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Phylogenetically distant but cohabiting: Fungal communities of fine roots in *Diphasiastrum complanatum*, *Pinus sylvestris*, and *Vaccinium myrtillus* in a Lithuanian pine forest

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ABSTRACT

Throughout evolution, distinct plant lineages independently established mutualistic relationships with various fungal taxa. However, the extent to which these relationships are conserved across different plant and fungal lineages remains unclear. In this study, we compared fungal communities associated with the fine roots of three phylogenetically distant yet cohabiting plant species: Diphasiastrum complanatum, a member of lycophytes, the most basal extant vascular plant lineages; Pinus sylvestris, a gymnosperm; and Vaccinium myrtillus, an angiosperm, an evolutionary relatively young lineage. To minimize environmental variability, fine roots of three species were collected from each of 19 five-square-meter plots within a Scots pine forest in Lithuania. Using metabarcoding and microscopic techniques, we observed significant differences in the fungal community composition and diversity among the three plant species. We detected no signs of arbuscular mycorrhiza in any species. Samples of D. complanatum showed significantly higher taxonomical diversity, while P. sylvestris showed lowest diversity, with ectomycorrhizal fungi being most abundant. Samples of V. myrtillus had a prevalence of putative ericoid mycorrhiza taxa, classes Sebacinales and Trechisporales, likely forming hyphal coils detected through microscopy. In contrast, no mycorrhiza was detected in D. complanatum sporophytes. This, along with the presence of well-developed root hairs and similarity to the fungal community inhabiting soil, suggest a low dependency of D. complanatum sporophytes on mycorrhizal associations and a more opportunistic fungi-plant relationship. This is the first study of fungi associated with the sporophytes of D. complanatum. Our findings provide valuable insights into the complex interactions between fungi and plants from diverse phylogenetic lineages in natural environments.

1. Introduction

The widespread symbiotic relationships between fungi and plants profoundly influence ecosystem structure and function (Grime et al. 1987; Clay and Schardl, 2002; Stone et al. 2004; Frank and Trappe, 2005; Smith and Read, 2008; Brundrett and Tedersoo, 2018). Endophytic and mycorrhizal fungi are key examples of these relationships, they coexisted with plants for over 400 million years and play an important role in driving their evolutionary trajectory (Remy et al. 1994; Krings et al. 2007; Redecker et al. 2000). Endophytic fungi inhabit plant tissues internally remaining asymptomatic and not causing visible disease (Petrini, 1991). In exchange for nutrition and protection against abiotic and biotic stresses provided by the host, they can enhance the host's fitness by increasing plant resistance to drought, heat, and salinity, and by producing secondary metabolites that protect against pathogens (Rodriguez et al. 2004; Rho et al. 2017). Mycorrhizal fungi, in turn, colonize not only plant roots but also the rhizosphere. This mutualistic association benefits both partners: the fungi supply the plant

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Abbreviations: RAF, Root-associated fungi; AM, Arbuscular mycorrhiza; OTU, Operational taxonomic unit; ECM, Ectomycorrhiza; ERM, Ericoid mycorrhiza; NTC, Non-template control.

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with organic or inorganic phosphates and nitrogen compounds, while the plant provides carbon for the fungi. As a result, approximately 80 % of plant species form mycorrhizal relationships and thrive best with specific fungal partners (Wang and Qiu, 2006; Park and Eom, 2007; Smith and Read, 2008; Delavaux et al. 2017).

There are several distinct types of mycorrhizal associations, each with a unique physiology, resource usage and specific fungal taxa (Wang and Qiu, 2006; Smith and Read, 2008; Brundrett and Tedersoo, 2018; Rimington et al., 2020). Arbuscular mycorrhiza (AM) evolved first around 400 Mya through associations between early land plants and Glomeromycota and Endogonomycetes fungi, improving carbon and nutrient exchange via intracellular structures called arbuscules (Remy et al. 1994; Orchard et al. 2017; Strullu-Derrien et al. 2018). Ectomycorrhiza (ECM) evolved independently around 200 Mya from saprotrophic ancestors within various fungal lineages (Tedersoo and Brundrett, 2017; Tedersoo and Smith, 2013). The first detection of ectotrophic mycorrhiza with angiosperms dates back to a fossil from Tertiary amber (52 Mya; Beimforde et al. 2011). ECM fungi develop a hyphal mantle around the fine roots of a plant, where nutrient and carbon exchange take place within the Hartig net, a hyphal network that develops between the root's epidermal and cortical cells. Due to their extensive hyphal systems, ECM fungi scavenge more effectively and at greater distances from the host roots compared with arbuscular mycorrhizal (AM) fungi (Teste et al. 2020). It was also shown that ECM symbiosis exhibits greater host specificity than AM symbiosis, which may be attributed to the enhanced dispersal capabilities of ECM fungi (Smith and Read, 2008). Ericoid mycorrhizal (ERM) symbiosis originated around 117 Mya in Ericales initially with Ascomycota fungi, followed by Basidiomycota fungi (Schwery et al. 2015). At present, it is the least explored type of mycorrhiza (Vohník, 2020). ERM fungi form intracellular hyphal coils in the plant roots and can also act as non-symptomatic endophytes in non-ericoid plants and as saprotrophs in soil and organic matter (Kohout, 2017). Although ERM has been considered the most host-specific of all mycorrhizae, an increasing number of studies highlights its surprisingly promiscuous nature (Perotto et al. 2002; Kjøller et al. 2010; Walker et al. 2011).

Recent findings depict plant-fungus interactions as a complex and dense network, linking different plant taxa to fungi from various lineages (Simard et al. 2012; Verbruggen et al. 2012). The main fungal phylogenetic and functional groups are present in all ecosystems worldwide, though their relative proportions vary among biomes (Tedersoo et al. 2012). Plant-fungal associations are not random; plants select specific fungal symbionts, and fungi show preferences for certain host plants (Kennedy et al. 2003; Kiers et al. 2011; Bruns et al. 2002; Tedersoo et al. 2008; Öpik et al. 2009; Walker et al. 2011; Davison et al. 2011). At the same time, phylogenetically distant plant lineages can exhibit substantial overlap in fungal taxa (Öpik et al. 2009; Toju et al. 2013; Perez-Lamarque et al. 2022). These findings help to elucidate how these preferences influence plant and fungal species composition and contribute to the formation of different biomes.

