Plant Soil (2024) 496:319–340
https://doi.org/10.1007/s11104-023-06364-1

RESEARCH ARTICLE

Temperate tree microbiomes: divergent soil and phyllosphere microbial communities share few but dominant taxa

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Received: 23 June 2023 / Accepted: 16 October 2023 / Published online: 3 November 2023

Abstract

Aims The phyllosphere and soil are crucial and distinct microbial spheres in forests, connected through trees that interact with both. As part of the tree's holobiont, these communities are vital to the fitness and evolution of the host. Differences between the spheres may be particularly evident at the two extreme ends of tall and long-lived trees of natural temperate forest; the top-canopy and the soil. Here, we evaluated the connectivity between the top-canopy and soil microbial communities of European beech and Norway spruce trees to determine the significance of tree-sphere and host-species identity, and to assess the contribution of taxa inhabiting both spheres.

Methods Bacterial and fungal community composition was determined through metabarcoding analysis of linked top-canopy leaf and bulk soil samples collected from tall (old) trees in the natural forest of Bavarian Forest National Park.

Results This study shows sphere-specific communities in European temperate forests, characterized by low connectivity. Results highlight that spheres exert stronger influence than host identity. Only a few taxa inhabited both spheres, yet they accounted for the bulk of the (relative) abundance in each sphere.

Conclusion Analysing the divergence and shared characteristics of these interlinked communities redefines the tree holobiont concept and enhances our understanding of the evolution of plant-associated microbial communities in a sphere-specific manner. This study emphasizes the importance of examining multiple microbiome components for a thorough understanding of temperate forest ecology, while also highlighting the existence of a small group of overlapping taxa that may play a bigger role than previously anticipated.

Keywords Temperate forest microbiome · Phyllosphere · Bulk soil · 16S rRNA gene · ITS rRNA region · eDNA

Responsible Editor: Luz E. Bashan.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-023-06364-1.

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Introduction

Temperate forests are structurally complex ecosystems containing a great variety of habitats. Biotic and abiotic variables exhibit a high level of spatial

heterogeneity within forest ecosystems, both horizontally (e.g., between different forest stands) and vertically, from the soil to the top canopy. The microbial communities living in these habitats play important ecological roles at multiple spatial scales (Baldrian 2017). They impact individual plant health as either plant-promoters or pathogenic taxa (Terhonen et al. 2019), and also provide ecosystem services due to their role in, among others, nutrient cycling, and carbon sequestration (Graham et al. 2016). Forest microbial communities generally display high diversity, encompassing both r-strategists (higher growth-rates in resource-rich environments) and K-strategists (slow growth but adept energy source utilization) (MacArthur and Wilson 1967). These communities can be can be shaped by both deterministic (where species occurrence and abundance stem primarily from abiotic and biotic factors) and stochastic processes (influenced by random and probabilistic events) (Dini-Andreote et al. 2015). Soils host some of the densest microbial communities on earth, with high taxonomical and functional diversity (Pulleman et al. 2012). Forest soils provide a vast habitat for microbes, being characterised by high nutrient levels and extensive spatial heterogeneity of microhabitats (Kadowaki et al. 2014; Martins et al. 2013; Zhou et al. 2022). The canopy also covers a massive surface area which is characterized by an oligotrophic environment in combination with rapidly fluctuating environmental stressors such as ultraviolet (UV) radiation and desiccation (Lindow and Brandl 2003). The soil and canopy are thus important "spheres" in forest ecosystems, depicting the extremities of tall and long-lived trees. In the context of plants, we define sphere as a term to describe the zone of influence or the area of interaction between the plant, environmental components, and microbial communities. Examples of plant spheres are the phyllosphere (the microenvironment on aerial plant surfaces), rhizosphere (the soil zone surrounding/adhering to a root), endosphere (habitats within the plant's tissues) and the spermosphere (zone around the seeds of a plant) (Bais et al. 2006; Lemanceau et al. 2017; Vacher et al. 2016). The host plants connect these different spheres, forming a complex ecological unit with the microbial communities living within these spheres. The plant, as host organism, and its associated symbiotic microbial community are often considered as a holobiont in which the microbes play a central role in host biology, ecology, and evolution, and vice versa (Simon et al. 2019).

The concept of the holobiont emphasizes the idea that host organisms and their associated microorganisms are interdependent and co-evolve over time (Zilber-Rosenberg and Rosenberg 2008). Plants actively select microbial communities via plant exudation and anatomical properties (Whipps et al. 2008; Zhou et al. 2022). The plant is also a prominent factor in the (cross-)colonization of the different spheres, for example via xylem and phloem transport, leaf fall or seed germination (Barret et al. 2015; Chi et al. 2005; Guerreiro et al. 2017). Other colonization pathways include transport via air, rainfall/stemflow, rain splash or organisms (insect, bird, animal) (Bittar et al. 2018; Coluccio et al. 2008; Levetin and Dorsey 2006; Zarraonaindia et al. 2015; Zhou et al. 2020). In the soil, trees can influence microbial communities up to several meters from the tree stem, as shown by studies on soil microbial communities under European beech and Norway spruce trees (Nacke et al. 2016). Within the tree-associated soil habitat, rhizosphere communities can be considered as a subset of bulk soil communities (bare soil next to the plant/roots), with the latter showing a lower density but higher diversity of microbes (Bulgarelli et al. 2013; Zhou et al. 2022). The rhizosphere has, compared to the bulk soil, a limited spatial influence beyond immediate adjacency to the direct fine root material since it only covers nanometers or milimeters from the roots.

Analysing overlapping taxa and connectivity between the different spheres of a plant can provide essential insight in plant health, holobiont evolution, and ecosystem response to environmental change (Coince et al. 2014; Zilber-Rosenberg and Rosenberg 2008). Microbial communities do not solely affect processes within a sphere but can also exert effects throughout various plant compartments, either by influencing plant health (i.e., via biomass, metabolites or pathogen resistance) (Berlanga-Clavero et al. 2020) or by microbe-microbe interactions via the secretion of secondary metabolites (Liu et al. 2017). While different spheres can be controlled by dissimilar environmental variables resulting in divergent communities (Coince et al. 2014), abundant generalist microbes occurring in multiple spheres may be part of a core microbiome; being on the one hand actively selected by the host plant (Bai et al. 2022) and on the other hand shaping microbe-microbe and microbe-plant

interactions throughout the host (Hassani et al. 2018). Via these interactions, these shared taxa may play a central role in host ecology, fitness and evolution (Simon et al. 2019). Studies on crops and annuals show varying levels of similarities and connectivity between the above-ground and below-ground plant associated communities (Bai et al. 2015; Tkacz et al. 2020; Zarraonaindia et al. 2015), but information about the connectivity of different spheres in natural temperate forests is scarce (some relevant examples: Coince et al. 2014; Haas et al. 2018; Rodríguez-Rodríguez et al. 2023). The little research on the connectivity of these spheres in European forests is often either experimental (e.g. Haas et al. 2018; Potthast et al. 2022) or focuses on a small number of plants or plots (e.g. Beule et al. 2017; Coince et al. 2014). Connectivity and taxonomic similarities between these different microbial communities may be especially complex in trees due to the tree's longevity and large size, resulting in sphere-specific variation in environmental stresses and host-interactions (Flessa et al. 2012; Herrmann et al. 2021), calling for a deeper comprehension of the overlap and divergence within the tree holobiont.

In the current paper, we analyse and compare the bacterial and fungal communities in the canopy and soil of the temperate forests of Bavarian Forest National Park (Germany), in order to assess their connectivity and divergence. We will focus on the communities located in top canopy and tree-associated bulk soil (i.e., under the canopy and in the zone of influence of the individual trees; Nacke et al. 2016) since these spheres are located at the most extreme, and environmentally dissimilar ends of the tree holobiont. Our hypotheses are: (i) Variation in European beech and Norway spruce leaf and soil bacterial and fungal communities can be primarily attributed to tree sphere rather than host-species identity. (ii) Taxa possessing the capability to inhabit both spheres demonstrate a competitive advantage, leading to heightened (relative) abundances. Insights gained on the complex interactions of these distinct microbiomes have high relevance for developing strategies to manage and conserve forest ecosystems. Our study highlights the necessity to incorporate microbial diversity indices of multiple spheres in ecosystem assessments since this will provide a more all-inclusive picture of variation and vulnerability of microbial communities and ecosystem functions in European temperate forests.

