Mycological Progress (2018) 17:925–939
https://doi.org/10.1007/s11557-018-1405-6

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ORIGINAL ARTICLE

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Fungal guilds are evenly distributed along a vertical spruce forest soil profile while individual fungi show pronounced niche partitioning

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Received: 26 June 2017 / Revised: 29 April 2018 / Accepted: 3 May 2018 / Published online: 19 May 2018

Abstract

Saprotrophic and ectomycorrhizal (EcM) forest fungi decompose organic matter and mobilize nutrients for host plants, respectively. Competition between the two guilds may cause the so-called Gadgil effect, i.e., decreased litter decomposition rates resulting in increased carbon storage in soil. The Gadgil effect was supposed to even affect global climate, highlighting the necessity to understand fungal distribution and interactions in soil. Searching for evidence of competition between saprotrophic and mycorrhizal fungi, we analyzed the distribution of fungi along a well-stratified vertical spruce forest soil profile in two seasons, i.e., autumn and the following spring. The different soil strata (i.e., two mineral horizons and two organic layers) underneath the litter layer were colonized by distinct fungal communities, which included roughly consistent proportions of all fungal guilds and phyla at each time. However, the community composition changed quantitatively between the sampling dates. Along the vertical soil profile, it differed mostly between the organic layers and the mineral soil, which is supposed to be due to differences in the predominant energy sources (i.e., aboveground litter and rhizodeposition, respectively). Network analyses revealed co-occurrences (i.e., positive correlations of individual abundances) to outweigh mutual exclusions (i.e., negative correlations) between individual fungi in each soil stratum and season. This also applied for interactions between saprotrophic and EcM fungi. Network analyses therefore provided no indications for a possible Gadgil effect. However, considering individual nutrient use efficiencies might refine insights from network analyses in future studies and facilitate linking community dynamics to ecosystem processes.

Keywords Ectomycorrhizal fungi · Saprophytic fungi · Co-occurrence networks · Seasonal shifts

Introduction

Forest soil fungi are key players in nutrient cycling and therefore essential for forest ecosystem functioning (Swift et al. 1979; Schneider et al. 2012b; Tedersoo et al. 2014). They decompose dead organic matter (Dix and Webster 1995; Boddy et al. 2008) and provide nutrients to living trees (Smith and Read 2008). These functions are fulfilled by at least two different fungal guilds, i.e., saprotrophic and mycorrhizal fungi. Competition between fungi of the two guilds in soil may have an impact on ecosystem functioning (Cairney and Meharg 2002), because nutrient cycling may shift towards decomposition by saprobes or nutrient exploitation by mycorrhizal fungi as a result. The latter may slow down decomposition of organic matter in forest soils, which is known as the "Gadgil effect" (Gadgil and Gadgil 1971). Recent field experiments revealed the magnitude of this effect: soil respiration, i.e., mineralization of soil carbon, was reduced by 67% where

Section Editor: Marc Stadler

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11557-018-1405-6) contains supplementary material, which is available to authorized users.

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abundance of ectomycorrhizal (EcM) fungi was high (Averill and Hawkes 2016). Reduced decomposition due to nitrogen (N) depletion from organic matter by EcM fungi was hypothesized to generally result in an accumulation of carbon in temperate and boreal forest soils, which are the major EcM habitats (Averill et al. 2014). Due to soil carbon-climate feedbacks (Bradford et al. 2016), the Gadgil effect may be also important for predicting future climate (Averill and Hawkes 2016). Magnitude and even direction of the Gadgil effect are, however, variable across experiments and challenging to assess in natural ecosystems (Fernandez and Kennedy 2016). While the effect is mainly discussed for the litter layer, profound impacts on ecosystem processes are also conceivable to arise from competition between saprotrophic and mycorrhizal fungi in the underlying organic layers and mineral horizons.

The two fungal guilds were spatially separated along a vertical pine forest soil profile in Central Sweden (Lindahl et al. 2007), in which saprotrophic fungi dominated in recently fallen litter (Oi layer) on top of the forest floor, while mycorrhizal taxa were more abundant and active in the underlying layers of increasingly decomposed litter (Oe and Oa). Anderson et al. (2014), however, found the highest frequency of mycorrhizal mycelia in the Oi layer of a Scottish pine forest and Dickie et al. (2002) revealed certain mycorrhizal fungi to even preferably colonize recently fallen pine needles at a site in Pennsylvania. This suggests that mycorrhizal fungi may be involved in decomposition of fresh litter, although their activity is not related to carbon acquisition, but to nitrogen and phosphorous assimilation (Lindahl and Tunlid 2015; Bödeker et al. 2016; Tunlid et al. 2016). Seasonal variation, which is rather pronounced in soil fungal communities in general (Voříšková et al. 2013) and mycorrhizal communities in particular (Bahram et al. 2015; Courty et al. 2008), may explain distribution patterns deviating between studies (Anderson et al. 2014). The spatial separation of fungal guilds in soil therefore appears not generalizable. Seasonal variation in niche selection along vertical soil profiles has, however, not been studied concertedly for saprotrophic and mycorrhizal fungi so far.

The goal of our study was to assess niche partitioning and co-occurrence patterns of individual fungi and fungal guilds along a vertical soil profile. We hypothesized that (1) individual fungi preferably occur in certain seasons and soil strata (i.e., mineral horizons and organic layers), while (2) fungal guilds and phyla (i.e., the sum of individuals) are more evenly distributed throughout space and time. Furthermore, (3) mutual exclusions (i.e., negative correlations between individual abundances) were assumed to be more frequent between fungi of different guilds than between fungi of the same guild. We analyzed the fungal communities at two points in time to account for temporal variation. Autumn and the following spring were chosen to embrace the winter season, which affects ecosystem dynamics most in coniferous forests (Havranek and Tranquillini 1995). In each season, four strata of four spruce forest soil cores were sampled. The fungal community was assessed by next-generation sequencing (NGS) of fungal barcoding regions in DNA extracts. Compositional differences in fungal communities among the soil strata were analyzed for fungal species, functional guilds, and higher taxa. Network analyses were conducted to assess the ratio of co-occurrences (i.e., positive correlations) to mutual exclusions (i.e., negative correlations) among individual fungi in general and between saprotrophic and mycorrhizal fungi in particular.

Material and methods

Sampling sites and sample collection

Soil samples were collected in an 80-year-old spruce forest in the "Naturpark Solling-Vogler" (51.7657° N, 9.5778° E, 530 m alt.), at a non-roofed control site ("D0") of the "Solling roof project" (Bredemeier et al. 1998). The soil is classified as Dystric Cambisol (FAO 1998). Beneath the layer of fresh litter (Oi), which was removed before sampling, two organic layers (Oe and Oa) of moder type and 1–2 and 1–3 cm depth, respectively, covered the mineral soil with an A horizon of 7–10 cm above the B horizon. Acidity of the soil was slightly higher in the Oa (pH = 2.7) than in the Oe layer (pH = 2.9), but decreases with further soil depth (A 3.2, B 4.1) (Dörr et al. 2010; Kandeler et al. 2009; Theuerl et al. 2010). The content of organically bound nitrogen (N) consistently decreased from 16 (Oe) to 14 (Oa) to 2 (A) to 1 mg N per kg of dry soil (B). The same trend was found for inorganic N (314, 189, 8, and 4 mg kg^{-1}, respectively) and organic carbon (418, 357, 38, and 14 mg kg^{-1}, respectively). The ratio of (organic) C to N accordingly slightly decreased from roughly 26 in the organic layers to about 21 in the A and 18 in the B horizon. While total microbial and its fraction of fungal biomass both decreased with soil depth, the decrease was clearly more pronounced for fungal than for microbial biomass (Enowashu et al. 2009).

