Mycorrhiza
https://doi.org/10.1007/s00572-022-01088-z

ORIGINAL ARTICLE

# Exploring fine-scale assembly of ectomycorrhizal fungal communities through phylogenetic and spatial distribution analyses

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Received: 13 January 2022 / Accepted: 5 July 2022


## Abstract

Ectomycorrhizal fungi (EMF) form symbiotic relationship with the roots of host plants. EMF communities are composed of highly diverse species; however, how they are assembled has been a long-standing question. In this study, we investigated from a phylogenetic perspective how EMF communities assemble on *Pinus densiflora* seedlings at different spatial scales (i.e., seedling scale and root tip scale). *P. densiflora* seedlings were collected from different habitats (i.e., disturbed areas and mature forests), and their EMF communities were investigated by morphotype sequencing and next-generation sequencing (NGS). To infer assembly mechanisms, phylogenetic relatedness within the community (i.e., phylogenetic structure) was estimated and spatial distribution of EMF root tips was analyzed. The EMF communities on pine seedlings were largely different between the two habitats. Phylogenetically restricted lineages (*Amphinema*, /suillus–rhizopogon) were abundant in the disturbed areas, whereas species from diverse lineages were abundant in the mature forests (*Russula*, *Sebacina*, /tomentella–thelephora, etc.). In the disturbed areas, phylogenetically similar EMF species were aggregated at the seedling scale, suggesting that disturbance acts as a powerful abiotic filter. However, phylogenetically similar species were spatially segregated from each other at the root tip scale, indicating limiting similarity. In the mature forest seedlings, no distinct phylogenetic signals were detected at both seedling and root tip scale. Collectively, our results suggest that limiting similarity may be an important assembly mechanism at the root tip scale and that assembly mechanisms can vary across habitats and spatial scales.

**Keywords** Assembly mechanism · Disturbance · Fungal interaction · Limiting similarity · *Pinus densiflora* · Point pattern analysis

## Introduction

Ectomycorrhizal fungi (EMF) form intercellular symbiotic structures (i.e., ectomycorrhiza) in the roots of various host plants, providing water and nutrients to their hosts while receiving photosynthetic products in return (Smith and Read 2008). Although EMF colonize only a small portion of plant species (Brundrett and Tedersoo 2018), they can account for as much as 40% of the total microbial carbon biomass in a pine forest (Högberg et al. 2010). EMF play important ecological roles in forest communities, including reducing atmospheric $CO_2$ concentrations through carbon sequestration (Clemmensen et al. 2013; Averill et al. 2014) and circulating nutrients through the decomposition of diverse organic substrates (Sterkenburg et al. 2018; Nicolás et al. 2019). Given their prevalence and roles in forest ecosystems, their diversity is not surprising. Up to 200 EMF species can colonize an adult *Populus tremula* tree (Bahram et al. 2011), and tens of EMF species can coexist even on a single conifer seedling (Obase et al. 2009; Yoshida et al. 2014). However, how diverse EMF species assemble on a single host is largely understudied.

Community assembly is a process in which species assemble into a community from a regional species pool (HilleRisLambers et al. 2012). The process is largely driven by two contrasting assembly mechanisms: habitat filtering and limiting similarity. Habitat filtering selects species with suitable traits for a habitat, thus promoting the spatial aggregation of ecologically similar species (Weiher and Keddy 1995). In contrast, limiting similarity results in the spatial segregation of ecologically similar species, as species with overlapping niche preferences competitively exclude

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Published online: 21 July 2022

each other (MacArthur and Levins 1967). The assembly mechanisms can be inferred from the ecological similarity of the species within the community, but it is challenging to estimate the ecological traits of some organisms. To overcome such limitation, phylogenetic relatedness has been used as a proxy for multidimensional ecological traits as phylogenetically similar species are likely to have similar ecological traits (Swenson and Enquist 2009; Kraft and Ackerly 2010; Wiens et al. 2010). Therefore, phylogenetic relatedness of the species within a community (i.e., the phylogenetic structure) can reflect the assembly mechanisms: spatial aggregation of phylogenetically similar species (i.e., phylogenetic clustering) reflects habitat filtering, whereas spatial segregation of phylogenetically similar species (i.e., phylogenetic overdispersion) reflects limiting similarity (Webb 2000).

These contrasting assembly mechanisms can occur simultaneously within a community, but with different strengths across spatial scales (Cavender-Bares et al. 2006; Silvertown et al. 2006; Emerson and Gillespie 2008). Phylogenetic structure of EMF communities has been investigated at varying size of sampling units, i.e., bulk soil (Peay et al. 2010; Pena et al. 2017), bulk root (Schröter et al. 2019) or individual seedling level (Rincón et al. 2014). However, the assembly mechanism at finer spatial scales has not been investigated from a phylogenetic perspective. Although a few studies have explored spatial distributions of individual EMF root tips on the root system to infer root tip scale assembly mechanisms (Yoshida et al. 2014; Thoen et al. 2019), these did not consider phylogenetic structure and the sample size was limited.

In this study, the main objective was to investigate from a phylogenetic perspective how EMF communities assemble on a single host plant at two different spatial scales: seedling scale represents the entire EMF community of a seedling, whereas root tip scale represents the EMF community composed of several neighboring root tips. To this end, *Pinus densiflora* seedlings were collected from two regions × two habitat types (disturbed area and mature forest). The EMF community of each seedling was investigated using morphotype sequencing and next-generation sequencing (NGS) methods. Then, assembly mechanisms on two different spatial scales were inferred. At the seedling scale, only the phylogenetic structure was estimated. At the root tip scale, we evaluated whether the phylogenetic structure could explain the spatial distribution of individual EMF root tips.