Methods

Site description and sampling design

Top-canopy and bulk soil samples were collected in twenty-nine square plots of 30×30 m in Bavarian Forest National Park (Fig. 1). The park is located in south-eastern Germany and is part of the Bohemian Forest, one of the most extensive contiguous natural forest ecosystems in Central Europe (Křenová and Kiener 2013). The mountainous forests in the study area (altitudes between 300–1400 m a.s.l.) are dominated by European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*) (Bässler et al. 2015). The park was established in 1970 and has a 40-year no-intervention management strategy (since 1983), resulting in a gradual decline in spruce (mainly due to bark beetle infestations) and an increase in abundance of beech (as well as, to some extent, other tree species such as *Abies alba*) (van der Knaap et al. 2020).

Plots were stratified over stands (>75% canopy dominance) of European beech ($N=15$) and Norway spruce (N = 14), and were situated at altitudes ranging from 665 to 1160 m. Sampling was conducted in the same season, during July–August 2020, while broad leaves were fully mature and before senescence (Laforest-Lapointe et al. 2016). At each 300 m^2 plot, leaves were collected from three representative trees. Per tree, a leaf sample consisted of 10 individual broadleaves from different branches or 20–30 needles from 10 separate cohorts (internodes of the same age). Leaf samples were collected from the sun-exposed top canopy using a large slingshot (Tree runner Big-Shot) and a rope (Youngentob et al. 2016). A modified crossbow was used for tall trees (Ali et al. 2016). Wearing disposable gloves, falling leaves were caught before touching the ground to avoid contamination and stored in zip-lock bags. Leaves were pooled per tree to reduce between-leaves variation (Cordier et al. 2012). Per tree, a composite bulk soil sample was collected from the topsoil (0–10 cm depth, after removal of litter) in a 9 m^2 subplot located under the canopy of the sampled tree (max 2 m distance from the tree trunk). Per subplot, 9 cores were collected in a 3×3 grid using a 5 cm Ø×5 cm height corer. Composite samples were collected by pooling the nine cores in a sterile bag, removal of roots and stones, manual homogenization, and subsequently transferring a subsample into sterile 50 ml tube. Samples were

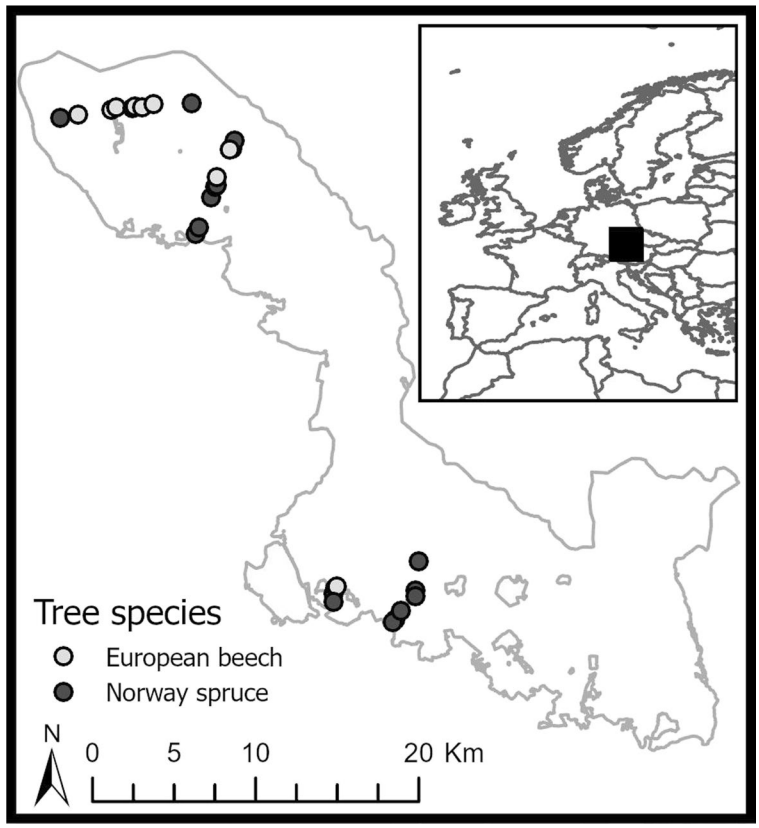

Fig. 1 Map of Bavarian Forest National Park showing the sample locations and park boundary

transported on ice to the laboratory where they were stored at -20 °C until further processing. Between samples, the soil corer was sterilized with 10% bleach followed by deionised water to avoid cross-contamination. For each fifth plot, an aliquot of the deionised water rinse of the corer was collected as field control.

DNA extraction and amplification

A sterile paper hole puncher (0.6 cm Ø) was used to punch leaf disks from broadleaf samples. For each tree, 0.1 g of broadleaf leaf-disks or needles were combined (representing 10 broadleaves or 10 needle cohorts) and homogenized using a Benchmark Beadbug™ Mini Homogenizer (D1030). Leaf-disks were cut-out and needles were selected from different parts of the leaves/cohorts to warrant a representative sample. Leaf total DNA (endophytes and epiphytes) was extracted using Qiagen Plant Pro extraction kit and the Qiagen Qiacube Connect extraction robot, following the manufacture's instructions. No sterilization was conducted prior to DNA extractions; total DNA from combined endophyte and epiphyte taxa was extracted (Zarraonaindia et al. 2015). Soil DNA was extracted following the phosphate extraction protocol of Taberlet et al. (2012), using minor modifications. Briefly, 15 g of well-homogenised soil sample was mixed with 15 ml saturated phosphate buffer (Na_2HPO_4; 0.12 m; pH ≈8) in order to extract extra-cellular DNA. After mixing and centrifugation, 2 mL of supernatant was purified using the NucleoSpin® soil extraction kit following the manufacture's instructions, but omitting the lysis step. Using the saturated phosphate buffer enabled processing larger soil volumes compared to the kit's lysis step, minimizing the effects of local heterogeneity. Negative extraction controls were included for each batch of 22 (leaf) or 25 (soil) samples. DNA concentrations were quantified on a Biotek Synergy HTX Multi Mode Reader, using the Quant-iT PicoGreen dsDNA Assay Kit, and standardised to 5 ng μl^{-1} (samples < 5 ng μl^{-1} were not standardised). Soil samples were further 100 × diluted to reduce polymerase chain reaction (PCR) inhibition. Prior to PCR, the field and

extraction controls for each sample and control type were combined.

DNA extraction and amplification were conducted at separate laboratories to reduce contamination risks. Bacterial (16S rRNA gene) and fungal (ITS rRNA region) DNA was amplified using the 515F/806R (Apprill et al. 2015; Parada et al. 2016) and ITS86/ITS4-ngs (Tedersoo et al. 2014; Turenne et al. 1999) primer sets, respectively. Amplification protocols and polymerase chain reaction (PCR) recipes are shown in supplementary table 1. Peptide Nucleic Acid (PNA) clamps were used (PNA Bio Inc.) to block the amplification of host chloroplast and mitochondrial DNA in the leaf 16S PCR reactions (Lundberg et al. 2013). Amplicons were sent to Genome Quebec (Montreal, Canada) for library preparation and Next Generation paired-end sequencing. Primers contained a CS1 (forward primer) or CS2 (reverse primer) adaptor sequence at the 5'-end to allow for multiplexing using the Fluidigm Access Array System (Fluidigm, South San Francisco, CA). An indexing PCR was used to attach the indexes and i5/i7 Illumina adapter sequences to the amplicons. Sequencing was performed on one lane of the Illumina NovaSeq 6000 SP platform using the PE250 kit.

