Short communication

Shifts in ectomycorrhizal fungal community composition during the early establishment of native and exotic pine seedlings

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ABSTRACT

Exotic pine trees are often introduced to novel places worldwide. How exotic pines interact with local microbial communities, such as symbiotic ectomycorrhizal (ECM) fungi, during early establishment compared to interactions by their native counterparts remains to be examined in detail. In this study, native masson pine (*Pinus massoniana*) and exotic slash pine (*Pinus elliottii*) seedlings were grown in field-collected soil cores. ECM root tips were collected 3- and 6-months after planting and fungi were identified using high-throughput sequencing. We found that host identity was a key factor determining ECM fungal community assembly after 3-months: native pines associated with *Rhizopogon* while exotic pines associated primarily with ECM generalists. After 6-months growth, however, ECM fungal communities in native and exotic pines were both dominated by *Rhizopogon* species. We also found that host selections of several pioneer ECM fungi shared by both native and exotic pines may facilitate an exotic pine's establishment. These findings indicate that more attention should be paid to ECM fungal community shuffling by exotic trees during early establishment and, in turn, the influence of the potential changes in ECM fungal communities on local forest ecosystems.

1. Introduction

Pine (*Pinus*) trees have been widely used in forestry and afforestation initiatives because of their relatively short generation time, high quality wood, and local pathogen resistance (Richardson and Higgins, 1998). When introducing non-native pines in new areas, the interactions between pines and symbiotic soil microbes, such as in ectomycorrhizal (ECM) symbiosis, may play an important role in the local naturalization process (Smith and Read, 2008; Moyano et al., 2020). Most studies have sought to understand the abiotic and biotic factors controlling native versus non-native pine establishment. For instance, non-native pines associate with a relatively low number of ECM species in the non-native system, and the ecological traits of these ECM fungi (e.g. exploration strategy, dispersal, nutrient uptake) can enhance the success of pines in new ranges (Policelli et al., 2019). The reported evidence also indicates that the ECM fungal communities in native and exotic pines may be regulated by phylogenetic distance of the trees (Kohout et al., 2011; Trocha et al., 2012; Nguyen et al., 2016; Ning et al., 2019). For example, various pine species formed distinctive ECM fungal community even when grown with the same ECM species pool

(Rusca et al., 2006). In addition, the subgenera of *Pinus* (hard pine) and *Strobus* (soft pine) have been shown to express different sets of functional genes when associating with compatible/incompatible ECM fungal partners (Liao et al., 2016). Finally, abiotic (e.g. drought, Patterson et al., 2018) and biotic factors (e.g. host root structure, Velmala et al., 2014; ECM fungal life history variations, Kennedy et al., 2011), as well as ECM symbiotic functioning (Hortal et al., 2017; Ning et al., 2020) may impact the composition of recruited ECM communities in exotic pines. However, we have considerably less information on the temporal patterns of ECM-pine interactions, especially on how introduced species do host-specific selections for native (local) during their early establishment.