## Materials and methods

### Seedling collection

*P. densiflora* seedlings were collected from four sampling plots (two regions × two habitats): Ganghwa disturbed area (GD), Ganghwa mature forest (GF), Inje disturbed area (ID), and Inje mature forest (IF). Ganghwa and Inje are about 170 km apart in South Korea. Ganghwa is a low-altitude (avg., 124 m) region, whereas Inje is a high-altitude (avg., 645 m), mountainous region. GF comprised of a single tree species, *P. densiflora* (70–130 years old), whereas IF was a *P. densiflora* dominant forest (60–80 years old) with a few *Quercus mongolica*. GD was an anthropogenically disturbed area where all *P. densiflora* seedlings were less than one-year-old. ID was recovering from a landslide caused by heavy rains, and *P. densiflora* seedlings were up to seven years old (Fig. S1a). In total, 160 healthy pine seedlings under one-year-old (40 seedlings per sampling plot) were collected (Fig. S1b) with at least a 3-m distance between each other to avoid autocorrelation (Pickles et al. 2012). To retrieve intact seedlings during the collection, 30 × 30 × 30 cm soil cube was sampled with the seedling and then the soil was removed carefully. The seedlings were placed separately in plastic bags and transported to the laboratory in an icebox. The entire root systems were cut from the stems, and soil debris was rinsed off with water using a bottle with beak-shaped nozzle. The root systems were stored in sterile water at − 20 °C until observation and DNA extraction. The root systems from each sampling plot were grouped into two halves: one half for morphotype sequencing, the other half for NGS.

### Morphotypegrouping and sequencing

Eighty root systems (20 roots × 4 plots) were placed separately on a water-filled lid of the Petri dish (90 by 15 mm or 150 by 20 mm, depending on the size of the root system). Root segments were carefully unfolded with forceps to minimize overlapping. The root topology was fixed by placing water-filled bottom of the Petri dish on the root system, and photographs of each root system were taken on a white background (Fig. S1c). The photographs of the root systems were used as a basis for counting and marking EMF morphotypes.

All vital EMF root tips per seedling were morphologically grouped and counted at their original position under a dissecting microscope at magnifications of 20–60 × (Agerer 1991). Vital EMF root tips were mantle covered, turgescent, and fresh, thus distinguished from non-vital or non-mycorrhizal root tips. Four seedlings were excluded from further processing because non-vital EMF root tips were dominant. To improve the accuracy of each morphotype, three or more representative root tips were longitudinally hand-sectioned and their outer mantle surfaces were observed under an Eclipse 80i light microscope (Nikon, Tokyo, Japan) at magnifications of 400 × and 1000 ×. Following morphological features were used for morphotyping: color, texture, outer surface of mantle; presence, abundance, and shape of emanating hyphae or cystidia; presence, abundance, ramification, and differentiation of rhizomorphs; tightly attached debris (plant litter, soil particles or minerals) (Agerer 1991). The EMF root tip branching

directly from the lateral root was counted as one, and when one infected individual root tip was branched and enlarged, it was also counted as one. No attempts were made to relate the presence of EMF species between seedlings until molecular identification was done.

To confirm the consistency of morphotypes, molecular identification was performed. DNA was extracted from three or more representative root tips of each morphotype on each seedling. If one morphotype grew on another, DNA was extracted separately from the apical and basal parts of the root tip. Individual root tips were ground in microtubes filled with 40 µL of Instagene™ Matrix (Bio-Rad, Hercules, CA, USA), boiled for 10 min, and centrifuged at 6000 rpm for 5 min. One microliter of the supernatant was used as a template DNA for PCR. The internal transcribed spacer (ITS) region was amplified with NSI1/NLB4 primers (Martin and Rygiewicz 2005). Amplification conditions were set as described by Cho et al. (2021). PCR products were sequenced at Macrogen (Seoul, South Korea). The sequences were edited using Geneious Prime® 2020.2.2 (https://www.geneious.com). Morphotype consistency was validated by assembling all sequences using the de novo assembly function (minimum overlap identity = 99%, minimum overlap = 100 bp) in Geneious Prime®. The resulting consensus sequences were considered representative sequences of each root tip operational taxonomic unit (rtOTU). All representative sequences were subjected to BLAST using UNITE v.8.2 (Nilsson et al. 2019) and the Seoul National University Fungus Collection (SFC) database. According to the closest species hypothesis (SH) reported by Kõljalg et al. (2013), if a query matched the closest species hypothesis (SH) with 97%, 90%, 85%, and 80% similarity, the taxonomic name of species, genus, family, and order was assigned, respectively.

## NGS

The remaining 80 root systems (20 roots × 4 plots) were processed for NGS. Each root system was surface sterilized by sonication for 3 min, submerged in 3% $H_2O_2$ for 1 min, and then washed with sterile distilled water three times. The root systems were dried overnight on a Petri dish with filter paper on a clean bench at room temperature. Dried root systems were gently rubbed in sterile oil paper to collect root tips. Collected root tips were ground in liquid $N_2$ using a mortar and pestle. DNA was extracted from 10 mg of root powder using the DNeasy® PowerPlant® Pro Kit (Qiagen GmbH, Hilden, Germany). The ITS2 region was amplified using 5.8S-Fun and ITS4-Fun primers (Taylor et al. 2016). PCR and NGS preparation were conducted using the protocol described by Park et al. (2021). One seedling was excluded from further procedure because of PCR failure. Sequencing was performed on an Illumina MiSeq platform (Macrogen, Seoul, South Korea). The sequences were processed with QIIME v.1.8.0. (Caporaso et al. 2010). The paired-end sequences were merged (maximum mismatch = 3%, minimum overlap = 50 bp), and low-quality sequences were filtered out ($Q < 30$). The sequences were clustered into OTUs with 97% similarity using Vsearch v.2.6.2 (Rognes et al. 2016), and chimeric sequences were removed based on the UCHIME database (Edgar et al. 2011). The most abundant sequences from each OTU were considered representative sequences. The sequences were identified using BLAST, and EMF OTUs were filtered based on the EMF lineages described in UNITE v.8.2. Only EMF OTUs representing more than 0.005% of the total read counts were used for further analysis.