Bioinformatic and data analyses

Bioinformatic analyses were performed using the QIIME 2™ software suite (Bolyen et al. 2019) and all statistical analyses were performed in R version 4.2.3 (https://www.R-project.org/). Post-clustering curation was conducted using LULU (Frøslev et al. 2017), and the SILVA (Quast et al. 2012) and UNITE (Nilsson et al. 2018) databases were used for Taxonomical assignment. ASV tables were further (i) blank corrected (removal criteria: max reads in blanks ≥ max reads in samples), (ii) filtered to retain only bacterial and fungal reads, and (iii) corrected for tag-switching (following: Taberlet et al. 2018). All taxa present with less than 10 reads in total were removed to reduce low frequency noise (Alsos et al. 2016; Polling et al. 2022). Details of the bioinformatic pipeline can be found in supplementary table 2. Curated ASV tables were rarefied to 15,000 reads (supplementary Fig. 1) using the 'rrarefy' function of the Vegan v. 2.6–2 R package (Oksanen et al. 2022). The average of 100 rarefactions was used to reduce stochastic effects (Cordier et al. 2019). The leaf and soil sample pairs of two subplots/trees were removed from the ITS dataset due to a read count lower than 15,000, and one plot only contained information of 2 trees/subplots. The package 'Vegan' was also used to visualise and test for differences in community composition between spheres (leaf vs. soil samples) and tree species, using Principle Coordinate Analyses (PCoA) and PERMANOVA (999 permutations) on Bray–Curtis dissimilarities constructed from Hellinger-transformed read counts. Pearson correlations between communities were tested using Mantel tests. Permutations were restricted per plot to account for within plot pseudo-replication in PERMANOVA and Mantel tests. Differences in alpha diversity were visualised using ASV accumulation curves and tested using linear mixed models ('nlme', v. 3.1–162) (Pinheiro et al. 2022) using plot ID as random effect. Venn diagrams were constructed ('ggvenn' v. 0.1.9) (Yan 2021) to visualise differences in ASV overlap between spheres and tree species and differences in the fraction of ASVs overlapping per sample were determined using linear mixed models (random effect: plot ID) on logit-transformed proportions. Connectivity between the spheres was assessed using SPIEC-EASI (Sparse InversE Covariance estimation for Ecological Association and Statistical Inference) co-occurrence networks (Kurtz et al. 2015) incorporated in the package 'NetCoMi' (Peschel et al. 2020). Soil and leaf data were combined per tree to allow for the construction of across-sphere networks, and taxa occurring in both spheres were allocated multiple nodes for each respective sphere. Data were filtered to remove any ASVs with < 10% prevalence, and read counts were centered log-ratio (clr) transformed as part of the SPIEC-EASI algorithm. Networks were constructed and compared using the 'NetCoMi' package, and the adjacency matrix was used to classify edges as within-spheres or between-spheres associations. Hub taxa ("key-species") were assigned based on highest eigenvector centralities (> 95% quantile), representing nodes that have a central position in the network (Peschel et al. 2020). Discriminant analyses ('Maaslin2' v. 1.12) (Mallick et al. 2021) on Hellinger-transformed read counts were ran, with Plot ID as random effect, to determine sphere specificity of bacterial and fungal families. Families were classified as unique (occurrence in only one sphere), dominant (discriminant [$p < 0.05$] to a specific sphere), or non-specific (not discriminant [$p > 0.05$] to a specific sphere). Differences in relative read abundances of discriminant taxa

(ASVs with > 10% prevalence) between spheres and tree species were further visualised over multiple taxonomic levels using heat trees analyses ('metacoder' v. 0.3.5) (Foster et al. 2017).

Results

Data summary

A total of 31,620,621 bacterial and 34,032,206 fungal reads remained after bioinformatic processing (supplementary table 2), with a mean (± SD) read count of 92,089 (± 42,250) 16S rRNA and 147,280 (± 86,560) ITS rRNA in leaf and 275,592 (± 41,831) 16S rRNA and 248,443 (± 36,380) ITS rRNA in soil samples. Rarefaction to 15,000 reads per sample resulted in a total of 38,538 bacterial ASVs (mean ± SD leaf: 389 ± 194; mean ± SD soil: 2235 ± 507) and 5,168 fungal ASVs (mean ± SD leaf: 246 ± 96; mean ± SD soil: 101 ± 33), al loss of 856 bacterial (2.2%) and 62 Fungal (1.2%) ASVs compared to the non-rarefied dataset. Phyllosphere bacterial communities were dominated by Proteobacteria (mean relative abundance in European beech samples: 55%; Norway spruce samples: 58%), Bacteroidota (beech: 34%; spruce: 9%) and Acidobacteriota (beech: 5%; spruce: 18%) while soil bacterial communities were dominated by Acidobacteriota (beech: 33%; spruce: 35%), Proteobacteria (beech: 22%, spruce: 19%) and Actinobacteriota (beech: 11%, spruce: 16%). Phyllosphere fungal communities mainly consisted of Ascomycota (beech: 97%, spruce: 93%) while the most abundant phyla in soil fungal communities were Basidiomycota (beech: 77%, spruce: 71%) and, to a lesser extend, Ascomycota (beech: 22%, spruce: 27%).

Diversity and community composition differences between compartments and host tree species

Bacterial and fungal communities differ both between plant spheres and host tree species (Fig. 2). PERMANOVA and Principle Coordinate (PCoA) analyses showed a significant ($p < 0.01$) influence of sphere and tree species, and a significant ($p < 0.01$) interaction between them for both 16S and ITS (Fig. 2; supplementary table 3). Samples clustered among spheres along the first PCoA axis, which explained 40% and 21% of the variance in community structure for 16S and ITS respectively. Clustering based on tree-species was along the second PCoA axis for the leaf samples (explaining 11% and 14% of the variance for 16S and ITS respectively) and the third axis for the soil samples (explaining 6% and 5% of the variance for 16S and ITS respectively). PERMANOVA analyses (supplementary table 3) confirmed the attribution of a larger proportion of variation to tree species in leaf samples (bacterial R^2: 0.34; fungal R^2:

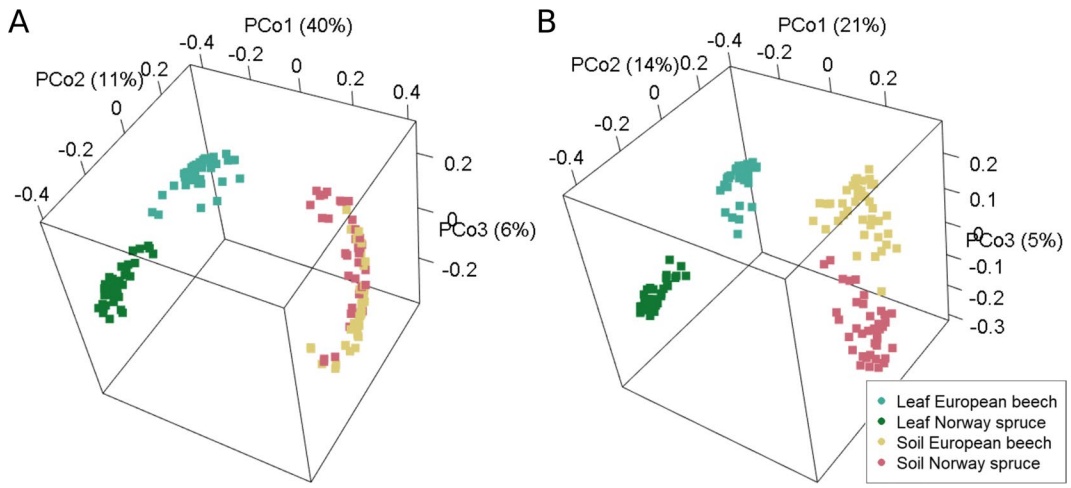

Fig. 2 Principal Coordinates Analysis of bacterial (**A**) and fungal (**B**) communities, based Bray–Curtis dissimilarities constructed from Hellinger-transformed read counts of Amplicon Sequence Variants (rarefied to 15,000 reads)

0.39) than in soil samples (bacterial R^2: 0.06; fungal R^2: 0.09).

Total bacteria richness was several orders of magnitude higher in soil samples than in leaf samples and only the ASV accumulation curves of leaf samples showed no overlap in 95% confidence intervals between tree species (Fig. 3A). Total fungal richness was a factor 10 lower compared to total bacterial richness, which could mainly be attributed to a substantially lower diversity detected in the soil. Differences in fungal richness between spheres varied per tree species (Fig. 3B). Linear mixed model analyses of Shannon diversity on the subplot level (Fig. 3C-D) confirmed these patterns with significant differences between groups for both bacterial ($F_{83} = 524.8$, $p < 0.0001$, marginal R^2 [R^2m] = 0.80) and fungal ($F_{81} = 54.9$, $p < 0.0001$, R^2m = 0.52) ASVs. Pairwise-comparisons showed significantly ($p < 0.001$) higher bacterial Shannon diversity in soil versus leaf samples and beech leaf versus spruce leaf samples, while soil bacterial diversity did not differ significantly ($p = 0.12$) between tree species (Fig. 3C). Spruce leaf samples contained a significant ($p < 0.0001$) higher fungal Shannon diversity than beech leaf samples and soil samples, independent of tree species. Soil fungal diversity did not differ significantly between tree species (Fig. 3D; $p = 0.19$).

