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Research

Community assembly of ectomycorrhizal fungi along a subtropical secondary forest succession

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Summary

- Environmental selection and dispersal limitation are two of the primary processes structuring biotic communities in ecosystems, but little is known about these processes in shaping soil microbial communities during secondary forest succession.
- We examined the communities of ectomycorrhizal (EM) fungi in young, intermediate and old forests in a Chinese subtropical ecosystem, using 454 pyrosequencing.
- The EM fungal community consisted of 393 operational taxonomic units (OTUs), belonging to 21 EM fungal lineages, in which three EM fungal lineages and 11 EM fungal OTUs showed significantly biased occurrence among the young, intermediate and old forests. The EM fungal community was structured by environmental selection and dispersal limitation in old forest, but only by environmental selection in young, intermediate, and whole forests. Furthermore, the EM fungal community was affected by different factors in the different forest successional stages, and the importance of these factors in structuring EM fungal community dramatically decreased along the secondary forest succession series.
- This study suggests that different assembly mechanisms operate on the EM fungal community at different stages in secondary subtropical forest succession.

Introduction

The interplay between aboveground and belowground biotic communities drives the stability and function of ecosystems (Wardle *et al.*, 2004; van Dam & Heil, 2011). Mycorrhizal fungi are important soil microorganisms, forming symbiotic associations with terrestrial plant species in many ecosystems (Smith & Read, 2008). In these associations, the plants provide photosynthetic carbon to support the growth and function of fungi, and thus can influence the fungal community (Wardle, 2006; Dickie, 2007). In return, mycorrhizal fungi improve plant nutrient uptake and resistance to abiotic stresses, and they therefore influence plant diversity, productivity and ecosystem functioning (van der Heijden *et al.*, 1998, 2008). The ecological consequence of the plant–fungus interaction is complicated by the temporal and spatial heterogeneity of natural ecosystems, including factors such as forest succession (Bardgett *et al.*, 2005; Twieg *et al.*, 2007). Due to differences in body size, life history strategy and habitat dependence, changes affecting plants and fungi may not always be synchronized during the course of forest succession (Bardgett *et al.*, 2005). However, to our knowledge, there are few published reports on the plant–fungus interplay that occurs during forest succession.

Environmental selection by both biotic and abiotic factors and dispersal limitation are two of the primary processes structuring biotic communities in ecosystems (Cottenie, 2005). The influences of various environmental factors on mycorrhizal fungal communities have been investigated in previous studies (Dickie *et al.*, 2009; Dumbrell *et al.*, 2010). For example, ectomycorrhizal (EM) fungal communities have been shown to be affected by host plant species composition (e.g. Ishida *et al.*, 2007; Tedersoo *et al.*, 2008a; Roy *et al.*, 2013), productivity (Kranabetter *et al.*, 2009) and root density (Peay *et al.*, 2011) in many temperate forests. By contrast, no relationship between EM fungal community and host plant community was found in a Guyanese neotropical rainforest (Smith *et al.*, 2011) or in two out of three temperate forests investigated by Bahram *et al.* (2012). In addition to host plant traits, EM fungal communities can also be influenced by surrounding nonhost plant communities (Taniguchi *et al.*, 2007), as well as by other characteristics of the forest vegetation

such as canopy cover and composition (DeBellis *et al.*, 2006; Wallander *et al.*, 2010), shrub layer cover (Wubet *et al.*, 2012) and herb layer plant proportion (Dickie *et al.*, 2009) in temperate forests. The distribution of the EM fungal community in natural forest ecosystems may also be related to the heterogeneity of certain abiotic factors such as soil pH (Põlme *et al.*, 2013), soil nutrients (Cox *et al.*, 2010) and elevation (Bahram *et al.*, 2012).

Stochastic dispersal of individuals from one site to another is limited by the geographic distance between the site where the dispersers come from and the site where they disperse to, and a biotic community assembly pattern therefore can be predicted by geographic distance (Hubbell, 2001). Dispersal processes have often been shown to shape communities of plants (Vellend, 2010) and microorganisms such as arbuscular mycorrhizal (AM) fungi (Dumbrell *et al.*, 2010), soil ascomyceteous fungi (Green *et al.*, 2006) and bacteria (Martiny *et al.*, 2011). Recently, the role of dispersal processes in shaping EM fungal communities has been investigated at different spatial scales and ecosystems (Bahram *et al.*, 2013; Gao & Guo, 2013). For example, EM fungal communities were influenced by dispersal limitation in studies of systems including *Alnus* species at the global scale (Põlme *et al.*, 2013) and four tropical forests in Africa and three temperate Hyrcanian forests in Iran at the regional scale (Tedersoo *et al.*, 2011; Bahram *et al.*, 2012). At local scale, EM fungal communities were influenced by dispersal limitation in tropical forests in Ecuador and Guyana (Tedersoo *et al.*, 2010b; Smith *et al.*, 2011) and temperate primary successional ecosystems in Japan, Norway and USA (Nara *et al.*, 2003; Peay *et al.*, 2010; Blaalid *et al.*, 2012), characterized by low EM plant density; whereas EM fungal communities were not found to be influenced by dispersal limitation within each of the three Hyrcanian forests characterized by high EM plant density (Bahram *et al.*, 2012). In addition, because environmental factors are often geographically aggregated, the role of dispersal limitation in structuring EM fungal community should be investigated by excluding the influences of plant, soil and topography. However, the relative importance of environmental selection and dispersal limitation in structuring EM fungal communities during forest succession is still less well documented.

Secondary forest successions in woody plant communities following disturbance have been intensively investigated; such series of community changes are important in the study of plant community assembly and diversity maintenance (Anderson, 2007; Bruelheide *et al.*, 2011). During secondary forest succession, several vegetative and abiotic characteristics, such as plant community and soil nutrient status, exhibit systematic patterns of change (Both *et al.*, 2011; Bruelheide *et al.*, 2011). As symbiotic partners of plants, EM fungal communities also show temporal variation during secondary forest succession (e.g. Last *et al.*, 1987; Mason *et al.*, 1987; Gebhardt *et al.*, 2007; Ishida *et al.*, 2007; Twieg *et al.*, 2007) and primary succession (Nara *et al.*, 2003; Blaalid *et al.*, 2012) in temperate ecosystems. Furthermore, the mechanism of assembly of EM fungal communities may vary between different forest successional stages. For example, EM fungal community composition in young forests is often strongly influenced by various biotic factors such as host plant identity (Bent *et al.*, 2011) and abiotic factors such as soil nutrient content (Pietras *et al.*, 2013). By contrast, EM fungal communities in some mature forests were not found to be associated with EM plant identity and soil factors, but they were affected by dispersal limitation (Iotti *et al.*, 2010; Smith *et al.*, 2011; Tedersoo *et al.*, 2011). However, as most studies to date have focused on temperate forests, the mechanism by which the mycorrhizal fungal community assembles during forest succession is still largely unknown in subtropical and tropical ecosystems (Reverchon *et al.*, 2012; Sun *et al.*, 2013).

Subtropical forests widely distributed in South and East China, have high EM plant and non-EM plant species diversity that may generate various niche spaces to accommodate diverse EM fungi (Dickie, 2007; Bruelheide *et al.*, 2011). Furthermore, subtropical forests are more abundant in non-EM plants than EM plants (Bruelheide *et al.*, 2011), and thus dispersal processes may be of particular importance for EM fungi between scattered distributed EM plant individuals surrounded by non-EM plants (Peay *et al.*, 2010; Tedersoo *et al.*, 2010b; Bahram *et al.*, 2013). Previous studies have shown systematic patterns of change of plant community and soil variables along a Chinese subtropical secondary forest succession (Both *et al.*, 2011; Bruelheide *et al.*, 2011). Therefore, in this subtropical forest successional series we hypothesized that: (1) the EM fungal community could be structured by environmental selection and/or dispersal limitation; and (2) the EM fungal community is influenced by different factors in different forest successional stages. Besides, as EM fungal community assembly seemed to be more predictable in young forests than in old forests in temperate ecosystems (Iotti *et al.*, 2010; Bent *et al.*, 2011), we hypothesized that (3) the explained variation in the EM fungal community decreases with subtropical secondary forest succession. To test these hypotheses, in this study we examined an EM fungal community from a subtropical forest chronosequence consisting of young, intermediate and old forests using 454 pyrosequencing technique. The correlation and causality between EM fungal community, plant, soil, topography and geographic distance were statistically analysed.