## EMF community composition and phylogenetic structure

The ITS2 region sequences obtained by both sequencing methods were aligned using MAFFT ver. 7.450 (Katoh and Standley 2013), and nucleotide positions containing more than 30% gaps were removed using the mask alignment function in Geneious Prime®. A phylogenetic tree was constructed using BEAST v.2.6.3 (Bouckaert et al. 2019), with a Yule tree prior and a relaxed lognormal clock model (Drummond et al. 2006). *Kuzuhaea moniliformis* (NR111188) was used as an outgroup. To overcome the limitations of constructing a deep phylogenetic tree using ITS2 sequences, topological and chronological constraints were applied from phylum to family level based on fossils or previous multigene or genome-scale phylogenetic studies on the kingdom Fungi (Lepage et al. 1997; Garnica et al. 2016; Zhao et al. 2017; Chang et al. 2019; Varga et al. 2019; Miyauchi et al. 2020) (Table S1). Markov chain Monte Carlo sampling was performed for every $10^3$ generations over a total of $10^7$ iterations. The 10,000 resulting trees were summarized into a maximum clade credibility (MCC) tree.

EMF community composition was summarized into an OTU table and then normalized using the scaling with ranked subsampling method (Beule and Karlovsky 2020). The dissimilarity in community composition between all possible pairs of seedlings was determined by weighted UniFrac distance (Lozupone and Knight 2005; Lozupone et al. 2007). Phylogenetic structure was estimated for all seedlings using the nearest taxon index (NTI) (Webb 2000), except those with a single EMF species (23 seedlings from the morphotype sequencing). The NTI is a standardized value of mean nearest taxon distance (MNTD), which considers the phylogenetic distance between every pair of phylogenetically closest taxa in the community. The NTI was calculated based on the MCC tree, using the *picante* package (Webb et al. 2008; Kembel et al. 2010). A random null model was generated by randomly drawing species from the MCC tree, with 999 permutations.

### Spatial distribution analysis

Spatial distribution of EMF root tips was analyzed for 47 of 76 seedlings investigated by morphotype sequencing (Fig. S2). Twenty-nine seedlings were excluded from the spatial distribution analysis, including seedlings with a single EMF species (23 seedlings), low number of vital root tips (less than 35 root tips), or low density of vital root tips (less than 1.5 root tips per cm on average). Root systems were reproduced as a linear network using the Smartroot plugin (Lobet et al. 2011) implemented in Fiji (Schindelin et al. 2012; Rueden et al. 2017). Each morphotype was marked with a different color on the linear network using the multipoint function in Fiji (ImageJ). Finally, all EMF root tips were mapped on the linear network using the *spatstat* package implemented in R (Baddeley et al. 2015). To estimate the spatial segregation among EMF species on the root systems, the species segregation index (SSI) was calculated for each seedling. SSI is an index originally developed to estimate the degree of spatial segregation among multivariate tree species in a research plot, which is irrelevant to the alpha diversity of the research plot (Pommerening and Uria-Diez 2017). SSI can also be applied to the EMF community within the root system and was calculated as follows. First, mingling index is obtained by calculating the mean fraction of heterospecific root tips among the $k$ nearest neighbors of a given root tip $i$ (Eq. 1). After calculating the mingling index for every root tip on the root system, expected mingling value (E$M$) was calculated (Eq. 2, $n$ = the number of species, $N$ = the total number of root tips in a seedling, $N_s$ = the number of species $s$). Finally, SSI was obtained by dividing the mean mingling index ($\overline{M}^{(k)}$) with E$M$ (Eq. 3). Through this normalization, SSI is normalized by the alpha diversity of EMF community. In this study, the $k$ value was fixed to three, that is, to consider three nearest neighbors to infer the mingling index of a given root tip. The three nearest neighboring root tips were determined for every root tip using the "nnwhich.lpp" function in the *spatstat* package.

$$M_i^{(k)} = \frac{1}{k}\sum_{j=1}^{k} 1\left(\text{species}_i \neq \text{species}_j\right) \tag{1}$$

$$\text{E}M = \sum_{s=1}^{n} \frac{N_s\left(N - N_s\right)}{N(N-1)} \tag{2}$$

$$\text{SSI} = 1 - \frac{\overline{M}^{(k)}}{\text{E}M} \tag{3}$$

### Statistical analysis

Statistical analyses were conducted using R v.4.1.1 (R Core Team 2021). Differences in community composition according to habitat and region were evaluated by PERMANOVA with 999 permutations, using the "adonis" function in the *vegan* package (Oksanen et al. 2018). Significantly differentially abundant OTUs between habitats were detected using the multinomial species classification method (CLAM) test (Chazdon et al. 2011) in the *vegan* package with a default setting. Significant deviation of the NTI and SSI from 0 (null expectation) was tested using the one-sample Wilcoxon test. Differences in the NTI among sampling plots were evaluated using the Kruskal–Wallis rank sum test and Nemenyi test with Bonferroni correction in the PMCMR package (Pohlert 2014). Differences in the SSI among sampling plots were evaluated using one-way ANOVA and Tukey's honestly significant difference test. Simple linear regression was performed between the NTI and SSI to investigate whether the phylogenetic structure could explain the spatial segregation among multiple EMF species. Numerical data were presented as mean ± standard deviation.