Soil cores (8 cm diam.) were drawn from four plots in October 2006 and April 2007, each. Samples were collected from the two organic layers (Oe, Oa) and two mineral horizons (A, B) of each soil core and after removal of roots transferred to sterile 15 ml reaction tubes, transported to the laboratory in liquid nitrogen, and stored at −80 °C until further processing.

DNA extraction, library preparation, and sequencing

Total DNA was extracted from 0.5 g of each soil sample (Supplementary Table S2) according to Peršoh et al. (2008). To flocculate humic substances, $Al_2(SO_4)_3$ was added to the samples from the Oe (45–85 μmol of $Al_2(SO_4)_3$), Oa (45–115 μmol), A (90–130 μmol), and B (30–50 μmol) horizons. After pH adjustment, cells were disrupted in a nucleic acid

extraction buffer (0.4 M LiCl, 100 mM Tris-HCl, 120 mM EDTA 2.5% SDS, pH 8), by adding 0.8 g of differently sized glass beads (0.1–0.4 mm diam.) and using a FastPrep™ Instrument (Bio 101) 5.5 ms^{-1}. DNA was purified using a standard phenol-chloroform protocol, as detailed by Peršoh et al. (2008).

Amplicon libraries were prepared by two consecutive PCRs, with TAG and index sequences introduced in the first (PCR1) and second PCR (PCR2), respectively (Supplementary Table S1). TAG sequences were selected from those published by Poland et al. (2012) and appended to the 5′-end of the fungi-specific primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Different TAG lengths were selected to achieve an AC to GT ratio ≥ 0.25 in the first eight sequenced positions, thus facilitating cluster recognition by the MiSeq sequencer (Lundberg et al. 2013). The PCR1 primers were further appended at the 5′-end of the TAG sequences by 21 bp of the Illumina sequencing primer, as binding site for the PCR2 primers. These consisted of the P5/P7 adapter sites, the index sequences, and the sequencing priming sites (all sequences © 2007–2012 Illumina, Inc.). Differently indexed PCR2 primers were used for PCR1 products with identical TAG sequences. Primers were synthesized by Microsynth AG (Switzerland).

Amplification was achieved using the MangoTaq kit (Bioline GmbH, Germany). Each PCR reaction of 25 μl in total contained 15.64 μl H_2O, 5 μl 5× reaction buffer, 1.7 μl $MgCl_2$ (50 mM), 0.5 μl dNTP (10 mM), 0.5 μl of each primer (10 mM), 0.16 μl polymerase (5 U μl^{-1}), and 1 μl of DNA extract. Following denaturation at 94 °C for 3 min, the ITS regions were amplified in 33 cycles (94 °C for 27 s, 56 °C for 60 s, 72 °C for 90 s) and a final extension step at 72 °C for 7 min. PCR products were purified using ExoSAP-IT® as recommended by the manufacturer (Affymetrix, UK), but diluted 1:5. Of the purified products, 5 μl was amplified in 5 cycles as detailed above, but with an annealing temperature of 53 °C and 10 min of final extension. PCR2 also differed slightly by applying 0.75 μl of the PCR2 primers, 0.13 μl of polymerase, and 11.17 μl of H_2O.

Images from gel electrophoresis (0.8% agarose, 120 V for ca. 20 min) were evaluated using ImageJ (Schneider et al. 2012a), to calculate the relative concentration of each PCR2 product (excluding primer dimers) in relation to a 1 kb ladder (Bio-Budget, Germany). Between 20 and 22 PCR2 products of similar concentration were equimolary pooled. The volume of pools with low concentration was reduced in a centrifugal evaporator (UNIVAPO 100 ECH, UniEquip, Germany). The pools were purified using the Dynabeads Sequencing Clean Up Kit (Invitrogen, Germany) as specified by the manufacturer. Selection of amplicons between 450 and 1500 bp in length by preparative electrophoresis (Blue Pippin, Sage Science, USA) ensured that primer dimers and contaminates were removed. DNA concentrations in the pools were determined using the Qubit 2.0 fluorometer (Life Technologies) and Bioanalyzer 2100 (Agilent Technologies, USA). Based on these data, the pools were equimolarly pooled to a total concentration of 4 nM. The Illumina MiSeq platform (Illumina Inc.) was used for paired-end sequencing (2 × 250 bp; Kit v3 Chemistry) of this final amplicon library.

Sequence processing

The raw sequence reads were quality filtered and demultiplexed (i.e., assigned to samples) retaining the quality scores according to the incorporated of indices and TAGs using the Qiime pipeline (Caporaso et al. 2010) and deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB18588). Only the forward-orientated reads, representing the ITS1 rRNA gene region, were further processed, because earlier studies showed that sequence information of the ITS1 region alone is well suited to infer ecological patterns (Guerreiro et al. 2017; Röhl et al. 2017a). To compensate for length differences in the TAG sequences, ITS1 reads of samples with identical TAGs were extracted and trimmed at the 5′-end to a conserved region comprising the final 11 bp of the SSU rRNA gene region using the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Files with length-adjusted reads were merged and the ITS1 reads trimmed at the 3′-end to a length of 172 bp. The trimmed ITS1 reads were subjected to CD-HIT-OTU (Li et al. 2012) (http://weizhongli-lab.org/cd-hit-otu/) for clustering. The algorithm was chosen because of its outstanding performance in previously conducted comparative analyses (Röhl et al. 2017b). A similarity threshold of 97% was applied and a table generated, which lists the reads in each cluster for each sample. Representative sequences for each cluster were assigned to taxa using Qiime and the UNITE database v7 (Kõljalg et al. 2013) as reference. Unassignable clusters were assigned following Peršoh et al. (2010) (Supplementary Table S5) and yeasts by comparison of sequences with those of reliably identified strains from cultivation-based soil surveys (Yurkov et al. 2012b, a, 2016a, b). Sequences not assignable to fungi were excluded from further analyses. Clusters representing identical "species hypotheses" according to UNITE were summarized. The resulting clusters were treated as operational taxonomic units (OTUs). OTUs were grouped to functional guilds (Supplementary Table S4) according to their taxonomic affiliation, as indicated by Agerer (2006), Cannon and Kirk (2007), and Kirk et al. (2011) and in the "Faces of Fungi" (Jayasiri et al. 2015) and "FUNGuild" (Nguyen et al. 2016) databases.

Data analyses

The OTU table (Supplementary Table S3) was imported into Primer7 (Plymouth Routines) and standardized by the total

number of reads per sample. For analyses of subgroups, i.e., distributions of OTUs belonging to a certain guild or taxon (e.g., Supplementary Fig. S8), the respective subsets of OTUs were re-standardized by the (sub)total. The data analyses were accordingly based on relative abundances of OTUs in samples. While this approach is commonly applied in NGS-based studies of microbial communities, relative abundances are prone to different biases (Bálint et al. 2016) and unlikely to reflect natural abundances (Peršoh 2013). Comparisons among similar samples are, however, considered to be reliable, because the samples were subjected to the same bias during processing (Bálint et al. 2016; Peršoh 2013). To assure that the results are not biased due to insufficient sequencing depth, all analyses were conducted in addition based on a dataset rarefied to 13,221 reads using the rarefy function of the vegan R package (https://CRAN.R-project.org/package=vegan). One sample with 5719 reads, originating from the B horizon in spring 2007, was excluded from the analyses of the rarefied data.