Conducting studies of the root-associated fungi (RAF) of plants in natural biomes presents multiple challenges. One major issue is the close association between roots and soil, making it difficult to distinguish mutualistic fungi from free-living or transient fungi. At current, no method can reliably reveal whether adjacent growing plants are connected via a common mycorrhizal network within a natural community (Karst et al. 2023). However, using a metabarcoding approach, it has become possible to obtain a large amount of data describing fungal species present in soil communities (Tedersoo et al. 2010), which allows for comparative studies on fungal species composition. Previous metabarcoding studies have shown that fungal diversity depends on multiple factors, such as soil properties, elevation and humidity (Veresoglou et al. 2013; Sun et al. 2016; Zhang et al. 2021; Perez-Lamarque et al. 2022; Luo et al. 2023; Tedersoo et al. 2024). The composition and structure of RAF communities are highly influenced by local abiotic and biotic conditions (Alzarhani et al. 2019). However, the extent to which

inter-species variation contributes to RAF community dynamics in naturally homogeneous environments remains uncertain. For instance, in an oak forest, roots of different plant species hosted distinct mycorrhizal and endophytic fungal communities, despite growing in proximity (Toju et al. 2013). In a tropical forest, Schroeder et al. (2019) found that the RAF community was more strongly associated with plant phylogeny than with spatial distance, with a stronger relationship observed for pathogens than for mutualists. At the same time, a metabarcoding study of different tropical communities revealed frequent fungal sharing between phylogenetically distant plant lineages at a fine scale, with significant variation in the level of specialization across different fungal lineages (Perez-Lamarque et al. 2022). The authors proposed that interaction of plants with fungi is determined by the intrinsic properties of each fungal lineage, but not by environmental conditions.

Within this study, we explore fine-scale variation in RAF communities among three cohabiting vascular plant species in a hemiboreal forest in Lithuania, dominated by Pinus sylvestris L. (Scots pine, Pinaceae). Alongside P. sylvestris, we studied the fine roots of two phylogenetically distant vascular plant species: the angiosperm Vaccinium myrtillus L. (European blueberry, Ericaceae) and the lycophyte Diphasiastrum complanatum (L.) Holub (northern groundcedar, Lycopodiaceae). Like many boreal forest trees, P. sylvestris is an obligate mutualist that relies highly on association with ECM fungi. As a member of the Ericaceae family, V. myrtillus is expected to form an obligate partnership with ERM fungi (Smith and Read, 2008). The species composition of mycorrhizal fungi associated with P. sylvestris and V. myrtillus is relatively well studied (e.g., Rudawska et al. 2018; Sietiö et al. 2018; Olchowik et al. 2021; Vohník, 2020; Bzdyk et al. 2022). However, there is limited knowledge about the fungal partners of lycophytes, the most basal extant lineage of vascular plants, particularly D. complanatum (Wikström and Kenrick, 2001; Rimington et al. 2020). Gametophytes of lycophytes are often achlorophyllous and fully dependent on their symbiotic AM fungi for nutrition (and this is the case for D. complanatum; Horn et al. 2013), while the sporophytes may have a low dependence on their mycorrhizal partners when mature (Rimington et al. 2020). Lycophytes grow in boreal and hemiboreal forests alongside other vascular plants that evolved much later. The extent to which early and late diverging phylogenetically distant plant lineages share fungal partners remains a subject of ongoing debate (Rimington et al. 2018).

In this study, we conducted a metabarcoding analysis in combination with a light microscopic investigation to check two hypotheses: (i) each of the studied plant species hosts a distinct fungal species composition, with a prevalence of ECM fungi for *P. sylvestris*, ERM fungi for *V. myrtillus*, and AM fungi for *D. complanatum* sporophytes; (ii) the fungal communities associated with *V. myrtillus* fine roots exhibit the lowest species diversity due to the highest host specificity of ERM fungi.

2. Material and methods

2.1. Study area

Our study area is located in Dzūkija National Park of Lithuania, with the investigated forest located on the north and south banks of the Merkys River (Fig. 1, Suppl. Table 1). The soils in this area have developed on non-carbonaceous quartz under sandy glacial plains, exhibiting podzolic characteristics with a mild podzolization, and are notably dry with severe deficiency in nutrients (Gulbinas and Samuila, 2002). The annual gross solar irradiance in the study area is approximately 3300–3400 MJ/m², the soil surface albedo is lower than 17.5 %. The mean annual temperature is +6-7 °C, with an absolute maximum of +35.6 °C and an absolute minimum of -35.9 °C, and the temperature remains above 0 °C for 252 days a year. Mean annual precipitation is around 650 mm (National Land Service under the Ministry of Agriculture of the Republic of Lithuania, 2016). The predominant tree species in the studied forest is Scots pine (*Pinus sylvestris*) (Augustaitis and Bytnerowicz, 2008). According to the forest cadastre data (State Forest



Fig. 1. (a) Location of 19 plots (red circles) analysed via metabarcoding. Each plot was examined for fungi associated with soil and fine roots of *Pinus sylvestris*, *Vaccinium myrtillus*, and *Diphasiastrum complanatum* sporophytes. Labels with asterisks represent ten plots where fine roots of *D. complanatum* sporophytes were additionally analysed via microscopy; underscored labels indicate three plots where fine roots of all three plant species were investigated. (b) Location of the sampling region in southern Lithuania (red rectangle).

Service under the Ministry of Environment, 2023), the age of the forest on the study area ranged from 76 to 126 years (average \pm sd: 107 \pm 15 years) and the height varied from 20.7 to 28.1 m (average \pm sd: 26.0 \pm 1.8 m) (Suppl. Table 1).

collected in August 2021. Samples were taken from 19 plots (5×5 m each), where *Pinus sylvestris* L. co-occurred with *Vaccinium myrtillus* L. and *Diphasiastrum complanatum* (L.) Holub (Suppl. Table 1). In each plot, three samples of fine roots (5–10 cm length) of *P. sylvestris, V. myrtillus,* and *D. complanatum* sporophytes were collected from three different ramets/trees per species (Fig. 2). Typically, each plot contained a single individual of *D. complanatum* consisting of multiple shoots connected by subterranean rhizomes. Previous study revealed that these individuals

2.2. Sampling design

For metabarcoding analysis, samples of plant material and soil were



Fig. 2. Collection scheme of samples for metabarcoding analysis of the soil and fine roots of *Pinus sylvestris*, *Vaccinium myrtillus*, and *Diphasiastrum complanatum* in each studied plot. Created in BioRender. Kuprina, K. (2025) https://BioRender.com/t92b024.

genetically differed between plots (Schnittler et al. 2019). Additionally, soil samples were analysed to identify free-living or transient fungi and determine the "background" trophic modes characteristic of the local soil type: one teaspoon of soil was taken from three spots at a depth of 10 cm from the surface, and all visible root fragments were sorted out. The collection spoon was pre-treated with soil from the plot before sampling. Root samples were cleaned of large substrate fragments through gentle shaking and then washed by immersion in distilled water. Samples of the same plant species or soil from one plot were combined, resulting in a total of 76 samples. All samples were air-dried at room temperature (RT) and sealed in plastic zip-lock bags.