Materials and Methods

Study site and sampling

This study was conducted in a subtropical forest in the Gutianshan National Nature Reserve (GNNR) in southeast China (29°08′18″–29°17′29″N, 118°02′14″–118°11′12″E). The GNNR is a subtropical monsoon climate zone *c.* 81 km^2 in area, with an annual mean temperature of 15.38°C and annual mean precipitation of 1964 mm (Bruelheide *et al.*, 2011; Gao *et al.*, 2013). A research site consisting of plots stratified by successional age was established in the GNNR in 2008 (Bruelheide *et al.*, 2011). The plots were divided into three forest successional stages (young, 10–40 yr; intermediate, 41–80 yr; and old, > 80 yr) representing intervals of 40 yr as described by Wu *et al.* (2012), in this case corresponding to different periods of time elapsed since the most recent forest harvesting, which is carried out manually by the local population to obtain timber and firewood. In this

study we selected 24 out of all 27 plots (each 30 m × 30 m ground area) belonging to three successional stages with eight replicates in each successional stage (Supporting Information Fig. S1). All plots were between 251 and 903 m above sea level, and the geographic distances between two plots ranged from 180 to 8630 m (Table S1; Fig. S1). We identified 18 plant species belonging to four families as being EM plants, by observing the root morphology of suspected EM plants under a dissecting microscope. The species were *Pinus massoniana* (Pinaceae), *Betula luminifera* and *Carpinus viminea* (Betulaceae), *Tilia endochrysea* (Tiliaceae), and *Castanea henryi*, *Castanopsis carlesii*, *C. eyrei*, *C. fargesii*, *C. sclerophylla*, *C. tibetana*, *Cyclobalanopsis glauca*, *C. gracilis*, *C. myrsinaefolia*, *C. nubium*, *C. stewardiana*, *Lithocarpus glaber*, *Quercus phillyreoides* and *Q. serrata* (all Fagaceae). For each plot, Bruelheide *et al.* (2011) had determined the woody plant (> 1 m high) species composition, abundance, species richness, basal area, upper tree layer cover, lower tree layer cover and shrub layer cover, and a series of abiotic factors including location, elevation, and soil total carbon [C], total nitrogen [N], total phosphorus [P], pH and soil moisture. In a central 10 m × 10 m subplot of each plot, Both *et al.* (2011) had determined the biomass of the herb layer functional groups: dicotyledonous herbs, monocotyledonous herbs, gramineae, legumes, ferns, climbers and woody seedlings (≤ 1 m high). Information about biotic and abiotic variables for the young, intermediate and old forests is summarized in Table S2.

In total, 12 soil samples (each 10 cm in diameter × 10 cm deep) were randomly collected from each plot in October 2010, with a minimum distance of 7.5 m between any two samples. A total of 288 soil samples were collected from 24 plots. Roots within each sample were washed free from soil over a 1-mm sieve in running tap water. All fine roots (< 2 mm diam.) were cut into fragments *c.* 1 cm in length, and EM root tips from the root fragments were identified on the basis of morphological characteristics, such as shape, colour, size and texture, using a stereomicroscope. In total, 150 ± 20 EM root tips per sample were randomly picked, resulting in the collection of a total of 1800 ± 200 root tips from the 12 samples from each plot. The root samples were washed with sterilized distilled water and stored at −80°C until DNA extraction.

Molecular analysis

Total DNA was extracted from each of the 12 EM root samples per plot using the cetyltrimethyl-ammonium bromide method (Gardes & Bruns, 1993). For direct unidirectional 454 sequencing of amplicons of the fungal internal transcribed spacer (ITS) region, forward 454 primers were constructed to contain a DNA capture bead anneal adaptor (B adaptor, for emulsion PCR) and an ITS1OF primer (a combination of the two primers ITS1OF-C and ITS1OF-T; Taylor & McCormick, 2008); reverse 454 primers were constructed to combine a 454 sequencing adaptor (A adaptor), a 9-base sample tag (Table S1) and an ITS4 primer (White *et al.*, 1990). The fungal ITS region was amplified using a one-step PCR method in a Gene Amplification PCR System (East Win, Beijing, China) with initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 50 s and 72°C for 1 min, and a final extension at 72°C for 10 min. Each amplification was carried out in a 25-μl reaction mixture containing 1 U *Taq* (Takara, Dalian, China), ×1 PCR buffer, 2 mM $MgCl_2$, 0.2 μM dNTP, 0.4 μM primers and 1 μl DNA template. Amplicon libraries were produced from a pool of six different PCRs generated from two dilution levels (×10 and ×100 template DNA solution) with three PCR replicates for each dilution. PCR products from 12 root samples from the same plot were pooled and purified using an Axygen PCR Product Gel Purification Kit (Axygen, Union City, CA, USA). The yields of purified PCR products were measured using a fluorescence spectrophotometer (TBS 380; Promega, USA), and 50 ng of DNA from each of the 24 samples was pooled and adjusted to 10 ng μl^{-1}. The pooled products were subjected to pyrosequencing in 1/4 region of a Roche Genome Sequencer FLX Titanium (454 Life Sciences, Branford, CT, USA). The raw sequence data have been submitted to the Sequence Read Archive of the GenBank database under accession no. SRA057902.

Bioinformatic analysis

The noise signals generated during sequencing process were detected and removed using the shhh.flow command in Mothur 1.31.2 (Schloss *et al.*, 2009). Subsequently, sequences with no valid primer sequence or DNA tag; containing ambiguous bases, homopolymers > 8 bases, < 250 bp in length; or with an average quality score < 25; were removed using the trim.seqs command in Mothur (Schloss *et al.*, 2009). Because the highly conserved 5.8S gene flanking the ITS2 region may influence the assignment of operational taxonomic units (OTUs), the ITS2 regions of the remaining sequences were extracted using the software package Fungal ITS Extractor (Nilsson *et al.*, 2010). For these ITS2 sequences, potential chimeras were detected using the chimera.uchime command in Mothur (Schloss *et al.*, 2009), using the unified system for the DNA based fungal species (UNITE) and international nucleotide sequence databases (INSD) fungal ITS databases as sources of reference data (Abarenkov *et al.*, 2010). The remaining nonchimeric ITS2 sequences were clustered into OTUs at a 97% similarity level using cd-hit as implemented in Quantitative Insights Into Microbial Ecology (QIIME, Caporaso *et al.*, 2010). The most abundant sequence in each OTU was selected as being representative of that OTU, and the taxonomic placements of these sequences were identified by a basic local alignment search tool (BLAST; Altschul *et al.*, 1990) search against the UNITE and INSD database. The OTUs were assigned to EM fungi when they showed high similarity to known EM fungi in the returned top BLAST results. Then EM fungal OTUs were assigned into phylogenetic lineages according to the nomenclature of Tedersoo *et al.* (2010a). As Helotiales, *Peziza*, *Sebacina* and *Meliniomyces* contain both EM and non-EM fungi, the EM status of OTUs belonging to these groups was further determined by constructing a neighbour-joining tree based on the representative OTU sequences and sequences from reference EM fungi in Helotiales, *Peziza*, *Sebacina* and *Meliniomyces* as given in Tedersoo *et al.* (2010a). To reduce the

potential influence of artificial OTUs derived from sequencing errors, OTUs with fewer than five reads were removed before further statistical analyses (Lindahl *et al.*, 2013). To eliminate the effects of different read numbers among the plots on the deduced EM fungal community composition, the number of sequences per plot was normalized to the smallest sample size using the normalize.shared command in Mothur (Schloss *et al.*, 2009).

Statistical analysis

A one-way ANOVA followed by a pairwise *t*-test adjusted using the Bonferroni method was carried out to explore the differences in abundance (read numbers, square root transformed) of each OTU and each lineage among the young, intermediate and old forests. EM fungal richness among the young, intermediate and old forests was tested by one-way ANOVA. The EM fungal community was analysed by ordination using nonmetric multidimensional scaling (NMDS) with the Bray–Curtis dissimilarity, and the three forest successional stages were fitted as centroids onto the NMDS graph using the envfit function.