A Bray-Curtis similarity matrix was calculated from the OTU table. It served as basis for statistical analyses (analysis of similarity, ANOSIM) and non-metric multidimensional scaling (nMDS) conducted in Primer7. Sequences of vertical strata and sampling dates were considered as ordered for two-way crossed and separate one-way ANOSIM analyses. Abundances of OTUs were summarized per taxon and functional group to test for compositional differences among strata at different taxonomic levels and of ecological guilds. Spider diagrams and bar charts were generated in Microsoft Excel, using preprocessed data exported from Primer7. Co-occurrence networks (Supplementary file Networks_Solling.cys) were calculated based on abundances coded in the OTU table using CoNet (http://systemsbiology.vub.ac.be/conet) as detailed by Faust et al. (2012). To prioritize interactions between fungi over environmental effects, the analyses were conducted separately for each sampling category (horizon × date), following the approach by Guerreiro et al. (2017). Co-occurrence networks were calculated for those sampling categories for which four samples were successfully sequenced (see below), resulting in six separate networks. The applied parameters and selected similarity and correlation measures (i.e., Bray-Curtis and Kullback-Leibler (dis)similarity, Pearson and Spearman correlation) followed Faust et al. (2015) and the online tutorial of CoNet (http://psbweb05.psb.ugent.be/conet/microbialnetworks/conet.php). However, no threshold was applied, i.e., all positive and negative correlations (edges) were considered for the initial network. In the following (permutation and bootstrapping), only edges were retained, which represented according to at least two measures significant correlations, as indicated by Benjamini-Hochberg-corrected p values. OTUs occurring in less than three samples of the respective category were removed from the networks for downstream analyses.

Data availability Accession numbers: Sequence reads were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession PRJEB18588.

Results

The sequencing data were first filtered for sufficient coverage and tested for technical biases to ensure reliability of the downstream analyses. Library preparation was successful for 30 of the 32 samples. Sequencing provided 1,749,216 reads assignable to these samples. Of these, 1,407,660 were clustered to 383 OTUs, which represent proxies for species. Each cluster of the remaining and discarded 314,556 reads contained less than 66 reads. These clusters were estimated to represent noise by the clustering software. After excluding two samples covered by less than 5 k reads, the remaining 28 samples contained > 50 k reads on average (Supplementary Table S2). Taxonomic assignment revealed 325 OTUs (1,400,804 reads) to represent fungi. Each fungal OTU was covered on average by 4310 reads, i.e., by 154 reads per sample. The number of fungal OTUs detected in mineral soil horizons (A and B) was clearly lower than that in the covering organic layers (Oe, Oa) (Fig. 1, Supplementary Fig. 1). While a weak correlation between read and OTU counts was observed across all samples (Spearman $p = 0{,}015$, $R^2 = 0.21$), this was not observed separately for samples of the mineral horizons ($p = 0.27$) and organic layers ($p = 0.62$). Excluding three samples with less than 30 OTUs, which all originated from the B horizon in spring (Fig. 1), already prevented a significant correlation between OTU and read numbers across the remaining samples ($p = 0.20$). Even though these results indicated that sequencing depth is not a major issue, we

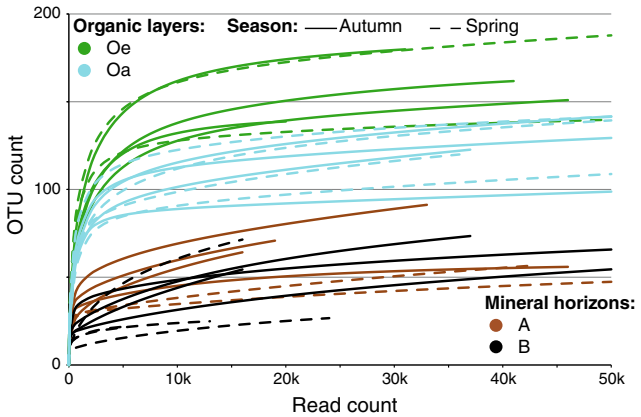

Fig. 1 OTU richness in dependence of sequencing depth. Rarefaction curve plotting the number of operational taxonomic units (OTUs) detected in each sample against the number of (rarefied) sequence reads. Reads were clustered to OTUs based on a 97% sequence-similarity threshold. Line colors encode strata and line types seasons for each sample

conducted all analyses in addition based on the rarefied dataset to assure reliability of the results.

Vertical stratification of fungal taxa and functional guilds

Presence and abundance of the entirety of OTUs representing each fungal phylum and guild, respectively, were analyzed to uncover their distribution patterns. Averaged abundance and totalized occurrence are presented in the following, while results of the analyses of the rarefied data are shown in Supplementary Figs. S2 and S3. In addition, the distribution patterns are depicted for each plot separately in the Supplementary Figs. S4–S7. Each OTU of Ascomycota and Basidiomycota was represented on average by 3104 and 7573 sequence reads, respectively (Supplementary Tables S3 and S4). Amplicons of Basidiomycota were therefore more abundant than those of Ascomycota (Fig. 2(a)), but represented by fewer OTUs (Fig. 2(c)). Relative abundance of Ascomyota slightly increased in autumn from the Oe layer (25%) to the A horizon (36%), but consistently decreased with soil depth from 39% (Oe) to 11% (B) in spring. Basidiomycota showed the reverse trend: they marginally decreased from the Oe layer (58%) to the B horizon (55%) in autumn but clearly increased in abundance from 34 to 74% in spring. OTU richness of both phyla was clearly higher in the organic layers (Oe and Oa) than in the mineral soil (horizons A and B; Fig. 2(c) and Supplementary Fig. 3C). Disregarding seasonal variations (see below), abundance and OTU richness differed only marginally between the two organic layers and between the two mineral horizons. Apart from higher abundances in the Oe layer and in the A horizon in spring, Zygomycota showed only minor variations along the soil profile, concerning abundance and OTU richness (Fig. 2(a, c)).

Ectomycorrhizal (EcM) fungi were represented by only few OTUs (Fig. 2(d), Supplementary Fig. 3D) but constituted the most abundant functional guild (Fig. 2(b), Supplementary Fig. 3B), except for the Oe layer in spring. Apart from temporal variations (see below), they varied negligibly in abundance throughout the soil profile. The number of EcM OTUs found in the organic layers (i.e., 16–21) was, however, slightly higher than that in the mineral soil (11–16). Saprotrophic yeasts and filamentous fungi also occurred at consistent abundances, but more OTUs occurred in the organic layers. The consistent decrease of fungi with other or unknown biology with soil depth is mostly caused by fungi known to be animal

