Biodiversity and assembly processes of soil fungal communities in Chinese subtropical forests with variable tree diversity

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Biodiversity and assembly processes of soil fungal communities in Chinese subtropical forests with variable tree diversity

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Dissertation

143 pages, 318 references, 29 figures, 28 tables

This cumulative dissertation unravels important drivers of soil fungal community assembly and soil fungal / tree species interaction patterns in relation to tree species diversity in young subtropical forests. Fungal communities were assessed by molecular methods amplifying and subsequently pyrosequencing a genomic fungal marker region. The work was conducted within the "Biodiversity and ecosystem-functioning experiment China" (BEF China) which is currently the largest tree diversity experiment worldwide. Manuscript 1 investigated the suitability of freeze-drying for the short-term conservation of soil samples subjected to the high-throughput sequencing of soil microbial communities. Freeze-drying of soil samples proved to be an effective, non-toxic, relatively cheap and feasible method for the conservation of bacterial and soil arbuscular mycorrhizal fungal communities for short-term storage of up to seven days at ambient temperature as analyzed by both RNA and DNA level. Manuscript 2 presents the investigation of the relative contribution of biotic and abiotic drivers on fungal community composition and richness in the BEF China experiment. We analyzed the effects of tree diversity at the level of actual neighborhood diversity of the sampling position. We found that at the early stage of forest development, environmental and stochastic processes dominated the assembly of the soil fungal community. Tree related variables, such as tree community composition, significantly affected arbuscular mycorrhizal and plant pathogen fungal community structure, while differences in tree host species and host abundance affected ectomycorrhizal fungal community composition. Manuscript 3 inferred the dependence of the degree of fungal specialization from forest tree diversity. Here, tree-fungal bipartite networks based on co-occurrences were constructed. Connectance and fungal generality values were highest for the two tree species mixtures. Tree monocultures showed the highest specialization in fungal OTUs.

ZUSAMMENFASSUNG

Unser Planet Erde ist Heimat einer gewaltigen Vielzahl an Organismen. In den letzten Jahrzehnten wurde sichtbar, dass anthropogene Einflüsse diese Diversität erheblich bedrohen und ein Artensterben in beispielloser Rate eingesetzt hat. Schätzungen zufolge ist die Hälfte aller Baumarten vom Aussterben bedroht. Die oberirdisch sichtbare Diversität der Pflanzen und die unterirdische Diversität der Bodenpilze sind eng miteinander verbunden. Der Großteil dieser Bodenpilze bleibt dem bloßen Auge verborgen und Schätzungen zufolge umfassen sie bis zu 5 Millionen Arten. Bodenpilze leisten einen erheblichen Beitrag zu einer Vielzahl von Ökosystemfunktionen wie beispielsweise dem globalen Nährstoffkreislauf, der Nährstoffversorgung und Gesunderhaltung der Pflanzen sowie der Bodenfruchtbarkeit und dem Bodenschutz. Um konkrete Vorhersagen über die ökologischen Konsequenzen des Baumsterbens in Bezug auf die Bodenpilzgemeinschaft anstellen zu können, ist ein besseres Verständnis der komplexen Beziehung von Baumdiversität und Bodenpilzgemeinschaft Voraussetzung.

Das „Biodiversität und Ökosystemfunktionen Experiment China“ (BEF China) zielte auf die Untersuchung „der Rolle von Baum- und Strauchdiversität für Produktion, Erosionsschutz, Nährstoffzyklus und Artenschutz in chinesischen subtropischen Waldökosystemen“ ab. Im Rahmen dieses Projektes wurden die Zusammenhänge zwischen der manipulierten Baumartenvielfalt und der Tier- und mikrobiellen Vielfalt untersucht und ebenfalls in Bezug zu abiotischen Faktoren gesetzt. Diese Doktorarbeit umfasst die erste Aufnahme der Bodenpilzgemeinschaft im BEF China Projekt, durchgeführt nach der dritten Wachstumsphase der Baumsetzlinge. Die Bodenproben wurden 2011 in insgesamt 31 Waldstücken der BEF China Site A genommen, die im Rahmen des „zufälligen Baumsterben Szenarios“ entsprechend einer sogenannten „broken stick“ Auswahl gepflanzt wurden. Unsere Studien basierten auf der molekularen Technik der hochsensitiven Pyrosequenzierung von Pilz-DNA aus Bodenproben. Die Dissertation besteht aus drei Manuskripten wissenschaftlicher Publikationen, wovon bereits zwei in internationalen Zeitschriften erschienen sind.

Manuskript 1, “Konservierung von Nukleinsäuren durch Gefriertrocknung für die Hochdurchsatz-Sequenzieranalyse von bodenmikrobiellen Gemeinschaften“ untersuchte die Eignung der Gefriertrocknung für die Kurzzeitkonservierung von Bodenproben. Eine erfolgreiche Konservierung der in China gesammelten Bodenproben stellte die Basis für die nachfolgende Analyse in Deutschland dar. Wir nahmen an, dass der effektive Wasserentzug durch die Gefriertrocknung weiteres Wachstum der Mikroorganismen in den Bodenproben verhindern und Enzyme inaktivieren würde (vor allem solche mit degradierenden Fähigkeiten gegenüber Nukleinsäuren). Für diese Studie wurden Bodenproben im Deutschen Biodiversitätsexploratorium Hainich-Dün auf zwei Graslandflächen gesammelt und verschiedenen Lagertemperaturen und Lagerzeiten nach der Gefriertrocknung unterzogen. Die detektierten mikrobiellen Gemeinschaften der gefriergetrockneten Bodenproben und durchgängig gefrorenen Kontrollproben wurden verglichen. Die Gefriertrocknung bewies sich als effektive, ungiftige, relativ preiswerte und leicht verfügbare Methode zur Konservierung von RNA und DNA für spätere molekularbiologische Analysen von Bodenbakterien und arbuskulären Mykorrhizapilzen.

Manuskript 2: “Experimentelle Beweise funktioneller gruppenabhängiger Effekte der Baumartenvielfalt auf Bodenpilze in subtropischen Wäldern“ stellt die Untersuchung des relativen Einflusses von biotischen und abiotischen Faktoren auf die Bodenpilzgemeinschaft im BEF China Experiment dar. Wir untersuchten die Pilzgemeinschaften im Diversitätsgradienten von 1, 2, 4, 8 und 16 Baumarten je Waldparzelle. Wir analysierten die Effekte von Baumartenvielfalt auf der Basis der tatsächlichen Nachbarschaftsdiversität an jeder Beprobungsstelle. Hierbei bezogen wir die Identität und die Mykorrhizierungsart des Probenbaumes und seiner acht benachbarten Bäume mit ein. Wir unterteilten die Bodenpilzgemeinschaft in die funktionellen Gruppen von Saprotrophen, Pflanzenpathogenen und Mykorrhizapilzen (arbuskuläre und Ectomykorrhiza). Wir fanden heraus, dass zum Zeitpunkt des frühen Waldentwicklungsstadiums Umweltfaktoren und stochastische Prozesse die Zusammensetzung der Bodenpilzgemeinschaft maßgeblich bestimmten. Baumbezogene Variablen wie die Baumartenzusammensetzung beeinflussten die Zusammensetzung der arbuskulären Mykorrhizapilze und der pflanzenpathogenen Pilzgemeinschaft signifikant. Hingegen beeinflussten Unterschiede in der Baumwirtspezies und Häufigkeit der Baumwirte die Gemeinschaft der Ektomykorrhizapilze.

Manuskript 3 “Der Zusammenhang von Pilzspezialisierungsgrad und Baumvielfalt in jungen subtropischen Chinesischen Wäldern” wandte einen eher systemischen Ansatz an und es wurden Muster von gemeinsamen Pilz- und Baumvorkommen durch bipartite Netzwerkanalysen untersucht. Des Weiteren wurde die Pilzspezialisierung in drei Baumdiversitäsgraden ermittelt. Hierzu wurden Netzwerkmaßzahlen und der Spezialisierungskoeffizient phi ausgewertet. Wir fanden heraus, dass Pilze die höchste Spezialisierung in Baummonokulturen aufwiesen. Entgegen unserer Erwartungen war die Spezialisierung der Pilze am geringsten in den Wäldern mit zwei Baumarten, statt in den Wäldern mit einer hohen Baumartenvielfalt. Dementsprechend war auch die Netzwerkmaßzahl Konnektivität in den Wäldern mit zwei Baumarten am größten. Die Artenvielfalt der Bodenpilze war signifikant höher in den Wäldern mit hoher Baumdiversität.

Wir haben also signifikante Einflüsse der Baumartenvielfalt auf die Bodenpilzgemeinschaft und deren Spezialisierungsmuster zu einem frühen Entwicklungsstadium der subtropischen experimentellen Wälder gefunden. Somit unterstreichen die Ergebnisse eine erhebliche Rolle der überirdischen Baumartenvielfalt auf die unterirdische Pilzgemeinschaft.

Die molekularen Methoden, die die Grundlage dieser Dissertation bildeten, ermöglichten eine umfassende Analyse der Pilzgemeinschaft. Weiterführende Arbeiten sollten ebenso funktionelle Zusammenhänge bspw. auf die Baumartenproduktivität, Baumgesundheit oder Laubabbau mit einbeziehen. Des Weiteren sollten mehrere Bodenorganismen in ökologischen Studien mit einbezogen werden wie bspw. Bakterien, Archäen und Protisten. Meta-omics Daten (Metagenomik, Metatranskriptomik und Metaproteomik) bieten darüber hinaus Möglichkeiten funktionelle Fähigkeiten der untersuchten mikrobiellen Gemeinschaft zu ermitteln.

SUMMARY

The Earth harbors a huge diversity of organisms. In the recent decades it became apparent that due to anthropogenic impacts this biodiversity is severely threatened and species go extinct at an unprecedented rate. Tree species loss was estimated to threaten half of the tree species. The aboveground diversity of plants and the belowground diversity of soil fungi are tightly connected. Estimates of global soil fungal diversity vary with up to 5 million species. Soil fungi contribute to a large extent to global element cycling, plant nutrition and performance, and to a multitude of other ecosystem functions. Hence, deforestation and tree species loss e.g. due to climate change or conversion of forests to plantations with low tree species richness are expected to also affect belowground fungal diversity and the ecological processes it conveys. A better understanding of the tree diversity – fungal community relationship will help to improve soil fungal conservation measures and the prediction of the consequences of tree species loss to soil fungal mediated ecosystem processes.

The "Biodiversity and Ecosystem-Functioning experiment China" (BEF China) aimed to investigate "the role of tree and shrub diversity for production, erosion control, element cycling, and species conservation in Chinese subtropical forest ecosystems". Within this project, the relationship between manipulated tree species diversity and animal and microbial diversity was assessed and also related to abiotic components. We aimed to analyze the huge diversity of soil fungi in relation to tree diversity by the highly sensitive and powerful molecular method of high-throughput next generation sequencing. This thesis constitutes the first assessment of the soil fungal community in the BEF China project, undertaken after the third growing season of planted tree saplings. The soil samples collected in 2011 on 31 forest plots at BEF China Site A from the "random-extinction scenario" treatment planted in a broken-stick-design built the basis for this thesis.

Manuscript 1, "Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities" investigated the suitability of freeze-drying for the short-term conservation of soil samples subjected to the high-throughput sequencing of soil microbial communities. The successful conservation of soil samples collected in China was the basis for the subsequent molecular analyses done in Germany.

We hypothesized that freeze-drying would be a suitable method to conserve samples for short-term transportation. By efficient water removal microbial communities would be prevented from growth and enzymes (especially nucleic acid degrading enzymes) rendered inactive in the dried state. For this study, samples were collected in the German Biodiversity Exploratory Site Hainich-Dün at two grassland sites and subjected to different storage temperatures and storage times after freeze-drying. Detectable microbial communities were compared for the freeze-dried treatments and frozen control samples. Freeze-drying of soil samples proved to be an effective, non-toxic, relatively cheap and feasible method for the conservation of soil RNA and DNA and molecular analyses of soil bacteria and soil arbuscular mycorrhizal fungal communities for a short-term storage of up to seven days at ambient temperature.

Manuscript 2: "Experimental evidence of functional group-dependent effects of tree diversity on soil fungi in subtropical forests" presents the investigation of the relative contribution of biotic and abiotic drivers on soil fungal community composition and richness in the BEF China experiment. We studied fungal communities in a tree diversity gradient of 1, 2, 4, 8 and 16 tree species. We analyzed the effects of tree diversity at the level of actual neighborhood diversity of the sampling position, by considering the identity and mycorrhizal status of the associated sampling tree and its eight neighboring tree species individuals. We investigated the main fungal functional groups of saprotrophic, plant pathogenic and mycorrhizal (arbuscular mycorrhizal and ectomycorrhizal) fungi separately. We found that at the early stage of forest development, environmental and stochastic processes dominated the assembly of the soil fungal community. Tree related variables, such as tree community composition, significantly affected arbuscular mycorrhizal and plant pathogen fungal community structure, while differences in tree host species identity and host abundance affected ectomycorrhizal fungal community composition.

Manuscript 3 "Linking soil fungal generality to tree diversity in young subtropical Chinese forests" took a rather systemic approach analyzing co-occurrence patterns of soil fungi and tree species by bipartite network analysis and inferred the dependence of the degree of fungal specialization on forest tree diversity. Network metrics like *fungal generality* were evaluated as well as the impact of tree diversity level on the specialization coefficient phi. We found that fungal OTUs showed the highest

specialization for the monocultures. Against our expectation, the degree of specialization was lowest at the two tree species mixtures instead for the high tree species mixtures. Accordingly, *connectance* and *fungal generality* values were highest for the two tree species mixtures. Fungal richness was highest for the high tree species mixtures.

Concluding our results, we found significant effects of tree species richness on soil fungal richness, community composition and specialization patterns even at this early developmental stage of the subtropical forest plots. Although the relative contribution of tree species richness and community composition on the soil fungal community was lower compared to the contribution of environmental variables (except for EcM fungi) and the amount of remaining unexplained variance, this underpins a tremendous role of aboveground tree species diversity on belowground soil fungal communities.

The molecular methods chosen in this work enabled a comprehensive investigation of the fungal communities. Future works should further include the investigation of functional aspects like relation to tree species productivity, tree health or litter decomposition and combine data of multiple soil biota e.g. bacteria, archaea or protists. Furthermore, meta-omics data (metagenomics, metatranscriptomic and metaproteomics) would also allow for a more functional approach.

INTRODUCTION

The Earth harbors a huge diversity of organisms. In the recent decades it became apparent that due to anthropogenic impacts this biodiversity is severely threatened and species go extinct at an unprecedented rate (Díaz *et al.*, 2019). Fungi represent one kingdom in the domain of Eukaryota and are in particular present in soils. Estimates of global soil fungal diversity vary with up to 5 million species (Blackwell, 2011) or even more (Larsen *et al.*, 2017). One gram of soil can harbor about 200 m of fungal hyphae (Leake *et al.*, 2004) representing up to several hundred species (Wagg *et al.*, 2014). Soil fungi contribute to a large extent to global element cycling, plant nutrition and performance, and to a multitude of other ecosystem functions. The belowground diversity of soil fungi and the aboveground diversity of plants are tightly connected (Hooper *et al.*, 2000). Hence, deforestation (Díaz *et al.*, 2019) and tree species loss e.g. due to climate change or conversion of forests to plantations with low tree species richness (Paquette *et al.*, 2018) are expected to also affect belowground fungal diversity and the ecological processes it conveys.

Rapid developments of molecular analysis methods in the recent decades have opened avenues for the study of the vast diversity of soil fungi. In this dissertation, we investigated the relative importance of tree species diversity on soil fungal diversity, assembly processes and potential fungal-tree interaction patterns in young subtropical Chinese forests with an experimentally established tree species richness gradient. A better understanding of the tree diversity – fungal community relationship will help to improve soil fungal conservation measures and the prediction of the consequences of tree species loss to soil fungal mediated ecosystem processes.

In this introductory part I will present the background I shortly summarized above in more detail and subsequently each of the three studies that constitute the result chapters. Finally, the overall achievement of this thesis for the field of soil fungal ecology is stated in the discussion, which also reviews limitations of the currently applied methods and future research directions.

I-1 Biodiversity

Biological **diversity** (biodiversity) is a multilevel concept, encompassing genetic, ecological, taxonomic and functional diversities over different space and time scales (Naeem, 2002; Isbell *et al.*, 2017). Therefore, there exist several measures of diversity for

a given community. Thereof, investigating the number of species at a certain place and time (alpha diversity) is the most intuitive approach (Figure 1). Biodiversity can also be measured as the difference in species between individual samples (beta-diversity, the difference in communities) or across several locations (regional or gamma-diversity) (Peay *et al.*, 2016). Furthermore, the relative abundances of species to each other, the evenness of community composition, or their functional differences can be assessed as well (Purvis & Hector, 2000).

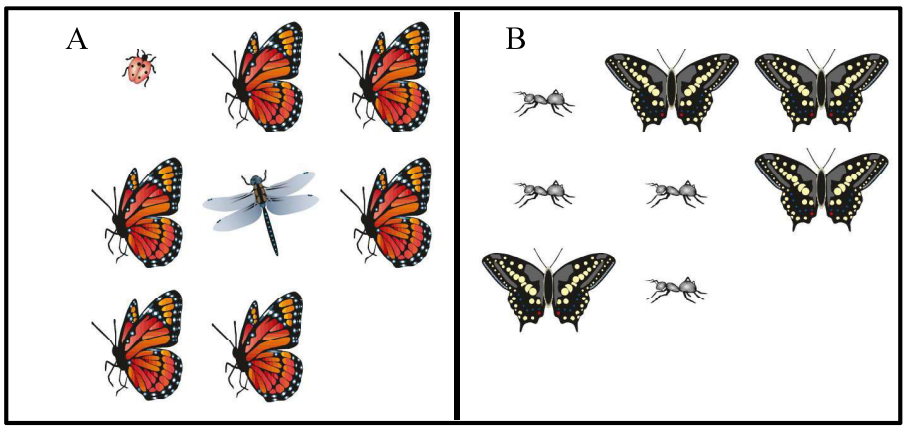

Figure 1| Illustration of two measures of diversity: species richness and species evenness. (A) and (B) depict two samples of insects from different locations. (A) could be ascribed as being more diverse as it contains three species in comparison to only two species in sample (B). However, there is a higher chance in sample (B) than in sample (A) that two randomly chosen individuals will be of different species. Thus, sample (B) shows a higher evenness. Picture and description were slightly modified from Purvis and Hector (2000).

In the terms of biomass, plants dominate our planet Earth with 80% of global biomass (Bar-On *et al.*, 2018). However, the microbes dominate in terms of species diversity. An estimated fungal/plant species ratio of 17:1 extrapolates to 6 million fungal species (Blackwell, 2011; Taylor *et al.*, 2014), which is even outnumbered by 2-3 times higher bacterial species numbers (Peay *et al.*, 2016). Fungal species diversity is highest in soils (Peay *et al.*, 2016), which are the most heterogeneous parts of the biosphere, with an extremely high differentiation of properties and processes within nano- to macroscales (Kuzyakov & Blagodatskaya, 2015; Totsche *et al.*, 2018).

Biodiversity, in particular the biodiversity of soils, is of major concern in the recent global development. Biodiversity is declining faster than at any time before in earth history with an estimated extinction of animal and plant species of around 1 million

species in the coming decades (Isbell *et al.*, 2018; Díaz *et al.*, 2019). Land-use change was attributed the largest relative negative impact on nature since 1970 (Díaz *et al.*, 2019). To raise public awareness and resolve a biodiversity strategic plan (United-Nations, 2010) 2011-2020 was declared the International Decade of Biodiversity (https://www.cbd.int/2011-2020/). Additionally, the year 2015 was declared as the International Year of Soils (United-Nations, 2014), acknowledging the importance of soils as foundation for agriculture and provisioning of essential ecosystem functions (United-Nations, 2014; McBratney *et al.*, 2019), which are mainly facilitated by microorganisms.

I-2 Biodiversity and ecosystem functioning

Why does biodiversity matter? Why does the biodiversity of microorganisms matter that are not even detectable with the naked eye? The metabolic activities of organisms like respiration, photosynthesis, nitrogen fixation and decomposing of organic matter generate fluxes of matter and energy in nature. By this, hundreds of gigatons ($1x10^{15}$g) of matter are moved between organic and inorganic pools annually (Butcher *et al.*, 1992; Schlesinger, 1997). The loss of species can alter these fluxes of materials and energy and thus processes such as the carbon, nitrogen and phosphorus cycling, and the rate of processes like respiration and biomass production might change (Floudas *et al.*, 2012; Isbell *et al.*, 2017) and affect ecosystem stability (Isbell *et al.*, 2017).

In general, a positive correlation between biodiversity and ecosystem functioning was reported from studies performed in natural and experimental ecosystems (Purvis & Hector, 2000; Balvanera *et al.*, 2006; Duffy *et al.*, 2017; Guerrero-Ramírez *et al.*, 2017; Eisenhauer *et al.*, 2018). For example, diverse mixtures of plants are often more productive than their respective monocultures (Tilman, 1999; Spehn *et al.*, 2000). There are several possible underlying mechanisms for the observed positive biodiversity-ecosystem functioning relationship (Tilman *et al.*, 2014). On the one hand, diverse mixtures have an increased probability to include a species with strong impacts on ecosystem function, meaning that a strong performing species for the current conditions is present within the mixture (sampling effect, Huston, 1997). On the other hand, resource exploitation in mixtures is more efficient (niche complementarity, Naeem *et al.*, 1994; Tilman, 1999; Naeem, 2002). For example, different rooting depths of the plant species can fill and exploit the soil volume more thoroughly (Sun *et al.*, 2017). Furthermore,

species richness positively contributes to ecosystem functioning at different times (insurance effect or asynchrony, Craven *et al.*, 2018; Isbell *et al.*, 2018) and for multiple ecosystem processes (multifunctionality, Isbell *et al.*, 2011; Lefcheck *et al.*, 2015; Soliveres *et al.*, 2016; Eisenhauer *et al.*, 2018). Also biotic interactions across trophic levels contribute to the biodiversity-ecosystem functioning relationship (Maron *et al.*, 2011; Schnitzer *et al.*, 2011; Eisenhauer *et al.*, 2012).

I-3 Evolution of soil fungal groups

True fungi evolved approximately 1 billion years ago (Taylor & Berbee, 2006). Most soil fungi form microscopic hyphae that can take up nutrients from soil compartments that are smaller than the pore spaces accessible to plant roots. Fungi become visible when these fine hyphae densely aggregate to form fruiting bodies, rhizomorphs and sclerotia.

Plants and soil fungi share an intimate evolutionary history (Figure 2). About 400 million years ago, fungi are thought to have facilitated the transition of plants from the aquatic to the terrestrial environment. As the first plants lacked root structures to take up minerals, hyphal arbuscular mycorrhizal (AM, “fungus roots”) fungi amended this function, forming a symbiosis, that is still observed in about 80% of all plant species (Smith & Read, 1997; Blackwell, 2011), comprising mainly grasses and shrubs but also trees. AM fungi are ancient and comprise one fungal phylum, the Glomeromycota, with a limited species richness of about 300 species known today and no clear established potential to decay organic substrates. In contrast, other soil fungi (e.g. in the Asco- and Basidiomycota) evolved diverse mechanisms to degrade plant litter and make it available for plant nutrition. These saprotrophic fungi are able to degrade leaf litter, root litter and dead wood. Pathogenic necrotrophic fungi couple such degradation capabilities to the potential to first attack and kill living plant tissue, while biotrophic parasitic fungi reside within living plants from which they derive nutrients. Hemiobiotrophic fungi can switch from the biotrophic to the necrotrophic life strategy. From the saprotrophic fungi, another group of mycorrhizal fungi evolved several times starting from 135 million years ago, the ectomycorrhizal fungi. They lost some of their lytic enzymes for carbon decomposition and optimized the acquisition of nitrogen, which is the limiting resource in most soil systems (Kohler *et al.*, 2015).

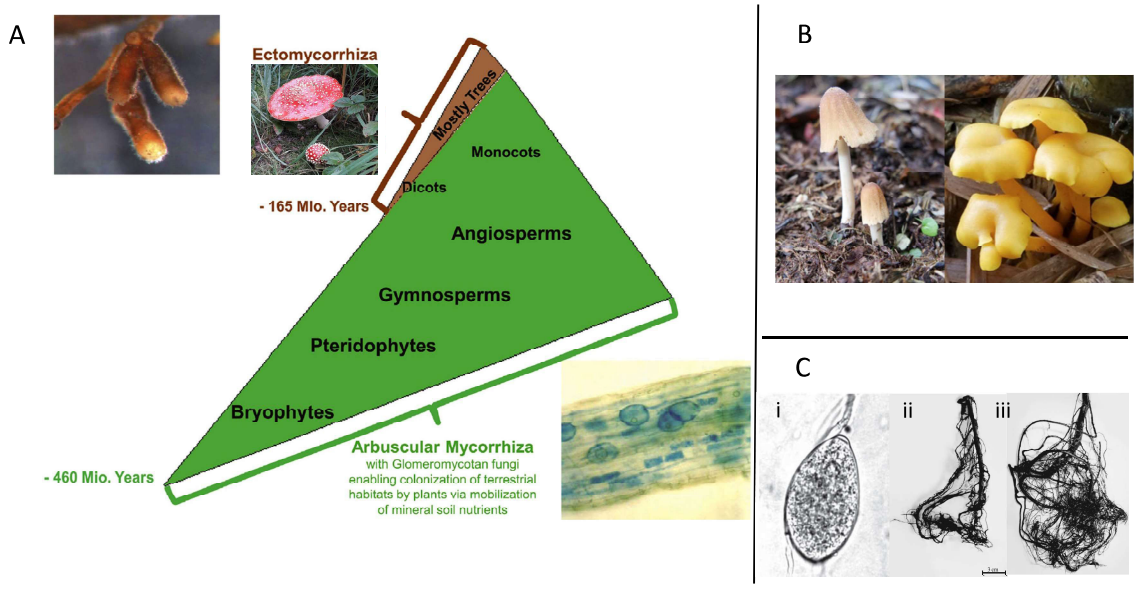

Figure 2| Soil fungal functional groups. (A) Evolution of arbuscular mycorrhizal fungi, which form visible (blue stain) structures within the plant root cells, and ectomycorrhizal fungi, which form a dense hyphal mantel around plant fine roots (picture modified from Buscot, 2015). *Amanita muscaria* is shown as an exemplary EcM fungus (photo by Steffen Wittig). (B) Saprotrophic fungi growing on decaying litter (picture modified from Degreef *et al.*, 2016) (C) Negative effects of the fungal plant pathogen *Phytophtora* spp. (i, asexual sporangia Nicholls, 2004) on a *Quercus robur* root system (ii) in comparison to an uninfected root system (iii, Jönsson, 2004).

The two main mycorrhizal types differ anatomically. Arbuscular mycorrhiza form minute tree-like structures (Latin: arb – tree) within the plant cells for the exchange of phosphorus (delivered by the fungus) while carbon (photosynthesis products of the plant) is exchanged via the intercellular fungal hyphae in the plant root. The ectomycorrhizal fungi form a dense hyphal mantle around the plant root (therefore the name "ecto"), from which hyphae develop in the apoplast of the root cortex to form an apposition structure called the Hartig net where the partners exchange photo-assimilates against soil nutrients. These soil nutrients are mobilized by the fungal mycelium that extends to large soil areas. Ectomycorrhizal symbioses are mainly formed with temperate, boreal and some (sub)tropical tree species where species richness of tree and fungal partners is about the same, with 6000 species (Alexander & Lee, 2005; Tedersoo *et al.*, 2010; in about 10% of all plant families, Blackwell, 2011; McGuire *et al.*, 2013), while single tree species are known to associate with up to one hundred EcM fungi at a time (Bahram *et al.*, 2011). AM and EcM fungi form huge networks underground that are estimated to derive one

third of the global photoassimilation and to provide plants with most of their mineral compounds, in particular phosphorus and nitrogen.

I-4 Contribution of soil fungi to ecosystem functioning

Soil fungi promote many ecosystem services, among which are decomposition, element cycling, prevention of nutrient leaching, carbon sequestration, soil formation and aggregation, plant nutrition and plant protection, and plant community assembly.

Fungi are the primary decomposers of plant and root litter as they are able to produce and secrete various enzymes which break down the highly complex plant material matter of cellulose, hemicellulose and lignin (Talbot *et al.*, 2008; Baldrian, 2009). Distinct fungal communities are involved in the early and late stages of decomposition (Purahong *et al.*, 2016). The decomposition of belowground carbon and subsequent respiration are estimated to generate carbon fluxes to the atmosphere which are about one order of magnitude larger than that of anthropogenic CO_2 emissions (Raich & Potter, 1995; IPCC, 2000). Next to the efficient decomposition of complex carbon compounds by predominantly saprotrophic fungi, ectomycorrhizal fungi are able to assess macromolecular nitrogen due to their proteolytic capabilities (Abuzinadah & Read, 1986). Due to the efficient scavaging of nitrogen by the ectomycorrhizal fungi, losses of nitrogen by leaching are minimized and nitrogen is conserved in the forest ecosystem (Leake *et al.*, 2004).

Mycorrhizal fungi from a huge part of the underground biomass and constitute an important carbon sink and pool of nitrogen. Högberg and Högberg (2002) estimated that 32% of microbial biomass is constituted by extramatrical EcM mycelium. While the turnover rate of arbuscular mycorrhizal fungi is quite high (several days, Friese & Allen, 1991; Staddon *et al.*, 2003), ectomycorrhizal fungi can form long-living rhizomorph strucutres (about 11 months, Treseder *et al.*, 2005) which are quite recalcitrant to degradation and thus promote the stable organic carbon pool in the soil. Fungal hyphae enmesh and entangle soil primary particles, organic materials and small aggregates, facilitating macroaggregate formation (Rillig & Mummey, 2006). Glomalin, produced by AM fungi, and glomalin-related soil proteins were also attributed a major role on soil aggregation and stability (Rillig & Mummey, 2006). Furthermore,the production of citric and oxalic acids by EcM fungi contribute to mineral weathering and soil formation (van Breemen *et al.*, 2000).

Another important ecosystem function of soil fungi, especially mycorrhizal fungi is the nutrition and protection of their plant hosts. Within mycorrhizal symbiotic systems, the fungal partner supports its host by the provision of macro (nitrogen and phosphorus)- and micronutrients (e.g. copper, iron and zinc), in return receiving carbon compounds from the plant (Smith & Read, 1997). Up to 80% of all plant nitrogen in boreal forests is derived from EcM fungi, and AM fungi contribute for up to 90% of plant phosphorus and up to 50% of plant nitrogen uptake (Hawkins *et al.*, 2000; van der Heijden *et al.*, 2008). The fungal hyphae are much finer (about 2µm) than plant fine roots (100-500 µm) and root hairs (10-20 µm) and thus can better access the soil pore space and exhibit a much larger surface (Leake *et al.*, 2004). EcM are better capable of hydroloyzing phosphorus from organic sources (Perez-Moreno & Read, 2001) but can also assess inorganic phosphorus, while AM fungi have a greater affinity for the uptake of inorganic forms of phosphorus (Liu *et al.*, 2018).

Mycorrhizal fungi are able to generate 'mycorrhiza-induced resistance' within their hosts which confers protection against a wide range of attackers, including biotrophic pathogens, necrotrophic pathogens, nematodes, and herbivorous arthropods (Cameron *et al.*, 2013). Plant signals after herbivore attack can also be channeled by the mycorrhizal network to induce defence strategies in neighboring plants (Barto *et al.*, 2012; Babikova *et al.*, 2013).

Last but not least, mycorrhizal and also pathogenic fungi both contribute to plant competition, plant community assembly and plant richness (Bell *et al.*, 2006; Bever *et al.*, 2010; Liang *et al.*, 2015). Mycorrhizal fungi can alter the competition between plants via the directed channeling of nutrients and signalling compounds across the mycorrhizal network. For example, allelopatic substances produced by plants, limiting the growth of neighboring plants, were reported to be efficiently transported to target plants via the common mycorrhizal network (Barto *et al.*, 2011). In addition, AM networks were observed to channel nutrients to sunlit target plants, which are more able to support the fungal symbiont with photoassimilates compared to shaded plants (Weremijewicz *et al.*, 2016). The promotion of seedlings by the mycorrhizal network was reported to have positive, negative and neutral effects, highly depending on soil type, nutrient availability and plant species identity (van der Heijden & Horton, 2009). EcM common mycorrhizal networks are known to support conspecific tree seedlings in temperate and tropical

ectomycorrhizal trees. However, also a negative feedback acting on conspecific tree seedlings in tropical forests and temperate forests was reported. This effect was ascribed to the action of host specific soil pathogens (Bever *et al.*, 2010; Mommer *et al.*, 2018) and it is estimated that 70–80% of all plant diseases are caused by fungi (Zeilinger *et al.*, 2016).

I-5 The investigation of biodiversity- ecosystem functioning relationships

Knowledge about biodiversity- ecosystem functioning relationships can be gained on one hand from observational studies which compare (natural) environments/communities with different levels of biodiversity. However, they bear the disadvantage that confounding factors are correlated or causal to the evolved species richness (Augusto *et al.*, 2002; Ayres *et al.*, 2009; Condit *et al.*, 2013; Zemunik *et al.*, 2016) and can hardly be excluded (Peay *et al.*, 2010; Martínez-García *et al.*, 2015; Schappe *et al.*, 2017), e.g. heterogeneity of soil and climatic conditions. Experimental manipulation of biodiversity can erase confounding factors for a large part or at least enable to identify the relative contribution of the process/ treatment of interest and the environmental conditions. Considering plant biodiversity experiments in the field, grassland experiments were started all over the world two to three decades ago (Tilman, 1997; Balvanera *et al.*, 2006). Positive effects of plant diversity on multiple ecosystem functions (plant root biomass, soil respiration and microbial biomass carbon) have been recently reported in several studies. Evaluating 18 experimental grassland studies (Eisenhauer *et al.*, 2012), effects of plant diversity on soil organisms became significant about four years after the establishment of the experiments. The authors found that species-rich grasslands benefit in the long term from positive facilitative net effects by soil biota promoting plant growth (e.g. arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria). Evaluating 39 grassland studies, Craven *et al.* (2018) found that high plant species richness and phylogenetic diversity stabilized plant biomass production via enhanced asynchrony in the performance of co-occurring species. Also, Isbell *et al.* (2018) found positive temporal insurance effects of biodiversity in a long term (18 years) grassland experiment. Duffy *et al.* (2017) conducted a meta-analysis of 67 natural grassland field studies and statistically achieved the separation of effects of plant biodiversity and abiotic factors on plant biomass production. They concluded that increases in plant biomass with biodiversity are even stronger in nature than has been previously reported by experiments.

However, results obtained from grassland studies cannot directly be extrapolated to forest ecosystems, as plants (grasses and shrubs vs. trees) and associated microorganisms (e.g. AM symbioses in grasslands vs. EcM dominance in boreal forests) as well as soil characteristics (typically acidic soil pH in forest soils) and nutrient dynamics differ severely. Compared to grasslands, forests are more complex and heterogeneous environments, including several layers of vegetation cover. 30% of the Earth surface is covered by forests, which provide the habitat for a high diversity of organisms and facilitate a broad spectrum of ecosystem functions and ecosystem services for human well-being (Brockerhoff *et al.*, 2017) including carbon sequestration (Canadell & Raupach, 2008), nutrient cycling (Scherer-Lorenzen *et al.*, 2007), protection against soil erosion (Labrière *et al.*, 2015) and increased water retention (Guo & Gan, 2002). Tree species richness loss in forests broadly occur due to the conversion to low diversity forestry plantations for timber production or are induced by climate change, threatening approximately one half of the tree species worldwide (Fleming *et al.*, 2011; International Union for Conservation of Nature (IUCN), 2012; ter Steege *et al.*, 2015). While planting restores tree cover, most industrial plantations are single species monocultures, often of fast-growing cultivars or hybrids of pine, eucalypt, acacia, spruce, poplar or larch (Paquette *et al.*, 2018).

The study of tree biodiversity-ecosystem functioning experiments was initiated in 1999 with the "Satakunta Experiment" which was set up in Finland. Tree experiments take much more effort, space and time to establish, but the number of forest experiments is steadily growing. Currently, 26 forest experiments covering six continents and comprising about 230 tree species were set up all over the world (http://www.treedivnet.ugent.be/), including up to over 1 million trees planted for this purpose (Paquette *et al.*, 2018).

I-6 High-throughput sequencing methods in soil fungal community investigations

Soil fungi were initially identified by their fruiting bodies (sporocarps, "mushrooms"), spores and mycelial morphology as well as mating type analysis for species determination (Blackwell, 2011). However, identification of fungi with these methods is not possible in the light of the diversity of existing fungi.

