community structure and C dynamics. Differences in tissue quality (e.g. C:N ratio, melanin content) among ECM fungal species can influence degradation dynamics and residue persistence in long term soil organic carbon (SOC) stocks (Fernandez et al. 2016; Siletti et al. 2017), which means that any change in the symbiosis could lead to profound effects on soil C accumulation. Additionally, Weight et al. (2012) have noted that long-distance exploration type ECM fungal taxa, i.e. those that produce cords or rhizomorphs, can provide ~15 times more biomass than ECM fungi with shorter distance exploration types. Rhizomorphs or hyphal cords can also persist for months to years and are thus more persistent than those of diffuse hyphae of short/contact taxa (Treseder et al. 2005; McCormack et al. 2010; Certano et al. 2018). This may be due to fact that the rhizomorphs or hyphal cords of long-exploration types generally have a hydrophobic surface and a relatively low contact surface to surrounding soil (Koide et al. 2014). Therefore, in our study, the ECM fungal community structural differences between hosts could feed back to influence the quantity and quality input of fungal necromass to soil C pool, in which a recovered community of native masson pine dominated by *Rhizopogon* (long-distance) rhizomorphs may be better contributors to SOC accumulation than the Thelephoraceae-Russulaceae (short/contact-distance) dominated communities in exotic slash pine.

Bioassay studies provided controlled conditions for both host seedlings and ECM fungi since factors such as spatial autocorrelation of ECM fungal communities in adult tree roots (Lang et al. 2013; Craig et al. 2016) and canopy shade effects (Peay et al. 2015) may complicate interpreting mutualistic patterns in field studies. Even so, field studies are needed to confirm the results reported here. Our estimates of differences between fungal communities and enzymatic function between native/non-native pines are based on the two most common host species in south China. Additional investigations using other native and non-native pines are warranted to test if the results in our study can be broadly extrapolated or if other strategies might be involved in those novel symbioses with local ECM fungal taxa.

Conclusions

Using a greenhouse experiment with natural soils, we found that the assembly and functioning of ECM fungal communities were linked to native/exotic host tree identity. Differences in enzyme activity of the ECM fungi associated with the two hosts were also documented with the ECM fungal communities on native masson pines having relatively higher potential exoenzyme activities than those with exotic slash pines, which may indicate a host-specific advantage of plant-mycorrhiza symbiosis for nutrient uptake and cycling. Given the large-scale utilization of exotic pine trees for afforestation in China (SFA 2014), one can predict that ECM fungal communities in non-native pine systems (compared to native counterparts) would shift towards less nutrient use efficiency, resulting in changes in the fungal necromass input to long-term C pool. Our data support the findings of Schaefer (2011) that the long-term introduction of a non-native host tree can promote further introduction of peripheral symbionts or companion taxa that may compromise the establishment, restoration, or maintenance of original native systems — resulting in loss of ecological memory. Such prospects point to the need for a better understanding of the roles of ECM functional traits, their interactions with native/exotic host growth, and nutrient availability and C stabilization in afforested ecosystems.

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Author's contributions CN, GMM and LEW conceived the ideas and designed methodology; CN collected the data; CN analyzed the data; CN, WX, LEW, and GMM led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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