

Genotype, Tannin Capacity, and Seasonality Influence the Structure and Function of Symptomless Fungal Communities in Aspen Leaves, Regardless of Historical Nitrogen Addition

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Abstract

Background

Plant mycobiomes play a crucial role in plant health, growth, and adaptation to changing environments, making their diversity and dynamics essential for agricultural and environmental contexts, including conservation efforts, climate change mitigation, and potential biotechnological applications. Horizontally transferred mycobiomes are established in plant organs, yet the dynamics of their colonization and establishment remain unknown. New molecular technologies offer a deeper insight into the establishment and dynamics of plant-associated mycobiomes. In this study, we investigated leaf-associated mycobiomes in cloned replicates of aspen (*Populus tremula*) with naturally varying phenolic profiles and a history of nitrogen fertilization.

Main findings

Using ITS2 metabarcoding of 344 samples collected from a ca ten-year-old common garden with small aspen trees at various time points over two consecutive years, we identified 30,080,430 reads in our database, corresponding to an average of 87,448 reads per sample clustered into 581 amplicon sequence variants (ASVs). Analysis of ASV patterns revealed changes in both richness and abundance among genotypes and across the seasons, with no discernible effect of fertilization history. Additionally, the number of reads was negatively correlated with the ability of the genotypes to synthesize and store condensed tannins.

Background

Microorganisms play an important role in higher life, as increasingly demonstrated in metagenomic DNA profiles from microbiomes associated with animals, plants, soils, and water bodies, collectively known as environmental DNA (eDNA). Healthy microbiomes have become a concept, yet the precise meaning of a healthy microbiome remains complex [1, 2]. In plants, some microorganisms are vertically transferred between generations and organs, such as systemic fungal endophytes in cold-season grasses, which provide their host with both tolerance and resistance benefits [3, 4, 5, 6]. This has paved the way for commercial use of endophyte-infected grass seeds [7]. However, most plant-associated fungi appear to be nonsystemic and horizontally transferred with functions ranging from beneficial to antagonistic for the plant [8, 9, 10, 11].

The most well-known and studied symbiotic association between plant roots and fungi is probably mycorrhizae, which provides both plants and fungi with advantages, primarily in terms of energy and nutrient supplies, respectively [12, 13, 14, 15, 16, 17]. Fungal endophytes, which inhabit the inter- and intracellular spaces of various plant organs (such as stems, roots, petioles, leaves, bark, seeds, and latex) without causing visible symptoms [18] are also increasingly recognized for their direct and indirect positive potential for the host and as a source of commercial products. For example, they may produce chemicals such as phytohormones and substances that support photosynthesis [19, 20, 21], enhance nitrogen assimilation mechanisms [22], solubilize minerals [23, 24], produce siderophores with iron-retaining properties [25, 26], and generate substances that enhance defense and tolerance properties [27, 28, 29, 30]. In return, the endophytes benefit from the sheltered life inside the plant, which is surrounded by nutrients, and they gain both competitive and dispersal advantages inside their host.

There may be millions of endophytic fungi Worldwide, yet only a fraction of which are known [31, 32] and an even smaller fraction has been isolated and studied for their biology and potential [33, 34, 35]. Fungal endophytes were previously discovered in plants in the 19th century [36, 37], and they were first isolated from sterilized plant parts; consequently, only culturable fungal endophytes could be studied and described based on their morphology [38, 39, 40]. Sanger sequencing subsequently enabled the molecular determination of culturable colonies [41, 42]. Today, metabarcoding and shotgun sequencing of eDNA has widely replaced culturing as a way to assess endophyte community composition independently of culturing; and high-throughput metagenomic sequencing techniques are now standardly used to characterize the presence of fungal DNA strands in environmental samples [43]. The amplification and sequencing of conserved DNA regions with high interspecific and low intraspecific variation, are followed by downstream bioinformatic analyses, to reveal the diversity, composition, and function of leaf-associated fungal communities [42]. The conserved regions to be targeted vary among organism groups, and the nuclear ribosomal internal transcribed spacer (ITS) region (500 base-pairs) is the formal fungal barcode and the most sequenced genetic marker in mycology [44, 45]. Based on its degree of conservation, the ITS region is divided into subunits, that with adequate primers may be targeted singly or in combination (i.e. ITS1 to ITS4); providing varied opportunities to estimate fungal richness and diversity in a sample [46, 47].

While these techniques are highly competent generating diverse DNA sequence reads in an environmental sample, the ability to recognize the true diversity is limited by the lack of reference databases, the inability to distinguish endophyte DNA from that of epiphytes [48], and the inability to culture reads back to colonies that would allow for morphology description and chemical screening to characterize the potential function of the endophyte. Analytical tools and models are being developed to suggest ecological function and interrelated guild structures (FUNGuild: [49]; FungalTraits: [50]; FunFun: [51]), and while these models provide insights into an endophytic community, they are also limited by the depth of taxonomic determination and sequencing efforts and the interpretation of ecological function thus builds on known relationships among related taxonomic groups.

Trees are long-lived organisms that are exposed to biological and abiotic stresses for many years. The ability of trees to grow and stay healthy has been associated with their ability to directly fight back by own means [52, 53], and indirectly through symbiotic associations with endophytic fungi, for example. These associations may rely on chemical and physiological mechanisms, as well as on ecological processes [27, 28, 29, 30]. The biosynthesis of chemical specialized products may for example be shared between the endophyte and its host (i.e. the production of camptothecin by the tree host *Camptotheca acuminata* and its endophyte *Fusarium solani* [54]). The host tree appears to select or filter the fungal community that established in its organs, as reported for the leaves of beech [11], cacao [55], oak [56], elm [57], aspen [58, 59, 60], *Populus trichocarpa* [61], temple trees [62], and eucalyptus [63]. Specific associations with organs and growing sites are further demonstrated for e.g. beech [11], oak [56], and tropical forest trees [64]. However, our understanding of what makes an endophyte community “healthy” as well as processes underlying the inclusion and exclusion of fungal taxa by trees, is limited, although host substrate conditions have been suggested to play a role [57, 60].

With European aspen (*Populus tremula*) as an example we explored the effect of plant genotype and its inherent ability to produce, and store condensed tannin phenolic polymers as a factor in determining the community of fungal endophytes across the short growth season in northern Sweden. In addition, this study allows for an assessment of the long-term effects of historic nutrient additions to a population of ca ten-year-old trees on the fungal endophyte community hidden in their leaves.

Methods

Study system

To delineate the fungal communities associated with aspen foliage, we obtained aspen leaves from the TanAsp garden, and selected six replicated aspen genotypes with either a high or low capacity to generate and retain condensed tannins, as highlighted in the work of Bandau et al. [65, 66]. The trees were planted in 2010, and our study aimed to assess the seasonal composition and dynamics of mycobiomes linked to the leaves of young trees within and between the years 2020 and 2021. To monitor the seasonal composition and dynamics of the mycobiomes associated with the leaves and to compare patterns over time, we revisited the same trees repeatedly. This included twelve replicates of each genotype in 2020, resulting in 72 trees. The number of trees was reduced to four genotype replicates in 2021, totaling 24 trees per sampling event.