ASV connectivity between phyllosphere and soil

The majority of bacterial and fungal ASVs were unique to either the soil or to the leaf samples (Fig. 4

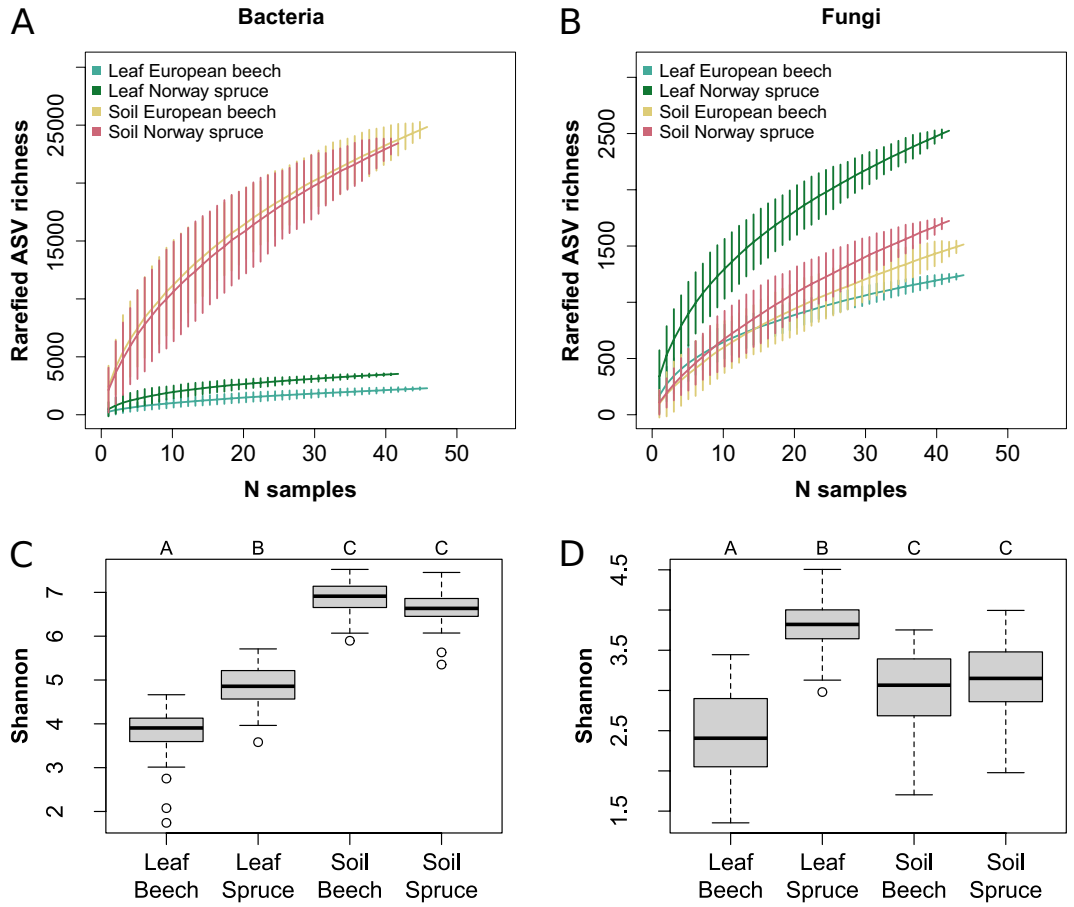

Fig. 3 Variation in bacterial (**A**-**C**) and fungal (**B**-**D**) accumulative ASV richness (±95% confidence interval) and mean Shannon diversity of leaf and soil samples (rarefied to 15,000 reads) collected from two tree species (European beech and Norway spruce). Different letters indicate significantly different groups (linear mixed model with Benjamini–Hochberg pairwise comparisons, $p < 0.05$)

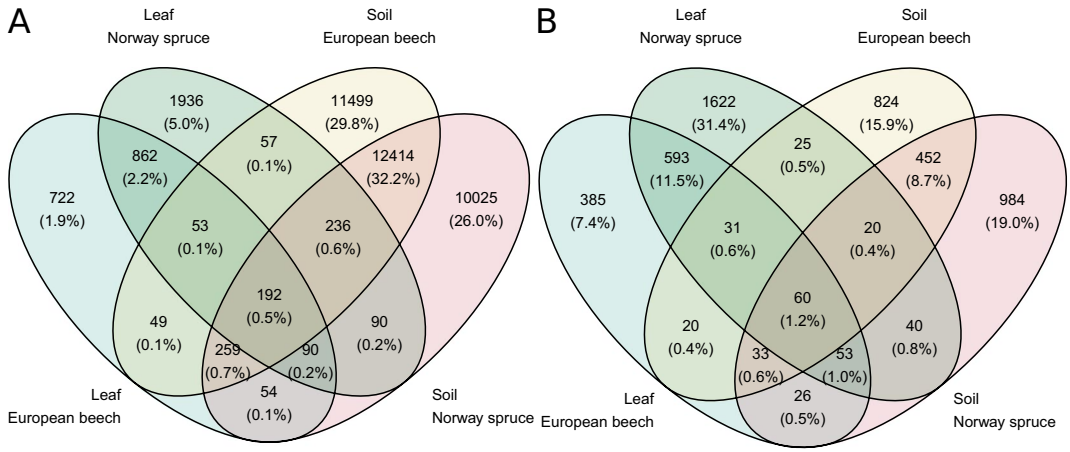

Fig. 4 Venn diagrams showing shared bacterial (**A**) and fungal (**B**) ASVs in bulk-soil and phyllosphere compartments in European beech and Norway spruce forests. Percentage of total number of ASVs is shown between brackets

& supplementary table 4). A substantially lower proportion of families was unique to either of the spheres compared to ASV-level taxa specificity (supplementary table 4), indicating that most variation in unique taxa is at lower taxonomic ranks. Only a small proportion of ASVs occurred in both spheres (bacterial: 2.8%; fungal: 6.0%). See supplementary table 5 for the top 10 overlapping taxa per kingdom. A total of 448 bacterial ASVs were detected in both soil and leaf samples within the same subplot, with a significantly higher mean proportion of shared bacterial ASVs in spruce compared to beech subplots (LMM: $F_{27}=22.4$, $p<0.001$, $R^2m=0.23$). Although when comparing proportions separately per sphere, this difference was only significant for the soil communities (supplementary Fig. 2A-C). The number of fungal ASVs detected in both soil and leaf samples within the same subplot was 119, and no significant difference was detected in the mean proportion of shared fungal ASVs per subplot between tree species (supplementary Fig. 2D-F; LMM: $F_{27}=1.52$, $p=0.23$, $R^2m=0.02$). A significant positive correlation was found in the community composition of bacterial phyllosphere and soil communities in Norway spruce forests (Mantel test: $R=0.32$ $P<0.01$). No significant correlations, though, were detected in European beech forest communities (supplementary table 6). Also no correlations were shown for Alpha diversity between the leaf and soil samples, independent of tree species or microbial kingdom (supplementary table 6). Correlation analyses of the relative abundance of individual families between spheres did only detect three families with significant (BH-adjusted) Spearman correlations in Norway spruce forest stands (*Xanthobacteraceae*: $R=0.56$, $P<0.05$; *Pleomassariaceae*: $R=0.56$, $P<0.01$; and *Sclerococcaceae*: $R=0.64$, $P<0.001$), while none of the bacterial or fungal families showed significant correlations between the two spheres in European beech forest stands.

Co-occurrence network analyses showed no explicit clustering of taxa (nodes) per sphere in either the bacterial nor the fungal networks (Fig. 5). Global network statistics (supplementary table 7) showed differences between bacterial and fungal networks, especially in terms number of nodes (2068 vs. 142), modularity (0.2 vs. 0.6) and positive edge percentages (57–58% vs. 64–74%), indicating kingdom specific variation in ecological associations within the host tree. Fungal networks showed more pronounced differences in network structure between host tree species compared to bacterial networks. Particularly the number of components (48 vs. 16), clustering coefficient (0.13 vs. 0.07) and positive edge percentage (74% vs. 64%) were higher in the fungal beech network than the fungal spruce network. Taxa detected in soil samples were dominant in the bacterial networks, as shown by the high number of nodes and hubs (i.e. keystone species), while the fungal networks were dominated by taxa from leaf samples. The low number of edges (6–12%) between taxa from different spheres indicates low connectivity between the spheres (Table 1). Even for the few taxa that do occur in