## Results

### EMF community composition

In total, 155 seedlings were investigated by morphotype sequencing (76 seedlings) or NGS (79 seedlings) (Table S2). For the 76 seedlings investigated with morphotype sequencing, 6532 root tips were counted and 679 representative root tips were sequenced, yielding 59 EMF species (Table S3). On average, 85.9 ± 42.2 root tips were observed per seedling, and the number of EMF species per seedling was similar between the disturbed areas (2.4 ± 1.2) and mature forests (2.5 ± 1.3). For the 79 seedlings investigated with NGS, a total of 16,136,380 reads were generated. After removing untargeted reads, 5,797,730 sequences were assigned to 61 EMF species (Table S3). Each seedling yielded 73,389.0 ± 30,618.6 EMF reads, which accounted for 87.7% ± 20.8% of the total fungal reads. After normalization, the disturbed area seedlings harbored 6.9 ± 3.0 EMF species, whereas the mature forest seedlings harbored 10.6 ± 4.2 EMF species. Through both sequencing methods, a total of 90 EMF species was detected, with 30 EMF species overlapping between the two methods (Table S3). Species in Ceratobasidiaceae, *Amanita*, *Clavulina*, and *Tomentellopsis* were only detected by morphotype sequencing, whereas species in *Cortinarius*, *Endogone*, *Hydnotrya*, *Inocybe*, and *Wilcoxina* were only detected using NGS.

EMF community compositions differed significantly between habitats and regions ($p = 0.001$). Habitat had a greater influence on community composition than region in both sequencing methods (Table 1). In the disturbed area, species in *Amphinema* and /suillus–rhizopogon accounted for most of the richness and abundance (Fig. 1). This

**Table 1** ADONIS results showing differences in community composition when using different sequencing methods

| Sequencing method | Variable | Df | SumsOfSqs | MeanSqs | F.Model | $R^2$ | $p$ |
|---|---|---|---|---|---|---|---|
| Morphotype sequencing | Region | 1 | 0.64 | 0.64 | 4.78 | 0.04 | 0.001 |
| | Habitat | 1 | 2.60 | 2.60 | 19.47 | 0.17 | 0.001 |
| | Subplot | 16 | 4.78 | 0.30 | 2.23 | 0.31 | 0.001 |
| | Residuals | 57 | 7.62 | 0.13 | – | 0.49 | – |
| | Total | 75 | 15.64 | – | – | 1.00 | – |
| NGS (Illumina MiSeq) | Region | 1 | 0.42 | 0.42 | 11.85 | 0.07 | 0.001 |
| | Habitat | 1 | 0.90 | 0.90 | 25.16 | 0.15 | 0.001 |
| | Subplot | 16 | 2.53 | 0.16 | 4.43 | 0.42 | 0.001 |
| | Residuals | 60 | 2.15 | 0.04 | – | 0.36 | – |
| | Total | 78 | 6.00 | – | – | 1.00 | – |

resulted in phylogenetically restricted EMF communities in the disturbed areas. *Amphinema* species showed plot specificity for the ID seedlings (Fig. S3). Many species in diverse lineages were exclusively abundant in the mature forests, including species in Agaricales, *Coltriciella*, *Endogone*, *Russula*, *Sebacina*, and /tomentella–thelephora (Fig. 1). In the mature forest seedlings, species of /suillus-rhizopogon were rare in morphotype sequencing data but abundant in NGS data (Fig. S3).

### Phylogenetic structure of EMF community at the seedling scale

The phylogenetic structure was analyzed using the NTI for each seedling with more than one EMF species. The NTI was estimated from both morphotype sequencing (53 seedlings, Fig. 2a) and NGS (79 seedlings, Fig. 2b) data. Positive NTI values indicate phylogenetic clustering, whereas negative values indicate phylogenetic overdispersion. In the disturbed areas, the NTI positively deviated from the null model (phylogenetic clustering) in both sequencing methods (Fig. 2a, b). In the mature forests, the NTI did not significantly differ from the null model in the morphotype sequencing data (Fig. 2a), but showed phylogenetic clustering in the NGS data (Fig. 2b). On average, the NTI was higher (phylogenetically more clustered) in the disturbed areas than in the mature forests, regardless of the region and sequencing method (Fig. 2a, b). GD showed the highest NTI value among the sampling plots, and its NTI was significantly higher than that of the mature forests in both sequencing methods (Fig. 2a, b).