With the development of molecular tools, the genomic information of each fungal cell can be detected at a high resolution. There are regions in the genomes of fungi that appear very suitable for the separation of fungal "species" (Schoch *et al.*, 2012). For example, the genomic regions that separate the genes encoding each of the three RNAs in ribosomal subunits (essential for the synthesis of proteins in all cells) are called ´intergenic transcribed spacer´ (ITS) regions (Figure 3). There are parts of the genes encoding the ribosomal RNAs that are highly conserved among and specific for fungi which can be employed for molecular analyses of the fungal sequences and not the whole DNA sequence information in the soil that comprises many other cells of plants, bacteria, archaea and animals. Besides selecting for a fungal specific region, the molecular analysis needs also be able to differentiate between fungi to enable the analysis of the community composition. The ITS region is highly variable in terms of the sequence of nucleotides and the length of the ITS fragments within the fungal kingdom. To analyze fungal DNA in a soil sample, the cells are destroyed by mechanic (bead beating or sonication) and/or chemical means (chemical lysis), released genomic DNA is purified and the ITS sequence part of the fungal cells in the sample is amplified by the method of polymerase chain reaction using the primers matching the conserved rRNA region (PCR, Innis *et al.*, 1990; White *et al.*, 1990). In this thesis, sequencing of the amplified fungal marker region was done by the high-throughput sequencing technology 454 pyrosequencing.

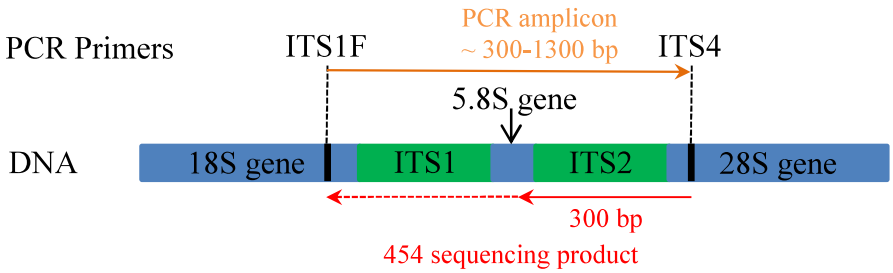

Figure 3| Intergenic transcribed spacer region 2 (ITS2) as genomic marker region for fungal community analysis. The ITS region is highly variable in its lengths across the lineages of the fungal kingdom (Feibelman *et al.*, 1994; Schoch *et al.*, 2014). Sequencing quality decreases with sequencing length, thus we trimmed the investigated sequences to the first 300 bp upstream of the ITS4 PCR primer target region, which included the ITS2 region and parts of the flanking 5.8S and 28S rRNA gene.

Comparing the determined sequences with databases can find matches with known fungi to determine the probable identity of the inspected sequences and thus determine which fungus was present in the respective soil sample. Sequences that are very similar (97% identity) to each other are typically considered as belonging to the same taxon, termed in molecular words as “operational taxonomic units” (OTUs). For many fungal OTUs found in soil environmental studies an identification with reference databases is often not possible due to the currently insufficient information content compared to the vast diversity of soil fungi. Still the abundance of fungal OTUs within a study can be compared between treatments (e.g. a tree biodiversity gradient) to infer the treatments´ impact on the fungal community. By means of 454 sequencing, hundreds to thousands of fungal sequences in a sample can be determined and the analysis of hundreds of samples in one sequencing run is possible.

I-7 The current gap in soil fungal ecology research addressed by the BEF China project

Soil fungal communities have been studied in several parts of the world in natural and experimental systems to better understand their assembly processes and identify the ecological and environmental drivers. It is now known, that several mechanisms can act in concert in shaping species communities of which neutral and deterministic processes are the two ends of the assembly continuum (Gravel *et al.*, 2006). Neutral processes assume that species are equivalent and abundance distributions are dependent on proliferation, death, immigration (e.g. dispersal), speciation (reference), the number of individuals in the community and the number of species in the external species pool (Bell, 2001). The deterministic neutral model (Tilman, 2004; Jabot *et al.*, 2008) acknowledges a selective influence of the environment for the establishment of a species in a new environment but argues that after successful establishment, neutral processes lead to community assembly. The deterministic model on the other hand assumes that environmental conditions like soil pH and soil nutrient content (“habitat filtering”) as well as biotic interaction (e.g. resource partitioning, competition) are the main cause for observed community patterns (Dumbrell *et al.*, 2010).

The variability of conditions on Earth acting on the soil microbial community is tremendous. The soil habitat itself is very heterogeneous and there is not one soil but one needs to speak of “soils” (Buscot, 2005). In general, biodiversity- ecosystem functioning

relationships are strongly context-dependent (Guerrero-Ramírez *et al.*, 2017; Guerrero-Ramírez & Eisenhauer, 2017) also with regard to spatial and temporal scales investigated. Although a lot of knowledge has been gained on the mechanisms of soil microbial community assembly, also in context of global environmental change, this currently available information lacks far behind to describe a comprehensive picture. Especially tropical as well as subtropical regions remained understudied in the recent years (Cameron *et al.*, 2018).

This thesis adds knowledge about fungal community assembly in subtropical forests at an early developmental state in relation to tree species diversity. The complexity of ecosystem functioning requires integrated research approaches to fully identify and understand ongoing processes. Thus, this thesis formed one of many subprojects within the larger and long-term Biodiversity and Ecosystem functioning (BEF) China project. The BEF China experiment aimed to investigate "the role of tree and shrub diversity for production, erosion control, element cycling, and species conservation in Chinese subtropical forest ecosystems" (Bruelheide, 2010). Within this project, the relationship between manipulated tree species diversity and animal and microbial diversity was assessed and also related to abiotic components. Furthermore, biotic interactions (e.g. among trophic levels) formed a research focus (Bruelheide, 2010). Finally, results of BEF China will contribute to resolve how plant diversity may maintain vital services in forest ecosystems in times of global change (Bruelheide, 2010). The subtropical region is extremely species rich, also in tree species. BEF China comprises natural ("Comparative study plots") and experimental forest sites ("Site A" and "Site B"). From the natural forests, 42 native tree species were selected for the experimental study sites which manipulate a broad and unprecedented diversity gradient of 1, 2, 4, 8, 16 and 24 tree species mixtures. While forest loss in China was globally one of the severest in the recent century, with devastating ecological consequences like soil erosion and biodiversity loss (Wenhua, 2004), China also initiated the largest reforestation program in history about 20 years ago (China´s Grain for Green Program, Wenhua, 2004; Delang & Yuan, 2015). The setting up of the world´s largest BEF experiment is in line with these huge efforts to mitigate the severe effects of the decline in forests and forest diversity and to gain knowledge about forest ecosystem functioning and consequently improve forest management practices.

BEF China comprises several strengths compared to other forest BEF experiments at the time of establishment and some of them are still unique. Until today, BEF China is the only forest BEF experiment in the species rich subtropics. The subtropics harbor a balance of AM and EcM forming tree species which is not the case in any other climatic region worldwide. Tropics are dominated by AM trees while temperate regions are dominated by EcM trees. Due to a nested study design all tree species were present at each diversity level and thus each tree species was present in mixtures and also as monoculture. The plot size (about 25 m x 25 m) was chosen to sustain tree diversities of grown tree individuals in the future. While most terrestrial ecosystems are nitrogen limited, the subtropical region is mainly restricted by phosphorus. Forests in China are mainly planted on hillsides and due to clear cutting soil erosion is a major threat, as the subtropics have concentrated precipitation in summer time. Thus soils are shallow, nutrient poor, acidic and, due to human activities, also contaminated with heavy metals.

Within the BEF China project, we analyzed the interdependency of the soil fungal community and biotic (e.g. tree diversity and tree identity) and abiotic (soil physico-chemical conditions) drivers and quantified their relative contribution on fungal community assembly and richness. Furthermore, we investigated the fungal co-occurrence with the subtropical native tree species at different diversity levels to infer the dependency of fungal specialization and tree diversity.

I-8 Aim of this thesis

We aimed to analyze the huge diversity of soil fungi in relation to biodiversity and ecosystem functioning in a forest ecosystem by the highly sensitive and powerful molecular method of high-throughput next generation sequencing. This thesis constitutes the first assessment of the soil fungal community in the BEF China project, undertaken after the third growing season of planted tree saplings. At this early stage of forest development, the average tree species height ranged from 52 to 301 cm (Li *et al.*, 2014) and most forest plots did not show a closed canopy yet. Still, early tree-soil fungal interactions and host specialization patterns have been reported from pot experiments and field experiments with tree saplings (Ding *et al.*, 2011). Thus, we wanted to assess the pattern of fungal community assembly in these young subtropical forests. To our best knowledge, there is no such information available from another subtropical forest system with this span of tree diversity gradient as analyzed in this thesis. The results of this work

will profoundly contribute to the existing knowledge of fungal community assembly in forest ecosystems and furthermore, analytical methods developed during this thesis will substantially contribute to future research undertaken in the frame of the Biodiversity and Ecosystem Functioning (BEF) China experimental platform (http://www.bef-china.de/index.php/en/) and soil fungal ecology in general.

BEF China includes a multitude of biodiversity manipulations scenarios within the experiment (Bruelheide, 2010; Bruelheide *et al.*, 2014). It would have been impossible to analyze all settings within one PhD thesis, so we had to select for a suitable biodiversity treatment for our research question and develop a reasonable sampling design. In 2011, we collected soil samples of 31 forest plots associated to the "random extinction scenario" treatment at site A planted in a broken-stick-design (Figure 4). These soil samples built the basis for this thesis.

The BEF China experimental site is located in a remote area in south-east China in the vicinity of the small village Xingangshan, Jiangxi Province (Figure 4). As is often the case for microbial environmental studies, facilities and infrastructure were not available to perform the highly sensitive molecular methods (high-throughput sequencing) that formed the analytical basis of this study. On site, we had to establish a method to preserve the soil samples collected in China for the long-distance transport to Germany. The storage of soil samples prior to analysis is a highly critical step as soil microbial communities can rapidly change (e.g. those containing fast growing molds) and nucleic acids that were the main target of the analysis applied in this thesis are prone to rapid enzymatic degradation. The optimal procedure is the flash freezing of soil samples in liquid nitrogen (about -170°C) and subsequent storage of samples at -80°C (for RNA based analysis studies) or at least -20°C (for DNA based studies). The costs and risks to ensure these low temperature storage conditions for hundreds of soil samples were too high for the long-distance transportation from China to Germany. We had to select and develop a sample treatment method that had to be available at the sampling site, reasonably cheap, enable a high throughput of samples (several hundred samples were collected) and be save for transportation (for example no fixation by toxic preservatives).

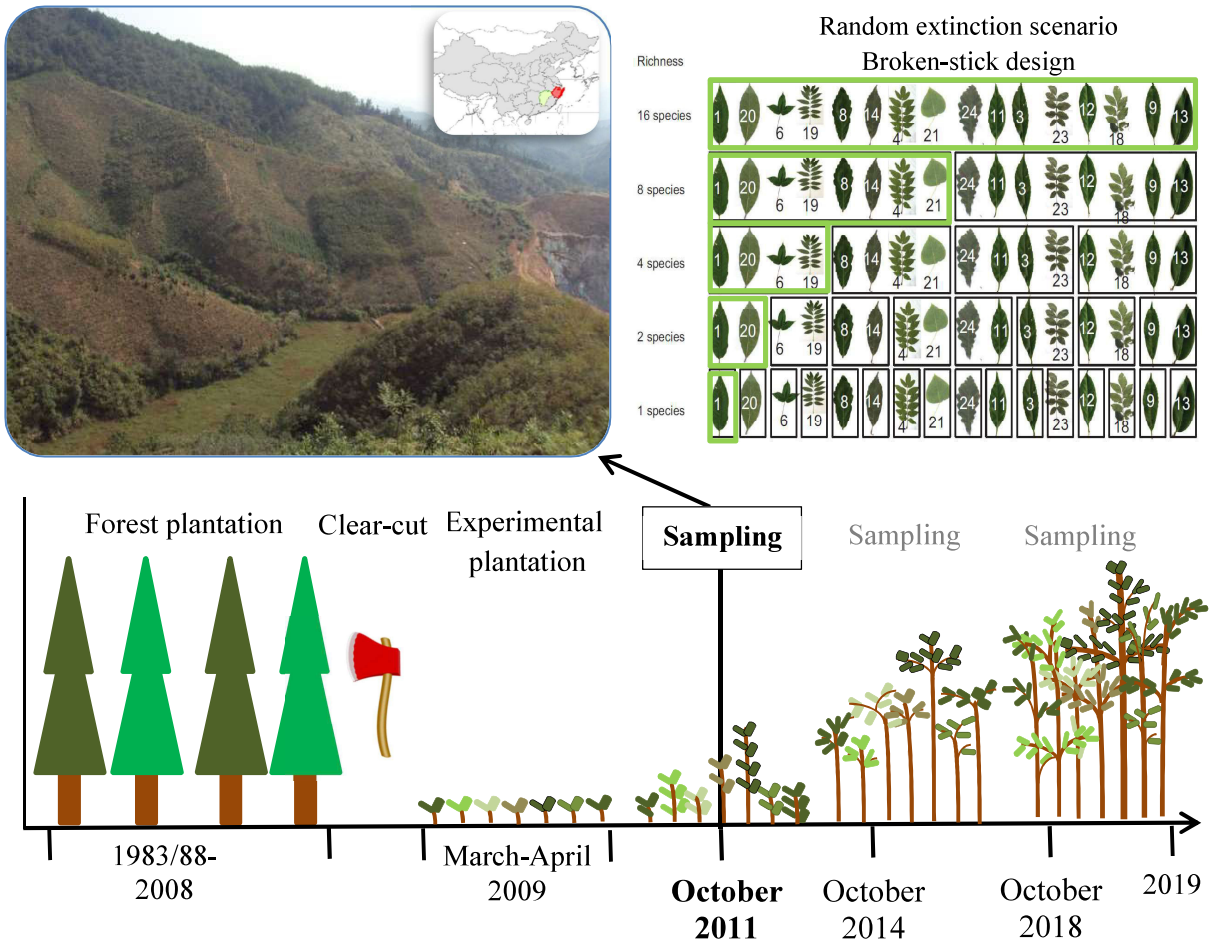

Figure 4| BEF China study site A, 2011. In 2009, forest plots were established in Jiangxi province (green). Plots were established according a broken-stick design (Bruelheide, 2010) after clear-cutting of the previous forest plantation *(Cunninghamia lanceolata* and *Pinus sylvestris*). Sampling for my thesis was accomplished in October 2011, while subsequent samplings were realized in 2014 and 2018 by successor research groups.

We had to select and establish the appropriate statistical methods for the evaluation of the collected data on the soil fungal community to i) determine the kind of statistical analysis enabling us to confer the insights on fungal community composition we aimed for andthat our data structure allowed to apply ii) select the appropriate scale of analysis and iii) adapt for the specific sampling design.

I-9 Working hypotheses

We hypothesized that AM and EcM fungi would show strong, but distinct, correlations with both plant community composition and abiotic soil properties. In contrast, necrotrophic parasites and saprotrophic fungi do not depend directly on living plants, and

we expected communities of these groups to be more strongly affected by abiotic soil properties than by the plant community. Furthermore, we hypothesized that a) fungi specialized to a certain set of environmental conditions will be more prevalent in monoculture and low tree species forests (more homogenous environment) and b) generalist fungi, able to cope with a broad range of environmental conditions, will be able to outcompete specialized fungi in higher tree species forests (more heterogeneous environment) (Kassen, 2002; Vitorino & Bessa, 2018). The successful conservation of soil samples collected in China was the basis for the subsequent analyses and conclusions to draw. We hypothesized that freeze-drying would be a suitable method to conserve samples for short-term transportation as by efficient water removal microbial communities would be prevented from growth and enzymes (especially nucleic acid degrading enzymes) rendered inactive in the dried state.

I-10 Presentation of the result manuscripts

The results of this thesis are presented along three chapters that correspond to three articles (Figure 5). The first two of them have already been published, while the third one was submitted to the journal "Microorganisms".

Manuscript 1 (Weißbecker *et al.*, 2017), "Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities" investigated the suitability of freeze-drying for the short-term conservation of soil samples subjected to the high-throughput sequencing of soil microbial communities. For this study, samples were collected in the German Biodiversity Exploratory Site Hainich-Dün at two grassland sites and subjected to different storage temperatures and storage times after freeze-drying. Detectable microbial communities were compared for the freeze-dried treatments and frozen control samples. As our main study took place in China, we had to ensure a save (no toxins for conservations), feasibly cheap and available method (in the remote village of the sampling site) to conserve our soil samples for the transport to Germany and successive molecular analysis.

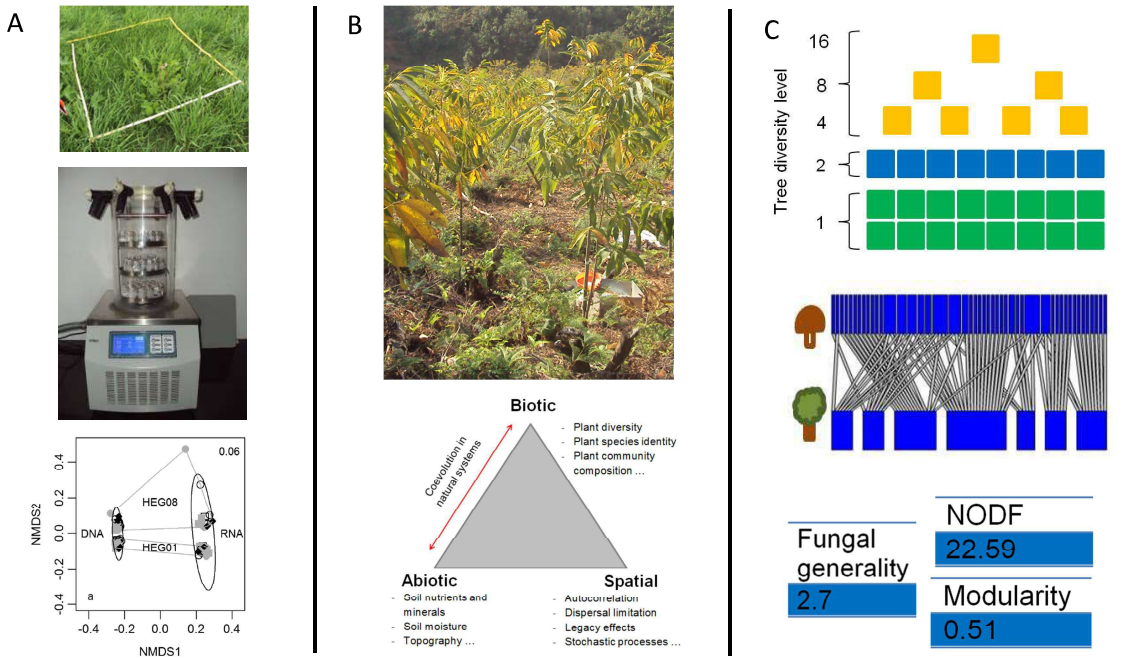

Figure 5| Research topics of the three manuscripts. (A) Freeze-drying as an effective method to conserve soil samples for next generation sequencing analysis of soil microbial communities. Sampling was done in two German grasslands. (B) Fungal communities in young experimental Chinese forests were analyzed in relation to biotic, abiotic and spatial variables. (C) Tree-fungal interactions were analyzed by bipartite network analysis in relation to tree diversity.

Manuscript 2 (Weißbecker *et al.*, 2018): "Experimental Evidence of Functional Group-Dependent Effects of Tree Diversity on Soil Fungi in Subtropical Forests" presents the investigation of the relative contribution of biotic and abiotic drivers on fungal community composition and richness in the BEF China experiment. We studied fungal communities in a tree diversity gradient of 1, 2, 4, 8 and 16 tree species. We analyzed the effects of tree diversity at the level of actual neighborhood diversity of the sampling position, by considering the identity and mycorrhizal status of the associated sampling tree and its eight neighboring tree species individuals.

Manuscript 3 (submitted to "Microorganisms"): "Linking soil fungal generality to biodiversity in young subtropical Chinese forests" inferred the dependence of the degree of fungal specialization and forest tree diversity. Here, tree-fungal bipartite networks based on co-occurrences were constructed and network metrics were evaluated as well as the impact of tree diversity level on the specialization coefficient phi.

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CHAPTER 1

Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities

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Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities

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Abstract

Aims
Soil sample preservation is a challenging aspect in molecular studies on soil microbial communities. The demands for specialized sample storage equipment, chemicals and standardized protocols for nucleic acid extraction often require sample processing in a home laboratory that can be continents apart from sampling sites. Standard sampling procedures, especially when dealing with RNA, comprise immediate snap freezing of soils in liquid nitrogen and storage at −80°C until further processing. For these instances, organizing a reliable cooling chain to transport hundreds of soil samples between continents is very costly, if possible at all. In this study we tested the effect of soil sample preservation by freeze-drying with subsequent short-term storage at 4°C or ambient temperatures compared to −80°C freezing by comparative barcoding analyses of soil microbial communities.

Methods
Two grassland soil samples were collected in Central Germany in the Biodiversity Exploratory Hainich-Dün. Samples were freeze-dried or stored at −80°C as controls. Freeze-dried samples were stored at 4°C or ambient temperature. Investigated storage times for both storage temperatures were 1 and 7 days. Total DNA and RNA were extracted and bacterial and arbuscular mycorrhizal (AM) fungal communities were analyzed by amplicon 454 pyrosequencing of the 16S (V4-V5 variable region) and 18S (NS31-AM1 fragment) of ribosomal RNA (rRNA) marker genes, respectively.

Important Findings
Bacterial communities were sufficiently well preserved at the rDNA and rRNA level although storage effects showed as slightly decreased alpha diversity indices for the prolonged storage of freeze-dried samples for 7 days. AM fungal communities could be studied without significant changes at the rDNA and rRNA level. Our results suggest that proper sampling design followed by immediate freeze-drying of soil samples enables short-term transportation of soil samples across continents.

Keywords: lyophilization, soil preservation, biodiversity, microbial communities

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INTRODUCTION

Biodiversity research on soil microorganisms is conducted throughout the world (Ramette and Tiedje 2007; Tedersoo *et al.* 2012). This interest is triggered by the pivotal contributions of microorganisms to ecosystem functioning (Torsvik and Øvreås 2002; van der Heijden *et al.* 2008), and the vast diversity of bacterial and fungal species (Curtis *et al.* 2002; Dykhuizen 1998; O'Brien *et al.* 2005). In depth analysis of microbial communities is realized by high-throughput sequencing generating millions of nucleic acid reads using next generation sequencing (NGS) platforms (Caporaso *et al.* 2012; Shokralla *et al.* 2012; Taberlet *et al.* 2012). However, nucleic acids are prone to degradation (Wackernagel 2006) and optimal sampling and sample processing methods include the immediate freezing of soil samples until processing.

Whenever sampling location and processing laboratories are distantly apart, the reliable freezing of samples in liquid nitrogen tank or dry ice during transportation is challenging, costly and not always realizable.

Though the advance in NGS and the possibility to analyze large number of samples lead to large scale and integrated biodiversity studies at a global scale, soil sample storage and transportation across continents still remain a big challenge. Storage of samples at elevated temperatures presumably after chemical preservation, air-drying or freeze-drying are potential alternatives. In several molecular studies, storage of untreated soil samples at ambient temperatures resulted in only minor changes of microbial communities (Rubin *et al.* 2013; Tzeneva *et al.* 2009) or none at all (Brandt *et al.* 2014; Klammer *et al.* 2005; Lauber *et al.* 2010; Tatangelo *et al.* 2014). Nevertheless sample- and microbial type dependent changes were observed (Cui *et al.* 2014; Rissanen *et al.* 2010). Chemical preservatives directly interact with the sampled materials, and discrepancies in preservation efficiencies for variable sample characteristics (Rissanen *et al.* 2010; Tatangelo *et al.* 2014) might be inherently expected.

Freeze-drying is the process where water is removed via sublimation from the frozen sample due to the application of vacuum (Adams 2007). Nucleic acids in soils are liable to degradation by microbial nucleolytic enzymes (Antheunisse 1972; Greaves and Wilson 1970; Wackernagel 2006). Water removal by freeze-drying prohibits diffusion of molecules in the soil matrix and withdraws the protein hydrate shell synced diminishing enzyme activity (Ball 2008; Kurkal *et al.* 2005). The freeze-drying process is non-toxic. Dried samples do not require temperature control during transportation, are reduced in weight, harbor no risk of solution leakage and can be declared as inactivated samples (Adams 2007). Freeze-dryers have a wide application in industry and science. In the vicinity of the specific sampling site they could be accessible via collaborations or bought in variable configurations. To our knowledge only two studies evaluated freeze-drying of soil samples in relation to investigations of bacterial communities. Larson *et al.* (2013) successfully applied pyrosequencing in a DNA based study on several freeze-dried soil samples. Sessitsch *et al.* (2002) accomplished RNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis on a single freeze-dried soil substrate. Both studies indicate promising potential of freeze-drying for soil sample preservation. However, their investigations were not comprehensive as restricted to only one microbial target (bacteria) and one soil sample in the RNA study. Furthermore, the effect of storage conditions of freeze-dried samples for sample transportation was not investigated.

In the present study we assessed the application of freeze-drying as soil storage and safe sample transportation method. We investigated the DNA and RNA based bacterial and arbuscular mycorrhizal (AM) fungal communities on two freeze-dried grassland soils using pyrosequencing. Sample transportation across continents is fastest by airplane. However, an airport might be several hours or days apart from the sampling site. We assumed that transportation from field site to processing lab could be accomplished in a minimum of 1 and a maximum of 7 days. Transportation across temperate regions could be done without additional cooling while in subtropical and tropical regions storage of freeze-dried samples in refrigerated boxes at 4°C could be necessary. But even across temperate regions cooling could be required during very hot summer weathers. Therefore, we analyzed the effect of freeze-drying and subsequent short-term storage (1 day or 7 days) at different temperatures (4°C or room temperature) on microbial community recovery, Shannon diversity and community composition. We hypothesized that freeze-drying is a suitable soil sample treatment prior to short-term storage and transportation to (i) preserve both microbial DNA and RNA and (ii) enable unbiased detection of fungal and bacterial communities using NGS approaches.

METHODS

Sampling site and sample processing

In August 2011, soil samples were collected in the German Biodiversity Exploratory Hainich-Dün (Fischer *et al.* 2010; Solly *et al.* 2014). The Hainich-Dün region is located in Central Germany (Thuringia) and is characterized by large spruce forests of various age classes and cultivated grasslands. Two grassland plots of different soil and land use types (Table 1, online supplementary Fig. S1) were selected. HEG01 was a fertilized meadow, mown twice a year, and HEG08 an unfertilized pasture grazed by cattle. On both plots, a subplot of 1 m × 1 m area was defined. In total, five soil cores with a diameter of 5 cm were collected in the edges and the center of each subplot in a depth of 0–10 cm. The rooted surface layer was removed and the five soil cores of one subplot were combined to a composite sample. The soil was sieved through a 2 mm mesh

Table 1: sampling site characteristics

	HEG01	HEG08
Area	Großenlupnitz	Unstruttal
Land-use	fertilized meadow	unfertilized pasture grazed by cattle
Coordinates	N50° 58.29983, E10° 24.32067	N51° 16.2765, E10° 25.07533
LUI (2006–2010)	High (2.8)	Medium (1.6)
Soil type	Cambisol	Stagnosol
Soil texture	Silty clay	Silty clay
pH	6.65	7.17
Water content	31%	27%
Total C (g kg^{-1} soil)	54.78	60.63
Total N (g kg^{-1} soil)	5.46	5.78
CN ratio	9.89	9.86

Land-use intensity (LUI) category was assigned according to Wiesner *et al.* (2014).

and mixed with a sterilized spoon. For each plot, HEG01 and HEG08, 14 replicate sample flasks (30 ml HDPE wide-mouth screw cap bottles purchased from VWR International GmbH, Germany) were filled with approximately 10 g homogenized soil from the respective composite soil sample. Samples were snap frozen in liquid nitrogen and transported on dry ice to the laboratory. Two replicate samples of each plot were stored as controls at −80°C until extraction and 12 replicate samples of each plot were freeze-dried immediately. In total, four soil samples were stored at −80°C as controls and 24 soil samples were freeze-dried (online supplementary Fig. S2).

Freeze-drying and subsequent storage conditions

The freeze-dryer (ALPHA 2–4, Martin Christ Gefriertrocknungsanlagen, Germany) was run for 39 h at 0.021 mbar at an ice condenser temperature of −84°C. For the first 22 h, utility space was set to 0°C and afterwards increased to 15°C for another 17 h. The soil samples had a temperature of −75°C at the start of the freeze-drying process, which rapidly settled to −35°C. At the end of the freeze-drying process a sample temperature of 20°C was reached. Freeze-dried samples were stored in the presence of blue silica gel within sealed plastic bags. Six freeze-dried replicates of each plot were stored either at room temperature or 4°C. For each temperature treatment three replicates were stored for either 1 day or 7 days (online supplementary Fig. S2). Freeze-dried samples were subsequently stored at −80°C which is the standard procedure for the storage of environmental samples after their transportation from the field to the laboratory if they are subjected to be analyzed at the RNA level. Nucleic acids of all samples were extracted in the same run.

Nucleic acid extraction and reverse transcription

Total RNA and DNA were co-extracted from 1 g dry weight soil using the Power Soil RNA Isolation Kit and RNA Power Soil DNA Elution Accessory Kit (MoBio Laboratories, Carlsbad, CA). For the withdrawal of soil sampling material, soil sample flasks were kept on ice and relocated to the −80°C storage as soon as possible. Sample material could be gained from control samples without prior thawing. Thus, the 24 freeze-dried soil samples resulted in 24 DNA and 24 RNA extracts, a total of 48 molecular samples. Furthermore, two DNA and two RNA extracts were obtained from frozen control samples of each plot. Summing up the number of nucleic acid extracts of control samples and freeze-dried samples, we analyzed 28 DNA and 28 RNA extracts, in the following referred to as a total of 56 samples. RNA extracts were treated with RQ1 RNase-Free DNase (Promega, USA) and purified by phenol-chloroform extraction. Nucleic acid extracts were quantified with the NanoDrop ND-8000 (Peqlab, Germany). Complementary DNA (cDNA) was synthesized from 25 ng RNA with the Monster-Script 1st strand Kit (Epicentre Biotechnologies, USA) using random nonamer primers. For each experimental treatment nucleic acid extracts of one sample replicate were subjected to quality analysis by gel electrophoresis. DNA extracts were loaded on an 1.5% Agarose gel, stained with Ethidium Bromide and photographed in a GeneGenius Gel Bio Imaging System (Syngene, Cambridge, UK). RNA extracts were loaded onto an Eukaryote Total RNA Nano Chip (Agilent Technologies, USA) and analyzed in an Agilent 2100 Bioanalyzer with software version 2.6 (Agilent Technologies, USA). Schroeder *et al.* (2006) described the sophisticated software algorithm of the instrument that considers a plethora of electropherogram features e.g. peak areas, peak heights and peak ratios to calculate an integrity (quality) value for the RNA sample ranging from 1 (most degraded) to 10 (most intact).

Multiplexed amplicon pyrosequencing

Amplicon libraries were prepared with pyrosequencing fusion primers. Polymerase chain reaction (PCR) primer sequences are shown in online supplementary Table S1. The bacterial 16S rRNA gene was amplified with the reverse primer 907R coupled to a barcode and the pyrosequencing adapter B. The forward primer 341F was coupled to pyrosequencing adapter A. PCR reactions were done in triplicate in a final volume of 50 µl and consisted of 1× GoTaqGreen Master Mix (Promega, USA), 25 pmol primers each and 10 ng DNA or 1 µl cDNA. Cycling conditions for primers 907R/341F were: initial activation at 98°C for 1 min, 95°C for 45 s, 57°C for 45 s, 72°C for 1.5 min and PCR cycle repeated 30 times ending with a final extension of 72°C for 10 min. The AM fungal 18S rRNA gene was amplified using a nested PCR approach, see Morris *et al.* (2013) for details. In short, the first PCR was performed using the primer pair GlomerWT0/Glomer1536 followed by two parallel nested PCR setups with the primer NS31 paired either with AM1A or AM1B. The forward primer NS31 was fused to the barcode and the pyrosequencing adapter B while both PCR reverse primers were coupled with the adapter A. One microlitre of a 10-fold dilution of the first PCR reaction was used as template for the nested PCR. Amplicon PCR replicates were pooled and purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Quantitation was done with Quant-iT-PicoGreen ds DNA Assay Kit (Invitrogen). Equimolar sample pools were sequenced on a 454 GS FLX Titanium machine (Roche, Branford, USA). The sequencing plate was divided into four lanes. A pool of all AM fungal community samples comprising both DNA and cDNA amplicons was sequenced on one of the four lanes. Bacterial DNA and cDNA amplicon libraries were pooled separately and sequenced on one lane each.

Bioinformatics

Quality filtering of raw sequences was done with the Mothur software v.1.31.2 (Schloss *et al.* 2009). Sequences were trimmed to 300 nt length (v4–v5 region) after removal of reads with an average quality value below 20, occurrence of ambiguous nucleotides or if barcodes exceeded more than one mismatch. As the bacterial rRNA gene was sequenced starting with the gene reverse primer, bacterial sequences were flipped. Dereplicated sequences were globally aligned to the

SILVA 119 reference database (Quast *et al.* 2013), release April 2015. Therefore, the reference database was either truncated for the BAC341F/BAC907R or NS31/AM1A-B primers for the respective bacterial and AM fungal target and thus two reference alignments were obtained to align our sequences against. Sequences which aligned at unusual alignment positions compared to 95% of the sequences were removed and the alignment was filtered. In case the alignment still showed end gaps as for the bacterial dataset, uniform start and end positions were explicitly set for a second screening step. Chimera check was done with uchime (Edgar *et al.* 2011) as implemented in Mothur and the remaining sequences were subsampled. Quality sequences of uniform length were clustered by USEARCH (Edgar 2010) version 8.0.1623 after sorting them by abundance and excluding singletons in the clustering step which follows the manual recommendations. Thus, representative sequences obtained by USEARCH are based on abundance. Bacterial representative sequences of each operational taxonomic unit (OTU) were taxonomically assigned using the GAST algorithm (Huse *et al.* 2008) against the v4–v5 truncated SILVA 111 database (Quast *et al.* 2013), release July 2012 and non-bacterial OTUs were removed from the dataset. The AM fungal sequences were quarried against the MaarjAM database (Öpik *et al.* 2010) on 10 February 2016. AM fungal representative sequences and their respective OTUs were removed from the dataset if the best blast hit showed less than 90% coverage or an E-value larger than 1e-50. Rare OTUs with less than four reads were removed from both datasets. Sequence reads were submitted to the European Nucleotide Archive with accession number PRJEB8238.

Statistics

Statistical analyses were done with R version 3.1.2 (R Core Team 2014). The experimental treatment effects were evaluated for the yield of nucleic acids and the alpha diversity indices observed species richness, Shannon diversity and Pielou's evenness. Nucleic acid yields were log transformed. The outlier function of the outliers package was applied to identify datapoints that potentially needed removal from the dataset prior to alpha diversity analysis of variances (ANOVA). Identified outlying datapoints were only removed if a visible deviation appeared in diversity index plotting and Non-metric multidimensional scaling (NMDS) ordination plotting or if the violation of test assumptions (normality of model residuals and homogeneity of variances) could be avoided. The three treatment contrasts (i) Freeze-drying versus control storage, (ii) 4°C storage of freeze-dried samples versus room temperature storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples versus 7 days storage of freeze-dried samples were analyzed in linear regression models. Specific formulation of treatment contrasts is shown in online supplementary Table S2. Linear regression models included the plot as fixed factor and the treatment with defined contrasts as fixed factor while interaction terms were only included if the model fit was much better as determined by a lower Akaike Information Criterion (AIC) value. Univariate ANOVA was applied to assess significant differences for the five single storage treatments which are (1) control samples stored at −80°C, (2+3) freeze-dried samples stored at 4°C for either 1 or 7 days and (4+5) freeze-dried samples stored at room temperature for either 1 or 7 days. Homogeneity of variance was assessed by Levene test, while normal distribution of model residuals was inspected by Shapiro tests. In case significant ANOVA results were found, Tukey HSD *post hoc* test was applied as implemented in the agricolae package by the HSD.test function to determine significant pairwise treatment comparisons and variance partitioning with the varpart function of the vegan package (Oksanen *et al.* 2013) was done to assess the effect size of the significant factors identified in the linear regression analysis of treatment contrasts. NMDS was done with the metaMDS function of the vegan package. For NMDS and Permanova, OTU count data was Hellinger transformed and converted to a Bray–Curtis dissimilarity matrix. Permanova analysis was carried out by the adonis function (vegan package) to determine the significance of the factors sampling plot, freeze-drying, storage duration and storage temperature on the bacterial and AM fungal community.

