Community and neighbourhood tree species richness effects on fungal species in leaf litter

Naili Zhang[1*], Helge Bruelheide[3,4], Yinong Li[5], Yu Liang[2], Tesfaye Wubet[4,6], Stefan Trogisch[3,4], Keping Ma[2]

[1] *Research Center of Forest Management Engineering of State Forestry and Grassland Administration, Beijing Forestry University, Beijing 100083, China*

[2] *State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, the Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Beijing 100093, China*

[3] *Institute of Biology/Geobotany and Botanical Garden, Martin Luther University Halle-Wittenberg, Am Kirchtor 1, 06108 Halle (Saale), Germany*

[4] *German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany*

[5] *Key Laboratory of Vegetation Ecology of Ministry of Education, Northeast Normal University, 5268 Renmin Street, Changchun 130024, China*

[6] *Department of Community Ecology, UFZ-Helmholtz Centre for Environmental Research, Theodor-Lieser-Str. 4, D-06120 Halle (Saale), Germany*

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* Corresponding author: Naili Zhang

Tel: +86-10-62337989

Email: zhangnl1978@163.com

Abstract

Investigating the effects of individual tree species on fungal species in leaf litter allows a mechanistic understanding of how tree diversity affects the diversity and composition of fungal species at the community level. We collected freshly-fallen leaves of eight focal tree species at four tree species richness levels in a large-scale subtropical forest diversity experiment to estimate tree species richness effects on fungal species diversity and community composition at two spatial scales: at the local tree neighbourhood and at the tree community level. The identity of focal tree species affected both the diversity and composition of the fungal community in freshly-fallen leaves, particularly structuring the composition of both the pathogenic and non-pathogenic fungal community. Furthermore, we found that the effects of community tree species richness on fungal OTU composition were tree species-specific. Besides community tree species richness, the neighbour tree community had significant effects on the structure of the entire fungal community and of functional groups in freshly-fallen leaves. These findings highlight that the response of fungal species assemblages to tree species richness depends on fungal-associated tree species identity, and suggest that heterospecificity of local tree neighbours is an important driver of tree richness effects on litter fungal community.

Keywords: BEF-China; Community tree species richness; Freshly-fallen leaf litter fungi; Next generation sequencing; Neighbour tree species richness; Subtropical forest

1. Introduction

Tree diversity loss and the ensuing changes in leaf characteristics and microenvironments (Díaz & Cabido, 2001) may have effects on fungal communities surviving in fresh leaves and leaf litters (Kembel et al., 2014; Nguyen et al., 2016a). Potential factors affecting fungal species composition in leaves of a certain tree species are microclimatic conditions (Prakash et al., 2015), species identity and functional traits (Prescott & Grayston, 2013; Vincent et al., 2016), and even neighbour tree characteristics. Resolving these drivers at the level of the leaves of an individual tree is essential to advance our mechanistic understanding of how tree species loss affects leaf litter-inhabiting fungal species and the resulting implications for decay processes and nutrient cycling at the community scale (Di Lonardo et al., 2013; Zhang et al., 2018).

Tree species identity is of importance for structuring leaf litter-inhabiting fungi due to distinct litter quality and quantity of tree species (Prescott & Grayston, 2013). Symbiotrophic and pathogenic fungi in fresh leaves that depend on carbon supply from the host plant (Petrini, 1991; Vincent et al., 2016) are expected to continue occurring in host leaf litters (Untersehe et al., 2013). The diversity and community composition of these fungi may particularly depend on host tree identity at the species level, or even at the family level. Saprotrophs in leaf litters can show some degree of host preference (Aneja et al., 2006; Kubartová et al., 2009; Prescott & Grayston, 2013), because of their preference of tree species-specific chemical leaf characteristics (Prescott & Grayston, 2013; Chamagne et al., 2016; Nguyen et al., 2016b), indicating the significance of the identity of the focal tree species for them. However, it remains unclear whether the responses of these leaf litter-inhabiting fungi to tree species loss at the neighbourhood or community levels would be dependent

upon tree species identity or not.

The mechanisms at the level of the leaves of an individual tree would add up to richness effects at the neighbourhood and community levels, and provide an explanation for reports that tree diversity can alter the diversity and composition of fungal species inhabiting a certain tree species (Nguyen et al., 2016a). In a plant community that is richer in tree species, there is a higher chance that a particular tree species is surrounded by a higher number of heterospecific neighbour species. More dissimilar tree neighbours can alter microenvironment, have facilitative effects on focal tree growth for resource complementarity (Potvin & Dutilleul, 2009; Chen et al., 2016; Fichtner et al., 2017) or inhibitory effects due to allelophathic effect from a certain neighbour tree species (Weidenhamer, 2006), and probably change the focal tree architecture (Lang et al., 2010; Valverde-Barrantes et al., 2013; MacFarlane & Kane, 2017) and traits. As such, dissimilar neighbors would affect litter inhabiting fungi of the focal tree individual. A second major impact on fungal species composition in the leaves of a particular tree species is an increased fungal species pool at the local neighbourhood. Fungi surviving in leaves, especially in the leaf litter of one particular tree species may be transferred from neighbour tree leaf litter (Hättenschwiler et al., 2005). Such an exchange may be passive, by undirected transfer of spores and hyphae of fungal species by chance in mixed leaf litter on the forest floor through wind or animals, or active when hyphae-based nutrient transfer between mixed litter of a focal and neighbour tree species is involved (Tiunov, 2009). Therefore, an increasing tree species richness in the local neighbourhood or community levels could consequently structure fungal community in leaf litters of a certain tree species.

In this study, we addressed the effects of tree species loss at the community and

neighbourhood levels on fungal communities of freshly fallen leaves of the focal tree. First, we hypothesized that there are significant effects of tree species identity on the litter-inhabiting fungal community. Second, assuming that tree species identity also affects the responses to the neighbourhood, we expected that the magnitude of tree species richness effects on litter fungal species diversity and composition are tree species-specific. Third, we tested the hypothesis that tree species richness more prominently affects the composition and diversity of fungal species in freshly-fallen leaves when being studied at the level of the local neighbourhood than at the community level, given that stronger tree-tree interactions commonly occur at the scale of the local neighbourhood (Valverde-Barrantes et al., 2013; MacFarlane & Kane, 2017).

2. Materials and methods

2.1. Study area

Our study was conducted in a large-scale tree diversity experiment (BEF-China) established in Xingangshan, Dexing, Jiangxi Province of P.R. China (29°08'–29°11' N, 117°90'–117°93' E, Bruelheide et al., 2014). The climate is subtropical with a mean annual temperature of 16.7 °C, the coldest temperature (0.4 °C) in January and the warmest temperature (34.2 °C) in July (Hu & Yu, 2008; Yang et al., 2013). Annual mean precipitation is 1821 mm (Yang et al., 2013). Soils in the tree diversity experiment are classified as dystric and siltic (Scholten et al., 2017).

2.2. Experimental design

The tree diversity experiment consists of two sites (A and B), which were established in 2009 and 2010, respectively, and covers a total net area of 38.4 ha (Bruelheide et al., 2014; Trogisch et al., 2017). Preceding land use was a plantation

with *Pinus massoniana* and *Cunninghamia lanceolata* that was harvested every 20 y. Briefly, a total of 566 plots were set up in both site A (271 plots) and B (295 plots). Plot size is 25.8 × 25.8 m (1 mu of the Chinese traditional unit of area) in horizontal projection and were planted with 400 trees (20 × 20 individuals) using a planting distance of 1.29 m. The species pool of the experiment is composed of 40 native subtropical broad-leaved tree species (Bruelheide et al., 2011, 2014). Here, we make use only of the random extinction scenario of the main set of species at site A. There are thirty-one very intensively studied plots (VIP) within site A, forming a gradient of tree species richness (i.e., sixteen monocultures, eight 2-species mixtures, four 4-species mixtures, two 8-species mixtures and one 16-species mixture, Fig. S1). A broken-stick design ensured that every tree species occurs the same number of times at each tree species richness level. In the mixture plots each tree species was equally represented in terms of planted tree individuals with species randomly assigned to planting positions.