To characterize the vegetation of each plot, the coverage percentage of five strata, as well as of each plant and lichen species, was recorded.

For the microscopic analysis, additional sampling was conducted in August 2023, at ten previously investigated plots on both sides of the Merkys river (Fig. 2). Soil samples and fine roots of *D. complanatum* sporophytes were collected using the same sampling scheme, as for the metabarcoding analysis. Additionally, as positive standards for the preparation and staining, fine roots of *P. sylvestris* and *V. myrtillus* were sampled in three of these plots ("2", "78", "30B"). The root material was preserved in 30 % ethanol and stored at 6–8 °C for three weeks. Soil samples were air-dried at RT and stored in paper bags.

The pH of each soil sample was measured according to DIN ISO 10390, after incubating 5 g of the soil in 25 mL of 0.01M CaCl₂ overnight, with pH/mV pocket meter WTW pH330i and electrode WTW SenTix41 (both Xylem Analytics, Weilheim i. OB, Germany).

2.3. Metabarcoding library preparation

To avoid the batch effect, samples from a single plot were processed jointly during DNA extraction and amplification. Throughout the library preparation, the samples were quasi-randomly distributed across two plates.

For DNA extraction from plant material, 40 fragments of fine roots with ca. 2 mm length were selected from a single sample and pooled, resulting in an average of 15 mg of dry material. In cases where visible ECM root tips were present, they were preferentially chosen. Regarding soil samples, 300 mg of dry material was utilized. The DNA extraction procedure employed the NucleoSpin Soil extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol with specific adjustments: root samples were dry homogenized in 2 mL tubes with a 5 mm steel ball, while soil samples were homogenized in provided tubes with added lysis buffers (SL and SX). The BeadBlaster 24TM rotorstator homogenizer (Benchmark, Almelo, Netherlands) was employed for two cycles at 4.0 rpm for 1 min, with a 10 s break. The supernatant was transferred to a new tube before the precipitation of contaminants (step 4), and DNA was eluted in 50 µl of Elution buffer. In each of the four DNA extractions, a negative control of extraction was included. DNA extracts were quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, USA).

The amplification of the ITS2 region as a barcode was conducted using primers gITS7F (Ihnmark et al. 2012) and ITS4ngsR (Tedersoo et al. 2014), each incorporating Illumina overhang adapters (underlined): 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCA TCGARTCTTTG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGTTCCTSCGCTTATTGATATGC-3', respectively. The PCR was carried out in a 25 µl reaction mix comprising 12.5 µl of Taq 2x Master Mix RED with 1.5 mM MgCl₂ (Amplicon, Odense, Denmark), 0.2 µM of each primer, and 70 ng of DNA. The PCR protocol included the following steps: (i) 5 min at 94 $^\circ$ C; (ii) 25 cycles of 30 s at 94 $^\circ$ C, 30 s at 48 $^\circ$ C, and 30 s at 72 °C; (iii) 7 min at 72 °C. A non-template negative control (NTC) was included for each of the two PCR runs. All samples (n = 76) and both types of negative controls (n = 5) were amplified; additionally, for all four samples from plot "2," three technical replicates were utilized (n = 8). One NTC for NGS sequencing was also included, giving 90 libraries in total. Resulted PCR products were examined in 1.5 % agarose gel with

RotiSafe stain. All amplicon libraries were barcoded using the Illumina MiSeq v3 reagent kit for 2×300 bp sequencing, following the manufacturer's protocol. After pooling, the libraries were sequenced in a single run at the Section of Microbiology (Copenhagen University, Denmark) where the sequences were also de-multiplexed, and the barcodes were removed.

2.4. Microscopic analysis

The preparation of root samples for microscopic analysis was conducted through Trypan blue staining following the modified protocol of Chabaud et al. (2006): (i) boiling at 90 °C in 15 ml of 10 % KOH (w/v) for 150 min; (ii) rinsing roots in tap water; (iii) bleaching in 15 ml of 10 % H₂O₂ (v/v) with a few drops (0.2–0.3 ml) of 25 % NH₃ (v/v) for 2 h; (iv) rinsing in tap water; (v) acidification of roots in 80 % lactic acid for 1 h; (vi) boiling at 90 °C in 15 ml of 0.2 % Trypan blue solution in lactoglycerol for 3 min. Stained roots were preserved in lactoglycerol at 4–6 °C. Subsequent mycorrhiza detection was conducted using a Leica DM2500 light microscope with FLEXACAM C1 camera (both Leica Camera, Wetzlar, Germany) and the associated program Leica Application Suite v4.8.0.

Since *D. complanatum is* the least studied species in this context, we focused our microscopic investigation primarily on this species. We examined a total of 300 fine root fragments from ten samples of its sporophytes (Suppl. Fig. 1c). Additionally, we examined 90 fine root fragments from three samples each of *P. sylvestris* and *V. myrtillus*, primarily as positive controls for sample preparation and staining. Each fragment, minimum 1 cm long, was analysed for the presence/absence of ECM, AM, or ERM via presence of arbuscles, hyphal mantles, or hyphal coils. To identify ECM, we looked for fine root tips that exhibit swollen, chromatically shiny tips, typically dichotomously branched in *Pinus*. The presence of arbuscules or hyphal coils within root cells indicates AM or ERM, respectively.

2.5. Data analysis

Nextera adapters, as well as N and low-quality leading and trailing bases (<3, Phred-33), were trimmed using Trimmomatic v0.39 (Bolger et al. 2014). Quality of the raw and resulting sequences was checked by running FastQC v0.11.5 and MultiQC v1.19 tools (Andrews, 2010; Ewels et al. 2016). Demultiplexed and trimmed reads are available on NCBI (PRJNA1185013).