Plant materials

Nondestructive phenotyping

Visual inspections included: plant height measured in centimeters (from the plant-soil intersection to the tip of the canopy); counting of *Harmandiola* galls; assessment of symptoms of *Venturia* sp. as wilted branches relative to the total number of branches per canopy; other biotic stress symptoms, such as leaf hypersensitive response (HR), abundance of *eriophyid* mite colonies, damage from chewing insects, and rust pustules (*Melampsora pinitorqua* [47, 67]). Leaf damage symptoms were ranked on a 0–3 scale: 0 indicated no symptoms, 1 indicated low damage corresponding to symptoms on some leaves, 2 indicated moderate damage with symptoms on approximately half of the leaves, and 3 indicated a high level of damage with symptoms on more than half of the leaves.

Pigment assessments included

nitrogen balance, chlorophyll, anthocyanin, and flavanol indeces, which were measured with an optical meter ((DUALEX® Optical LeafClip meter, Force-A, Orsay, France). Three readings were performed on each of ten randomly collected leaves per canopy, generating a mean value per tree from 30 measurements.

Destructive phenotyping

Leaf harvest and sample preparation: In 2020, leaf sampling was conducted on the following dates: June 22, July 14, and August 6 and 23. In 2021, sampling occurred on June 23, July 14, and August 24. The selected trees were chosen in accordance with the original garden setup [65, 66], including low-tannin genotypes (GT50, GT60, GT115) and high-tannin genotypes (GT5, GT65, GT72). Additionally, trees were selected based on a history of NH_4NO_3 addition [66]. Therefore, during the 2020 sampling, three nitrogen treatment levels were considered, while in 2021, the sampling was limited to control trees that had not received fertilizer.

Each sample, representing one tree and one sampling event, consisted of ten undamaged mature leaves collected from one canopy with gloves to avoid contamination, at breast height. Each leaf was cut at the base with a clean pair of scissors, leaving the petiole on the twig, and then added to a labelled plastic bag. The sample bags were flash-frozen on dry ice in the field, transported to the laboratory for approximately one hour in coolers, and then stored at -80°C until lyophilization for 24 hours in a LABOGENE freeze dryer (3450 Lillerød, Denmark). The dry leaves were subsequently ground to a fine powder and kept in labelled Falcon tubes (15 mL) at -20°C until DNA extraction.

DNA extraction and library preparation

DNA extraction and amplicon library preparation were conducted as described in Siddique et al. (2022) [46]. In short, the E.Z.N.A. Plant DNA Kit (OMEGA-BIO-TEK Inc, PW, GA, USA) was used for DNA extraction, and the fungal-specific ITS2 region was amplified using extended primers. After

confirming the successful attachment of sample-specific index primers, the library was subjected to Illumina Miseq (300 bp) paired-end (PE) sequencing at NGI SciLifeLab (Stockholm).

Bioinformatic analyses

Amplicon sequencing analyses followed FAIR principles suggested by Ewels et al. (2020) [68] and Straub et al., (2020) [69]; the ampliseq pipeline (<https://github.com/nf-core/ampliseq>) was assessed on 14 April 2022).

In brief, Raw Illumina, MiSeq v3 2-300 bp, paired-end reads were demultiplexed by SciLifeLab and delivered as sample specific fastq files, which were then individually quality checked with FastQC. Fungal primers were removed with the CutAdapt tool. DADA2 was used to generate a curated dataset of amplicon sequence variants (ASVs). The ITS region was truncated (settings: truncLenf = 223 and truncLenr = 162); chimera and “noisy” reads were removed using the DADA2 algorithm, which distinguishes between biological variation and sequencing errors; mitochondria, chloroplast, and archaea reads were identified and removed with the Barrnap tool; and only sequences with fungal ITS2 regions were retained. Based on the ITS region, fungal taxonomy was then assigned with DADA2 and QIIME2 against the UNITE-fungal reference database v.8.3.

ASVs represented by fewer than five reads in a sample were removed to avoid rare sequences and singleton bias [70]. The fungal data are available as available as 06.1_rel-table-ASV_with-DADA2-tax.tsv scaled after total fungal DNA in a sample. The QC results and sample summary files were aggregated and visualized with MultiQC into a single report which is available at 04_multiqc_report.html.

The reproducible pipeline, tools, codes, and parameters can be found in GitHub repository: https://github.com/abu85/ampliseq_ITS and the bioinformatic summary, output and tools wise detail reference lists can be found in the Zenodo repository: [10.5281/zenodo.10839669] -as referred to in the data availability section. The entire bioinformatic pipeline and analysis were run on the UPPMAX HPC server (<https://www.uppmx.uu.se/>). The community analyses were built on the curated dataset.

Community analyses

The number of sequence reads (or ASVs) was used as a measurement of fungal abundance in a sample. The abundance of different ASVs was used as a metric of species richness. The identification depended on the taxonomic resolution that the ASVs allowed, and it was expected to vary among samples, genotypes, and sampling events. As the amplification threshold was set to 1000 reads per sequence in a sample, and to avoid including potential sequencing errors as unique reads [71, 72], we removed samples with low coverage (< 1000 reads) from the database.

Rarefaction curves

Randomized species accumulation curves (rarefaction curves) were compared after season, genotype, and treatment to assess fungal richness. The specaccum function with two segments, coleman and rarefaction, was employed ('coleman' finds the expected richness, and 'rarefaction' finds the mean when accumulating ASVs [73, 74]).

Species diversity indices: To assess biodiversity in foliar mycobiome communities, we employed a suite of indices: Fisher’s alpha, a measure of species richness; the Shannon index, considering both richness and abundance; and the three Hill numbers that evaluate diversity indices for relative community data [75] (Hill, 1973). The Hill numbers primarily scrutinize communities based on species abundances: N1, the exponent of the Shannon index, emphasizes abundant species; N2, the inverse Simpson index, focuses on highly abundant species; and N3 emphasizes diversity measurements by comparing species of similar abundance within the community.

Community structure

Community composition was analysed and visualized using nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) of square root transformed ASV counts [76] to handle nonlinear relationships between samples in dissimilarity space. The distinctiveness of fungal assemblages was statistically tested with permutational multivariate analyses of variance using Bray–Curtis and AltGower distance matrices. Dissimilarities among mycobiome communities were tested across seasons, between years, among treatments, and among genotypes with permutational multivariate analysis of variance, using AltGower dissimilarity indexes on standardized count data [PERMANOVA/adonis2 [77, 78]; number of permutations = 9999).