For the whole forest (including all 24 plots) and for each individual forest successional stage (eight plots each), Bray–Curtis dissimilarities were calculated to construct distance matrices of EM fungal community, forest successional stage, EM plant species composition, non-EM plant species composition, EM plant basal area, non-EM plant basal area, EM plant species richness, non-EM plant species richness, EM plant abundance, non-EM plant abundance, herb layer functional group composition, upper tree layer cover, lower tree layer cover, shrub layer cover, elevation, total C, total N, pH, total P and soil moisture. Mantel tests were carried out to explore the correlations among the distance matrices listed above.

In order to explore the independent influence of dispersal limitation on EM fungal community assembly, partial Mantel tests were carried out by demonstrating the correlation between EM fungal community and geographic distance, after excluding the effects of significant biotic and abiotic factors in Mantel tests. Conversely, partial Mantel tests were carried out to explore the relationships between EM fungal community and plant and abiotic factors, after excluding the influence of geographic distance.

In order to quantitatively test the extent to which the EM fungal community was influenced by these factors, multiple regression of distance matrices (MRM) models were constructed to include distance matrices that were significantly related to EM fungal community according to Mantel tests. The MRM models were then simplified using stepwise backward selection until $P < 0.05$ for all distance matrices. If more than one distance matrix was retained in the final MRM model, hierarchical partitioning was carried out to explore the independent contribution of each distance matrix. The partial residuals in EM fungal community were calculated by partialling out the effects of significant environmental factors retained in final MRM model. As the geographic distances between pairs of plots ranged from 180 to 8630 m (Fig. S1), all distances between plots were grouped into nine distance classes, that is 0–500 m (midpoint = 250 m), 500–1500 m (1000 m), 1500–2500 m (2000 m), 2500–3500 m (3000 m), 3500–4500 m (4000 m), 4500–5500 m (5000 m), 5500–6500 m (6000 m), 6500–7500 m (7000 m) and 7500–8700 m (8100 m). Mantel correlograms were calculated to test the correlation between the partial residuals in EM fungal community and geographic distance at different spatial scales.

Additionally, structural equation models (SEM) using Mantel *R* values as input were constructed in AMOS 20.0 (Arbuckle, 2011) to explore the causal relationships among forest succession, geographic distance, elevation, EM plant species composition, non-EM plant species composition, EM plant basal area, non-EM plant basal area, EM plant species richness, non-EM plant species richness, EM plant abundance, non-EM plant abundance, herb layer functional group composition, upper tree layer cover, lower tree layer cover, shrub layer cover, total P, soil moisture and EM fungal community. Based on *a priori* and theoretical knowledge, we assumed a conceptual model in which forest successional stage, geographic distance, elevation, total P and soil moisture affect the plant community, which in turn affects the EM fungal community. A maximum likelihood estimation method was used to compare the SEM models with observations. Model adequacy was determined by χ^2 tests, goodness-of-fit index (GFI), Bollen-Stine bootstrap (BSB), Akaike Information Criteria (AIC), and root square mean errors of approximation (RSMEA). Adequate model fits are indicated by nonsignificant χ^2, high GFI, high BSB P, low AIC and low RSMEA (< 0.05).

NMDS and envfit were carried out in the package vegan (Oksanen *et al.*, 2007). Mantel tests, partial Mantel tests, Mantel correlograms and MRM were carried out in the package ecodist (Goslee & Urban, 2007). Hierarchical partitioning was carried out in the package hier.part (Walsh & Mac Nally, 2013). All the above statistical analyses with the exception of SEM were carried out in R 2.15.1 (R Development Core Team, 2011).

Results

General characterization of 454 sequence data

After controlling for sequence quality, 71 522 nonchimeric ITS2 sequences were obtained and clustered into 3524 OTUs at a 97% similarity level; 1519 OTUs (27 770 reads) were assigned to EM fungi (OTUs ≥ 5 reads listed in Table S3) and 2005 OTUs (43 752 reads) were of non-EM fungal origin, including endophytes, pathogens and unidentified OTUs (OTUs ≥ 5 reads listed in Table S4). Among the 1520 EM fungal OTUs, 1126 (1597 reads) with < 5 reads were removed from the dataset. The remaining 393 EM fungal OTUs (26 182 reads) with ≥ 5 reads were used in the following analyses. As the EM fungal read numbers ranged from 379 to 2336 among the 24 plots, the read numbers were normalized to 379, resulting in a normalized dataset containing 393 EM fungal OTUs (9050 reads). The 100 most abundant OTUs accounted for 79.6% of the EM fungal reads (Fig. 1a). The frequency distribution of EM fungal OTUs had a long tail, with 314 OTUs occurring in no more than three plots (Fig. 1b). All EM fungal OTUs belonged to 21 fungal lineages dominated by /tomentella-thelephora (38.2% of the total EM

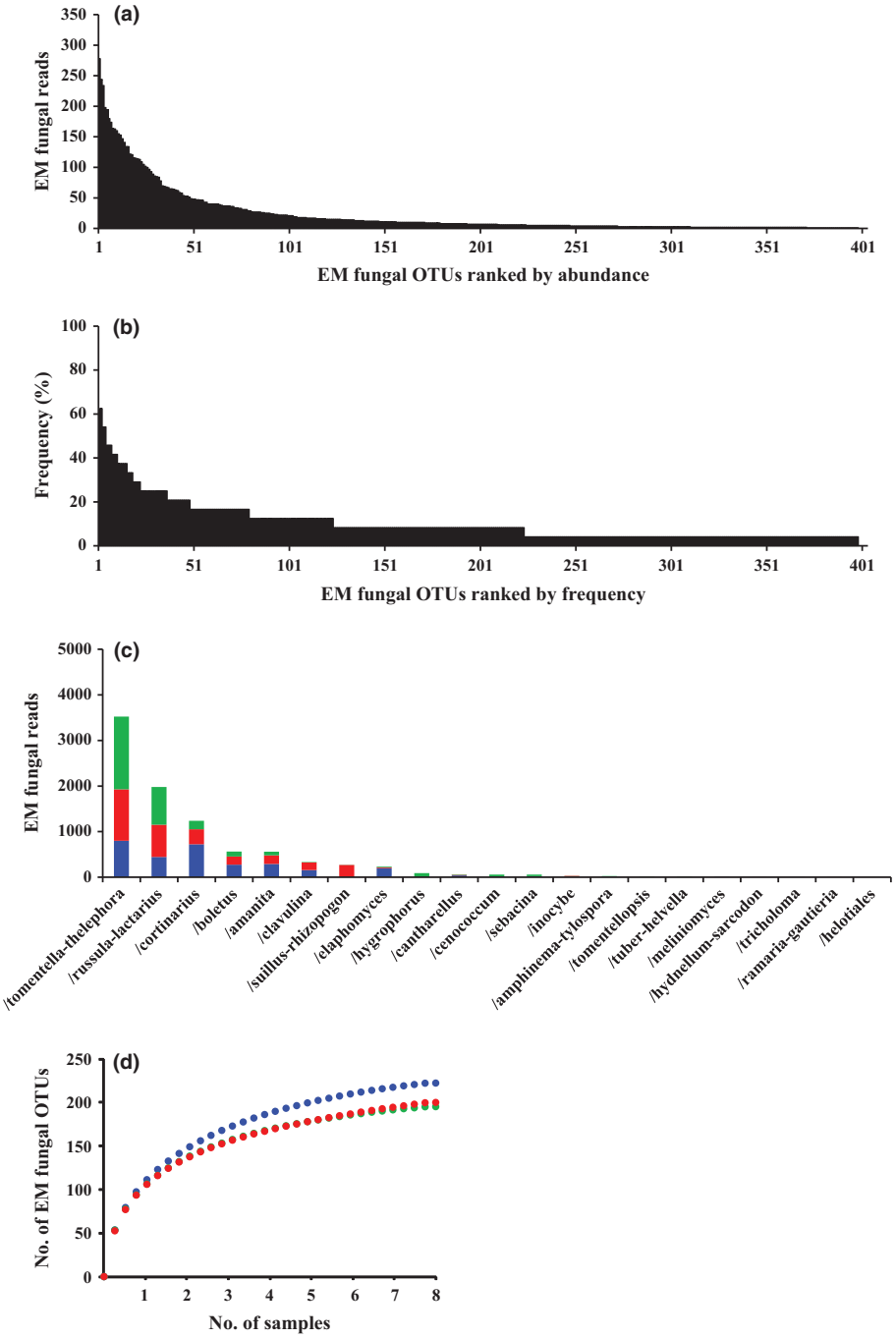


Fig. 1 Ectomycorrhizal (EM) fungal operational taxonomic units (OTUs) ranked by abundance (a) and frequency (b). (c) EM fungal lineages ranked by abundance in young (blue bars), intermediate (red bars) and old (green bars) forests. (d) Rarefaction of EM fungal OTUs in young (blue circles), intermediate (red circles) and old (green circles) forests.

fungal reads), /russula-lactarius (22.0%) and /cortinarius (13.9%; Fig. 1c).

