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ORIGINAL ARTICLE

Phylogenetic and functional traits of ectomycorrhizal assemblages in top soil from different biogeographic regions and forest types

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Abstract Ectomycorrhizal (EM) fungal taxonomic, phylogenetic, and trait diversity (exploration types) were analyzed in beech and conifer forests along a north-to-south gradient in three biogeographic regions in Germany. The taxonomic community structures of the ectomycorrhizal assemblages in top soil were influenced by stand density and forest type, by biogeographic environmental factors (soil physical properties, temperature, and precipitation), and by nitrogen forms (amino acids, ammonium, and nitrate). While α-diversity did not differ between forest types, β-diversity increased, leading to higher γ-diversity on the landscape level when both forest types were present. The highest taxonomic diversity of EM was found in forests in cool, moist climate on clay and silty soils and the lowest in the forests in warm, dry climate on sandy soils. In the region with higher taxonomic diversity, phylogenetic clustering was found, but not trait clustering. In the warm region, trait clustering occurred despite neutral phylogenetic effects. These results suggest that different forest types and favorable environmental conditions in forests promote high EM species richness in top soil presumably with both high functional diversity and phylogenetic redundancy, while stressful environmental conditions lead to lower species richness and functional redundancy.

Keywords Beech (*Fagus sylvatica*) · Exploration type · Ectomycorrhiza · Drought · Nitrogen · Spruce (*Picea abies*) · Pine (*Pinus sylvestris*)

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Introduction

Mycorrhizal fungi are an important component of belowground biodiversity and required for maintaining ecosystem multifunctionality (Wagg et al. 2014). In temperate forest ecosystems, ectomycorrhizal (EM) fungi constitute the prevalent form of fungal symbiosis with huge taxonomic diversity (Tedersoo et al. 2010). EM fungi colonize the root tips of their hosts forming an ectomycorrhiza and pervade the soil with a dense network of extramatrical hyphae. These hyphae mine the soil for mineral nutrients, which they trade for plant-derived carbohydrates (Habib et al. 2013). The emanating extramatrical fungal hyphae of the EM fungi enlarge the soil volume accessible for host nutrient acquisition and overcome nutrient depletion zones (Cairney 2011). Thus, EM fungi constitute a direct link for matter flux between the above- and belowground compartment. Moreover, they provide important ecosystem functions such as soil carbon sequestration and nutrient cycling

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(Godbold et al. 2006; Clemmensen et al. 2013, 2015; Phillips et al. 2014; Bödeker et al. 2014; Valtanen et al. 2014).

Different fungal taxa forage in different soil volumes because the morphology of hyphae differs among EM species (Rosling et al. 2003; Weigt et al. 2011; Agerer et al. 2012). Hyphal morphology has been used to classify EM taxa according to their soil exploration strategy as long distance, medium distance, medium distance fringe, short distance, or contact exploration types (Agerer 2001). Hyphal morphology is an important trait because it determines access to distinct nutrient sources, for example to nitrogen (N) (Hobbie and Agerer 2009; Tedersoo et al. 2012; Pena et al. 2013; Tedersoo and Smith 2013). EM fungi without or with short emanating hyphae mainly use soluble N forms such as amino acids, ammonium, and nitrate in the vicinity of the plant mycorrhizosphere, while medium and long distance EM fungi proliferate in the organic layer and access insoluble N substrates (Lilleskov et al. 2002; Hobbie and Agerer 2009; Tedersoo and Smith 2013); long distance mycelial fungi possess cords and rhizomorphs that enable an efficient long distance transport of nutrients (Cairney 1992).

While the taxonomic structure of EM communities has often been studied across a wide range of ecosystems and environmental conditions such as forest type (Buée et al. 2005; Courty et al. 2005), climate, soil properties (Courty et al. 2008; Tedersoo et al. 2010; Lang et al. 2011), nitrogen (Lilleskov et al. 2002; Kranabetter et al. 2009; Cox et al. 2010), and biomes (Bahram et al. 2015), EM exploration types have rarely been addressed (but see Pena et al. 2013; Pena and Polle 2014; Moeller et al. 2014). Studies on trait diversity across larger biogeographic areas are missing.

Here, we used the "Biodiversity Exploratories," a large scale infrastructure in three biogeographic regions along a north-to-south gradient in Germany (Fischer et al. 2010), to determine phylogenetic and functional trait variation of EM community structures in relation to environmental factors. The three Exploratories, Schorfheide-Chorin (SCH), Hainich-Dün (HAI), and Schwäbische Alb (ALB), differ in edaphic and climatic conditions. The climate in SCH is warmer and drier than in HAI and ALB; in the ALB region, the climate is moister and cooler than in the other areas (Fischer et al. 2010). The SCH soils are sandy with low pH, while those in the ALB and HAI regions have high silt and clay contents with higher pH (Fischer et al. 2010). Because the structure of the forest floor differs among the sites, the present study was conducted with top soil to avoid confounding effects of different soil layers. Because high nitrogen availability decreased EM species richness (Lilleskov et al. 2002; Kranabetter et al. 2009; Cox et al. 2010; Horton et al. 2013), the impact of inorganic and organic soluble N compounds on EM community structure was considered. In each region, we selected two forest types. One is dominated by the deciduous European beech (*Fagus sylvatica*) and the other by conifers (*Picea abies* in HAI and ALB and *Pinus sylvestris* in SCH). We determined the soil extractable nitrogen compounds (nitrate, ammonium, amino acids) and the EM fungal species associated with the roots in the top soil. We hypothesized that (i) EM species richness in top soil does not vary among large-scale biogeographic areas because the host species form similar habitats for root colonization, but that (ii) biogeographic factors, plant-available soil nitrogen, and forest type drive the taxonomic structures of the EM communities and trait diversity, and that (iii) forests with high phylogenetic diversity of EM assemblages exhibit functional overdispersion compared to forests with phylogenetic clustering.