In this study, we examined the interactions between local ECM fungal communities and a widespread plantation pine (*Pinus elliottii*) in China during early establishment through a greenhouse experiment. In subtropical areas of China, plantations comprise nearly 69 million hectares (~36%) of the total forest coverage. Native masson pine (*Pinus massoniana*) is one of the most dominant native species in silviculture. However, nearly 2.2 million hectares have been planted with non-native slash pine (*Pinus elliottii*; SFA, 2014). Therefore, monitoring how
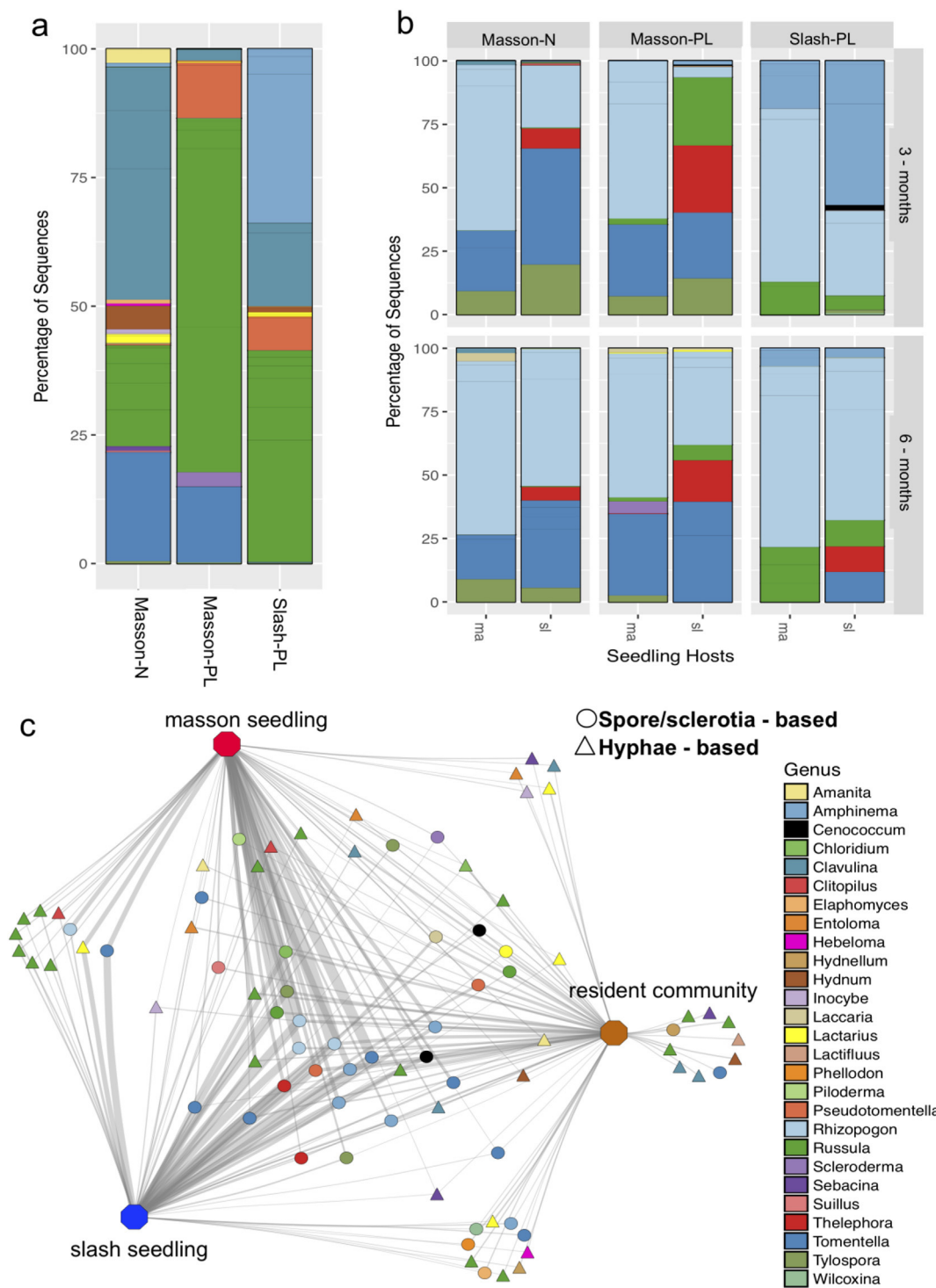

Fig. 1. Relative abundance of ectomycorrhizal genera in (a) resident communities in soil samples and (b) colonization of pine seedlings in greenhouse experiments comparing combinations of *Sites* (Masson-N – masson pine natural forest, Masson-PL - masson pine plantation, Slash-PL - slash pine planation), *Hosts* (masson-ma, slash-sl) and collecting times. Each bar represents the total proportion of DNA sequences in each treatment combination assigned to each genus. (c) OTU network map of ectomycorrhizal fungal communities among resident soil and seedling roots. Each point represents an independent fungal OTU. Edge thickness was weighted by the frequency (proportion of present samples to total samples) from the OTU to the specific sampling scenario.

exotic pines impact on local ECM fungal communities and whether they can induce new ecological problems is a vital task for local forest management. Here, we applied Illumina MiSeq sequencing methods to investigate the resident (soil) and recovered seedling ECM fungal communities in native and slash pines to address two questions: (1) to what extent does the ECM fungal community on establishing exotic pine seedlings diverge from that on establishing native pine seedlings?

(2) are there specific fungi that disproportionately survive the early establishment process of native versus exotic pine hosts?