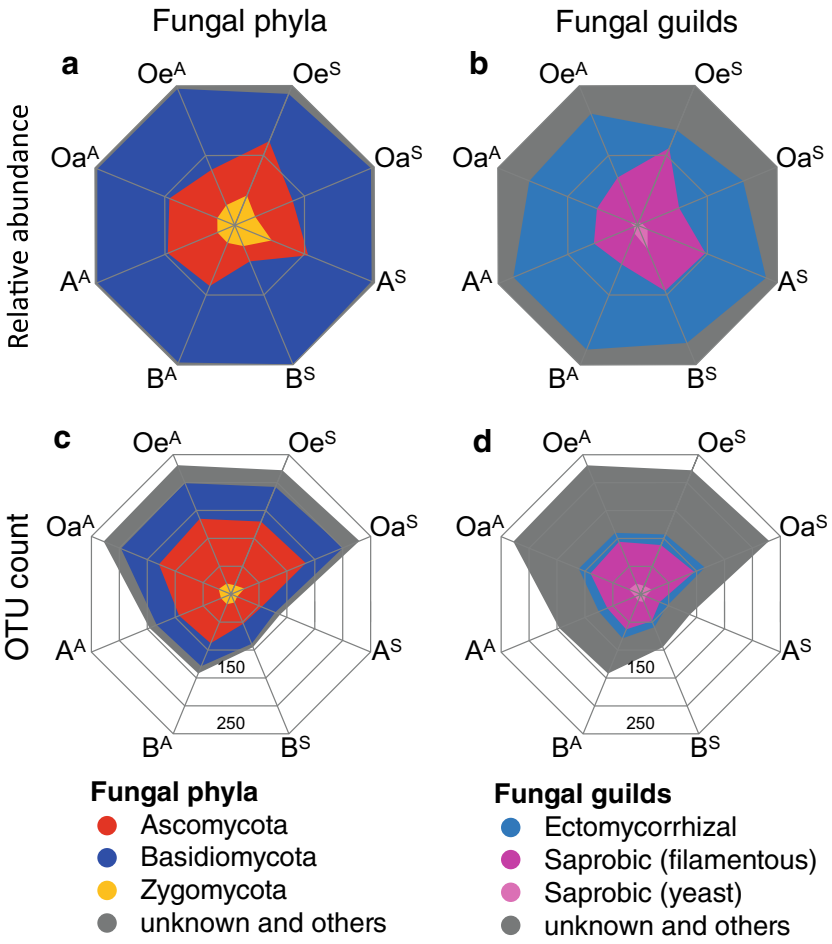

Fig. 2 Distribution of fungal phyla (a, c) and guilds (b, d) along the vertical soil profile. Stacked plot of averaged relative abundances (a, b) and OTU counts (c, d) in the four the strata (Oe, Oa, A, and B; top to bottom in of each diagram) in autumn 2006 (left part, superscript "A") and spring 2007 (right, superscript "S"). Averaged relative abundances amount to 100% (a, b); OTU counts (c, d) are indicated on the axis. Variations among plots are depicted in Supplementary Figs. S4–S7. Colors code fungal phyla and guilds, respectively

and phytopathogenic or epiphytic, which predominantly occur in the upper strata (Supplementary Tables S3 and S4).

Phyla and functional guilds, represented by the total abundance of comprised OTUs, were not differentially distributed between strata, neither across (ANOSIM $R < 0.01$, $p > 0.47$; Fig. 3 and Supplementary Fig. S2) nor within seasons ($R < 0.12$, $p > 0.18$). Only in spring 2007, the abundance of fungal phyla differed slightly ($p = 0.273$), but significantly ($p = 0.038$) across the strata. Above species level, taxonomic composition of communities otherwise only differed significantly among the strata when summarized on order and genus level ($p = 0.043$ and 0.012, respectively), and then just marginally ($R \leq 0.19$, Fig. 3). While species and OTU compositions of the fungal communities reflected stratification of the soil in general ($R \geq 0.75$, $p = 0.0001$), Oa and A were the only adjacent strata inhabited by significantly different communities across seasons (Fig. 4(a)). However, omitting one sample of the B horizon (i.e., the sample with the lowest read count) indicated a significant difference between the A and B horizons ($R = 0.427$, $p = 0{,}037$), as also revealed by analyses of the rarefied data (Supplementary Fig. S2). OTU richness of all three major phyla peaked in the organic layers (Fig. 4(b)). Basidiomycota were accordingly most abundant in the (lowermost) B horizons, but most OTU-rich in the (uppermost) Oe layer. A similar pattern was observed for EcM fungi and saprotrophic yeasts; the relative abundances of both were highest in the mineral horizons, while most OTUs of these groups are found in the organic layers. Filamentous saprotrophic fungi were most abundant and OTU-rich in the Oe layer, even though abundance was only weakly but OTU richness strongly correlated with the NMDS axis (Fig. 4(b)).

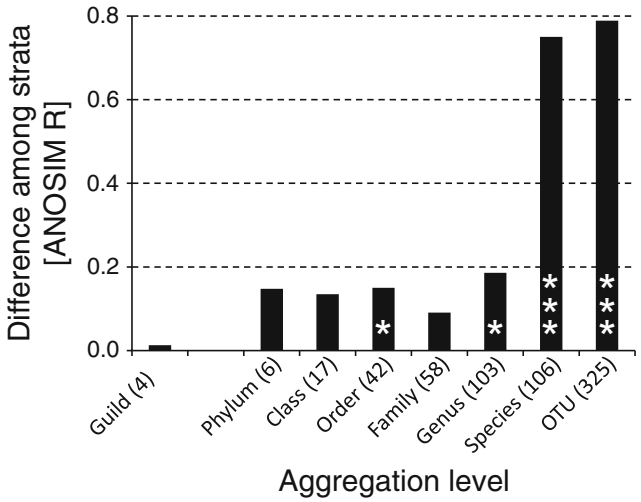

Fig. 3 Differences among soil strata according to fungal communities aggregated at taxonomic and functional levels. Abundances were summarized for OTUs assigned to the same functional guilds and taxa, respectively. ANOSIM analyses are based on Bray-Curtis similarities among samples according to total abundances of OTUs, taxa, and guilds, respectively. ANOSIM *R* values (according to two-way crossed analyses of stratum and season) indicate the magnitude of resolution, i.e., difference among strata. Significance levels are coded by asterisks: $p < 0.05$ (*), $p < 0.001$ (**), and $p \leq 0.0001$ (***). The number of categories considered in each analysis is indicated in parentheses. Categories include the taxa with OTU assignments at the respective taxonomic level and four guilds (ectomycorrhizal and non-ectomycorrhizal root-associated fungi, as well as saprotrophic yeasts and saprotrophic filamentous fungi), as detailed in Supplementary Table S4

In contrast to the total abundance of each functional guild, their composition of individual OTUs mostly differed significantly among strata (Supplementary Fig. S8). While the ectomycorrhizal communities in adjacent upper strata (Oe, Oa, A) were composed of similar, i.e., statistically indistinguishably (ANOSIM $p > 0.35$), fungal OTUs, they differed clearly in composition between the mineral horizons ($p = 0.004$, $R = 0.596$). The yeast community, by contrast, differed in OTU composition significantly between the organic layers ($p = 0.008$, $R = 0.650$) and by trend between Oa layer and A horizon ($p = 0.078$, $R = 0.215$), but not between the A and B horizons ($p = 0.571$). The community of filamentous saprotrophic fungi differed between all adjacent strata ($p \leq 0.015$, $R > 0.48$).

To assess if the preferences of OTUs for certain strata were quantitative or qualitative, occurrences were analyzed for the most abundant 52 OTUs, i.e., OTUs accounting for ≥ 1.5% of the whole community in any stratum and season (Fig. 5, Supplementary Figs. S9–S12). The vast majority (i.e., 77%) of these occurred in all strata throughout the soil profile. Only two OTUs (i.e., *Bullera coprosmae* and a Tremellales sp.) and five OTUs (*Hygrocybe mucronella*, *Vishniacozyma tephrensis*, and a species of *Candida*, *Hymenoscyphus*, and Tremellales, each) were restricted to one and two strata, respectively. However, relative abundances indicated clear preferences for a certain stratum or at least for either the organic layers or the mineral horizons for 46 out of the 52 most abundant OTUs (Fig. 5, Supplementary Figs S9–S12).