Taxonomic composition

Species abundances were visualized using normalized relative abundance data, and guild analyses were used to reveal the ecological function of annotated fungal species in the samples. The FUNGuild [49] package was used for those analyses.

Results

Sequencing output and quality

The Amplicon Sequence Variant (ASV) dataset obtained from 344 aspen leaf samples comprised 30,081,923 reads, which were clustered into 581 ASV variants as detailed in the 06_asv_table.tsv. The average library size was 87,448 reads per sample, with a range from seven to 957,090 reads. Based on our criterion of having more than 1000 reads in a sample, we excluded seven samples. The final reads resulted in a refined dataset of 337 samples with 30,080,430 reads, equivalent to 99.99% of the initial total reads.

Fungal richness and abundance across seasons

The refined dataset allowed for the generation of rarefaction curves, indicating an increase in the number of fungal ASVs throughout the season in both 2020 (Fig. 1a) and 2021 (Fig. 1b). The pace at which the fungal community developed in the leaves exhibited similar trends in both years although the trajectory differed somewhat between the years (Fig. 1c).

However, a notable distinction among genotypes consistently emerged (Fig. 1d), and fungal richness, expressed as the number of reads, also appeared to be greater in aspen leaves categorized as low-tannin genotypes than in those categorized as high- tannin genotypes (Fig. 1d), corroborating the findings of Bandau et al. (2021) [66].

Fungal richness and abundance among Aspen genotypes

Diversity indices characterize community structures based on aspects of species richness and abundance. Using five indices to calculate the diversity based on ASV reads, we obtained insights into these structures. We observed a significant effect of aspen genotype on each index, underscoring the importance of genotype for both species' richness and abundance. The pattern of relative index differences was remarkably consistent among sampling events and years (Fig. 2). Pairwise comparisons further indicated a stronger differentiation of genotypes when assessing diversity with the Shannon index than with the Fisher's alpha index (Fig. 3), which suggests that effects of both richness and abundance separate fungal communities in aspen leaves.

Table 1

ANOVA summaries of genotype and seasonal effects from two consecutive years on five measures of fungal biodiversity (five diversity indices, also see text) in aspen leaves from the TanAsp collection [65, 66]. The results are provided by year with sample sizes $N_{2020} = 72$ and $N_{2021} = 24$. Significant results ($P < 0.05$) are shown in bold. Also see Fig. 2.

Diversity indices																
		Fishers' alpha			Shannon			Hill N1			Hill N2			Hill N3		
Year	Effect	SS	Dev	P	SS	Dev	P	SS	Dev	P	SS	Dev	P	SS	Dev	P
2020	Genotype	172.24	3.1	0.001	7.47	0.3	0.004	40.32	0.73	0.001	25.66	0.39	0.001	23.55	0.3	0.001
	Season	188.46	3.1	0.001	0.39	0.3	0.44	4.04	0.82	0.061	0.10	0.50	0.58	0.01	0.4	0.84
2021	Genotype	15.5	1.4	0.013	1.95	0.2	0.15	9.93	0.51	0.001	7.59	0.31	0.001	7.27	0.3	0.001
	Season	48.69	1.2	0.001	0.08	0.3	0.75	0.65	0.64	0.40	0.03	0.46	0.77	0.01	0.4	0.87

Table 2

Summary of permutational multivariate analysis of variance (PERMANOVA) tests for 2020 and 2021, partitioning effects of season, historic nitrogen treatment, aspen genotype, and ability to synthesize and store condensed tannins (CTs) on the mycobiome (ASV abundances) in aspen leaves. The samples from 2021 included aspen trees that had not received any nitrogen fertilization. Significant responses are shown in bold ($p < 0.05$). See also Fig. 4.

Year	Effect	df	SS	R ²	F	P
2020	Season	3	-0.39	-0.05	-3.97	1.00
	Treatment	2	0.08	0.01	1.24	0.20
	Genotype	5	2.35	0.28	20.74	0.0001
	CTs	1	0.34	0.04	11.47	0.0001
2021	Season	2	0.24	0.03	1.01	0.20
	Genotype	5	3.35	0.42	9.11	0.0001
	CTs	1	0.64	0.08	5.81	0.0001

Fisher's alpha confirmed a seasonal effect, as did Hill N1, suggesting that there was both a change in species richness across the season and a change in the organization of abundant species. However, neither the Shannon index, nor Hill's N2 or N3 suggested a significant seasonal impact on the leaf-associated fungal community composition (Table 2).

Association stability between aspen genotype and mycobiome

To visualize the stability of the fungal association with genotype, two kinds of multivariate projections were performed. Both methods (the iterative hypothesis generating NMDS and the analytical correlative projection PCoA) reached similar conclusions from the data that included all samples from the refined dataset. In both cases the ASV reads strongly clustered after genotype, with two high tannin genotypes (72 and 5) being more distant from the other genotypes. The projection of tree phenotypic traits, collected nondestructively during sampling events, indicated that height and susceptibility to infection with *Venturia* (a necrotrophic fungal pathogen) were opposite vectors with significance for the projection of the samples in the multivariate space (Fig. 4), corroborating suggestions by Robinson et al (2012) [67]. Apart from the genotypic effect, neither the impact of seasonal variation nor the historical nitrogen addition treatment appeared to significantly influence the projection, as evidenced by permanova tests (Table 2, also see Fig. S1).

Fungi taxonomic resolution

The taxonomic resolution for this study at the phylum level was 99.98%. However, only 1.44% of the ASVs could be classified at the class level, and 0.98% could be classified to the genus level (summing up to 49 genera, Fig. 5a). The mycobiome of the aspen leaves was dominated by four orders within the Ascomycota phylum (Dothideales, Helotiales, Capnoidiales and Saccharomycetales) with three genera dominating: *Aureobasidium* (Dothideales), *Candida* (Saccharomycetales) and *Cladosporium* (Capnoidiales) (Fig. 5b). Among 24 identified fungal orders, 13 belonged to Ascomycota, 10 to Basidiomycota and one to Dothideomycetes (data to be found in the Zenodo repository: 10.5281/zenodo.10839669). Interestingly Helotiales was more abundant in the database and included more genera compared to Dothideales, Capnoidiales and Saccharomycetales (Fig. 5c).

Representation of trophic modes in the mycobiome

Out of 581 ASVs only 183 were determined to genus level and could be assigned to functional guilds. Despite this limited ability to identify fungi at lower taxonomic levels, we detected representatives of seven ecological guilds: pathotrophs, patho-saprotrophs, patho-symbiotrophs, patho-sapro-symbiotrophs, saprotrophs, sapro-symbiotrophs, and symbiotrophs. Patho-sapro-symbiotrophs clearly dominated the total dataset that included all sampling events (Fig. 5d).