EM fungal diversity in different forest successional stages

Of the 393 EM fungal OTUs, 221 were recovered from the young forest, 194 from the intermediate forest and 196 from the old forest, respectively (Fig. 1d). EM fungal OTU richness was not significantly different among the young, intermediate and old forests ($F_{2,81} = 1.188$, $P = 0.325$). Of these EM fungi, three EM fungal lineages and 11 OTUs showed significantly biased occurrence among the three forest successional stages (Figs 2, 3). Lineages /cortinarius and /elaphomyces significantly decreased in abundance from young to intermediate and/or old forests (Fig. 2a,b), whereas /tomentella-thelephora significantly increased in abundance from young to old forests (Fig. 2c). Of these 11 OTUs, one /cortinarius (accounting for 1.7% of total read numbers of /cortinarius), three /russula-lactarius and two /tomentella-thelephora OTUs increased in abundance from young to intermediate and old forests (Fig. 3a,e–i); whereas one /elaphomyces, two /russula-lactarius and two /tomentella-thelephora OTUs (accounting for 0.1% and 0.3% of total read numbers of /tomentella-thelephora) significantly decreased in abundance from young to intermediate and old forests (Fig. 3b–d,j,k).

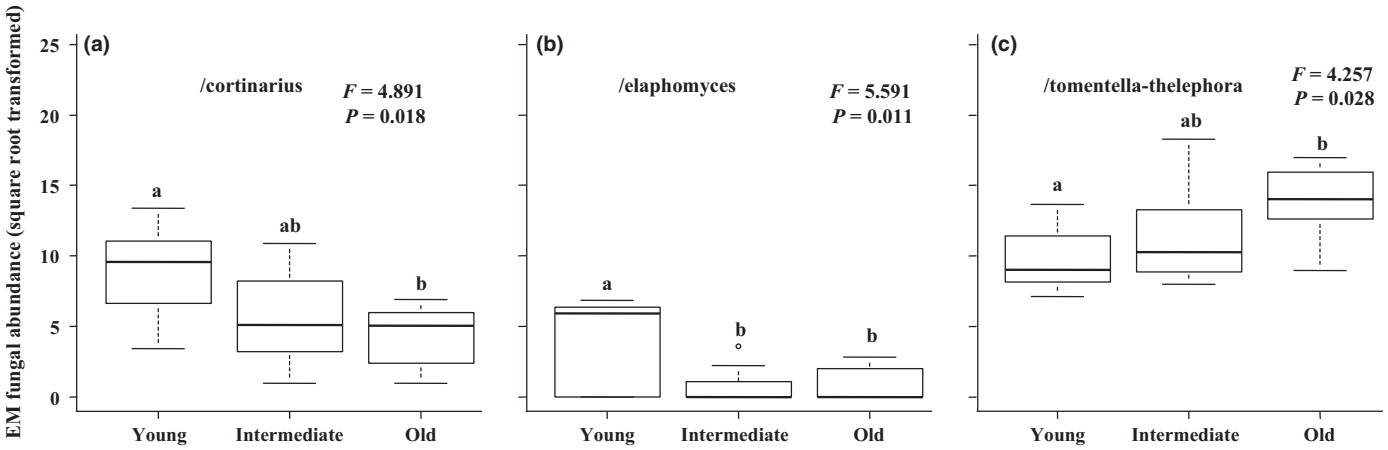


Fig. 2 Abundance (read numbers, square root transformed) of the ectomycorrhizal (EM) fungal lineages /cortinarius (a), /elaphomyces (b) and /tomentella-thelephora (c) in young, intermediate and old forests as demonstrated by boxplot with median and 95% confidence intervals displayed. Bars without shared letters indicate significant differences after adjustment by the Bonferroni method.

Effect of biotic and abiotic factors on the EM fungal community in the forest as a whole

NMDS followed by envfit analysis showed that the EM fungal community was significantly different among forest successional stages (Fig. 4). Mantel tests showed that 64 out of the 210 pairs of matrices were significantly interrelated (Table S5). Of these pairs of matrices, the EM fungal community was significantly correlated with forest successional stage, geographic distance, EM plant species composition, non-EM plant species composition, EM plant species richness, non-EM plant basal area, herb layer functional group composition, upper tree layer cover, shrub layer cover, elevation, soil moisture and total P (Table 1). Geographic distance was not significantly related to the EM fungal community after the effects of these variables that were significant in Mantel tests had been partialled out. After partialling out the effect of geographic distance, the EM fungal community composition was significantly correlated with forest successional stage, EM plant species composition, non-EM plant species composition, EM plant species richness, herb layer plant functional group composition, upper tree layer cover, shrub layer cover, elevation, soil moisture and total P (Table 1).

The final MRM model ($F = 15.835$, $P = 0.001$; Table 2) showed that variation in the EM fungal community was explained by forest successional stage (6.1% variation explained), herb layer functional group composition (4.7%), upper tree layer cover (4.2%), elevation (3.8%) and total P (3.9%). In addition, the partial residuals in EM fungal community were calculated by partialling out the effects of forest successional stage, herb layer functional group composition, upper tree layer cover, elevation and total P. A Mantel correlogram demonstrated no significant spatial correlation between the partial residuals in EM fungal community and geographic distance at any distance scales (Fig. 5a).

The final SEM model adequately fitted the data describing the pathways of interaction among succession, EM fungal community, and plant and abiotic variables ($\chi^2 = 38.209$, df = 30, $P = 0.144$, GFI = 0.976, BSB $P = 0.175$, AIC = 110.209, RSMEA = 0.032; Fig. 6a). This final model explained 25.7% of the variation in EM fungal community (Fig. 6a). The EM fungal community was also found to be significantly affected directly by forest successional stage, herb layer functional group composition, elevation, total P, shrub layer cover and upper tree layer cover (Fig. 6a). Taking these findings from the Mantel test, MRM and SEM analyses as a whole, the EM fungal community was influenced by environmental selection but not by dispersal limitation in the secondary forest succession.

Effect of biotic and abiotic factors on the EM fungal community within young, intermediate and old forests

Mantel tests showed that 51, 23 and 16 out of 190 pairs of biotic and abiotic matrices were significantly interrelated in the young, intermediate and old forests, respectively (Table S5). Of these pairs of matrices, the EM fungal community was significantly correlated with geographic distance, EM plant species composition, non-EM plant species composition, EM plant basal area, herb layer functional group composition, elevation, soil moisture and total P in the young forest; with upper tree layer cover in the intermediate forest; and with geographic distance, EM plant species richness and non-EM plant species richness in the old forest (Table 1). Geographic distance was significantly related to EM fungal community in the old forest after excluding the effects of EM plant species richness and non-EM plant species richness; but the relationship was not significant after excluding the effects of EM plant species composition, non-EM plant species composition, EM plant basal area, herb layer functional group composition, elevation, soil moisture and total P in the young forest, and excluding the effect of upper tree layer cover in the intermediate forest (Table 1). After partialling out the effect of geographic distance, the EM fungal community was significantly correlated with EM plant species composition, non-EM plant species composition, EM plant basal area, herb layer functional group composition, elevation, soil moisture and total P in the young forest; with upper tree layer cover in the intermediate forest; and with EM plant species richness and non-EM plant species richness in the old forest (Table 1).