Materials and methods

Study areas—Exploratories

The study was conducted in 30 forests located in three regions in Germany as part of the Biodiversity Exploratories program (www.biodiversity-exploratories.de) (Table 1). In each forest, an experimental plot had been established, for which encompassing data collections are available including continuous recordings of weather data, soil analyses, data for plant diversity and composition, and for forest management (Fischer et al. 2010; Schall and Ammer 2013; Boch et al. 2013; Herold et al. 2014; Solly et al. 2015). We used geographic, weather and climate data (mean annual temperature, sum of annual precipitation, mean soil temperatures in 10 cm depth from April to September 2010, mean air temperatures from April to September 2010 at 2 m height, elevation, and geographic position), data for the vascular plant diversity in forest experimental plots 2009 (number of herbaceous plant species, shrub and tree species, percentage ground cover by each plant group), tree species composition (percentage of conifers and European beech as basal stem area per total area), a standardized forest management intensity index (SMI) (Schall and Ammer 2013), and information on soil properties (C and N in the organic layer and in the mineral top soil, fractions of sand [coarse, medium, fine], silt [coarse, medium, fine], clay, and pH, Herold et al. 2014). Soil humidity and the concentrations of extractable soil amino acids, nitrate and ammonium were determined in soil samples collected for this study (see below). All data are available in the BEXIS database (https://www.bexis.uni-jena.de).

Sampling and sample processing

In each plot, five soil samples were collected in each corner and in the center of a 20 m × 20 m area. The upper layer with coarse decomposing material was removed. The organic layer with strongly decomposed material (Oa) had a depth of 0.03 m in SCH forests and was absent in the other forests. To avoid confounding effects of different contributions of different soil

Table 1 Location, climatic conditions, and sampling design in three biogeographic regions (Exploratories) in Central Europe (Germany). Source: http://www.biodiversity-exploratories.de/exploratories

Region	Longitude east-to-west	Latitude north-to-south	Size (km^2)	Elevation above sea level (m)	Mean annual temperature (°C)	Mean annual precipitation (mm)	Plots with beech	Plots with conifers
ALB	9.58024–9.02362	48.53435–48.34996	315	462–858	6.5–8.0	938–963	6	3
HAI	10.77917–10.17332	51.37872–50.93735	1561	300–400	6.5–7.5	750–800	9[a]	3
SCH[b]	14.14796–13.39094	53.22390–52.79023	1300	10–140	8.0–8.4	520–600	5	4

[a] Three beech forests were managed as selection forests, the other as age class forests

[b] Conifers were spruce (*Picea abies*) in ALB and HAI, and pine (*Pinus sylvestris*) in SCH

layers, the Oa layer was also removed. The soil was sampled using a motor-driven auger (diameter 8.3 cm, length 1.0 m, Eijkelkamp, Giesbeek, The Netherlands). Subsequently, the soil column was separated by soil horizons. For this study, samples of the A_h horizon were used. The A_h horizon, also called top soil, has a coarser texture and a darker color than the lower horizons because it contains humified organic matter and is characterized by high biological activity (Wilkinson and Humphreys 2005). All five top soil samples of each plot were pooled to obtain one composite sample per plot. An amount of 200 g was stored at 4 °C until further processing in the laboratory.

All roots of each soil sample were removed, gently washed, weighed, and stored at 4 °C between moist tissue papers. Fresh soil was sieved (2 mm mesh) and mixed carefully. Aliquots of 4 g fresh soil were collected to determine soil water content by weighing the soil samples before and after drying at 105 °C until no further weight loss was detected.

Further aliquots of fresh soil (about 40 g) were extracted by mixing for 10 min in 40 ml 1 mM $CaCl_2$. The slurry was filtered through a fluted filter (MN 280, Macherey-Nagel, Düren, Germany) for 1 h. If the filtered solution still contained small particles, it was filtered through a glass fiber filter (pore size 1 μm, PALL Life Sciences, Ann Arbor, USA) and then through a sterile filter (pore size 0.2 μm, Sarstedt, Nümbrecht, Germany). If the solution was clear, it was directly filtered through the sterile filter. The volume of the filtrate was determined, freeze-dried, and dissolved in 0.5 ml double-deionized water. The concentrated filtrate was used for the determination of nitrate, ammonium, and amino acids.

Root analyses and determination of ectomycorrhizal morphotypes

The roots were inspected under a compound microscope and sorted according to the target tree species (after Lang et al. 2011). The fresh mass of the target root sample was determined. All root tips of each target root sample were counted and classified as vital mycorrhizal, vital non-mycorrhizal, or non-vital. Non-vital root tips were distinguished from vital root tips by their shrunken, shriveled, and dry appearance. Earlier physiological studies showed that these root tips were inactive for N uptake (Pena and Polle 2014). The vitality index (VI) was calculated as follows:

$$\mathrm{VI} = (\text{vital non-mycorrhizal root tips} + \text{vital mycorrhizal root tips}) \times 100/(\text{total number of root tips})$$

Mycorrhizal colonization (MC) was calculated as follows:

$$\mathrm{MC} = (\text{vital mycorrhizal root tips}) \times 100/(\text{vital non-mycorrhizal root tips} + \text{vital mycorrhizal root tips})$$

Vital ectomycorrhizal root tips were further subjected to morphotyping according to a simplified scheme after (Agerer 1987–2012) on the basis of color and texture of the ectomycorrhizal mantle, branching, abundance of external hyphae, and rhizomorphs under the compound microscope (Stemi SV 11, Zeiss, Jena, Germany). The number of root tips colonized by each morphotype was recorded for the whole target root sample.

About 10 to 20 root tips of each morphotype were collected and stored frozen at −80 °C for molecular analysis. The non-target roots and the target roots were dried at 60 °C for 1 week and used to determine the dry mass.

Determination of the mycorrhizal species and exploration types

The frozen morphotype samples were ground (Type MM2, Retsch, Haan, Germany) and used for DNA extraction with Innu PREP Plant DNA Kit (Analytik Jena, Jena, Germany) according to the manual. For ectomycorrhizal analyses, the internal transcribed spacer (ITS) region of the fungal rRNA was amplified by using the primers ITS1-F (5′CTTGGTCA TTTAGAGGAAGTAA-3′) and ITS4 (5′TCCTCCGC TTATTGATATGC-3′) (MWG, Biotech, Ebersberg, Germany) after Gardes and Bruns (1993) and White et al. (1990). The polymerase chain reaction (PCR), cloning, cleaning, and labeling with the Big Dye Terminator Kit (Applied Biosystems Foster City, USA) were performed as described before (Lang et al. 2011). The ITS regions were sequenced on an ABI Prism 3100 Genetic Analyser (36 cm capillary, Matrix Pop 6, Applied Biosystems Foster City,

USA). For fungal identification, Basic Local Alignment Search Tool (BLAST) searches were carried out against the National Center for Biotechnology Information (NCBI) and UNITE public sequence databases (http://www.ncbi.nlm.nih.gov/, http://unite.ut.ee). Sequences were assigned matching species names when the BLAST matches showed sequence identity higher than 97% and a bit score higher than 800. If no appropriate match was found, the sequence was assigned to the next higher level taxonomic name followed by its morphotype number (e.g., Russula sp._MT213). The sequences have been deposited in NCBI GenBank (for accession numbers, see supplement, Table S1). Exploration types of the species were determined after Agerer (2001), Lilleskov et al. (2002), and the DEEMY database (www.deemy.de/) and listed in Supplement Table S1. In the further statistical analyses, the exploration type was used as a fungal trait.