Table 2: nucleic acid yields of frozen and freeze-dried soil samples

	Sample	Mean DNA (μg g^{-1} soil)	SD	Mean RNA (μg g^{-1} soil)	SD
Control	1	61.0	17.2	12.3	5.7
	8	52.9	1.2	14.7	12.1
FD 4°C 1 day	1	78.0	29.2	7.2	2.0
	8	45.6	13.0	7.5	4.5
FD RT 1 day	1	49.0	2.4	8.1	3.2
	8	64.0	41.1	6.3	1.3
FD 4°C 7 days	1	53.9	5.2	6.3	2.7
	8	37.9	7.2	9.3	3.1
FD RT 7 days	1	40.5	9.5	6.1	1.9
	8	29.9	7.1	8.9	1.2

Abbreviation: SD = standard deviation. Freeze-dried (FD) soil samples were stored at room temperature (RT) or 4°C for 1 or 7 days.

RESULTS

Quality and quantity of nucleic acids

High-molecular weight DNA was recovered from frozen and freeze-dried soil samples (online supplementary Fig. S3a). DNA yields (Table 2) were not affected by freeze-drying but a significant decrease of DNA yields ($P < 0.05$) was detected with the prolonged storage time of 7 days for freeze-dried samples (Table 3). RNA yields were neither affected by freeze-drying nor by storage time or temperature. RNA integrity numbers (RIN) were about 7 for all treatments and electropherograms clearly showed an 18S and 23S rRNA peak (online supplementary Fig. S3b). The cDNA transcription and PCR amplification of target microbial communities could be accomplished for all samples.

Bacterial and AM fungal community analysis

From the total of 56 nucleic acid samples, 159 010 bacterial 16S raw sequences were obtained. After quality filtering, the number of bacterial sequences was normalized to the minimum number of sequences per sample resulting in 1646 bacterial reads per sample, which clustered into 1114 bacterial abundant OTUs containing at least three reads. The true bacterial diversity still exceeded the recovered OTUs as indicated by rarefaction curves (online supplementary Fig. S4a). About 68% of the bacterial OTUs could be assigned to family level. The bacterial community comprised 14 phyla and six candidate divisions (online supplementary Table S3). Proteobacteria (40%), Actinobacteria (16%), Bacteroidetes (13%), Acidobacteria (10%) and Chloroflexi (9%) were the dominant phyla accounting for 87% of the bacterial OTUs found. In terms of sequence abundance, the top ten bacterial phyla contributed to 99% of all bacterial sequences and were dominated by Proteobacteria (38%), Actinobacteria (23%), Acidobacteria (20%), Chloroflexi (7%), Bacteroidetes (6%), Firmicutes (2%) and 1% of each Gemmatimonadetes, Nitrospirae, Candidate division WS3 and Verrucomicrobia. The 10 most abundant bacterial classes contributed to 80% of total bacterial sequence reads and were composed of Acidobacteria and Alphaproteobacteria (each 17%) followed by Deltaproteobacteria (13%), Thermoleophilia (8%), Acidimicrobia (8%), Actinobacteria (6%) and the Betaproteobacteria, Gammaproteobacteria, Sphingobacteria and Cytophagia each contributing less than 5%.

The AM fungal dataset of 18S reads comprised 83 796 sequences. After quality filtering, the number of AM fungal sequences was normalized to the minimum number of sequences per sample resulting in 730 AM fungal reads per sample, which clustered into 66 abundant OTUs. Most AM fungal rarefaction curves (online supplementary Fig. S4b) did not reach saturation but came closer to saturation level than the bacterial samples. The dominant AM fungal orders were Glomerales (48%), Archaeosporales (35%), 15% Paraglomerales and (2%) Diversisporales based on the total number of OTUs. Based on the relative abundances of sequences reads, AM fungi were dominated by Glomerales (79%), followed by Archaeosporales (13%), Diversisporales

Table 3: linear regression analysis of nucleic acid yields of the three treatment contrasts (i) freeze-drying vs. control, (ii) 4°C storage of freeze-dried samples vs. room temperature (RT) storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples vs. 7 days storage of freeze-dried samples and ANOVA of specific treatment conditions followed by Tukey HSD *post hoc* test

	Significance of treatment contrasts as model coefficients in linear regression models						ANOVA		Tukey HSD	
	(i) Controle vs. Freeze-drying		(ii) 4°C vs. RT storage		(iii) 1-day vs. 7-day storage		Specific storage treatment		Specific storage treatment	
	t	*P*	*t*	*P*	*t*	*P*	*F*	*P*	Sign. treatment differences	Mean
DNA	0.9	—	-1.4	—	**2.3**	**<0.05**	2.8	—	—	
RNA	1.6	—	0.0	—	—	—	0.9	—	—	

Linear regression models formulated as yield ~ Plot origin + Treatment. Nucleic acid yields were log transformed prior to analysis. Statistical significant *P* values ($P < 0.05$) are given in bold.

Table 4: comparison of OTU richness between frozen (C) and freeze-dried soil samples stored under different time (1 day or 7 days) and temperature conditions (room temperature or 4°C)

		Mean OTU richness					Shared OTUs with control			
Bacteria	C mean OTUs	RT_1d	RT_7d	4°C_1d	4°C_7d	Total OTUs C	RT_1d	RT_7d	4°C_1d	4°C_7d
HEG01 DNA	371	372 (100%)	344 (93%)	365 (98%)	371 (100%)	506	396 (78%)	379 (75%)	396 (78%)	398 (79%)
HEG01 RNA	362	382 (106%)	367 (101%)	387 (107%)	384 (106%)	496	410 (83%)	387 (78%)	399 (80%)	400 (81%)
HEG08 DNA	383	386 (101%)	374 (98%)	388 (101%)	369 (96%)	520	415 (80%)	400 (77%)	415 (80%)	386 (74%)
HEG08 RNA	372	382 (103%)	395 (106%)	385 (103%)	362 (97%)	509	392 (77%)	410 (81%)	405 (80%)	388 (76%)
		Mean OTU richness					Shared OTUs with control			
AM fungi	C mean OTUs	RT_1d	RT_7d	4°C_1d	4°C_7d	Total OTUs C	RT_1d	RT_7d	4°C_1d	4°C_7d
HEG01 DNA	44	39 (89%)	42 (95%)	39 (89%)	41 (93%)	48	43 (90%)	46 (96%)	45 (94%)	45 (94%)
HEG01 RNA	16	15 (94%)	16 (100%)	21 (131%)	13 (81%)	22	13 (59%)	14 (64%)	16 (73%)	13 (59%)
HEG08 DNA	41	40 (98%)	43 (105%)	43 (105%)	41 (100%)	53	45 (85%)	49 (92%)	48 (91%)	46 (87%)
HEG08 RNA	14	19 (136%)	27 (193%)	21 (150%)	27 (193%)	23	21 (91%)	22 (96%)	21 (91%)	22 (96%)

Percentage values are given in brackets.

(6%) and Paraglomerales (2%). We detected six AM fungal families which were dominated in sequence abundance by Claroideoglomeraceae (41%), Glomeraceae (39%), followed by Archaeosporaceae (8%), Diversisporaceae (6%), Ambisporaceae (5%) and Paraglomeraceae (2%).

One Paraglomus OTU could be identified as *Paraglomus majewskii* by BLAST (Altschul *et al.* 1990) nucleotide search.

Impact of freeze-drying, storage time and temperature conditions on microbial diversity

On average, 79% of the bacterial OTUs detected in frozen soil samples were shared by freeze-dried samples (Table 4), while mean OTU richness was equal. At the DNA level, observed bacterial species richness was statistically higher ($P < 0.01$) on plot HEG08 with an average of 380 OTUs compared to an average of 364 OTUs on plot HEG01 while no significant difference could by found at the RNA level. Bacterial diversity was not affected by freeze-drying of soil samples itself but by a prolonged storage duration of 7 days (Table 5, online supplementary Fig. S5). At the DNA level, the observed species number and Shannon diversity were significantly lower for freeze-dried samples stored for 7 days than for freeze-dried samples stored only for 1 day. At the RNA level, this phenomenon was observed for the Pielou's evenness index. At the DNA level, 13% of explained variance in observed bacterial species numbers could be independently attributed to storage time while 26% were explained by the sample plot origin as well as 10% of explained variance in bacterial Shannon diversity could be independently attributed to storage time while 69% were explained by the sample plot origin. At the RNA level, 18% of explained variance in Pielou's evenness could be independently attributed to storage time while 42% were explained by the sample plot origin.

About 85% of AM fungal OTUs were shared between frozen and freeze-dried soil samples while mean OTU richness was about 116% (Table 4). At the RNA level the mean OTU richness of freeze-dried samples compared to control samples and the number of shared OTUs with the control varied strongly between sampling plots and treatments. For example, the number of shared OTUs between freeze-dried and control samples reached a minimum of 59% while the mean OTU richness of freeze-dried samples reached a maximum of even 193% compared to the control samples. Nevertheless, neither freeze-drying nor tested storage conditions were found to significantly affect the detected alpha diversity of AM fungi in the soil samples (Table 6, online supplementary Fig. S6). At the RNA level, AM fungal OTU numbers were higher on plot HEG08 than HEG01 ($P = 0.01$).

Impact of freeze-drying, storage time and temperature conditions on microbial community composition

NMDS ordination plots showed a clear clustering of bacterial communities in respect to plot and nucleic acid origin (Fig.1a). In the RNA based analysis bacterial communities were enriched for Deltaproteobacteria (online supplementary Fig. S7, online supplementary Table S4). Freeze-dried samples clustered with respective controls in general. Permanova analysis showed a significant effect of the sample plot origin on the detected bacterial community but no significant effect of freeze-drying, storage time or storage temperature was found (Table 7).

The NMDS ordination plots showed that, AM fungal communities clustered on the plot at DNA level but exhibited no clear pattern in the ordination of RNA-based AM fungal communities (Fig. 1b). Permanova analysis showed a significant effect of the sample plot origin on the detected AM fungal community but no significant effect of freeze-drying, storage time or storage temperature was found (Table 7).

DISCUSSION

Freeze-drying preserved high quality nucleic acids in the soil samples with high molecular weight DNA recovered and RNA extracts showing RIN with number of about 7. Fleige and Pfaffl (2006) recommended RIN values greater than 5 as good total RNA and RIN larger than 8 as perfect total RNA

Table 5: linear regression analysis of the bacterial alpha diversity indices, observed species number, Shannon diversity and Pilou's evenness, of the three treatment contrasts (i) freeze-drying vs. control, (ii) 4°C storage of freeze-dried samples vs. room temperature (RT) storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples vs. 7 days storage of freeze-dried samples and ANOVA of specific treatment conditions followed by Tukey HSD *post hoc* test

		Significance of treatment contrasts as model coefficients in linear regression models						ANOVA		Tukey HSD	
		(i) Controle vs. Freeze-drying		(ii) 4°C vs. RT storage		(iii) 1-day vs. 7-day storage		Specific storage treatment		Specific storage treatment	
	Index	*t*	*P*	*t*	*P*	*t*	*P*	*F*	*P*	Sign. treatment differences	Mean
DNA	S.obs.	0.8	—	−0.8	—	**2.6**	**0.01**	2.8	—	—	
	Shannon	1.0	—	−1.2	—	**3.4**	**<0.01**	**4.8**	**<0.01**	**4°C_1d-RT_7d**	**5.29–5.21**
	Pielou	0.4	—	−0.77	—	1.8	—	1.3	—	—	
RNA	S.obs.	−2.0	—	−1.4	—	1.4	—	—	—	—	
	Shannon	−0.2	—	−0.05	—	0.7	—	—	—	—	
	Pielou	1.0	—	0.20	—	**3.3**	**<0.01**	**4.1**	**<0.05**	**RT_1d-RT_7d**	**0.889–0.878**
										RT_1d-4°C_7d	**0.889–0.880**

Abbreviation: S. obs. = observed richness. Linear regression models formulated as alpha diversity index ~ Plot origin + Treatment. For the RNA dataset the samples HEG08-4°C-7days-b replicate was removed as outlier from the dataset for all three analyzed alpha diversity indices, while additionally HEG08-4°C-1day-a replicate and HEG01-RT-1day-c was removed as outlier for the Pilou's evenness analysis. HEG08-4°C-7days-b and HEG08-4°C-1day-a samples were confirmed as apparent outliers in NMDS ordination plot (Fig. 1a). Statistical significant *P* values ($P < 0.05$) are given in bold.

Table 6: linear regression analysis of AM fungal alpha diversity indices, observed species number, Shannon diversity and Pilou eveness, of the three treatment contrasts (i) freeze-drying vs. control, (ii) 4°C storage of freeze-dried samples vs. room temperature (RT) storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples vs. 7 days storage of freeze-dried samples and ANOVA of specific treatment conditions followed by Tukey HSD *post hoc* test

		Significance of treatment contrasts as model coefficients in linear regression models						ANOVA		Tukey HSD	
		(i) Controle vs. Freeze-drying		(ii) 4°C vs. RT storage		(iii) 1-day vs. 7-day storage		Specific storage treatment		Specific storage treatment	
	Index	*t*	*P*	*t*	*P*	*t*	*P*	*F*	*P*	Sign. treatment differences	Mean
DNA	S.obs.	0.9	—	−0.2	—	−1.3	—	0.9	—	—	
	Shannon	1.9	—	−1.0	—	−0.9	—	2.0	—	—	
	Pielou	−1.2	—	−0.1	—	1.5	—	0.6	—	—	
RNA	S.obs.	−0.0	—	−0.5	—	1.1	—	1.2	—	—	
	Shannon	−1.2	—	−0.1	—	−0.3	—	0.5	—	—	
	Pielou	−0.8	—	0.7	—	0.2	—	0.4	—	—	

Abbreviation: S. obs. = observed richness. Linear regression models formulated as alpha diversity index ~ Plot origin + Treatment while the model including the interaction terms was applied for Pielou's evenness at the DNA level and the observed species number at the RNA level as determined by more favorable AIC values.

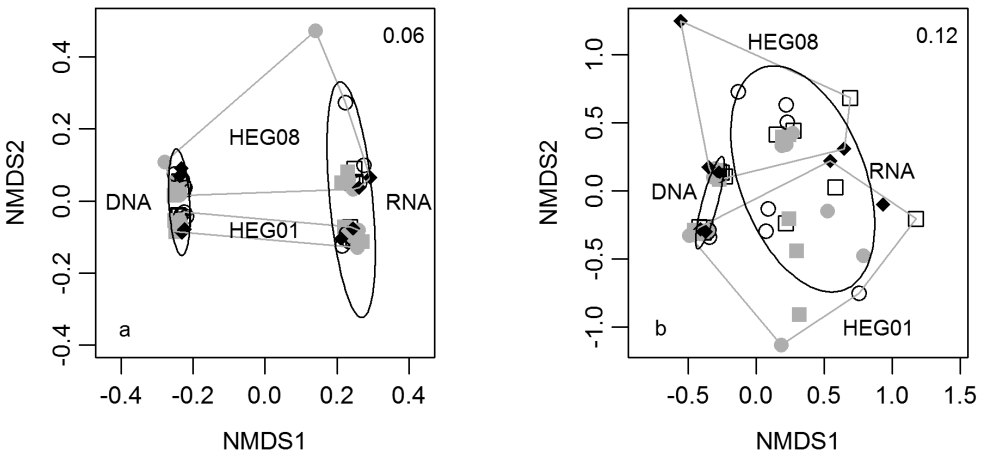

Figure 1: non-metric multidimensional scaling plots of bacterial (a) and AM fungal (b) communities. Frozen control samples: filled diamonds, freeze-dried samples stored under different conditions: room temperature (square), 4°C (circle), 1 day (open symbols), 7 days (grey-filled symbols). Polygons indicate plot origin while elipses indicate DNA or RNA derived microbial communities.

Table 7: permanova analysis of treatment effects on bacterial and AM fungal community composition at the DNA and RNA level

	Bacteria				AM fungi			
	DNA		RNA		DNA		RNA	
Factor	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sampling plot	5.5	**0.001**	4.8	**0.001**	46.5	**0.001**	9.9	**0.001**
Freeze-drying	1.5	—	1.1	—	1.5	—	1.7	—
Storage duration	0.96	—	0.95	—	0.67	—	1.2	—
Storage temperature	0.95	—	0.93	—	1.5	—	0.8	—

Statistical significant *P* values ($P < 0.05$) are given in bold.

for downstream applications like real time PCR or gene expression studies. DNA yields decreased with storage time. Rehydration of desoxyribonucleases (Dnases) from air moisture could have occurred while freeze-dried Ribonuclease (Rnase) A was described to form insoluble precipitates during storage (Townsend and DeLuca 1991). Optimal exclusion of air moisture could be achieved by closing sample flasks directly in the freeze-dryer after purging them with an inert gas like nitrogen. Our bench top freeze-dryer did not provide this sophisticated feature and our sample flasks probably did not seal air-tight. As cheap alternative method we had stored the closed sample flasks in sealed plastic bags with blue silica gel.

Impact of freeze-drying, storage time and temperature conditions on soil microbial diversity

Soil microbes appear in patchy distributions (Mummey and Rillig 2008; Raynaud *et al.* 2014) inhabiting mechanically resistant micro-aggregates (<250 µm) (Tisdall and Oades 1982; Vos *et al.* 2013). A true homogenization of soil samples with complete coverage of OTUs between replicate sample flasks is thus impossible. Therefore, 70–80% overlap of OTUs between control and treatment samples can be considered as satisfactory. Our sequencing effort of the bacterial community did not completely assess the whole bacterial diversity present in the soil which also accounts for an incomplete recovery of OTUs. Considering this, a recovery of OTUs with an average of 79% still proves the validity of the study. Sequencing of AM fungi was closer to saturation level and explained the enhanced recovery rate of 85% of the OTUs between control and freeze-dried samples. A major factor influencing bacterial species richness and community composition is soil pH (Tripathi *et al.* 2012). At the DNA level, bacterial OTU numbers were indeed highest on the unfertilized pasture with near neutral pH. At the RNA level no difference could be found, indicating that the pH difference between both plots is quite small and the fertilized plot with a pH of 6.65 still reasonable neutral. Storage of freeze-dried samples for 7 days showed a statistical significant reduction of bacterial OTU numbers and Shannon diversity at the DNA level and of Pielou's evenness at the RNA level. However, the effect size of this reduction was small as at least 93% of bacterial OTU numbers were recovered from freeze-dried samples compared to the control and the explained variance in Shannon diversity attributed to storage duration was only 10% in comparison to 69% of variance explained by plot origin.

At the RNA level, total AM fungal OTU richness was higher on the unfertilized pasture than on the fertilized meadow. A higher diversity of AM fungi in sites with lower anthropogenic impact as HEG08 compared to the more intensively used site HEG01 was reported before (Lumini *et al.* 2010). Several direct and indirect mechanisms affiliated with fertilization

were identified (Alguacil *et al.* 2014). We found no effect of freeze-drying or subsequent storage conditions on the AM fungal alpha diversity measures.

Impact of freeze-drying, storage time and temperature conditions on microbial community composition

Relative abundances of the five most abundant bacterial phyla were similar for the fertilized meadow and the unfertilized pasture. Riber *et al.* (2014) also found bacteria to be unaffected at the phylum level for the application of animal, urban and waste fertilizers. Nevertheless, NMDS ordination plots showed distinct clusters for both sampling sites. As we investigated only two soil samples, the major environmental drivers for this distinction cannot be identified. In terms of storage conditions, we found no significant effects of freeze-drying, storage temperature or storage time on the detected bacterial communities.

Several studies found Glomerales to be a widespread and a dominant class in AM fungal communities, which was also the case for the investigated grasslands. Gosling *et al.* (2014) reported a potential negative impact of intensive agricultural management on Paraglomus spp. and we found Paraglomerales on both grassland plots (medium and high land use index) in low relative abundances of about 2–3%. AM fungal communities were well separated for sampling plots in NMDS analysis, which could be due to the differing land use of mowing and grazing (Morris *et al.* 2013). AM fungal community composition was not affected by freeze-drying, storage time or storage temperature.

Our findings strongly advocate the use of freeze-drying prior to short-term storage and long-distance transportation of soil samples for molecular studies. Furthermore, the sample transportation is non-hazardous and even huge sample numbers can be transported cost efficiently and reliably across countries and continents. Projects with huge sampling efforts in remote areas, such as the one of Shi et al. (2017), will benefit from using lyophilizaton. Using lyophilization would also allow projects on large-scale soil chararacteristics (see Scholten et al. 2017) or litter decomposition (see Li et al. 2017) to include microbial charateristics among the traditionally analyzed chemical properties.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology online*.

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Supplementary Material

Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities

Christina Weißbecker, Tesfaye Wubet, Guillaume Lentendu, Peter Kühn, Thomas Scholten, Helge Bruelheide and François Buscot

Supplementary Tables

Table S1 PCR primer sequences.

Name	Sequence (5'-3')	Reference
907R	CCGTCAATTCMTTTGAGTTT	Lane *et al.* (1985)
341F	CCTACGGGAGGCAGCAG	Muyzer *et al.* (1993)
NS31	TTGGAGGGCAAGTCTGGTGCC	Simon *et al.* (1992)
GlomerWT0	CGAGDWTCATTCAAATTTCTGCCC	Wubet *et al.* (2006)
Glomer1536	AATARTTGCAATGCTCTATCCCCA	Wubet *et al.* (2006)
AM1A	CTTTGGTTTCCCRTAAGGYGCC	modified after Helgason *et al.* (1998)
AM1B	CTTTGGTTTCCCATARGGTGCC	modified after Helgason *et al.* (1998)

Table S2 Formulation of treatment contrasts for linear regression model analysis by contrasts function in r. C: Controle samples, RT: freeze-dried samples stored at room temperature, 4°C: freeze-dried samples stored at 4°C.

	Contrast 1) Freeze-drying versus controle	**Contrast 2)** 4°C versus RT storage of freeze-dried samples	**Contrast 3)** 1 day versus 7 days storage of freeze-dried samples
Controle	4	0	0
RT stored 1day	-1	1	1
RT stored 7 days	-1	1	-1
4°C stored 1 day	-1	-1	1
4°C stored 7 days	-1	-1	-1

Table S3 Overview of detected bacterial phyla, occurrence of Proteobacteria subphyla and classes of arbuscular mycorrhizal fungi.

	Phylum	Number of OTUs
1	Proteobacteria	448
2	Actinobacteria	178
3	Bacteroidetes	142
4	Acidobacteria	107
5	Chloroflexi	96
6	Firmicutes	36
7	Gemmatimonadetes	25
8	Candidate division WS3	15
9	Cyanobacteria	15
10	Verrucomicrobia	13
11	Nitrospirae	10
12	Chlorobi	9
13	Elusimicrobia	7
14	Candidate division BHI80-139	3
15	Planctomycetes	3
16	Candidate division JL-ETNP-Z39	2
17	Fibrobacteres	2
18	Candidate division BRC1	1
19	Candidate division TM6	1
20	Candidate division WCHB1-60	1

	Class	Number of OTUs
1	Glomerales	32
2	Archaeosporales	23
3	Paraglomerales	10
4	Diversisporales	1

	Proteobacteria	Number of OTUs
1	Delta-	221
2	Alpha-	114
3	Gamma-	67
4	Beta-	39
5	Candidate divisions	5
	unclassified	2

Table S4 Treatment specific relative abundances of the twelve most abundant bacterial phyla. Visualization by bar plots Fig. S7. RT: storage of freeze-dried samples at room temperature, 4°C: storage of freeze-dried samples at 4°C, C: control, storage time one day (1d) or seven days (7d).

		DNA					RNA				
		C	**RT1d**	**RT7d**	**4°C1d**	**4°C7d**	**C**	**RT1d**	**RT7d**	**4°C1d**	**4°C7d**
HEG01	Proteobacteria	0.31	0.28	0.29	0.31	0.31	0.48	0.47	0.46	0.45	0.49
	Actinobacteria	0.29	0.32	0.37	0.30	0.31	0.17	0.15	0.16	0.17	0.14
	Acidobacteria	0.21	0.20	0.17	0.21	0.19	0.21	0.19	0.19	0.21	0.20
	Bacteroidetes	0.08	0.05	0.04	0.05	0.04	0.04	0.05	0.05	0.05	0.05
	Chloroflexi	0.07	0.09	0.08	0.09	0.09	0.06	0.08	0.09	0.07	0.07
	Gemmatimonadetes	0.03	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01
	Nitrospirae	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	Candidate division WS3	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01
	Verrucomicrobia	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01
	Chlorobi	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01
	Firmicutes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Cyanobacteria	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00
HEG08	Proteobacteria	0.31	0.31	0.29	0.30	0.30	0.54	0.48	0.44	0.47	0.41
	Actinobacteria	0.25	0.22	0.19	0.21	0.21	0.14	0.15	0.19	0.12	0.13
	Acidobacteria	0.22	0.26	0.30	0.27	0.28	0.18	0.20	0.19	0.18	0.16
	Bacteroidetes	0.09	0.07	0.06	0.08	0.07	0.06	0.07	0.06	0.08	0.10
	Chloroflexi	0.06	0.08	0.10	0.08	0.08	0.04	0.05	0.08	0.06	0.05
	Gemmatimonadetes	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01
	Nitrospirae	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00
	Candidate division WS3	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00
	Verrucomicrobia	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01
	Chlorobi	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.01
	Firmicutes	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.06	0.11
	Cyanobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S5 Treatment specific relative abundances of arbuscular mycorrhizal fungi classes. Visualization by bar plots Fig. S7. RT: storage of freeze-dried samples at room temperature, 4°C: storage of freeze-dried samples at 4°C, C: control, storage time one day (1d) or seven days (7d).

		DNA					RNA				
		C	**RT1d**	**RT7d**	**4°C1d**	**4°C7d**	**C**	**RT1d**	**RT7d**	**4°C1d**	**4°C7d**
HEG01	Glomerales	0.65	0.67	0.70	0.66	0.68	0.73	0.76	0.82	0.69	0.84
	Archaeosporales	0.28	0.27	0.26	0.26	0.26	0.24	0.20	0.11	0.10	0.12
	Diversisporales	0.04	0.04	0.02	0.04	0.04	0.03	0.02	0.07	0.18	0.01
	Paraglomerales	0.03	0.02	0.02	0.04	0.02	0.00	0.03	0.00	0.02	0.03
HEG08	Glomerales	0.74	0.79	0.80	0.78	0.82	0.87	0.96	0.88	0.93	0.93
	Archaeosporales	0.24	0.19	0.18	0.19	0.17	0.05	0.01	0.04	0.02	0.03
	Diversisporales	0.01	0.02	0.01	0.02	0.01	0.04	0.02	0.06	0.03	0.04
	Paraglomerales	0.01	0.00	0.00	0.01	0.00	0.03	0.01	0.02	0.03	0.00

Supplementary Figures

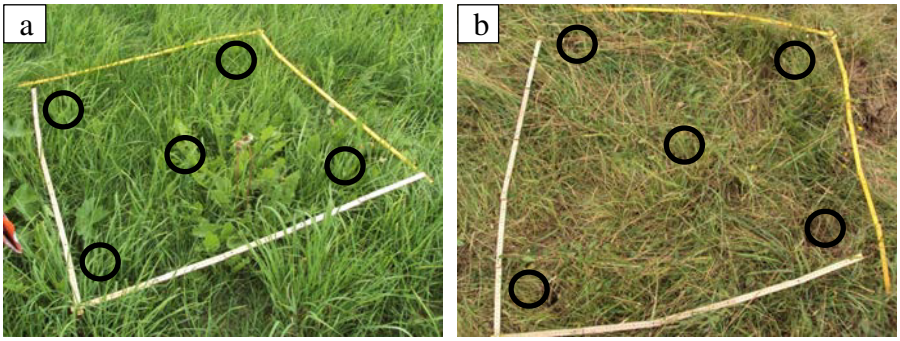

Fig. S1 Sampling site: subplots 1 m x 1 m on a) a fertilized meadow, HEG01, and b) an unfertilized pasture grazed by cattle, HEG08. Circles indicate the location of the five soil cores on each subplot that were combined to a composite soil sample at each plot.

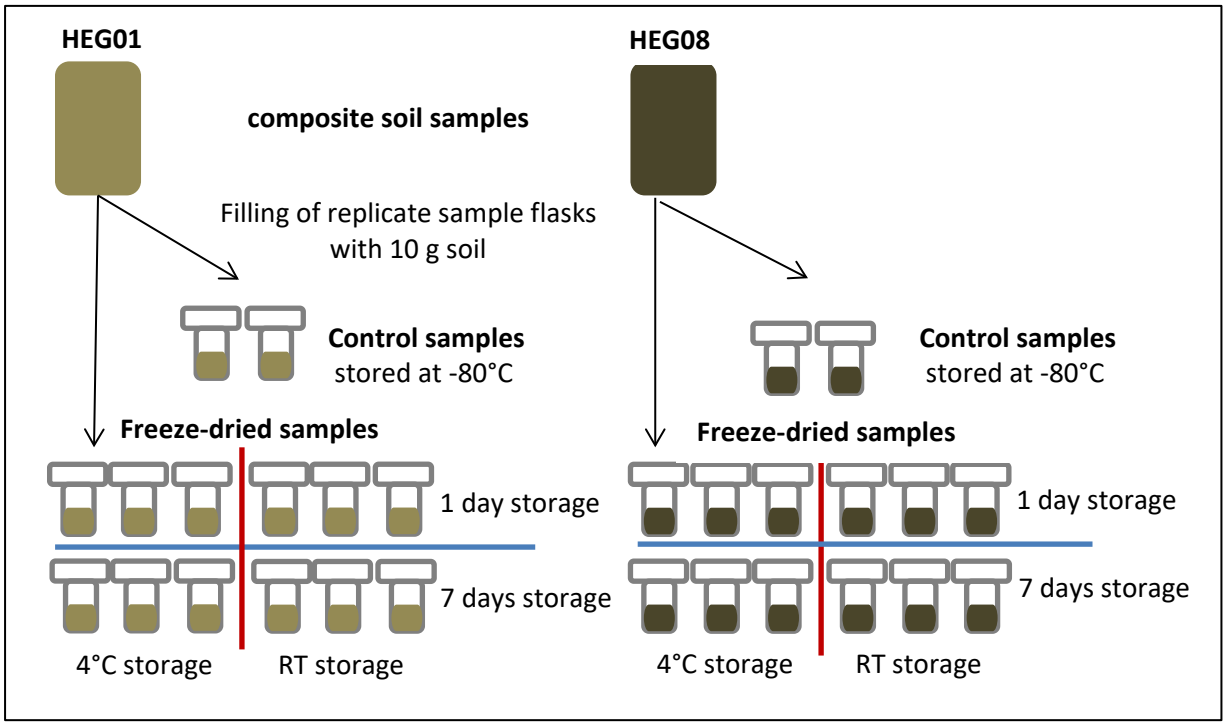

Fig. S2 Experimental design. RT: room temperature.

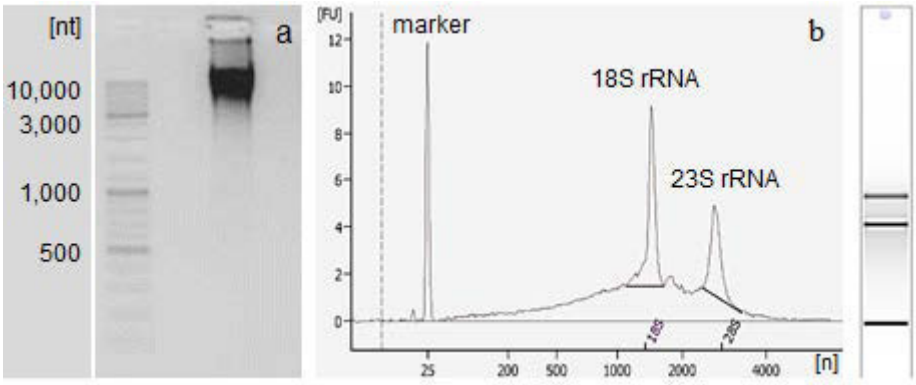

Fig. S3 Quality assessment of extracted nucleic acids a) DNA extract of a freeze-dried soil sample stored at 4°C for 7 days loaded on an agarose gel with GeneRuler DNA ladder mix b) RNA extract (RIN 7.5) of a freeze-dried soil sample stored for 7 days at room temperature analyzed by capillary gel electrophoresis on an Agilent2100 Bioanalyzer.

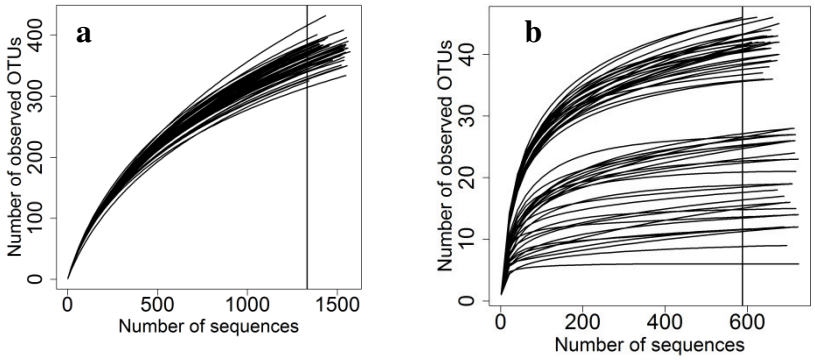

Fig. S4 Rarefaction curves a) bacterial OTUs b) AM fungal OTUs.

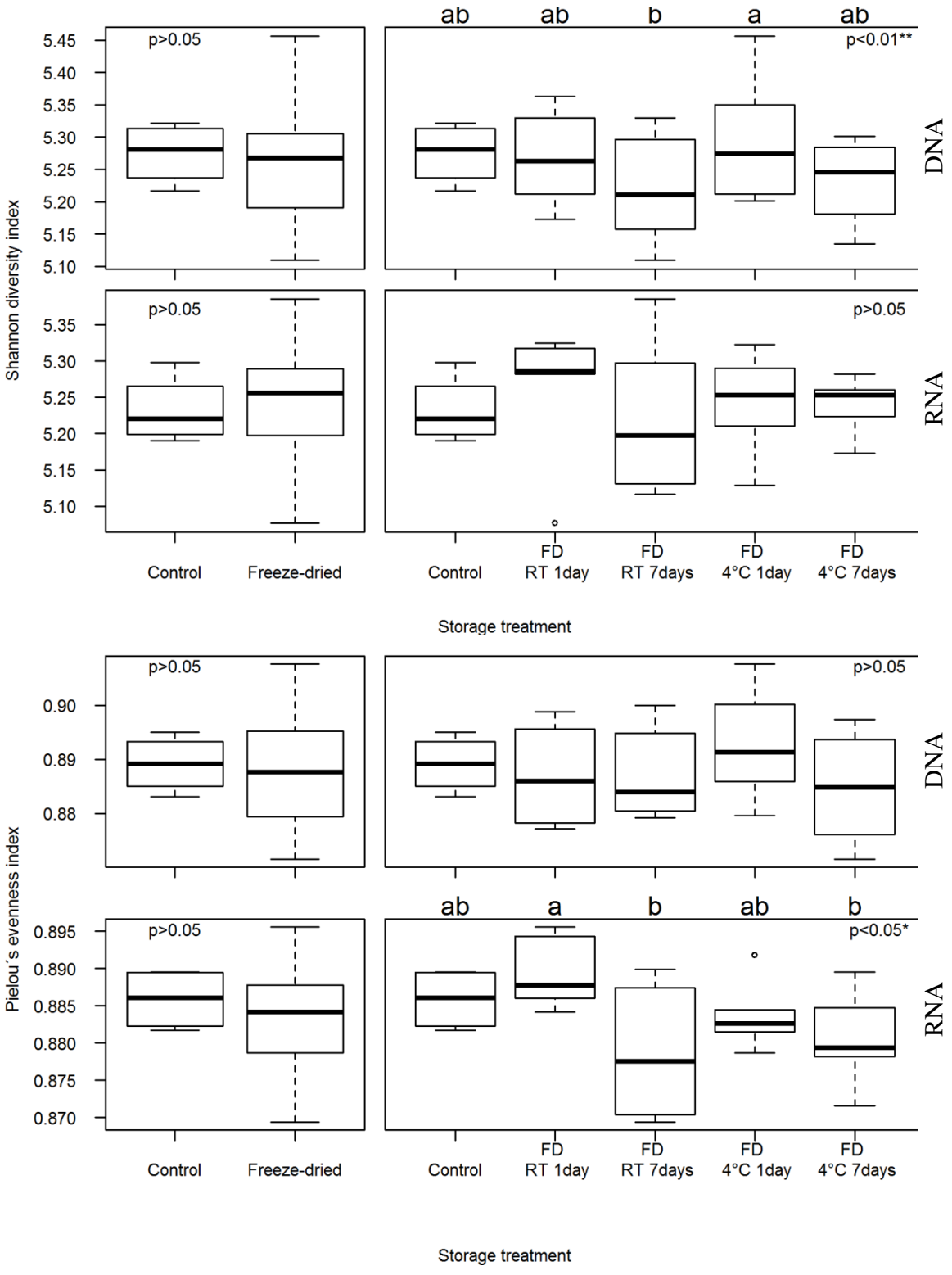

Fig. S5 Boxplots of the Shannon diversity and Pielou`s evenness index for bacterial communities. Control (C) samples were frozen and stored at -80°C while freeze-dried (FD) samples were stored under different conditions: room temperature (RT) or 4°C for one or seven days. In case letters are shown, treatments without shared letters indicate significant differences at $p<0.05$.