Discussion

Recent molecular advances have enabled us to study hidden microbial communities in plant organs. However, the worldwide number of tree-associated microorganisms is vast, their diversity is underexplored, and their functions are complex and largely unknown, warranting basic insight into their composition and dynamics [79, 80]. Using Illumina sequencing, we revealed a seasonal increase in the abundance of fungal eDNA in aspen leaves for two consecutive years. We also demonstrated genotype differences in the abundance and composition of the foliar fungal microflora, which were negatively related to the presence of polyphenolic condensed tannin complexes in the leaves. Additionally, our study strongly indicated that fertilization with ammonium nitrate did not have any long-term effects on the mycobiome (Fig. S1), suggesting that genotype differences in host trees may be a strong factor in the maintenance of forest biodiversity.

Genotype and environmental effects on aspen leaf-associated mycobiomes

We have previously proposed a significant impact of host genotype on leaf-associated fungal communities in aspen trees cultivated within a greenhouse environment [58], as well as when comparing clones repeatedly grown indoors and outdoors [81]. This study, which was conducted on six cloned young aspen trees in a common garden setting, further reinforces the influence that hosts may exert on shaping the mycobiome within their leaves. Similar genotypic effects have also been observed in foliage from several studies of poplar trees including *Populus angustifolia* [82], and *P. balsamifera* [83] and in the roots of a selection of mainly hybrid poplar clones [84] as well as in willows [85].

It is also becoming evident that different organs may filter fungal-associated communities differently, as observed in studies on alder and hazel [86], beech [11], and oak [87]. The composition of the mycobiome may further depend on the general health condition of a tree [87] and, in some cases, may determine the severity of damage from leaf pathogens [88]. The growth environment may also affect the richness and abundance of the mycobiome [89, 90]; however, this influence is not consistently corroborated by empirical data, as highlighted by Siddique et al. (2021) [11]. This assertion is further supported by the absence of long-term effects resulting from nitrogen fertilization, as reported in Fig. S1. While tree species are often assumed to play a crucial role in biodiversity within ecosystems, our study thus highlights the diversity shaped by individual variation in a key player species such as aspen [91] thereby supporting the fundamental implication that genetic variation in primary producers may have for understanding terrestrial ecosystems [82].

The mycobiome reported here primarily identified ASV reads belonging to the Ascomycota phylum, predominantly representing the orders Dothideales, Helotiales, Capnoidiales, and Saccharomycetales. Among these genera, three dominated: *Aureobasidium* (Dothideales), *Candida* (Saccharomycetales), and *Cladosporium* (Capnoidiales). This division aligns with similar screenings reported in previous studies (e.g. [11]). Helotiales comprises a diverse array of fungi, encompassing numerous species that are yet to be formally described. These genera however have various lifestyles, many of which involve leaf and wood decay processes [92], and some species associated mainly with roots as endophytes act as facilitators of plant growth through promotion effects, while others exhibit pathogenic behavior [93]. Notably, within this order, *Fraxinus excelsior* stands out as a well-known pathogen responsible for inducing Ash dieback. However, Helotiales is also recognized for its potential for pharmaceutical discoveries [93].

Diversity indices could be calculated based on reads, supporting the genotype-specific mycobiome diversity findings, as both species richness and abundance significantly varied among genotypes in similar ways during both study years. Various diversity indices yielded similar suggestions, and projections in multivariate space repeatedly indicated that some genotypes might be closer to each other than others in terms of their impact on associated mycobiomes.

What shapes mycobiomes and their functions?

It is suggested that leaves may serve as a chemical landscape, and the metabolic dynamics of the leaf may determine the endophytes that colonize its interior [60]. Additionally, physical structures, such as bark surfaces, have been reported to create environments more conducive to the attachment of certain cryptogams and fungi than other structures e.g. [94]. The TanAsp common garden, utilized for the present study, was established to investigate the potential effects of aspen tannins on their association with surrounding organisms [66]. Here we observed differences in the abundance of fungal ASV reads corresponding to relative variations in the expected tannin profiles of the trees. Additionally, qualitative distinctions in the genet-related mycobiome were apparent, as demonstrated by multivariate projections. While two of the high tannin genotypes clustered separately, the third high-tannin genotype clustered together with the low-tannin genotypes. Hence, while our study suggested a negative impact of condensed tannins on the abundance of foliar-associated mycobiomes, it is premature to infer a simple relationship.

Condensed tannins, for example, were reported to have a negative correlation with twig endophyte infection in poplars within a hybrid zone [95] as well as with the abundance of molecular markers of the biotrophic pathogen *Melampsora pinitorqua* [47], and the symptoms of necrotrophic pathogen *Venturia* infection in *Populus tremula* [66]. Moreover, in a review by Bhat et al (1998) [96], degradation primarily by filamentous fungi such as *Aspergillus* and *Penicillium*, along with yeasts, was found to degrade condensed tannins, potentially using them as substrate. Additionally, the ectomycorrhizal fungus *Laccaria bicolor* was reported to colocalize with condensed tannins in the roots of *Populus tremula* [97]. The function of mycobiomes evidently varies depending on the substrate composition of a plant organ, such as a leaf. Niche overlap may result in competition for substrates among the endophytic community [98], occurring in both the inter- and intracellular spaces of various plant organs where endophytes reside [18], which could be advantageous for some fungal taxa and disadvantageous for others.

The increase in fungal species abundance observed throughout the season was a notable finding of this study. Speculations regarding mycobiome function in plant parts suggest that the establishment of degrading fungi could benefit litter decomposition of plant material and contribute to nutrient recycling in forest habitats potentially affected by tannins [65]), their resistance to degradation by soil microbes, and their ability to bind and stabilize microbial enzymes [99, 100]. Despite the negligible species-level determination coverage of the mycobiome in this study, valuable insights were gained into the diverse functions that were represented in the leaves across two seasons. Different combinations of patho-, -sapro-, and -symbiotroph- related lifestyles were suggested based on the data. The dominating *Aureobasidium*, a sooty mold, is a taxonomic complex usually assigned to a saprotroph lifestyle similar to that of *Candida* [50]. In our study, symbiotrophic fungi were mainly lichenized and ectomycorrhizal endophytes, while pathotropic members were primarily associated with *Curvularia*.

Methodological challenges and way to go

The selection of appropriate taxonomic markers is crucial for successful DNA metabarcoding [101]. Primers should ideally encompass all members of an organism group in a sample while efficiently distinguishing nucleotide variability within that group to estimate taxonomic diversity. For fungi, the internal transcribed spacer region (ITS) of ribosomal RNA, including the ITS1, 5.8S, and ITS2 loci, is commonly utilized as the primary marker sequence [102]. ITS2, known for its low taxonomic bias, is frequently targeted for metabarcoding through Illumina MiSeq, the widely used high-throughput sequencing platform [45].