Temporal patterns

To disentangle temporal inconsistencies between patterns of fungal phyla and guilds, these were analyzed in more detail, with a stronger focus on single OTUs, which apparently resolve the vertical stratification better than superordinate units. While seasonal differences in community composition were neither statistically significant across strata (Figs. 3 and 4, Supplementary Fig. S2) nor within single strata (ANOSIM $R < 0.3$, $p > 0.13$), individual taxa clearly differed in abundance between spring and autumn (Fig. 5, Supplementary Figs. S9–S12).

Temporal differences in the totalized relative OTU abundance of Ascomycota were obvious for the Oe layer and the mineral horizons (Fig. 2(a), Supplementary Fig. S3A). In the Oe layer, their proportion was higher in spring at the expense of Basidiomycota OTUs. This resulted in pronounced (ANOSIM $R = 0.929$) and by trend significantly ($p = 0.067$)

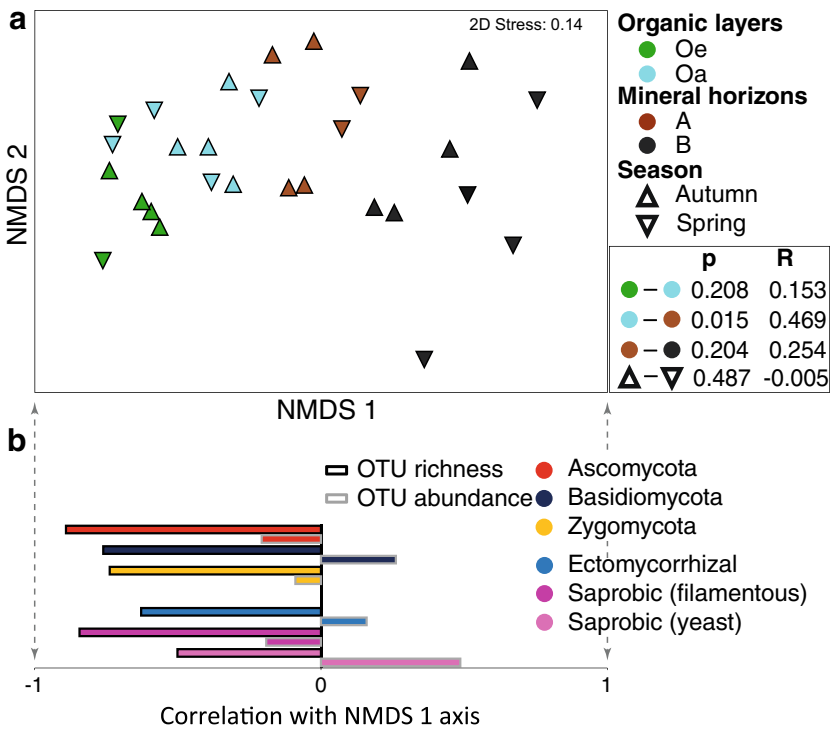

Fig. 4 Fungal communities along the vertical soil profile. (a) Similarity among samples according to community composition. Ordination of Bray-Curtis similarities according to standardized OTU abundances by non-metric multidimensional scaling (nMDS). Colors code the strata, symbol orientation the sampling date. Significance (p) and magnitude (R) of differences according to according to two-way crossed (stratum × season) analysis of similarity (ANOSIM) between adjacent strata and seasons are given below the legend. (b) Pearson correlation of abundance (gray frames) and OTU richness (black frames) of major fungal phyla and guilds (coded by colors) with the nMDS 1 axis of (a)

different phyla compositions between autumn and spring in the Oe layer communities. Most strikingly, two OTUs representing the Herpotrichiellaceae and Helotiales (both Ascomycota), respectively, doubled in abundance, while the most abundant Basidiomycete in autumn (*Tylospora fibrillosa*) decreased in relative abundance from 30 to 6% between the sampling dates (Fig. 5). The increase of Zygomycota in the Oe layer was mostly due to an unidentified *Mortierella* sp. (SH180124) and *M. gemmifera*, both of which were twice as abundant in spring. The lower strata showed no significant differences according to phyla proportions between the sampling dates (ANOSIM $R < 0.1$, $p > 0.4$). Nevertheless, several individual fungi changed in abundance. In the A horizon, two species of *Elaphomyces* (Ascomycota) were less abundant in spring, when *Mortierella macrocystis* and *M. longigemmata* (Zygomycota) were more abundant. The OTU assignable as *Cenococcum geophilum* contributed most to the decline of Ascomycota in the B horizon between the sampling dates; it accounted on average for 14% (54% in a single sample) of the community in autumn, but was almost absent (i.e., < 0.01%) in spring. The simultaneous increase in Basidiomycota was predominantly due to considerably high abundances of OTUs absent in autumn (e.g., *Bullera coprosmae* 3%, *Vishniacozyma tephrensis* 8%, *Hygrocybe mucronella* 8%, Tremellales sp. (SH466030) 5%; Supplementary Figs. S10 and S12). These OTUs were also mostly restricted to single samples.

The higher proportion of saprotrophic fungi in the Oe layer in spring (Fig. 2(b)) resulted in a, by trend significant (ANOSIM $p = 0.067$), seasonal difference ($R = 0.786$) in the composition of functional guilds in the Oe layer, while temporal changes in guild proportions were insignificant ($p > 0.6$) and negligible ($R < 0.1$) in the underlying strata. The EcM fungus *Tylospora fibrillosa* decreased in abundance between the sampling dates from ≥ 30 to 4% in both organic layers and from 24 to 9% in the A horizon (Fig. 5). Three EcM fungi (*Piloderma sphaerosporum*, *Russula ochroleuca*, *Thelephora terrestris*) were most abundant in the Oe and the Oa layers in spring and autumn, respectively. Increasing abundance of *Hygrophorus pustulatus* partly compensated for the decline of *Tylospora fibrillosa* in the A horizon between the sampling dates. In the B horizon, the decrease of EcM fungi was mostly attributable to *Cenococcum geophilum* (see above). The most abundant EcM fungus in the B horizon (*Tylospora asterophora*) showed no temporal variation. Saprotrophic fungi consistently showed higher abundances in spring, except for the Oa layer. In the Oe layer, the majority of filamentous saprobes of all three phyla (see above) increased in abundance, including representatives of Basidiomycota taxa Trechisporales and *Mycena*. This was less consistent in the Oa layer, in which most saprotrophic fungi occurred at low abundance. Two representatives of the Zygomycota (i.e., *M. macrocystis* and *M. longigemmata*) contributed most to the increase of saprotrophic fungi in the A horizon. Abundances of filamentous saprobes and particularly of yeasts of the Basidiomycota predominantly caused the higher proportion of saprotrophic fungi in the B horizon in spring than in autumn (see above).