### Spatial distribution of EMF species at the root tip scale

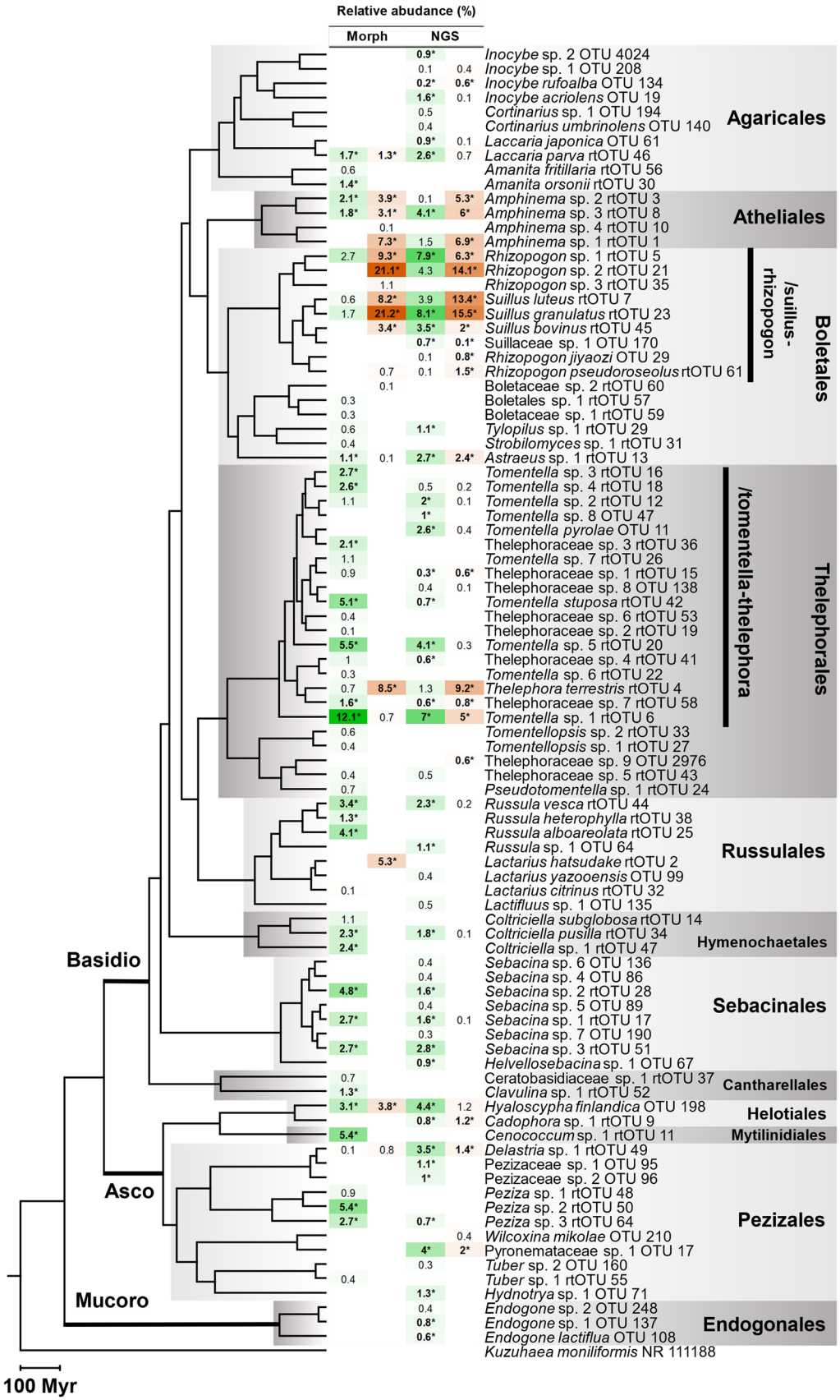
Maps for calculating the SSI were reproduced from 47 seedlings used in morphotype sequencing (Fig. 3a, b). The total length of the root system ranged from 7.0 to 69.7 cm, and the mean length of the root system for each plot was as follows in cm: $42.1 \pm 14.3$ (GD), $32.8 \pm 14.5$ (GF), $27.9 \pm 10.4$ (ID), $20.1 \pm 8.6$ (IF). On average, $2.9 \pm 1.3$ EMF root tips were found within a centimeter. Complete segregation among different species resulted in SSI = 1, whereas random distribution of the root tips led to SSI = 0. Generally, different EMF species were spatially segregated from each other, as the SSI values of all sampling plots were significantly higher than 0 (Fig. 3c). The SSI values of the disturbed area seedlings were higher than those of the mature forest seedlings, with the highest values noted for the GD seedlings (Fig. 3c).

### Effect of phylogenetic structure on the spatial distribution of EMF species at the root tip scale

Simple linear regression showed that the SSI significantly increased as the NTI increased (adjusted $R^2 = 0.20$, $p < 0.001$) (Fig. 4a). When regression was performed for each habitat, a significant positive linear relationship was found for the disturbed area seedlings ($n = 25$, adjusted $R^2 = 0.19$, $p < 0.05$), whereas no relationship was detected in the mature forest seedlings ($n = 22$, adjusted $R^2 = 0.021$, $p = 0.24$) (Fig. 4b). Competitive displacement events (i.e., one EMF species displacing another at the root tip) were observed between several pairs of phylogenetically dissimilar species (Fig. S4). The seedlings with competitive displacement showed low SSI ($0.40 \pm 0.21$) and NTI ($0.36 \pm 0.62$) (Fig. 4a).

## Discussion

To understand how EMF communities assemble at a fine scale, we investigated the phylogenetic structure and spatial distribution of EMF root tips on the root systems of *P. densiflora* seedlings. By using standardized indices (NTI and SSI) independent of alpha diversity, we could statistically compare EMF assembly mechanisms across different spatial scales, habitats, and regions. Phylogenetic structure enabled