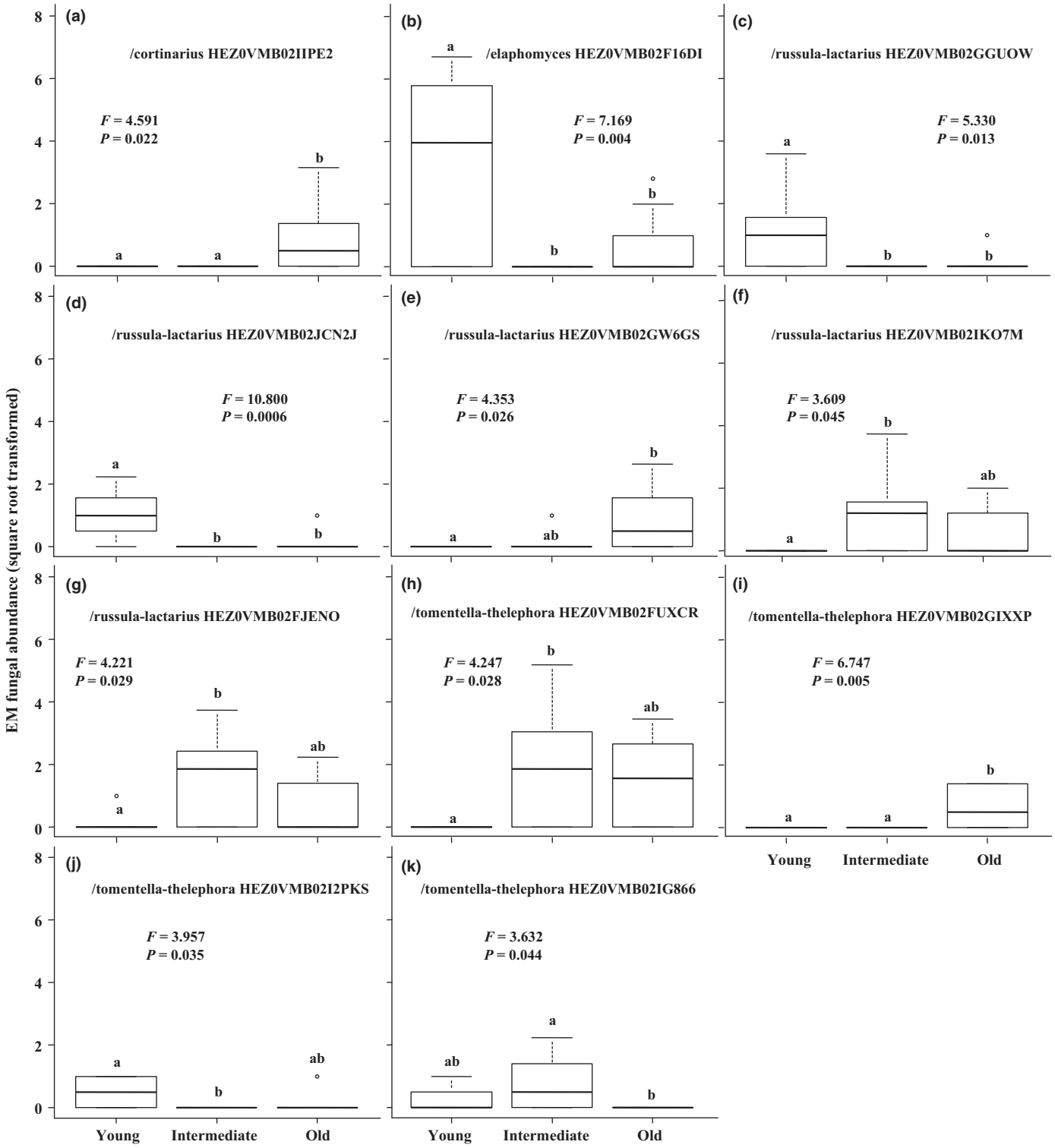


Fig. 3 Abundance (read numbers, square root transformed) of each of the 11 ectomycorrhizal (EM) fungal operational taxonomic units (OTUs)/cortinarius HEZ0VMB02IIPE2 (a), /elaphomyces HEZ0VMB02F16DI (b), /russula-lactarius HEZ0VMB02GGUOW (c), /russula-lactarius HEZ0VMB02JCN2J (d), /russula-lactarius HEZ0VMB02GW6GS (e), /russula-lactarius HEZ0VMB02IKO7M (f), /russula-lactarius HEZ0VMB02FJENO (g), /tomentella-thelephora HEZ0VMB02FUXCR (h), /tomentella-thelephora HEZ0VMB02GIXXP (i), /tomentella-thelephora HEZ0VMB02I2PKS (j) and /tomentella-thelephora HEZ0VMB02IG866 (k), in young, intermediate and old forests as demonstrated by boxplots with median and 95% confidence intervals displayed. Bars without shared letters indicate significant differences after adjustment by the Bonferroni method. The codes after the lineage names are the representative reads of EM fungal OTUs.

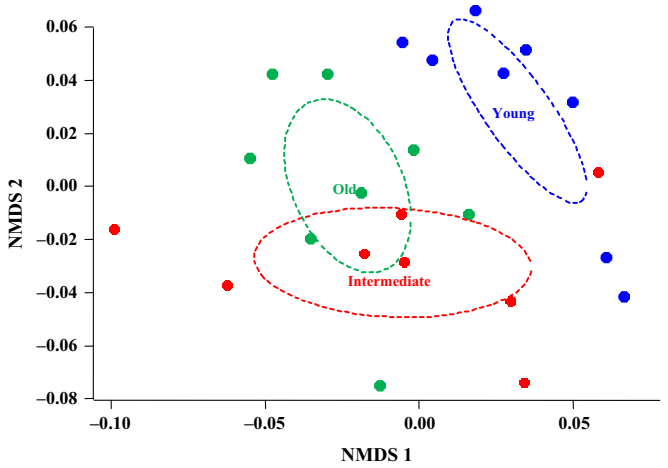


Fig. 4 Nonmetric multidimensional scaling (NMDS) of the ectomycorrhizal (EM) fungal community composition. Three forest successional stages were fitted as centroids onto the NMDS graph (stress = 0.239, $R^2 = 0.333$, $P = 0.004$). Dotted ellipses indicate 95% confidence intervals around centroids of young (blue), intermediate (red) and old (green) forests.

The final MRM models showed that variation in EM fungal community was explained by non-EM plant species composition (59.4% variation explained) in the young forest ($F = 38.070$, $P = 0.002$), by upper tree layer cover (27.6%) in the intermediate forest ($F = 9.923$, $P = 0.026$), and by EM plant species richness (10.5%) and geographic distance (17.2%) in the old forest ($F = 4.791$, $P = 0.030$; Table 2). In addition, the partial residuals in EM fungal community were calculated by partialling out the effects of non-EM plant species composition in the young forest, upper tree layer cover in the intermediate forest and EM plant species richness in the old forest. Mantel correlograms demonstrated significant spatial correlation between the EM fungal community and geographic distance at a spatial scale of 500–1500 m (midpoint = 1000 m) in the intermediate and old forests, but not in the young forest (Fig. 5b–d).

The final SEM models adequately fitted the data describing interaction pathways among EM fungal community and plant and abiotic variables in the young forest ($\chi^2 = 8.116$, df = 15, $P = 0.919$, GFI = 0.936, BSB $P = 0.950$, AIC = 50.116, RSMEA = 0.000; Fig. 6b), intermediate forest ($\chi^2 = 9.122$, df = 10, $P = 0.521$, GFI = 0.899, BSB $P = 0.627$, AIC = 31.122, RSMEA = 0.000; Fig. 6c) and old forest ($\chi^2 = 9.591$, df = 10, $P = 0.477$, GFI = 0.912, BSB $P = 0.542$, AIC = 31.591, RSMEA = 0.000; Fig. 6d). These final SEM models explained 70.2%, 27.0% and 32.8% of the EM fungal community variation in young, intermediate and old forests, respectively (Fig. 6b–d). In addition, the EM fungal community was directly significantly influenced by non-EM plant species composition in the young forest, by upper tree layer cover in the intermediate forest, and by EM plant species richness and geographic distance in the old forest (Fig. 6b–d). Taken these findings from the Mantel test, MRM and SEM analyses as a whole, the EM fungal community was influenced only by environmental selection in the young and intermediate forests, but by environmental selection and dispersal limitation in the old forest.