2. Materials and methods

Our sampling sites are located in Longli Forest Farm, Guizhou, China (26°22′ ∼ 26°45′ N, 106°45′ ∼ 107°11′E). Soil cores from three

20 year-old forests that differed in origin and management regimes were collected: (1) a semi-natural forest of *P. massoniana* (subsect. *Pinus*; abbreviated as Masson-N) that spontaneously regenerated after a forest fire, (2) *P. massoniana* plantation (Masson-PL), and (3) *P. elliottii* plantation (subsect. *Australes*; Slash-PL). Details of soil sampling, experiment design and seedling planting can be found in Ning et al. (2020). The total number of soil samples for this study was 108 (4 cores × 3 focal trees × 3 plots × 3 sites) plus 6 additional soil cores from each site that served as controls (18 cores). At the beginning of the experiment, a sub-sample of soil from each 'focal' tree was collected (3 trees × 3 plots × 3 sites = 27 samples) for subsequent DNA extraction and analysis of the resident ECM fungal community. All potted soil samples were seeded with either native masson pine or exotic slash pine seeds and thinned after germination to keep a total of 8 seedlings per pot. Because masson and slash pines are fast-growing, the seedlings were harvested at 3- and 6-months after germination to quantify ECM colonization and examine fungal communities through Illumina MiSeq sequencing (see Supplemental methods for fungal community sampling and sequencing). Sequences of sample data are deposited in the NCBI sequence read archive with accession number SRP140037.

3. Statistical analysis

Patterns in ECM fungal community composition were analyzed and visualized with nonparametric multi-dimensional scaling (NMDS) using Bray-Curtis distance. To examine variation in composition of ECM fungi with respect to *Site*, *Host* as well as *Site × Host* interactions, we used program Primer v7 with PERMANOVA+ (PRIMERE Ltd., Lutton, Ivybridge, UK) on Bray-Curtis dissimilarity matrices. *Plot* identity nested in *Site* and 'focal' tree identity nested in *Plot* as random factors were included in the mixed model. By using the presence-absence data, we conducted network-based visualization (Cytoscape 3.7.1; Cline et al., 2007) to identify the relative proportion of ECM fungi shared between native and exotic pines, and between seedlings and the resident ECM community. The ECM exploration types, colonization type ('spore/sclerotia-based' or 'hyphae-based') and possible native ranges (based on UNITE species hypothesis v8.0) of ECM taxa were listed in Table S1 based on previous greenhouse/field bioassay studies (e.g. Taylor and Bruns, 1999).

We also followed a multinomial probability distribution method in Glassman et al. (2016) to determine if the frequency of certain ECM fungi increased or decreased in response to host-specific selection between 3-months and 6-months harvests. By transferring the presence data to probabilities (number of pots each ECM fungus gained, lost or had no change/total number of pots) of those three aforementioned transitions, chi-squared goodness-of-fit tests were applied to screen for ECM species where losses and/or gains between two harvest phases exceeded 10%. Three possible transitions using presence of each ECM fungal taxon in each bioassay seedling pot as the reference condition: loss (present 3-months, absent 6-months), no change (present or absent in both two collecting times) and gain (absent 3-months, present 6-months). Significant probabilities of gain, loss or no change are not mutually exclusive, suggesting that fungal species can have variation in response to certain conditions.

4. Results

All seedlings were well-colonized by ECM fungi. Root colonization increased in masson pine seedlings from 37.2 ± 2.5% (3-months) to 64.8 ± 3.6% (6-months), and in slash pine (56.9 ± 2.2% after 3-months, 75 ± 2.9% after 6-months).

After quality filtering there were 10,267,131 sequences for downstream analysis, with an average of 36,800 ± 279 (mean ± SE) reads per sample, and clustered into 517 OTUs. Sixty eight percent of the sequences (6,970,167) were identified as 83 putative ECM fungal OTUs. Species accumulation curves showed that most samples were well

saturated for ECM fungal species (Fig. S1), with average richness of 21 ECM fungal OTUs per soil sample and 8 OTUs per seedling.

Species of *Russula* were well represented in all resident ECM fungal communities (Fig. 1a), and abundances ranged from 19.4% sequencing reads in Masson-N to 40.4% in Slash-PL, and 64% in Masson-PL. Masson pines hosted *Tomentella* (12–25.6%) in combination with *Clavulina* (47.3% Masson-N) or *Pseudotomentella* (16.8% Masson-PL), whereas the slash pine resident community (Slash-PL) was dominated by *Amphinema* (37.9%) and *Pseudotomentella* (11%).