2.3. Sampling

At each diversity level (monocultures and mixtures of 2, 4 and 8 tree species), we collected freshly-fallen leaves of eight focal tree species: *Castanea henryi*, *Castanopsis sclerophylla*, *Choerospondias axillaris*, *Liquidambar formosana*, *Nyssa sinensis*, *Quercus serrata*, *Sapindus saponaria* and *Triadica sebifera* (Table 1). The eight tree species randomly create a subset drawn from the total species pool according to the broken-stick design of the experiment (Bruelheide et al., 2014). Four individuals per focal tree species were randomly selected in each of the respective VIPs. Sampling was conducted in early October 2014 when leaf litter production was the largest (Huang et al., 2017). Specifically, we collected freshly-fallen leaves under the canopy of each of the focal tree individuals for measuring fungal species

composition in the leaf litter. A 30 × 30 cm quadrat was placed under the canopy of the focal tree individual to collect freshly-fallen leaf litter for measuring litter biomass. Closely adjacent to the quadrat, we collected three to five leaves of the focal tree species from the top of the litter layer to analyse fungal species composition and chemical traits of the leaf litter. We selected intact, brightly-colored leaves with fresh and plump petioles and without any sign of decomposition. Based on a 10-y experience of litter collection and classification during weekly leaf sampling in a 24-ha long-term monitoring subtropical forest, we are confident that these leaves have fallen within about 1 week. These samples were stored with dry ice until further processing. In total, we collected 128 individual samples (8 species × 4 diversity levels × 4 replicates) of freshly-fallen leaves. With sampling freshly-fallen leaves, we also recorded the eight neighbour tree individuals surrounding each focal tree individual in the eight compass directions. The number of neighbour tree species was 1 in the monoculture plot, 1-2 in the two-species plots, 1-4 in the four-species plots and 1-8 in the eight-species plots. As all trees were planted in a grid, the four neighbour tree individuals at the east, south, west and north directions were located at a distance of 1.29 m from the focal tree individual, while the other four neighbour tree individuals at the southeast, southwest, northwest and northeast directions were 1.82 m away from the focal tree individual (Fig. S1). We recorded the detailed information about neighbour tree species and their survival conditions.

2.4. Topographic properties and chemical analyses

Mean plot aspect and inclination were calculated using a digital elevation model (Bruelheide et al., 2013), with the aspect of the slope divided into a north-south (cosine aspect) and an east-west component (sine aspect). Litter organic carbon (C) and total nitrogen (N) were measured by CHNOS Elemental Analyzer (vario EL III,

CHNOS Elemental Analyzer, Elementar Analysensysteme GmbH, Germany). Leaf litter phosphorus (P), calcium (Ca), potassium (K), magnesium (Mg) and iron (Fe) were determined using an Inductive Coupled Plasma Emission Spectrometer (ICAP 6300 ICP-OES Spectrometer, Thermo Scientific, USA).

2.5. DNA extraction, PCR, and next generation sequencing

After collection, litter samples were immediately transferred to the laboratory in a portable container with dry ice and stored at -80 ºC until DNA was extracted. We used plant Genomic DNA Kit (Tiangen Biotech Co., Ltd, China) to perform DNA isolation according to the manufacturer's instruction. The resultant DNA products were used to carry out Polymerase Chain Reaction (PCR)-amplification for fungal ribosomal internal transcribed spacer 1 (ITS1). The PCR amplification was conducted in a total volume of 20 µl with 10 ng template DNA, 4 µl 5× FastPfu Buffer, 2 µl 2.5 mM dNTPs, 0.4 µl FastPfu Polymerase, and 0.8 µl 5 µM primer ITS1F (5'-barcode-CTTGGT CATTTAGAGGAAGTAA-3') and 0.8 µl 5 µM primer ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'). The PCR condition was set to 3 min at 95 ºC, followed by 35 cycles of 30 s at 95 ºC, 30 s at 55 ºC, and 45 s at 72 ºC and a final extension at 72 ºC for 10 min. The PCR products were extracted from 2 % agarose gels and purified by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.). The purified PCR products were sequenced following the paired-end amplicon sequencing protocol by Majorbio Bio-Pharm Technology Co. Ltd (Shanghai, China) using the Illumina MiSeq platform (Illumina, San Diego, USA).

2.6. Bioinformatic analysis

Raw fastq data obtained from Illumina MiSeq sequencing were demultiplexed and quality-filtered by Mac QIIME 1.8.0 (QIIME http://qiime.org/index.html) based on the following criteria: (1) over a 10 bp sliding window, we truncated the reads at

any site with less 20 score of an average quality, and discarded the resultant truncated reads if they were shorter than 50 bp; (2) we removed the reads consisting of ambiguous characters, and those with no accurate barcode matching, 2 nucleotide mistake in primer matching; (3) the sequences with the overlap longer than 10 bp were assembled, and those that could not be assembled were excluded. As a result, the quality-filtered fungal ITS reads were a total of 1,849,051 that were used to extract operational taxonomic units (OTUs).

The pick_open_reference_otus.py in Mac QIIME was used for selecting fungal OTUs, and finally 2389 OTUs were obtained. The representative sequences of each OTU have been submitted to EMBL (accession no. **PRJEB28409**). The filter_otus_from_otu_table.py was used to remove singletons of the OTU table. The assign_taxonomy.py was carried out for taxonomic assignment on the basis of the ITS 12-11 dataset (QIIME http://qiime.org/home_static/dataFiles.html). Additionally, the ITSx filter was performed for the removal of plant sequences (Bengtsson- Palme et al., 2013). To level out sampling depth heterogeneity and to retain as many sample as possible, we used single_rarefaction.py to rarefy the OTU table at a sampling depth of 2,000 OTUs. Finally, there were 112 samples subjected to the following statistical analysis. Based on the taxonomic assignments of OTUs, we assigned our fungal OTUs to functional guilds using the online database FUNGuild (http://www.stbates.org/guilds/app.php).

2.7. Statistic analyses

All data analyses were performed using R 3.4.3 (R core team 2015). Linear mixed models were fit using lmer function of the lmerTest package (Kuznetsova et al., 2017) to test the effects of either community or neighbourhood tree species richness on fungal diversity in freshly-fallen leaves. While community richness

referred to the number of tree species planted in a plot, neighbourhood richness was the number of tree species in the eight planting positions around a focal tree. In the model testing for a community tree species richness effect we related the response variable Shannon diversity of litter fungi to the fixed variables community tree species richness, tree species identity, litter chemical traits (i.e., litter C/N ratio, P, Ca, Fe, K and Mg) and topographic variables, while community tree species composition and plot nested in species composition were included as random factors. Similarly, in the model testing the effect of neighbourhood tree species richness, we regressed Shannon diversity of litter fungi on the fixed variables neighbourhood tree species richness, tree species identity, litter chemical traits (i.e., litter C to N ratio, P, Ca, Fe, K and Mg) and topographic variables, while neighbourhood species composition and plot nested in species composition were used as random factors. Linear mixed models were also fit to estimate the effects of the focal tree family, comparing the family that was represented with three species (Fagaceae) with all other species, and neighbour tree species and family (here it refers to the relative proportion of neighbour tree individuals belonging to Fagaceae) on fungal Shannon diversity and relative abundance.

A heat map was produced using heatmap.2 of gplots package to relate the 35 dominant fungal OTUs, i.e., those with at least 5‰ of relative abundance, to tree species richness. The relative OTU abundance is the percent of each OTU detected in a sample. After testing for normality and homogeneity of variance using the shapiro.test and bartlett.test functions, we employed Kruskal-Wallis tests (a non-parametric method) to assess the effect of tree species richness on each dominant OTU. A similar heat map and Kruskal-Wallis tests were made for testing difference in abundance of the dominant fungal OTUs with respect to the eight focal tree species.

Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function to test whether the OTU composition of the entire fungal community and the composition of fungal guilds depended on focal tree species identity, community and neighbour tree species richness. We also tested effects of tree species families of focal tree species and neighbourhood species families on fungal community in litter leaves using PERMANOVA. Distance-based redundancy analysis (dbRDA) was subsequently performed to analyse significant predictors interpreting the overall OTU composition of litter fungal community, and also applied separately to those in the litter of each tree species. These dbRDA models relevant to litter fungi were all based upon Bray-Curtis distance, which was selected using rankindex function of vegan package.

To test neighbour tree species effects, we first calculated the Bray-Curtis dissimilarity of fungal communities per focal tree individual in tree species mixture plots to those inhabiting litters of the same tree species in the monoculture using the vegdist function in the vegan package (Oksanen et al., 2018). Then the dissimilarity values were regressed on community tree species richness, neighbourhood tree species richness and the proportion of heterospecific tree species in the neighbourhood surrounding each focal tree individual to test how tree species richness affect litter-inhabiting fungal species at the local neighbourhood scale.

3. Results

3.1. Chemical traits of freshly-fallen leaves

We first compared chemical traits of the eight focal tree species before testing tree species identity effects on fungal species in freshly-fallen leaves. The chemical traits such as litter C/N ratio, P, K, and Mg concentration did not differ among focal

tree species (Table S1). However, Fe concentration showed significant differences between the litter of the different tree species, with *C. sclerophylla, T. sebifera, and L. formosana* displaying significantly lower Fe concentrations than *C. axillaris* (Table S1).

3.2. Tree species identity and family effects

Among all 2389 OTUs, the proportion of fungal species from the phylum Ascomycota were the highest compared to others in freshly-fallen litter of all focal tree species (Fig. S2). There were 35 dominant OTUs with more than 5‰ relative abundance (18 OTUs more than 1 %) within all OTUs. The relative abundance of 14 dominant fungal OTUs significantly differed among eight focal tree species. These dominant fungi include *Cercospora apii*, *Mycosphaerella holualoana*, *Ramichloridium cerophilum*, Botryosphaeriaceae sp., Chaetosphaeriaceae sp., Dothideomycetes sp., *Mycosphaerella* sp.2 and sp.3, Mycosphaerellaceae sp., Pleosporales sp., *Pseudocercospora* sp., Teratosphaeriaceae sp.1 and sp.2, and Ascomycota sp.2 (Fig. 1A).

Our results showed that not only the relative abundance of dominant fungal species (Fig. 1A) but also the diversity and composition of fungal OTUs in leaf litter were significantly affected by focal tree species identity (Fig. 2, Table 2). Based on a linear mixed effect model, we found that the Shannon diversity of litter-inhabiting fungi significantly depended on tree species identity (Table 2). In line with the Shannon diversity, the composition of fungal OTUs was significantly affected by tree species identity (Table S2). Moreover, the dbRDA results confirm the significant effect of tree species identity on the composition of all fungal OTUs in these freshly-fallen leaves (Fig. 2B).

We did not find any effect of tree species family (paired family groups, i.e., Fagaceae vs others) of focal tree species on the Shannon diversity and the relative abundance of fungal community in leaf litter (linear mixed effect model, both $P > 0.05$), but observed a significant interaction of tree species family with community-level tree species richness. Although the three focal tree species belonging to Fagaceae shared only a few dominant OTUs (Fig. 1A), the family of focal tree species pronouncedly influenced the OTU composition of the entire fungal, pathogenic and non-pathogenic fungal community (Table S2).

3.3. Community-level tree richness effects

Among all 35 dominant fungal species, six species had a positive and five species had a negative Spearman correlation with community-level tree species richness (Fig.S3). The relationship to tree species richness at the community level was linear as revealed by Kruskal-Wallis tests showed significant effects of tree species richness on Sordariomycetes sp., Teratosphaeriaceae sp.2, unidentified sp.2, Ascomycota sp.1 and *Ramichloridium cerophilum*, and in addition, slightly influenced ($P < 0.10$) Agaricomycetes sp.3, *Mycena plumbea*, Basidiomycota sp., Mycosphaerellaceae sp. and *Mycosphaerella holualoana* (Fig. 1B).

Tree species richness effects disappeared when all fungal OTUs were considered simultaneously, as the linear mixed models based either on community-level tree species richness or neighbourhood tree species richness showed that Shannon diversity of litter fungi was unaffected by tree species richness (Table 2). Instead, the diversity of the entire fungal communities showed significant associations with certain tree species and litter chemical traits such as Fe and K concentration (Table 2). The Shannon diversity index of the entire fungal community and non-pathogenic fungi in freshly-fallen leaves did not change with community-level tree species

richness (both $P > 0.05$). However, Shannon diversity of pathogenic fungi showed a gradual increase with increasing tree species richness (Fig. 3A). In contrast, the relative abundance of fungal pathogens did not significantly change with community-level tree species richness (Fig. 3B).

The composition of leaf litter fungal community, in general, was significantly affected by community-level tree species richness (Fig. 2A). The results of permutational multivariate analysis of variance confirmed the significance of tree species richness effects (Table S2). Furthermore, there was an interaction of tree species richness with focal tree species identity in affecting fungal OTUs composition (Table S2). The separate dbRDA models for each focal tree species further showed that the response of fungal OTUs composition in freshly-fallen leaves to tree species richness was focal tree species-dependent (Table 3). Specifically, fungal species composition in litter of *C. sclerophylla* that was dominated by *Mycosphaerella holualoana*, *Ramichloridium cerophilum*, Teratosphaeriaceae sp.1, and Dothideomycetes sp. (Fig. 1), was significantly affected by community tree species richness (Table 3). Similarly, the fungal community in the litter of *T. sebifera* was also influenced by tree species richness and mainly dominated by *Mycosphaerella holualoana*, Teratosphaeriaceae sp.2, Botryosphaeriaceae sp., *Mycosphaerella* sp.2 and Ascomycota sp. 2 (Table 3). Moreover, we also found a significant interaction of tree species richness with the family of focal tree species in affecting the composition of all fungal OTUs and pathogenic fungi, but not of non-pathogenic fungi (Table S2).

3.4. Neighbourhood tree species richness and family effects

In accordance with community tree species richness, neighbourhood tree species richness showed a significant effect on the OTU composition of fungi in freshly-fallen leaves (Table S2). Moreover, neighbor tree species richness interacted with the

identity of focal tree species affecting fungal OTU composition (Table S2). Specifically, the fungal community composition of freshy-fallen leaves of *T. sebifera* was significantly influenced by neighbourhood tree diversity, but those of other focal tree species were not (Table 3). Neighbour tree family, as assessed by the proportion of neighbour individuals belonging either to Fagaceae or to other families, showed significant effects on the OTU composition of the entire fungal, pathogenic and non-pathogenic fungal community (Table S2).

The Bray-Curtis dissimilarity of fungal community per focal tree individual in tree mixture plots to those inhabiting the same tree species in monoculture plots, generally increased with increasing community tree species richness (Fig. 4A), neighbour tree species richness (Fig.4B), and proportion of species that were heterospecific to the focal species in the local neighbourhood (Fig. 4C). These three relationships had similar correlation coefficients (r = 0.250, 0.209 and 0.256, respectively), showing that they explained differences in fungal community composition equally well. As to focal tree species, the dissimilarity of fungi occurring in litter of *C. sclerophylla*, *L. formosana*, *N. sinensis*, and *T. sebifera* was significantly different from those in litter of *C. henryi* (all $P > 0.05$, Fig. 4D). Except for *S. saponaria* and *C. axillaris* (Fig. 4D), the dissimilarity of litter fungal communities tended to increase with the proportion of heterospecific neighbour tree species (Fig. 4D).