Temporal changes in the numbers of observed OTUs also reflected differences among the strata. Between the sampling dates in autumn and the following spring, OTU richness

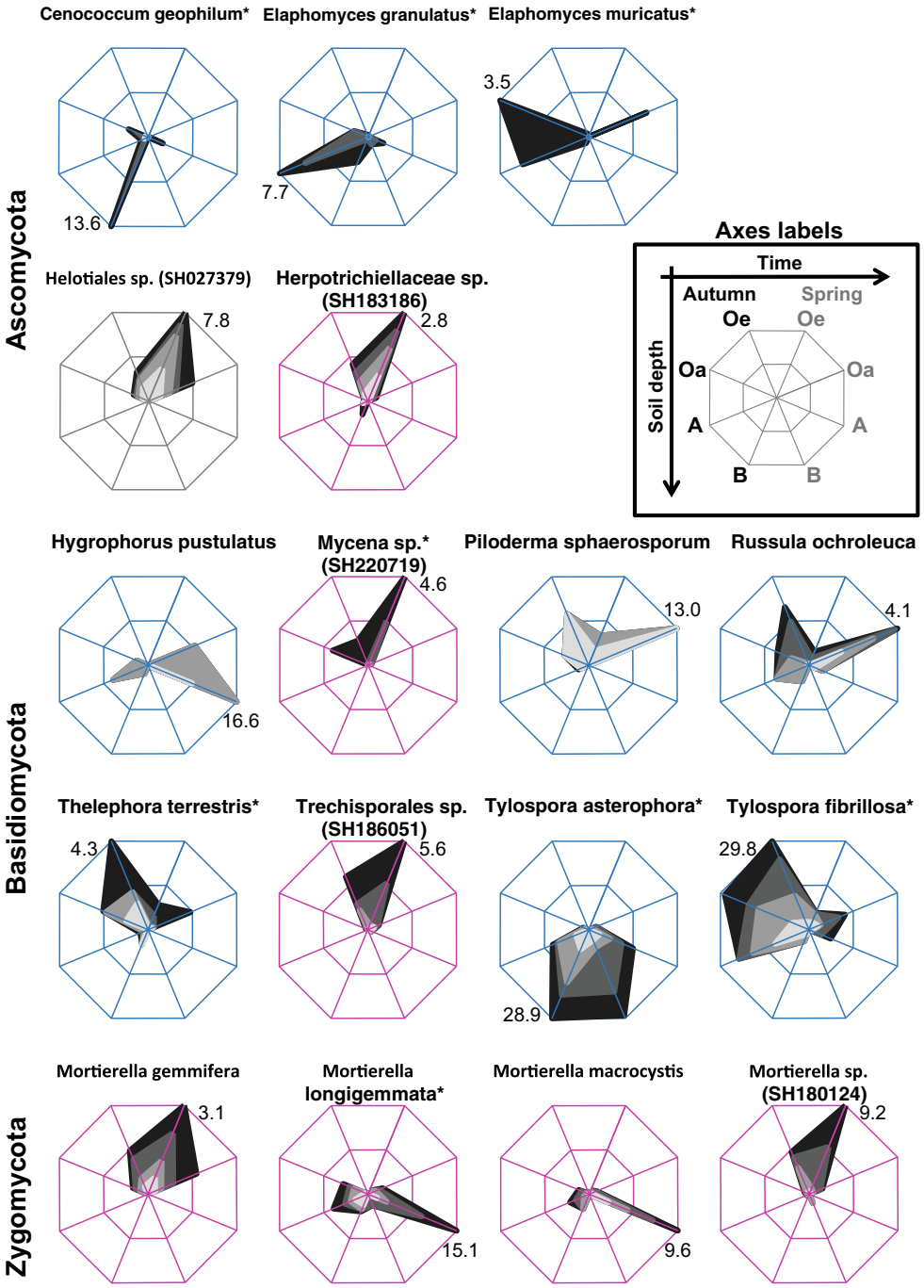

Fig. 5 Distribution of selected fungi along the vertical soil profile. Relative abundances of OTUs in the four strata (Oe, Oa, A, and B; top to bottom in of each diagram) in autumn 2006 (left part) and spring 2007 (right) are shown. The maximum average abundance is specified for each OTU. Contributions of single samples to overall average abundance per category are indicated by different shades of gray. For reasons of clarity, samples of categories with only two samples (Oe and A in spring) were split and represented as four virtual samples. The UNITE species hypothesis ID (http://unite.ut.ee) is noted for OTUs not assignable to species. Diagrams are alphabetically arranged for OTUs within phyla. Grid colors differentiate between ectomycorrhizal (blue), saprotrophic (pink), and ecologically diverse (gray) taxa. Asterisks indicate names assigned based on searches for similar sequences in the NCBI database (http://www.ncbi.nlm.nih.gov) as detailed in Supplementary Table S5

remained largely constant in the organic layers, but clearly decreased in the mineral horizons (Fig. 2(c, d)). In the Oe and Oa layers, 191 and 185 OTUs persisted and 40 and 43 were not recovered in spring, while 31 and 45, respectively, occurred only in spring. The transient OTUs accounted for up to 20% of all OTUs, but for less than 2% of the amplicon reads (i.e., abundance). Of all 146 OTUs detected in the A horizon in autumn, 77 were no longer recovered there in spring, while only 19 were exclusively present in the A horizon in spring. The irrecoverable OTUs represented 5% of the reads obtained from the A horizon in autumn, the newly occurring ones 2% in spring. From the B horizon, 72 OTUs (6% of the reads) of the 142 OTUs in autumn were no longer detected in spring and 25 (31% of the reads) were only found there in spring.

Co-occurrence and mutual exclusions among and between saprotrophic and ectomycorrhizal fungi

Network analyses were conducted to identify interactions among individual fungi, which may not be reflected on the level of integrated superordinate units. Each OTU was connected on average to 5.5 other OTUs in the Oe layer in

autumn. Connectedness, i.e., the total number of pairwise co-occurrences, decreased slightly towards the Oa layer (5.3 connections per OTU) and, in accordance with species richness, sharply towards the mineral horizons A (1.9) and B (1.3). Positive correlations (i.e., co-occurrences) between abundances of OTUs prevailed in all strata (Fig. 6). The proportion of negative correlations (i.e., mutual exclusions) was highest in the Oa layer (21%) and decreased towards the B horizon (15%), but was lowest in the Oe layer (13%). Correlations among EcM and saprotrophic fungi, respectively, and between fungi of the different guilds were largely consistent with the overall pattern. The proportion of negative correlations (mutual exclusions) between abundances of OTUs of different guilds was lower than among EcM (Oa layer) and among saprotrophic fungi (Oe and A). Only in the B horizon, more negative correlations (21%) were found between EcM and saprotrophic fungi, than within each guild (18 and 14%, respectively).

Connectedness of fungal OTUs in the Oa layer was by roughly 10% lower in autumn than in spring, but decreased between the sampling dates by 69% in the B horizon. The proportion of mutual exclusions thereby decreased throughout all categories in the Oa layer and within EcM fungi in the B horizon. While the proportion of mutual exclusions remained roughly the same among saprotrophic and between saprotrophic and EcM fungi, it was higher in spring than in autumn regarding the entire community of the B horizon.

Discussion

Our results are in agreement with our first two hypotheses, assuming clear preference of individual fungi for certain strata (Figs. 3, 5) while the total of the fungi representing higher taxa or functional guilds were equally distributed along the soil profile (Figs. 2, 3). In disagreement with the hypotheses, temporal shifts appeared, at least in the uppermost Oe layer, statistically less pronounced in the community of individuals than in proportions of taxonomic and functional groups representing the sum of individuals. Considering this more differentiated picture, the spatial and temporal distribution patterns are discussed in the following with regard to their agreement with ecosystem processes. The communities are first compared between and then within organic layers and mineral soil horizons.