Significant differences among assembled ECM communities were determined by *Sites* (p = 0.018, PERMANOVA) and *Host* identity (p = 0.014, PERMANOVA) after 3-months, whereas only *Host* identity (p = 0.008, PERMANOVA) was key factor influencing ECM community composition of seedlings after 6-months (Table S2, Fig. S2). There were also significant spatial effects (tree location; p = 0.001 for 'focal' tree identity nested in *Plot*) on recruited ECM community composition of seedlings. In soils collected from Masson-N and Masson-PL sites, ECM communities of masson pine seedlings in both Phases were characterized by an abundance of *Rhizopogon* (56.8–68.3% of sequencing reads), *Tomentella* (17.5–32.1%) and *Tylospora* (2.6–9.2%), whereas communities in slash pine were composed of *Rhizopogon* (4.2–54%), *Tomentella* (25.9–45.8%), *Thelephora* (5.3–26.4%), and *Russula* (0.3–26.9%) (Fig. 1b). In Slash-PL soils, ECM communities in masson pine were dominated by *Rhizopogon* (68.3–71.3%), whereas communities in slash pines were initially dominated by *Amphinema* (56.8%) but switched to *Rhizopogon*-dominated (64%) after 6-months. There was negligible colonization (< 10%) in the 18 sterilized control pots due to a common greenhouse ECM contaminant species, *Thelephora terrestris* (OTU1214, see also Velmala et al., 2014).

By the end of 6-months, we recovered 70% (51/73) of ECM fungal communities for masson pine and 80% (56/73) for slash pine seedlings of the community documented at the sampling sites. Exotic slash pine seedlings also seemed to adapt well to subset of either local or cosmopolitan ECM fungal partners (Table S1). Ten ECM fungal OTUs (*Russula* OTU1352, *Russula* OTU1357, *Elaphomyces* OTU325, *Hydellum* OTU1257, *Hebeloma* OTU1193, *Tomentella* OTU1225, *Amphinema* OTU1112, *Lactarius* OTU1171, *Wilcoxina* OTU531, and *Phellodon* OTU1462) were only found in slash pine, whereas 5 OTUs (*Sebacina* OTU1430, *Clavulina* OTU1184, *Lactarius* OTU1341, *Inocybe* OTU1316, and *Entoloma* OTU1300) were only found in masson pine seedlings (Fig. 1c).

According to the Monte Carlo multinomial probability simulation, 16 OTUs associated with masson pine (e.g. all four *Rhizopogon* OTUs) and 10 OTUs associated with slash pine (e.g. *Rhizopogon* OTU1297) had stable associations with their host species (ranging from 59% to 91% probability) (Fig. 2; Supplementary Tables S3–S6). On the other hand, of those taxa associating with masson pine seedlings, the frequency of *Chloridium* OTU711 significantly decreased after 6-months, whereas 8 OTUs (e.g. *Russula* OTU1367) had an 11–54% probability of increasing. In contrast, 5 OTUs associating with slash pine declined in frequency from 3-months to 6-months (e.g. *Tylospora* OTU1494) and 6 OTUs (e.g. *Rhizopogon* OTU1463) identified had a 13–72% probability of increasing. The frequencies of *Rhizopogon* OTU1298, *Tomentella* OTU1219, and *Amphinema* OTU1288 varied among samples suggesting that these taxa varied in response to certain conditions.

5. Discussion

In the early stage (3-months) of seedling establishment, *Sites* provided various inocula pools that satisfied the host preference, e.g., consistent dominance of *Rhizopogon* in masson pine and optional combinations between slash pine and local ECM fungi (e.g. *Russula* and *Thelephora* in Masson-PL site versus *Amphinema* in Slash-PL site). By 6-months, however, only *Host* identity had a significant influence on ECM composition and species' dominance.