Discussion

The EM fungal community was significantly different among secondary forest successional stages in this subtropical ecosystem. Changes in EM fungal community have also been demonstrated in temperate forests undergoing secondary and primary succession (e.g. Last *et al.*, 1987; Nara *et al.*, 2003; Twieg *et al.*, 2007). Furthermore, our study found that the EM fungal community changed significantly from young to intermediate and old forests, but there was no significant difference between intermediate and old forests (Fig. 4). Similarly, the EM fungal community only changed significantly from 5- to 65-yr forests, but not from 65- to 100-yr forests in USA (Twieg *et al.*, 2007). The change of EM fungal community during forest succession may be due to the change of plant characteristics such as EM plant abundance, upper tree layer cover, lower tree layer cover and shrub layer cover that significantly changed from young to intermediate and old forests, but not from intermediate to old forests (Table S2). In addition, the fungal lineages /cortinarius and /elaphomyces were abundant in the young forest, but lineage /tomentella-thelephora was abundant in the old forest in this subtropical ecosystem. Similar results were reported in many temperate forests (e.g. Ishida *et al.*, 2007; Twieg *et al.*, 2007; Tedersoo *et al.*, 2008b; Bahram *et al.*, 2012). These results suggest that the /cortinarius and /elaphomyces fungi may adopt a 'ruderal strategy', whereas the /tomentella-thelephora fungi may employ a 'stress-tolerant or combative strategy' (Bruns, 1995).

We found that the EM fungal community in the whole forest was structured by environmental selection factors such as plant characteristics, total P and elevation, as reported in previous studies (e.g. Bahram *et al.*, 2012; Berner *et al.*, 2012; Põlme *et al.*, 2013). Furthermore, the EM fungal community was affected by different environmental factors in the young, intermediate and old forests in this subtropical ecosystem. For example, the key environmental factor in the young forest was non-EM plant species composition. The starting point for secondary forest succession in the GNNR is post-logging old-growth forest (Bruelheide *et al.*, 2011), thus there is likely to be a large EM fungal 'propagule bank' inherited from the preceding late successional stage (Jones *et al.*, 2003). However, not all soil fungal propagules can successfully establish on EM plant roots; they are selected by host plants in order to gain an advantage in response to environmental stresses (Jones *et al.*, 2003). This study suggests that the most important environmental stresses in the early successional stage derive from the direct effect of non-EM plants and the interaction between EM plants and non-EM plants (Fig. 6b). Due to their symbiotic nature, EM fungi and host plants are reciprocally selected during the course of evolution (Halling, 2001); preferences for different EM fungi by different hosts would lead to distinct EM fungal communities being harboured by different host species (e.g. Dickie, 2007; Morris *et al.*, 2008; Smith *et al.*, 2009; Wang *et al.*, 2011; Põlme *et al.*, 2013). In this study site, there are a large number of non-EM plant species (accounting for, on average, 74.3% of woody plant individuals; Bruelheide *et al.*, 2011), and most of these plants form mutualistic symbioses with AM fungi (Smith & Read, 2008). The role played by AM fungi

Table 1 Mantel tests and partial Mantel tests of the relationships between ectomycorrhizal (EM) fungal community composition and various biotic and abiotic variables in whole, young, intermediate, and old forests

	Whole forest				Young forest				Intermediate forest				Old forest			
	Mantel test		Partial Mantel		Mantel test		Partial Mantel		Mantel test		Partial Mantel		Mantel test		Partial Mantel	
Distance matrix	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*R*	*P*
Forest successional stage	0.278	0.001	0.269	0.001												
Geographic distance	0.216	0.008	0.035	0.320	0.515	0.020	−0.091	0.623	0.148	0.252	0.262	0.193	0.377	0.048	0.432	0.02
EM plant species composition	0.302	0.001	0.239	0.005	0.629	0.003	0.418	0.015	0.193	0.244	0.137	0.248	0.227	0.151	0.255	0.128
Non-EM plant species composition	0.335	0.001	0.276	0.004	0.774	0.002	0.676	0.006	−0.033	0.535	−0.129	0.678	0.200	0.181	0.230	0.155
EM plant species richness	0.148	0.033	0.143	0.041	0.163	0.205	−0.002	0.463	0.195	0.173	0.259	0.105	0.274	0.049	0.397	0.022
Non-EM plant species richness	0.084	0.183	0.100	0.141	0.055	0.297	−0.101	0.632	−0.157	0.668	−0.131	0.645	0.362	0.036	0.360	0.042
EM plant abundance	0.157	0.083	0.086	0.197	−0.019	0.493	−0.088	0.656	−0.174	0.777	−0.158	0.767	0.249	0.12	0.197	0.205
Non-EM plant abundance	0.123	0.091	0.102	0.152	−0.031	0.482	−0.189	0.835	0.245	0.181	0.224	0.21	0.121	0.289	−0.013	0.547
EM plant basal area	0.119	0.113	0.105	0.162	0.684	0.004	0.518	0.012	−0.232	0.811	−0.238	0.827	0.055	0.426	0.177	0.257
Non-EM plant basal area	0.136	0.025	0.107	0.081	0.008	0.434	0.011	0.444	0.230	0.197	0.240	0.187	0.169	0.183	0.088	0.347
Herb layer functional group composition	0.223	0.006	0.206	0.011	0.576	0.018	0.568	0.015	0.107	0.278	0.068	0.378	0.117	0.308	0.120	0.323
Upper tree layer cover	0.246	0.002	0.236	0.002	0.052	0.316	0.131	0.205	0.526	0.026	0.548	0.014	−0.095	0.696	−0.130	0.737
Lower tree layer cover	0.089	0.124	0.091	0.148	0.207	0.084	0.061	0.306	−0.026	0.542	−0.01	0.492	0.138	0.345	−0.192	0.809
Shrub layer cover	0.245	0.008	0.168	0.036	0.391	0.055	−0.002	0.499	0.314	0.153	0.334	0.169	0.250	0.168	0.062	0.396
Elevation	0.206	0.006	0.196	0.022	0.708	0.003	0.592	0.009	0.072	0.400	−0.048	0.574	0.252	0.144	0.272	0.129
Total N	−0.058	0.711	−0.124	0.908	0.360	0.060	0.343	0.082	0.112	0.336	0.087	0.367	−0.094	0.661	−0.176	0.795
Total C	−0.048	0.685	−0.124	0.897	0.408	0.051	0.371	0.077	−0.081	0.573	−0.074	0.586	−0.042	0.570	−0.208	0.861
pH	0.017	0.396	0.003	0.469	−0.146	0.751	−0.181	0.817	0.279	0.212	−0.049	0.577	−0.022	0.568	0.087	0.372
Soil moisture	0.207	0.033	0.176	0.048	0.556	0.015	0.503	0.013	0.211	0.247	0.265	0.215	0.299	0.110	0.156	0.287
Total P	0.188	0.024	0.146	0.055	0.463	0.030	0.356	0.056	−0.279	0.884	0.178	0.25	0.177	0.223	0.296	0.106

Table 2 Multiple regression of distance matrices (MRM) of ectomycorrhizal (EM) fungal community composition against independent variables for whole, young, intermediate and old forests

