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Blessing Alabi

`blessing.durodola@forpath.uni-freiburg.de`

University of Freiburg

Abiodun Abeebe Azeez

University of Helsinki

Emma Lührs

University of Freiburg

Frederik Loup Siebert

University of Freiburg

Kathrin Blumenstein

University of Freiburg

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Beyond pathogen enrichment: restructuring of the foliar mycobiome in declining giant sequoia

Blessing Alabi^{1*}, Abiodun Abeeb Azeez², Emma Lührs¹, Frederik Loup Siebert¹, Kathrin Blumenstein¹

¹Chair of Pathology of Trees, Institute of Forestry, Faculty of Environment and Natural Resources, University of Freiburg, Bertoldstr. 17, 79098 Freiburg, Germany

²Department of Forest Sciences, University of Helsinki, Latokartanonkaari 5, P. O. Box 27, 00014, Helsinki.

Corresponding author: Blessing Alabi

E-mail addresses of all authors:

Blessing Alabi: blessing.durodola@forpath.uni-freiburg.de

Abiodun Abeeb Azeez: abeeb.azeez@helsinki.fi

Emma Lührs: emma.luehrs@gmail.com

Frederik Siebert: loup.siebert@posteo.de

Kathrin Blumenstein: Kathrin.Blumenstein@forpath.uni-freiburg.de

Authors ORCID IDs:

Blessing Alabi: 0000-0002-1128-2440

Kathrin Blumenstein: 0000-0002-0677-7799

Abstract

Background

Sequoiadendron giganteum is increasingly affected by decline symptoms caused by the fungal disease *Botryosphaeria* tip blight and associated fungal pathogens. However, very little is known about the contribution of fungal communities, as well as their associations, to disease occurrence. Here, we present the first characterisation of the foliar fungal mycobiome of giant sequoia using high-throughput amplicon sequencing, integrated with culture-based isolation methods to examine how fungal community composition, diversity, and interaction patterns relate to tree health status.

Results

Fungal phyla consisted of Ascomycota (75.1%), Basidiomycota (24.2%), Mucoromycota (0.4%), Zoopagomycota (0.1%) and unidentified (0.2%). These phyla differed significantly between symptomatic and asymptomatic trees (PERMANOVA: $R^2 = 0.158$, $p = 0.001$). Metabarcoding analysis showed significant enrichment of pathogenic genera, most notably *Botryosphaeria* ($\log_2FC = 7.82$), in symptomatic trees. Additionally, *Peltaster* was significantly more abundant in asymptomatic trees and occurred at very low abundance in symptomatic samples. Using the classical isolation method, *Neofusicoccum* was recovered exclusively from symptomatic trees. Co-occurrence network analysis showed that asymptomatic trees formed a single integrated network with greater overall connectivity, whereas symptomatic trees were fragmented into four modules. Pathogenic genera such as *Botryosphaeria* showed contrasting association patterns between asymptomatic and symptomatic trees, suggesting a restructuring of fungal association patterns as host health declines.

Conclusion

Collectively, these findings indicate that giant sequoia decline is associated with broader changes in mycobiome organisation rather than the dominance of a single pathogen, providing a baseline for future studies of fungal community dynamics and disease monitoring in this species across its introduced European range. Key pathogens, including *Botryosphaeria*, showed contrasting co-occurrence patterns in asymptomatic versus symptomatic trees, suggesting a restructuring of fungal interactions associated with disease. These patterns indicate that the declining health of giant sequoia reflects a broader restructuring of mycobiome organisation rather than a single dominant pathogen, providing a baseline for future studies on fungal community dynamics and disease monitoring in this species across its European introduced range.

Keywords: *Sequoiadendron giganteum*; Botryosphaeriaceae; culture-dependent isolation; co-occurrence networks; ITS metabarcoding; fungal community; pathobiome; plant-microbe interactions

Background

Trees harbour a hidden community: a complex composition of fungi that influences their growth, defence, stress tolerance, and disease dynamics. Together, these fungi form the mycobiome, an integral component that can shape tree performance and support tree health and resilience in a changing environment [1, 2]. The relationship between biodiversity and ecosystem functioning has become a major focus in ecology [3, 4]. In particular, the 'insurance hypothesis' postulates that diverse communities are better able to maintain ecosystem functioning when conditions fluctuate, because species differ in how they respond to environmental change [5, 6]. Extending this idea to host-microbe systems, diverse fungal communities and functions may collectively enhance host defence and stability through complementary interactions, nutrient exchange, and the competitive antagonism of pathogens [7–9].