The strong effect of host on ECM fungal community recruitment in
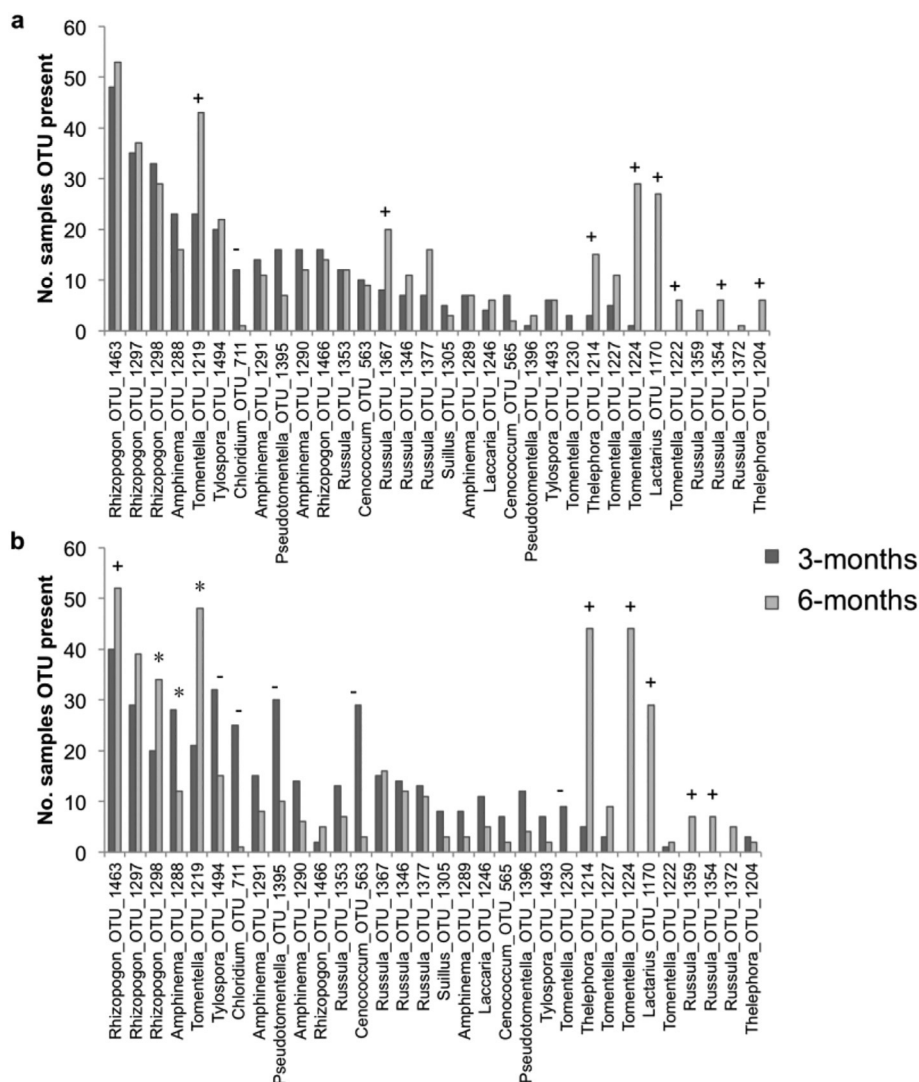
Fig. 2. Rank abundance curve by frequency for ectomycorrhizal fungal OTUs (total frequency > 6) recovered with **(a) masson pine** and **(b) slash pine** seedlings in 3-months and 6-months. The axes represent each ECM fungal OTU per total number of samples for either 3-months or 6-months bioassay seedlings. Upside marks indicate the probability of gain (+), loss (−), and gain and loss (*) of each OTU two collecting time changes based on transition analysis. Other OTUs had a significant probability of no change, i.e., a significant probability of no gain or loss.

soils containing the same species pools is in general agreement with other studies, especially for those comparing pine hosts (e.g. Rusca et al., 2006; Kohout et al., 2011; Liao et al., 2016; Ning et al., 2020). Such variation among pine species may be driven by the (in)compatible interaction between host and fungal partner, which was attributed to their co-evolutionary history (Liao et al., 2016). Our study also provides strong evidence that even at the subsection-level, different hosts (subsect. *Pinus* versus *Australes*) can recruit different ECM fungal communities.