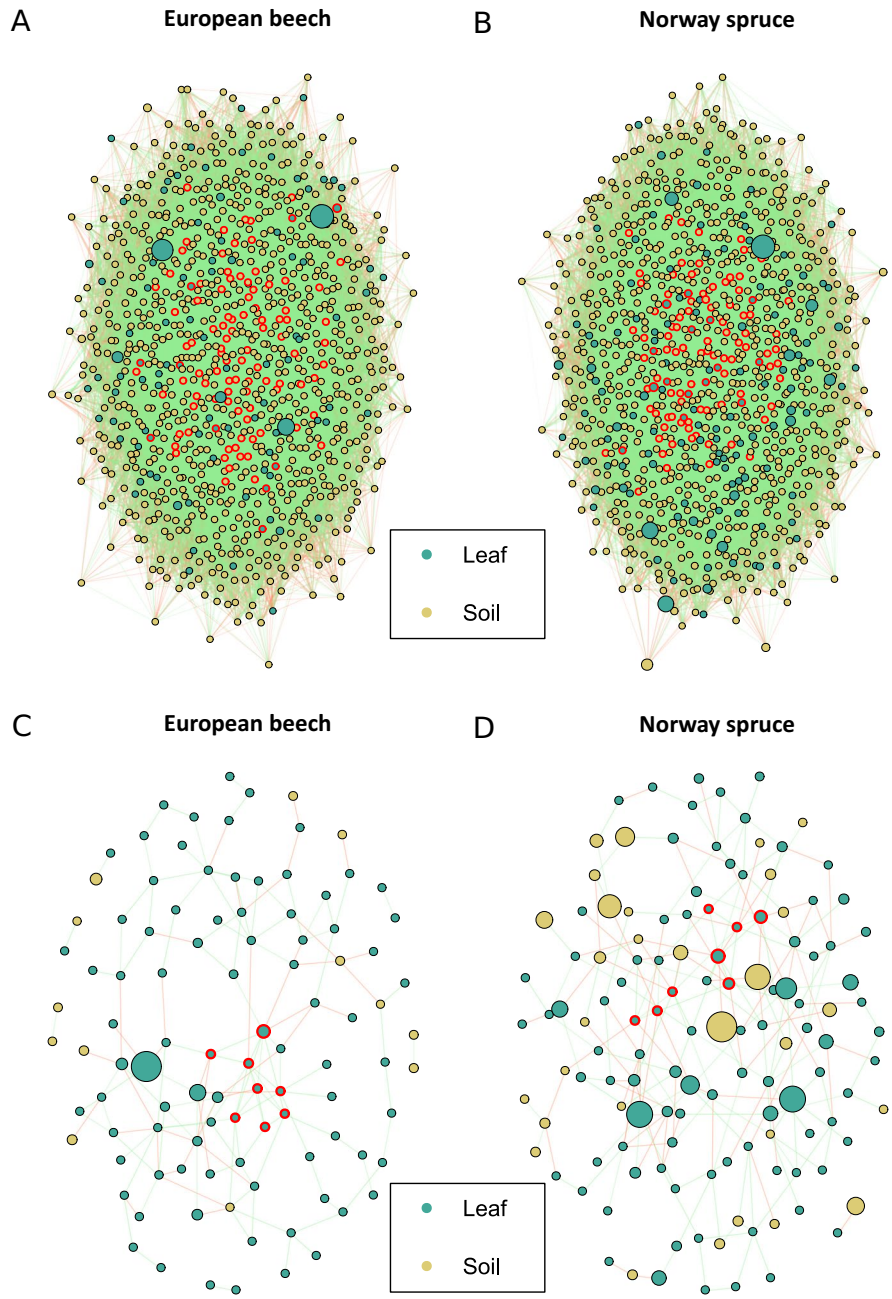

Fig. 5 Co-occurrence networks of bacterial (**A**-**B**) and fungal (**C**-**D**) ASVs (> 10% prevalence) detected in forest bulk soil and phyllosphere samples. Data from soil and leaf samples was combined per tree prior to SPIEC-EASI network construction, and taxa occurring in both spheres were allocated multiple nodes for each respective sphere. Edge colour represents negative (red) and positive (green) associations. Hub nodes (based on eigenvector centralities) have a red outline. Node size represents differences in relative read abundance. Maximum 1000 nodes with the highest degree are shown

both spheres, the nodes were not linked, i.e. occurrence patterns did not correlate between spheres.

Taxa specificity and relative abundance in phyllosphere and soil

Even though the majority of ASVs detected were unique to either the soil or the leaf samples (Fig. 6A), the contribution of these unique taxa was low in terms of relative abundance (Fig. 6B). Discriminant taxa (significant higher relative abundance in leaf or soil samples based on Maaslin2 discriminant analyses) showed a substantially lower ASV richness compared to unique taxa but contributed most to the microbial communities in terms of relative read abundance, especially in leaf samples (Fig. 6). Variation in taxa

Table 1 Number of edges between nodes of similar and different spheres in bacterial and fungal co-occurrence networks generated by SPIEC-EASI

	Edge type	Positive	Negative	Total
Bacteria Beech	Soil-soil	41,858	29,388	71,246
	Leaf-leaf	2066	300	2366
	Soil-leaf	2797 (6.0%)	3758 (11.2%)	6555 (8.2%)
Bacteria Spruce	Soil-soil	45,812	31,546	77,358
	Leaf-leaf	1636	934	2570
	Soil-leaf	3928 (7.6%)	4047 (11.1%)	7975 (9.1%)
Fungi Spruce	Soil-soil	20	4	24
	Leaf-leaf	208	94	302
	Soil-leaf	19 (7.7%)	25 (20.3%)	44 (11.9%)
Fungi Beech	Soil-soil	4	2	6
	Leaf-leaf	198	56	254
	Soil-leaf	8 (3.8%)	9 (13.4%)	17 (6.1%)

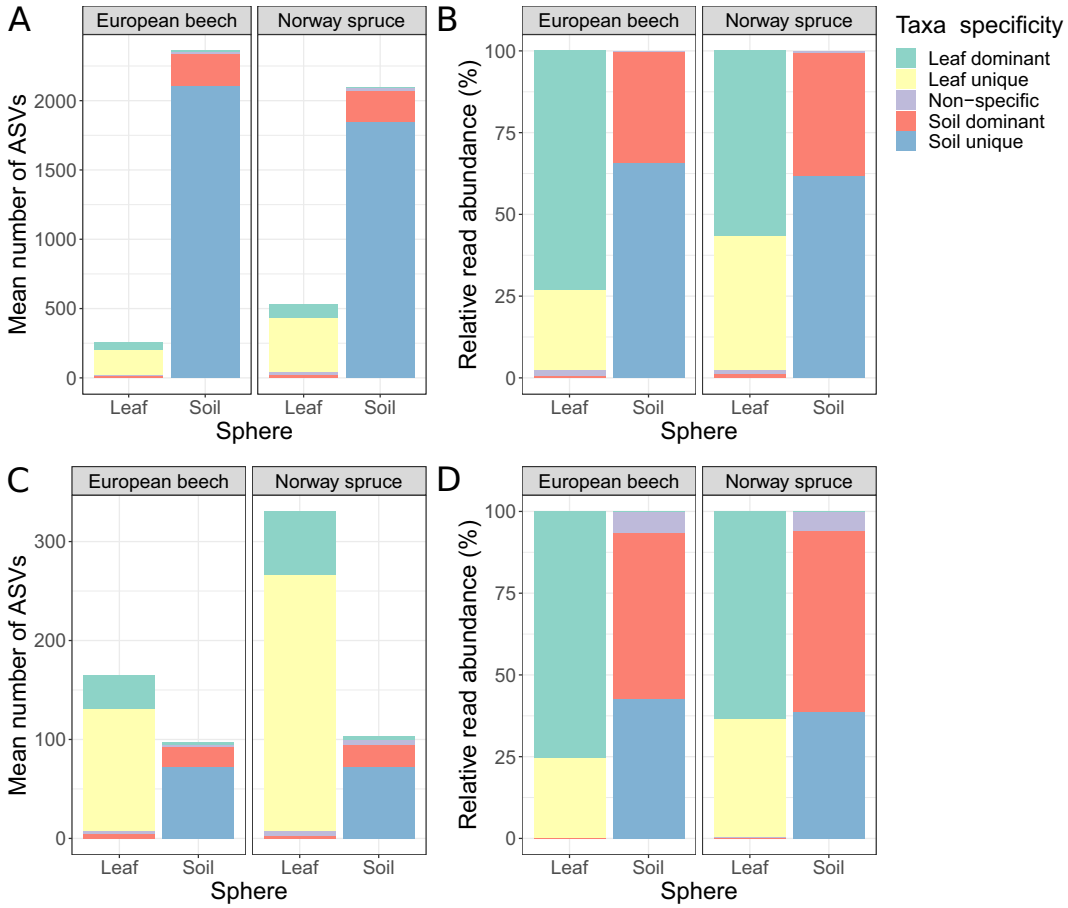

Fig. 6 Variation in ASV specificity of bacterial (**A** & **B**) and fungal (**C** & **D**) detected in leaf and soil samples in European beech and Norway spruce forests stands. Panels A and C show variation in ASV richness and panels B and D show variation in mean relative read abundances. Samples have been rarefied to 15,000 reads and specificity was determined by Maaslin2 discriminant analyses

specificity between tree species was most pronounced in the phyllosphere. Spruce stands show a higher contribution of unique leaf bacterial and fungal ASVs, both in terms of richness and relative abundance (Fig. 6). Soil profiles did not show pronounced differences in the ratio unique:dominant taxa of both microbial kingdoms, either in terms of richness or relative abundance (Fig. 6). The phyllosphere communities also showed clear patterns in variation in the relative abundance of leaf-discriminant taxa between the beech and spruce phyllosphere, while this was much less apparent in the soil (Fig. 7, supplementary Figs. 3 and 4).