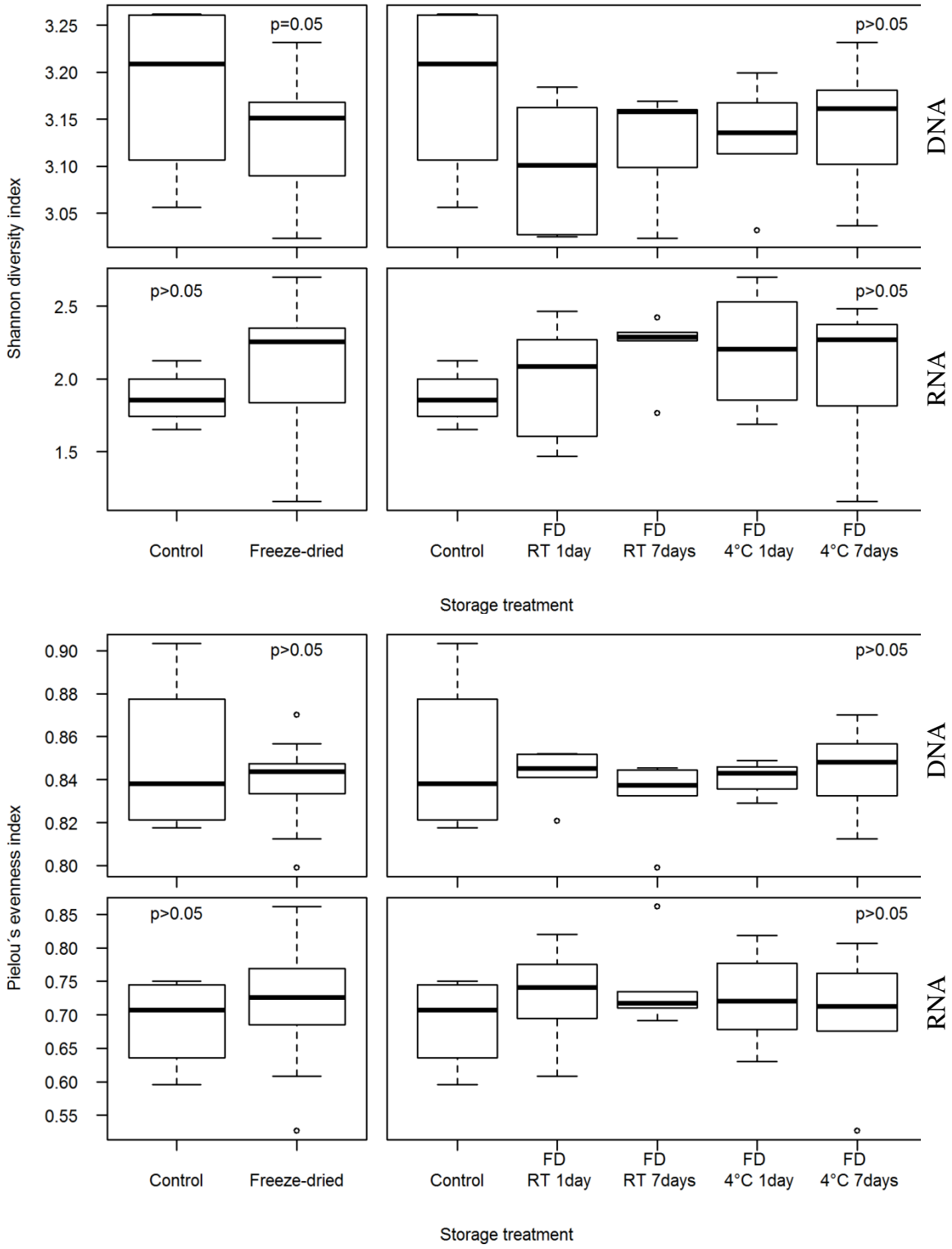

Fig. S6 Boxplots of the Shannon diversity and Pielou`s evenness index for AM fungal communities. Control (C) samples were frozen and stored at -80°C while freeze-dried (FD) samples were stored under different conditions: room temperature (RT) or 4°C for one or seven days upon extraction.

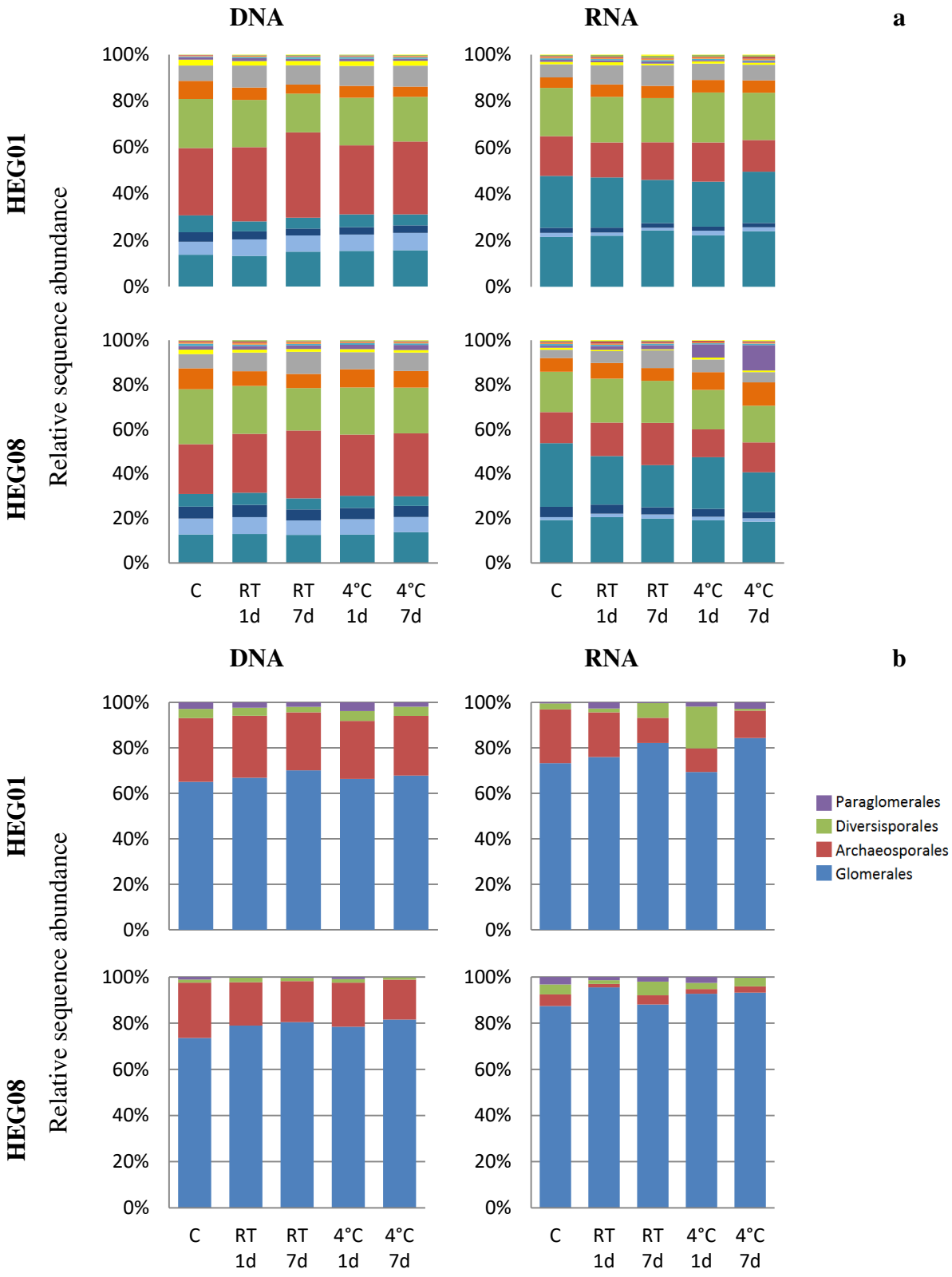

Fig. S7 Taxonomic distribution of microbial communities at different soil sample storage conditions. a) twelve most abundant bacterial phyla b) four classes of AM fungi. C: frozen control samples, RT: freeze-dried and stored at room temperature, 4°C: freeze-dried and stored at 4°C.

CHAPTER 2

Experimental evidence of functional group-dependent effects of tree diversity on soil fungi in subtropical forests

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Experimental Evidence of Functional Group-Dependent Effects of Tree Diversity on Soil Fungi in Subtropical Forests

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Deconvoluting the relative contributions made by specific biotic and abiotic drivers to soil fungal community compositions facilitates predictions about the functional responses of ecosystems to environmental changes, such as losses of plant diversity, but it is hindered by the complex interactions involved. Experimental assembly of tree species allows separation of the respective effects of plant community composition (biotic components) and soil properties (abiotic components), enabling much greater statistical power than can be achieved in observational studies. We therefore analyzed these contributions by assessing, via pyrotag sequencing of the internal transcribed spacer (ITS2) rDNA region, fungal communities in young subtropical forest plots included in a large experiment on the effects of tree species richness. Spatial variables and soil properties were the main drivers of soil fungal alpha and beta-diversity, implying strong early-stage environmental filtering and dispersal limitation. Tree related variables, such as tree community composition, significantly affected arbuscular mycorrhizal and pathogen fungal community structure, while differences in tree host species and host abundance affected ectomycorrhizal fungal community composition. At this early stage of the experiment, only a limited amount of carbon inputs (rhizodeposits and leaf litter) was being provided to the ecosystem due to the size of the tree saplings, and persisting legacy effects were observed. We thus expect to find increasing tree related effects on fungal community composition as forest development proceeds.

Keywords: BEF-China, experimental forest, forest biodiversity experiment, fungal functional groups, host preference, metagenomics, mycorrhizal fungi, soil

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INTRODUCTION

Soil fungi are a highly diverse group of organisms (possibly including several million species; Blackwell, 2011; Taylor et al., 2014), providing many ecosystem services such as organic matter decomposition, element cycling, plant nutrition and plant protection (van der Heijden et al., 2015). They can be assigned to functional guilds based on the primary classes of resources they exploit (Nguyen et al., 2016a), and the composition of their communities is governed by multiple,

strongly interacting abiotic, biotic and spatial variables (**Figure 1**). Unraveling the relative contributions of these potential drivers to fungal community composition will greatly facilitate predictions about ecosystem functioning in response to environmental changes, particularly reductions in plant diversity.

Land plants and fungi share intimately associated natural histories (Buscot, 2015). From the onset of the colonization of terrestrial habitats, plants have gained crucial support from arbuscular mycorrhizal (AM) fungi (Humphreys et al., 2010), which provide their plant symbionts with substantial proportions of their phosphorus requirements (Smith and Smith, 2011). Saprotrophic fungi evolved to be the most prominent group of microorganisms capable of decomposing complex plant residues (Floudas et al., 2012), and their descendants include ectomycorrhizal (EcM) fungi. The latter mainly decompose nitrogen-rich polymers, and trade nitrogen and micronutrients against photosynthates with their plant symbionts, thereby greatly extending the plants' ability to acquire both mineral and organic soil resources (Bruns and Shefferson, 2004). Pathogenic fungi can substantially impair plant growth and fecundity (Zeilinger et al., 2016), or even destroy large populations of their hosts, but they also play important roles in maintaining plant diversity and mediating plant succession in forest ecosystems (Gilbert, 2002).

In natural ecosystems, biotic and abiotic components often show strong interdependencies as plant communities coevolve with the abiotic soil matrix, and they interact to affect the physico-chemical conditions of the soils (Augusto et al., 2002; Ayres et al., 2009; Condit et al., 2013; Zemunik et al., 2016). Hence in observational studies it is methodologically difficult to assess the contributions made by each specific factor to the development of ecosystems and their responses to environmental changes (Peay et al., 2010; Martínez-García et al., 2015; Schappe et al., 2017). Correlations between biotic and abiotic factors must be taken into account in order to allow inferences about causal relationships underlying community assemblies and

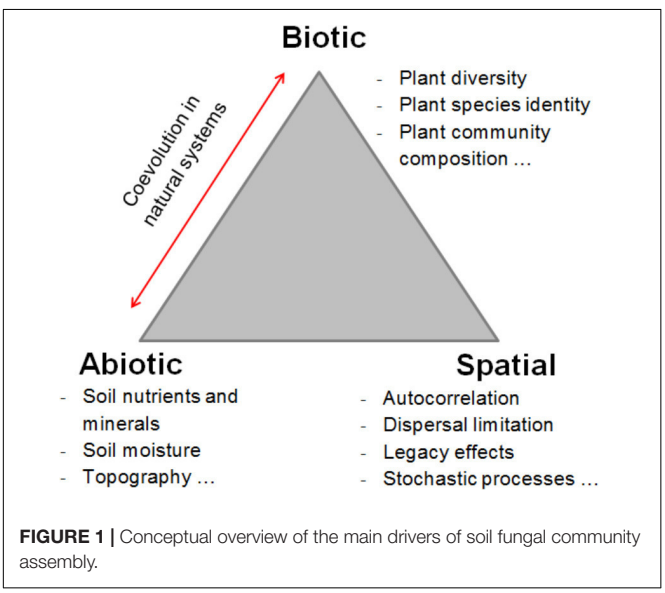

FIGURE 1 | Conceptual overview of the main drivers of soil fungal community assembly.

confirmation of insights gained by the multitude of observational studies conducted on regional (e.g., Gao et al., 2015; Martínez-García et al., 2015; Urbanova et al., 2015; Tedersoo et al., 2016; Schappe et al., 2017) and global (Tedersoo et al., 2014; Davison et al., 2015; Prober et al., 2015) scales. While Tedersoo et al. (2014) and Prober et al. (2015) postulated that plant and fungal alpha diversity are independent on the global scale (EcM fungi being an exception), several studies have detected significant regional relationships between these variables (tropics: Peay et al., 2013; subtropics: Liang et al., 2015; temperate: Martínez-García et al., 2015; Urbanova et al., 2015; Tedersoo et al., 2016). In terms of community composition, Prober et al. (2015) found a strong correlation between AM fungal and plant beta-diversity in grasslands. Moreover, regional correlations between subtropical tree and AM fungal communities and between tropical tree and non-AM fungal communities have been found by, respectively, Liang et al. (2015) and Schappe et al. (2017). In contrast, EcM fungal communities have been reported to be related to host identity (Ishida et al., 2007; Tedersoo et al., 2008, 2010) and host richness and abundance (Tedersoo et al., 2014), at both host species and higher phylogenetic levels, including host genera (Gao et al., 2013) and families (Tedersoo et al., 2012).

Experimental assembly of host plant species makes it possible to separate the effects of plant community composition (biotic components) and soil properties (abiotic components) on the plant-fungus relationships and associated functional responses of ecosystems. Information about microbial communities in experimental forests will increase in the coming years as many experimental forest platforms have been established recently (Verheyen et al., 2016; Grossman et al., 2018; Paquette et al., 2018). Currently, though, published studies on soil fungal diversity are scarce. To the best of our knowledge, the only published studies on soil fungi conducted in tree biodiversity experiments are those by Nguyen et al. (2016b) and Tedersoo et al. (2016). Nguyen et al. (2016b) focused on relationships among tree and EcM fungal and saprotroph communities in young temperate-boreal forests in the Cloquet IDENT experiment (United States), which includes both American and European tree species. These were assembled in 12 monoculture and 4 six-species mixture plots (replicated four times in a block design, resulting in a total of 64 plots). They detected significant correlations between the beta-diversities of the trees and both the EcM and saprotroph soil fungal communities, but no significant correlations between fungal and tree alpha diversities. Tedersoo et al. (2016) found context-dependent tree diversity and species identity effects on soil fungi among tree experimental forest sites in Finland (the Satakunta experiment) and Estonia. Tree richness was positively correlated with soil fungal groups in Estonia and with EcM fungi in Finland. Communities of soil biota were generally driven by spatial eigenvectors in Finland and soil properties in Estonia. Furthermore, fungal richness was most strongly associated with herb cover and tree basal area.

Here, we extend these findings by reporting the results of a study of fungal communities in plots in a young large-scale subtropical experimental forest in China planted with 1, 2, 4, 8, or 16 native tree species included in a Biodiversity-Ecosystem Functioning experiment designated BEF-China

(Bruelheide et al., 2014). We assessed the contributions made by biotic (tree community variables), abiotic (soil properties and topography) and spatial information to the soil fungal richness and community patterns of the four main fungal functional groups: saprotrophic, pathogenic, AM and EcM fungi.

The interplay between plant-driven and abiotic processes and soil fungal communities is likely to vary in strength among fungal functional groups, since they differ in their degree of association with particular plant species. We hypothesized that AM and EcM fungi would show strong, but distinct, correlations with both plant community composition and abiotic soil properties. This is because both groups associate directly with plant roots and the soil matrix, but the ratio of host to symbiont diversity differs between the two types of mycorrhiza (Buscot, 2015). In contrast, necrotrophic parasites and saprotrophic fungi do not depend directly on living plants (Zeilinger et al., 2016), and we expected communities of these groups to be more strongly affected by abiotic soil properties than by the plant community.

MATERIALS AND METHODS

Sampling Site

The samples analyzed in this study were collected from plots hosting 1–16 native subtropical tree species at the BEF-China site (Bruelheide et al., 2014), which was established in 2009 on a hillside in Southeast China, Jiangxi Province (29°07′26.0″N 117°54′29.0″E). The climate there is subtropical with warm wet summers and cold dry winters. January and July are the coldest and warmest months, with mean temperatures of 0.4 and 34.2°C, respectively. The mean annual temperature is 17.4°C and mean annual rainfall amounts to 1635 mm (Yang et al., 2013). Before 2008, the site was in forestry use and hosted an approximately 20-year-old mixed planted stand of trees of the economically important timber species *Pinus massoniana* (EcM) and *Cunninghamia lanceolata* (AM), which were clear-cut directly before establishment of the experiment. Monoculture plots of both tree species were present in the BEF-China experimental plantation scheme but were not included in the sampling design of the present study. The planted forest plots are located in the hill altitudinal zone, spanning elevations from 105 to 275 m a.s.l. and varying considerably in inclination, with an average slope of 25° (Scholten et al., 2017). The soils are mainly Cambisols and Cambisol derivatives, falling into the reference soil groups Regosols, Cambisols, Acrisols, and Gleysols (IUSS Working Group WRB, 2014), with Cambisols and Regosols on ridges, spurs and crests, Cambisols and Acrisols along slopes and colluvic Cambisols and Gleysols occurring predominantly on foot slopes and in valleys (Scholten et al., 2017). Soil samples collected for this study had pH values (H_2O) of 4.1–5.6, total nitrogen contents of 0.08–0.31%, carbon to nitrogen ratios of 10–24, effective cation exchange capacities of 35–91 μmol g^{-1} soil and base saturation values of 6–92%.

A broken-stick design determined the experimental planting schemes of the 31 forest plots investigated here, i.e., the set of 16 native subtropical tree species was repeatedly sub-divided into subsets of eight, four, two, and one species to establish communities with lower diversity levels (**Supplementary Figure S1**). Each of the 16 tree species was represented once at each diversity level (monocultures, and mixtures of 2, 4, 8, and 16 tree species) and less diverse plots were nested within more diverse plots (Bruelheide et al., 2014). The total tree species pool has equal numbers of AM- and EcM-forming tree species, but lower diversity plots do not necessarily have equal proportions of AM and ECM trees (see **Supplementary Figure S1**). Each forest plot covers 25.8 m × 25.8 m, and tree species compositions were randomly assigned to plots. In each plot, 400 trees were planted at 1.29 m spacing, in a regular grid with assigned species being randomly allocated planting positions within the plot. We took samples for this study in October 2011 after the third growing season following planting. At this time the mean total tree height ranged from 52 to 301 cm depending on tree species (Li et al., 2014). Before sampling for our study, the herb layer was removed by weeding.

Soil Sampling

In October 2011, we randomly selected five trees per species in each plot (where possible) for sampling of soil in their root zones. Thus we planned to collect five replicate samples of soil in root zones of all 16 tree species at each diversity level (400 samples in total). However, tree mortality prevented collection of six samples in *Castanopsis eyrei* root zones (five in the four-tree species plot and one in the 16-tree species plot), resulting in a final number of 394 samples.

Soil samples were collected within 7 days. Loose stones and litter were removed from the soil surface and the upper 10 cm of the mineral soil was sampled, by removing four cores (6 cm in diameter and 10 cm deep) at points 20–30 cm from each selected tree trunk in cardinal compass directions using an auger. The four soil cores obtained from the root zone of each selected tree were mixed, sieved (2 mm mesh size) and homogenized to form a composite soil sample. Two 15 g subsamples were immediately flash-frozen in liquid nitrogen for molecular analysis. One was stored permanently at −80°C as a backup. The other was freeze-dried for 48 h and subsequently stored at −80°C in a vacuum-sealed plastic bag containing silica gel prior to transportation to the processing laboratory in Germany. Freeze-dried soil samples were transported by airplane within 4 days, following the recommendation of Weißbecker et al. (2017), and immediately stored at −80°C until required for molecular analysis.

Soil Chemical Analysis

Soil samples were air-dried, and a 50 g subsample of each sample was ground with a ball mill. The pH of sieved samples (<2 mm) resuspended in 25 ml double-distilled water was measured potentiometrically using a Sentix 81 electrode (WTW, Weilheim, Germany). Total organic carbon (C_{tot}) and total nitrogen (N_{tot}) contents of ground samples were measured using a Vario El III CN-analyzer (Elementar, Hanau, Germany). Because of the acidic soil conditions, no inorganic carbon was present, so C_{tot} represented the soil organic carbon content. The sieved soil samples were percolated with an unbuffered 1 M NH_4Cl solution and the effective cation exchange capacity

(CEC) of the extracts was measured with a DV 5300 inductively coupled plasma atomic emission spectrometer (PerkinElmer). Soil moisture content was assessed from water loss after freeze-drying.

Nucleic Acid Extraction and Multiplexed Amplicon Pyrosequencing

Microbial DNA was extracted with a PowerSoil® htp 96 Well Soil DNA Isolation Kit, RNA using a PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, United States). When using the first of these kits, 0.25 g samples of freeze-dried soil were extracted twice. When using the second kit, 1 g samples of freeze-dried soil were re-wetted with 1 ml clean water for 5 min before extraction. After RNA extraction, an RNA PowerSoil® DNA Elution Accessory Kit was used according to the manufacturer's instructions. A negative control with no soil was included in each batch of samples subjected to nucleic acid extraction. Initially, we intended to produce a cDNA dataset based on the extracted RNA as reported by, for example, Baldrian et al. (2012). However, we did not succeed in generating high quality sequences based on cDNA.

The nucleic acid extracts were quantified with a NanoDrop ND-8000 spectrophotometer (Peqlab, Germany). Fungal ITS rDNA amplicon libraries were generated using the fungal-specific ITS1F primer (Gardes and Bruns, 1993) containing Roche 454 pyrosequencing adaptor B, the universal primer ITS4 (White et al., 1990), Roche 454 pyrosequencing adaptor A and a sample-specific multiplex identifier sequence (MID). The ITS region has been proposed as an universal fungal barcode in metagenomic studies (Schoch et al., 2012) but it has also been reported to lack optimal resolving power for AM fungi (Stockinger et al., 2010). Nevertheless, Berruti et al. (2017) found similar patterns of diversity in AM fungal communities assessed by means of an ITS2 and an AM fungal specific 18S primer pair. The community-structuring effects of location and environment could be resolved correctly by the ITS2 targeting primers. Similar numbers of AM fungal operational taxonomic units (OTUs) have been found in Panamanian rainforest soils using fungal ITS and AM fungus-specific primers (Schappe et al., 2017). We amplified the ITS region sequences by PCR using 50 µl reaction mixtures containing 10 ng DNA template in 1 µl extraction buffer, 25 µl GoTaq Green Master Mix (Promega, Mannheim, Germany) and 1 µl of a solution providing 25 pmol of each of the ITS region-specific primers, as described by Wubet et al. (2012). All samples were amplified in triplicate. The PCR replicates were pooled, then purified using a gel extraction kit (Qiagen, Hilden, Germany). DNA concentrations of the purified amplicon products were measured using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Waldbronn, Germany). Equimolar pools of c. 60 sample amplicons were produced and processed according to instructions supplied with the GS FLX+ sequencing kit (Roche, Mannheim, Germany). The sequencing plate was divided into four lanes and one processed amplicon library pool was assigned to each lane. The amplicons were then sequenced by unidirectional pyrosequencing from the ITS4 ends using a Roche GS-FLX+ 454 pyrosequencer (Roche, Mannheim, Germany) at the Department of Soil Ecology, Helmholtz Centre of Environmental Research (UFZ, Halle, Germany).

Bioinformatics Analysis

Multiple levels of sequence processing and quality filtering were applied using an in-house metabarcode analysis pipeline for grid engines based mainly on the MOTHUR (version 1.39.5, Schloss et al., 2009) and OBITools (version 1.2.11, Boyer et al., 2016) software suites. Initially, sequences with any barcode mismatches or four primer mismatches were removed. All primer and barcode sequences were discarded. Sequences with any ambiguous bases or homopolymers exceeding eight nucleotides were removed. Flows were denoised and reads were trimmed, using FlowClus (Gaspar and Thomas, 2015), to uniform 360 bp long read fragments spanning the ITS2 and the 5.8S rRNA gene. Chimeric reads were detected and removed from each sample using the UCHIME algorithm as implemented in MOTHUR (Edgar et al., 2011). Dereplicated quality-filtered sequences were sorted by decreasing abundance and clustered into OTUs using the vsearch algorithm (version 2.4.4, Rognes et al., 2016) with a sequence similarity threshold of 97%. Representative (the most abundant) sequences for each OTU were taxonomically assigned based on reference sequences from the UNITE database (version v7_2, Kõljalg et al., 2013) using the naïve Bayesian classifier (Wang et al., 2007), as implemented in MOTHUR, at a consensus threshold of 60%. The sequences identified as fungal were further classified against the full version of the unite.v7_2 database augmented with non-fungal eukaryotic ITS sequences retrieved from GenBank to improve taxonomic annotation and detect non-target OTUs. In addition, taxonomic assignments of the first 2500 OTUs were manually refined by inspection of the first 15 INSDC database blast search results. Assignments with *E*-values smaller than eˆ-50 were assumed to be reliable and sequence similarity thresholds of 75, 80, 85, 90, and 95% were applied for class, order, family, genus and species classifications, respectively. Putative functions were annotated using the FUNGuild fungal database (version 1.1, Nguyen et al., 2016a). Functional annotations were further refined using information accessible through the APSnet search engine of the American Phytopathological Society[1] and the MycoBank database (Robert et al., 2013).

Statistical Analysis

Fungal OTUs with at least four sequence reads were included in the statistical analyses; singletons, doubletons and tripletons were discarded. Non-metric multidimensional scaling (NMDS) ordinations based on 30 random starts were calculated from the abundant fungal OTU dataset (containing at least four sequences) and the original dataset containing all OTUs. Procrustes correlation analysis conducted on both ordinations revealed that fungal community composition was not significantly affected by the presence or absence of rare fungal and potentially artificial OTUs (Procrustes correlation coefficient = 0.9987, $p < 0.001$). Procrustes analysis was carried out applying the protest function (Peres-Neto and Jackson, 2001) of the vegan package (Oksanen et al., 2013). Zhan et al. (2014) found OTUs generated

[1] http://www.apsnet.org

by pyrosequencing containing more than three sequences to be highly reproducible between sequence runs whereas the reproducibility of OTUs containing three (tripletons), two (doubletons) or one (singletons) sequence read(s) was drastically lower.

Statistical analyses were performed using R version 3.4.2 (R Core Team, 2014). The data matrices for taxonomic information, environmental variables measured and OTU abundances were merged using the phyloseq package (McMurdie and Holmes, 2013) to facilitate further splitting of the dataset into data pertaining to each of the fungal functional groups under consideration. For the individual fungal functional group analyses, samples were included in the statistical analysis only if an arbitrary read count of 20 (215 samples), 40 (178 samples), 210 (190 samples) and 250 (361 samples) was met for AM, pathogenic, EcM and saprotrophic fungi, respectively. These sequence thresholds correspond to approximately a tenth of the maximum read count for the respective functional groups in a sample. Due to missing tree community data, three samples had to be excluded from the statistical analysis.

We applied linear regression analyses to determine the contributions of tree community variables, soil properties, topography and spatial variables to fungal alpha and beta-diversity relationships. The tree species of the selected tree at each sampling position and its eight nearest neighbors were recorded. The tree community variables assessed included the following: tree species richness, Shannon and Simpson diversity indices of the trees, abundance and richness of EcM and AM trees, tree species identity, their mycorrhizal (ecto vs. arbuscular) type and tree community composition. The abiotic variables included the following soil properties: pH; total nitrogen (N_{tot}), total carbon (C_{tot}), and moisture contents; *C*:*N* ratio; effective cation exchange capacity (CEC); and base saturation (BS); all of these are important indicators of soil fertility (Lincoln et al., 2014; Scholten et al., 2017; Bünemann et al., 2018). In addition, two major topographical variables (altitude and slope) were taken into consideration because the experimental site is located on steep hills. The GPS coordinates of sampling locations were included in the statistical analysis as pairwise sampling distances or spatial eigenvectors.

All analyses were carried out at plot level. For all samples taken from the root zone of the same tree species in a plot, fungal read information was summed for richness analysis, and Hellinger-transformed fungal abundance counts were averaged. Due to the sequence thresholds applied, not all statistical sampling units contained the sequence information from five core replicates (per tree species per plot). While most samples were retained for the analysis of saprotrophic fungi, the number of core replicates was nearly evenly distributed from one to five sampling cores for the remaining fungal groups, AM, EcM and plant pathogenic fungi (**Supplementary Table S1**).

Fungal Alpha Diversity Analysis

Species richness information in the fungal count data was derived using the vegan package (Oksanen et al., 2013) and regressed against the square root of the number of reads it was based upon. The resulting fungal richness residuals were included in model calculations. This approach is an alternative method for sequence normalization which is applied to avoid severe loss of valid sequence information (McMurdie and Holmes, 2014; Tedersoo et al., 2014, 2016). We applied a forward model selection procedure to identify significant drivers of fungal alpha diversity. Soil properties, topographic, and tree richness variables (excluding Shannon and Simpson diversity indices) were transformed by the natural logarithm. Coordinates of sampling locations were transformed into principal coordinates of neighborhood matrices (PCNM, Legendre et al., 2009) using the vegan package (Oksanen et al., 2013). The resulting vectors were incorporated into mixed effect models with the variable forest plot identity as a random factor. Correlations of the selected variables were inspected by variance inflation analysis (vif) carried out with the fmsb package using a threshold of 10. We applied a forward model selection procedure with linear mixed-effect models using the lme4 package (Bates et al., 2015) based on the Akaike Information Criterion corrected for small sample sizes (MuMIn package, Barton, 2018). The significance of model parameters was assessed by linear mixed effect models using the nlme package (Pinheiro et al., 2017) followed by Analysis of Variance (ANOVA) type II tests implemented in the car package (Fox and Weisberg, 2011). Shapiro–Wilk tests were applied to confirm that model residuals met normal distribution assumptions. The VarCorr function of the lme4 package was applied to derive the extent to which fungal richness variation was attributable to the random factor forest plot. Explained variance was partitioned to fixed effect factors using the hier.part package (Walsh and MacNally, 2013). The conditional and marginal coefficients of determination for the mixed effect models were calculated using the MuMIn package (Barton, 2018). The marginal coefficients of determination represent the amount of variance explained by the fixed factors while the conditional coefficients of determination indicate the amount of variance explained by both fixed and random factors (Nakagawa et al., 2013).

Fungal Beta-Diversity Analysis

Beta-diversity values for the fungal and tree communities were calculated in terms of pairwise Bray–Curtis dissimilarities based on averaged (per tree species per plot) Hellinger-transformed abundance counts, representing percentage differences in community composition (Legendre and De Cáceres, 2013). Tree community abundance counts were based on the sampled trees and their eight neighboring tree individuals. Geographic distances between sampling locations, soil properties and topographic variables were standardized by natural logarithm transformation and averaged data were transformed to Euclidean distances.

To identify the environmental variables that explained the most fungal beta-diversity, multiple regressions of distance matrices were applied using the MRM function in the ecodist package (Goslee and Urban, 2007). The identity of forest plots was included as a fixed factor to account for the differences in pairwise sample comparisons within and between plots. Only variables showing significant correlation with fungal beta-diversity in partial Mantel tests after accounting for

variations in geographical distances between sampling locations were considered for multiple regression analysis. Furthermore, variables were excluded if they had a variance inflation factor (vif) greater than 10, calculated using the vif function in the fmsb package (Nakazawa, 2014). Best subset model selection was carried out to identify the parameters that best explained fungal community turnover. The complete list of best model subsets is shown in **Supplementary Tables S4–S7**. The percentages of explained variance contributed by the remaining variables were calculated using the varpart function in the vegan package (Oksanen et al., 2013).

RESULTS

Fungal Taxonomic Assignment

Pyrosequencing generated 1,155,299 raw sequences in total from the 394 soil samples collected. Clusters of least four of the 737,907 reads that passed the quality filtering were assigned to 5,665 fungal OTUs. Rarefaction curves for each fungal functional group investigated are available in **Supplementary Figure S2**. In total, 72, 56, and 49% of the fungal OTUs were classified at the order, family and genus levels, respectively.

We could assign 54% of the fungal OTUs to a functional group: 31% (1,768 OTUs), 7% (410 OTUs), 5% (320 OTUs), and 5% (310 OTUs) to saprotrophic, EcM, AM, and pathogenic fungi, respectively (**Supplementary Figure S3**). On the basis of the OTU numbers, saprotrophic fungi were predominantly Ascomycota (57%), followed by Basidiomycota (37%), Mucoromycota (5%) and Chytridiomycota (1%). Agaricales was the most diverse order of saprotrophic fungi, accounting for about 20% of the OTUs (**Supplementary Table S2**). Pathogenic fungi were predominantly plant pathogens (76%), followed by mycoparasites (11%) and animal parasites (11%). This group was strongly dominated by Ascomycota (87% of the OTUs), followed by Basidiomycota (10%) and Chytridiomycota (2%). The five most diverse orders of pathogenic fungi were the Capnodiales (26%), Pleosporales (14%), Hypocreales (12%), and Cantharellales (5%, **Supplementary Table S3**). AM fungi, which are monophyletic Glomeromycota, were clearly dominated by members of the order Glomerales (76% of the OTUs), followed by Archaeosporales (10%), Diversisporales (4%) and Paraglomerales (4%). EcM fungi were almost completely made up of Basidiomycota (86% of the OTUs) and Ascomycota (13%). The most diverse orders of these fungi were Agaricales (24%) and Thelephorales (22%).

Effects of Spatial, Soil Property and Tree Diversity Variables on Soil Fungal Alpha Diversity

Mean numbers of saprotrophic, pathogenic, AM and EcM fungal species per tree species within a forest plot were 78, 20, 14, and 9, respectively. The relative abundances of EcM fungal sequences were greater in the rooting zones of EcM-forming tree species than in those of AM-forming trees, especially in the tree monocultures (**Figure 2**). Linear regression analysis of fungal richness residuals and tree alpha diversity (Simpson indices) revealed no significant correlations (**Figure 3**). For the four fungal groups investigated, the final models selected by the forward selection procedure did not include any tree richness related variables (**Table 1**). In total, spatial variables and soil properties explained 75, 53, 46, and 44% of AM, saprotrophic, EcM and pathogenic fungal richness residual variance, respectively. Thus about 28–57% of the variation in fungal richness remained unexplained. Most of the explained variance of the fungal richness residuals was attributable to the PCNM spatial eigenvectors: 47, 27, 26, and 18% for AM, EcM, saprotrophic and pathogenic fungi, respectively. Of the soil properties tested, N_{tot} contributed significantly to the explained variation in saprotrophic fungal richness residuals (8%), soil water content to EcM fungal (16%) and soil water content, slope and effective cation exchange capacity to AM fungal richness residuals (25%). The residual richness of pathogenic fungi was correlated only with the spatial PCNM eigenvectors. The variable forest plot, which was included as a random factor in the linear mixed effect models, contributed strongly to the total amount of variance explained by the final models for saprotrophic (17%) and pathogenic fungi (13%). It did not affect the model strength for the mycorrhizal fungi richness residuals.

Effects of Differences in Spatial, Soil Property and Tree Community Variables on Soil Fungal Community Structure

Partial Mantel tests, after accounting for differences in geographic distances between samples, showed that at least one of the tree community variables investigated was significantly correlated with differences in pathogenic, AM and EcM fungal community structure (**Table 2**). The correlation between tree and saprotrophic community composition was close to the Bonferroni-corrected $\alpha = 0.05$ significance level. Differences in AM fungal and pathogen community structure were significantly correlated with tree community composition. EcM fungal community structure was significantly correlated with sample tree identity, sample tree mycorrhiza type (EcM vs. AM), EcM tree abundance and EcM tree richness.