Analysis of nitrate, ammonium, and amino acids

Nitrate and ammonium were determined spectrophotometrically (Shimadzu UV 1602) using commercial kits (Nitrate Test, Ammonium Test, Spectroquant, Merck, Germany) according to the instruction of the company.

Amino acids were determined by high pressure liquid chromatography as described by Tilsner et al. (2005). Briefly, amino acids and ammonium were derivatized with o-phthaldialdehyde and separated on a reversed phase RP 18 end-capped column (Merck, Darmstadt, Germany) with a gradient of phosphate buffer and acetonitrile. Data acquisition and processing were accomplished with the Peaknet software (version 5.2, Dionex, Idstein, Germany).

Data analyses

Venn diagrams including all species data for all plots ($n = 30$) were generated with a Venn diagram generator (http://www.bioinformatics.lu/venn.php). Diversity analyses were conducted with the PAST free software package 2.17c (http://folk.uio.no/ohammer/past/, Hammer et al. 2001). The number of available beech respective conifer plots per region was not balanced (Table 1). Because diversity analyses are sensitive to differences in sampling intensity, we used $n = 5$ plots for beech using plots with similar number of root tips and $n = 3$ for the conifers for each region to compare diversities. Because of different numbers of root tips in our samples, we rarefied species number for $n = 300$ roots tips per sample. The correlation between measured and rarefied data was strong and the slope close to 1 ($R = 0.985$, slope 0.90 ± 0.03, $P < 0.001$). Because of the absence of significant deviations from linearity, we used the measured species data for our analyses. The nearest taxon index (NTI) and standardarized trait variation (SESmetric) were calculated with the software PHYLOCOM v 4.2 using the COMSTRUCT function (http://phylodiversity.net/phylocom/, Webb et al. 2008). The phylogenetic tree (Newick format) was reconstructed from the ITS rDNA sequences using MEGA5 software (Tamura et al. 2004). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004).

For the comparison of means, data were tested for normal distribution (variance check by Levene's test) and analyzed by a two-factorial ANOVA (STATGRAPHICS Centurion, Statistical Graphics Corp., Warrenton, USA). When significant models were obtained, a *post hoc* test (Fischer's test) was conducted. Means were considered to differ significantly when $P < 0.05$. When normal distribution was not achieved by log transformation, the Mann-Whitney or Kruskal-Wallis test was applied to compare samples.

A data matrix was compiled for the ordination of abundance data of EM taxa and fungal traits (long distance [LD], short distance [SD], medium distance [MD], medium distance fringe [MDF], contact [CO], and unknown [unk]). To reduce the number of explanatory variables, the parameters were grouped according to functional categories: soil (with the parameters specified above), climate ([Clim] with geographic location, climate, and weather data as specified above), vegetation ([Veg] with the parameters specified above), forest management ([FM] with number trees per hectare, ground area of stems per hectare, percentage of basal stem area of beech, percentage of basal stem area of conifers, management indices SMI_d, SMI_r, and SMI obtained from Schall and Ammer 2013), root data ([R] with vitality, mycorrhizal colonization, root tip numbers, root mass in the soil sample, root mass of the target tree in the soil), and soluble nitrogen compounds in soil ([SolN] with aspartate (asp), glutamic acid (glu), asparagine (asn), serine (ser), histidine (his) glutamine (gln), glycine (gly), threonine (thr), arginine (arg), alanine (ala), tyrosine (tyr), valine (val), isoleucine (ile), leucine (leu), lysine (lys), phenylalanine (phe), γ-amino butyric acid (gaba), sum of amino acids, nitrate, ammonium). For each group, a principle component analyses were conducted using normalized data (PAST, Hammer et al. 2001), and scores and loadings are compiled in Supplement Table S2. Significant principle components (PCs) were determined by Scree plots with broken stick analysis. EM species abundance data (counts of root tips per EM species) and trait abundance data (counts of root tips per exploration type) were ordinated by non-metric multidimensional scaling (NMDS) based on the Morisita metrics (PAST, Hammer et al. 2001) using the significant PCs of each functional group as explanatory vectors.

To compare the similarities of taxon-based community structures or trait-based community, structures analyses of similarities (ANOSIM) were conducted using the Morisita similarity index and 9999 permutations (PAST, Hammer et al. 2001).

Factors related to EM species richness were analyzed by testing linear models with the environmental variables for vegetation, management intensity, climate, nitrogen, and soil

properties reported above as continuous fixed factors using the standard statistical function *lm ()* in R (R Core Team 2015). The assumptions of linearity between EM species richness and the independent predictive variables and data homoscedasticity were addressed by visual inspection of the residual plot (residuals versus fitted value). To address potential collinearity among the predictive variables, the variance inflation factor (VIF) was calculated for each predictor. The highest VIF predictor variables were sequentially dropped, recalculated, and repeated until all VIF values were below the threshold (i.e., VIF <10, Montgomery and Peck 1992). The procedure was run using the R package "usdm" (Naimi 2015). The number of non-collinear variables used in the model was reduced from 62 to 41 (Table S3). The best model was stepwise selected based on the Akaike information criterion (AIC) function in the package "MASS" using direction "forward" (Venables and Ripley 2002).

Results

Mycorrhizal species richness in three biogeographic regions

Across all forest plots, the mean dry mass of the fine roots was 19.6 ± 4.0 g kg^{-1} of dry soil and contained 69.9% root mass of the target tree species. All root tips of the target tree species were inspected but showed no pronounced variation in vitality among the regions and forest types (mean VI = 42.2 ± 2.2%, $F_{5,24} = 2.38$, $P = 0.068$). Mycorrhizal colonization was lower on pine roots (86.6 ± 15.3%) than on beech (99.7 ± 0.5%) or spruce roots (99.6 ± 0.3%, Kruskal-Wallis test: $P = 0.008$).