Sphere-specific patterns of dominant taxa

Abundant families mainly varied in terms of relative abundance between spheres (Fig. 8). Soil-discriminant bacteria were found in a wide range of phyla (Fig. 8A) while soil-discriminant fungi mainly belonged to one phylogenetic group, the order Agaricomycetes in the phylum Basidomycota (Fig. 8B). In case of leaf-discriminant taxa, both the bacterial and fungal taxa belonged to a wide range of phylogenetic groups (Fig. 8). Acidobacteriota were underrepresented in the phyllosphere with *Acidobacteriaceae* as notable exception (Fig. 8A). Abundant (> 5%) ASVs in the leaf samples were assigned to genera 1174–901-12 (*Beijerinckiaceae*) and *Mucilaginibacter* while the most abundant (> 1%) ASVs in the soil samples were all uncultured Acidobacteriales (supplementary table 8). Abundant (> 5%) fungal ASVs in the leaf samples were assigned to the genera *Naevala* and *Erysiphe*, and the family *Phaeosphaeriaceae*. In the soil samples, the most abundant (> 5%) fungal taxa were *Russula cyanoxantha*, *Russula vesca*, *Imleria badia* and an unassigned ASV in the order Atheliales (supplementary table 8).

Discussion

The top canopy phyllosphere and bulk-soil bacterial and fungal communities of European beech and Norway spruce trees in Bavarian Forest National Park showed distinct microbial communities, with little overlap. In line with our first hypothesis, tree spheres had a multitude stronger (2–7 times) influence than host-species on both the fungal and bacterial communities, respectively, indicating that the microbiome 'worlds' in the soil under a tree and in the leaves of the upper crown are detached in terms of community composition, both across tree species as well as within a host species.

Host identity effects were sphere and kingdom specific, with main differences between beech and spruce fungal communities in the phyllosphere. While the majority of taxa were rare and specialised in either the soil or top-canopy, the few taxa possessing the capability to inhabit both spheres were relatively abundant, as postulated in our second hypothesis. Divergent microbial communities between plant spheres have been shown in a large variety of plant species (Fonseca-García et al. 2016; Yang et al. 2022), including a limited number of studies on temperate European natural forests (e.g. Beule et al. 2017; Coince et al. 2014; Haas et al. 2018; Lynikiene et al. 2020; Schneider et al. 2021). Our study adds to this knowledge by providing a comprehensive representation of the microbial communities across multiple spheres of the same individual tall forest tree, revealing patterns in two environmental spheres and among two microbial kingdoms sampled from 86 trees distributed over 29 plots in one of the largest contiguous natural forest ecosystems in Central Europe (Křenová and Kiener 2013). By sampling the top-canopy and bulk soil, our study focusses on the two most extreme environmental spheres of the tree's holobiont which have distinct functions in the forest ecosystem (Bulgarelli et al. 2013).

Limited connectivity and sphere-specific community composition

The phyllosphere and soil are two unique ecological spheres that are linked by the same host plant (Vandenkoornhuyse et al. 2015). The host plant and associated microbial communities can be viewed as an holobiont in which the microbes play a central role in host biology, ecology, and evolution (Simon et al. 2019). The host tree has been traditionally postulated to be an important constant in this assemblage, not only by physically connecting the different microbial components, but also by actively selecting and shaping these communities by selective pressure via root exudates, secondary metabolites, and leaf chemical and topological properties (Bais et al. 2006; Cesarz et al. 2013; Karamanoli et al. 2005). Our results,

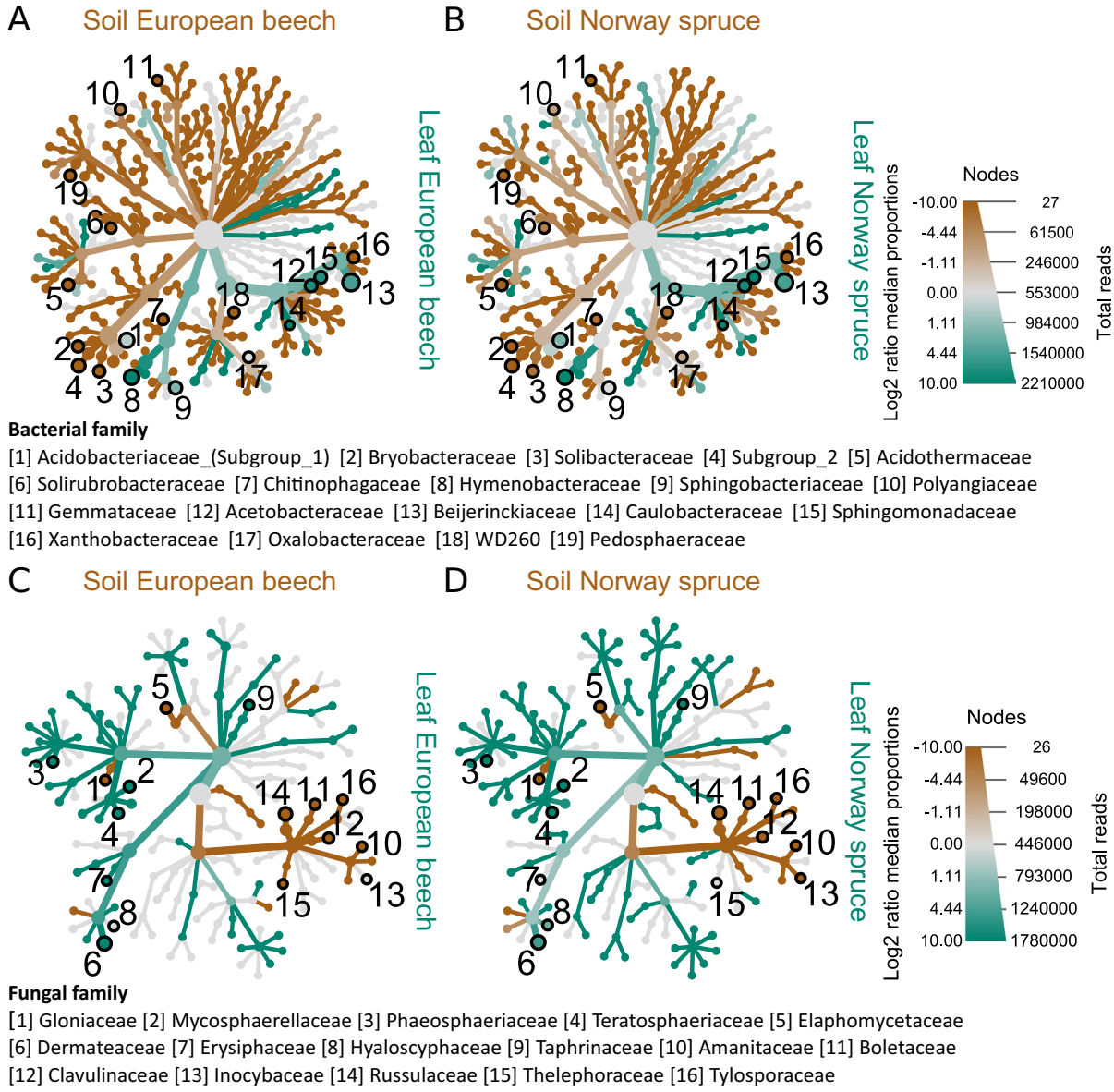

Fig. 7 Heat trees summarising differences in relative read abundance of bacterial (**A**-**B**) and fungal (**C**-**D**) taxa detected in soil and leaf samples over different taxonomic levels. Nodes highlighted in brown and green were significantly ($p < 0.05$) discriminant for the soil and leave compartments, respectively (based on Maaslin2 discriminant analyses). Colour intensity represent differences in Log2 ration median proportions of relative read abundances and node size represents total read count of the taxon. Abundant families (> 1% mean relative abundance) are highlighted. Full taxonomical key is provided in supplementary Figs. 3–4. Samples were rarefied to 15,000 reads prior to analysis

however, indicate that the environmental sphere has a larger influence on tree-associated communities than host-selective pressures. Additionally, network analyses showed limited connectivity between the spheres, with only a fraction (6–12%) of associations detected across spheres. Concerning these few co-occurrences that span across spheres, it is improbable that they originated from direct interactions among microbiome species. Instead, they are more likely the outcome of indirect interactions facilitated through the host tree. Known examples of these indirect interactions within host plants are the transfer of