In our study, we also targeted the ITS2 region using the ITS3F and ITS4R primers [46]. However, we encountered coverage issues, with less than 1% of reads identifiable to the genus level, necessitating methodological improvements in subsequent studies, particularly during the experimental design phase of molecular work. The initial step of primer design may introduce biases in estimating major fungal taxa [102]. ITS primers, mostly adapted from Sanger's sequencing, may favour mono- or oligo-specific sequences, potentially mismatching or suboptimally amplifying the entire fungal community in an environmental DNA (eDNA) sample [103, 104]. Moreover, the primers used in our study might suffer from mismatches with the target site, favouring certain fungal taxa over others in the amplicon. The use of degenerate primers specifically targeting ITS2 regions can mitigate primer bias and enhance taxonomic coverage [104]. However, some ITS2-specific degenerate primers, such as fITS7/ITS4 and gITS7/ITS4, may also amplify nontargeted host DNA, reducing sequencing depth and read length [48]. Although ITS1 and ITS2 information was considered, ITS1 was ultimately discarded due to the occurrence of undesired PCR bands and primer-dimers [46].

Another source of error is the potential coamplification of plant-fungal DNA in aspen leaves, as highlighted in recent literature. Utilizing peptide nucleic acid (PNA) clamps alongside primer pairs can effectively increase fungal reads and associated diversity by blocking the amplification of nontargeted organisms, such as the plant host [48]. Furthermore, achieving genus or species-level taxonomic resolution is challenging when targeting only the ITS2 region, which may be too short to distinguish closely related fungal species, potentially resulting in unassigned fungal taxonomy at the class-to-genus level (Fig. 5a, [44]). Long-read high-throughput amplicon sequencing methods, such as Pacific Biosciences (PacBio), which map the entire ITS region and highly conserved flanking sites (small subunit-SSU and large subunit- LSU), offer an alternative for comprehensive fungal community analysis with improved taxonomic resolution [105]. However, taxon-specific PCR amplification biases can obscure community diversity in amplicon sequencing. PCR-free metagenomic sequencing presents a promising solution for overcoming this issue [106, 102]. Despite these advancements, our knowledge of microbial life remains limited. While the UNITE databases contain entries for 3,846,536 ITS sequences, only 195,696

fungal species are hypothesized to have DOIs at the 1.5% threshold (<https://unite.ut.ee/>). This is due to the unavailability of reference genomes for every organism.

Thus, the scientific community faces the significant challenge of exploring potential beneficial organisms among the diverse unknown fungal communities associated with plants.

In the realm of plant-associated microorganisms, inquiries into the internal, external, or combined lifestyle persist. Various microorganisms can access plant tissues through openings like stomata, residing within the apoplast or penetrating host cells as genuine endophytes. Conversely, some organisms primarily exist as external partners or epiphytes. This differentiation is fundamental, yet fully comprehending the physical association becomes challenging with eDNA applications, as both living and deceased DNA are amplified. In our investigation, we sterilized a subset of samples to explore potential sequencing discrepancies, and we were unable to differentiate between surface-sterilized and unsterilized leaves (Fig. S2). Consequently, we omitted this additional handling step from our study samples.

Localization studies, such as immunoblotting or fluorescence in situ hybridization (FISH) techniques, which label target organisms with molecular markers, may be integrated into metagenome studies [106] to gain insights into the physical connection between the plant host and its associated mycobiome. However, this approach is currently meaningful only for fungal associations of particular interest.

Conclusion

In summary, our study emphasized that the impact of fungal microbes associated with aspen trees varies with genetic background, suggesting that within-population genetic variability of aspen trees may hold considerable importance for hidden forest biodiversity. Our study also exemplifies the need not only to study these hidden communities but also to optimize metagenome sequencing to refine the understanding of tree-associated mycobiome composition and function.

Abbreviations

ASV
Amplicon sequence variant
CTs
Condensed tannins
DNA
Deoxyribonucleic Acid
eDNA
environmental DNA
FASTQ
refers to text-based biological sequence (usually nucleotide sequence) files. These files have the following extension: fastq
ITS
Ribosomal internal transcribed spacer
LSU
rDNA large subunit
NMDS
Nonmetric multidimensional scaling
PCoA
Principal coordinate analysis
PCR
polymerase chain reaction
PNA
Peptide nucleic acid
RNA
Ribo nucleic acid
SRA
Sequence read archive
SSU
rDNA small subunit

Declarations

Ethics approval: Not applicable

Consent for publication: Not applicable.

Availability of data and material:

The authors declare that links to the data supporting the findings of this study are available within the paper and its Supplementary Information files.

The sequencing reads (FASTQ) were registered in the Sequence Read Archive (SRA) at SRP496938, as BioProject:PRJNA1090416 with accession numbers SRX24011776 to SRX24012154.

The dataset information can be found as part of the Zenodo repository: [10.5281/zenodo.10839669]. The short 'readme' file contains the data and material content.

Bioinformatic codes and R scripts are stored at https://github.com/abu85/ampliseq_ITS. Should any raw data files be needed in another format they are available from the corresponding author upon request.