Obtained sequences were processed using the VSEARCH v2.15 Python package (Rognes et al. 2016). The following steps were undertaken: merging of forward and reverse reads (fastq_minovlen = 150, fastq_*maxdiffs* = 15), quality filtering of reads (*fastq_maxee* = 0.5, *fastq_minlen* = 250, fastq maxns = 0), dereplication of reads across the samples and removal of singletons (*minuniquesize* = 10), pooling of the samples, and eliminating reads with putative PCR errors (denoising; fastq eeout parameter). The VSEARCH v2.15 package was also utilized for the identification and removal of chimera sequences using the UCHIME algorithm, applying both de novo and reference-based detections (Edgar et al. 2011). All resulting sequences with a similarity lower than 97 % were clustered to the different Operational Taxonomic Units (OTUs) using "greedy" clustering in the function cluster-size. Each OTU was taxonomically assigned by comparing it to the UNITE v9.0 database with a 96 % identity threshold (Abarenkov et al. 2023). As a result, multiple OTUs could be assigned to the same fungal taxa. The assignment of the bottom ten OTUs with the lowest match values was additionally verified by blasting them against the GenBank database (megablast, nr/nt). For ecological characterization of revealed genera and species, FUNGuild v1.1 database was implemented to assign them to guilds and trophic modes (Nguyen et al. 2016). If a taxon was assigned to multiple guilds, including ECM, it was categorized as "ectomycorrhizal". Taxa assigned to multiple guilds exclusively within the saprotrophic category, including pathogenic and endophytic, were categorized as

"saprotrophic". Guilds and trophic modes were assigned to taxa only when confidence levels were "highly probable" or "probable".

Statistical evaluation and visualization of results were conducted in R 4.4.2 using RStudio 2023.12.1 (RCore Team, 2024; Posit Team, 2024).

Prior to conducting the data analysis, OTUs potentially associated with contamination were excluded. This process involved removing OTUs with total counts from NTCs that exceeded a half of the normalized total sample counts, obtained by dividing the total sample counts by the ratio of the number of samples to the number of NTCs (84/6). NTCs were then removed from the dataset. Following this treatment, the counts underwent normalization for sampling depth through a variance stabilizing transformation implemented in the R package *DESeq2* v1.46 (Love et al. 2014). After this step, each count value within a sample underwent subtraction by the lowest count value in that sample, resulting in zero for absent counts.

To visualize variations in taxonomic composition among sample types, a Non-metric Multidimensional Scaling (NMDS) with Bray distances, as well Principal Coordinate Analysis (PCoA) with Euclidean distances (with and without technical replicates) were performed utilizing the R package *phyloseq* v1.50 (McMurdie and Holmes, 2014). Three types of datasets were used for NMDS: (1) one containing all revealed 278 OTUs, (2) one containing only OTUs assigned to ectomy-corrhizal functional guilds of fungi (86 OTUs), (3) and one with only OTUs of saprotrophic fungi (108 OTUs). The outcomes were graphically represented using the R packages *ggplot2* v3.5.1 and *ggrepel* v0.9.6 (Wickham, 2016; Slowikowski, 2024). In the subsequent data analysis steps, technical replicates were excluded.

Taxonomic relative abundance per sample type was visualized using the R packages microeco (v1.11.0) and ggplot2 (Liu et al. 2021). The statistical significance between groups of samples at each taxonomic rank was determined using the Wilcoxon Rank Sum test, with the false discovery rate p-value correction for multiple comparisons (Benjamini and Hochberg, 1995; Haynes, 2013) using the R package metacoder v0.3.7 (Rice et al. 2000). Four types of pairwise comparisons of relative abundances were conducted: (1) between different sample types, (2) between P. sylvestris samples categorized by high and low tree age, (3) between P. sylvestris samples categorized by high and low tree height, and (4) between D. complanatum samples categorized by large and small colony diameters. Samples were classified into high or low groups based on whether their respective values were above or below the corresponding average. To achieve this, normalized counts of OTUs across all ranks of every taxon were aggregated within a sample type. Only taxa represented by at least two OTUs were included in the analysis to simplify visualization and interpretation of the results. In the case of comparison of the sample type, results were depicted on a heat tree.

To visualize OTU diversity within each sample type, sample-based rarefaction and extrapolation curves were generated for Hill numbers (OTU richness, Shannon, and Simpson diversities) using the R package *iNEXT* v.3.0.1 (Hsieh et al. 2022). Additionally, this package was employed to construct a sample completeness curve to assess the adequacy of the sample size. A Venn diagram was built to show the number of unique and shared OTUs across sample types using the online tool *jvenn* (Bardou et al. 2014).

The abundance of trophic modes and guilds among sample types was statistically tested. Prior to analysis, the dataset was assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Bartlett's test. Normally distributed data with homogeneous variances were analysed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test (Tukey, 1949), while data not meeting these assumptions were analysed using the Kruskal-Wallis test with Dunn's post-hoc test, implemented using the R package *rstatix* v0.7.2 (Kassambara, 2023). For both tests, Bonferroni correction was applied to adjust *p*-values for multiple comparisons at a significance level of $\alpha = 0.05$.

A MultiQC report on the raw and trimmed reads, along with raw and normalized read counts, taxonomic assignment of OTUs, and scripts in bash (Trimmomatic, VSEARCH, and FUNGuild steps) and R are available on GitHub: https://github.com/kuprinak/lithuania-metabar.

3. Results

3.1. Plot characteristics

All sampling plots had acidic soils, with pH varying from 2.9 to 4.0 across two sampling years (average pH \pm sd: 3.3 \pm 0.18 in 2021, 3.4 \pm 0.25 in 2023, Suppl. Table 1).

The vegetation survey conducted on sampling plots revealed *P. sylvestris* as the dominant tree species with 28.0 % surface coverage, followed by *Betula pendula* Roth (6.1 % coverage) (Suppl. Table 2). The dominant shrubs and subshrubs observed were *Juniperus communis* L. with 9.3 % coverage, *Vaccinium vitis-idaea* L. with 6.6 %, *D. complanatum* with 6.3 %, and *Vaccinium myrtillus* with 4.6 %. The tree and shrub strata comprised both typical AM plants, such as *Prunus serotina* Ehrh., *Juniperus communis, Sorbus aucuparia* L., as well as ECM plants like *Pinus sylvestris, Picea abies* (L.) Karst., *B. pendula*, and *Quercus robur* L. (Akhmetzhanova et al. 2012).

3.2. Metabarcoding analysis

3.2.1. DNA library

Sample sequencing produced a total of 13,938,499 raw read pairs, with an average of 165,934 read pairs per sample and standard deviations of 29,378 read pairs across samples and 6100 read pairs across sample types. After processing reads with Trimmomatic, 10,128,361 read pairs remained, with an average of 120,500 read pairs per sample. The average sequence length of the libraries ranged from 220 to 273 bases, with a mean of 252 bases.

After processing the data with the VSEARCH package, 283 distinct OTUs were initially identified. Four OTUs were excluded due to potential contamination, and one was excluded because no counts remained after NTC removal, reducing the total to 278 OTUs.