4. Discussion

Fungal species in freshly-fallen leaves were analysed at the individual level of eight focal tree species, allowing us to disentangle the effects of community and neighbourhood tree species richness on them. Basically, our results demonstrate significant effects of tree species identity on the Shannon diversity and OTU

composition of fungal community inhabiting freshly fallen leaves. Furthermore, species-dependent and family-dependent community tree species richness effects were encountered on fungal OTU composition. The Shannon diversity of pathogenic fungal guild but not the other functional guild significantly changed with community-level tree species richness. Dissimilarity in the composition of fungal species in focal tree litter was equally well explained by community tree species richness, neighour tree species richness, and proportion of heterospecific tree neighbourhoods, suggesting that heterospecificity of the local tree neighbourhood is one of the underlying mechanisms of tree diversity effects.

4.1. The significant effects of tree species identity on fungal species

Our results confirm the effects of tree species identity as reported by previous studies. Saprotrophic fungal species are prone to develop on leaf litters of particular tree species (Prescott & Grayston, 2013). Fungal species inhabiting freshly-fallen litter can physiologically or evolutionarily adapt to use litter nutrients of a particular tree species and to cope with specific secondary compounds although they usually show a less strong host plant specificity compared to symbiotic and pathogenic fungi (Lodge, 1997). Alternatively, a considerable proportion of epiphytic and endophytic fungi in fresh leaves are predicted to occur in leaf litters due to their saprotrophic potentials (Herre et al., 2005; Unterseher et al., 2013; Guerreiro et al., 2018). Among the dominant fungal species in our study, some fungi such as *Mycosphaerella* spp. are both endophytes and efficient early saprotrophs (Sun et al., 2012; Voříšková & Baldrian, 2013). They are usually more strongly affected by host tree species identity due to their host specialization (Crous & Braun, 2003). The host specialization of these dominant fungal species may thus contribute to the response of the composition of litter-inhabiting fungal species to tree species identity (Kubartová et al., 2009). The

host preference of pathogenic fungi (Hantsch et al., 2014a), at least within a defined study area (Thomas et al. 2019), may be another explanation for the dependence of the entire fungal community in freshly-fallen leaves on tree species identity. We also found family identity effects of Fagaceae, but only on the fungal community composition in freshly-fallen leaves and not on Shannon diversity or abundance of fungi in leaf litter. This indicates that the leaf litter-inhabiting fungal community probably depends more on the identity of tree species rather than on that of families. Overall, our findings highlight the importance of tree species identity in modifying fungal community in freshly-fallen leaves of subtrophical tree species, in support of our first hypothesis.

4.2. The contrasting effects of tree species richness on the diversity of different fungal guilds

The Shannon diversity of the entire fungal community in freshly-fallen leaves was not affected either by topographic factors or by community-level tree species richness. The results suggest that chemical traits and/or abiotic factors rather than community tree species number per se are more important drivers, as observed in previous studies (McGuire et al., 2012; Prakash et al., 2015). Litter chemical traits including Fe and K may play a role in affecting the diversity of these fungal species. The positive relationship between fungal diversity and litter K is in accordance with the abundance hypothesis stating that nutrient-based increase in abundance of fungal species would reduce their risk of becoming locally lost from the fungal community (Srivastava & Lawton, 1998; Kaspari et al., 2017). In contrast, following the competition hypothesis, high availability of litter Fe may suppress fungal diversity by promoting competitive and fast growing specialists (Tilman, 1982; Kaspari et al., 2017).

It is worth noting that the plant pathogenic fungal guild showed a stronger sensitivity to tree species loss at the community level than of non-pathogenic fungi. The finding that richness of pathogenic fungi increased with community tree species richness in our study was opposite to the observation of the Kreinitz forest biodiversity experiment in Germany, where the authors found decreasing foliar pathogen richness with increasing tree species richness (Hantsch et al., 2014b). As in living leaves tree species richness acts on host-specific fungi through host dilution effects also in freshly-fallen leaves, brought about by a decreasing proportion of host species in a more diverse tree community (Keesing et al., 2006; Hantsch et al., 2014b). From this perspective, pathogenic fungal specialists showing stronger host tree preference may be less important compared to those fungal generalists in response to tree species richness in our study. Pathogenic fungal generalists that can be associated with multiple host tree species may benefit from transmission from other hosts in the neighbourhood, thus explaining the increased pathogenic fungal richness with community tree species richness in our study.

4.3. Species (family)-dependence of community-level tree species richness effects on fungal community composition

In line with our second hypothesis, the magnitude of tree species richness effect on fungal community composition depended on the identity of focal tree species, as well as on the family to which the focal tree species belong. Furthermore, fungal species only in leaf litter of *C. sclerophylla* and *T. sebifera* significantly changed with community tree species richness. Except that the litter of both species was characterized by low Fe concentration, no other differences in litter chemical characteristics from the other species were observed in this study. Yet litter-inhabiting fungal species of *L. formosana* did not respond to community tree species richness

loss though it has likewise lower Fe concentration in litter, indicating that these litter chemical traits are not, at least, direct and compelling reasons for the species-specific tree species richness effects. Some other traits that we did not measure may have structured the fungal community inhabiting the two focal tree species, such as concentration of phenolics, tannins (Eichenberg et al., 2015) and lignin (Donnelly et al., 1990; Fioretto et al., 2005).

The fungal community in freshly-fallen leaves of *C. sclerophylla* and *T. sebifera* was dominated by *Mycosphaerella holualoana*, *Ramichloridium cerophilum* and Teratosphaeriaceae sp. 2. As our results have indicated, they were also more sensitive to the loss of community-level tree species richness. These fungal species are able to survive as endophytes in living leaves and have a stronger host specificity than common saprotrophic fungi (Crous & Braun, 2003; Douanla-Meli et al., 2013; Voříšková & Baldrian, 2013). The stronger linkages of these fungal species to the host tree species may, to some extent, contribute to species-dependent tree species richness effects on the fungal community composition in leaf litter. Our results showed that plant pathogenic fungi that are host specific (Konno et al., 2011; Hantsch et al., 2014a), also contributed to the tree species-specific divesity effect in our study. Overall, these findings provide insight into species-dependent and family-dependent tree species richness effects on the fungal community in freshly-fallen leaves.

4.4. Neighbour effects on fungal community in freshly-fallen leaves of focal tree species

In the third hypothesis, we expected that neighbour tree species richness would more strongly affect the fungal community in freshly-fallen leaves of the focal tree species than community tree species richness. Although we could not confirm this hypothesis as community and neighbourhood tree species richness effects were of the

same magnitude, we found richness effects equally well explained by the proportion of heterospecific trees in the local neighbourhood. The dissimilarity of fungal species inhabiting freshly-fallen leaves of a given focal tree species between non-monoculture plots and monoculture plots increased with the increasing proportion of non-focal tree species in the local neighbourhood. We found that neighbour tree species richness and the proportion of tree individuals belonging to Fagaceae affected the fungal OTU composition, indicating phylogenetic effects of the neighbourhood. There are numerous cases of plant pathogens that can attack a range of closely related plant species of the same family (Santana et al., 2005; Gilbert & Webb, 2007). Thus, in addition to heterospecificity as a key mechanism of tree diversity effects, the proportion of neighbour Fagaceae trees contributed to the focal tree-associated fungal community composition and also modified tree diversity effects on fungal community composition. Neighbour trees can alter the chemical traits of focal tree individual through interspecific nutrient transfer belowground (Klein et al., 2016) and/or in the litter layer (Hättenschwiler et al., 2005), thereby inducing transmission of fungal species from neighbour tree species (Hättenschwiler et al., 2005; Keesing et al., 2010; Hantsch et al., 2014b). Furthermore, the heterospecific neighbourhood can alter light transmittance and subsequently change the abiotic environment that promotes specific fungal species (Tremmel & Bazzaz, 1993; Chamagne et al., 2016). All of these may be plausible explanations for the effects of tree species richness at the local neighbourhood.