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Figures

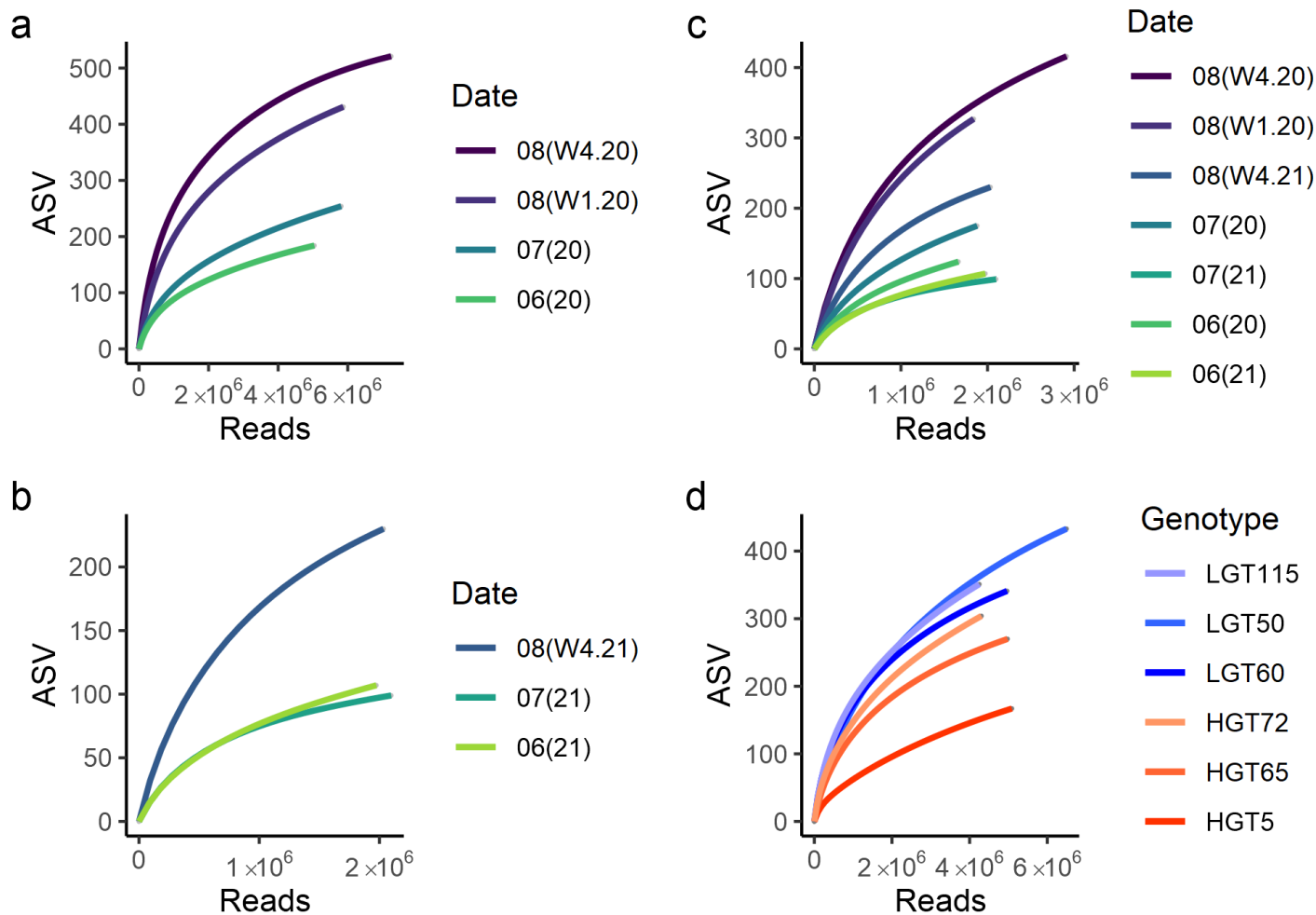


Figure 1

Rarefaction curves were generated based on amplicon sequence variant (ASV) counts to project seasonal patterns of fungal species richness in aspen leaves for **a)**2020, **b)** 2021, and **c)** across both years. The numbers 06, 07, and 08 refer to June, July, and August, respectively. w1 and w4 refer to sampling weeks in August. Years are indicated by the last digits 20 and 21. Additionally, rarefaction curves were generated for all samples, highlighting accumulation **d)** related to genotype, which were further divided according to Bandau et al. 2017 [65] into low tannin genotypes (LGT, blue colours) and high tannin genotypes (HGT, red colours). GT numbers (5, 50, 60, 65, 72, and 115) correspond to the identifiers defined in the SwAsp collection [67].

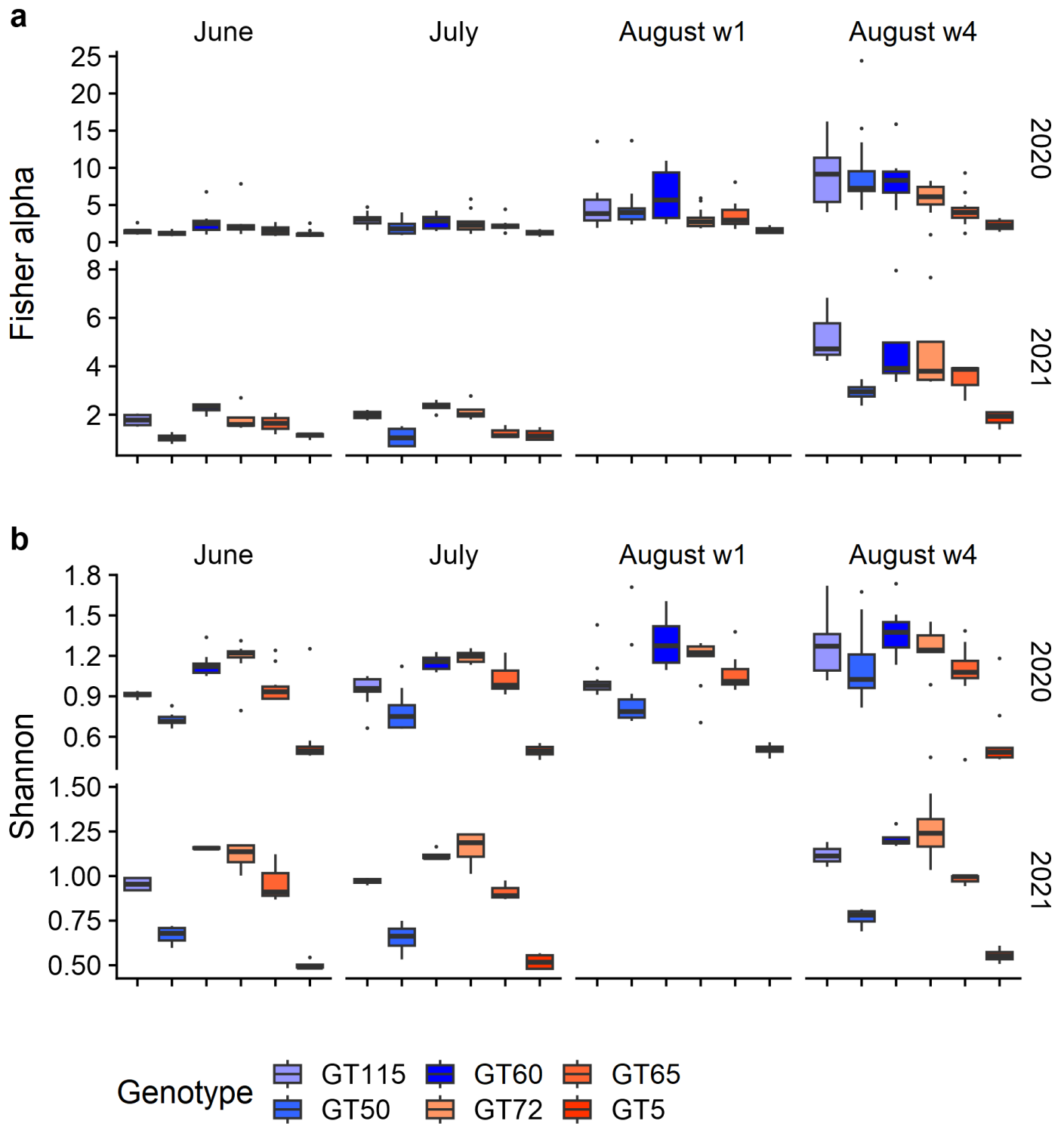


Figure 2

Diversity estimates, represented by **a**) Fisher's alpha diversity index and **b**) Shannon's diversity index, showcase the mycobiomes in aspen leaves, categorized by year, genotype, and sampling event. Genotypes are color-coded to reflect their extreme abilities to produce and store condensed tannins—blue for low tannin contents and red for high tannin contents. The impacts of genotype, seasonality, and historic nitrogen addition are elucidated in Table 2, while Figure 3 visualizes pairwise genotypic differences in diversity measurements. Sample sizes: $N_{2020} = 72$; $N_{2021} = 24$.