All vital mycorrhizal root tips ($n = 21{,}628$) were morphotyped. Samples of each morphotype for each forest type and region were sequenced resulting in 75 putative EM species and 7 morphotypes, for which no sequences were obtained. The morphotypes that were not specified were rare, colonizing only 2.5% of the mycorrhizal root tips and were, therefore, excluded from further analyses. Rarefaction curves showed that our sampling strategy resulted in EM species saturation at the scale of the regions and forest types (Fig. 1a). Seven EM species were shared among the three regions (Fig. 1b). The shared species were relatively abundant taxa (*Cenococcum geophilum*, *Genea hispidula*, *Russula ochroleuca* and *Russula* sp._MT212 + S12, *Lactarius blennius*, *Tomentella sublilacina*, and *Xerocomus pruinatus*) colonizing altogether about 25% of the mycorrhizal root tips of the trees in HAI and ALB and 47% in SCH. Only *C. geophilum* was present in each forest type and region. Beech and conifers shared approximately 30% of the EM taxa, while 40% were only associated with beech and 30% with conifers (Fig. 1c). The shared species colonized about 50% of the beech and 37% of the conifer root tips.

For the comparison of diversity indices, we used a subset of the plots ($n = 24$ instead of 30) to achieve equal numbers of replicate beech and conifer forests per region. This reduction in sample size resulted only in the loss of one EM species (74 instead of 75). EM fungal species richness was higher in the HAI and ALB regions than in the SCH region but did not differ between forest types (Poisson regression, $Chi^2_{(region)} = 18.15$, $P = 0.001$, $Chi^2_{(forest\ type)} = 0.32$, $P = 0.572$, Fig. 2a). Overall species richness was highest in ALB and lowest in SCH (permutation test, Fig. 2a). The mean Shannon diversity of EM fungi was also higher in the ALB and HAI forests than in the SCH forests but did not differ between the forest types (ANOVA main factor region $F_{2,18} = 18.17$, $P = 0.003$, main factor forest type $F_{1,18} = 0.15$, $P = 0.707$, interaction $F_{2,18} = 0.31$, $P = 0.734$, Fig. 2b). To exclude that this result was biased by different numbers of replicates for beech ($n = 5$) and conifer forests ($n = 3$), we tested also for differences in diversities using the same number of plots ($n = 3$), neither resulting in significant differences between conifer and beech forests. At the scale of the region, the Shannon diversity of EM was lower in SCH than in ALB or HAI (Fig. 2b). Overall, evenness was 0.512 ± 0.003 and did not vary within beech or conifer forests or among the regions.

We tested whether EM species richness was explained by environmental variables (Table 2). The annual sum of precipitation, the density component of SMI, and the soil concentrations of gaba yielded a significant model explaining about 50% of the variation of EM species richness (Table 2). The coefficients show that EM species richness increased with increasing annual precipitation and decreased with increasing gaba and increasing SMI_d, the density component of SMI. SMI_d is high if the basal area of a given stand strongly deviates from the potential maximum basal area of the stand at that site (Schall and Ammer 2013). Thus, the less trees were removed, and the older a stand is, the lower is SMI_d.

Mycorrhizal communities are structured by forest type and abiotic factors

NMDS analysis separated the EM communities according to their similarities in three major groups, one was formed by the conifer and the second by beech forest EM fungi of HAI and ALB (axis 1). The third group consisted of EM communities of SCH (beech and conifers, axis 2) (Fig. 3a). Axis 1 was mainly explained by forest management parameters (FM1), whose main factors were the percentage of basal area of beech and conifers, respectively, and soluble nitrogen compounds (SolN 3 with nitrate and arginine, Fig. 3a, Supplement Table S4). The nitrate concentrations were highest in soil of the beech forests in the ALB and HAI areas, whereas ammonium was highest in SCH (Fig. 4a–c). In soils of conifer forests, the concentrations of the soluble nitrogen compounds exhibited intermediate levels (Fig. 4).

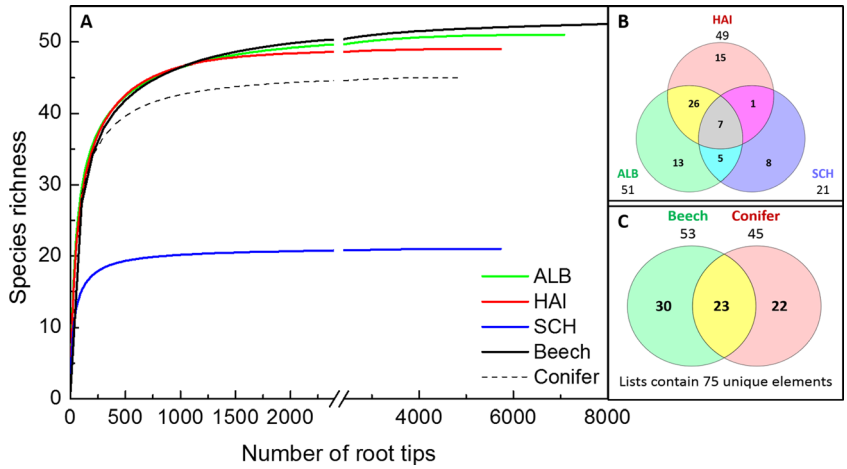

Fig. 1 Rarefaction curve of ectomycorrhizal species richness (**a**), species distribution among three biogeographic regions (**b**), and between conifers and beech as host species (**c**). Abbreviations indicate Schorfheide, SCH; Hainich, HAI; Schwäbische Alb, ALB