Scatterplots of pairwise Bray–Curtis dissimilarities showing correlations between tree beta-diversity and that of the specific fungal groups differed visibly (**Figure 4**). Saprotrophic fungal communities showed the least community turnover of all fungal groups, partly overlapping throughout the study site as the dissimilarity value for all pairwise community comparisons was <1 (which would represent 100% community dissimilarity). Community turnover within forest plots was much smaller than that between forest plots. There was no detectable trend in saprotroph community turnover associated with tree community composition. AM and pathogen fungal communities showed less pronounced separation of within- and between-forest plot community comparisons, implying some correlation with tree community structure. Strongly differing fungal communities were detected in some comparisons of soil samples from plots with ≥30% differences in tree community composition. EcM fungi formed very distinct

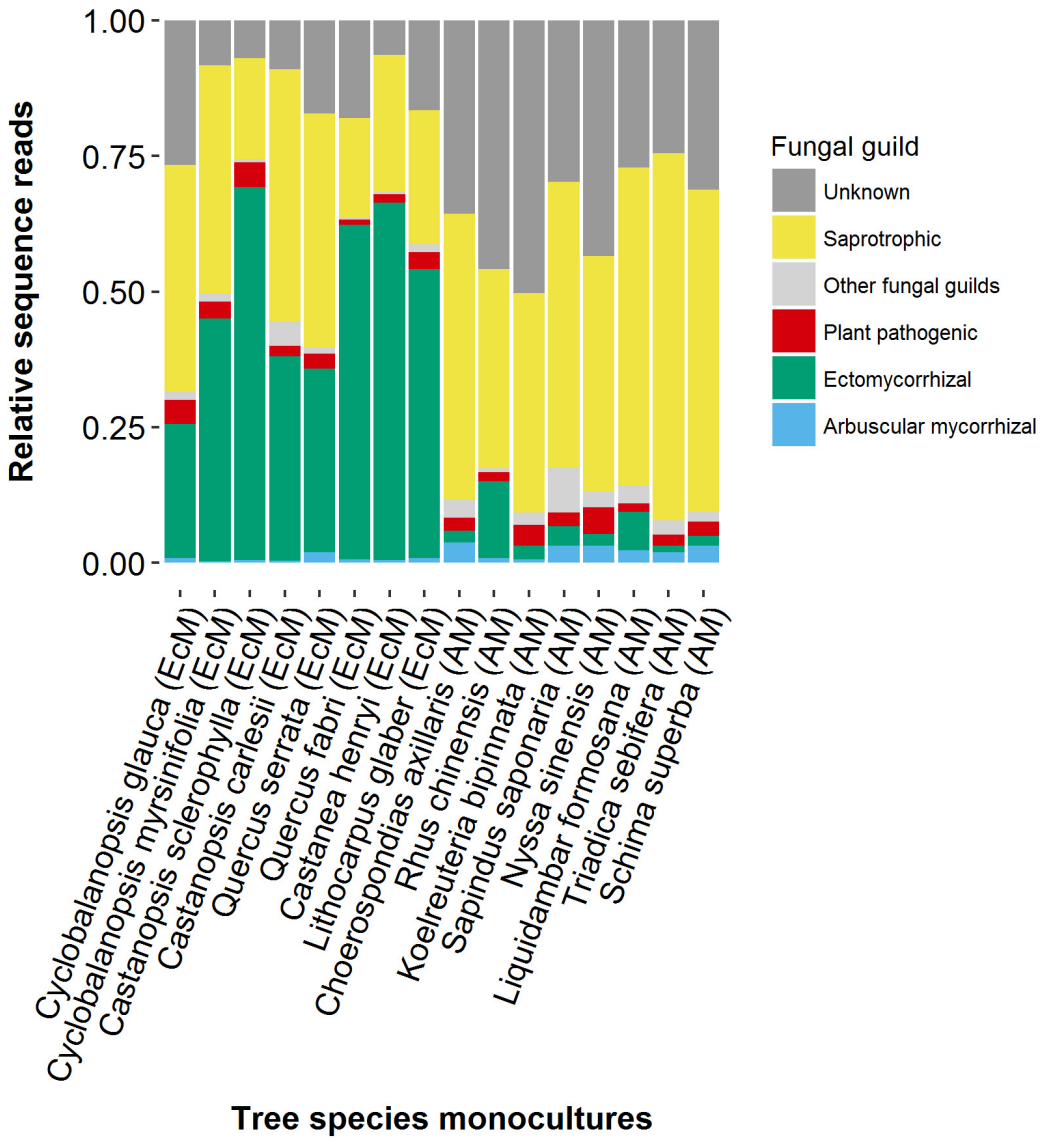

FIGURE 2 | Relative abundances of fungal functional groups in tree monocultures. The mycorrhizal type (EcM or AM) of each tree species is indicated in brackets. Atypically high numbers of EcM fungal sequences were found in two and one of the five replicates of *Liquidambar formosana* and *Sapindus saponaria* rooting zone soil samples, respectively (10-fold higher than in the other replicates). These numbers are not displayed in this graphic, but they were included in the statistical analyses.

communities and many pairwise sample comparisons showed no overlap of fungal species. EcM fungal communities showed the highest pairwise community dissimilarities of the four functional groups, with a mean Bray–Curtis dissimilarity of 0.89, followed by 0.78, 0.74, and 0.69 for AM, pathogenic and saprotrophic fungal communities, respectively. However, EcM fungal communities also showed the highest overlap of two sampled communities of a fungal functional group (approximately 80% EcM fungal community similarity). Tree communities with differences in composition as low as 13% had non-overlapping EcM fungal communities. There were no indications of any correlation between EcM fungal and tree community composition.

Following partial Mantel test analysis we selected the best model subsets for identifying the parameters that best explained differences in fungal community structure (**Table 3**). Abiotic soil properties explained the most variance in saprotrophic, pathogenic and AM community composition. The relevant abiotic variables for saprotroph community turnover were soil total carbon amount, carbon to nitrogen ratio, effective cation exchange capacity, base saturation and soil water content. Carbon to nitrogen ratio was also included in the final models of pathogenic and AM fungal community structure; in addition the latter model contained the abiotic soil properties pH and effective cation exchange capacity. The influence of tree community variables on fungal species turnover was

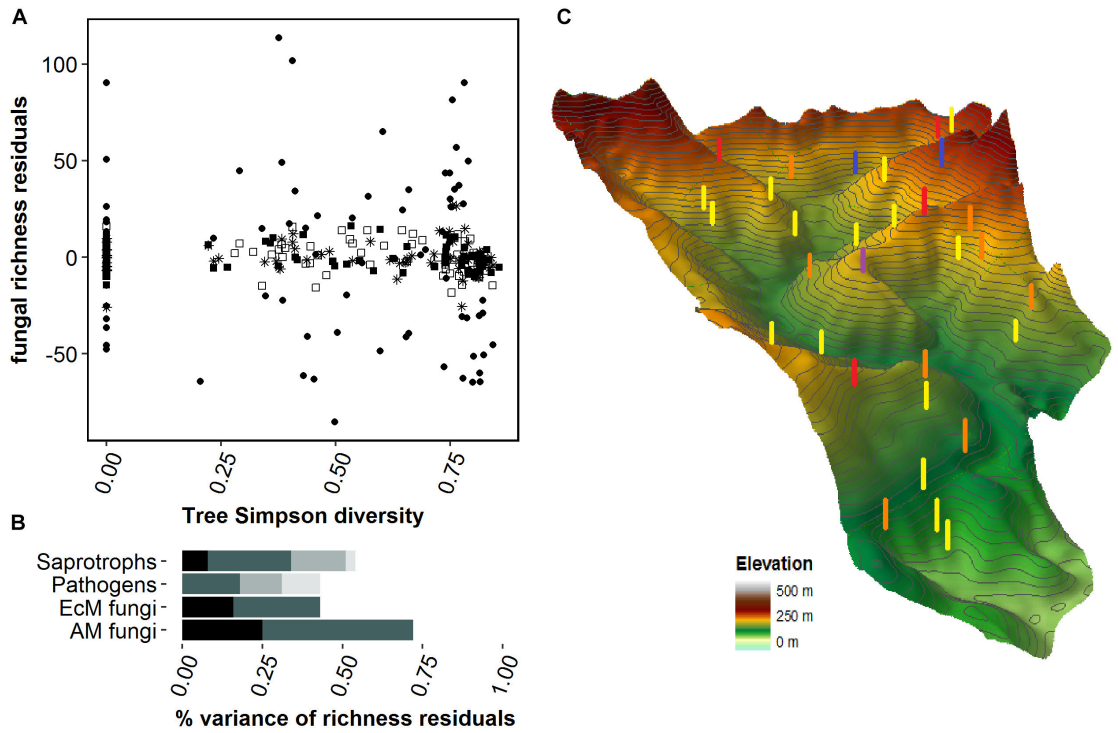

FIGURE 3 | Correlation of fungal richness residuals with tree diversity **(A)**. The scatterplots show residuals of saprotrophic (closed circles), pathogenic (star), ectomycorrhizal (filled squares) and arbuscular mycorrhizal (open squares) fungal richness. Bar plots **(B)** show contributions of the following abiotic and spatial variables to variations in fungal richness, in decreasing intensity of shading: soil chemistry, geographical principal component neighborhood matrix eigenvectors, forest plot and the joint contribution of the last two factors. The three-dimensional elevation map **(C)** of the study area highlights the locations of forest plots, with color coding indicating the diversity levels of monocultures (yellow), two-species mixtures (orange), four-species mixtures (red), eight-species mixtures (blue), and 16-species mixture (purple).

TABLE 1 | Final model explaining fungal richness residuals in correlations of saprotrophic, pathogenic, ectomycorrhizal and arbuscular mycorrhizal fungi as functions of spatial, environmental and tree diversity variables.

Saprotrophic fungi				Pathogenic fungi				Ectomycorrhizal fungi				Arbuscular mycorrhizal fungi			
	Chisq	**Df**	***P***		**Chisq**	**Df**	***P***		**Chisq**	**Df**	***P***		**Chisq**	**Df**	***P***
N_{tot}	6.2	1	0.013	**PCNM36**	10.7	1	0.00	**SWC**	19.7	1	0.000	**PCNM25**	9.2	1	0.002
PCNM8	8.5	1	0.004	**PCNM24**	5.4	1	0.02	**PCNM5**	9.7	1	0.002	**PCNM13**	18.8	1	0.000
PCNM21	6.4	1	0.011	**PCNM40**	5.2	1	0.02	**PCNM12**	6.2	1	0.012	**PCNM34**	4.8	1	0.029
PCNM3	6.0	1	0.014					**PCNM1**	6.0	1	0.014	**SWC**	30.9	1	0.000
								PCNM29	5.5	1	0.019	**PCNM3**	29.5	1	0.000
R^2_m	**0.36**			R^2_m	**0.19**			**PCNM14**	4.1	1	0.043	**PCNM4**	15.4	1	0.000
R^2_c	**0.53**			R^2_c	**0.44**							**SLOPE**	24.0	1	0.000
								R^2_m	**0.46**			**PCNM5**	10.0	1	0.002
								R^2_c	**0.46**			**PCNM9**	9.4	1	0.002
												CEC	7.1	1	0.008
												PCNM22	7.1	1	0.008
												PCNM35	4.6	1	0.031
												PCNM32	4.4	1	0.036
												R^2_m	**0.75**		
												R^2_c	**0.75**		

SWC, soil water content; CEC, effective cation exchange capacity; PCNM, Principal Component Neighborhood Matrices of geographical sampling locations. Linear mixed effect models include experimental forest plot as a random factor. Values reported are the marginal amounts of explained variance (R^2_m) attributed to the fixed variables only and the conditional amount of explained variance (R^2_c) attributed to the summed contributions of fixed and random factors (forest plot).

TABLE 2 | Partial Mantel correlations, after accounting for dissimilarities in geographic location, of fungal and environmental as well as tree community dissimilarities for the fungal functional groups indicated.

		Saprotrophic fungi		Pathogenic fungi		AM fungi		EcM fungi	
	Variable	***R***	***P***	***R***	***P***	***R***	***P***	***R***	***P***
(1)	Forest plot	0.21	0.0001	**0.16**	0.0001	**0.14**	0.0001	**0.16**	0.0001
(2)	Tree community composition	0.11	0.0024	**0.22**	0.0001	**0.15**	0.0003	0.07	0.0292
(3)	Tree species identity	0.01	0.1668	0.02	0.0738	0.03	0.0492	**0.13**	0.0001
(4)	Sample tree AM/EcM type	0.02	0.0677	0.00	0.306	0.03	0.0729	**0.32**	0.0001
(5)	Tree richness	0.00	0.8481	0.03	0.1732	0.01	0.3838	0.05	0.0901
(6)	Tree Shannon diversity	0.00	0.8736	0.03	0.1685	0.01	0.3561	0.05	0.0885
(7)	Tree Simpson diversity	0.00	0.8903	0.04	0.162	0.03	0.2673	0.03	0.2373
(8)	EcM tree abundance	0.00	0.5179	0.05	0.1991	0.00	0.7535	**0.21**	0.0004
(9)	AM tree abundance	0.00	0.5649	0.02	0.3418	0.08	0.0905	0.06	0.0863
(10)	EcM tree richness	0.00	0.7971	0.06	0.1652	0.00	0.9385	**0.19**	0.0003
(11)	AM tree richness	0.00	0.6219	0.00	0.4885	0.00	0.4695	0.00	0.1234
(12)	pH (H_20)	**0.38**	0.0001	0.20	0.0028	**0.41**	0.0001	0.12	0.0141
(13)	N_{tot}	0.13	0.0214	0.06	0.1714	0.08	0.1225	0.07	0.0979
(14)	C_{tot}	**0.22**	0.0001	**0.19**	0.0016	**0.26**	0.0004	0.09	0.0418
(15)	*C*:*N* ratio	**0.41**	0.0001	**0.31**	0.0001	**0.40**	0.0001	0.08	0.0497
(16)	BS	**0.35**	0.0001	0.19	0.0038	**0.35**	0.0001	0.04	0.2378
(17)	CEC	**0.23**	0.0001	0.07	0.0956	**0.26**	0.0002	0.11	0.0272
(18)	Soil water content	**0.18**	0.0005	0.18	0.0092	0.12	0.0368	0.09	0.0331
(19)	Altitude	0.00	0.96	0.00	0.8169	0.00	0.644	0.00	0.948
(20)	Slope	0.07	0.0798	0.00	0.4638	0.06	0.1671	0.00	0.6141

CEC, effective cation exchange capacity; BS, base saturation; N_{tot}, total nitrogen content; C_{tot}, total carbon content; C/N ratio, total carbon to total nitrogen ratio. Dissimilarity matrices of all the given variables were used in the partial Mantel test analysis. The significant alpha level (<0.05) was Bonferroni-corrected based on the number of variables tested ($\alpha < 0.0023$). Bolded values indicate variables that are significantly correlated.

strongly dependent on the fungal functional group investigated. Collectively, the tree-related variables that were found to be significant in the partial Mantel tests explained 11% of differences in EcM fungal community structure in the final MRM models. Although significant correlations were detected between tree community dissimilarity and both pathogenic and AM fungal community structure, the percentages of variance these correlations explained were very low: 5 and 2%, respectively. Spatial variables explained a large part of saprotroph and EcM fungal community variation but were only negligibly correlated with pathogenic and AM fungal community composition. Thus all of the three variable classes, biotic, abiotic and spatial variables, showed important correlations with soil fungal community structure, but the extent to which they did so greatly varied among the functional fungal groups. The best model subsets explained 54, 43, 26, and 23% of differences in saprotrophic, AM, EcM and pathogenic fungal community structure, respectively, leaving a large part of fungal community variation unexplained.

DISCUSSION

Soil fungi are a diverse (Blackwell, 2011; Taylor et al., 2014) and very heterogeneous group of organisms (Nguyen et al., 2016a). Our unprecedented study comprised the systematic analysis of four main fungal functional subgroups: saprotrophic, plant pathogenic, AM and EcM fungi. The subtropics constitute a zone of transition from temperate forests dominated by ectomycorrhizal symbiosis to tropical forests dominated by arbuscular mycorrhizal symbiosis. The subtropics thus harbor a high diversity of evergreen and deciduous tree species, and the number of AM and EcM forming trees occurring there is balanced, enabling the investigation of a broad range of fungal functional groups (Toju et al., 2014). Furthermore, the availability of a large-scale experimental assembly of native subtropical tree species enabled the quantification of the independent individual contributions made by tree community structure and soil properties and topography to soil fungal assembly. In contrast, tree community composition and richness (biotic variables) and soil properties (abiotic variables) are intermingled in naturally evolved forests. Our results provide evidence for a highly differentiated pattern in fungal-tree and fungal-environment (abiotic) relationships for all the fungal groups investigated. As expected, EcM fungal community assembly showed the strongest correlation with tree community variables, while saprotroph community assembly was driven only by abiotic spatial variables and soil properties. Against our expectations, AM fungal and tree community structure were significantly but weakly related. Fungal richness was not correlated with any of the tree community variables assessed. The strong influence of spatial variables and abiotic soil properties on fungal community assembly implies considerable early-stage environmental filtering and dispersal limitation.

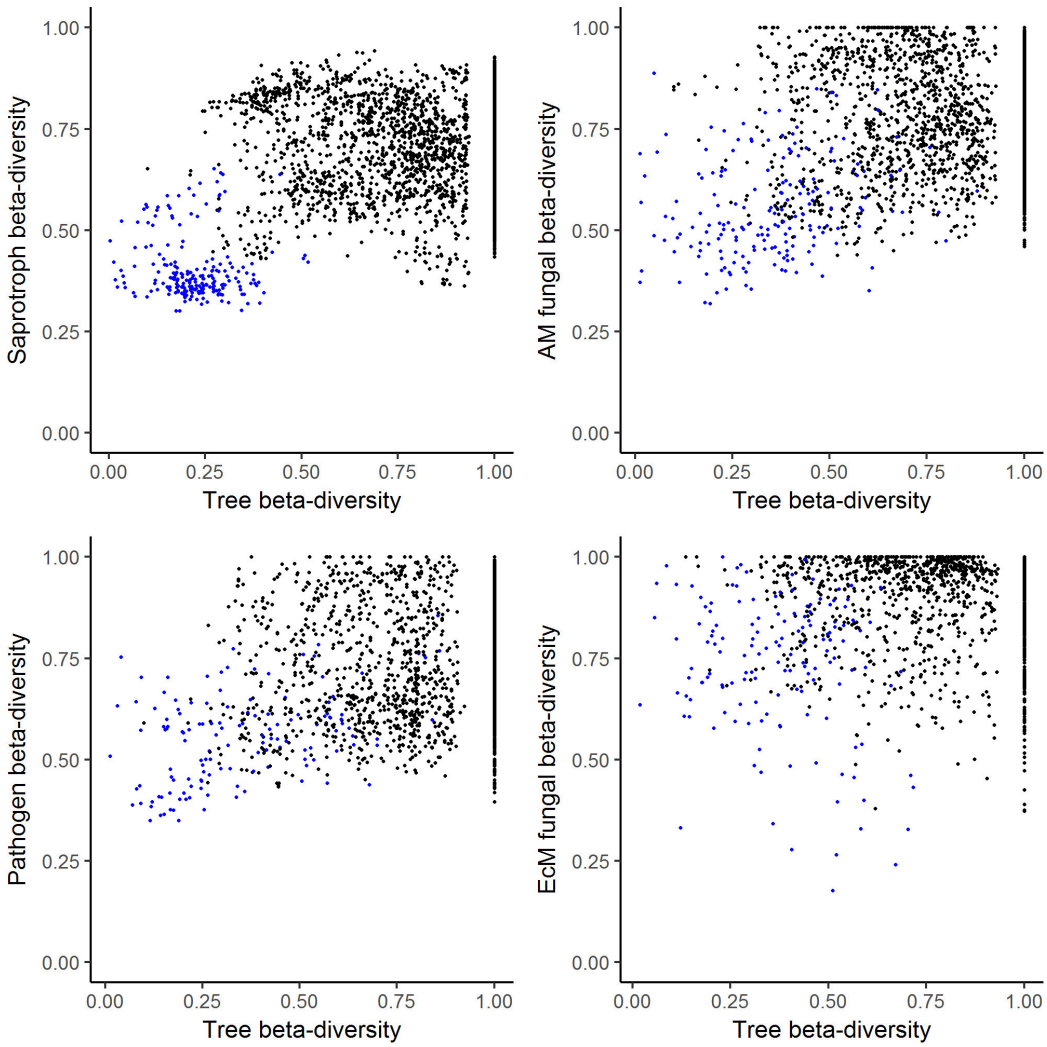

FIGURE 4 | Relationship between fungal and tree beta-diversity (expressed as pairwise Bray–Curtis dissimilarities). Black circles show results of across forest plot comparisons and blue circles results of within forest plot comparisons (between differing tree species).

Spatial Variables and Abiotic Soil Properties Contributed to Variation in Fungal Richness

It was predominantly spatial variables, but also soil properties, that influenced fungal richness. The strong spatial effects might result from variations in recent fungal spore inputs, and unknown legacy effects of the vegetation previously at the site may also contribute. Such effects would be extremely difficult to quantify. The experimental site was directly surrounded by forest plantations to the north, west and east. Inputs from these forests would depend on multiple factors, including their composition, maturity and climatic factors. Similarly, the two tree species that were dominant in the clear-cut forest plantation are known—*Cunninghamia lanceolata* (AM) and *Pinus massoniana* (EcM)—but they were randomly distributed and the exact previous positions of these species and other minor components of the previous stands were not recorded.

Abiotic soil properties moderately impacted the variation in fungal richness of saprotrophic (8%, total nitrogen content), EcM (16%, soil water content) and AM fungi (25%, soil water content, slope, effective cation exchange capacity). The significant effect of soil nitrogen on saprotroph richness could be explained by the major limitation of this soil resource in our forests, where plant-microbial competition for soil nitrogen was reported previously (Pei et al., 2016). AM fungi depend on carbon provided by their host plants. The link between AM fungal richness and the two abiotic variables slope and effective cation exchange capacity could be related to this dependency. At our experimental site, slope was one of the main predictors of soil fertility (Scholten et al., 2017) which might impact tree productivity, thereby influencing the amount of rhizodeposition by host plants. Cation exchange capacity can be attributed mainly to soil aluminum content and aluminum negatively affects tree height (Scholten et al., 2017). Aluminum stress has been reported

TABLE 3 | Best subsets of environmental dissimilarity models explaining fungal beta-diversity.

	Saprotrophic fungi		Pathogenic fungi		Arbuscular mycorrhizal fungi		Ectomycorrhizal fungi	
	Model	**Variance**	**Model**	**Variance**	**Model**	**Variance**	**Model**	**Variance**
	Plot location	16%	Location	2%	Location	3%	Plot location	14%
			Tree comp	5%	Tree comp	2%	Sample tree ID	11%
							Sample tree	
							AM/EcM type	
							EcM tree abundance*	
	C_{tot}	19%	CN	9%	pH	22%		
	CN				CN			
	CEC				CEC			
	BS							
	SWC							
R^2,P	**54%**	**0.0001**	**23%**	**0.0001**	**43%**	**0.0001**	**26%**	**0.0001**

*Environmental predictors are those that were retained in the best subsets of multiple regression models. All environmental variables and species information were transformed to dissimilarity matrices (see section "Materials and Methods"). CEC, effective cation exchange capacity; BS, base saturation; SWC, soil water content; Tree comp, tree community composition; variance, specific contributions of summarized soil variables, topography and tree composition to the total amount of variance. *EcM tree abundance could be replaced by the variable EcM tree richness, which showed very similar model performance.*

to hamper fine root growth and nutrient acquisition by trees (Marschner, 1991; Kinraide, 2008; de Wit et al., 2010). The detectable decrease in tree fitness due to metal toxicity might have led to fewer resources being translocated to the mycorrhizal fungal partner and fewer colonization sites due to negative impacts on root structure. None of the topographic variables and abiotic soil properties analyzed correlated with plant pathogen fungal richness. This could be due to the primary dependency of the pathogenic fungi on the living plant tissue. However, many pathogens spend their lifecycle partly as saprotrophs and thus a correlation with abiotic soil properties would have been expected (Kabbage et al., 2015).

None of the fungal groups analyzed, saprotrophs, pathogens, AM or EcM fungi, showed significant correlations between species richness and tree diversity. Similarly, no causal relationship of fungal richness and richness of fungal functional groups with plant diversity was found in a global study by Tedersoo et al. (2014). Only ectomycorrhizal fungal richness was globally correlated with the relative proportion and richness of EcM plants. In addition, in a regional study, Tedersoo et al. (2016) concluded that soil resources and tree species identity have greater effects on the diversity of soil biota than tree species richness *per se*. This is also supported by the observational study of Scheibe et al. (2015) conducted in German temperate broadleaved forests. Specific tree fungal richness relationships were found by Liang et al. (2015) and Nguyen et al. (2016b). Nguyen et al. (2016b) reported, from the American IDENT experimental site, a correlation between EcM fungal richness and plant phylogenetic diversity which was caused by the host specific EcM fungal species associated with gymno- and angiosperms. Several local and regional observational studies have reported strong tree species effects (Urbanova et al., 2015) and a correlation between plant and fungal richness (Gao et al., 2013; Martínez-García et al., 2015). Liang et al. (2015) found a negative relationship between AM fungi and tree diversity in subtropical restoration sites, which was attributed to a (presumably) higher carbon flux to the belowground compartment in less diverse and fast-growing forests compared to diverse but light-limited secondary forests.

We determined fungal richness based on the presence and absence of diagnostic sequences in DNA extracted from bulk soil samples. However, tree community effects might first be discernible in changes in fungal abundances, before fungal species disappear from the detectable soil DNA pool. Fungal species could be detectable for several years through DNA content extracted from inactive spores, dead mycelium and extracellular DNA (Levy-Booth et al., 2007; Nielsen et al., 2007) even when they are not actually living under present-day conditions. Furthermore, plants must be successfully colonized by fungi before differences in their fungal symbionts' efficiency can have any effect (Dickie et al., 2015), so relatively inefficient fungi may reside in habitats spanning fairly wide ranges of environmental conditions for considerable periods. In grassland experiments, time lags of several years were reported before changes in the plant community composition led to detectable changes in the composition of the soil microbial community (Eisenhauer et al., 2010). Thus the effects of tree species identity and tree species richness on fungal richness could still become detectable in future years of forest development. In comparison, the experimental forests in the study of Tedersoo et al. (2016) were well-grown with a closed canopy.

Differences in Tree Community Variables Significantly Affected Community Structure for Fungal Groups Other Than Saprotrophs

Consistent with their dominant influence on fungal richness, spatial variables and abiotic soil properties also had the strongest effects on fungal community assembly. These variables explained a large proportion of saprotroph beta-diversity without there being any effect of tree related variables. At the time of the study, tree saplings (including many evergreen tree species) provided only limited belowground carbon input through rhizodeposits

and leaf litter. As saprotrophic fungi depend on dead rather than living plant tissue, this community was likely sustained by the carbon stock residing in stumps and dead roots from the previous forest plantation. Several other studies have also revealed a strong influence of abiotic conditions on saprotroph and whole fungal communities (Wu et al., 2013; Prévost-Bouré et al., 2014; Tedersoo et al., 2014; Pei et al., 2016) while significant impacts of tree species community composition on saprotroph community structure have been found as well (Nguyen et al., 2016b; Schappe et al., 2017). This divergence in results regarding the impact of spatial, biotic and abiotic drivers on soil fungal community composition is also evident from studies focusing on the AM and EcM fungal subgroups (Öpik and Peay, 2016). Many AM fungi are distributed globally (Davison et al., 2015) with global AM fungal diversity (about 300 described to 3000 estimated species, Krüger et al., 2011; Buscot, 2015) being extremely low compared to that of AM host plants (several hundreds of thousands, Wang and Qiu, 2006). The AM fungi have therefore long been thought to be host generalists. Many studies report strong environmental filtering of AM fungal communities by soil properties such as pH (Dumbrell et al., 2010), distance and CN (Dumbrell et al., 2010; Davison et al., 2016), soil texture and soil moisture (Freitas et al., 2014), and temperature and soil P (Davison et al., 2016). The AM fungal communities in our young subtropical forests were strongly structured by abiotic (pH, CEC, and CN) and spatial variables. However, several studies found that host plant identity has effects on AM community assembly (Öpik et al., 2009; Wubet et al., 2009; Martínez-García et al., 2015) and it has been suggested that discrete regional and habitat specific fungal pools exist, indicating context dependent host specificity (Öpik and Peay, 2016). We found a significant but weak effect of tree community composition on AM fungal community structure. It should be noted that a rich herb layer, dominated in terms of biomass by ferns, developed at our experimental forest site in addition to the tree saplings planted there (Germany et al., 2017). In Southwest China, Zhang et al. (2004) found that the majority of the fern species they investigated were AM hosts. Substantial amounts of fern-associated AM fungi presumably persisted in the soil and could have impacted the tree-associated AM fungal community composition that we identified and potential relationships with tree variable effects.

The evolutionary development of EcM fungi from white and brown rot fungi took place convergently multiple times during the past 125 million years, reaching an EcM fungal species diversity approximately equal to that of EcM host plants (about 6000, Buscot, 2015). Thus EcM fungi have been assumed to be specific in nature (Öpik and Peay, 2016) and many studies, including our results, support a strong host effect (Ishida et al., 2007; Tedersoo et al., 2008, 2010; Ding et al., 2011). The linkage between tree and EcM communities, together with the high diversity of EcM fungi found, presumably reflects an early stage in the establishment of a complete EcM fungal community at our experimental site. However, the true host preference of EcM fungi may rely causally on the specific environmental conditions created by the host (Öpik and Peay, 2016), since strong environmental drivers of EcM community composition have been reported previously (Huang et al., 2014; Glassman et al., 2017; van der Linde et al., 2018), to the extent that EcM species can be indicators for key environmental variables (van der Linde et al., 2018). At our site, spatial and tree related variables structured EcM community composition while abiotic soil properties did not. Pathogenic fungal community composition was related to tree community structure, spatial and abiotic variables (CN content). The simultaneous lack of host effects (as indicated by a lack of correlation with differences in tree species identity) could indicate that local tree diversity and non-host neighboring tree species have played prominent roles. A similar pattern was found for biotrophic foliar pathogens in a young temperate experimental forest (Hantsch et al., 2014). Hantsch et al. (2014) concluded that particular non-host species (fast growing conifers in their study) in the vicinity of a target tree species (*Tilia cordata* and *Quercus petraea*) may impede or facilitate fungal pathogen infection depending on the identity of the species and its proportion in the local neighborhood.

A large proportion of the variation in fungal community structure and richness remained unexplained by the variables that we studied. There are numerous possible reasons for this finding (Bahram et al., 2015). Some significant effects may have been missed, because influential environmental variables were not measured. For example, Tedersoo et al. (2016) found herb cover and tree basal area to be strongly associated with fungal richness. These variables were not quantified within the framework of our study. In addition, manganese was present in high concentrations, and this has been reported to have a negative influence on tree height (Scholten et al., 2017) and potentially also to have a negative impact on EcM fungal diversity (Huang et al., 2014). However, stochastic processes may also have major effects on fungal community assembly (Powell et al., 2015; Bahram et al., 2016). Furthermore, our sequence-based data on fungal community composition may be insufficiently precise and representative, and this would certainly account for most of the unexplained variation.

CONCLUSION

We quantitatively assessed the independent contributions made by spatial, abiotic (soil properties and topography) and biotic (tree community structure) variables to soil fungal community structure in a study facilitated by the experimental set-up of the tree diversity forest plots that we investigated. Our results suggest that strong environmental filtering and dispersal limitation were the most important drivers of fungal community assembly in young subtropical forests. The influence of biotic tree community variables could already be detected in mycorrhizal and pathogen fungal groups. Due to the limited size of the tree saplings and thus of the carbon input to the ecosystem by rhizodeposits and leaf litter, we expect there to be increasingly strong tree related effects on fungal community composition as forest development proceeds. Despite focusing on an early stage of forest development, our study clearly indicates that different functional groups of soil fungi respond specifically to different soil and vegetation variables, and that these specific responses may be at either the species richness or the community

composition level. Ongoing studies on context-dependent community assembly of soil fungi should therefore take into account functional guilds within the fungi.

DATA AVAILABILITY

Relevant materials and protocols will be made available upon request. Datasets of the raw sequences generated for this study can be found in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/data/view/PRJEB12020) (Weißbecker et al., 2016). The bioinformatically processed sequence dataset and metadata can be found in the Zenodo repository (https://zenodo.org/record/1215505) (Weißbecker et al., 2018), as can the R scripts generated for the statistical analyses (https://zenodo.org/record/1401839) (Weißbecker and Wubet, 2018).