3.2.2. Taxon assignment and species composition among sample types

Taxonomic assignment successfully classified 99.6 % of these OTUs to the phylum, 95.1 % to the class, 87,6 % to the order, 80.9 % to the family, 73.5 % to the genus, and 47.0 % to the species level. In total, nine phyla, 21 class, 37 orders, 62 families, 77 genera, and 132 species of fungi were revealed (Suppl. Fig. 2). The most abundant phylum was shown to be Basidiomycota with Agaricomycetes as the most abundant class (Suppl. Fig. 3). The most species rich genera were *Cortinarius*, *Russula*, and *Mortierella* with 15, nine and eight species, respectively.

Two distinct clusters were identified through NMDS analysis: (1) soil samples grouped with *D. complanatum* and (2) *P. sylvestris* with *V. myrtillus* (Fig. 3a). PCoA, however, revealed three clusters: (1) soil samples, (2) *P. sylvestris* and *V. myrtillus*, and (3) *D. complanatum* positioned in between (Fig. 3d). Technical replicates for each sample were tightly clustered (Suppl. Fig. 5).

The number of observed OTUs varies among sample types, with the highest number (256 OTUs) found in soil samples and the lowest (201 OTUs) in *P. sylvestris* samples (Suppl. Fig. 6b). More than half of all OTUs (182 OTUs, 66 %) were present in all four sample types, while only soil samples contained OTUs that were not found in any other sample type (26 OTUs, 9 %) (Suppl. Fig. 6a).

Significant differences in relative abundances across the sample types were shown for multiple taxa (Fig. 4a–g). The highest number of significant differences was found between soil samples and those from *P. sylvestris* and *V. myrtillus*: soil had predominance across six Phyla; only the genus *Luellia* was more abundant in *P. sylvestris* and *V. myrtillus*, and the genus *Mycena* was dominant in *V. myrtillus* samples (Fig. 4e and f). Samples of *D. complanatum* showed only genus *Mucor* as a more prevalent taxon in comparison with the soil samples (Fig. 4g). At the same time, several taxa were dominant in *D. complanatum* samples in



Fig. 3. Ordination diagrams generated from Bray distances via NMDS for all (a) 278 OTUs, (b) 86 OTUs of ectomycorrhizal fungi, and (c) 108 OTUs of saprotrophic fungi. (d) Ordination diagram generated from Euclidean distances via PCoA for all 278 OTUs. Distances were calculated between normalized counts of OTUs found in soil, *Diphasiastrum complanatum* sporophytes (DIPcom), *Pinus sylvestris* (PINsyl), and *Vaccinium myrtillus* (VACmyr) fine root samples collected in 19 plots (83 samples in total).

comparison with *P. sylvestris* and *V. myrtillus* samples: the phyla Mucoromycota (classes Mucoromycetes and Umbelopsidomycetes), Ascomycota (classes Sordariomycetes), Rozellomycota, and Basidiomycota (classes Tremellomycetes, Geminibasidiomycetes, and the genera *Amanita* and *Ceratobasidium* from the class Agaricomycetes) (Fig. 4b and c). The lowest number of taxa with significant differences was found between samples of *P. sylvestris* and *V. myrtillus*: *P. sylvestris* had more reads of the genus *Russula*, while *V. myrtillus* had more abundant reads of the family Serendipitaceae (Sebacinales), the order Auriculariales, and the genus *Trechispora* (Trechisporales) (Fig. 4d).

No significant difference in taxa relative abundancies was found between samples from plots with high and low tree age or height for both types of datasets (all types of samples and only *P. sylvestris* samples), as well as between *D. complanatum* samples with "big" and "small" colony sizes.

3.2.3. Relative OTU diversity among sample types

All calculated diversity indices indicated a similar trend: soil samples exhibited the highest values of Hill numbers, followed by *D. complanatum* and *V. myrtillus* samples, and the lowest values recorded for *P. sylvestris* samples. (Fig. 5a–c). The sample completeness curves reached the plateau shape for each sample type, indicating an adequacy of the sample number for analysing species diversity (Suppl. Fig. 7). The pattern also remained consistent when only the 86 OTUs assigned to ECM fungi were considered (Suppl. Fig. 8).

3.2.4. Trophic modes

Of the 278 OTUS, FUNGuild assigned 218 OTUS to their functional guilds, with 86 categorized as ECM and 108 as saprotrophic fungi. The most abundant ECM genera were *Russula*, *Cortinarius*, *Tomentella*, and *Amanita*, while the most abundant saprotrophic genera included

Umbelopsis, Mortierella, Penicillium, Cladophialophora, and Trichoderma (Suppl. Fig. 3e).

Looking at the saprotrophs only, the NMDS plot shows a similar pattern as for all OTUs together: (1) soil samples grouped with *D. complanatum* and (2) *P. sylvestris* grouped with *V. myrtillus* (Fig. 3a–c). Clustering was less distinct for the ectomycorrhizal functional guilds (Fig. 3b).

The relative abundance of fungal trophic modes and functional guilds differed between sample types (Fig. 6a and b). Compared to the other sample types, roots of *D. complanatum* sporophytes showed significantly lower abundance of ECM fungi and symbiotrophs in comparison to *P. sylvestris* (adjusted *p*-values from the Kruskall-Wallis test: p = 0.0105 and p = 0.0007, respectively). Samples of *D. complanatum* also comprised a significantly higher abundance of OTUs with saprotrophic modes than *P. sylvestris* (adjusted *p*-value from the Tukey HSD test: p = 0.0025). Soil samples contained more saprotrophs than *D. complanatum*, *P. sylvestris* or *V. myrtillus* (adjusted *p*-values from the Tukey HSD test: 0.0274, 0.0205, 0.0275, respectively).

3.3. Microscopic analysis

A distinctive feature of *D. complanatum* fine roots was a dense cover of unicellular root hairs, visible to the naked eye (Suppl. Figs. 1b and c). Investigation of all 300 fine root fragments from *D. complanatum* sporophytes revealed an absence of arbuscles, hyphal mantles, or hyphal coils characteristic to AM, ECM and ERM, respectively (Fig. 7a and b). In contrast, all examined fragments of *P. sylvestris* fine root samples exhibited mantles of ECM hyphae (Fig. 7c), and all hair root fragments from *V. myrtillus* contained cells with hyphal coils of ERM (Fig. 7d and e).



Fig. 4. (a) Tree of fungal taxa identified during metabarcoding analysis of four sample types (only taxa with OTUs number ≥ 2 included): soil, fine roots of *Diphasiastrum complanatum* sporophytes (DIPcom), *Vaccinium myrtillus* (VACmyr), and *Pinus sylvestris* (PINsyl) in 19 studied plots (76 samples in total). The genus labels are colour-coded according to guilds defined using the FUNGuild v1.1 database. (b–g) Heat trees illustrating the comparison of abundance of normalized counts of OTUs assigned to the corresponding taxa among sample types. The colour of each taxon node represents the log-2 ratio of median proportions of reads observed at sample type determined by a Wilcox rank-sum test with a false-discovery rate correction for multiple comparisons. Taxa coloured green have significantly higher number of reads in the sample type shown in the row and the taxa coloured brown are prevalent in the sample type shown in the column.