5. Conclusions

Our study elucidates the consequences of community tree species richness and local tree neighbourhoods on fungal communities in freshly-fallen leaves of eight focal tree species. Our results point to the significance of focal tree species identity

structuring the diversity and composition of fungal groups and entire fungal community in freshly-fallen leaves. Furthermore, the identity of the host tree species strongly affected the response of fungal species in freshly-fallen leaves, particularly of pathogenic fungi, to community and neighourhood tree species richness. We suggest that a key mechanism of tree species richness effect is the proportion of heterospecific tree species in the local neighbourhood. An increasing proportion of heterospecific tree neighbours could affect litter fungal species composition of the focal tree individual either by fungal species dispersal between litter types or biotic- and/or abiotic modifications of leaf litter traits. These findings highlight the importance for teasing apart the underlying principles of tree species richness effects at the local neighbourhood and community scales.

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Data accessibility We confirm that the data supporting the results will be archived on

the BEF-China project data base http://china.befdata.biow.uni-leipzig.de/ and will be available upon request from the corresponding author. The representative sequences obtained from Illumina MiSeq sequencing have been submitted to EMBL (accession no. **PRJEB28409**).

Author contributions N.Z. together with K.P.M. conceived the ideas and designed the experiment. Y.N.L. and N.Z. conducted sample collection and measurement. N.Z., H.B., T.W. and Y.L. contributed to data analysis. N.Z., H.B and S.T. performed paper writing. All authors gave final approval for publication.

Conflict of interest The authors declare no conflict of interest.

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Figure legends

Figure 1 Heat map of 35 dominant fungal OTUs showing their different abundance among (A) eight focal tree species and (B) the four levels of plot tree species richness. Hierarchical clustering was performed for the dominant fungal OTUs. The Z values show the magnitude of treatment effects. Kruskal-Wallis test was used to test the difference per dominant OTU among (A) eight focal tree species and (B) the four tree species richness. Symbols †, *, **, *** represent significance at $P < 0.1$, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. R1, R2, R4 and R8 refer to 1, 2, 4 and 8 tree specie richess, respectively. Cahe, *Castanea henryi*; Casc, *Castanopsis sclerophylla*; Chax, *Choerospondias axillaris*; Lifo, *Liquidambar formosana*; Nysi, *Nyssa sinensis*; Quse, *Quercus serrata*; Sasa, *Sapindus saponaria*; Trse, *Triadica sebifera*.

Figure 2 Distance-based redundancy analysis (dbRDA) showing significant predictors affecting the overall fungal community composition in freshly-fallen leaves. The pattern of litter-inhabiting fungal community with respect to (A) community tree species richness and (B) significant predictors. TreeR, community tree species richness; C/N, litter carbon to nitrogen ratio; Ca, litter calcium; NS, north-south component of slope aspect; slope, slope inclination. Cahe, *Castanea henryi*; Casc, *Castanopsis sclerophylla*; Chax, *Choerospondias axillaris*; Lifo, *Liquidambar formosana*; Nysi, *Nyssa sinensis*; Quse, *Quercus serrata*; Sasa, *Sapindus saponaria*; Trse, *Triadica sebifera*.

Figure 3 The (A) Shannon diverity index and (B) relative abundance of fungal pathogens in freshly-fallen leaves. TreeR, community tree species richness; NeighbourR, neighbourhood tree species richness; SP, focal tree species. There was

no significant effects of tree richness, neighbour tree richness, species and their interactions on the relative abundance of pathogenic fungi, and thus did not show the relevant results in the right panel of the figure.

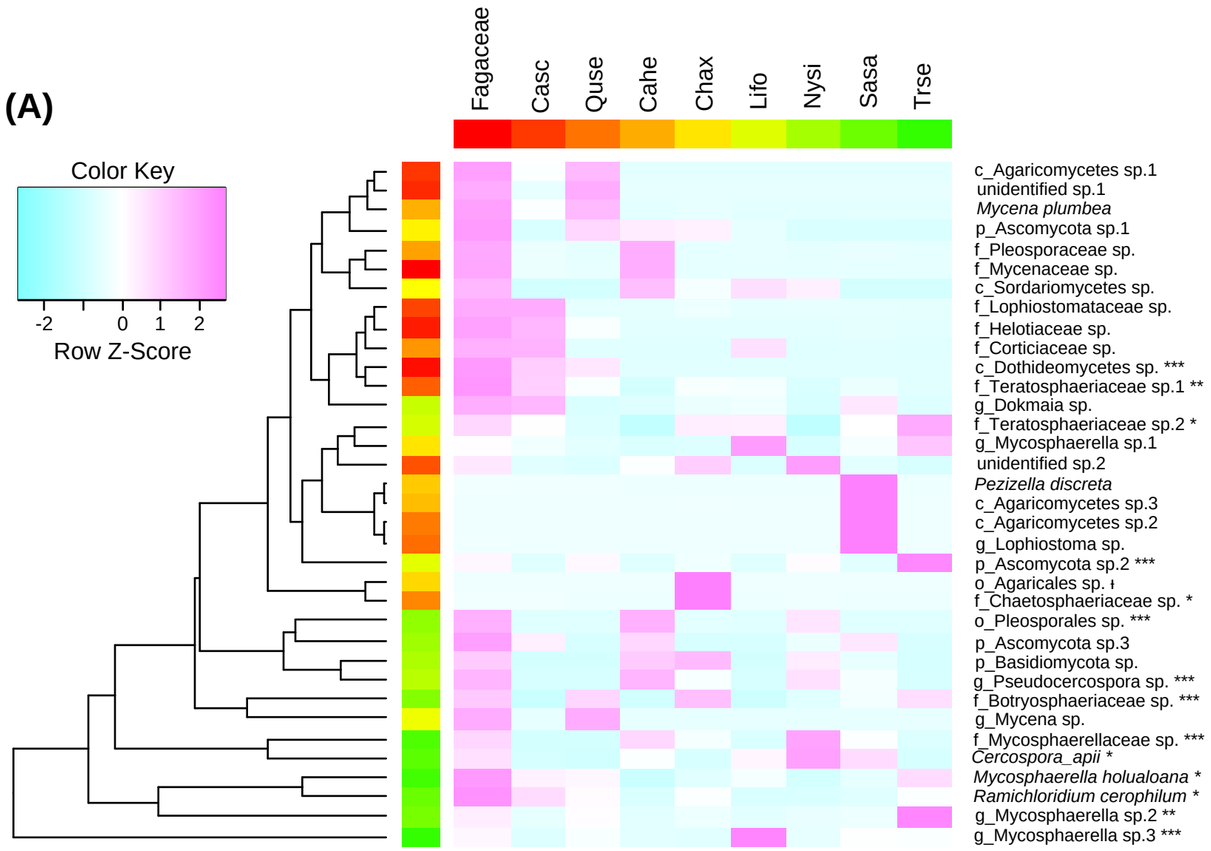
Figure 4 The effects of (A) community tree species richness, (B) neighbour tree richness and (C,D) the proportion of heterospecific tree species in the neighbourhood surrounding each target individual on the Bray-Curtis dissimilarity of litter-inhabiting fungal communities per target individual. Cahe, *Castanea henryi*; Casc, *Castanopsis sclerophylla*; Chax, *Choerospondias axillaris*; Lifo, *Liquidambar formosana*; Nysi, *Nyssa sinensis*; Quse, *Quercus serrata*; Sasa, *Sapindus saponaria*; Trse, *Triadica sebifera*.