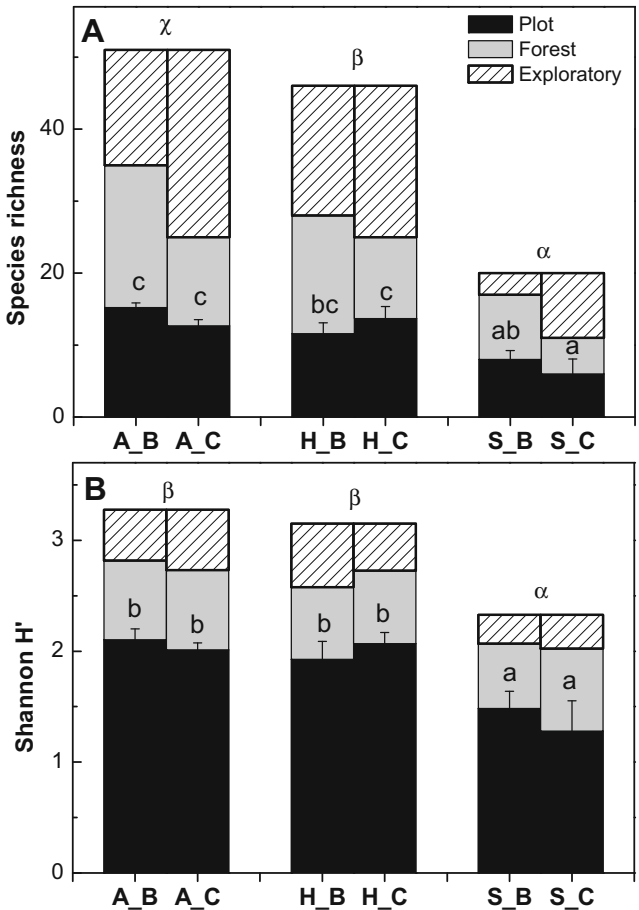

Fig. 2 Ectomycorrhizal fungal species richness (**a**) and Shannon index (**b**) in three biogeographic regions. Data indicate means of EM species richness per plot (α diversity), EM species richness per forest type in a region (β diversity), and cumulated species richness of a region (γ diversity) (C = conifer, B = beech, S = Schorfheide; H = Hainich; A = Schwäbische Alb). Mean values are indicated for $n = 5$ for beech forests and $n = 3$ for conifer forests per region. In each forest, all root tips in defined soil volume were analyzed. *Different letters* indicate significant differences with $P < 0.05$

Further vectors with strong relation to the EM communities of beech forests in HAI and ALB were Veg 2 with parameters related to area covered by woody species and FM_2 with SMI_d and number of stems (Fig. 3a, Supplement Table S4).

NMDS axis 2 was mainly explained by biogeographic variables (soil properties and climate) but was less important than axis 1. The EM community assemblages in ALB and HAI (negative direction of axis 2) were explained by soil 1 with silt (positive) and sand (negative) as main factors and Clim 1 with soil water content (positive) and annual temperate (negative) and Veg 1 (the number of vascular plants (positive) and cover sum of trees (negative), Fig. 3a, Supplement Table S4). EM community structures in SCH (positive direction of axis 2) were explained by SolN 2 [leucine (positive) and asparagine (negative), Fig. 3a, Supplement Table S4].

In addition to the taxonomic composition, we studied the composition of exploration types of the forest types and regions (Fig. 5). Contact and short distance exploration types were most abundant across all conditions, with the exception of beech forests in HAI, where medium distance exploration types were more abundant than short distance (Fig. 5). In contrast to the separation of the taxonomic composition of the EM fungal communities (Fig. 3a), the exploration type-based community structures could be distinguished less well

Table 2 Linear mixed model for EM species richness. Measured EM species richness = Annual.Precipitation.mm. + SMI_d + gaba

Parameter	Coefficient	SE	*t* value	Pr (>|*t*|)
Intercept	0.084	2.947	0.029	0.9774
Annual precipitation	0.020	0.004	5.647	6.15×10^{-6}
SMI_d	−7.466	3.109	−2.401	0.0238
gaba	−0.192	0.091	−2.112	0.0444