Fig. 5. Rarefaction and extrapolation curves with 95 % confidence intervals of three Hill numbers describing OTU diversity: (a) OTU richness, (b) Shannon diversity, (c) Simpson diversity. The numbers are calculated using normalized counts of 278 OTUs for soil or fine roots of *Diphasiastrum complanatum* sporophytes (DIPcom), *Pinus sylvestris* (PINsyl) and *Vaccinium myrtillus* (VACmyr) from 19 studied plots (76 samples in total).



Fig. 6. Relative abundance of normalized counts of 278 OTUs assigned to (a) functional guilds and (b) fungal trophic modes using FUNGuild v1.1 database for soil of or fine roots of *Diphasiastrum complanatum* sporophytes (DIPcom), *Pinus sylvestris* (PINsyl) and *Vaccinium myrtillus* (VAC myr) from 19 studied plots (76 samples in total). Each bar represents one sample (n = 76). Boxplots show differences between sample types for the two most abundant (b) trophic modes and guilds (c). Samples of the boxes with the same letter code are not significantly different (T – Tukey Post-hoc test, K - Kruskal-Wallis test with the Dunn post-hoc test).

4. Discussion

4.1. Fungal community

In the studied hemiboreal Scots pine forest, a diverse fungal community is present, with Basidiomycota as the most prevalent phylum and saprotrophic fungi as the dominant trophic mode. In boreal forests, ECM is the predominant mycorrhizal type, playing a crucial role in nutrient cycling (Smith and Read, 2008; Clemmensen et al. 2013; Kyaschenko et al. 2019). Previous studies have shown that ECM fungi are most abundant in soils of forests dominated by Pinaceae and can comprise about 34 % of all fungal taxa (Tedersoo and Smith, 2013; Tedersoo et al. 2012). In our study, only 20 % of fungal taxa from the soil samples were assigned to the ECM functional guild, and almost 50 % to saprotrophic fungi (Fig. 6a). However, functional overlap between these groups is possible: saprotrophic fungi can form facultative symbiotrophic relationships with plant roots, while some ECM fungi exhibit capabilities for facultative saprotrophy and decomposition (Koide et al. 2008; Bödeker et al. 2014; Smith et al. 2017). Additionally, the fungal community is a dynamic system which can change over time and especially after rapid environmental changes (Rudawska et al. 2018; Taniguchi et al. 2018; Olchowik et al. 2021). Various genera of ECM fungi are associated with different forest or host ages. For example, the genus *Russula* was shown to be more diverse and abundant in older *P. sylvestris* stands (above 50 years), while the genera *Lactarius* and *Suillus* were associated with younger stands (Dahlberg and Finlay, 1999; Hutchison,



Fig. 7. (a, b) Fine root of *Diphasiastrum complanatum* sporophyte; (c) fine root of *Pinus sylvestris*; (d, e) hair root of *Vaccinium myrtillus*. RH – root hair, H – hypha of fungi, T - tracheid, Ma – mantel of hyphae, My – mycelium, HC – hyphae coils. Scale bar - 100 µm.

1999; Brundrett, 2002; Rudawska et al. 2018). Given the relatively old age of the trees in the studied plots (average 102 years), our results are comparable with data from an 87-year-old *P. sylvestris* forest in Poland, which also showed a predominance and highest species-richness of *Russula* and *Cortinarius* (Rudawska et al. 2018). A metabarcoding study of the boreal forest in Finland identified the ECM-forming Basidiomy-cetes *Cortinarius*, *Russula*, *Piloderma*, and *Tomentella* as the most abundant genera in the soil (Sun et al. 2016). Similarly, in our study, *Cortinarius*, *Russula*, and *Tomentella* were the most abundant ECM genera, with the exception of *Piloderma*, *which* ranked after *Tomentella* and *Amanita* (Suppl. Fig. 3e). Overall, however, saprotrophic *Umbelopsis* and *Mortierella* were the most species-rich, likely representing the core fungal community in the studied forest.

Despite being most abundant in temperate grasslands and tropical rainforests, AM fungi have also been found in temperate forests, including those dominated by P. sylvestris (Vandenkoornhuyse et al. 2002; Onguene and Kuyper, 2001; Öpik et al. 2003). However, our microscopic analysis did not reveal any signatures of AM colonization in any type of samples. Tedersoo et al. (2016) reported that 2 a.m. genera, Glomus and Endogone, have a lower number of rDNA copies than the other studied fungi taxa, leading to low amplification of their ITS regions. In our study, three OTUs were assigned to the phylum Glomeromycota (Suppl. Fig. 2), but no significant difference in the relative abundance were found between sample types (Fig. 4a-g). However, the Mucoromycota was relatively abundant and included the species Umbelopsis dimorpha, U. angularis, Mucor hiemalis, M. moelleri, M. sylvaticus, and Bifiguratus adelaidae. The latter species is frequently sequenced from soil in northern temperate zones and may have a symbiotic function, having been detected in orchid and chestnut roots (James and Seifert, 2017). Umbelopsis dimorpha was also described as an endopyte (Quin et al. 2018).

Despite previously reported difficulties in detecting closely related Mucoromycota and Mortierellomycota fungi using the ITS2 marker (Tedersoo et al. 2016; Perez-Lamarque et al., 2022; 2023), these phyla were the third and fourth most abundant in our dataset, respectively (Suppl. Fig. 3a). This lower amplification bias among phyla can be attributed to using a different primer combination, gITS7F-ITS4ngsR, instead of ITS86F-ITS4. Different genetic markers and primer combinations can lead to biased detection of specific fungi in a sample due to varying primer specificities (Waud et al. 2014; Op De Beeck et al. 2014). Moreover, mycorrhizal root colonization can fluctuate seasonally and has a limited lifespan, potentially making it undetectable if not sampled at the right time (Majdi et al. 2001; Kemp et al. 2003; Mandyam and Jumpponen, 2008; Meddad-Hamza et al. 2017). Therefore, it is important to be cautious when comparing the results of the studies utilizing different sampling and DNA library preparation techniques.

4.2. Species composition among sample types

Multiple factors shape RAF community composition, leading to both variations and similarities within the same habitat. Abiotic factors, particularly soil pH and nutrient availability, along with plant species, age, and phenological status, play a significant role (Alzarhani et al. 2019; Unuk et al. 2019; Hofmann et al. 2023). These factors influence fungal taxa and their functional groups through dynamic responses to environmental conditions and host characteristics.