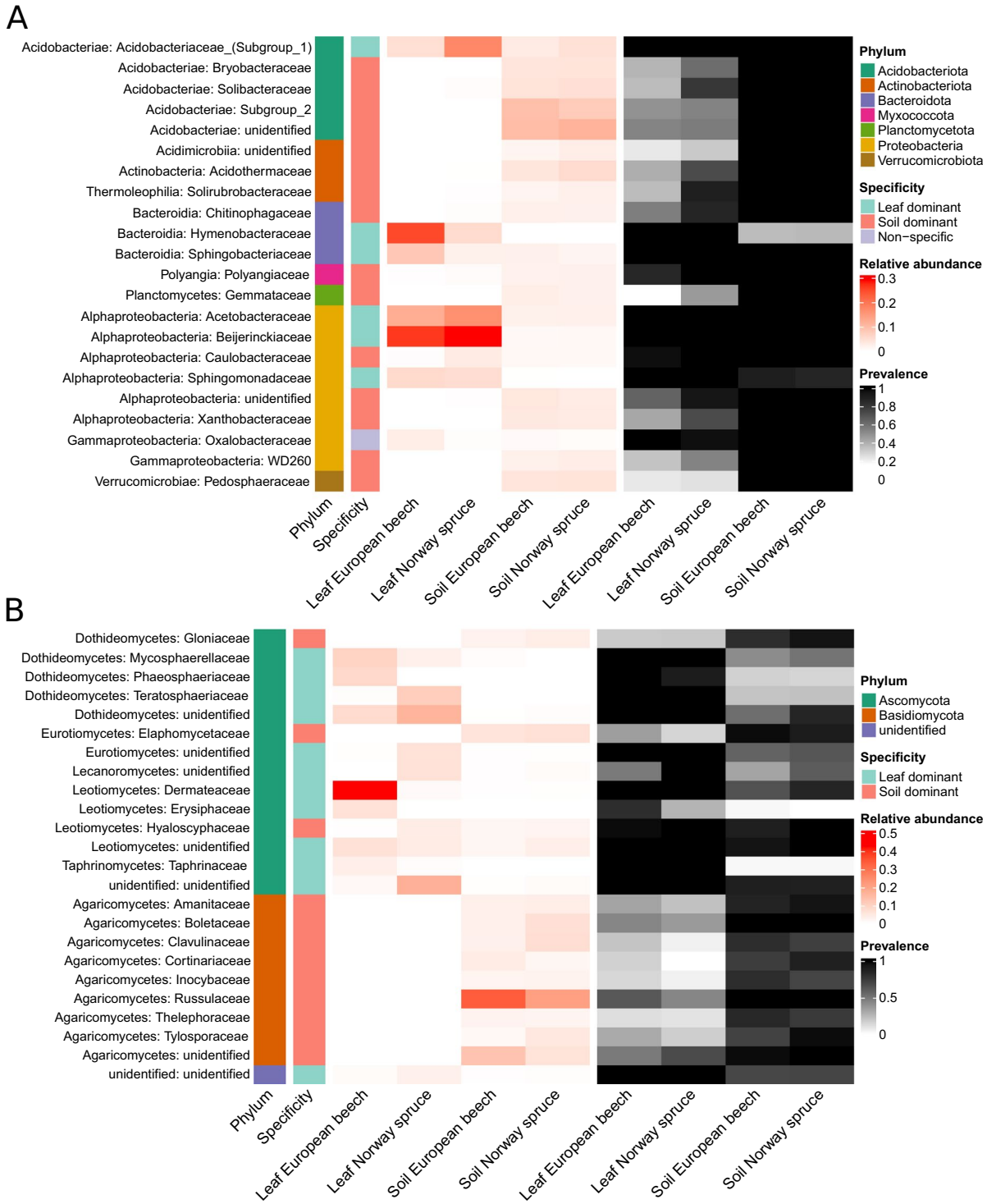

Fig. 8 Heatmaps of bacterial (**A**) and fungal (**B**) taxa identified in phyllosphere and soil samples in European beech and Norway spruce forest stands, showing variation in mean relative read abundance and prevalence (proportion of samples present) of abundant families (> 1% mean relative abundance). Family specificity was determined by Maaslin2 discriminant analyses and samples were rarefied to 15,000 reads prior to analysis

genes, fluctuations in the host's C-dynamics and the production of phytohormones (Chen et al. 2017; Liu et al. 2017; Potthast et al. 2022). The bacterial network exhibited a substantial contribution of taxa from soil samples, both in terms of number of nodes and hub taxa, while their role in the fungal network was comparatively minor. A similar pattern was observed in the heat trees, with soil-specific taxa dominating the bacterial trees and leaf-specific taxa being most apparent in the fungal trees. The leaf-taxa dominated fungal networks also showed a 3.8 times higher modularity compared to the soil-taxa dominated bacterial networks, indicating a higher subdivision of the network in different subcommunities which potentially inhabit different niches within the phyllosphere (Abdelfattah et al. 2016; Herrmann et al. 2021). Soil environments provide temporally stable, highly nutritious and micro-heterogenous habitats while the phyllosphere is characterised by oligotrophic habitats with harsher environmental conditions and large temporal variation (Kadowaki et al. 2014; Lindow and Brandl 2003; Zhou et al. 2022). As a result of these sphere-specific environmental influences, forest soil communities are driven by long-term and deterministic processes, such as long-term forest stand conditions and micro-scale variation in soil conditions (especially pH) (Hannam et al. 2007; Rodríguez-Rodríguez et al. 2023), while phyllosphere communities are largely influenced by host-identity (Laforest-Lapointe et al. 2016) and spatio-temporal variation in environmental and host variables such as climate and leaf senescence (Coince et al. 2014; Flessa et al. 2012). Limited cross-colonization may also further the establishment of sphere-specific communities, especially in the case of large trees (i.e. some trees were 30 m + as sampled in our study, and average height was 24 m) in which the top canopy is located at large distance from the soil, and is characterized by extreme conditions compared to the phyllosphere of shorter plants (Bodenhausen et al. 2013; Herrmann et al. 2021; Unterseher et al. 2007).

The sphere-specific adaptations of the microbial communities are exemplified by a distinction in the functions associated with the main observed taxonomic groups. The soil showed highly diverse communities (Dukunde et al. 2019; Wilhelm et al. 2023; Wubet et al. 2012), and the soil discriminant fungal taxa (e.g. *Russulaceae*, *Elaphomycetaceae* and *Gloniaceae*) were mainly ectomycorrhizal groups with known associations with a diversity of tree species (Castellano and Stephens 2017; Nacke et al. 2016; Spatafora et al. 2012). As indicated by our results, leaf communities showed a substantial lower bacterial alpha diversity (a factor 10 lower) and largely consisted of families which are well adapted to the phyllosphere. An example are the members of the bacterial family *Hymenobacteraceae,* which contain carotenoid pigments that can provide protection against the high levels of UV radiation typical for the phyllosphere (Munoz et al. 2016; Vacher et al. 2016). The identified leaf discriminant fungal taxa predominantly belonged to the very diverse fungal orders Dothideomycetes and Leotiomycetes, which are common endophytes of temperate trees (Delhomme et al. 2015; Lazarević et al. 2022; Lynikiene et al. 2020; U'Ren et al. 2012; Unterseher et al. 2013), and encompass many plant pathogens, saprobes and extremotolerant species that are associated with a broad range of hosts (Hujslová et al. 2012; Ohm et al. 2012; Zhang and Wang 2015).