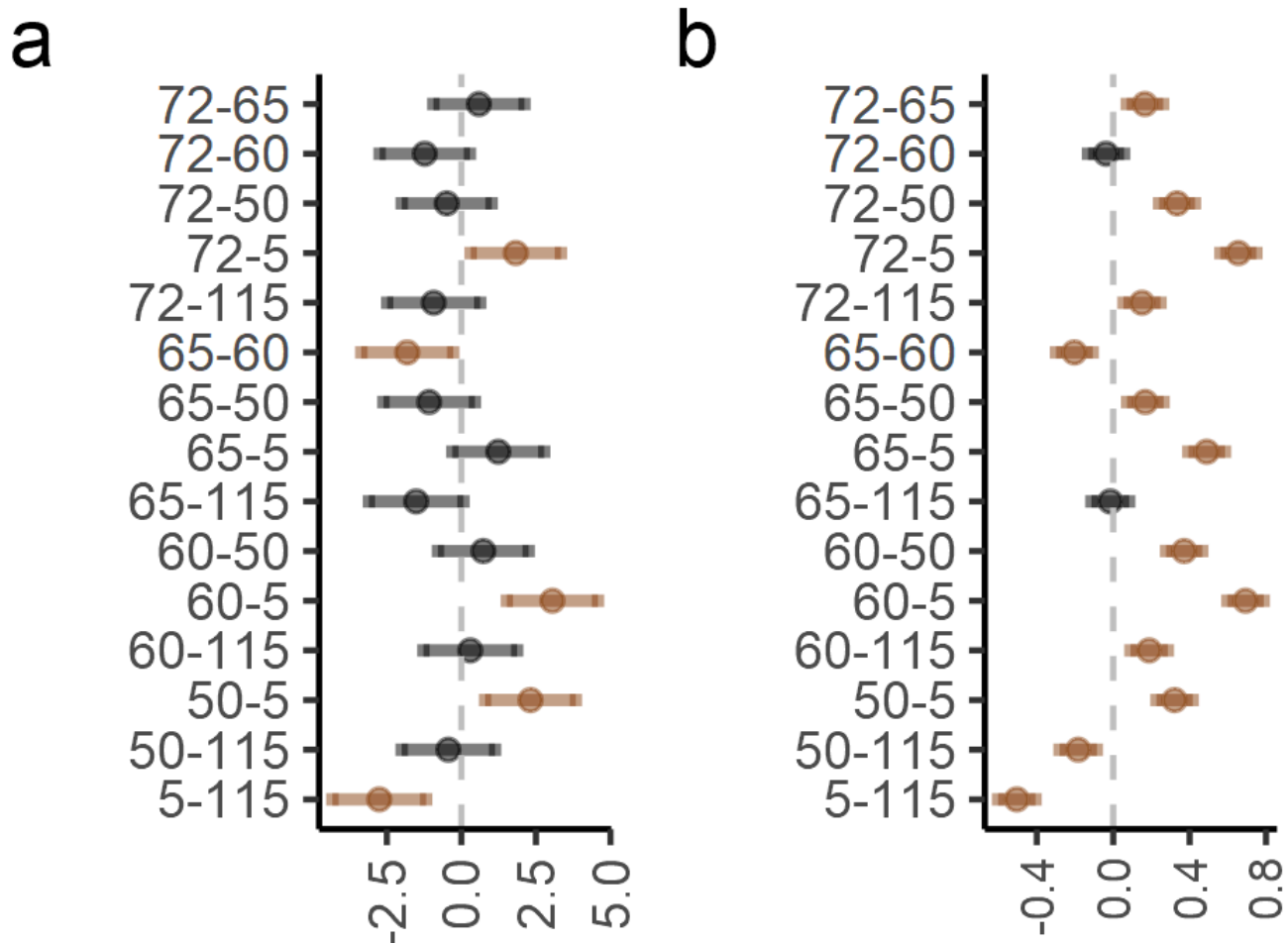


Figure 3

Comparison of genotype-specific diversity indices, including a) Fisher's alpha and b) Shannon's, based on data from seven (all) sampling events. Mean differences between genotypes are depicted with 95% confidence intervals. Significant differences ($p_{adj} < 0.05$) are highlighted in brown.