Despite analysing samples of different species collected within an area of five square meters, our study found significant differences in fungi composition among sample types, as well as variation inside the plots (Suppl. Fig. 4). Surprisingly, the smallest difference in the relative abundance of taxa was found between samples of *V. myrtillus* and *P. sylvestris* (Fig. 4d), two species known to form different types of mycorrhizal symbiosis.

4.2.1. Diphasiastrum complanatum

It has long been known that Lycopodiaceae species rely upon mycorrhizae for successful development of both sporophytes and gametophytes (Bruchmann, 1898; Whittier, 1977; Schmid and Oberwinkler, 1993; Winther and Friedman, 2008; Horn et al. 2013). Surveys of mycorrhizal associations in lycophyte sporophytes often report not only AM, but also ERM, orchid mycorrhiza, and microsclerotia with Ascomycota and Basidiomycota (Spessard, 1917; Freeberg, 1962; Treu et al. 1996). However, our knowledge about lycophyte fungi interactions and fungal sharing remains scarce (Horn et al. 2013; Perez-Lamarque et al. 2023). Rimington et al. (2020) reviewed publications on the fungal symbiosis status of lycophytes and found that only six species showed Mucoromycotina, 53 had Glomeromycota and one species Basidiomycota. However, the authors pointed out that Mucoromycotina symbionts have likely been misidentified as Glomeromycota and the number of species with Glomeromycota could be lower.

The ordination analyses (NMDA and PCoA) revealed that the fungi associated with D. complanatum sporophyte roots are a distinct community and more closely related to the fungal community in soil samples than to that associated with P. sylvestris and V. myrtillus (Fig. 4a-d). Moreover, the Hill numbers describing OTU diversity of ECM fungi were more similar to the soil samples, than other sample types (Suppl. Figs. 9a,b,c). This similarity to the soil samples might be connected to the fragility and high surface area of the fine roots, which made them difficult to separate from soil organic particles during the cleaning process. However, the Hill numbers calculated with all OTUs were significantly lower for *D. complanatum*, than for the soil (Fig. 5a,b,c). This observation could be related to the different weight of the analysed roots compared to the soil samples (15 mg vs. 300 mg), despite normalizing the amount of DNA used for amplification and library preparation. The sample coverage plot (Suppl. Fig. 7) showed that each sample type reached a plateau, but the soil samples exhibited significantly higher coverage than the root samples, merging only after 35 samples per type. Nonetheless, all root samples showed no significant differences in their coverage, supporting the robustness of the comparison.

Only the order Mucoromycetes was more abundant in *D. complanatum* samples than in the soil samples (Fig. 4a–g). However, in comparison with *P. sylvestris* and *V. myrtillus*, there was a predominance of such saprotrophic genera as *Solicoccozyma*, *Geminibasidium*, *Umbelopsis*, *Mucor*, *Trichoderma*, and *Hypocreales* (Fig. 6c–e). Only two ECM genera, *Ceratobasidium* and *Amanita*, were more abundant in *D. complanatum* samples compared to both *P. sylvestris* and *V. myrtillus*. However, soil samples also showed predominance of these ECM genera compared to *P. sylvestris* and *V. myrtillus* samples (Fig. 4e and 6f). There is no direct evidence of *Amanita* or *Ceratobasidium* forming symbiotic relationships with club mosses (Lycopodiophyta). It remains unclear whether these fungi participate in any types of mycorrhizal partnership with *D. complanatum* and, if so, they likely exhibit rather opportunistic relationship.

Perez-Lamarque et al. (2023) demonstrated that gametophytes and sporophyte roots for Diphasiastrum tristachyum, D. oellgaardii, D. zeilleri, and Lycopodium clavatum were colonized by a specific fungus from the clade Densosporaceae (Endogonales, Mucoromycotina). This fungus was abundant in the germinated spores suggesting that lycopod spores require this specific association for germination. For the roots of sporophytes, they found a dominance of Leotiomycetes, Archaeorhizomycetes, Endogonales and the parasitic genus Spizellomyces (Chytridiomycota). In our study, OTUs of Endogonomycetes and Spizellomyces were found, but their relative abundances in D. complanatum samples were not higher than in the other root samples (Fig. 4b and c) and were even lower than in the soil samples (Fig. 4g). This discrepancy between studies could be attributed to differences between studied biotopes: plants from the previous study were collected from artificially created subalpine heathland after the disturbance of a beech forest in the Hochfeld reserve, France. Perhaps another group of fungi plays a crucial

role in the germination of *D. complanatum* spores in the pine forests in Lithuania and it has yet to be identified.

The surprising observation was that the multiple root hairs of *D. complanatum* sporophytes stayed intact after boiling the roots at 90 °C in 10 % KOH for 150 min (Fig. 7a and b). Similarly, root hairs of *Lycopodiella inundata* stayed intact after 20 min of boiling (Kowal et al. 2020). The cell walls of lycophyte root hairs may differ structurally and be more resilient than those of angiosperm plants, potentially due to the parallel evolution of lycophyte roots alongside other seed plants (Raven and Edwards, 2001; Weng et al. 2008; Weng and Chapple, 2010). Further research into the root hair chemistry and anatomy of lycophytes could provide deeper insights into this unique structure.

In our study, we did not find any microscopic evidence for the presence of AM, ECM, or ERM in the fine roots of *D. complanatum* sporophytes. Multiple septated and non-septated hyphae could be observed around the root, which however did not form any mycorrhiza-like structures. Occasional vesicles were observed inside the fine root cells, resembling Mucoromycotina endophytes previously identified in the fine roots of *Lycopodiella inundata* sporophytes (Kowal et al. 2020). Future experiments, such as plant inoculation with axenic fungal isolates, will provide deeper insights into the relationship between *D. complanatum* and Mucoromycota.

The presence of long and dense root hairs was a peculiar and distinguishing feature of all *D. complanatum* fine root samples, visible even to the naked eye (Fig. 9a, Suppl. Fig. 1). The root hairs closely resemble 'root clusters' found in several non-mycorrhizal seed plants, which enhance phosphorus uptake from nutrient-poor soils (Lambers et al. 2006)—an adaptation that may have evolved independently in *D. complanatum*. We speculate that, unlike the obligatory mycorrhizal plants *P. sylvestris* and *V. myrtillus*, the lycophyte *D. complanatum* is less dependent on fungi partnership due to the increased surface resulting from numerous root hairs.