AUTHOR CONTRIBUTIONS

CW, FB, TW, and HB designed the study. CW performed the soil sampling and sample preparation. TS and PK provided soil measurements. GL, TW, and CW performed the bioinformatics analysis. CW and TW did the statistical analyses. CW, TW, FB, and HB outlined the manuscript, the first draft was written by CW. All authors contributed to revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02312/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Experimental evidence of functional group-dependent effects of tree diversity on soil fungi in subtropical forests

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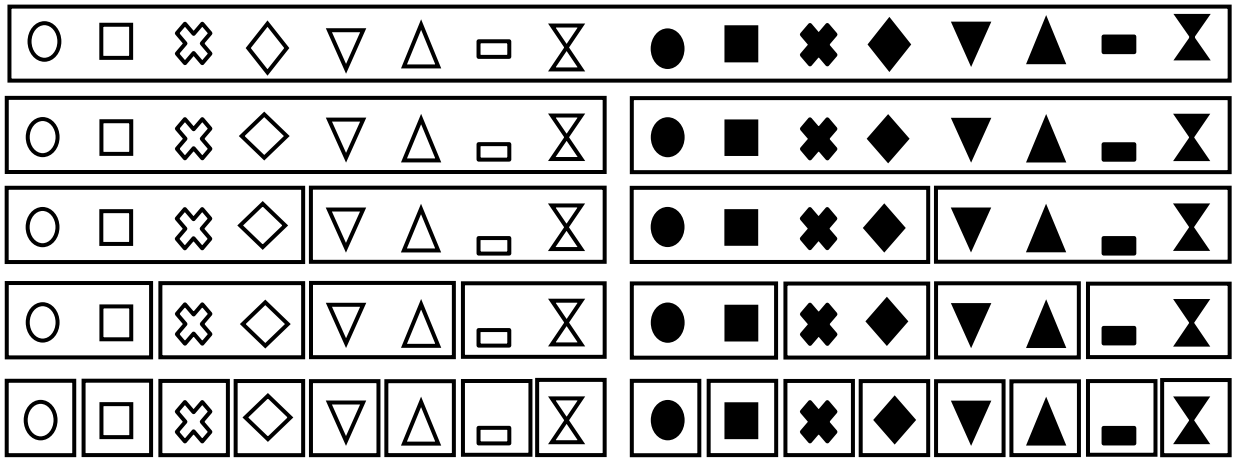
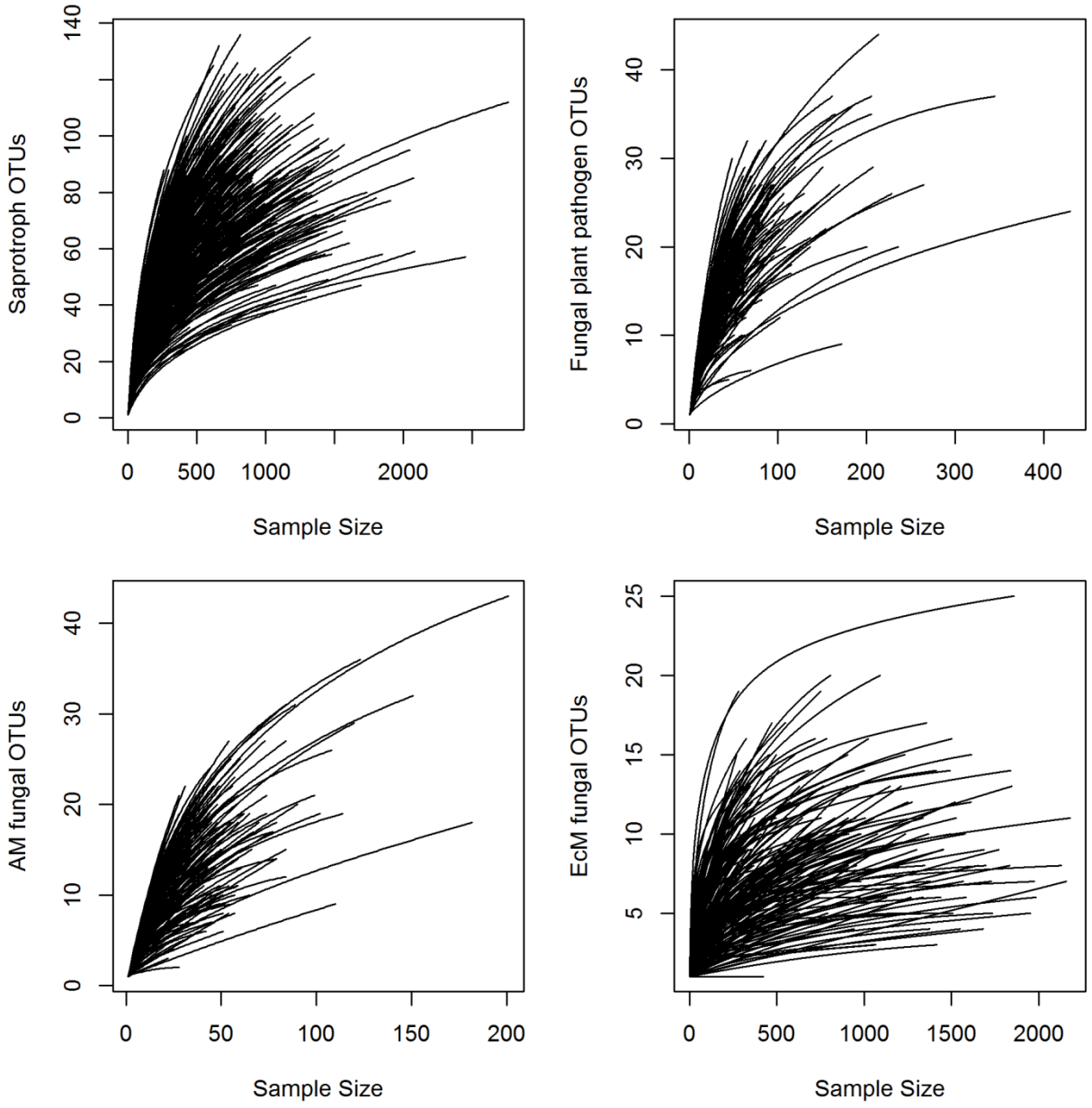
1.1 Supplementary Figures

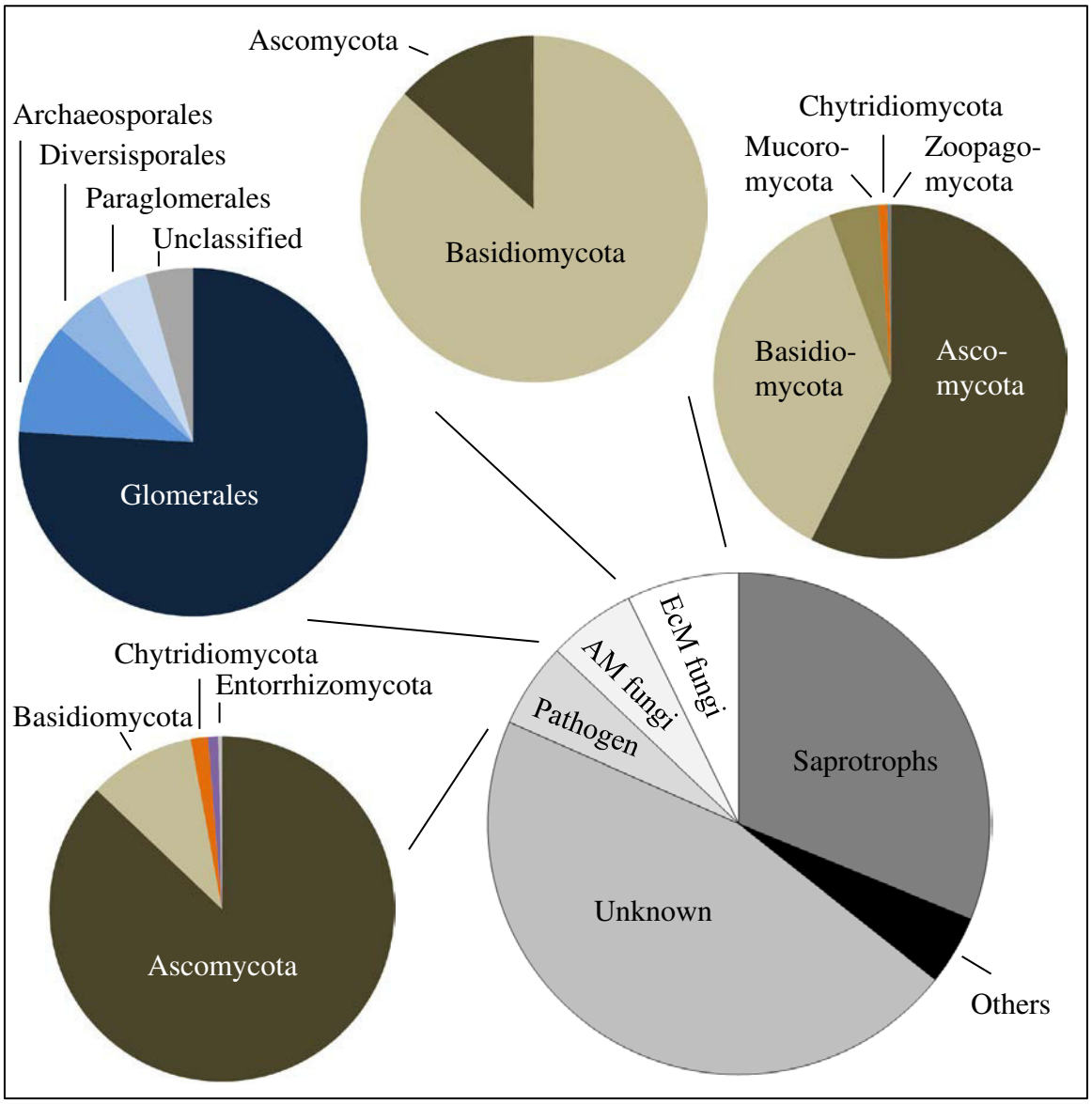


	Tree species	Type	No.		Tree species	Type	No.
○	*Castanea henryi* (Skan) Rehd. & Wils.	EcM	5 x 5	●	*Cyclobalanopsis glauca* (Thunb.) Oerst.	EcM	5 x 5
□	*Nyssa sinensis* Oliver	AM	5 x 5	■	*Quercus fabri* Hance	EcM	5 x 5
✕	*Liquidambar formosana* Hance	AM	5 x 5	✖	*Rhus chinensis* Mill.	AM	5 x 5
◇	*Sapindus saponaria* Linn.	AM	5 x 5	◆	*Schima superba* Gardner & Champion	AM	5 x 5
▽	*Choerospondias axillaris* (Roxb.) Burtt & Hill	AM	5 x 5	▼	*Castanopsis eyrei* (Champ.) Tutcher/ *C. carlesii* (Hemsl.) Hay.	EcM	5 x 5
△	*Triadica sebifera* (L.) Small	AM	5 x 5	▲	*Cyclobalanopsis myrsinifolia* (Blume) Oerst.	EcM	5 x 5
▭	*Quercus serrata* Murray	EcM	5 x 5	▬	*Lithocarpus glaber* (Thunb.) Nakai	AM	5 x 5
⧖	*Castanopsis sclerophylla* (Lindl.) Schott.	EcM	5 x 5	⧗	*Koelreuteria bipinnata* Franch.	AM	5 x 5
	Number of soil samples		**200**		**Number of soil samples**		**200**

Supplementary Figure 1. Broken-stick-design of the experimental forest plots. Plot design presented for the Biodiversity and Ecosystem Functioning (BEF) experiment China, study site A. The 16 species mix was sub divided in two times eight species mixtures. These were likewise partitioned in four, two and one tree species communities. Tree species are shown as symbols. Similarity of symbols was only chosen to emphasize the experimental design and does not imply any similarities or dissimilarities of tree species. No.: Number of samples.



Supplementary Figure 2. Rarefaction curves for the main fungal functional groups after sequence quality filtering and removal of singleton, doubleton and tripleton operational taxonomic units (OTUs).



Supplementary Figure 3. Taxonomic composition of the main fungal functional groups based on operational taxonomic unit (OTU) counts. Taxonomic composition of saprotrophic, pathogenic and ectomycorrhiza fungi is given by phyla while it is presented by orders for arbuscular mycorrhiza fungi.

1.2 Supplementary Tables

Supplementary Table 1. Frequencies of the actual number of soil samples replicates included in the statistical sampling units.

Number of replicates included in a statistical sampling unit	Number of sampling units containing the sequence information of the respective number of sample replicates			
	Saprotrophic fungi	**Plant pathogenic fungi**	**Ectomycorrhizal fungi**	**Arbuscular mycorrhizal fungi**
1	-	15	17	13
2	2	17	6	16
3	10	18	6	15
4	15	11	14	11
5	53	6	17	16

Supplementary Table 2. Taxonomic phyla and orders of saprotrophic soil fungi detected.

	Saprotrophic fungi	OTU count
1	Agaricales	378
2	Eurotiales	143
3	Hypocreales	139
4	Archaeorhizomycetales	127
5	Pleosporales	99
6	Helotiales	81
7	Tremellales	78
8	Mortierellales	75
9	Chaetothyriales	71
10	Sordariales	69
11	Xylariales	45
12	Trechisporales	40
13	Geminibasidiales	39
14	Orbiliales	26
15	Saccharomycetales	24
16	Venturiales	24
17	Auriculariales	24
18	Corticiales	21
19	Polyporales	20
20	Chaetosphaeriales	18
21	Coniochaetales	16
22	Geoglossales	14
23	GS31	14
24	Geastrales	14
25	Cantharellales	12
26	Capnodiales	11
27	Russulales	10
28	Umbelopsidales	10
29	Conioscyphales	9
30	Microascales	8
31	Spizellomycetales	8
32	Boletales	8
33	Dacrymycetales	7
34	Hymenochaetales	6
35	Kickxellales	6
36	Ophiostomatales	6
37	Pezizales	6

	Saprotrophic fungi	OTU count
38	Dothideales	5
39	Myrmecridiales	5
40	Ostropales	5
41	Phallales	4
42	Tritirachiales	4
43	Tubeufiales	4
44	Onygenales	4
45	Trichosporonales	4
46	Agaricostilbales	4
47	Boliniales	3
48	Sporidiobolales	3
49	Annulatascales	2
50	Atractiellales	2
51	Chytridiales	2
52	Filobasidiales	1
53	Hysteriales	1
54	Kriegeriales	1
55	Lichenostigmatales	1
56	Magnaporthales	1
57	Pleurotheciales	1
58	Pyrenulales	1
59	Rhizophydiales	1
60	Chytridiales	1
61	Filobasidiales	1

Ascomycota	1015
Basidiomycota	653
Mucoromycota	80
Chytridiomycota	14
Zoopagomycota	6

Supplementary Table 3. Taxonomic phyla and orders of pathogenic fungi, arbuscular mycorrhizal (AM) fungi and ectomycorrhizal fungi (EcM).

	Pathogenic fungi	OTU number
1	Capnodiales	80
2	Pleosporales	43
3	Hypocreales	36
4	NA	34
5	Cantharellales	16
6	Glomerellales	14
7	Xylariales	14
8	Helotiales	13
9	Magnaporthales	9
10	Diaporthales	8
11	Botryosphaeriales	7
12	Togniniales	6
13	Chaetothyriales	5
14	Polyporales	5
15	Entorrhizales	3
16	Platygloeales	3
17	Hymenochaetales	2
18	Phacidiales	2
19	Phaeomoniellales	2
20	Spizellomycetales	2
21	Coniochaetales	1
22	Ophiostomatales	1
23	Rhizophydiales	1
24	Rhytismatales	1
25	Ustilaginales	1
26	Venturiales	1
	Ascomycota	270
	Basidiomycota	31
	Chytridiomycota	5
	Entorrhizomycota	3

	AM Fungi	OTU numbers
1	Glomerales	243
2	Archaeosporales	33
3	Diversisporales	15
4	Paraglomerales	15
	Glomeromycota	320

	EcM Fungi	OTU numbers
1	Agaricales	98
2	Thelephorales	89
3	Sebacinales	61
4	Russulales	33
5	Cantharellales	31
6	Helotiales	23
7	Boletales	22
8	Corticiales	15
9	Chaetosphaeriales	9
10	Pezizales	7
11	Atheliales	4
12	Endogonales	1
13	Eurotiales	1
14	Hysterangiales	1
	Ascomycota	54
	Basidiomycota	355
	Mucoromycota	1

Supplementary Table 4. Best subsets model selection for saprotrophic fungal community composition. The lower gray marked model constitutes the one presented in the manuscript. It was chosen as all model variables were significant and for the number of included variables it showed the highest F value. The upper model was marked as there is additionally an increase in explained variance (R^2) of at least 5% for the addition of one variable compared to the best model subset with one variable less. No.: Running number of the model, V1: number of variables included in the model, Int: Intercept, Ctot: total carbon content, CN: carbon to nitrogen ratio, CEC: effective cation exchange capacity, BS: base saturation, SWC: soil water content.

No	V1	F	F.pval	R2	pval	Int	Plot	location	pH	Ctot	CN	CEC	BS	SWC
1	1	1556	1.00E-04	0.33	1.00E-04	1	1.00E-04	NA	NA	NA	NA	NA	NA	NA
2	1	1478	1.00E-04	0.32	1.00E-04	1	NA	1.00E-04	NA	NA	NA	NA	NA	NA
3	1	883	1.00E-04	0.22	1.00E-04	0.882	NA	NA	NA	NA	1.00E-04	NA	NA	NA
4	2	1258	1.00E-04	0.44	1.00E-04	1	1.00E-04	NA	NA	NA	1.00E-04	NA	NA	NA
5	2	1219	1.00E-04	0.44	1.00E-04	1	NA	1.00E-04	NA	NA	1.00E-04	NA	NA	NA
6	2	1212	1.00E-04	0.43	1.00E-04	1	1.00E-04	NA	1.00E-04	NA	NA	NA	NA	NA
7	3	1043	1.00E-04	**0.50**	1.00E-04	1	1.00E-04	NA	NA	NA	1.00E-04	NA	1.00E-04	NA
8	3	1003	1.00E-04	0.49	1.00E-04	1	1.00E-04	NA	1.00E-04	NA	1.00E-04	NA	NA	NA
9	3	984	1.00E-04	0.48	1.00E-04	1	NA	1.00E-04	NA	NA	1.00E-04	NA	1.00E-04	NA
10	4	838	1.00E-04	0.52	1.00E-04	1	1.00E-04	NA	NA	NA	1.00E-04	0.0011	1.00E-04	NA
11	4	832	1.00E-04	0.51	1.00E-04	1	1.00E-04	NA	NA	6.00E-04	1.00E-04	NA	1.00E-04	NA
12	4	816	1.00E-04	0.51	1.00E-04	1	1.00E-04	NA	1.00E-04	NA	1.00E-04	3.00E-04	NA	NA
13	5	690	1.00E-04	0.52	1.00E-04	1	1.00E-04	0.0011	NA	NA	1.00E-04	7.00E-04	1.00E-04	NA
14	5	690	1.00E-04	0.52	1.00E-04	1	1.00E-04	NA	NA	3.00E-04	1.00E-04	NA	1.00E-04	0.0054
15	5	687	1.00E-04	0.52	1.00E-04	1	1.00E-04	NA	NA	NA	1.00E-04	0.0014	1.00E-04	0.0242
16	6	598	1.00E-04	0.53	1.00E-04	1	1.00E-04	2.00E-04	NA	7.00E-04	1.00E-04	NA	1.00E-04	0.0019
17	6	594	1.00E-04	0.53	1.00E-04	1	1.00E-04	3.00E-04	NA	NA	1.00E-04	0.002	1.00E-04	0.0063
18	6	587	1.00E-04	0.53	1.00E-04	1	1.00E-04	NA	NA	0.0129	1.00E-04	0.0324	1.00E-04	0.0123
19	7	522	1.00E-04	**0.54**	1.00E-04	1	1.00E-04	2.00E-04	NA	0.0149	1.00E-04	0.0477	1.00E-04	0.0027
20	7	516	1.00E-04	0.53	1.00E-04	1	1.00E-04	2.00E-04	0.203	3.00E-04	1.00E-04	NA	0.0014	0.0023
21	7	513	1.00E-04	0.53	1.00E-04	1	1.00E-04	3.00E-04	0.1494	NA	1.00E-04	0.0019	0.0083	0.0087
22	8	460	1.00E-04	0.54	1.00E-04	1	1.00E-04	5.00E-04	0.1501	0.0147	1.00E-04	0.0397	0.0039	0.0037

Supplementary Table 5. Best subsets model selection for fungal plant pathogen community composition. The lower gray marked model constitutes the one presented in the manuscript. It was chosen as all model variables were significant and for the number of variables included it showed the highest F value. The upper model is marked as there was also an increase in explained variance (R^2) of at least 5% for the addition of one variable compared to the best model subset with one variable less. No.: Running number of the model, V1: number of variables included in the model, Int: Intercept, Ctot: total carbon content, CN: carbon to nitrogen ratio, CEC: effective cation exchange capacity, BS: base saturation, SWC: soil water content.

No	V1	F	F.pval	R2	pval	Int	Plot	location	Tree	Ctot	CN
1	1	308.06	1.00E-04	0.12	1.00E-04	0.9999	NA	NA	NA	NA	1.00E-04
2	1	265.93	1.00E-04	0.11	1.00E-04	1	1.00E-04	NA	NA	NA	NA
3	1	232.92	1.00E-04	0.10	1.00E-04	1	NA	NA	1.00E-04	NA	NA
4	2	291.93	1.00E-04	**0.21**	1.00E-04	1	NA	NA	1.00E-04	NA	1.00E-04
5	2	255.09	1.00E-04	0.19	1.00E-04	1	1.00E-04	NA	NA	NA	1.00E-04
6	2	236.75	1.00E-04	0.18	1.00E-04	1	NA	1.00E-04	NA	NA	1.00E-04
7	3	215.38	1.00E-04	**0.23**	1.00E-04	1	NA	3.00E-04	1.00E-04	NA	1.00E-04
8	3	213.20	1.00E-04	0.22	1.00E-04	1	1.00E-04	NA	1.00E-04	NA	1.00E-04
9	3	201.29	1.00E-04	0.21	1.00E-04	1	NA	NA	1.00E-04	0.1423	1.00E-04
10	4	165.61	1.00E-04	0.23	1.00E-04	1	NA	4.00E-04	1.00E-04	0.1844	1.00E-04
11	4	164.74	1.00E-04	0.23	1.00E-04	1	1.00E-04	NA	1.00E-04	0.1515	1.00E-04
12	4	162.57	1.00E-04	0.23	1.00E-04	1	0.3434	0.1789	1.00E-04	NA	1.00E-04
13	5	133.51	1.00E-04	0.23	1.00E-04	1	0.2849	0.2235	1.00E-04	0.1834	1.00E-04

Supplementary Table 6. Best subsets model selection for ectomycorrhizal fungi community composition. The lower gray marked model constitutes the one presented in the manuscript. It was chosen as all model variables were significant and for the number of variables included it showed the highest F value. The upper model is marked as there was also an increase in explained variance (R^2) of at least 5% for the addition of one variable compared to the best model subset with one variable less. No.: Running number of the model, V1: number of variables included in the model, Int: Intercept, Ctot: total carbon content, CN: carbon to nitrogen ratio, CEC: effective cation exchange capacity, BS: base saturation, SWC: soil water content.

No	V1	F	F.pval	R2	pval	Int	Plot	location	Sample_Tree	Tree Myco	EcM_ab	EcM_richness
1	1	290.94	1.00E-04	0.14	1.00E-04	1	1.00E-04	NA	NA	NA	NA	NA
2	1	245.56	1.00E-04	0.12	1.00E-04	1	NA	1.00E-04	NA	NA	NA	NA
3	1	171.37	1.00E-04	0.09	1.00E-04	0.1066	NA	NA	NA	1.00E-04	NA	NA
4	2	275.54	1.00E-04	**0.24**	1.00E-04	1	1.00E-04	NA	NA	1.00E-04	NA	NA
5	2	247.28	1.00E-04	0.22	1.00E-04	1	NA	1.00E-04	NA	1.00E-04	NA	NA
6	2	181.87	1.00E-04	0.17	1.00E-04	1	1.00E-04	NA	NA	NA	0.0013	NA
7	3	194.15	1.00E-04	0.25	1.00E-04	1	1.00E-04	NA	NA	1.00E-04	0.0452	NA
8	3	193.50	1.00E-04	0.25	1.00E-04	1	1.00E-04	NA	NA	1.00E-04	NA	0.016
9	3	187.17	1.00E-04	0.24	1.00E-04	1	1.00E-04	NA	0.003	1.00E-04	NA	NA
10	4	148.76	1.00E-04	0.25	1.00E-04	1	1.00E-04	0.0452	NA	1.00E-04	0.03	NA
11	4	148.39	1.00E-04	0.25	1.00E-04	1	1.00E-04	NA	0.0022	1.00E-04	0.0392	NA
12	4	148.32	1.00E-04	0.25	1.00E-04	1	3.00E-04	0.0454	NA	1.00E-04	NA	0.0112
13	5	**121.32**	1.00E-04	**0.26**	1.00E-04	1	1.00E-04	0.0405	0.0024	1.00E-04	0.0285	NA
14	5	**120.98**	1.00E-04	**0.26**	1.00E-04	1	3.00E-04	0.0406	0.0024	1.00E-04	NA	0.0092
15	5	120.75	1.00E-04	0.25	1.00E-04	1	2.00E-04	0.0313	NA	1.00E-04	0.1838	0.1034
16	6	102.60	1.00E-04	0.26	1.00E-04	1	1.00E-04	0.039	0.0019	1.00E-04	0.1752	0.0985

Supplementary Table 7. Best subsets model selection for arbuscular mycorrhizal fungal community composition. The lower gray marked model constitutes the one presented in the manuscript. It was chosen as all model variables were significant and for the number of variables included it showed the highest F value. The upper model is marked as there was also an increase in explained variance (R^2) of at least 5% for the addition of one variable compared to the best model subset with one variable less. No.: Running number of the model, V1: number of variables included in the model, Int: Intercept, Ctot: total carbon content, CN: carbon to nitrogen ratio, CEC: effective cation exchange capacity, BS: base saturation, SWC: soil water content.

No	V1	F	F.pval	R2	pval	Int	Plot	location	Tree	pH	Ctot	CN	CEC	BS
1	1	705.65	1.00E-04	0.22	1.00E-04	0.3903	NA	NA	NA	1.00E-04	NA	NA	NA	NA
2	1	647.55	1.00E-04	0.21	1.00E-04	0.8591	NA	NA	NA	NA	NA	1.00E-04	NA	NA
3	1	588.33	1.00E-04	0.19	1.00E-04	1	NA	1.00E-04	NA	NA	NA	NA	NA	NA
4	2	607.70	1.00E-04	0.33	1.00E-04	1	NA	1.00E-04	NA	1.00E-04	NA	NA	NA	NA
5	2	605.46	1.00E-04	0.33	1.00E-04	1	1.00E-04	NA	NA	1.00E-04	NA	NA	NA	NA
6	2	585.57	1.00E-04	0.32	1.00E-04	1	NA	1.00E-04	NA	NA	NA	1.00E-04	NA	NA
7	3	510.16	1.00E-04	0.38	1.00E-04	1	NA	1.00E-04	NA	2.00E-04	NA	1.00E-04	NA	NA
8	3	509.51	1.00E-04	0.38	1.00E-04	1	1.00E-04	NA	NA	NA	NA	1.00E-04	NA	1.00E-04
9	3	508.00	1.00E-04	0.38	1.00E-04	1	NA	1.00E-04	NA	NA	NA	1.00E-04	NA	1.00E-04
10	4	436.50	1.00E-04	0.41	1.00E-04	1	NA	1.00E-04	NA	2.00E-04	NA	2.00E-04	0.0011	NA
11	4	433.89	1.00E-04	0.41	1.00E-04	1	1.00E-04	NA	NA	NA	NA	1.00E-04	0.0015	1.00E-04
12	4	433.53	1.00E-04	0.41	1.00E-04	1	1.00E-04	NA	NA	1.00E-04	NA	2.00E-04	7.00E-04	NA
13	5	379.32	1.00E-04	**0.43**	1.00E-04	1	NA	1.00E-04	1.00E-04	1.00E-04	NA	2.00E-04	0.0015	NA
14	5	371.93	1.00E-04	0.43	1.00E-04	1	NA	1.00E-04	2.00E-04	NA	NA	1.00E-04	0.0014	3.00E-04
15	5	368.84	1.00E-04	0.43	1.00E-04	1	1.00E-04	NA	1.00E-04	2.00E-04	NA	1.00E-04	8.00E-04	NA
16	6	320.02	1.00E-04	0.44	1.00E-04	1	NA	1.00E-04	1.00E-04	0.0421	NA	1.00E-04	0.0012	0.172
17	6	319.34	1.00E-04	0.44	1.00E-04	1	NA	1.00E-04	1.00E-04	1.00E-04	0.2156	1.00E-04	0.0054	NA
18	6	316.94	1.00E-04	0.43	1.00E-04	1	0.3477	0.0054	2.00E-04	1.00E-04	NA	2.00E-04	0.0016	NA
19	7	277.21	1.00E-04	0.44	1.00E-04	1	NA	1.00E-04	1.00E-04	0.0325	0.2133	1.00E-04	0.0061	0.1659
20	7	275.34	1.00E-04	0.44	1.00E-04	1	0.2736	0.0066	1.00E-04	0.048	NA	1.00E-04	0.0018	0.1558
21	7	274.40	1.00E-04	0.44	1.00E-04	1	0.3712	0.0029	1.00E-04	1.00E-04	0.2293	1.00E-04	0.0044	NA
22	8	243.43	1.00E-04	0.44	1.00E-04	1	0.282	0.0063	1.00E-04	0.0392	0.2152	1.00E-04	0.0047	0.146

CHAPTER 3

Linking soil fungal generality to biodiversity in young subtropical Chinese forests

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Microorganisms,

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Linking soil fungal generality to tree richness in young subtropical Chinese forests

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Abstract: Soil fungi are a highly diverse group of microorganisms that provide many ecosystem services. The mechanisms of soil fungal community assembly must therefore be understood to reliably predict how global changes such as climate warming and biodiversity loss will affect ecosystem functioning . To this end, we assessed fungal communities in experimental subtropical forests by pyrosequencing of the ITS2 region, and constructed tree-fungal bipartite networks based on the co-occurrence of fungal OTUs and tree species. The characteristics of the networks and the observed degree of fungal specialization were then analyzed in relation to the level of tree species diversity. Unexpectedly, plots containing two tree species had higher *network connectance* and *fungal generality* values than those with higher tree diversity. Most of the frequent fungal OTUs were saprotrophs. The degree of fungal specialization were highest in tree monocultures. Ectomycorrhizal fungi had higher specialization coefficients than saprotrophic, arbuscular mycorrhizal, and plant pathogenic fungi. High tree species diversity plots with 4 to 16 different tree species sustained the greatest number of fungal species, which is assumed to be beneficial for ecosystem services because it leads to more effective resource exploitation and greater resilience due to functional redundancy.

Keywords: bipartite network; diversity; fungal community assembly, soil, specialization, subtropics

1. Introduction

Soil fungi are a highly diverse group of microorganisms [1,2] that are crucial for soil health [3] and provide many ecosystem services including decomposition, element cycling, plant nutrition, and plant protection [4]. The mechanisms of soil fungal community assembly must therefore be understood to reliably predict how global changes such as climate warming and biodiversity loss will affect ecosystem functionality. Fungal community assembly is influenced by abiotic, biotic and stochastic factors. Key drivers of fungal community composition and richness include soil moisture [5], soil nutrient content [6,7], precipitation [8], and vegetation [9,10]. Tree species loss is a likely consequence of global change, so it is important to determine how such losses could affect soil fungal communities. We have previously characterized the effects of tree diversity on specific functional groups of soil fungi in subtropical young forests [11]. Here, we extend this analysis by investigating the effects of tree diversity on fungal specialization and tree-fungal network patterns.

Tree species are known to strongly affect ecosystem conditions including soil properties [12-14] and microclimate [15,16]. Therefore, regions of high tree diversity have less homogeneous soil and environmental conditions than those with tree monocultures. Additionally, the local conditions in regions of high tree diversity depend strongly on the tree species that are present. The neighborhood conditions of a tree can also result in niche shifts. For example, niche differentiation based on crown height was observed in communities with high tree diversity [17,18]. Similarly, fine root niche complementarity [19] was shown to increase resource capture in mixed stands [20,21], and tree species richness was found to correlate positively with the filling of the soil volume by fine roots [19].

The performance of species under different environmental conditions can differ strongly [22]. Some species can cope with a broad range of environmental conditions and thus occur frequently in many different habitats. Other species, known as specialists, only perform well in a narrow range of environmental conditions. Therefore, it is assumed that well-adapted specialists will outperform generalists in homogenous environments, while the reverse will be true in heterogeneous environments. In molecular soil fungal ecology, the abundance of a fungal taxon can be regarded as a proxy for its performance because it is assumed that well-performing species will be more able to proliferate and will thus have a greater chance of being detected.

Network analysis is a technique that originated in the social sciences but has been widely used in community macroecology, for instance to characterize pollinator-plant or predator-prey interaction networks. The advent of molecular high throughput sequencing technologies has enabled this technique to also be used in microbiology to clarify the mechanisms that structure fungal communities in a way that complements descriptive investigations based on alpha and beta diversity relationships [23-25]. Network analysis can be used to assess the ecological interactions between functionally different partners and to deduce their ecosystem-level consequences in a more integrated manner than is possible by intraspecific investigation. Network analyses inherently account for the fact that all components of an ecosystem are interconnected [26]. Consequently, ecological network analyses are increasingly being used to evaluate the effects of environmental change on ecosystems [27,28]. For example, Tylianakis, *et al.* [29] found that anthropogenic habitat modification did not affect species richness but significantly influenced the network structure of bees, wasps, and their parasitoids, affecting parasitism rates and thus pollination. Plant-fungal networks have been analyzed to support or better understand disease management [30], ecosystem development [25], succession and seasonality [31], latitudinal gradients [32], and host preferences [33-37]. However, to our knowledge, this work is the first to examine the effects of tree species diversity on tree-soil fungal network structure and soil fungal specialization, and the likely consequences of global tree species loss. The data analyzed here were derived from the biodiversity and ecosystem functioning experiment China (BEF China) [38,39], which features plots having 1, 2, 4, 8, and 16 different tree species.

We performed a tree-fungal bipartite network analysis using a subsampling approach and evaluated the network metrics specified below in relation to three tree diversity levels. Additionally, we analyzed the specialization of fungal OTUs based on the phi coefficient [40] and assessed differences in the specialization of specific fungal functional groups. Our analysis is based on several network structure metrics, including the main metrics of *nestedness*, *modularity*, and *connectance*, as well as *generality* – a measure of network asymmetry. The latter metric was included because studies on consumer-prey networks have shown that environmental change can affect consumer-prey asymmetries without strongly affecting other network metrics [27]. The fungal *C score* was also computed to deduce possible mechanisms of fungal community assembly [41].

Nestedness measures the extent to which specialist species of higher trophic levels (e.g. pollinators) interact with generalist species of lower trophic levels (e.g. plants). Each generalist species typically interacts with many higher trophic level species [28]. Highly nested communities are assumed to be stable because most of the interactions involve generalist species, so the overall network structure will not be greatly affected if a disturbance removes a specialist species. Non-nested patterns may be either modular or checkerboard (anti-nested). In modular patterns,

there are sets of species that interact more strongly with one-another than with species outside the set. Such patterns may result from evolutionary processes that favor the emergence of highly co-adapted species (for example, species that form symbiotic interactions [42]) or modules that independently perform specialized functions [43]. To assess general network structure in terms of nestedness, computed nestedness values were compared to those for a randomized dataset (a null model). *Connectance* is the ratio of the number of interactions in the network to the total number of possible interactions, while fungal *generality* is defined as the mean number of tree species per fungal species.

We hypothesized that increasing tree diversity would increase connectance and fungal generality while reducing modularity, fungal C score, and fungal specialization as measured by the phi coefficient.

2. Materials and Methods

We used previously published amplicon sequencing data to construct interaction networks [11]. For details of the soil sampling, soil sample preparation, nucleic acid extraction, 454 pyrosequencing, and bioinformatics analysis procedures, please see the work of Weißbecker, Wubet, Lentendu, Kühn, Scholten, Bruelheide and Buscot [11]. Here we briefly outline the experimental design and major sample processing procedures, and describe in detail the data processing steps involved in the network and statistical analyses.

2.1. Sampling Site

Our study was conducted in the frame of the biodiversity and ecosystem functioning experiment China [BEF China, 38]. In 2009, experimental forest plots were established on a hillside in Southeastern China, Jiangxi Province (29°C07′26.0′′N 117°C54′29.0′′E). The site's climate is subtropical with warm wet summers and cold dry winters. A broken-stick design was used to determine the experimental planting schemes of the 31 forest plots investigated here: a set of 16 native subtropical tree species was repeatedly sub-divided into subsets of eight, four, two and one species to establish communities with lower tree diversity levels (Figure S1). The total species pool had equal numbers of AM- and EcM- forming tree species. Each forest plot covered 25.8 m x 25.8 m. In each plot, 400 trees were planted with a spacing of 1.29 m. In October 2011, the mean total tree height ranged from 52 to 301 cm depending on tree species [44]

2.2. Soil Sampling

In October 2011, we randomly selected five tree individuals per tree species in each plot (where possible) for root zone sampling, which was performed by using an augur to remove four soil cores (6 cm in diameter and 10 cm deep) at points 20-30 cm from the tree trunk in each of the cardinal compass directions. The four soil cores were then mixed, sieved (2 mm mesh size), and homogenized to form a composite soil sample. The experimental plots were planted according to a broken-stick design (Figure S1), and the number of experimental plots chosen for sampling decreased with increasing tree diversity while the number of samples collected per plot increased (Figure S1). Two 15 g subsamples from each pooled sample were immediately flash-frozen in liquid nitrogen. One subsample was then freeze-dried [45] and transported by airplane within 4 days to the processing lab in Germany, where it was immediately stored at -80°C until needed for molecular analysis.

2.3. Nucleic acid extraction and multiplexed amplicon pyrosequencing

Microbial DNA was extracted with a PowerSoil® htp 96 Well Soil DNA Isolation Kit or a PowerSoil® Total RNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, United States) in combination with a PowerSoil® DNA Elution Accessory Kit. Fungal ITS rDNA amplicon libraries were generated using the fungal-specific ITS1f primer [46] containing Roche 454 pyrosequencing adaptor B, the universal ITS4 [47], Roche 454 pyrosequencing adaptor A, and a sample-specific

multiplex identifier sequence (MID). All samples were subjected to three replicate PCR reactions. PCR products were cleaned, quantified, and processed using the GS FLX+ sequencing kit (Roche, Mannheim, Germany). The amplicons were sequenced by unidirectional pyrosequencing from the ITS4 ends using a Roche GS-FLX+ 454 pyrosequencer (Roche, Mannheim, Germany) at the Department of Soil Ecology, Helmholtz Centre of Environmental Research (UFZ, Halle, Germany).

2.4. Bioinformatic analysis

Multiple levels of sequence processing and quality filtering were applied using an in-house metabarcode analysis pipeline for grid engines based mainly on the MOTHUR [48] and OBITools [49] software suites. Sequences with ambiguous bases, barcode mismatches, or homopolymers exceeding eight nucleotides were discarded. FlowClus [50] was used to denoise flows and trim reads into uniform 360 bp long read fragments spanning the ITS2 region and the 5.8S rRNA gene. Chimeric reads were removed using UCHIME [51] and quality filtered sequences were clustered into operational taxonomic units (OTUs) using vsearch [52] with a sequence similarity threshold of 97%. OTUs were taxonomically assigned using the UNITE database [version v7_2, 53]. Putative functions were annotated using the FUNGuild fungal database [54].

2.4. Data processing

Data processing and statistical analyses were performed using R [version 3.5.2, 55]. The phyloseq package [56] was used to combine and process OTU count and environmental data. Rare fungal OTUs comprising only singleton, doubleton, and tripleton sequences were discarded [57]. Sequences were rarefied to 700 sequences per sample. All remaining OTUs with at least 10 sequences in the total rarefied dataset were considered in subsequent analyses [30]. The abundance data were transformed into incidence data. Other R packages used for data management and visualization included BiocManager [58], biomformat [59], dplyr [60], data.table [61], extrafont [62], gdata [63], ggplot2 [64], plyr [65], prodlim [66] and vegan [67].

2.5. Tree-fungal bipartite analysis in a subsampling approach

We performed a fungal-tree bipartite network analysis based on observations of fungal-tree co-occurrence using the bipartite package [68]. In accordance with our sampling design, we sampled each of the 31 forest plots of the broken-stick design. No replicates of tree species mixtures were sampled. For each tree species, we collected five samples at each diversity level. The number of collected samples per plot thus increased with the diversity level: five samples were collected from each monoculture plot, whereas 80 (16×5) samples were collected from the 16 tree species mixture plot. Because the number of forest plots decreased as the tree diversity level increased, we aggregated the data for the 4, 8, and 16 tree species mixture plots into a single "high tree diversity" dataset (Figure 1a). Thus, the "high tree diversity dataset" represented seven independent forest plots compared to eight two-tree species mixtures and 16 tree monoculture plots. We therefore constructed our bipartite networks (see Figure 1c) using a subsampling approach in which each subsample was based on seven plots per tree species diversity level and seven tree species This ensured that all networks were based on the same number of individual plots and the same number of samples within a plot.