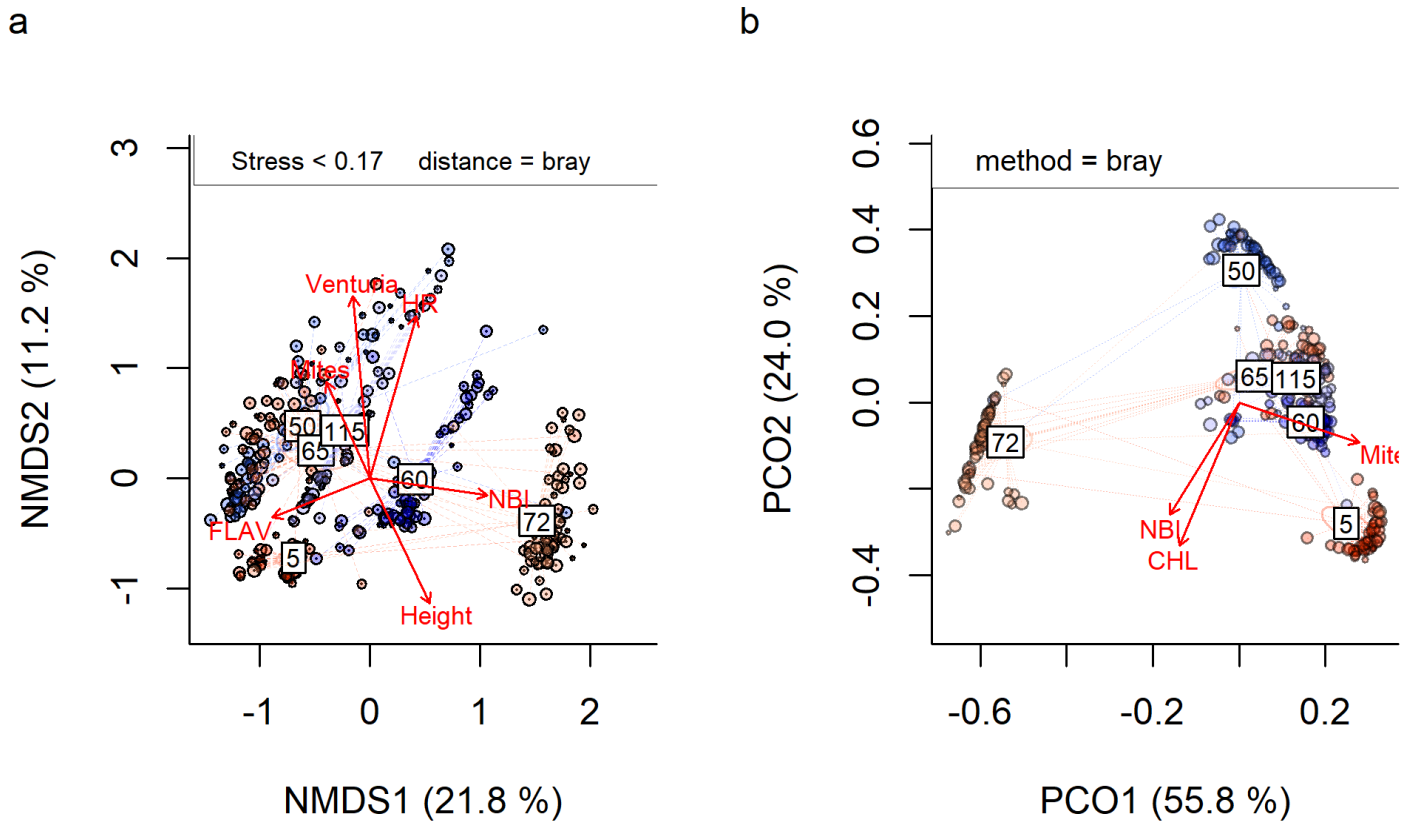


Figure 4

Multivariate projections integrating metagenomic and phenotypic data from aspen leaves. Analyses were performed on all data (including nondetermined ASVs, see text) and are represented through **a**) Nonmetric multidimensional scaling (NMDS) and **b**) principal coordinate analysis (PCoA), both displayed for the first two dimensions. Assemblages are clustered according to the aspen host genotype (indicated by Swasp/Tanasp numbers). Growth traits are illustrated with labelled vectors (height=plant height; chl=leaf chlorophyll content; nbi= nitrogen balance index; anth=anthocyanidins), while damage traits include mites (*Eriophyid* mite gall symptoms), *Venturia* (symptoms of the necrotrophic fungal pathogen on aspen, tentatively *Venturia radiosa*), Rust (tentatively *Melampsora pinitorqua*; Siddique et al. 2022, HR=hypersensitivity responses), and galls by *Harmandiola* wasps.

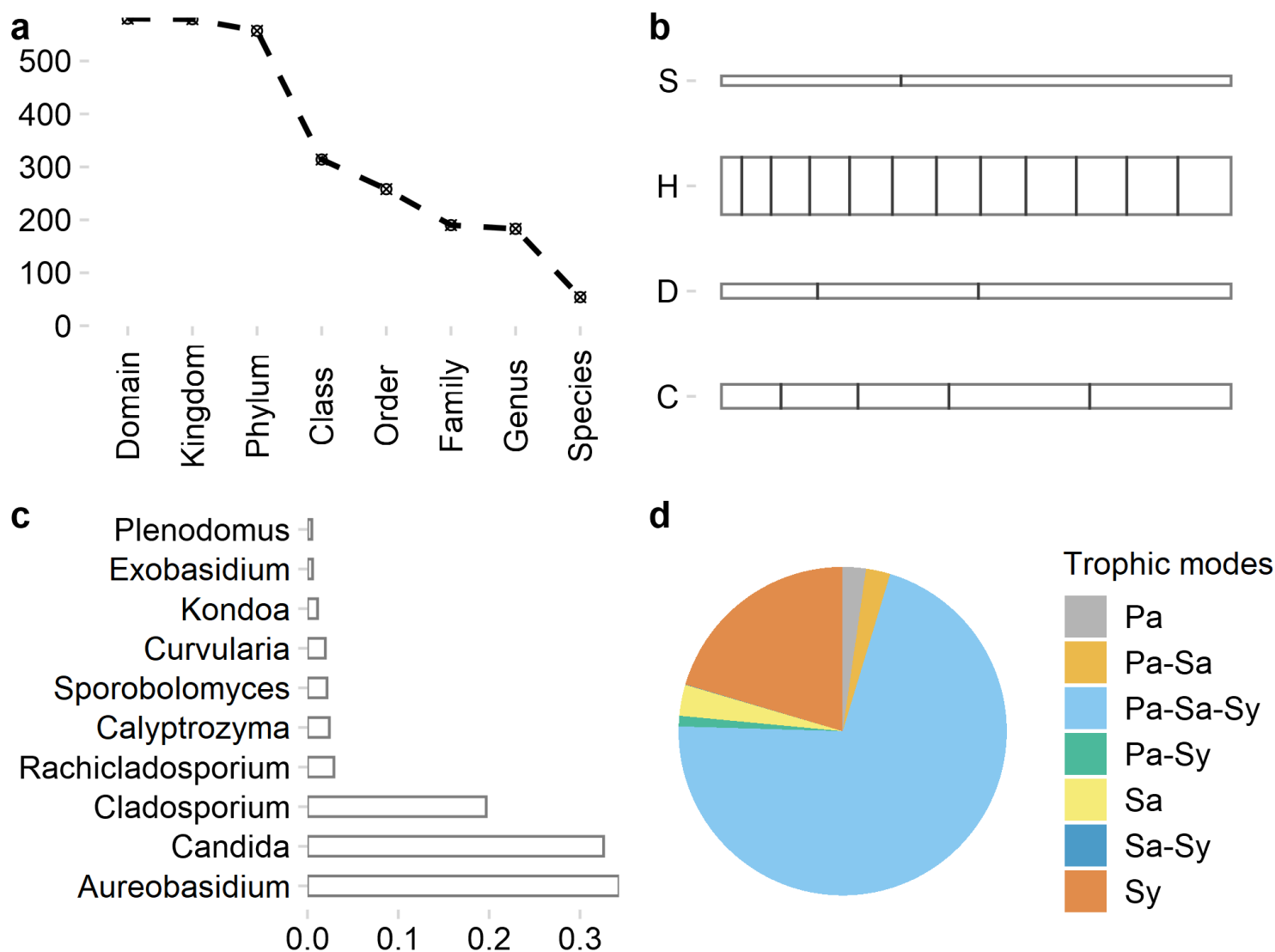


Figure 5

Taxonomic classification and trophic mode of the mycobiome in aspen leaves. **a)** The determination of success as the number of ASVs that could be determined to taxonomic level as indicated on the x-axis. **b)** Each bar represents one of the four most abundant orders S = Saccharomycetales, H = Helotiales, D = Dothideales, and C = Capnodiales in the database with bar height relative to the abundance of the order in the database. The segments indicate the genera richness of sizes relative to their abundance. **c)** The relative abundance of the ten most abundant genera was determined after their relative abundance in the database. **d)** The representation of trophic modes was assessed for determined ASVs using FUNGuild software [49]. P = pathotrophs; PaSa = patho-saprotrophs; PaSaSy = patho-sapro-symbiotrophs; PaSy = Patho-symbiotrophs; Sa = saprotrophs; Sy = Symbiotrophs.

Supplementary Files

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