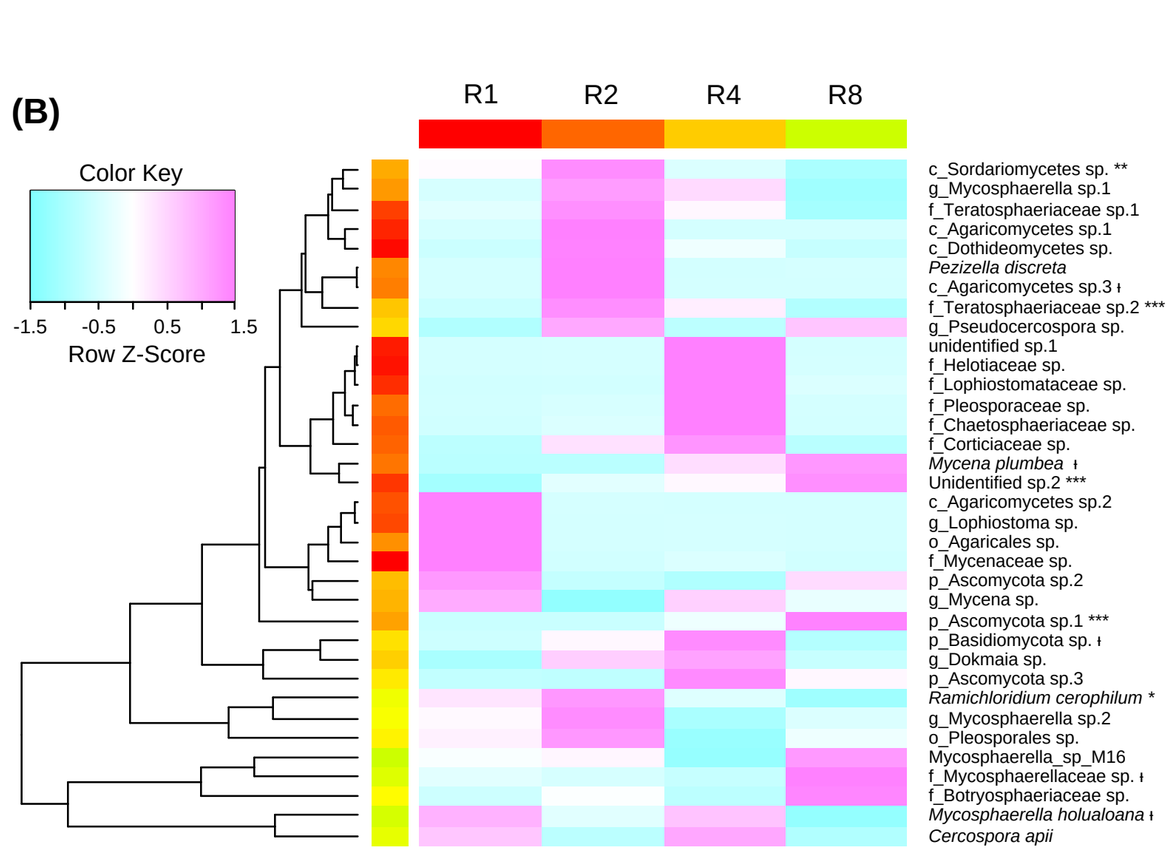
Figure S1 Geographical location of the very intensively studied (VIP) plots at site A and sampling design.

Figure S2 Phylogenetic assignment (phylum) of all fungal OTUs in freshly fallen leaves of eight tree species.

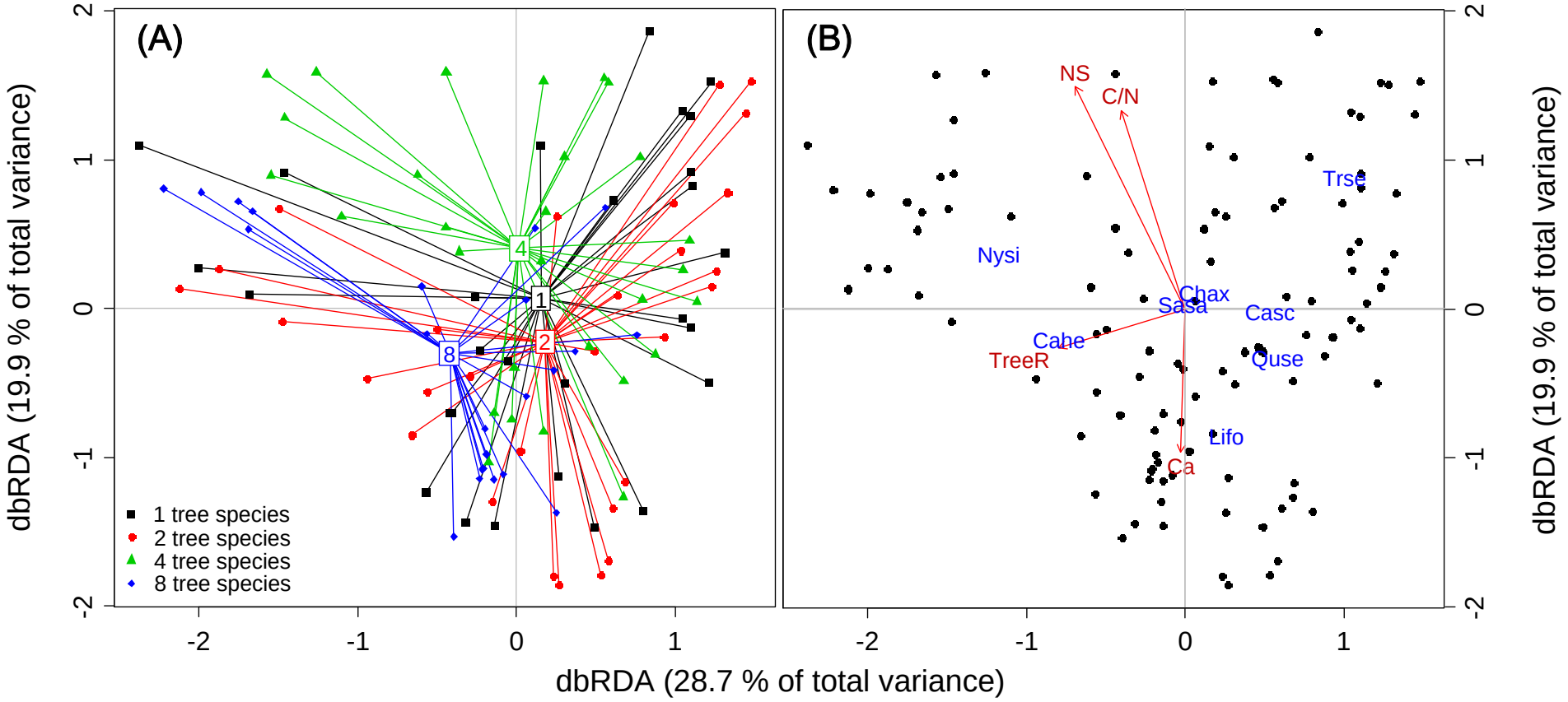
Figure S3 Dominant fungal species that had significant Spearman correlation with community (TreeR) and neighbourhood tree species richness (NeighbourR). Here we also showed the graphs of Dokmaia.sp and Lophiostomataceae.sp although they were not significantly correlated with neighbourhood tree species richness.