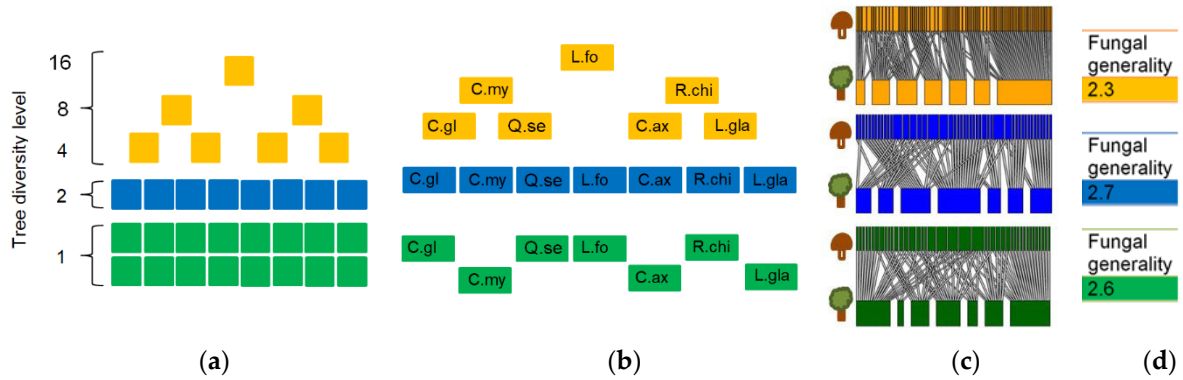

Figure 1. The bipartite network analysis procedure. Data were pooled into three tree diversity levels (a). An illustrative subsampling set (b). For each subsampling combination, a bipartite network was generated (c) and network characteristics such as fungal generality were computed (d). Statistical differences between the tree diversity levels could be analyzed by considering the combined network characteristics of 576 possible subsampling combinations at each tree species diversity level.

Within a given subsampling combination, the same seven tree species were investigated at all three diversity levels and only one tree species was sampled per plot (Figure 1b). For the two tree species mixtures, there were 1024 (8×2^7) valid subsamples based on seven independent plots with one tree species per plot. However, the tree species *Castanopsis eyrei* suffered severe mortality and comparatively few individuals of this species were planted initially. Therefore, at the time of sampling, only a few individuals of this species remained in the experiment, so it was excluded from our analysis. Consequently, there were 576 independent combinations of seven tree species and seven two-species plots that could be used to generate bipartite networks. Bipartite networks were generated based on tree-fungal co-occurrence (Figure 1c) for each of the possible co-occurrence thresholds. That is to say, networks were generated based on the observation of tree-fungus co-occurrence in 1, 2, 3, 4, or 5 of the five soil samples collected for each tree species at each diversity level. We only considered presence-absence data. Network topological characteristics (Figure 1d) were calculated at the network and fungal OTU levels using the networklevel and grouplevel functions of the bipartite package, respectively. For each tree diversity level, we calculated the fungal richness, Shannon diversity, and the following network characteristics: number of fungal OTUs, nestedness (NODF), network connectance, fungal generality, mean number of shared fungal partners, and fungal C score. The Kruskal-Wallis test was used to assess the statistical significance of differences in network characteristics between tree diversity levels based on the 576 data points generated by the subsampling approach. The Kruskal-Wallis test for multiple comparisons (as implemented in the pigrmess package [69]) was used as a post hoc test to perform pairwise group comparisons between the three tree diversity levels.

According to Almeida-Neto, *et al.* [70] the nestedness metric NODF (Nestedness metric based on Overlap and Decreasing Fill) is more robust than the nested temperature metric; higher NODF values indicate greater nestedness. NODF values of our data were statistically compared them to NODF values generated using a simulated null model. The null model was created by shuffling the OTU abundance data before it was divided into subsets corresponding to different tree diversity levels. The column and row sums of the data were kept constant during shuffling. We then used the nullmodel function of the vegan package with the "r2dtable" method to create the null models.

2.6. Specialization analysis

To complement the bipartite network analysis, we assessed the degree of fungal specialization across the tree diversity levels and among the fungal functional groups. The specialization of each fungal OTU for each tree species was assessed by computing the φ (phi) specialization coefficient based on presence/absence data using Equation 1 [40].

$$\Phi = \pm \sqrt{(X^2/N)} = (a \cdot d - b \cdot c) / \sqrt{((a+b) \cdot (c+d) \cdot (a+c) \cdot (b+d))} \quad (1)$$

Equation 1: X^2 is the chi-square statistic for a 2 x 2 contingency table with N being the total number of observations, *a* the number of occurrences of a fungal OTU in a plot containing a particular tree species, *b* the number of occurrences in plots without that species, *c* the number of times the fungal OTU is absent in plots containing that species, and *d* the number of times the fungal OTU is absent in all other plots. The phi coefficient ranges from -1 to 1; the extrema of this range indicate a fungal OTU that always avoids the tree species in question and one that is only found in association with that tree species, respectively.

We determined the median phi coefficient for each of the 576 subsampling combinations (see section 2.6), generating seven plots for each of the three tree diversity levels. The median value of the tree-specific positive phi coefficients of the present OTUs was then calculated for each subsampling combination. Boxplots were used to visualize the median phi coefficients of the subsampling combinations for each tree diversity level. We also determined whether the phi coefficient differed between fungal functional groups and analyzed the differences in the calculated positive phi values. The Kruskal-Wallis test and the Kruskal-Wallis test for multiple comparisons with the Bonferroni correction were used to assess the statistical significance of observed differences. For each fungal OTU, we calculated the maximum phi coefficient across all tree species and identified the 200 fungal OTUs with the highest maximum phi coefficients. The phi coefficients of these fungal OTUs were visualized in a heatmap and clustered using Euclidean distance-based hierarchical clustering dendrograms. The R packages used for this purpose were gplots [63], colorspace [71], and dendextend [72]. We also determined the taxonomic identities of the 20 fungal OTUs with the highest positive phi coefficients.

To complement the specialization pattern analysis, we also assessed the taxonomic identity of the most frequent fungal species in all the subsampling combinations. We defined a fungal OTU as being frequent if it occurred in all seven plots of at least one subsampling combination. All fungal OTUs showing this high occurrence pattern at all three diversity levels in at least one subsampling combination were identified taxonomically. In addition, we identified all of the fungal OTUs that were only frequent at one diversity level (which we termed "unique frequent fungal OTUs") and investigated their occurrence patterns at the diversity levels in which they were not frequent.

3. Results

Taxonomic assignments of fungal OTUs, the assignments of OTUs to functional groups, and the effects of environmental, spatial, and biotic factors on fungal community composition and diversity were reported by Weißbecker, Wubet, Lentendu, Kühn, Scholten, Bruelheide and Buscot [11]. Briefly, pyrosequencing generated 1,155,299 raw sequences from the 394 collected soil samples. Among the major fungal functional groups, saprotrophic fungi dominated, accounting for 31% of the detected OTUs. Less common functional groups were EcM fungi (7% of all OTUs), AM fungi (5%), and plant pathogens (5%); 46% of the fungal OTUs could not be assigned to a functional group. The final dataset for the following analyses (rarefied to a uniform number of 700 sequences per sample and pruned to exclude OTUs not containing at least 10 sequence reads) comprised 248,026 sequences that were clustered into 1,926 fungal OTUs. The analysis was based on three data subsets representing: i) tree monoculture plots ii) two tree species mixture plots, and iii) high tree diversity plots (i.e. plots with 4, 8, or 16 tree species). Rarefaction curves for these data subsets are shown in Figure S2.

3.1. Tree-fungal bipartite network analysis with a subsampling approach

The network analysis was based on a subsampling approach (see Method section 2.6 and Figure 1), which was used to generate all the results presented below. The topological characteristics of the tree-fungal bipartite network were calculated at the network and group levels for all possible

tree-fungal co-occurrence link thresholds (Table S1). Although the specific values of the network parameters depended on the choice of link threshold, the general trends between tree diversity levels were robust (Figure 2). Increasing the link threshold generally reduced the number of fungal OTUs retained in the bipartite networks (Figure 2a) from about 1000 fungal OTUs for a threshold of 1/5 to about 50 OTUs for a threshold of 5/5. Table 1 presents the full set of results obtained using a link threshold of 3/5 (meaning that the bipartite network only included a link between an OTU and a tree species if at least three of the five samples collected for that tree species showed the presence of that fungal OTU). The fungal richness, fungal Shannon diversity, and fungal C score for the monocultures and the two tree species mixtures did not differ significantly but were significantly lower than those for the high tree diversity mixtures (Table 1, Figure S3).

At the network level, we analyzed *nestedness*, *network modularity*, and *network connectance*. Tree-fungal networks were less nested (i.e. had lower NODF values) than the null model (Table 2). The two tree species diversity level had the lowest *network modularity* value and the highest *network connectance* and fungal *generality*. All calculated networks consisted of a single module.

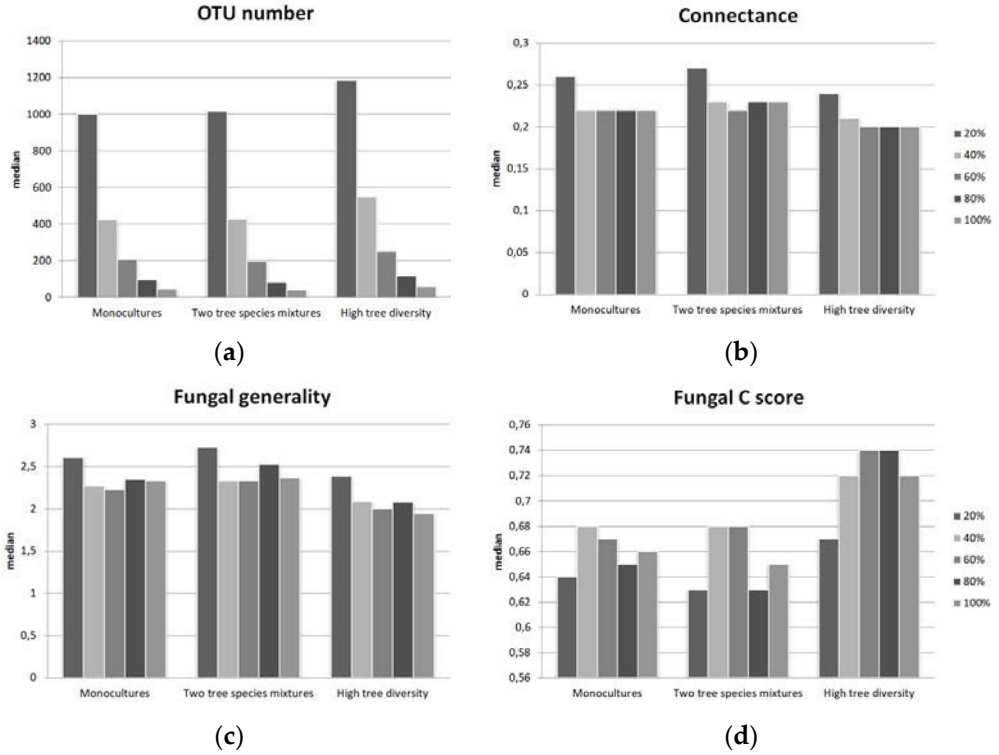

Figure 2. Dependence of the calculated network characteristics on the link threshold for tree species – fungal OTU co-occurrence in the bipartite network analysis. The charts show the median values (based on 576 subsamples) of four key network characteristics: fungal OTU number (a), network connectance (b), fungal generality (c) and fungal C score (d). A table showing all of the computed network characteristics is available in the Supplementary Material (Table S1).

Table 1. Calculated network metrics for different levels of tree species diversity based on 576 tree-fungal bipartite subsamples and three tree diversity levels: monocultures ("Mono"), two-tree species mixtures ("Two mix.") and high tree diversity mixtures ("High"). The Kruskal-Wallis test was used to identify significant differences in network values across the tree diversity levels. The median values of the network characteristics are reported for each tree diversity level. The Kruskal-Wallis test for multiple comparisons with the Bonferroni-Holmes correction was used to assess the significance of pairwise differences in network characteristics across tree diversity levels (n.s.: no significant difference detected). Numbers indicate the tree diversity levels: 1-monocultures, 2-two tree species mixtures, 3-high diversity tree species mixtures. Results are shown for networks generated using a tree species-fungal OTU co-occurrence threshold of 3/5.

	number OTUs	modularity	connectance[1]	fungal generality[2]	fungal C score[3]	mean number of shared fungal partners[4]	Fungal OTU richness	Fungal Shannon diversity
Kruskal.p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Median								
Mono	206	0.52	0.22	2.26	0.67	9.43	1004	4.99
Two mix.	198	0.51	0.22	2.33	0.68	9.57	1017	5.06
High	251	0.58	0.2	2	0.74	8.48	1187	5.34
Pairw.p								
1-2	<0.001	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.	n.s.
1-3	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2-3	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Network connectance: Realized proportion of possible links, [2]Fungal generality: Mean effective number of tree species per fungal species, [3]Fungal C score: Average degree of co-occurrence for all possible pairs of fungal OTUs. Values close to 1 indicate evidence for disaggregation, e.g. through competition. Values close to 0 indicate aggregation of species (i.e. no repelling forces between species), [4]Mean number of shared fungal partners: Mean number of fungal species that interact with at least two tree species.

Table 2. Median nestedness (NODF) values for three tree diversity levels (monocultures, two tree species mixtures, and high tree diversity mixtures) based on null models and bipartite networks generated for 576 subsamples. Networks were generated using a tree species-fungal OTU co-occurrence threshold of 3/5.

	NODF median	Wilcox.p
Tree monocultures	21.51	<0.001
Null model	57.6	
Two tree species mixtures	22.59	<0.001
Null model	57.48	
High tree species mixtures	15.66	<0.001
Null model	57.32	

3.2. Fungal specialization patterns as evaluated using the phi coefficient

The specialization of the fungal community at the three tree diversity levels was assessed by computing the median phi coefficients for the 576 subsampling combinations. Specialization was lowest in the two tree species mixtures plots and highest in the tree monocultures (Figure 3). The

EcM fungi exhibited a greater degree of specialization than the other fungal functional groups (Figure 4; a table showing the phi coefficients of all the fungal OTUs is available at the zenodo archive); the degrees of specialization of the other groups (saprotrophs, plant pathogens, and AM fungi) did not differ significantly. Additionally, the degree of specialization of saprotrophic fungi in plots with AM tree species was significantly higher than in those with EcM tree species (data not shown). We visualized the distributions of the 200 most frequently identified fungal OTUs in a heatmap covering all the studied tree species (Figure 5). Taxonomic identifications of the 20 most highly specialized fungal OTUs are presented in Table S2; eight of these OTUs were EcM fungi, four were saprotrophs, one was an orchid mycorrhizal OTU, and seven belonged to unknown fungal functional groups. Fifteen fungal OTUs were identified as frequent fungal species at all three tree diversity levels (Table S3). Most (nine) of these frequent fungal OTUs were saprotrophs (9 OTUs), but two were plant pathogens and one arbuscular mycorrhizal fungal OTU was also identified. All fungal taxa that were frequent at only one tree diversity level also occurred at the other tree diversity levels at lower frequencies (Figure S4).

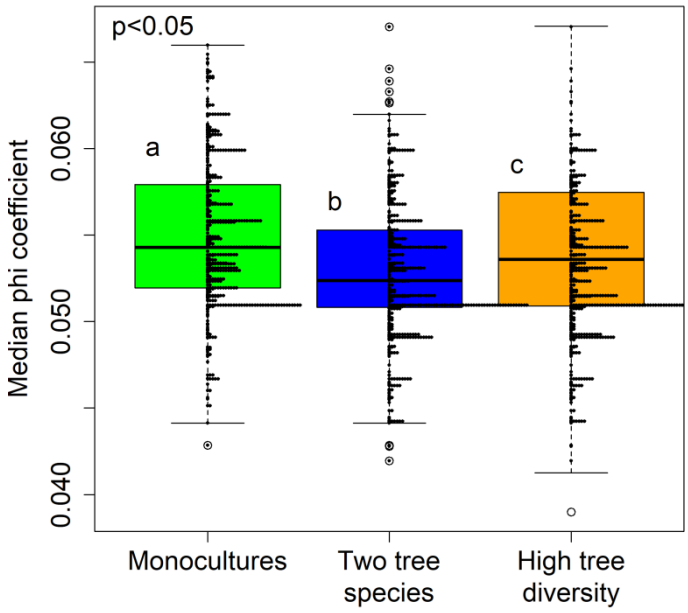

Figure 3. Boxplots of phi coefficients for the three tree diversity levels. The Kruskal-Wallis rank sum test and Kruskal-Wallis test for multiple comparisons were used to evaluate the significance of group differences.

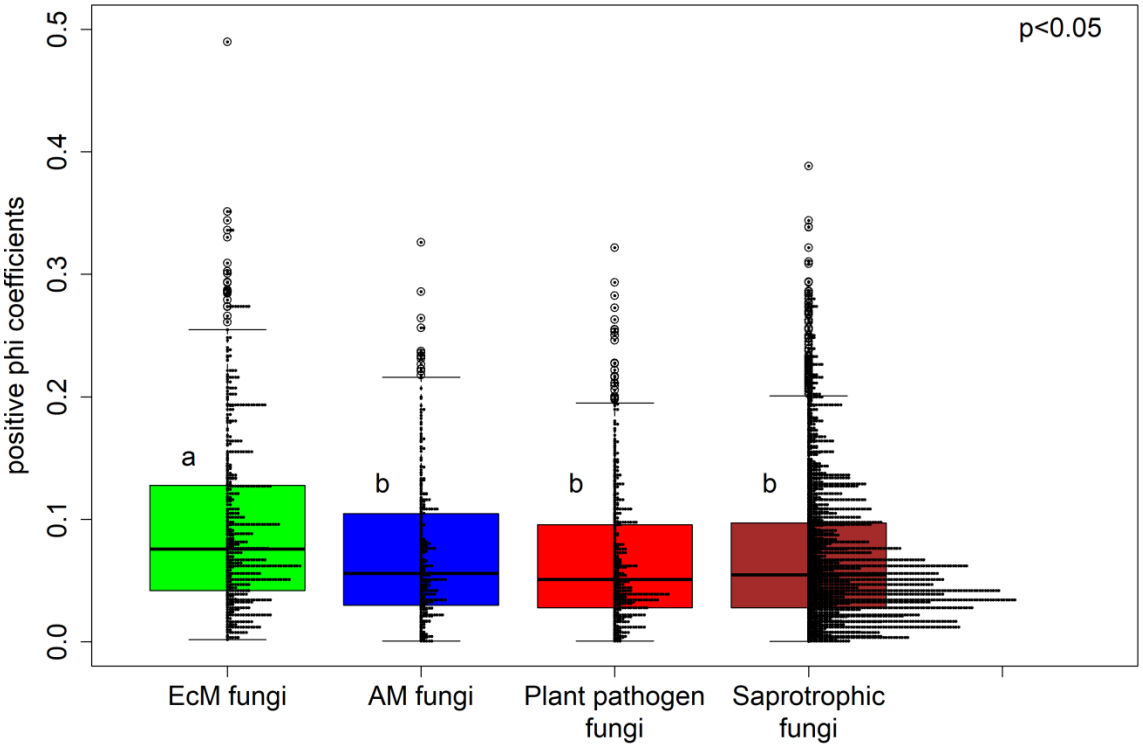

Figure 4. Boxplot showing phi specialization coefficients for the main fungal functional groups.

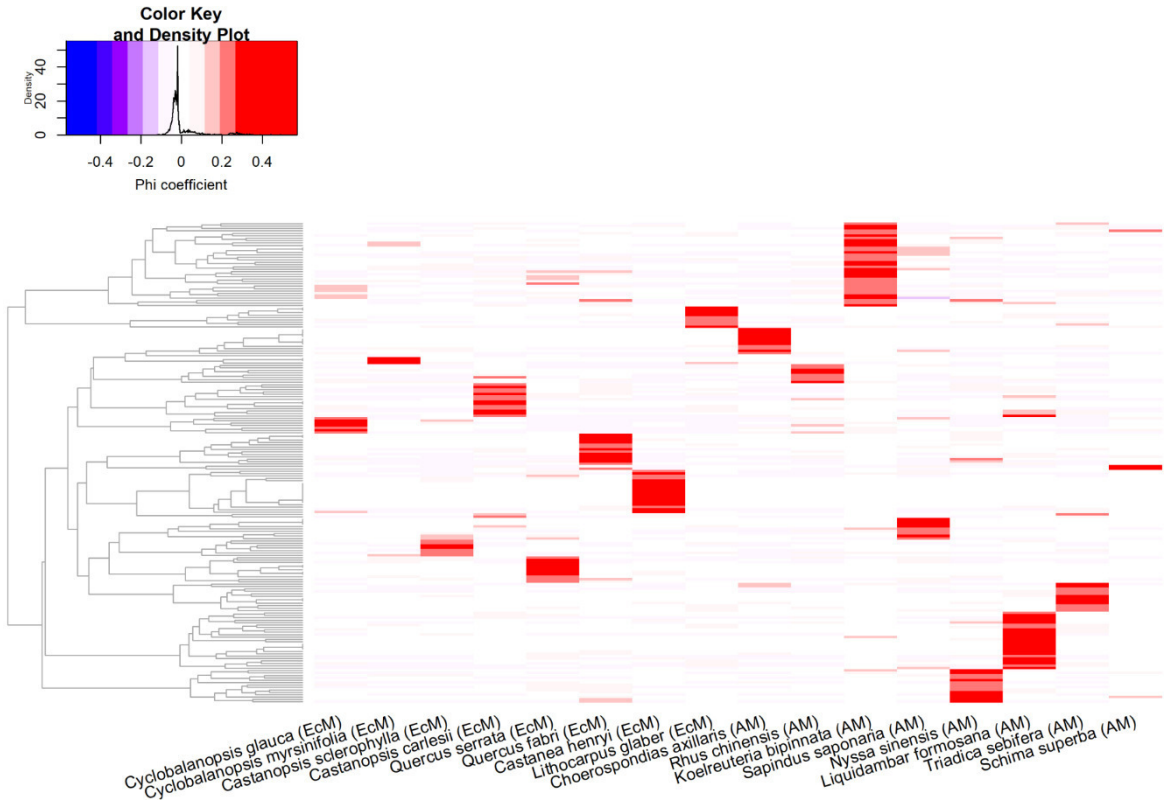

Figure 5. Heat map showing the distribution patterns of the 200 fungal OTUs with the highest phi specialization coefficients among the 16 tree species.

4. Discussion

In this study, we analyzed the relationship between tree diversity, tree-fungal bipartite network structure, and fungal specialization in young subtropical forest plantations. Weißbecker, Wubet, Lentendu, Kühn, Scholten, Bruelheide and Buscot [11] previously found that local tree species richness had no effect on soil fungal OTU richness. Here, using a network analysis approach that combines tree diversity levels, we found that plots with high tree species diversity (i.e. plots containing 4 to 16 different tree species) exhibited increased fungal diversity. We also observed differences in the network structure of fungal-tree bipartite networks and differences in the degree of fungal specialization between tree diversity levels.

4.1. Increased fungal alpha diversity in plots with high tree species diversity

The fungal alpha diversity (richness and Shannon diversity) was significantly greater for the high diversity tree species mixtures than for tree monocultures and two tree species mixtures. Tree species richness enhances forest productivity [73-75] and can thus yield higher productivity compared to monocultures (overyielding). Therefore, in addition to providing a greater variety of distinct niches, increasing tree species diversity could increase the quantity of resources (e.g. rhizodeposits, litter input, and fine root turnover) available to fungi, thereby increasing the fungal diversity that can be sustained. A more diverse fungal community might also enhance tree productivity; the two effects could thus be complementary. Accordingly, in a separate study conducted at the site considered here, Fichtner, *et al.* [76] found that local neighborhood tree species richness increased tree community productivity due to facilitation and competitive reduction. Our previous study [11] revealed no comparable positive effects of tree species diversity on fungal richness. However, that study was conducted at the local neighborhood scale, with tree species diversity values ranging from one to eight because only one focal sampling tree and its eight nearest neighbors were considered. In this work, we instead focused on abundant fungal OTUs (i.e. those represented by at least 10 sequence reads) and binned data representing five diversity levels into three wider diversity categories, increasing the statistical power of our analysis. This resulted in the detection of a positive effect of tree species richness on the abundance of fungal taxa.

4.2. The connectance and fungal generality of tree-fungal bipartite networks are highest at the two tree species diversity level

Next to investigate the relationship between fungal and tree species diversity, we investigated tree species - fungal OTU co-occurrence patterns. The computed network characteristics revealed significant differences between the low tree diversity plots (monocultures and two tree species mixtures) and those with high tree diversity (4, 8, or 16 tree species; see Table 1). Independently of the chosen link threshold, none of the network characteristics of the tree-fungal bipartite networks differed significantly between the monocultures and the two tree species mixtures. However, contradicting our hypotheses relating to network characteristics, we found that the high tree diversity plots had i) lower median connectance and fungal generality values than the monoculture and two tree species mixture plots as well as ii) higher modularity values and fungal C scores.

Our hypothesis about fungal specialization patterns was supported by the finding that monoculture plots had the highest degree of fungal specialization, which suggests that fungal specialists outcompete generalists in the relatively homogeneous environments created when only one tree species is present. The two tree species networks had higher network connectance and a lower degree of fungal specialization than those for monocultures. These observations also support our hypothesis that generalist fungi can cope adequately with the more heterogeneous environments created by the presence of two tree species, and outcompete specialist fungi that only perform well in one of the two niches created by the two tree species. However, our initial hypothesis was not supported by the finding that high tree diversity mixtures had a greater degree of specialization and lower network connectance than the two tree species mixtures. Planting several tree species together presumably creates more environmental niches than are present in monocultures due to both species diversity and interaction effects/processes. Additionally, highly

diverse tree species mixtures may offer habitats suitable for fungi specialized in connecting different tree species, i.e. those fungi that need resources from different trees to which they are connected.

Frequent species are believed to provide crucial network structure support and resilience [77] because they are not limited by resource or partner availability [78]. Therefore, a high number of frequent species is sometimes taken as an indicator of ecosystem stability. On the other hand, specialist species broaden functionality and resource use. Of the three tree species diversity levels considered in this work, the high diversity level may be ecologically preferable in terms of fungal richness and the number of specialist and frequent taxa for three reasons. First, it has the highest number of fungal species. Second, these fungal species include more specialized fungal taxa than are present in plots with less diverse tree species mixtures, meaning that the fungal community's resource usage is broader. Third, the frequent species found at the lower tree diversity levels are also present at the high diversity level, albeit at reduced frequencies. In the event of tree diversity loss from the high tree diversity plots (which could cause the loss of some specialized fungi), these fungal species may increase in abundance and become frequent, taking over ecological processes such as decomposition, tree protection, and tree nutrition.

EcM fungal OTUs exhibited a significantly higher degree of specialization than the other fungal functional groups (saprotrophic fungi, AM fungi, and plant pathogens). Moreover, unlike other fungal functional groups, EcM fungal communities reportedly exhibit significant host effects [11]. In general, evolutionary history suggests that EcM fungi are more highly specialized than AM fungi [79]. The number of plant host species and EcM fungal species is similar [about 6000 species,1,80,81,82], while AM plants comprise around 80% of all plant species [1,83] but only around 300 AM fungal morphospecies have been described [84]. Nevertheless, some degree of host preference has been reported for AM fungi [85-87]. Bennett, *et al.* [88] found a higher degree of specialization in tree-AM fungal networks of old forests (>130 years) than in young forests (25 years), and proposed that specialization in AM fungi will become more pronounced as a forest develops after clear-cutting. These authors also suggested that post-disturbance (clear-cutting) associations might reflect the local availability of fungal taxa rather than the intrinsic host preferences of AM fungi.

4.3. Comparison with other bipartite network studies

In general, our tree-fungal bipartite networks exhibited low to moderate network connectance (0.20-0.27), high modularity (0.41-0.58), and non-nested structures. For comparative purposes, the network characteristics determined in other plant-soil fungal studies are presented in Table 2. Network metrics depend strongly on the number of nodes included, and care must be taken when comparing network metrics from different studies. For example Fodor [41] found a high network connectance in mature forests (55-100 years old) and concluded that mycorrhizal fungi (which were predominantly generalists) acted as connector organisms linking the tree species. This pattern did not exist in our young forests (which were sampled in the third growing season after planting) even though many EcM tree species and tree individuals were present. The fungal communities at our sampling site were characterized by limited dispersal and a high beta-diversity across and within plots [11]. This indicates that mycorrhizal networks had not yet been established at the plot scale; the fungal communities (especially those of EcM fungi) differed strongly between samples within the same plot [11]. Whereas the tree-EcM networks analyzed by Fodor [41] showed a nested pattern, Bahram, *et al.* [89] found tree-EcM networks to be non-nested. While some studies suggest that mutualistic networks have inherently nested structures [e.g. 90], nestedness patterns in soil fungal communities span the full spectrum of possible structures, ranging from nested [23,41,90] to non-nested [91; this study] and even anti-nested [32] (Table 2). Nestedness is a core network metric because it has been suggested to be related to network persistence [92].

In contrast to previous bipartite network studies, all our networks consisted of a single module with high modularity, "indicating the possible presence of community structure" [93]. A modular structure indicates that groups of nodes perform different functions with some independency from one-another [43]. For example, Toju, Guimaraes, Olesen and Thompson [91] found eight

interconnected modules with differing fungal functional group compositions. The high modularity [>0.4, 94] of our networks may indicate the presence of different fungal functional groups that assemble in different ways relative to the tree community [11]. The computational method used in this work only divides networks into multiple modules if the number of edges between communities is lower than expected [93], which was not the case for our networks. The low number of modules per network (one) may be another indicator of the early developmental state of our networks, indicating that they have yet to form densely interconnected modules.

Table 3. Network metrics reported in previously published plant-fungal network studies.

	This study	[90]	[32]	[91]	[41]	[88]* [24]*	[23]
Study system	16 subtropical tree species in a forest biodiversity experiment	Semi natural grasslands, 33 plant species	cool-temperate, warm-temperate and subtropical forests	Temperate forest with 33 tree species	Temperate forests, mainly Quercus and Carpinus	33 understory plant species in temperate spruce forest	Xeric shrubland
Country	China	Estonia	Japan	Japan	Romania	Estonia	Mexico
Age	3 years				55-100 years	25 years 130 years and 130 years	
Treatment	Tree species diversity	Host plant functional group	Latitudinal gradient			Succession and seasonality	
Samples	Soil within tree rooting zone	Root samples	Root samples	Root samples	aboveground EcM fructifications	Root samples	Root samples
Study target	Soil fungi	AM fungi	Soil fungi, fungal groups	Soil fungi	EcM fungi	AM fungi	AM fungi
Nestedness	Less nested (15.66-29.42,) than random (53.87-60.04) NODF	More nested than random (27) nestedness temperature)	Anti-nested (-9 – 4) weighted NODF)	Less nested (25-35,) than random (32-40) weighted NODF	More nested (16) than random (38, 31) nestedness temperature)		More nested (14.36-54.83) than random, NODF
Number of modules	1	5		8	4	5-9	
Modularity	0.41-0.58	Higher than random 0.18		Moderate to low modularity (0.35-0.42), higher than random (0.32-0.38)	Low modularity 0.24	0.3-0.44	Modular 0.30-0.57
Connectance	0.20-0.27	Less connected than random 0.52	0.07	0.1-0.55	High connectance 0.42		Low connectance 0.05-0.15
Fungal generality	1.95-2.73					2.25-4.0	
Fungal C score	0.63-0.74				No difference of observed		

		(0.59) and random value (0.58)		

* these studies re-evaluated the data from [86]; empty fields indicate no available information

5. Conclusions

In accordance with our hypothesis, tree monocultures had the highest frequencies of specialist fungi. However, the degree of fungal specialization and network segregation were higher in plots with high tree diversity than in those with only two tree species. There is ongoing and global interest in clarifying the impact of tree diversity on sustainable forest plantations [95,96]. Plots with high tree diversity (i.e. those with 4 to 16 different tree species) supported the greatest number of fungal species, which is assumed to be beneficial for ecosystem service provision because greater fungal diversity enables more effective resource exploitation and confers greater resilience due to functional redundancy.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Broken-stick-design of the experimental forest plots, Figure S2: Rarefaction curves, Figure S3: Fungal richness, Figure S4: Occurrence patterns of unique frequent fungal OTUs. Table S1: Network characteristics for tree-fungal bipartite analysis based on co-occurrence, Table S2: Top 20 specialist fungal OTUs based on maximum phi coefficient values, Table S3: Frequent fungal OTUs at all three tree diversity levels.

Data availability: Relevant materials and protocols will be made available upon request. Datasets of the raw sequences generated for this study can be found in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/data/view/PRJEB12020) [97]. The bioinformatically processed sequence dataset and metadata can be found in the Zenodo repository (https://zenodo.org/ record/1215505)[98]. **The R scripts generated for the statistical analyses and the table with the fungal OTU phi coefficients will be made available in a public repository during the revision process.**

Author Contributions: conceptualization, C.W., T.W., F.B. and H.B.; formal analysis, C.W., A.HB., T.W. and H.B.; investigation, C.W.; data curation, C.W.; writing—original draft preparation, C.W.; writing—review and editing, C.W., A.HB, F.B., H.B. and T.W.;

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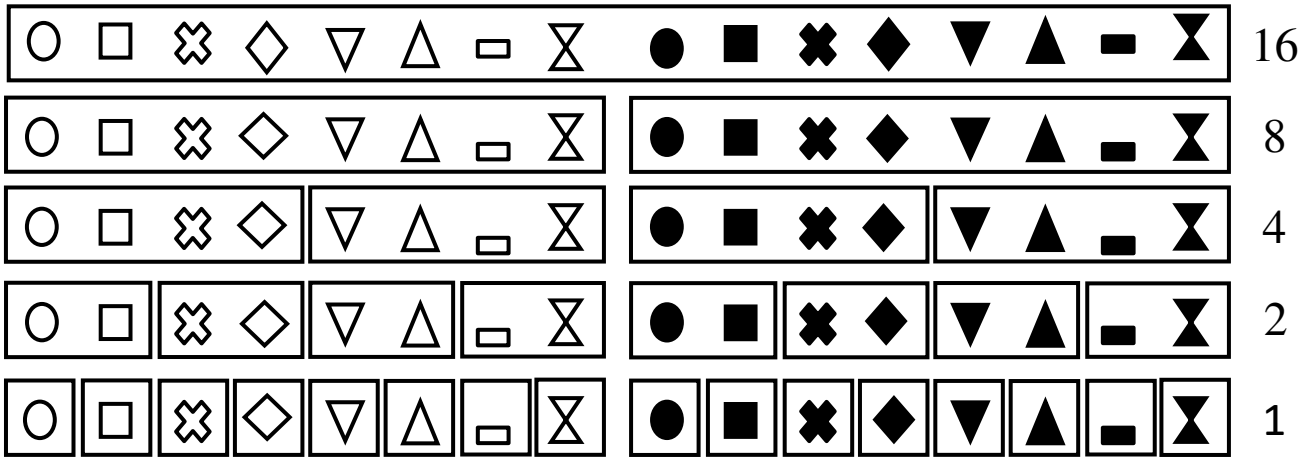
Supplementary Material

Linking soil fungal generality to tree richness in young subtropical Chinese forests

Christina Weißbecker, Anna Heintz-Buschart, Helge Bruelheide, François Buscot and Tesfaye Wubet



	Tree species	Type	No.		Tree species	Type	No.
○	*Castanea henryi* (Skan) Rehd. & Wils.	EcM	5 x 5	●	*Cyclobalanopsis glauca* (Thunb.) Oerst.	EcM	5 x 5
□	*Nyssa sinensis* Oliver	AM	5 x 5	■	*Quercus fabri* Hance	EcM	5 x 5
⊠	*Liquidambar formosana* Hance	AM	5 x 5	✖	*Rhus chinensis* Mill.	AM	5 x 5
◇	*Sapindus saponaria* Linn.	AM	5 x 5	◆	*Schima superba* Gardner & Champion	AM	5 x 5
▽	*Choerospondias axillaris* (Roxb.) Burtt & Hill	AM	5 x 5	▼	*Castanopsis eyrei* (Champ.) Tutcher/ *C. carlesii* (Hemsl.) Hay.	EcM	5 x 5
△	*Triadica sebifera* (L.) Small	AM	5 x 5	▲	*Cyclobalanopsis myrsinifolia* (Blume) Oerst.	EcM	5 x 5
▭	*Quercus serrata* Murray	EcM	5 x 5	▬	*Lithocarpus glaber* (Thunb.) Nakai	AM	5 x 5
⧖	*Castanopsis sclerophylla* (Lindl.) Schott.	EcM	5 x 5	⧗	*Koelreuteria bipinnata* Franch.	AM	5 x 5
	Number of soil samples		**200**		**Number of soil samples**		**200**

Figure S1. Broken-stick-design of the experimental forest plots. Plot design presented for the **b**iodiversity and **e**cosystem **f**unctioning (BEF) experiment China, study site A. The 16 species mix was sub-divided in two times eight species mixtures. These were likewise partitioned into four, two and one tree species communities. Tree species are shown as symbols. Similarity of symbols was only chosen to emphasize the experimental design and does not imply any similarities or dissimilarities of tree species traits. No.: Number of samples.