Adjusted $R^2 = 0.5325$, $F_{(3,26)} = 12.01$, P value 4.029×10^{-5}

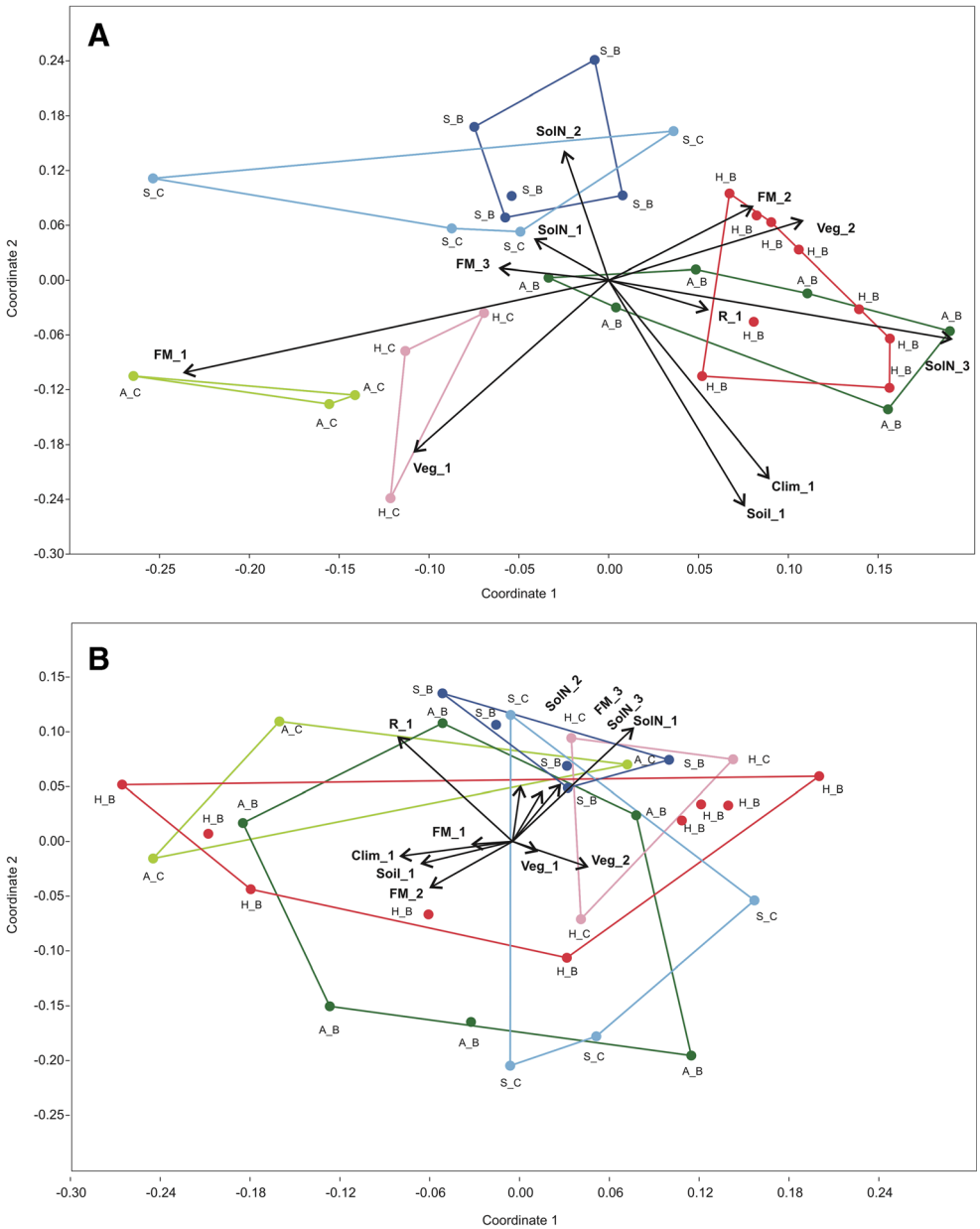

Fig. 3 Non-metric multidimensional scaling (NMDS) ordination for the taxonomic similarities (**a**) and trait similarities (**b**) of the ectomycorrhizal assemblages in conifer and beech forests in the biogeographic regions. The Morisita index was used a similarity measure. The scores of significant axis determined by PCA for the variables soil properties (Soil_1), climate (Clim_1), vegetation parameters (Veg_1, Veg_2), forest management parameters (FM_1, FM_2, FM_3), soluble nitrogen (SolN_1, SolN_2, SolN_3) and root parameters (R_1) are shown as explanatory variables. The data and the main factors are found in Table S4. Abbreviations: *S* Schorfheide, *H* Hainich, *A* Schwäbische Alb, *C* conifers, *B* beech

by NMDS and the explanatory power of the vectors with environmental information was generally low (Fig. 3b, Supplement Table S4). The positive direction of the first and second coordinate was determined by nitrogen (SolN 1 with sum of amino acids and nitrate) and the negative direction by root parameters and climate (Clim 1 with soil water content and annual mean temperature) for coordinate 1 and forest management parameters for coordinate 2 (Fig. 3b). Although the data (Fig. 5) suggest some differences in the abundances of the exploration types among regions and forest types, these could not be clearly specified by NMDS ordination.

Phylogenetic and trait signatures distinguish EM communities of different biogeographic regions

The EM communities of spruce in ALB and HAI exhibited taxonomic similarity, while all other community structures differed significantly from each other (ANOSIM, Table 3). Since taxonomically different communities can be composed of phylogenetically closely related species, with presumably similar functions, we also investigated the phylogenetic community structures of EM communities by the NTI (Fig. 6a). Differences of the NTIs among regions or forest types were not observed (main effects: region $F_{2,29} = 1.85$, $P = 0.179$, forest type $F_{1,29} = 0.91$, $P = 0.348$, interaction $F_{2,29} = 0.58$,

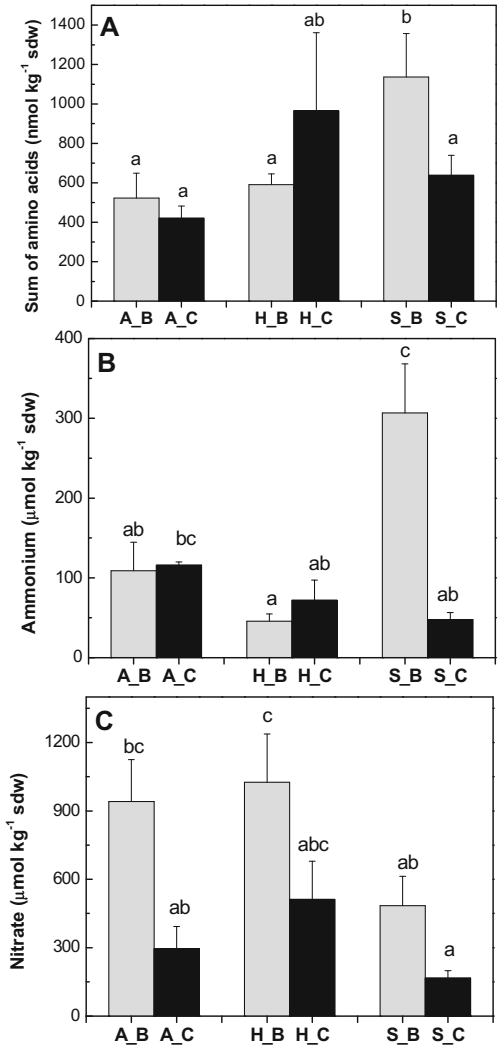

Fig. 4 Soil extractable concentrations of amino acids (**a**), ammonium (**b**), and nitrate (**c**). Data indicate means for $n = 5$ to 9 beech plots and $n = 3$ to 4 conifer plots per region. *Different letters* indicate significant differences with $P < 0.05$. Abbreviations: *S* Schorfheide, *H* Hainich, *A* Schwäbische Alb, *C* conifers, *B* beech

$P = 0.566$, Fig. 6a). When the null hypothesis was tested combining all forests per region, NTI of EM species in the ALB forests was significantly greater than zero ($P = 0.019$) indicating phylogenetic clustering of the EM taxa of the ALB forests (Fig. 6a). NTIs of EM assemblages in HAI and SCH indicated neutral effects (Fig. 6a).

ANOSIM using exploration types as trait revealed similarity among most of the EM communities but showed that traits of the EM communities from SCH conifers differed from those of ALB beech and SCH beech (Table 3). Analyses of standardized trait variation (SESmetric) revealed differences among the regions (SCH ≠ HAI and ALB), but not between the forest types (main effects: region $F_{2,29} = 11.05$, $P = 0.0004$, forest type $F_{1,29} = 0.00$, $P = 0.985$, interaction $F_{2,29} = 1.35$, $P = 0.278$, Fig. 6b). Test of the null hypothesis for the SESmetrics revealed trait clustering in the SCH

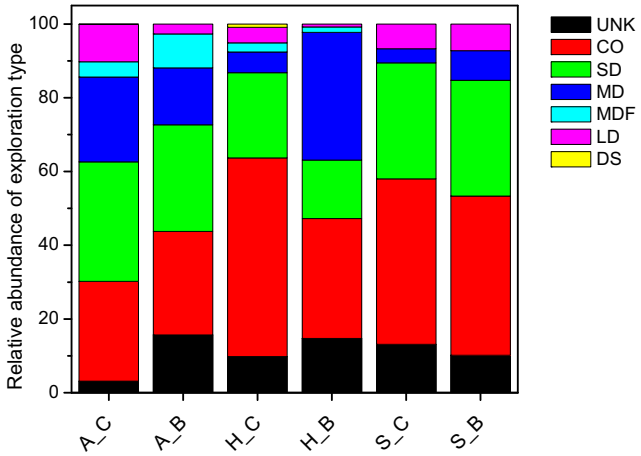

Fig. 5 Relative abundance of ectomycorrhizal exploration types according to region (*A* ALB, *H* HAI, *S* SCH) and forest type (*C* conifer, *B* beech). *UNK* unknown, *CO* contact, *SD* short distance, *MD* medium distance, *MDF* medium distance fringe, *LD* long distance, *DS* dark septate

exploratory ($P = 0.012$) and marginal trait overdispersion in ALB ($P = 0.059$, Fig. 6b).