Forest	Model type	Distance matrix	Slope	*P*	Independent contribution	Model parameters
Whole forest	Original model	Forest successional stage	0.022	0.001		$R^2 = 0.287$, $F = 8.835$, $P = 0.001$
		Geographic distance	0.001	0.694		
		EM plant species composition	−0.010	0.777		
		Non-EM plant species composition	0.005	0.923		
		EM plant species richness	0.004	0.149		
		Non-EM plant basal area	−0.009	0.464		
		Herb layer functional group composition	0.052	0.022		
		Upper tree layer cover	0.001	0.079		
		Shrub layer cover	0.001	0.198		
		Elevation	0.000	0.049		
		Soil moisture	0.002	0.050		
		Total P	0.104	0.119		
	Final model	Forest successional stage	0.022	0.001	0.061	$R^2 = 0.227$, $F = 15.835$, $P = 0.001$
		Herb layer functional group composition	0.064	0.006	0.047	
		Upper tree layer cover	0.001	0.022	0.042	
		Elevation	0.000	0.013	0.038	
		Total P	0.162	0.008	0.039	
Young forest	Original model	Geographic distance	0.000	0.969		$R^2 = 0.713$, $F = 5.911$, $P = 0.024$
		Elevation	0.000	0.367		
		EM plant species composition	0.037	0.724		
		Non-EM plant species composition	0.243	0.231		
		EM plant basal area	−0.043	0.760		
		Herb layer functional group composition	0.089	0.229		
		Soil moisture	0.005	0.345		
		Total P	0.084	0.725		
	Final model	Non-EM plant species composition	0.481	0.002		$R^2 = 0.594$, $F = 38.07$, $P = 0.002$
Intermediate forest	Original model	Upper tree layer cover	0.003	0.026		$R^2 = 0.276$, $F = 9.923$, $P = 0.026$
	Final model	Upper tree layer cover	0.003	0.026		$R^2 = 0.276$, $F = 9.923$, $P = 0.026$
Old forest	Original model	Geographic distance	0.017	0.070		$R^2 = 0.300$, $F = 3.429$, $P = 0.055$
		Non-EM plant species richness	0.002	0.451		
		EM plant species richness	0.010	0.221		
	Final model	Geographic distance	0.018	0.044	0.172	$R^2 = 0.277$, $F = 4.791$, $P = 0.030$
		EM plant species richness	0.015	0.040	0.105	

in resource mineralization and absorption can result in altered soil resource availability such as P, and this can have a competitive or facilitative influence on the EM fungal community (Taniguchi *et al.*, 2007; Dickie *et al.*, 2009). However, our study showed no significant influence of total N on EM fungal community, whereas the role of soil N has been demosntrated in European pine forests (Cox *et al.*, 2010). The difference may be due to the steeper gradient of N and greater geographic scale in the study of Cox *et al.* (2010) than that in this study.

As succession proceeds in this study, upper tree layer cover became an important environmental factor influencing the EM fungal community in the intermediate forest. It has also been reported that the EM fungal community in mature forests was influenced by overstory tree composition in Canadian fir-birch forests (DeBellis *et al.*, 2006) and canopy closure in Swedish spruce forests (Wallander *et al.*, 2010). As a high level of upper tree layer cover is characteristic typical of mature forest rather than young forest in this subtropical ecosystem (Table S2), the influence of the upper tree layer on the EM fungal community may be due to the increase of EM plant abundance in the upper tree layer, suggesting that secondary forest succession introduces a niche axis for the EM fungal community. Unfortunately, the effect of EM plant abundance in upper tree layer cover on EM fungal community was not tested in this study, as the proportion of EM plants and non-EM plants in the upper tree layer was not measured (Bruelheide *et al.*, 2011). However, EM plant richness became an environmental factor influencing the EM fungal community in the old forest in this study. One explanation is that the plant 'richness effect' only operates in a stable ecosystem that has been established for a long time (Eisenhauer *et al.*, 2011). Thus, the EM fungal community experiences a shift in the selection regime to which it is subject along the secondary forest succession series in this subtropical ecosystem.

The variation in the EM fungal community explained by environmental factors dramatically decreased from young to intermediate and old forests in this study. The relatively high proportion of EM fungal community variation explained in young forest highlights the importance of the plant characteristics investigated in this study in shaping the EM fungal community, as reported that EM fungal community on tree seedlings was strongly structured by the identity of EM plants in an Alaskan boreal forest (Bent *et al.*, 2011). Furthermore, the small amount of community variation explained in intermediate and old forests in this subtropical ecosystem is consistent with the findings of some

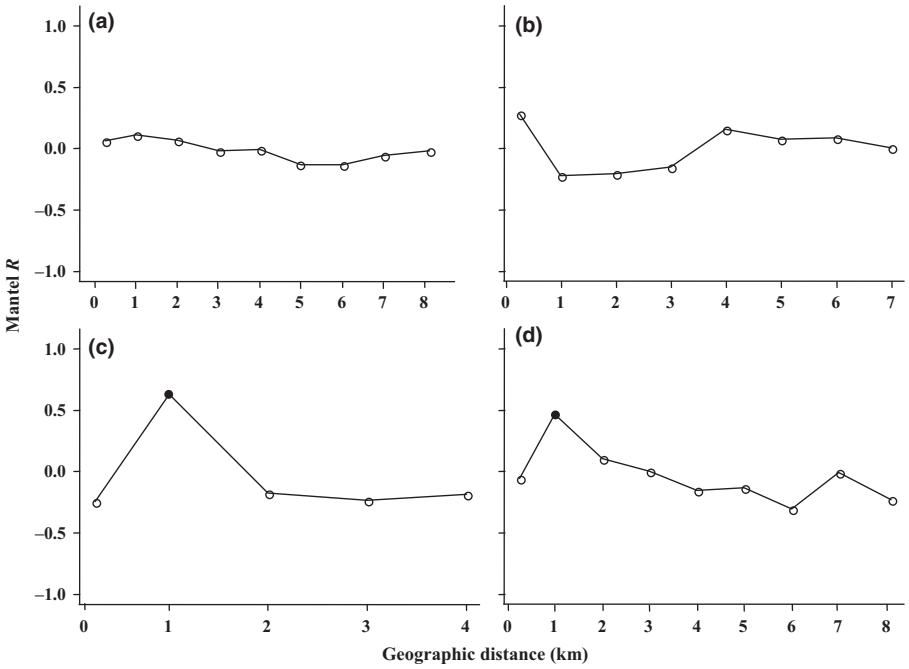
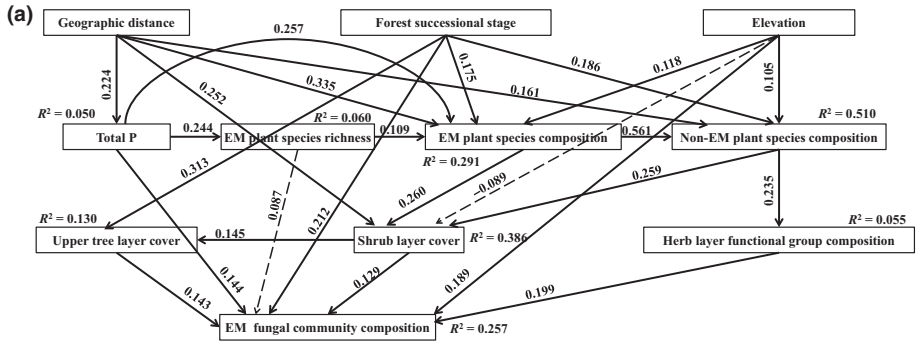


Fig. 5 Mantel correlograms showing the correlation between partial residuals in ectomycorrhizal (EM) fungal community composition and spatial proximity for plots within different distance classes in whole (a), young (b), intermediate (c), and old (d) forests. Closed circles, $P < 0.05$; open circles, $P > 0.05$.

previous studies, in which 6.53–23.1% of the variation in EM communities was explained by EM plants in mature temperate forest ecosystems (e.g. Ishida *et al.*, 2007; Morris *et al.*, 2009; Iotti *et al.*, 2010) and a neotropical rainforest (Tedersoo *et al.*, 2010b). The decreasing influence of the plant community on the EM community as forests age may be due to the stability of the plant community being greater in intermediate and old forests than in young forest (Bruelheide *et al.*, 2011). In addition, it may be the case that the EM fungal community, once established in the early successional stage, becomes relatively stable during the subsequent course of succession due to the huge abundance of mycelia (Högberg & Högberg, 2002), the formation of a common mycorrhizal network (Selosse *et al.*, 2006), the legacy effects of their spatial distributions (van der Putten *et al.*, 2009), and their idiosyncratic ecological interactions (Kennedy, 2010). However, as many biotic and abiotic factors had been investigated in this study, the low percentage of EM fungal community variation explained in intermediate and old forests suggests a large stochastic component operating on these systems.