Relative abudance (%)
Morph
NGS
Inocybe sp. 2 OTU 4024
Inocybe sp. 1 OTU 208
Inocybe rufoalba OTU 134
Inocybe acriolens OTU 19
Cortinarius sp. 1 OTU 194
Cortinarius umbrinolens OTU 140
Laccaria japonica OTU 61
Laccaria parva rtOTU 46
Amanita fritillaria rtOTU 56
Amanita orsonii rtOTU 30
Agaricales
Amphinema sp. 2 rtOTU 3
Amphinema sp. 3 rtOTU 8
Amphinema sp. 4 rtOTU 10
Amphinema sp. 1 rtOTU 1
Atheliales
Rhizopogon sp. 1 rtOTU 5
Rhizopogon sp. 2 rtOTU 21
Rhizopogon sp. 3 rtOTU 35
Suillus luteus rtOTU 7
Suillus granulatus rtOTU 23
Suillus bovinus rtOTU 45
Suillaceae sp. 1 OTU 170
Rhizopogon jiyaozi OTU 29
Rhizopogon pseudoroseolus rtOTU 61
/suillus-rhizopogon
Boletaceae sp. 2 rtOTU 60
Boletales sp. 1 rtOTU 57
Boletaceae sp. 1 rtOTU 59
Tylopilus sp. 1 rtOTU 29
Strobilomyces sp. 1 rtOTU 31
Astraeus sp. 1 rtOTU 13
Boletales
Tomentella sp. 3 rtOTU 16
Tomentella sp. 4 rtOTU 18
Tomentella sp. 2 rtOTU 12
Tomentella sp. 8 OTU 47
Tomentella pyrolae OTU 11
Thelephoraceae sp. 3 rtOTU 36
Tomentella sp. 7 rtOTU 26
Thelephoraceae sp. 1 rtOTU 15
Thelephoraceae sp. 8 OTU 138
Tomentella stuposa rtOTU 42
Thelephoraceae sp. 6 rtOTU 53
Thelephoraceae sp. 2 rtOTU 19
Tomentella sp. 5 rtOTU 20
Thelephoraceae sp. 4 rtOTU 41
Tomentella sp. 6 rtOTU 22
Thelephora terrestris rtOTU 4
Thelephoraceae sp. 7 rtOTU 58
Tomentella sp. 1 rtOTU 6
/tomentella-thelephora
Tomentellopsis sp. 2 rtOTU 33
Tomentellopsis sp. 1 rtOTU 27
Thelephoraceae sp. 9 OTU 2976
Thelephoraceae sp. 5 rtOTU 43
Pseudotomentella sp. 1 rtOTU 24
Thelephorales
Russula vesca rtOTU 44
Russula heterophylla rtOTU 38
Russula alboareolata rtOTU 25
Russula sp. 1 OTU 64
Lactarius hatsudake rtOTU 2
Lactarius yazooensis OTU 99
Lactarius citrinus rtOTU 32
Lactifluus sp. 1 OTU 135
Russulales
Coltriciella subglobosa rtOTU 14
Coltriciella pusilla rtOTU 34
Coltriciella sp. 1 rtOTU 47
Hymenochaetales
Basidio
Sebacina sp. 6 OTU 136
Sebacina sp. 4 OTU 86
Sebacina sp. 2 rtOTU 28
Sebacina sp. 5 OTU 89
Sebacina sp. 1 rtOTU 17
Sebacina sp. 7 OTU 190
Sebacina sp. 3 rtOTU 51
Helvellosebacina sp. 1 OTU 67
Sebacinales
Ceratobasidiaceae sp. 1 rtOTU 37
Clavulina sp. 1 rtOTU 52
Cantharellales
Hyaloscypha finlandica OTU 198
Cadophora sp. 1 rtOTU 9
Helotiales
Cenococcum sp. 1 rtOTU 11
Mytilinidiales
Delastria sp. 1 rtOTU 49
Pezizaceae sp. 1 OTU 95
Pezizaceae sp. 2 OTU 96
Peziza sp. 1 rtOTU 48
Peziza sp. 2 rtOTU 50
Peziza sp. 3 rtOTU 64
Wilcoxina mikolae OTU 210
Pyronemataceae sp. 1 OTU 17
Tuber sp. 2 OTU 160
Tuber sp. 1 rtOTU 55
Hydnotrya sp. 1 OTU 71
Asco
Pezizales
Endogone sp. 2 OTU 248
Endogone sp. 1 OTU 137
Endogone lactiflua OTU 108
Mucoro
Endogonales
Kuzuhaea moniliformis NR 111188
100 Myr

◂**Fig. 1** Phylogenetic community composition of *P. densiflora* seedlings from each habitat. An MCC tree was generated by Bayesian inference of an ITS2 sequence alignment from a total of 90 EMF species and one outgroup species (alignment length = 347 bp). The scale bar indicates 100 million years. The heatmap shows the relative abundance of each species in each habitat obtained by morphotype sequencing (Morph) and NGS. Differently colored blocks represent different habitats (brown = disturbed area, green = mature forest). Asterisks indicate significantly abundant species in each habitat detected by the CLAM test. Basidio = Basidiomycota, Asco = Ascomycota, Mucoro = Mucoromycota

us to infer relationships between species, which cannot be obtained from a simple taxonomic structure. In addition, for the uncultured microorganisms whose characteristics are difficult to measure, phylogenetic structure is useful to infer the overall assembly mechanism. Although the spatial distribution of the root tips on our 2D root system does not intactly reflect the 3D ordination of root system, we could infer the general spatial distribution pattern based on the fact that neighboring root tips on the root system are more likely to be adjacent in situ.

The two sequencing methods used in the present study showed little methodological bias, despite different seedlings being used between sequencing methods. Both revealed a similar pattern of EMF community composition: differed more strongly between habitats than between regions. A similar pattern was obtained between sequencing methods because seedlings from the same sampling plots were randomly shuffled before subjected to different sequencing

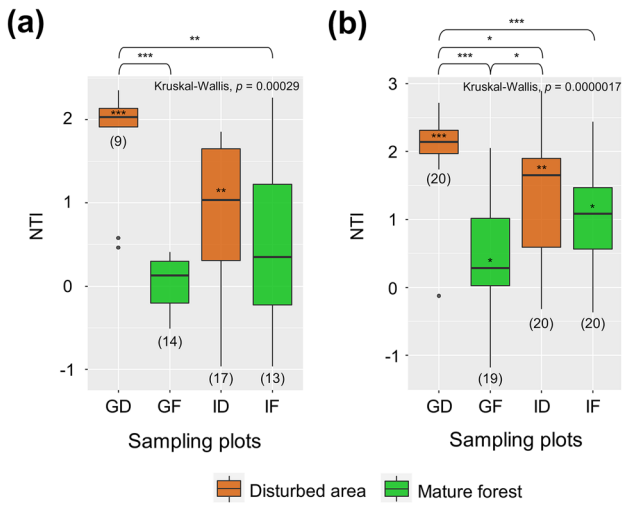



**Fig. 2** Phylogenetic structure analysis at the seedling scale. The nearest taxon index (NTI) was estimated from the EMF community on each *P. densiflora* seedling. Two different datasets were used: (**a**) morphotype sequencing ($n = 53$) and (**b**) NGS ($n = 79$). The numbers in parentheses below the box plot indicate the number of seedlings investigated per sampling plot. Asterisks in the box plot indicate significant deviation from the null expectation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