Discussion

Environmental filtering and forest management impact on ectomycorrhizal species richness

Our initial expectation was that EM fungal species richness on roots in top soil was similar among the three study regions, but this hypothesis had to be rejected because the EM assemblages on root tips of trees in the SCH region were less species-rich than the ones in the ALB and HAI regions. This finding is consistent with reports on lower diversity of soil-residing fungi in SCH than in ALB and HAI (total fungal richness in top soil: Wubet et al. 2012; mycorrhizal fungal richness in top soil: Goldmann et al. 2015). Since roots are colonized from the fungal reservoir in soil (Danielsen et al. 2012; Goldmann et al. 2016), the

Table 3 Similarities of EM species composition (upper part) and EM trait composition (lower part). The comparisons were conducted by ANOSIM based on Morisita indices

	EM Species					
	A_C	A_B	H_C	H_B	S_C	S_B
A_C		*0.0124*	0.6118	*0.0053*	*0.0280*	*0.0177*
A_B	0.5931		*0.0116*	*0.0093*	*0.0043*	*0.0023*
H_C	0.2993	0.4445		*0.0047*	*0.0306*	*0.0210*
H_B	0.4725	0.3340	0.7819		*0.0013*	*0.0005*
S_C	0.1441	*0.0338*	0.2332	0.1047		*0.0093*
S_B	0.2627	0.0889	0.7925	0.1848	*0.008*	

Significant *P* values are shown in italicized numbers

S Schorfheide, *H* Hainich, *A* Schwäbische Alb, *C* conifers, *B* beech

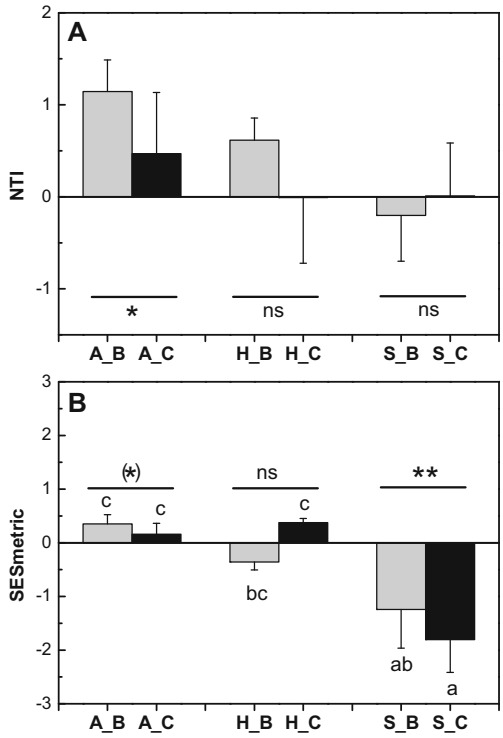

Fig. 6 Phylogenetic (**a**) and trait relatedness (**b**) in beech and conifer forests in three biogeographic regions. Phylogenetic relationship was determined as the nearest taxon index (NTI) and trait relationship as standardized trait variance (SESmetric) for the ectomycorrhizal community in each plot. Data indicate means for $n = 5$ to 9 beech plots and $n = 3$ to 4 conifer plots per region. *Different letters* above *bars* indicate significant differences between forest types and regions with $P < 0.05$. *Stars* above the *vertical line* indicate significant deviation from the null hypothesis ($*P < 0.05$, $**P > 0.01$). Abbreviations: *S* Schorfheide, *H* Hainich, *A* Schwäbische Alb, *C* conifers, *B* beech

correspondence of the fungal diversity between soil and root might have been expected. However, the "less-rich" fungal pool in top soil of SCH still contained almost tenfold higher species numbers of potential EM taxa (Goldmann et al. 2015) than those colonizing the roots (this study). Therefore, the reduction of species in the soil pool compared to other areas is a priori no reason for a lower EM species richness of the root-associated assemblages. The finding that both SCH root-associated and soil-residing EM fungal richness were lower than in ALB and HAI could either have been caused by the sampling method, which excluded the forest floor or might be the result of large-scale environmental factors that impeded EM diversity in SCH compared with the two other regions. Although the first possibility cannot be entirely ruled out, it is unlikely because other field and experimental studies across the whole profile including the organic layer in acid sand and calcareous silt/clay soils showed lower EM species richness in sandy than in other soil types (Leberecht et al. 2016; Zavišić et al. 2016). Our regression model suggests that water availability could be an overarching environmental factor affecting EM communities because a strong positive correlation of EM richness with annual precipitation was found. This assumption is further supported by the finding that summer drought decreased soil fungal diversity in heathlands (Toberman et al. 2008), and that experimental drought decreased EM species richness in sandy, acid as well as in silty, calcareous soil types (Leberecht et al. 2016).

Previous studies reported negative relationships between soil extractable N concentrations and EM species richness (Lilleskov et al. 2002; Avis et al. 2003; Cox et al. 2010), whereas the impact of phosphorus availability on EM richness is less clear (Horton et al. 2013; Teste et al. 2016; Zavišić et al. 2016). For example, EM species richness showed no correlation with increasing phosphorus availability in a gradient in soil similar to those of the present study with sandy as well as silt and clay-containing soils (Zavišić et al. 2016). Therefore, we focused here on nitrogen. The concentrations of NO_3^- in soil were higher in the ALB and HAI beech forests, while NH_4^+ was higher in the SCH beech forests, and both inorganic N forms were generally lower in conifer than in beech forests. These findings point to complex region- and forest type-specific effects, obviously precluding simple correlations of inorganic soluble N forms with fungal species richness. Remarkably, we found that the non-proteinogenic amino acid gaba was negatively correlated with fungal species richness of the colonized roots. Gaba is an abundant compound in the free amino acid pool of plant and fungal cells, has a role as a stress metabolite in plants, and can be induced by EM colonization (Luo et al. 2011). Whether external gaba has direct effects on the species richness of the EM communities or whether its accumulation in soil is a legacy of yet unknown physiological activities is unclear, but both possibilities open exciting routes for future research to better understanding the mechanisms governing the richness of EM fungi on roots.