Figure

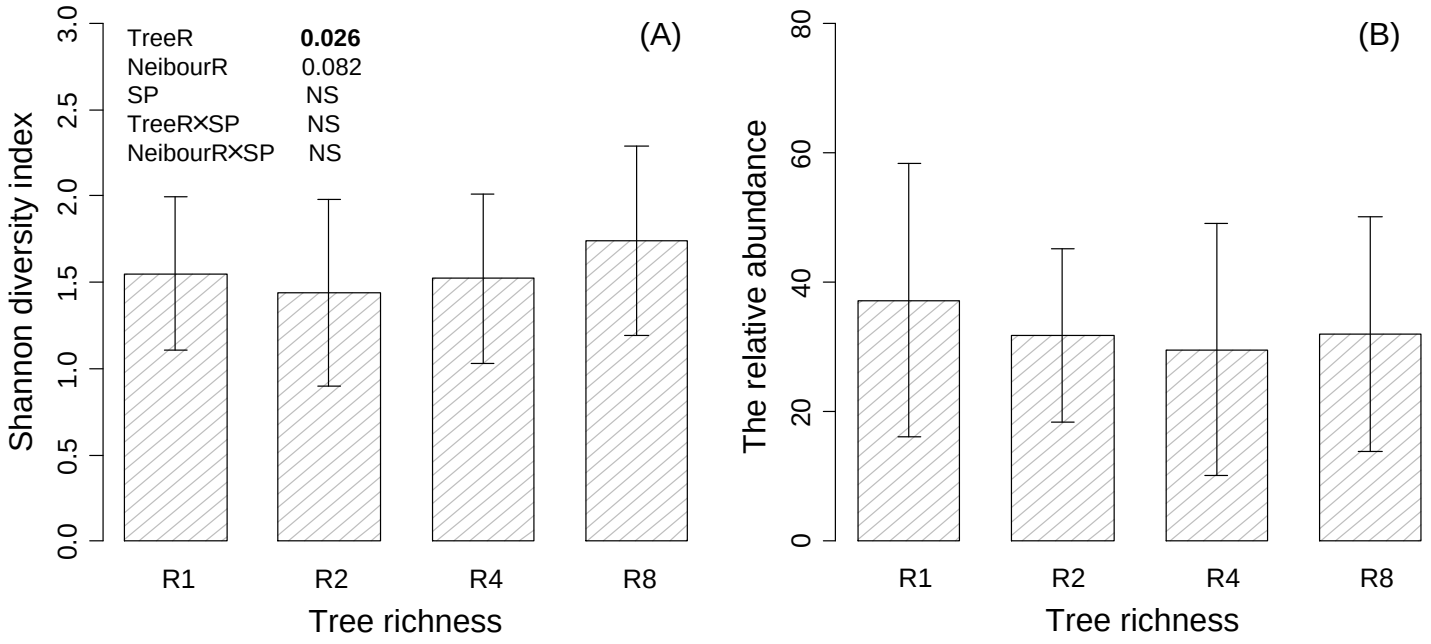

(A)
Fagaceae
Casc
Quse
Cahe
Chax
Lifo
Nysi
Sasa
Trse
Color Key
-2
0
1
2
Row Z-Score
c_Agaricomycetes sp.1
unidentified sp.1
Mycena plumbea
p_Ascomycota sp.1
f_Pleosporaceae sp.
f_Mycenaceae sp.
c_Sordariomycetes sp.
f_Lophiostomataceae sp.
f_Helotiaceae sp.
f_Corticiaceae sp.
c_Dothideomycetes sp. ***
f_Teratosphaeriaceae sp.1 **
g_Dokmaia sp.
f_Teratosphaeriaceae sp.2 *
g_Mycosphaerella sp.1
unidentified sp.2
Pezizella discreta
c_Agaricomycetes sp.3
c_Agaricomycetes sp.2
g_Lophiostoma sp.
p_Ascomycota sp.2 ***
o_Agaricales sp.
f_Chaetosphaeriaceae sp. *
o_Pleosporales sp. ***
p_Ascomycota sp.3
p_Basidiomycota sp.
g_Pseudocercospora sp. ***
f_Botryosphaeriaceae sp. ***
g_Mycena sp.
f_Mycosphaerellaceae sp. ***
Cercospora_apii *
Mycosphaerella holualoana *
Ramichloridium cerophilum *
g_Mycosphaerella sp.2 **
g_Mycosphaerella sp.3 ***


(B)
R1
R2
R4
R8
Color Key
-1.5
-0.5
0.5
1.5
Row Z-Score
c_Sordariomycetes sp. **
g_Mycosphaerella sp.1
f_Teratosphaeriaceae sp.1
c_Agaricomycetes sp.1
c_Dothideomycetes sp.
Pezizella discreta
c_Agaricomycetes sp.3
f_Teratosphaeriaceae sp.2 ***
g_Pseudocercospora sp.
unidentified sp.1
f_Helotiaceae sp.
f_Lophiostomataceae sp.
f_Pleosporaceae sp.
f_Chaetosphaeriaceae sp.
f_Corticiaceae sp.
Mycena plumbea
Unidentified sp.2 ***
c_Agaricomycetes sp.2
g_Lophiostoma sp.
o_Agaricales sp.
f_Mycenaceae sp.
p_Ascomycota sp.2
g_Mycena sp.
p_Ascomycota sp.1 ***
p_Basidiomycota sp.
g_Dokmaia sp.
p_Ascomycota sp.3
Ramichloridium cerophilum *
g_Mycosphaerella sp.2
o_Pleosporales sp.
Mycosphaerella_sp_M16
f_Mycosphaerellaceae sp.
f_Botryosphaeriaceae sp.
Mycosphaerella holualoana
Cercospora apii

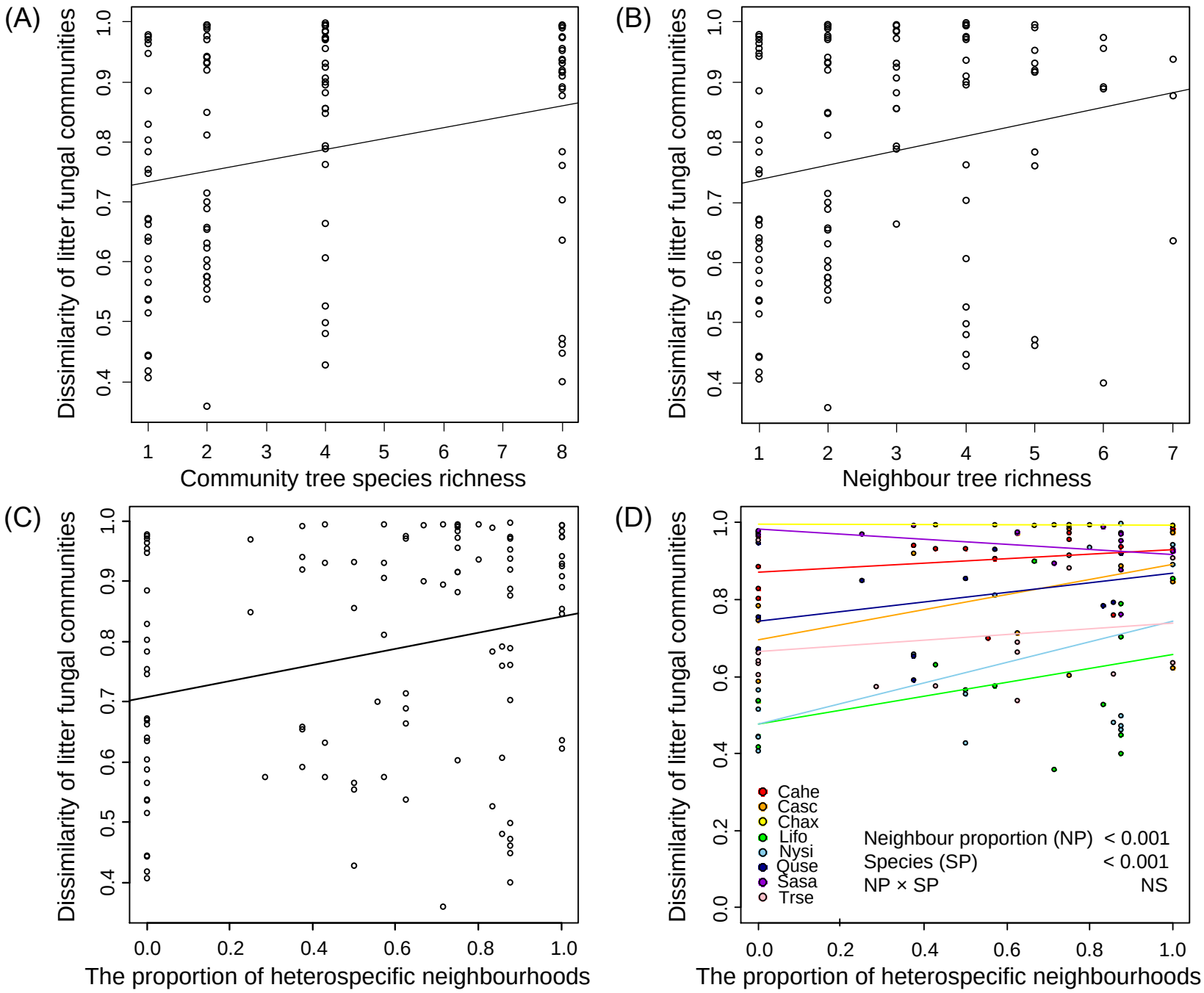
Figure


(A)
(B)
1 tree species
2 tree species
4 tree species
8 tree species
NS
C/N
Trse
Nysi
Chax
SaSa
Casc
Cahe
TreeR
Quse
Lifo
Ca
dbRDA (19.9 % of total variance)
dbRDA (28.7 % of total variance)
dbRDA (19.9 % of total variance)