Sporophytes of D. complanatum often grow radially outwards, forming circle-shaped colonies, known as "fairy rings" (Spalding et al. 1975). These colonies expand at a rate of 20-50 cm per year, with the central portions dying off. The diameter of these rings can be indicative of the colony's age (Oinonen, 1967), although re-invasion of the centre is possible. In our study, the diameter of the "fairy rings" ranged from 2 to 25 m (average \pm SD: 10 \pm 6 m; Suppl. Table 1), suggesting that only "adult" sporophytes with the age varying from 4 to 125 years, were investigated in this study, and no sporophyte that was still connected to its gametophyte was collected. While the comparison between the age groups did not reveal difference in fungal composition, it is possible that younger sporophytes might exhibit different fungi composition in their fine roots. Additionally, seasonal changes could also change the species composition. For instance, the colonization of Mucoromycota endophytes in Lycopodiella inundata roots showed strong seasonal variation, with 86 % colonization in autumn and only 14 % in spring (Kowal et al. 2020). Our data is limited to a single collection time in August. Studying younger sporophytes and conducting collections at multiple time points would offer better insights into the relationship between D. complanatum sporophytes and their fungal partners.

4.2.2. Pinus sylvestris

Considering a high host specificity of ECM symbiosis, especially increased by the high soil acidity and monodominance of the studied forest (Smith and Read, 2008; Tedersoo et al., 2024), we expected *P. sylvestris* to have highest abundance of ECM fungi together with the lowest overall OTU diversity numbers. Compared to *D. complanatum*, *P. sylvestris* samples had significantly higher relative abundances of both symbiotrophic and ECM fungi along with the weaker association with saprotrophic fungi (Fig. 6a and b). The same pattern was observed in comparison with *V. myrtillus*, although it was not supported statistically.

The comparison of relative abundances per taxa (Fig. 4c,d,e) showed a significantly higher association of *P. sylvestris* only with genera *Russula* and *Tomentellopsis* compared to *V. myrtillus* and *D. complanatum*, respectively. Additionally, the analysis did not reveal differences in abundance of any ECM fungi between *P. sylvestris* and soil samples, most probably due to the high content of mycelia and spores of ECM fungi in the soil. One saprotrophic genus, *Luellia*, was more abundant in *P. sylvestris* fine roots than in the soil. Additionally, both *Luellia* and *Exophiala* were more abundant in comparison with *D. complanatum* samples, possibly playing an important role in root turnover of *P. sylvestris*. Fortunately, no ASVs in the dataset were assigned to the genus *Heterobasidion* or its saprotrophic family Bondarzewiaceae, the widely distributed and most destructive disease agent of conifer trees, including *P. sylvestris* (Garbelotto and Gonthier, 2013).

As expected, the calculated Hill numbers revealed that *P. sylvestris* had the lowest values for OTU richness and the diversity of common and dominant OTUs compared to the other sample types, even when considering only ECM fungi (Fig. 5a–c, Suppl. Figs. 9a–c).

4.2.3. Vaccinium myrtillus

Despite the concept that ERM has the highest host-specificity among all types of mycorrhizae, *V. myrtillus* samples showed significantly higher values of OTU diversity than *P. sylvestris* samples (Fig. 7). OTU richness calculated using only ECM fungi the confidence intervals overlapped with those for *P. sylvestris*, although they remained higher (Suppl. Fig. 8). Similar to *D. complanatum* and soil samples, this higher diversity of ECM fungi may be attributed to the presence of ECM fungi that also function as endophytes or sporophytes.

In our study, all fragments of V. myrtillus fine roots investigated microscopically showed the presence of dense hyphal coils inside the rhizodermal root cells, and these structures are characteristic for ERM (Fig. 7d and e). These coils could be formed by an association with Serendipitaceae (Sebacinales). Sebacinales were previously isolated from hyphal complexes of V. myrtillus collected in Europe and the ability of the genus Serendipita to colonize the roots of this species was even proved experimentally (Selosse et al. 2007; Vohník et al. 2016). Additionally, we found this family to be significantly more abundant in V. myrtillus compared to P. sylvestris root samples (Fig. 4d). Interestingly, the order Trechisporales was more frequently found in V. myrtillus than in the other root samples, with its genus Trechispora being more abundant than in D. complanatum. This genus has been previously observed to form hyphal coils in the ericoid plants (Vohník et al. 2012). However, additionally to the intercellular structure, this fungus produces a hyphal sheath around the hair roots, which was not observed in our samples. The other genus potentially forming ERM, Cladophialophora (Herpotrichiellaceae, Chaetothyriales) (Allen et al. 2003), was also identified in our dataset, but did not show predominance in V. myrtillus samples. No other OTUs were assigned to the confirmed or putative ERM fungi listed in Leopold (2016). Fungi that form ERM are believed to exclusively associate with Ericaceae and no other non-Ericaceae plants (Smith and Read, 2008). This could explain why two out of three putative ERM families were predominantly found in V. myrtillus samples.

5. Conclusion

Both metabarcoding and microscopic approaches revealed differences in RAF communities among three closely growing yet phylogenetically distant plant species. *P. sylvestris* fine roots had a higher abundance of ECM, dominantly *Russula* and *Tomentellopsis*, while putative ERM fungi (Sebacinales and Trechisporales) were predominant in *V. myrtillus*. Neither ECM nor AM were dominant in *D. complanatum* samples. Metabarcoding revealed that 66 % of identified taxa were present across all sample types, but *P. sylvestris* had significantly lower overall fungal diversity and ECM fungal diversity supporting the high host specificity of the relationships. The fungal community of V. *myrtillus* root hairs was most similar to *P. sylvestris* but more diverse. No AM was detected by microscopy. The abundant presence of Mucoromycota in *D. complanatum* roots suggests a potential endophytic association, though its exact nature remains unclear. The absence of mycorrhiza, extensive root hair development, and similarity to soil samples together are pointing towards a low dependency of *D. complanatum* sporophytes on any known mycorrhiza type and an opportunistic fungal-plant relationship.

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CRediT authorship contribution statement

Kristina Kuprina: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Moana Wirth: Writing – review & editing, Investigation. Maria Sanchez Luque: Writing – review & editing, Methodology, Investigation, Conceptualization. Heike Heklau: Writing – review & editing, Methodology, Investigation. Radvilė Rimgailė-Voicik: Writing – review & editing, Resources, Methodology. Manuela Bog: Writing – review & editing, Supervision. Martin Schnittler: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2025.101053.

Data availability

Sequences are available on NCBI (PRJNA1185013), the R and bash scripts of the data analysis, as well as created datasets, are available on GitHub: https://github.com/kuprinak/lithuania-metabar

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