methods. The EMF community composition of each habitat was consistent with previously reported compositions in pine seedlings. In seedlings of the disturbed area, species of /suillus–rhizopogon and *Amphinema* as well as *Thelephora terrestris* were most abundant. These species are well known to dominate pine seedlings after a disturbance event (Horton et al. 1998; Baar et al. 1999; Buscardo et al. 2010; Rincón et al. 2014). However, *Amphinema* species showed some specificity on ID seedlings among disturbed area seedlings. It appears that ID seedlings were influenced by the older *P. densiflora* seedlings (up to seven years old) nearby, since Atheliaceae species preferred older pine seedlings over pine seedlings under one-year-old (Obase et al. 2009). In the mature forest seedlings, species from diverse ectomycorrhizal lineages were detected, including species of /tomentella–thelephora, *Coltriciella*, *Endogone*, *Russula*, and *Sebacina*. Species in these clades are well known to colonize *P. densiflora* seedlings in mature forests (Sim and Eom 2009; Ma et al. 2010). However, mature forest EMF community composition was different between the two sequencing methods in that /suillus-rhizopogon species were only abundant in NGS data. Although direct comparison is impossible because different samples were used for the two sequencing methods, it can be speculated that DNA was detected from degraded tissues of /suillus-rhizopogon species, which were replaced by other competitive EMFs in mature forest.

EMF communities from the disturbed areas showed strong phylogenetic clustering at the seedling scale, regardless of the sampling region and sequencing method. This suggests that disturbance acts as a powerful abiotic filter, which assembles phylogenetic clades that are suitable for the environment. Indeed, /suillus–rhizopogon species were highly diverse and abundant in the disturbed area seedlings. They share traits such as long-distance exploration type (Agerer 2001, 2006) and host specificity for Pinaceae seedlings (Molina et al. 1992; Molina and Horton 2015). Therefore, the clustering of phylogenetically and ecologically similar species strongly supports habitat filtering in the disturbed area seedlings. Disturbance has also been shown to be a dominant factor affecting other fungal communities, often leading to phylogenetic clustering at a bulk soil scale (Sigisfredo et al. 2013; Mykrä et al. 2016; Mikryukov et al. 2020). However, the phylogenetic community structure largely varied depending on the intensity or type of disturbance and fungal trophic mode (Geedicke et al. 2016; Schröter et al. 2019; Mikryukov et al. 2020).

At the root tip scale, phylogenetically similar EMF species were spatially more segregated from each other in the disturbed area seedlings. This trend is in line with the concept of limiting similarity, as phylogenetically closely related species spatially excluded each other. Such spatial segregation among EMF species has been considered a result of a "priority effect", as root tip monopolization by early-arriving