In this study, EM fungal richness did not differ between host species, but together conifer and beech forests hosted higher EM fungal species richness at the scale of the biogeographic regions. In other words, while α-diversity did not differ between forest types, β-diversity increased, leading to higher γ-diversity on the landscape level if both forest types are present. This observation is in agreement with other studies in temperate forests showing that the presence of additional tree species increases EM species richness because of fungal host preferences (Ishida et al. 2007; Lang et al. 2011; Trocha et al. 2012). In our study, about one third of the EM taxa were specific for beech, one third for conifers, and one third were shared between different host species. The latter fraction of generalists colonized a large fraction of root tips (50%) in accordance with other studies (Lang et al. 2011; Bahram et al. 2013).

EM species richness was negatively correlated with the tree density component of the forest management index SMI. Since SMI_d is measured as the relative deviation of the actual basal ground area of the trees from the maximum natural basal ground area of a stand at a given site (Schall and Ammer 2013), our

findings suggest that old and densely stocked stands resulting in high living tree biomass foster higher EM species richness. For forest management practice, our results suggest that EM species richness could be enhanced by creating mixed stands which keep the growing stock permanently above a certain minimum as suggested for the uneven-aged forest management system (O'Hara and Gersonde 2004).

Taxonomic, phylogenetic, and trait structures of EM communities along a biogeographic gradient

The abundant as well as the rare fungal taxa identified here belong to genera typically present as mycorrhizas in temperate forests (Courty et al. 2008; Pena et al. 2010; Lang et al. 2011; Trocha et al. 2012; Bahram et al. 2013). With few exceptions (*Genea* sp., *C. geophilum*), the dominant species of the root-associated EM assemblages had also been detected by pyrosequencing among the main fungal genera in top soil (*Russula* sp., *Lactarius* sp., *Tomentella* sp., *Xerocomus* sp. (Boletales) (Wubet et al. 2012; Goldmann et al. 2015). While ordination analyses separated the soil-residing fungi according to study regions (Wubet et al. 2012), the main factors explaining the ordination of the root-associated EM fungi in our study were the relative host abundance in the cool-moist regions of ALB and HAI resulting in distinct beech and conifer clusters on the one hand and climate, soil properties and soluble nitrogen compounds on the other hand separating the EM communities of SCH from those of the other fungal assemblages. These findings support the conclusion that host and environmental selection are better predictors of soil fungal community composition than dispersal constraints, at least at large spatial scales (Danielsen et al. 2012; Kivlin et al. 2014; Goldmann et al. 2016).

Besides the taxonomic distinctness, the phylogenetic relationship of the taxa is an important character of communities because closely related species often share functional properties, whereas phylogenetically distant species are thought to exhibit different functional properties (Maherali and Klironomos 2007). Maherali and Klironomos (2007) reasoned that if environmental filtering influenced community assembly, then co-occurring species should share functional characteristics that enable survival in a particular habitat (phylogenetic clustering), whereas phylogenetically even communities were expected, if competition was the major force driving community assembly. Field and experimental data support this theory for the assembly of arbuscular mycorrhizal fungi (Maherali and Klironomos 2007, 2012), but data for EM fungi were lacking. An important result of our study was that the EM communities in different forest types showed neutral phylogenetic effects along the biogeographic gradient, proposing in agreement with other studies (Maherali and Klironomos 2012; Pickles et al. 2012; Lang et al. 2013) that effects such as competition or host control may have prevented co-existence of closely related species at small spatial scales. Interestingly, phylogenetic clustering occurred in the species-rich EM assemblage at the regional scale (ALB), suggesting that at larger spatial scales environmental filtering can superimpose local effects.

In an apparent contrast to the above theoretical considerations, phylogenetic clustering did not result in trait clustering, but trait dispersal. This result was obtained in the ALB region with the most species-rich EM assemblages suggesting that in the presence of a high number of taxonomically distinct species both ecological mechanisms are not mutually exclusive. In our study, the *Tomentella* genus showed diversity of exploration types (supplement Table S1), while the other EM fungi displayed trait constancy. *Tomentella* sp. occur in N-rich ecosystems and strongly accumulate nitrogen (Lilleskov et al. 2002; Pena et al. 2013). Therefore, these species may have contributed to trait dispersal in the N-rich forests in ALB. Furthermore, there can be high trait diversity among taxonomically more distant species, while at the same time additional, phylogenetically more closely related species are present. In line with this assumption, the less species-rich community exhibited strong trait clustering. Trait clustering indicates environmental filtering, which was apparently stronger in the warm-dry than in the cool-moist habitats.

The trait analyzed in our study was the mycelial exploration type, which is linked to the fungal taxon's ability to forage for nutrients in the soil and thereby overcome nutrient depletion zones (Read and Perez-Moreno 2003; Helmisaari et al. 2009; Hobbie and Agerer 2009). Numerous field and experimental evidence support a role of EM in N nutrition, and N availability is one of the key factors structuring EM assemblages (Lilleskov et al. 2002; Cox et al. 2010). Here, ordination analyses identified soluble nitrogen compounds as a major explanatory variable for trait differences. This finding supports the important role functional traits in the EM community may play to effectively respond to soil N resources (Tibbett et al. 1998; Lilleskov et al. 2002). Although we show that environmental filtering affects the functional structure of EM communities, the differences were less pronounced than those found for the taxonomic structures.

In conclusion, this study shows that EM fungal species richness is related to the amount of annual precipitation, tree species mixture, and stand density and highlights that apart from environmental filtering, silvicultural management opens opportunities to increase EM richness. Biogeographic factors and forest type affected the taxonomic EM composition in a complex way. In contrast to our initial hypothesis, the species-rich EM communities exhibited trait dispersion along with phylogenetically clustering, while the species-poor EM communities showed trait clustering and phylogenetically neutral effects. This suggests that at larger spatial scales, different ecological mechanisms can be active. The clustering of the exploration types indicated environmental filtering with reduced N compounds such as amino acids in the soil solutions as an important structuring factor.

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Compliance of ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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