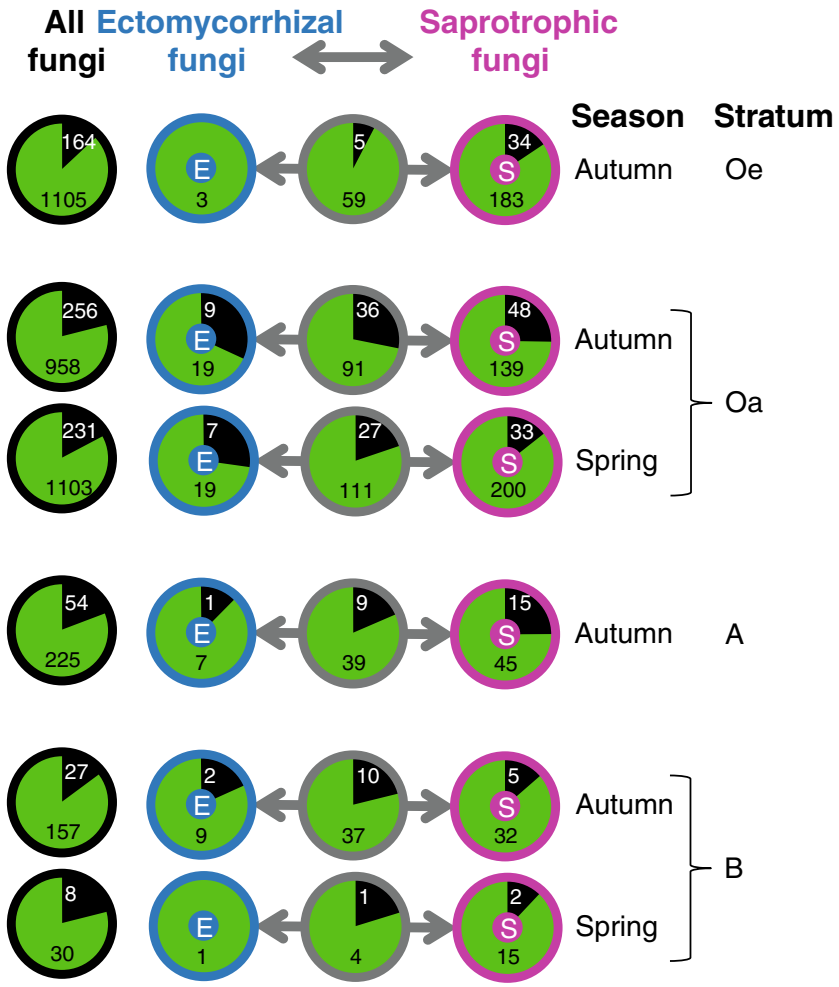

Fig. 6 Co-occurrences (green) and mutual exclusions (black) between fungal OTUs in the association networks. The proportions are indicated by the pie charts and the total counts of significant negative (black) and positive (green) correlations are given among all fungi (left column, black circle), among ectomycorrhizal (EcM, blue) fungi (2nd column), among saprotrophic filamentous and yeast fungi (right column, pinkish), and between EcM and saprotrophic fungi (3rd column, gray circle). Networks were calculated for strata and seasons of which 4 samples were successfully processed, as indicated to the right

The third hypothesis concerning positive (co-occurrences) and negative (i.e., mutual exclusions) correlations among abundances of OTUs representing saprotrophic and mycorrhizal fungi will be addressed thereafter.

Our results (Fig. 4) confirm that mineral soil is inhabited by significantly different fungal communities than the organic layers (Dickie et al. 2002; O'Brien et al. 2005; Lindahl et al. 2007; Taylor et al. 2014). However, niche partitioning appeared more distinct than in previous studies due to the abundance-based analyses applied here. The vast majority of the most abundant fungi (Supplementary Figs. S9–S12) occurred throughout the soil profile, but at negligible abundances in other than the preferred strata. Frequency-based distribution patterns would therefore coincide with the earlier reports. More distinct niche partitioning was already revealed based on relative abundances in a coniferous forest soil in Sweden (Rosling et al. 2003). Refinement of the taxonomic assignment of OTUs further increased the spatial resolution compared to the earlier study. The genus *Tylospora*, for example, occurred throughout the soil profile in Sweden. This also applies for our study, but we revealed that this pattern is due to opposed preferences of the two congeneric species at the Solling site (Fig. 5).

The reduced species richness in mineral horizons, compared to organic layers (Fig. 2), mirrors the decrease in microbial biomass, carbon content, and nitrogen content with soil depth at the study site (Enowashu et al. 2009; Kandeler et al. 2009; Dörr et al. 2010; Theuerl et al. 2010). However, the clear preferences of species for either mineral soil or litter layers (Fig. 5, Supplementary Figs. S9–S12) indicate also qualitative changes in community composition. Organic litter layers and mineral soil are generally quite distinct habitats regarding physicochemical conditions (Vries and Leeters 2001), which is reflected by the significant differences in community composition between the two adjacent strata A and O at the soil-litter interface (Fig. 4). This even resulted in fungal communities separated vertically by a few centimeter being less similar than communities of the same stratum which are separated horizontally by more than 100 km (Taylor et al. 2014). Litter and soil strata also differ according to energy sources, with aboveground litter serving as predominant carbon source in the former and plant roots in the latter (Kramer et al. 2010; Braakhekke et al. 2013; Spielvogel et al. 2014).

Progressing decomposition is supposed to shape fungal communities in the litter layer. Freshly fallen leaves are predominantly colonized by Ascomycota, and with progressing decomposition, the litter is increasingly colonized by Basidiomycota (Kendrick 1962; Hudson 1968; Ponge 2005; Kuramae et al. 2013; Voříšková and Baldrian 2012). The community succession is supposed to mirror the successive degradation of plant compounds (Frankland 1998), i.e., the most easily accessible ones become successively depleted (Berg and Staaf 1980; Moorhead and Sinsabaugh 2006; Šnajdr et al. 2011; Berg and McClaugherty 2014). Basidiomycota are better equipped to degrade recalcitrant compounds, such as lignin (Baldrian 2008; Lundell et al. 2010; Baldrian et al. 2012), but are outcompeted by faster growing fungi in the presence of more labile compounds (Dighton 2007). The high abundance of Basidiomycota in the analyzed Oe layer (Fig. 2) indicates an already advanced stage of fungal community succession and litter decomposition in the uppermost layer analyzed in this study. A further increase of Basidiomycota towards the humus layer (Oa) was only observed in spring, and then due to EcM taxa. This change was not accompanied by a significant shift in community composition, while composition of yeast and filamentous saprotrophic fungal communities changed significantly from the Oe to the Oa layer (Supplementary Fig. S8). In accordance with earlier studies (Lindahl et al. 2007; Peršoh et al. 2013; Žifčáková et al. 2016), saprotrophic Basidiomycota with ligninolytic capabilities, such as Trechisporales (Nagy et al. 2016) and *Mycena* spp. (Miyamoto et al. 2000; Žifčáková et al. 2011), were more abundant above the Oa layer (Fig. 5). Saprotrophic Basidiomycetes also predominantly colonized fresh litter and litter incubated above the humus layer in a field experiment (Bödeker et al. 2016). These results indicate concertedly that succession from Ascomycota to Basidiomycota is already completed in the Oe layer concerning the saprotrophic taxa. Opposed to the widely accepted scenario of a successive degradation of plant compounds, as outlined above, the previously reported attack of lignin during early stages of decomposition (Johansson et al. 1986) appears therefore well accordable to the distribution of ligninolytic fungi in stratified litter layers. It would be also compatible with the emerging idea that the molecular structure of plant litter may be less important than its microbial turnover for carbon pools in soil (Thevenot et al. 2010; Gougoulias et al. 2014; Kögel-Knabner 2016).