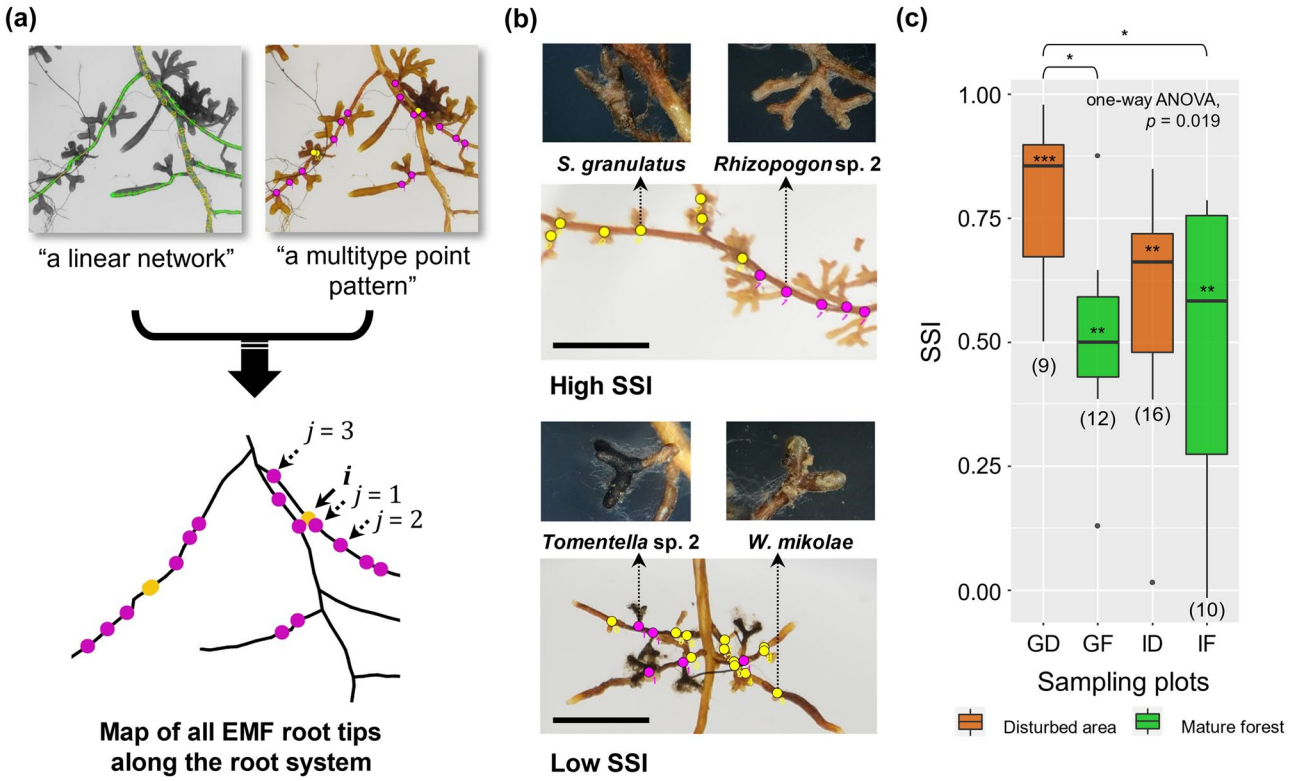



**Fig. 3** Spatial distribution analysis at the root tip scale. **a** Schematic diagram of the estimation of the species segregation index (SSI) from the map of all EMF root tips along the root system: "a linear network" indicates a root system of a *P. densiflora* seedling, and "a multitype point pattern" depicted by different colors indicates different species. The three nearest root tips were considered neighbors ($k=3$) for every root tip $i$. $j=k$ indicates $k$ nearest neighbor of an $i$. **b** Examples of high and low SSI values. Scale bar = 5 mm. **c** SSI estimated from the EMF community on *P. densiflora* seedlings ($n=47$). The numbers in parentheses below the box plot indicate the number of seedlings investigated per sampling plot. Asterisks in the box indicates significant deviation from the null expectation ($*p<0.05$, $**p<0.01$, $***p<0.001$)

species spatially excludes late-arriving species (Kennedy et al. 2009; Yoshida et al. 2014; Thoen et al. 2019). Previous in vitro studies on filamentous fungi and yeasts have shown a stronger priority effect between phylogenetically more similar species (Peay et al. 2012; Morales et al. 2016). Pairwise inoculation of 37 wood-decaying fungal strains also resulted in spatial segregation between phylogenetically similar species, which was termed a "deadlock" (Maynard

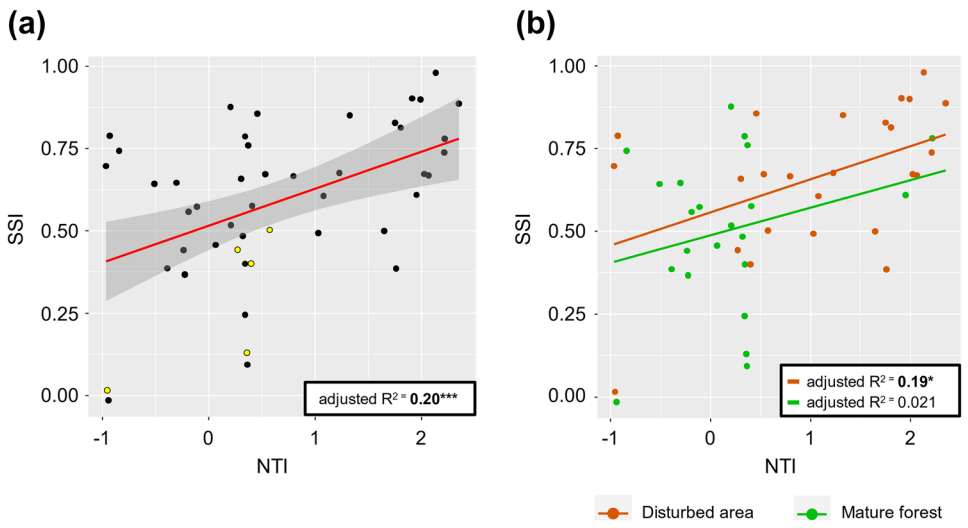



**Fig. 4** Simple linear regression between nearest taxon index (NTI) and species segregation index (SSI). Analysis was performed for (**a**) 47 *P. densiflora* seedlings and (**b**) the seedlings per habitat, respectively (disturbed area: $n=25$, mature forest: $n=22$). Each circle indicates a *P. densiflora* seedling, and yellow filled circles indicate seedlings for which competitive displacement events were observed. The number of asterisks indicates the degree of significant relationship ($*p<0.05$, $**p<0.01$, $***p<0.001$)

et al. 2017). Although different terminologies have been used to describe the spatial exclusion among fungal species, our results and previous studies generally support spatial segregation among phylogenetically similar fungal species.

Distinct phylogenetic signals were detected at both spatial scales (seedling scale and root tip scale) in seedlings of disturbed areas. However, mature forest seedlings did not show an obvious phylogenetic signal. Given that the EMF species richness of mature forests was higher than that of disturbed areas, more complex networks among species may have resulted in unpredictable spatial distributions. This speculation is based on a result reported by Maynard et al. (2017), who showed that the biodiversity of a fungal community can buffer competitive exclusion through a complex interaction network. However, as there are numerous assembly mechanisms that cannot be inferred through phylogenetic signals, experimental validation is required to prove EMF assembly mechanisms in mature forests.

## Conclusion

Collectively, the results highlight that assembly mechanisms vary across spatial scales (the seedling scale and the root tip scale). In the disturbed areas, EMF communities assembled through habitat filtering at the seedling scale, but rather through limiting similarity at the root tip scale. The lack of significant phylogenetic signals among EMF communities in the mature forests indicates that assembly mechanisms can also vary across habitat types. However, this study only inferred assembly mechanisms from a phylogenetic perspective. Therefore, further experimental validation is needed, e.g., by inoculating phylogenetically and functionally diverse EMF species into host plants. Nevertheless, the results are expected to contribute to the fundamental understanding of EMF community assembly and interaction among different EMF species.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00572-022-01088-z.

**Author contribution** Shinnam Yoo and Young Woon Lim designed the research. Shinnam Yoo, Yoonhee Cho, and Young Woon Lim collected the samples. Shinnam Yoo and Yoonhee Cho conducted the laboratory experiments. Shinnam Yoo analyzed the data with the help of Ki Hyeong Park. Shinnam Yoo wrote the manuscript draft, and all authors reviewed and edited the manuscript. Young Woon Lim supervised the research.

**Funding** This study was supported by the Korea National Arboretum (grant number KNA1-1–25, 19–2).

**Data availability** The amplicon sequence data generated in this study are available in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/), with links to BioProject accession number PRJNA796051. The sequences of EMF OTUs generated in this study are available in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/) under accession numbers OM236546–OM236665. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing interests** The authors declare no competing interests.

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