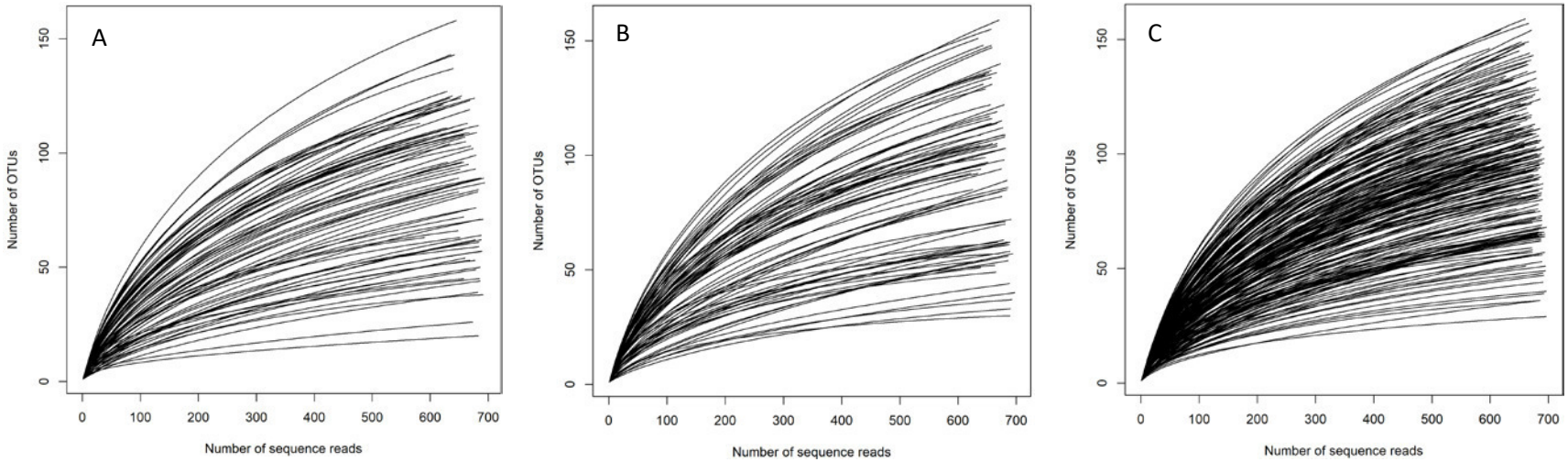


Figure S2. Rarefaction curves of monoculture (A) two tree species mixtures (B) high tree diversity mixtures (4, 8 or 16 tree species mixtures).

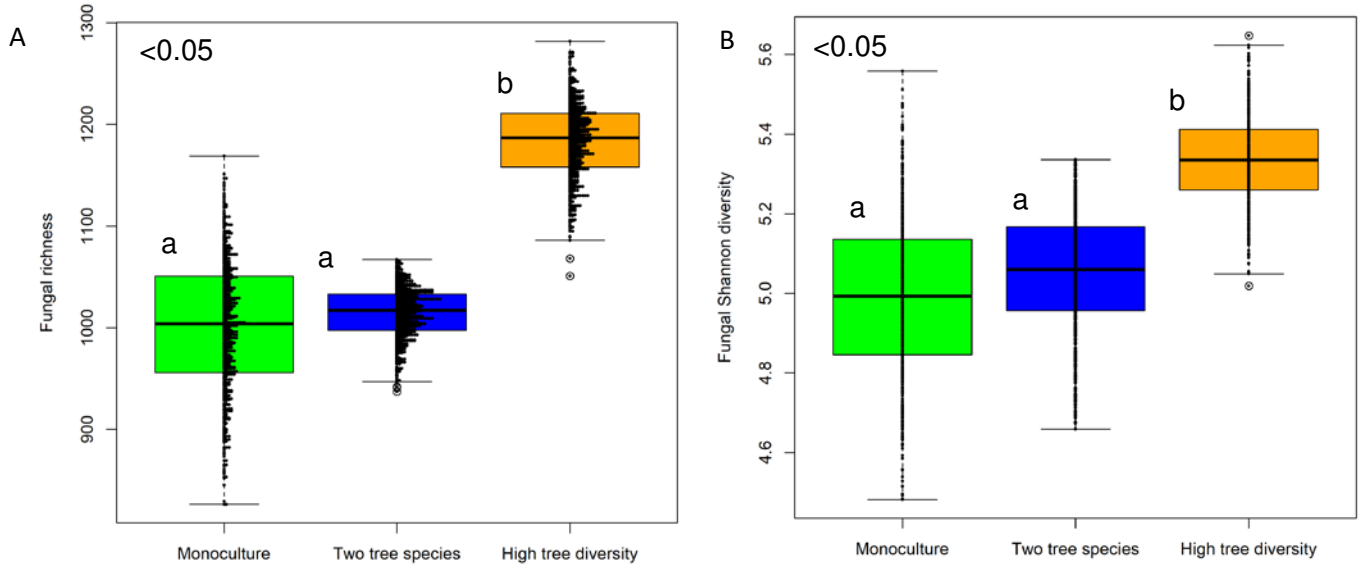


Figure S3. Fungal richness (A) and fungal Shannon diversity (B) across the 576 subsampling permutations.

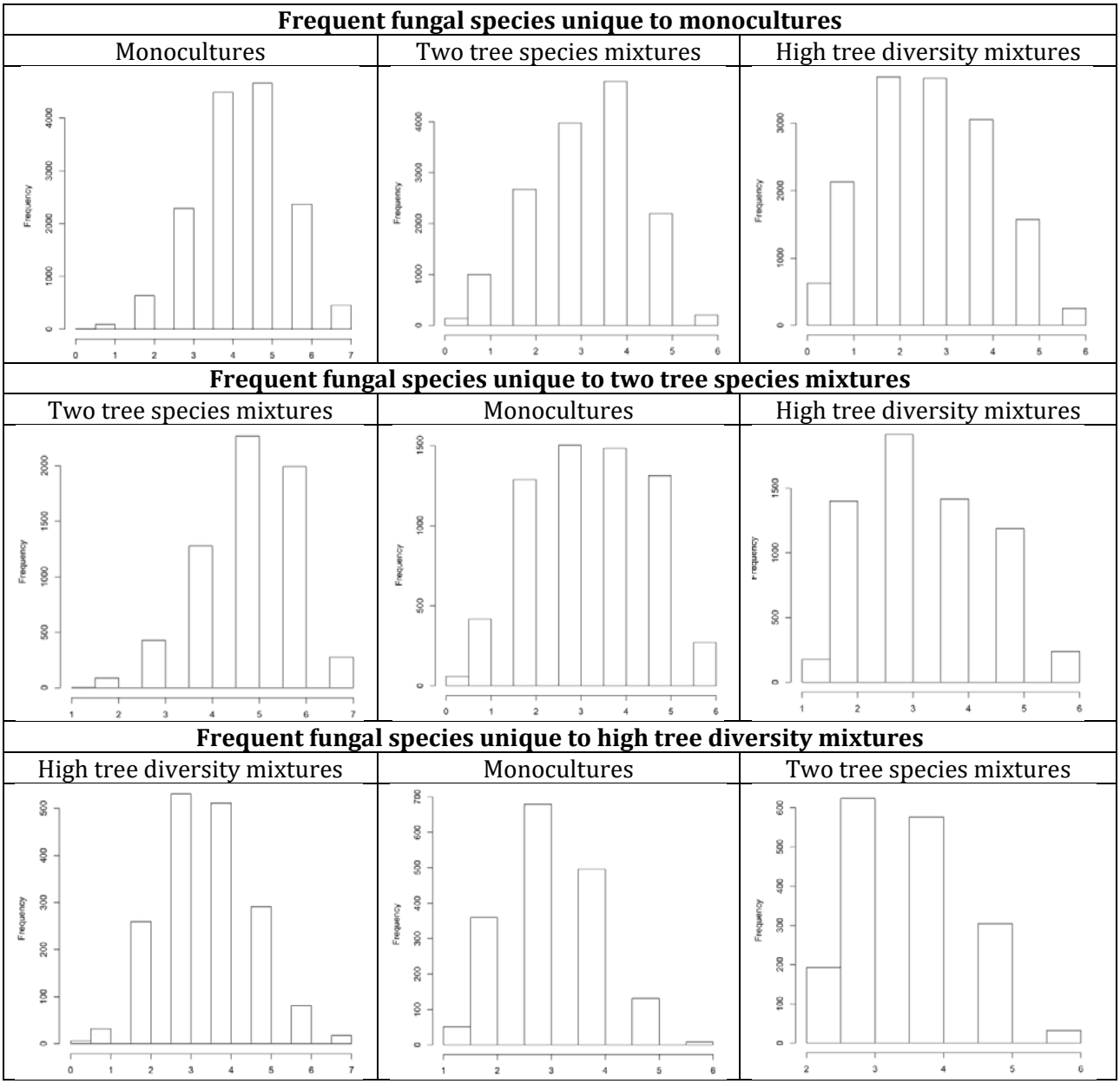


Figure S4. Occurrence patterns of unique frequent fungal OTUs of one tree diversity level across all tree diversity levels. Frequent fungal OTUs were defined as those, occurring on all seven plots in at least one permutation.

Table S1. Network characteristics for tree-fungal bipartite analysis based on co-occurrence. Network metrics (A) were compared between the tree diversity levels, while network nestedness (B) was compared against a null model. Networks were calculated for all possible link thresholds (one to five out of five samples) in which co-occurrence was observed. The Kruskal-Wallis test was applied to test for a significant difference of each network characteristic, the p value is reported. Median values of the 576 subsampling routines are given for the three tree diversity levels and below stated whether the Kruskal-Wallis test for multiple comparisons was significant (T=TRUE) or not (F=FALSE) for the group comparisons (indicated by numbers).

A	number OTUs	modularity	connectance	fungal generality	fungal C score	mean shared fungal partners
1 co-occurrence	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Median						
mono	1004	0.43	0.26	2.61	0.64	69.17
two mix.	1017	0.41	0.27	2.73	0.63	77.98
high	1187	0.46	0.24	2.39	0.67	66.67
1-2/1-3/2-3	F/T/T/	T/T/T/	T/T/T/	T/T/T/	T/T/T/	T/T/T/
2 co-occurrences	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Median						
mono	423	0.50	0.22	2.27	0.68	20.19
two mix.	427	49	0.23	2.33	0.68	21.14
high	550	0.55	0.21	2.06	0.72	20.71
1-2/1-/.2-3	F/T/T/	T/T/T/	T/T/T/	T/T/T/	F/T/T/	T/_F/T/
3 co-occurrences	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Median						
Mono	206	0.52	0.22	2.26	0.67	9.43
two mix.	198	0.51	0.22	2.33	0.68	9.57
high	251	0.58	0.2	2	0.74	8.48
1-2/1-3/2-3	T/T/T/	F/T/T/	F/T/T/	T/T/T/	F/T/T/	F/T/T/
4 co-occurrences	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Median						
mono	96	0.51	0.22	2.35	0.65	4.83
two mix.	83	0.49	0.23	2.53	0.63	4.86
high	117	0.56	0.2	2.08	0.72	4.43
1-2/1-3/2-3	T/T/T/	T/T/T/	T/T/T/	T/T/T/	T/T/T/	F/T/T/
5 co-occurrences	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Median						
mono	45	0.52	0.22	2.33	0.66	2.19
two mix.	39	0.51	0.23	2.37	0.65	2.05
high	56	0.58	0.2	1.95	0.72	1.9
1-2/1-3/2-3	T/T/T/	F/T/T/	T/T/T/	F/T/T/	F/T/T/	T/T/_F/

B		NODF median	NODF Wilcox.p
1 **co-occurrence**	Mono	27.83	<0.001
	Nullmodel	56.71	
	Two mix.	29.42	<0.001
	Nullmodel	56.83	
	High	24.61	<0.001
	Nullmodel	56.66	
2 **co-occurrences**	Mono	22.18	<0.001
	Nullmodel	54.74	
	Two mix.	23.67	<0.001
	Nullmodel	53.87	
	High	17.8	<0.001
	Nullmodel	54.24	
3 **co-occurrences**	Mono	21.51	<0.001
	Nullmodel	57.6	
	Two mix.	22.59	<0.001
	Nullmodel	57.48	
	High	15.66	<0.001
	Nullmodel	57.32	
4 co-occurrences	Mono	22.91	<0.001
	Nullmodel	59.76	
	Two mix.	26.3	<0.001
	Nullmodel	60.04	
	High	16.85	<0.001
	Nullmodel	58.71	
5 co-occurrences	Mono	21.47	<0.001
	Nullmodel	59.63	
	Two mix.	24.25	<0.001
	Nullmodel	58.67	
	High	14.98	<0.001
	Nullmodel	59.37	

Network modularity: A high value indicates stronger connections within than between modules, **Network connectance:** Realized proportion of possible links, **Fungal generality:** Mean effective number of tree species per fungal species, **Mean number of shared fungal partners:** Mean number of fungal species that two tree species interact with, **Fungal C score:** Average degree of co-occurrence for all possible pairs of fungal OTUs. Values close to 1 indicate that there is evidence for disaggregation, e.g. through competition. Value close to 0 indicate aggregation of species (i.e. no repelling forces between species); **NODF:** nestedness metric (**n**estedness metric based on **o**verlap and **d**ecreasing **f**ill.

Table S2. Top 20 specialist fungal OTUs identified by maximum phi coefficient values.

	Fungal_guild	Phylum	Class	Order	Family	Genus
Otu00046	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Agaricales	Hymenogastraceae	Hymenogaster
Otu00561	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Entoloma
Otu01026	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Tomentella
Otu01059	Unknown	Rozellomycota	GS11	NA	NA	NA
Otu01081	Unknown	NA	NA	NA	NA	NA
Otu01112	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Tomentella
Otu01197	Unknown	Rozellomycota	GS11	NA	NA	NA
Otu01234	Orchid Mycorrhizal	Basidiomycota	Agaricomycetes	Cantharellales	Tulasnellaceae	Epulorhiza
Otu01639	Saprotroph	Ascomycota	Archaeorhizomycetes	Archaeorhizomycetales	Archaeorhizomycetaceae	Archaeorhizomyces
Otu01746	Unknown	Ascomycota	Leotiomycetes	Helotiales	NA	NA
Otu01872	Saprotroph	Mucoromycota	NA	Mortierellales	Mortierellaceae	Mortierella
Otu01993	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidium
Otu02009	Unknown	Ascomycota	Eurotiomycetes	NA	NA	NA
Otu02292	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Tomentella
Otu02384	Unknown	Ascomycota	Xylonomycetes	GS34	NA	NA
Otu02663	Saprotroph	Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	Clavaria
Otu02893	Saprotroph	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium
Otu03239	Unknown	Ascomycota	NA	NA	NA	NA
Otu03384	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Entoloma
Otu04209	Ectomycorrhizal	Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Sebacina

Table S3. Frequent fungal OTUs at all three tree diversity treatments (monocultures, two tree species mixtures and high tree diversity mixtures). Frequent fungal species were defined as fungal OTUs appearing at least once on all seven plots of one subsampling permutation.

	Fungal_guild	Kingdom	Phylum	Class	Order	Family
Otu00002	Saprotroph	Fungi	Ascomycota	Archaeorhizomycetes	GS31	NA
Otu00003	Saprotroph	Fungi	Basidiomycota	Geminibasidiomycetes	Geminibasidiales	Geminibasidiaceae
Otu00005	Saprotroph	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae
Otu00009	Saprotroph	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae
Otu00012	Unknown	Fungi	Ascomycota	Leotiomycetes	Helotiales	NA
Otu00019	Saprotroph	Fungi	Mucoromycota	NA	Mortierellales	Mortierellaceae
Otu00034	Saprotroph	Fungi	Basidiomycota	Agaricomycetes	Trechisporales	Hydnodontaceae
Otu00038	Arbuscular Mycorrhizal	Fungi	Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae
Otu00068	Unknown	Fungi	NA	NA	NA	NA
Otu00080	Plant Pathogen	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae
Otu00084	Saprotroph	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae
Otu00203	Saprotroph	Fungi	Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae
Otu00211	Unknown	Fungi	Basidiomycota	Agaricomycetes	Agaricales	NA
Otu00273	Saprotroph	Fungi	Mucoromycota	NA	Mortierellales	Mortierellaceae
Otu00298	Plant Pathogen	Fungi	Ascomycota	Sordariomycetes	Xylariales	Sporocadaceae

DISCUSSION

We analyzed the impact of tree species diversity on soil fungal community assembly in a large subtropical tree biodiversity-ecosystem functioning experiment (BEF China). We selected molecular methods (PCR amplification of the genomic ITS region) and 454 pyrosequencing to comprehensively assess the soil fungal communities. Therefore, we developed a freeze-drying procedure to preserve the quality of collected soil samples during short-term transportation. We investigated the relative impact of environmental (e.g. soil pH and soil nutrient content), spatial (distance between samples) and biotic variables (e.g. tree species richness, tree species identity and tee species community composition) on soil fungal richness and community composition. Furthermore, we inspected the tree-fungal interaction patterns by bipartite network analysis in relation to three tree richness levels. In the following part we present and discuss the main findings of the result chapters, state the contribution of this thesis for the specific BEF China project and for fungal ecology research in general, present the current limitations and give some examples for future research directions.

D-1 Approaches and main findings of the result chapters

High-throughput molecular methods are highly sensitive. Soil microbial communities can rapidly change within the samples during storage, e.g. by fast growing molds. Also, nucleic acids are prone to degradation. Thus, a careful handling of samples is crucial for a subsequent analysis to be informative. The gold standard of sample storage after flash-freezing in liquid nitrogen is the constant storage at -80°C (for RNA analysis) or -20°C (for DNA analysis). Many soil environmental studies take place in remote areas across the world where facilities for molecular methods are not readily available. Furthermore, it is desired to analyze collected samples in the same laboratory to keep procedures as similar and reproducible as possible. Thus, with the main study in China ahead, we needed a reliable method for soil sample conservation for short-term transportation by aero plane to the processing laboratory in Germany. In Manuscript 1 (Weißbecker *et al.*, 2017), we presented that freeze-drying of soil samples proved to be an effective, non-toxic, relatively cheap and feasible method for the conservation of bacterial and soil arbuscular mycorrhizal fungal communities for a short-term storage of up to seven days at ambient temperature as analyzed by both RNA and DNA level.

In the main study, presented in Manuscript 2 (Weißbecker *et al.*, 2018), we analyzed soil fungal alpha and beta-diversity in relation to a range of environmental drivers and tree

diversity measures. Each soil sample was collected in the vicinity of a selected sampling tree. Analysis was done at the scale of the specific individual sampling tree neighborhoods, including the tree species information of the sampling tree and its eight tree individual neighbors. We analyzed the main fungal functional groups of saprotrophic, plant pathogenic and mycorrhizal (arbuscular mycorrhizal and ectomycorrhizal) fungi separately. We found that at the early stage of forest development, environmental and stochastic processes dominated the assembly of the soil fungal community. Tree related variables, such as tree community composition, significantly affected arbuscular mycorrhizal and plant pathogen fungal community structure, while differences in tree host species and host abundance affected ectomycorrhizal fungal community composition.

In a third study (Manuscript 3, submitted), we took a rather systemic approach analyzing soil fungal- tree co-occurrence patterns by bipartite network analysis. Also, we assessed the degree of fungal specialization across the tree diversity levels and among fungal functional groups. We found that fungal OTUs showed the highest specialization for the monocultures. Against our expectation, the degree of specialization was lowest at the two tree species mixtures instead for the high tree species mixtures. Accordingly, connectance and fungal generality values were highest for the two tree species mixtures. Fungal richness was highest in the high tree diversity forests (4, 8 or 16 tree species).

D-2 Implications of study findings

Concluding our results, we found significant effects of tree species richness on soil fungal richness, community composition and specialization patterns even at an early developmental stage of the subtropical forest plots, three seasons after its plantation. Although the relative contribution of tree species richness and community composition on the soil fungal community was lower compared to the contribution of environmental variables (except for EcM fungi) and the amount of remaining unexplained variance, this underpins a tremendous role of aboveground tree species diversity on belowground soil fungal communities.

In the years after our sampling campaign in 2011 we expect a further increasing influence of the tree species richness on the soil fungal communities. Plant-soil feedbacks will become stronger due to an increased microhabitat alteration of the soil by the plant (e.g. by accumulation of plant litter and root exudates, Eisenhauer *et al.*, 2010) and emerging

interactions between the plants that will increase complementary effects e.g. through the development of different rooting depths (Cardinale *et al.*, 2007). Indeed, several studies support the strong influence of tree species richness on ecosystem functioning and soil microbial community composition in the BEF China forests at later developmental stages (Ma *et al.*, 2017). Sun *et al.* (2017) reported an increased fine-root production with increasing tree richness, with fine roots being the essential root compartment for AM and EcM mycorrhizal symbiosis. Huang *et al.* (2018) evaluated productivity in multiple tree diversity treatments at both experimental sites in BEF China. They found that tree species richness strongly increased stand-level productivity eight years after planting of the tree saplings. The 16-species mixtures had accumulated over twice the amount of carbon found in average monocultures. Sampling the experimental forests in 2014, Dr. Witoon Purahong (personal communication) observed a declining amount of specialist fungal species and an increasing of generalist fungi with increasing tree species diversity. Last but not least, he also found that soil plant pathogen pressure by soil fungi increased in the tree monocultures.

D-3 Contribution of this thesis to fungal ecology research and the BEF China project

This thesis contributes to the field of fungal ecology and the BEF China research project in multiple ways. The development of the freeze-drying method to efficiently preserve soil samples at ambient temperatures that are intended for highly sensitive molecular analyses methods can be used by many researchers in the future. To apply this method within the BEF China project, we established a new laboratory near the Chinese sampling site with the freeze-drying facility, freezer storage capacities and basic laboratory equipment. An emergency power system with gasoline generators had to be established and secured against burglary to protect the laboratory freezers and the freeze-dryer against the frequent electricity dropouts in the region.

Concerning the data analysis, selecting the scale of soil fungal analysis at the sampling tree neighborhood, contributed to the further development of the BEF China project, to the follow-up project TreeDì (https://www.idiv.de/de/treedi.html) that focuses on the tree-tree interactions in local neighborhoods, acknowledging that the local tree neighborhood is a crucial determinant of community and ecosystem processes. Thus, preliminary results of this thesis did benefit TreeDì at the stages of project development and defense.

This dissertation work also acknowledges that the soil fungal community consists of very different life strategies so that the main fungal functional groups can underlie very different influences and assembly patterns. Analyzing fungal functional groups instead of the soil fungal community as a whole or just a single group is a recent approach, with the first databases on fungal functional group information being online since only a few years (Nguyen *et al.*, 2016) and still making manual literature research necessary. Mommer *et al.* (2018) even described this route of analysis to still be in its infancy. For the network analysis we developed a subsampling approach, to handle the limited number of plots sampled, which is a good example for handling this challenge within biodiversity studies.

D-4 Technical limitations of the study

Molecular methods, bioinformatics and statistical evaluation tools underlie a rapid development. This thesis was based on the PCR amplification of the ITS marker region followed by pyrosequencing. The results in this thesis are assumed to be solid and were generated with the best effort and conscience, applying many rounds of revisions to minimize biases. Still, the applied approaches and methods have some inherent limitations that should be considered for the interpretation of the generated results.

Soil is a heterogeneous habitat and care must be taken to achieve a representative sampling in respect to the aim of analysis. To achieve a representative soil sampling at each forest plot for each tree species, we collected five soil samples for each tree species across the plot. Sampling positions were randomly chosen. Each single soil sample itself constituted a composite sample from four soil cores extracted in cardinal compass direction around the respective sampling tree and was manually homogenized as best as possible. Currently, this approach is one of the standard procedures in soil ecology. However, the amount of the soil material for DNA extraction was very small. We extracted either 1g of freeze-dried soil once or 0.25g of freeze-dried soil twice, depending on the extraction kit applied. To ensure the representative DNA extraction of the collected soil sample, the number of extractions could be increased and pooled or even sequenced separately.

DNA extraction has its own inherent challenges. It captures not only DNA form active cells, but also from dormant (e.g. fungal spores) or dead cells and from extracellular DNA absorbed to the soil matrix particles originating of long deceased cells. Furthermore, cell lysis and DNA capture during extraction might be incomplete. Thus, our study based on

the DNA constitutes information about the potential microbial community in the soil. Kuzyakov and Blagodatskaya (2015) even argue that beside microbial "hotspots" and "hot moments" most microbial cells are inactive (Jones & Lennon, 2010; Carini *et al.*, 2017; Couradeau *et al.*, 2019). For the EcM fungi, we could on one hand detect the actively growing fungi that showed increased relative abundances at EcM trees, while the detection of some EcM fungal taxa at the AM trees indicated that those were rather related to the EcM propagule bank deposited in the soil originating from the previous forestry plantation (data not shown in the published manuscripts).

The selected marker region (ITS) is currently viewed as the standard tool for fungal community analysis. Although it is used as a "general" fungal marker, some (or even a lot) fungal species or lineages might escape detection and be underrepresented in the resulting dataset (Peay *et al.*, 2016). Furthermore, the sensitivity of the ITS region alone might not efficiently discriminate between all fungal taxa and some identical sequences might belong to in fact different fungal taxa (Kõljalg *et al.*, 2013). We determined fungal taxa by defining "operational taxonomic units" as sequences clustered that differed at 3% of each other. There are many sequence clustering algorithms available and there is the common challenge of overestimating species richness with these methods. Therefore, several quality filtering steps were applied to reduce spurious fungal OTUs as best as possible. Identification of fungal OTUs to named species or phylogenetic levels is especially challenging in the soil habitat as current databases do not sufficiently capture the vast diversity of fungi and we could classify only about 50% of the fungal OTUs to the genus taxonomic level (Weißbecker *et al.*, 2018).

D-5 Future research directions in soil fungal ecology

In the field of nucleic acid high-throughput sequencing there are many developments going on to overcome the above mentioned biases (Nilsson *et al.*, 2019). At the time of writing, pyrosequencing was already abandoned since several years. The current main sequencing platforms are offered by Illumina, PacBio and Nanopore industries and rely on distinct sequencing mechanisms. In general, developments try to achieve a greater sequencing depth, longer read fragments and improved sequencing error rates. Some techniques even do not need the PCR amplification step. New analysis tools like DADA2 (Callahan *et al.*, 2016) overcome the clustering of OTUs. This eliminates the bias of

species overestimation and enables the comparison of results (sequence variants) across studies.

Ecological studies should be formulated as more integrated research projects covering a multitude of organisms and trophic levels, and monitoring of associated functions. The soil environment can also be seen as a holobiont (Morris, 2018), with a variety of micro-, meso- and macro-organisms living together in close proximity and conferring ecological functions in concert. This could be achieved by including multiple organisms from above and belowground, prokaryotes and eukaryotes, in one network analysis. Morriën *et al.* (2017) for example combined observations and DNA-based identifications to infer a multi-organism network including spiders, earthworms, Enchytraeids, nematodes, plants, protists and microorganisms like bacteria, archea and fungi. Machine learning approaches could help to evaluate these complex networks. For example, Derocles *et al.* (2018) reports of a study by Tamaddoni-Nezhad *et al.* (2015) that could discover that 'big things eat small things' directly from a simulated, synthetic food web by a logic-based machine learning approach, called meta-interpretative learning.

Last but not least, enlarging the high-throughput sequencing of a marker gene to the scale of meta-omics (like metagenomics, metatranscriptomics and metaproteomics) will enable a more functional view on the investigated soil communities. Understanding the complex mechanisms of soil community assembly and mediated ecosystem functions across spatial and temporal scales will be an important factor to develop strategies to mitigate the effects of global change and protect soils and species.

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DATA AVAILABILITY

All manuscripts were published open access. The manuscript-related information (sequencing raw data, metadata of sampling location and samples, bioinformatically processed sequences, statistic R scripts) are available in public repositories and can be found by the digital object identifier (DOI) stated in the publications. Questions regarding the published analyses were always answered comprehensively.

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Oral presentations

Lachmann C, Wubet T, Bönn M, Pei Z, Buscot F. (2012): Patterns of soil fungal communities in subtropical Chinese forests in relation to plant diversity.; Jährliches Treffen der Gesellschaft für Ökologie Deutschland, Österreich und der Schweiz (42. GfÖ), 10. -14. September 2012, Lüneburg (Germany)

Poster presentations

Lachmann C, Wubet T, Bönn M, Buscot F (2012): Effective preservation of nucleic acids by freeze-drying for 454 sequencing analysis of soil microbial communities.; Jährliches Treffen der Gesellschaft für Ökologie Deutschland, Österreich und der Schweiz (42. GfÖ), 10. -14. September 2012, Lüneburg (Germany)

Lachmann C, Wubet T, Pei Z, Buscot F (2013): Patterns of soil fungal communities in subtropical Chinese forests in relation to plant diversity.; International Conference on Mycorrhiza (ICOM7), 06. – 11. Januar 2013, New Delhi (India)

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Weißbecker C, Heintz-Buschart A, Wubet T, Bruelheide H, Buscot F (2018): Fungal networks in subtropical Chinese forests.; Annual Conference of the Association for General and Applied Microbiology (VAAM), 17.-20. März 2018, Mainz (Germany)

STATUTORY DECLARATION

Hereby, I, Christina Weißbecker, confirm that I took note and accept the doctorate regulations of the Faculty of Life Science, Pharmacy and Psychology of the University of Leipzig from April, 29th 2015.

Furthermore I affirm that the presented thesis was prepared by me without inadmissible help. All aids used in this thesis and scientific ideas originating from other sources were cited at the respective point. All people that helped me to develop ideas, supported the field and lab work, and writing are named in the acknowledgments.

With exception of the namely mentioned people, no other persons were involved in the intellectual work. No PhD consultant service was employed. A language editing service (Sees-editing Ltd , 3 St. Peter's Avenue, Weston-Super-Mare, North Somerset, BS23 2JU, United Kingdom) was employed on the manuscripts prior to manuscript submission as was recommended by the international journals for non-native English speaking authors. Third parties did not receive benefit in money´s worth in conjunction with the content of this dissertation.

I declare that this dissertation has not been presented nationally or internationally in its entirety or in parts to any institution for the purpose of dissertation or other official or scientific examination. This is my first submission of a doctorate thesis, no unsuccessful dissertation attempts took place in the past.

The original document of the verification of the co-author parts are deposited in the office of the dean.

Leipzig, October 2019

Christina Weißbecker

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, Christina Weißbecker, dass ich die Promotionsordnung der Fakultät für Lebenswissenschaften, Pharmazie und Psychologie vom 29. April 2015 vollständig zur Kenntnis genommen und anerkannt habe.

Ich versichere weiterhin, dass diese Doktorarbeit von mir autonom und ohne unzulässige Hilfe angefertigt wurde. Alle verwendeten Hilfsmittel zur Erstellung dieser Doktorarbeit sowie wissenschaftliche Ideen entlehnt aus anderen Quellen, sind eindeutig an gegebener Stelle kenntlich gemacht. Alle Personen, die mich bei der Entwicklung meiner wissenschaftlichen Ideen, der Feld- und Laborarbeit sowie dem Schreiben unterstützt haben, sind in der Danksagung namentlich aufgeführt.

Mit Ausnahme der namentlich genannten Personen, waren keine weiteren Personen an der intellektuellen Arbeit im Rahmen dieser Dissertation beteiligt. Es wurde keine Hilfe eines Promotionsberaters in Anspruch genommen. Die wissenschaftlichen Veröffentlichungen in dieser Dissertation wurden vor dem Einreichen bei den internationalen Zeitschriften durch eine professionelle und bezahlte Spracheditierung korrigiert (Sees-editing Ltd , 3 St. Peter's Avenue, Weston-Super-Mare, North Somerset, BS23 2JU, United Kingdom) gemäß der Empfehlung der Zeitschriften für Autoren, deren Muttersprache nicht Englisch ist. Drittanbieter haben von mir weder mittelbar noch unmittelbar geldwerte Leistungen erhalten, die im Zusammenhang mit dieser Arbeit stehen.

I versichere, dass diese Doktorarbeit weder national noch international in ihrer Ganzheit oder in Teilen an einer anderen Institution zum Zweck der Dissertation oder einer anderen wissenschaftlichen Prüfung vorgelegt wurde. Es fanden keine vorigen erfolglosen Promotionsversuche statt.

Die Originalbelege über die Anteile der (Co-) Autorenschaften sind im Dekanat der Universität Leipzig hinterlegt.

Leipzig, Oktober 2019

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Verification of author parts:

Title:	**Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities**
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- Sample drawing and preparation
- Data analysis and interpretation
- Manuscript writing

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- Conception and study design
- Manuscript revision

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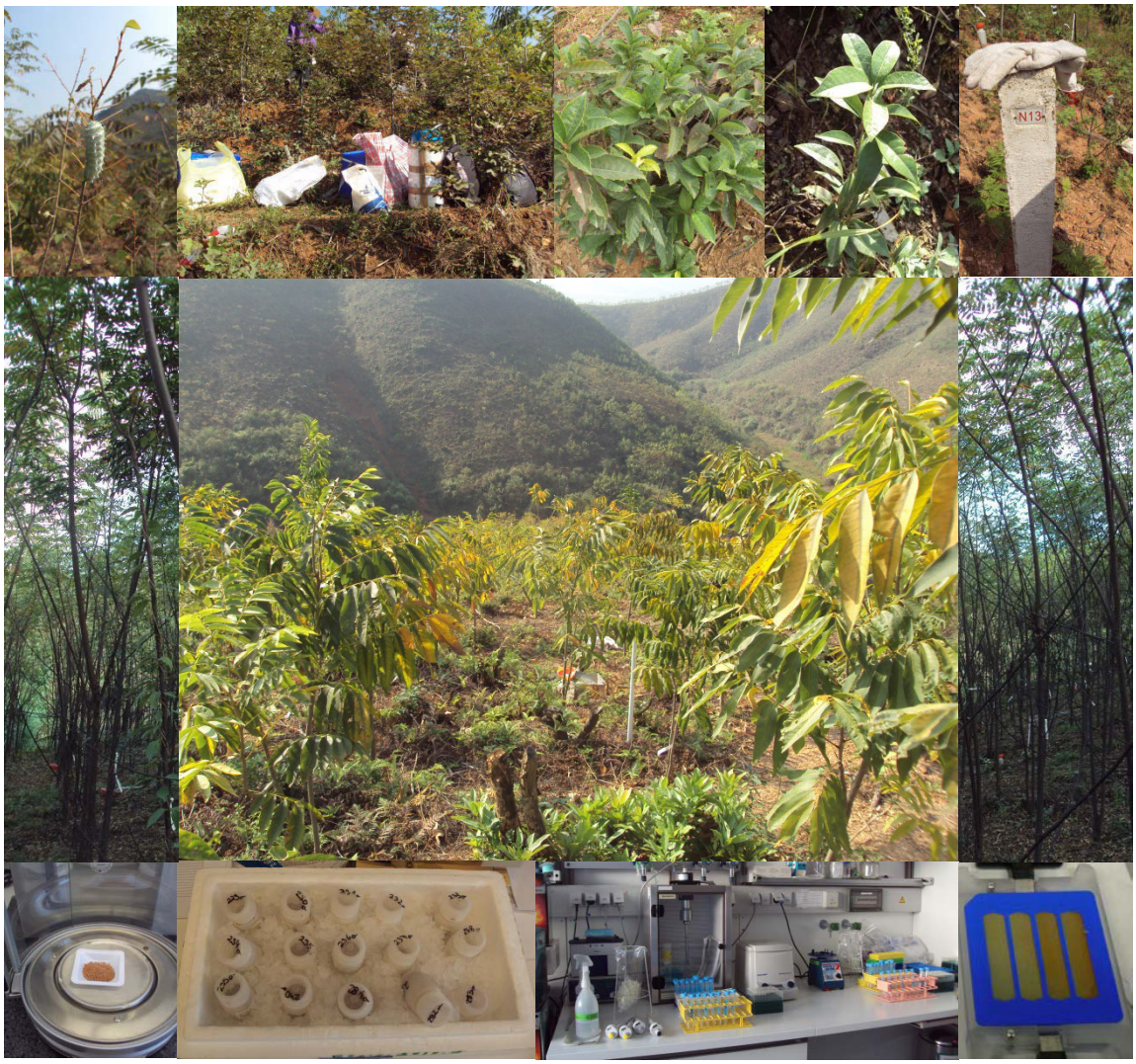
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