While ectomycorrhizal Basidiomycota occurring at notable abundance in the Oe layer in autumn concertedly preferred the Oa layer in the following spring, relative abundance of nearly all saprotrophic fungi increased in the Oe layer between the sampling dates (Fig. 5, Supplementary Figs. S9–S11). Voříšková et al. (2013) also found a clearly lower proportion of EcM fungi in the uppermost layer in spring than in winter, while fungal biomass was higher in spring. An increase of saprotrophic fungi over winter therefore appears plausible, in particular for representatives of the abundant genus *Mortierella*, which includes several psychrophilic species (Latter and Heal 1971; Carreiro and Koske 1992). The species are known to be capable of forming extensive mats below snow cover (Schmidt et al. 2008). However, ergosterol analyses indicated that fungal biomass decreased more than 5-fold in the Oe layer between autumn and spring at our sampling site (Enowashu et al. 2009). Since mycelia of *Mortierella* contain no ergosterol (Weete and Gandhi 1999), but represent key organisms in the observed temporal shifts, different biomass markers would be required to infer absolute shifts from the changes in relative abundances of barcoding genes.

Nevertheless, the preference shift of EcM fungi from Oe to Oa over winter might indicate withdrawal of EcM fungi from the Oe layer, thus evading the lower temperatures at the soil surface in winter. While frost hardiness and survival strategies at low temperatures are known to differ among EcM fungi (Tibbett and Cairney 2007; Qu et al. 2010; Timling and Taylor 2012), detailed studies on seasonal variation of distribution patterns of individual fungi in temperate forests are currently missing.

Despite pronounced temporal shifts of individual ectomycorrhizal Basidiomycota in the organic layers, their overall abundance was consistently high in the Oa layer (Fig. 2). Many ectomycorrhizal Basidiomycota possess the enzymatic machinery to degrade humified organic material (Kohler et al. 2015) and play a crucial role in long-term decomposition processes (Lindahl and Tunlid 2015; Keiluweit et al. 2015; Moore et al. 2015). That saprotrophic fungi with similar capabilities predominantly occurred in the covering Oe layer suggests that EcM fungi may substantially contribute to degradation of the recalcitrant compounds accumulated in the underlying Oa layer (Bödeker et al. 2016). Limited accessibility of nutrients may lead to increased competition in this layer, which is indicated by the exceptionally high proportion of mutual exclusions among EcM fungi of the Oa layer (Fig. 6).

In contrast to the organic layers, fungi of the mineral soil preferred the same horizon at both sampling dates (Fig. 5, Supplementary Figs. S9–S12). While substrate quality differs in the organic layers due to progressing decomposition of aboveground litter, roots are the predominant carbon source throughout the mineral soil (Kramer et al. 2010; Braakhekke et al. 2013). Fungal preferences for a certain mineral horizon are therefore not ascribable to different energy sources, but probably caused by distinct competitiveness under different environmental conditions (Mujic et al. 2016). Due to their dependence on rhizodeposition by the tree, it is reasonable to assume that seasonality of fungal communities in mineral soil is tightly linked to annual cycles in tree physiology. Increasing abundance and decreasing species richness of saprotrophic fungi in mineral soil over winter (Fig. 2) may indicate emergence of a relatively uniform carbon source. The decreasing ratio of live to dead tree roots over winter (Persson and Stadenberg 2010) suggests that dead roots may serve as energy source for the saprotrophic fungi. At the same time, root growth ceases over winter (Mainiero et al. 2010), indicating a lower rate of carbon allocation to the rhizosphere, which would reduce heterogeneity of the carbon input into mineral soil (Jones et al. 2009) and thus diversity of the fungal community depending on this energy source. However, the finding that predominantly saprotrophic Zygomycota increased in the A horizon, but saprotrophic Basidiomycota in the B horizon (Figs. 2, 5 and Supplementary Figs. S10–S11), suggests that different factors and mechanisms may control and underlie the temporal shifts, respectively. In addition, several fungi of the mineral soil were only detected at one sampling date (Supplementary Figs. S10 and S12), which resulted in a higher species turnover in the mineral soil than in the organic layers. The transient species were mostly detected only in a single sample, indicating also a more patchy distribution. These findings may be ascribed to the decreasing root density with soil depth (Bredemeier et al. 1998; Borken et al. 2007; Leppälammi-Kujansuu et al. 2014), resulting in increasingly discontinuous distribution of energy sources (Ota et al. 2013).

Metabolic interdependencies were recently reported to outweigh resource competition in bacterial communities (Zelezniak et al. 2015). The prevalence of co-occurrences (i.e., positive correlations) of fungi in all strata throughout the soil profile might indicate similar mechanisms to underlie community assembly in litter decomposing and soil fungal communities (Fig. 6). An exceptionally high proportion of mutual exclusions (i.e., negative correlations) was only found in the Oa layer, which indicates stronger competition for not readily accessible nutrients in the recalcitrant organic compounds accumulated there. Mutual exclusions were, however, more common among ectomycorrhizal (EcM) fungi than between saprotrophic and EcM fungi in this layer. In contrast to our third hypothesis, representatives of both guilds predominantly co-occurred throughout the soil profile and mutual exclusions were comparatively rare. The network analyses therefore provide no evidence for a particularly pronounced competition between saprotrophic and EcM fungi beneath the layer of fresh litter. Reduced litter decomposition rates related to the Gadgil effect (Gadgil and Gadgil 1971; Fernandez and Kennedy 2016) are accordingly not indicated by the results. It is possible that the effect did not occur in the analyzed horizons and/or during the study period. It may not even occur at the 80-year-old stands studied here, because the Gadgil effect was detected earlier only in forest stands older than 132 years (Averill and Hawkes 2016). However, the applied approach to infer interactions from co-occurences of barcoding genes may also be insufficient to detect indications for ecosystem processes. Nutrient use efficiency differs among individual fungi (Geyer et al. 2016; Lashermes et al. 2016) and changes with time (Mooshammer et al. 2014). Mutual exclusions between fungi cycling nutrients particularly efficiently may have a greater effect than indicated by the overall number of interactions counted here. Conclusive evidence for impacts of competition on ecosystem processes is therefore best obtained directly, i.e., by network analyses based on expression levels of genes and quantities of proteins involved in the process of interest (Peršoh 2015).

Conclusion

Ectomycorrhizal and saprotrophic fungi co-occurred at consistent proportions in the strata throughout the spruce forest

soil profile, but the share of saprobes was higher in spring than in the preceding autumn. This indicates similar functionality of the fungal communities in the different strata, but only at a certain time. Species composition of communities and functional guilds was rather distinct, in particular between mineral soil and organic layers. Differences in nutrient use efficiency of individual fungi may therefore result in different types and rates of ecosystem processes in the strata, despite similar overall abundances of functional guilds. This highlights the need for considering performance of individual fungi to infer the impact of fungal communities on ecosystem processes in future studies, either by complementary experimental studies or by directly targeting the relevant functional genes and/or proteins in addition. Temporal variations in community composition further suggest that reasonable time frames have to be covered, in particular to link long-term processes, such as carbon cycling, to fungal community structure (Peršoh 2015).

Acknowledgements We thank Dirk Böttger and Norbert Lamersdorf (both Göttingen) and our project partners (i.e., working groups of François Buscot, Halle; Georg Guggenberger, Hannover; Barbara Reinhold-Hurek, Bremen; Ellen Kandeler, Hohenheim) for help during the soil sampling and the good cooperation. Christopher Sadlowski (Bochum) supported the library preparation and Andrey Yurkov (Braunschweig) revised the molecular identification of the basidiomycetous yeasts.

The study was designed by DB, DP and GR. DP and GR performed the sampling. NS extracted the DNA and conducted preliminary analyses. AB and DP prepared the amplicon libraries. AB performed the sequencing. DP analyzed the data and wrote the manuscript. All authors discussed and contributed to the final version of the manuscript.

Funding information The project was partly funded by the Deutsche Forschungsgemeinschaft (DFG-project RA 731/9-1).

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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