We also found that the introduction of exotic slash pine appeared to facilitate the shift of the local ECM species pool with a positive interaction with *Rhizopogon*. This was especially notable in slash pine. The observation of *Rhizopogon* dominance combined with our previous field investigations (Fig. 1a and b; Ning et al., 2019) are consistent with other findings of the predominance of *Rhizopogon* spp. in greenhouse seedlings but sharply contrasts with data from forest soil communities (Taylor and Bruns, 1999; Glassman et al., 2016). Dominant ECM fungal species in bioassays usually correspond to the fungi that colonize naturally regenerating seedlings after severe disturbance in sites, such as fire and clear-cutting (e.g. Cowan et al., 2016; Glassman et al., 2016; Walker et al., 2016), and from extensive and long-lived soil spore banks

of resistant propagules. Thus, it is reasonable to suggest that seedlings planted into regenerating exotic pine plantations may show increased colonization by ECM fungi held in soil spore banks, with taxa such as *Rhizopogon* spp. becoming the predominant ECM fungi if seedlings are planted after large-scale disturbance (Bruns et al., 2009). This ability also appears to be an important factor in facilitating dispersal and expansion of exotic tree species by tens or hundreds of kilometers outside of their planted/managed ranges (Hayward et al., 2015; Urcelay et al., 2017).

On the other hand, this shift may reflect a change in the functional needs of the host since hosts can select beneficial mutualists from less beneficial counterparts (Hortal et al., 2017). In our previous study on enzymatic functioning in ECM fungal groups (Ning et al., 2020), we found that *Rhizopogon* spp. on slash pine seedlings showed a higher capacity for organic N and P mobilization (26–46% of relative contributions to the total root system enzymatic activities) in comparison with other ECM species, which tightly bounded to biomass accumulation and carbon content of hosts. Those high contributions enhanced the total enzymatic activities of exotic pine roots with a comparable level of native masson pines. Therefore, selection of high efficiency partners may be an important strategy for exotic slash pine to establish

in the local areas.

Host traits such as fine root growth patterns may also explain the different host-fungi interaction patterns between masson and slash pine. Slash pine seedlings provided a higher number of sites available for ECM fungal colonization as their mean short-root density was constantly higher than native masson pine's (3-months: 94 ± 12 for masson, 129 ± 16 for slash; 6-months: 233 ± 34 for masson, 494 ± 73 for slash), which may increase the potential for colonization by ECM fungi (Kennedy et al., 2009). This pattern of colonization could be expected to produce dense clusters of *Rhizopogon* ECM that prevented colonization by other ECM taxa. It is also possible that *Rhizopogon* (esp OTU1463) are very competitive, and part of the success may be due to fast rates of ECM root colonization (priority competitive effects; Kennedy et al., 2009) and/or as low-C consumers (Peay, 2016).

While *Rhizopogon* spp. were persistent in association with native masson pine, other ECM taxa, including *Russula*, *Tomentella* and *Tylospora*, were abundant on the roots of slash pine. The relatively 'stable' abundances of these fungi in this study and their broad host ranges (e.g. Looney et al., 2016) suggests that they are important fungi in the recovery of forest ecosystems. The use of 100% field collected soil core method ('mesocosm') for bioassay, with its full spectrum of fungal inocula including spores, hyphae and mycorrhizal roots, allowed us to detect these 'late successional' ECM fungi that disperse using vegetative mycelium to colonize new roots (Avis and Charvat, 2005; Kohout et al., 2011; Avis et al., 2017). Their considerable contributions to the total enzymatic activities of slash pine seedlings (e.g. *Russula*: 10–27%, *Tomentella*: 20–28%, *Tylospora*: 22–28%) and their distinct soil organic matter degradation patterns thus seems applicable for exotic tree establishment (Ning et al., 2020). However, cautions should be paid if some high efficiently functioning cosmopolitan/non-native ECM fungi (e.g. *Tomentella* OTU1214, *Tomentella* OTU1224) are preferentially selected by exotic pine, which may suppress the colonization chance of other local ECM species.

6. Conclusions

Using a bioassay method with natural soil cores, we found distinct ECM fungal community assembly patterns between native and exotic pine hosts in the early establishment process, even though communities of both hosts were largely dominated by *Rhizopogon* spp. Local *Rhizopogon* spp. are likely important mutualistic partners for forest regeneration of both native and exotic pine species in South China as has been reported elsewhere. Importantly, the introduction of an exotic pine was shown to potentially facilitate the shift of the local ECM fungal species pool by randomly or unstably interacting with available symbionts. In view of this result, caution is needed when using exotic hosts in afforestation process because the potential shift of assembled ECM fungal community under exotic trees may alter below ground functional capabilities and symbiosis-mediated successional trajectories.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2020.103722.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

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