Host-identity was more pronounced in the phyllosphere than the soil, both in terms of diversity and community composition. Within the soil, fungal communities showed a sharper distinction between host tree species than bacterial communities, likely related to the symbiotic association of these ectomycorrhizal fungi with specific tree species (Urbanová et al. 2015). Within the phyllosphere, the high diversity of fungi in coniferous trees is probably related to the longevity of needles compared to broadleaves, providing a more stable and longer-lived environment for the fungal community (i.e., filamentous ascomycete endophytes) to inoculate and reach a steady state (Abdelfattah et al. 2016; Flessa et al. 2012; Osono 2008). Norway spruce trees also showed a higher connectivity (number of positive network edges) and proportion of shared bacterial taxa between the bulk-soil and leaf samples, compared to European beech trees. The latter could mainly be attributed to a higher proportion of soil taxa found in the phyllosphere (Supplementary Fig. 2). Coniferous forest soils are characterised by a low pH and high concentrations of difficult to digest components such as lignin (Achilles et al. 2021; Berg 2008). Coniferous soil microbial communities are, consequentially, dominated by acidophilic taxa and saprophytic species which show resistance to environmental

stressors such as drought and season (Nacke et al. 2016; Wilhelm et al. 2023), characteristics that may increase survival in the phyllosphere. Coniferous-deciduous specific differences in multi-year versus single-year leaf fall (relating to host life history traits such as leaf age) may further be an especially relevant host-driven factor in the highly seasonal temperate forests sampled here which could influence host-specific connectivity between the phyllosphere and soil. This would include (i) increasing the rate of 'seeding' with bacteria and fungal taxa and spores from fallen leaves on the ground during leaf senescence in deciduous forests (especially in autumn) and (ii) providing a stable environment over time for leaf communities to develop, mature and evolve in coniferous trees due to the longevity (multi-year duration) of needle leaves (Flessa et al. 2012). Other relevant differences in host attributes may include leaf surface properties (e.g. leaf morphology and area), the excretion of soluble carbohydrates and (micro) nutrients, and the production of secondary metabolites and antibiotics (Bodenhausen et al. 2014; Kembel et al. 2014; Lajoie et al. 2020). Deciduous and coniferous trees also have distinct feedback loops between trees and the surrounding soil through the formation of humus (mor, moder and mull) (Handley 1954); influencing soil fertility and properties (Nacke et al. 2016; Ponge 2013). These humus forms are characterised by different soil characteristics including pH, C:N ratio and nutrient composition, which in turn drive soil microbial community structure (Cesarz et al. 2013; Choma et al. 2020; Dukunde et al. 2019; Nacke et al. 2016). The strong link to host identity might limit the dispersal of phyllosphere communities, increasing their sensitivity to habitat fragmentation (David et al. 2016; Helander et al. 2007). This is of less concern for bulk soil communities due to their weaker link between host and microbial phylogeny and the less discrete nature of the soil environment. Other soil-associated spheres as the rhizosphere may, though, be more sensitive to habitat fragmentation due to their stronger relationship with the host (Bai et al. 2022). Overall, host identity can be considered as an important driver of microbial communities. Though less influential than the tree sphere habitat, host identity exhibits sphere-specific influences on the microbial communities impacting the fitness and evolution of the whole tree holobiont.

The characteristics of shared taxa in forest soil and phyllosphere communities

Taxa inhabiting both spheres were a major component of both the phyllosphere and soil communities in terms of relative read abundance, especially considering that only a small subset of taxa were shared between the spheres. Even though most of these taxa showed sphere-specific preferences, the capability to inhabit multiple spheres within the holobiont indicates a competitive advantage, which could be related the plant-beneficial/pathogenic capabilities of these taxa. Taxa occurring in both spheres (supplementary table 5) shared characteristics of high habitat flexibility, allowing them to adapt to the divergent environment of both the soil and top-canopy. They largely belonged to taxonomic groups with ecological phenotypic diversity and occurrence in a wide range of habitats (Bozoudi and Tsaltas 2018; Heuchert et al. 2005; Kaur et al. 2017; White et al. 1996; Willems 2014). Many of these taxa are moreover capable of N-fixation or are in other ways involved in nutrient or carbon cycling, functions that can be either part of the microbe-plant symbiosis in the rhizosphere or provide a competitive advantage in the oligotrophic phyllosphere (Azcón-Aguilar and Barea 2015; Kielak et al. 2016; King and Weber 2007; Marín and Arahal 2014). Also adaptations such as the production of pigments can be beneficial in both spheres since these can reduce competition in the soil due to their anti-bacterial properties and provide protection against UV-radiation in the canopy (Asaf et al. 2020; Pankratov and Dedysh 2010; Rashid et al. 2014). Many of these sphere-overlapping taxa show a strong association with the host. The bacterial taxa *Burkholderiaceae, Conexibacter* and *Sphingomonas* have, for example, known plant-beneficial or plant-pathogenic capabilities (some exhibit both functions based on contextual factors) (Akinola et al. 2021; Asaf et al. 2020; Romero-Gutiérrez et al. 2020). Many of the overlapping Ascomycota fungal taxa (e.g. *Cladosporium* and *Aureobasidium pullulans*), on the other hand, come from plant pathogenic families (Cooke 1959; Heuchert et al. 2005). Remarkable was the inclusion of several fruitbody forming basicomicetes (e.g. *I. badia* and *R. cyanoxantha*) in the group of overlapping taxa, which are typical arbuscular mycorrhizal soil-associated fungi (Luptáková and Mihál

2020). These taxa were possibly detected as dormant spores or carried by wind or vectors. Field observations and laboratory studies show that Blascomicota generally produce more and smaller spores than Ascomycota which can abundantly be detected in aerial samples, including samples collected above the canopy of trees (David et al. 2016; Elbert et al. 2007; Womack et al. 2015). As a more transient habitat, the phyllosphere may provide niches suitable for colonization species that easily spread (e.g., via small spores) or by r-strategists (Maignien et al. 2014). These factors can also influence the variability of phyllosphere communities, which are more sensitive to seasonal variation than soil (Coince et al. 2014; Dukunde et al. 2019; Gomes et al. 2018; Haas et al. 2018). Sampling during multiple seasons or years was, unfortunately, not possible during this study. Samples were collected during one narrow two-month window during a leaf-on season, allowing us to generate a sufficient sample number to compare host species and connectivity, and minimizing the impact of seasonal, leaf-phenological, and year-to-year alterations. Also, no distinction was made between leaf endophyte and epiphyte taxa for which there is some evidence they may form distinct communities with different environmental susceptibilities (Bodenhausen et al. 2013). Phyllosphere and soil microbial communities are shaped by a combination of specific biotic and abiotic environmental constraints, plant genotype and phenotype, and plant selective pressures (Liu et al. 2020; Llado et al. 2018). Taxa occurring in multiple spheres could provide insight in these processes and the evolution of the tree holobiont, since these taxa are strongly associated with the host tree while keeping the environmental flexibility to survive in very dissimilar environments.

Conclusions

This paper confirms the primary influence of plant sphere for microbial communities within plant holobionts, specifically in the case of tall temperate trees. While the number of taxa present across spheres was limited, they exert a disproportionate role in their communities in terms of relative abundance. These taxa generally exhibit specific ecological traits, such as ecological flexibility, growth-promotion, or pathogenic properties, which likely account for their dominance within the plant holobiont. Considering plants and their associated communities as an holobiont or "individual unit of selection" (Zilber-Rosenberg and Rosenberg 2008) may assist in linking these communities to ecosystem processes, but glazes over these differences in microbial community composition and function of the different spheres, especially between the soil and above ground components of plants. Consequentially, plant-microbiome co-evolution likely vary across spheres, in which taxa with the capability to occupy multiple spheres may play a crucial role given their evident functional connection to the host's fitness (e.g. nutrient cycling, plant-beneficial or pathogenic traits). While this study provides a snapshot of the microbial composition at a single time point, it lays the groundwork for exploring temporal changes and deeper monitoring in subsequent research. This study highlights the need to carefully assess multiple components of the tree microbiome and their connectivity to understand and assess the ecology of temperate forests since each component tells an unique story.

Acknowledgements This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme [grant agreement number 834709]. We would like to thank M. Heurich and his team for the access to and support Bavarian Forest National Park. Our gratitude to D. Adiningrat, E. Neinavaz, T. Wang, R. Darvishzadeh, M. Lock, Y. Cheng and X. Zhu for their invaluable assistance in the field. We are also grateful to K. Zweers-Peter for her support with the DNA extractions, M. Polling for his help with the bioinformatics and H. Abdullah for checking the final manuscript. Amplicon sequencing was carried out by Genome Quebec (Montreal, Canada).

Author contributions AS: Conceptualization, Methodology, Software, Investigation, Writing—Original Draft. AKS: Funding acquisition, Conceptualization, Writing—Review & Editing. AdG: Conceptualization, Writing—Review & Editing. IL: Methodology, Investigation. MR: Methodology, Investigation. YD: Methodology, Investigation. All authors read and approved the final manuscript.

Funding This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme [grant agreement number 834709].

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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