Figure


TreeR 0.026
NeibourR 0.082
SP NS
TreeR×SP NS
NeibourR×SP NS
(A)
Shannon diversity index
0.0
0.5
1.0
1.5
2.0
2.5
3.0
R1
R2
R4
R8
Tree richness
(B)
The relative abundance
0
20
40
60
80
R1
R2
R4
R8
Tree richness

Figure


(A)
Dissimilarity of litter fungal communities
Community tree species richness
(B)
Dissimilarity of litter fungal communities
Neighbour tree richness
(C)
Dissimilarity of litter fungal communities
The proportion of heterospecific neighbourhoods
(D)
Dissimilarity of litter fungal communities
The proportion of heterospecific neighbourhoods
Cahe
Casc
Chax
Lifo
Nysi
Quse
Sasa
Trse
Neighbour proportion (NP) < 0.001
Species (SP) < 0.001
NP × SP NS

Figure

Site A

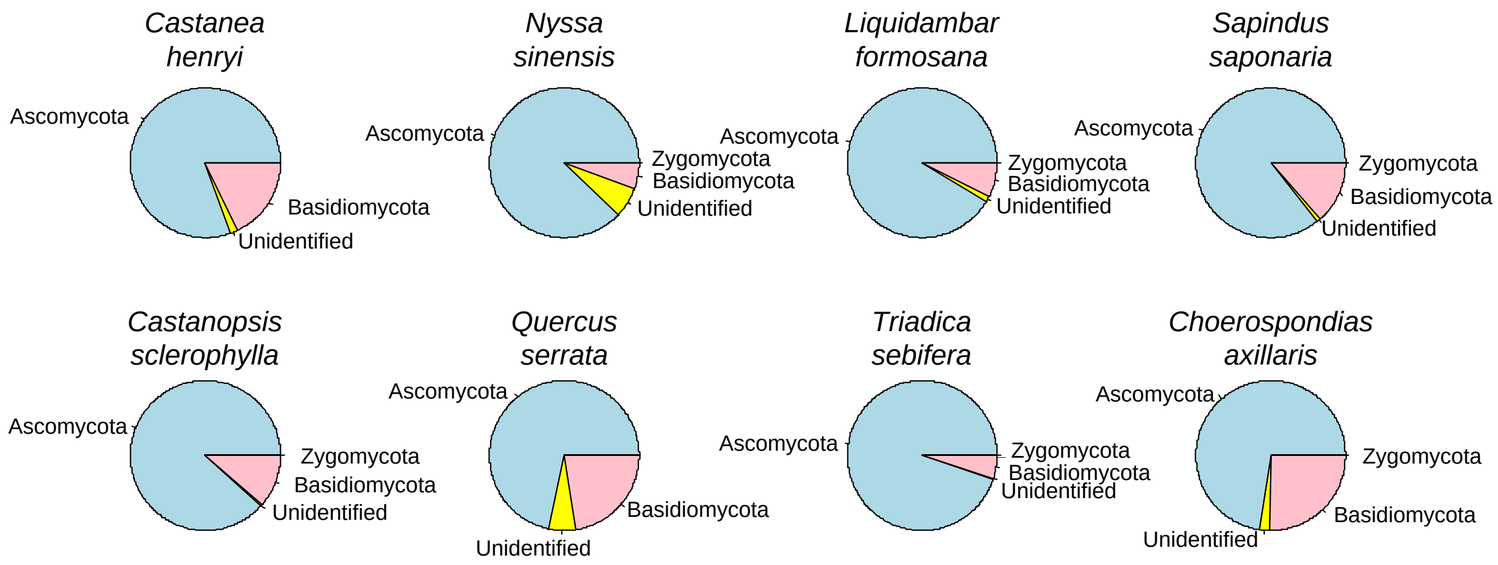
Monoculture
2-species mix
4-species mix
8-species mix
16-specis mix

Focal tree
Neighbour tree
Other trees within plot

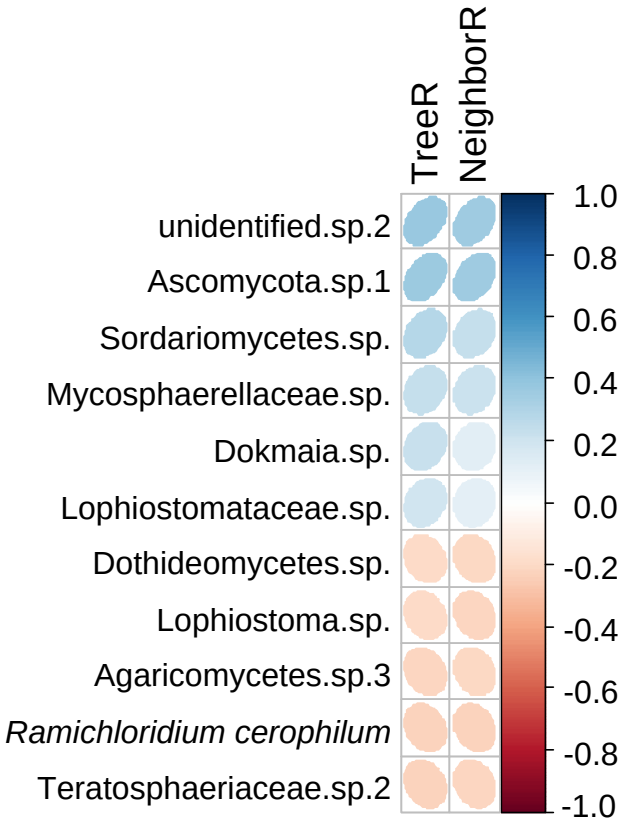
1.29m
1.82m

Plot (1 mu) - 400 individual tree in total

Figure


Castanea henryi
Ascomycota
Basidiomycota
Unidentified
Nyssa sinensis
Ascomycota
Zygomycota
Basidiomycota
Unidentified
Liquidambar formosana
Ascomycota
Zygomycota
Basidiomycota
Unidentified
Sapindus saponaria
Ascomycota
Zygomycota
Basidiomycota
Unidentified
Castanopsis sclerophylla
Ascomycota
Zygomycota
Basidiomycota
Unidentified
Quercus serrata
Ascomycota
Basidiomycota
Unidentified
Triadica sebifera
Ascomycota
Zygomycota
Basidiomycota
Unidentified
Choerospondias axillaris
Ascomycota
Zygomycota
Basidiomycota
Unidentified

Figure


TreeR
NeighborR
unidentified.sp.2
Ascomycota.sp.1
Sordariomycetes.sp.
Mycosphaerellaceae.sp.
Dokmaia.sp.
Lophiostomataceae.sp.
Dothideomycetes.sp.
Lophiostoma.sp.
Agaricomycetes.sp.3
Ramichloridium cerophilum
Teratosphaeriaceae.sp.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0

Table

Table

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Table

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