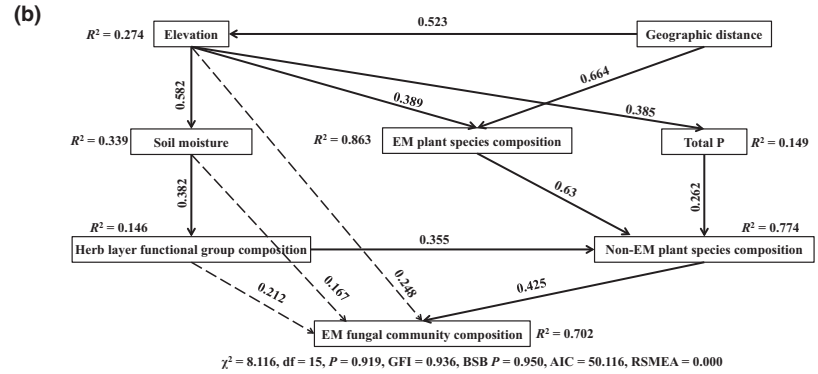
In the present study, the EM fungal community was also found to be structured by dispersal limitation in the old forest, but not in the young and intermediate forests. The difference between young and old forests may be due to the strong environmental selection acting in the young forest (59.4% of variation explained), in contrast to the relatively weak environmental selection in the old forest (10.5%) in this subtropical ecosystem. As environmental selection and dispersal limitation are two competing processes influencing the assembly of a biotic community, the influence of dispersal limitation is dependent on the strength of environmental selection (Leibold *et al.*, 2004). For example, the influence of environmental selection on a soil microbial community was found to be weak when dispersal limitation was strong, and *vice versa* (Hovatter *et al.*, 2011). Besides, as the direct independent role of geographic distance on EM fungal community had been demonstrated after partialling out the effect of plant community in the old forest, this study suggests that the spatial pattern of EM fungal community is not attributed to the spatial aggregation of plant and abiotic factors generating niches for EM fungi. An additional possible explanation for the difference between intermediate and old forests may be that the intermediate plots are spread over a relatively small area compared to that of old plots in this study (Table S2, Fig. S1). In support of this, EM fungal communities were shown to be significantly influenced by dispersal limitation in mature forests in studies carried out at the regional and global scales (e.g. Tedersoo *et al.*, 2011; Bahram *et al.*, 2012; Põlme *et al.*, 2013), but dispersal limitation had no effect on EM fungal communities at the local scale in temperate mature forests (Bahram *et al.*, 2012).

In contrast to our result in young forest, strong influences of dispersal limitation on EM fungal communities have been demonstrated in young forests along primary succession in Japan (Nara *et al.*, 2003) and USA (Ashkannejhad & Horton, 2006; Peay *et al.*, 2010, 2012). The difference between our and previous studies may be that aerial fungal dispersal is less difficult because the patches of young forest are small and interdigitated with older forest in this study rather than that far away from the main forests in Japan (Nara *et al.*, 2003) and USA (Ashkannejhad & Horton, 2006; Peay *et al.*, 2010, 2012); aerial dispersal is more abundant in this wetter subtropical region than in dry California (Peay *et al.*, 2010, 2012); the Thelephoraceae fungi dominated this study are better dispersers than the *Inocybe* in Japan (Nara *et al.*, 2003) and *Suillus* in USA (Ashkannejhad & Horton, 2006; Peay *et al.*, 2012) or because the young forest (10–40 yr) in this study is substantially older than the seedlings (≤ 5 yr) in Oregon (Ashkannejhad & Horton, 2006). Moreover, the young forest in this secondary succession may be not limited by propagule availability, as it has a large autochthonous EM fungal 'propagule



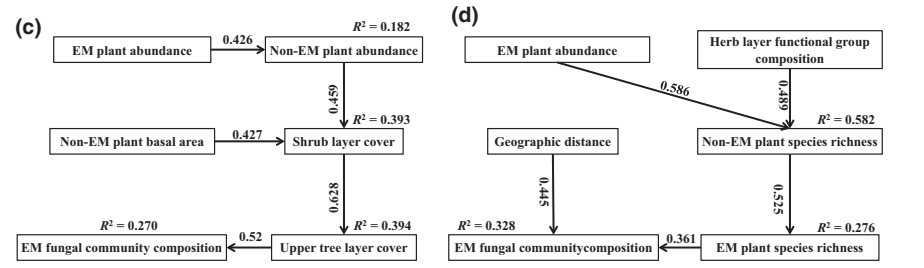
$\chi^2 = 38.209$, df = 30, $P = 0.144$, GFI = 0.976, BSB $P = 0.175$, AIC = 110.209, RSMEA = 0.032

	Direct path	Indirect path	Total effect
Forest successional stage	0.212	0.076	0.288
Herb layer functional group composition	0.199	0	0.199
Elevation	0.189	0.006	0.194
Total P	0.144	0.046	0.190
Shrub layer cover	0.129	0.021	0.150
Upper tree layer cover	0.143	0	0.143
Geographic distance	0	0.123	0.123
EM plant species richness	0.087	0.010	0.097
EM plant species composition	0	0.087	0.087
Non-EM plant species composition	0	0.086	0.086



$\chi^2 = 8.116$, df = 15, $P = 0.919$, GFI = 0.936, BSB $P = 0.950$, AIC = 50.116, RSMEA = 0.000

	Direct path	Indirect path	Total effect
Non-EM plant species composition	0.425	0	0.425
Elevation	0.248	0.325	0.573
Herb layer functional group composition	0.212	0.151	0.363
Soil moisture	0.167	0.139	0.306
Geographic distance	0	0.477	0.477
Total P	0	0.111	0.111
EM plant species composition	0	0.267	0.267



$\chi^2 = 9.122$, df = 10, $P = 0.521$, GFI = 0.899, BSB $P = 0.627$, AIC = 31.122, RSMEA = 0.000

	Direct path	Indirect path	Total effect
Upper tree layer cover	0.52	0	0.52
Non-EM plant abundance	0	0.15	0.15
EM plant abundance	0	0.064	0.064
Non-EM plant basal area	0	0.139	0.139
Shrub layer cover	0	0.326	0.326

$\chi^2 = 9.591$, df =10, $P = 0.477$, GFI = 0.912, BSB $P = 0.542$, AIC = 31.591, RSMEA = 0.000

	Direct path	Indirect path	Total effect
EM plant species richness	0.361	0	0.361
Geographic distance	0.445	0	0.445
Non-EM plant species richness	0	0.190	0.190
Herb layer functional group composition		0.093	0.093
EM plant abundance		0.111	0.111

Fig. 6 Structural equation models (SEM) showing the direct and indirect effects of forest successional stage, biotic and abiotic variables on ectomycorrhizal (EM) fungal community composition in whole (a), young (b), intermediate (c) and old (d) forests. The numbers above the arrows indicate path coefficients. Bold and dashed lines indicate significant and nonsignificant pathways, respectively. R^2 values represent the proportion of variance explained for each variable. Adequate model fits are indicated by a nonsignificant χ^2, high goodness-of-fit index (GFI), high Bollen-Stine bootstrap (BSB) P, low Akaike Information Criteria (AIC), and low root square mean errors of approximation (RSMEA < 0.05). The tables below the SEM graphs show the standardized direct, indirect and total effects of forest successional stage, biotic and abiotic variables on EM fungal community composition.

bank' inherited from the preceding late successional stage forest (Jones *et al.*, 2003).

In summary, the EM fungal community was significantly different among secondary forest successional stages in a Chinese subtropical ecosystem, an observation which is consistent with those of some previous studies on temperate forests undergoing secondary and primary succession (Nara *et al.*, 2003; Twieg *et al.*, 2007). The EM fungal community was structured by environmental selection as well as dispersal limitation in the old forest, but by environmental selection alone in the young, intermediate

and whole forests. Furthermore, the EM fungal community was affected by different factors at the different forest successional stages, and the importance of these factors in structuring the EM fungal community decreased along the secondary forest succession series. This study suggests that there are different mechanisms of assembly operating in the EM fungal community at different stages of secondary forest succession.

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Supporting Information

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

Fig. S1 Geographical distribution of the 24 plots in this study.

Table S1 The DNA tag, forest successional stage, elevation, latitude and longitude of plots in young, intermediate and old forests

Table S2 Biotic and abiotic variables among the young, intermediate and old forests

Table S3 Molecular identification of ectomycorrhizal (EM) fungi (OTUs ≥ 5 reads) in this study

Table S4 Molecular identification of nonectomycorrhizal (EM) fungi (OTUs ≥ 5 reads) in this study

Table S5 Interrelationships between ectomycorrhizal (EM) fungal community composition and various biotic and abiotic factors as determined by Mantel tests

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