Sequoiadendron giganteum [Lindl.] J. Buchholz, commonly known as the giant sequoia, is among the tallest and longest-lived trees on Earth, with individuals exceeding 90 m in height and surviving for more than 3,000 years [10, 11]. Native to the western slopes of California's Sierra Nevada, the species grows under a climate characterised by dry summers, mild winters, deep, moisture-retaining soils, and annual precipitation of about 900–1400 mm, with most of it as snow [11, 12]. *S. giganteum* possesses several traits associated with environmental resilience, including thick bark and decay-resistant heartwood [10, 11, 13]. These features have made this tree species a symbol of ecological resilience, yet its long-term persistence may depend on stable environmental conditions that are now shifting in its introduced range.

Since its introduction to Europe in the mid-nineteenth century, *S. giganteum* has been widely cultivated in parks, arboreta, and urban landscapes, where it grows under different climatic conditions that differ substantially from those of its native range [14]. In Central Europe, *S.*

giganteum is increasingly exposed to drought and heat stress episodes, with temperatures rising by more than 1°C over recent decades [15]. In Freiburg, Germany, where this study was conducted, mean annual temperatures have risen by approximately 0.8°C since the 1960s, accompanied by a growing frequency of extreme heat and drought events in recent years [16, 17]. Similar warming trends across Central Europe have been associated with soil moisture deficits and physiological stress in many tree species [18]. Such climate changes may compromise host vitality and disrupt plant-fungal interactions, potentially altering the foliar fungal community structure. During the 2012-2016 California drought, sublethal responses including foliage dieback, canopy thinning, and reduced canopy water content were observed in *S. giganteum* despite low mortality [19, 20]. Similar stress conditions in Europe may weaken trees and alter mycobiome composition, potentially increasing susceptibility to opportunistic fungi [21].

Trees are increasingly viewed as holobionts, (comprising the host and its associated microbial communities, including fungi, bacteria, archaea, and other microorganisms) in which host performance depends on stable interactions with the associated microbiota [2, 22]. When this balance breaks down, microbial community structure can shift, opportunistic pathogens may increase, and the holobiont can move toward a disease-associated state of dysbiosis. This process can be triggered by environmental stress, leading to reduced microbial stability and increased abundance of opportunistic taxa [22, 23].

Evidence from conifer pathosystems suggests that environmental stress can trigger precisely such changes in mycobiome structure, with consequences for pathogen resistance, as shown in Norway spruce where drought-altered fungal communities were linked to susceptibility to *Heterobasidion parviporum* [24]. Environmental stress can also alter individual host-fungus interactions, particularly among members of the Botryosphaeriaceae. Many species in this

family can persist asymptotically as endophytes or latent pathogens but cause disease following physiological stress or weakening of their hosts [25, 26].

Declining vitality and disease symptoms have become increasingly evident in European *S. giganteum* populations, with shoot dieback, branch cankers, sapwood discolouration, and crown dieback reported across several European countries. *Botryosphaeria* tip blight, caused by *B. dothidea*, is associated with shoot and branch dieback in *S. giganteum* [27, 28]. However, *B. dothidea* is not the only potentially pathogenic fungus reported from symptomatic trees. Fungal taxa isolated from symptomatic tissues include *B. dothidea*, *Neofusicoccum parvum*, *N. yunnanense*, *Amycosphaerella africana*, *Diaporthe rudis*, *D. nobilis*, and *D. eres* across Austria, Bulgaria, Croatia, Germany, and Switzerland [27–32]. Whether the occurrence of these fungi is accompanied by broader changes in the foliar mycobiome of declining *S. giganteum*, particularly in its introduced European range, remains unknown.

To date, studies investigating the microbiome of *S. giganteum* remain limited, with most focusing on bacterial endophytes or soil-associated fungi [33, 34]. To our knowledge, no study has comprehensively characterised the foliar fungal mycobiome of *S. giganteum* using high-throughput sequencing, particularly in relation to host health status. Culture-based isolation remains fundamental for understanding tree-associated fungi as it enables direct identification and functional testing of isolates. However, this approach captures only part of the true diversity, as many fungi remain dormant or unculturable under standard laboratory conditions [35]. Moreover, culture-dependent and molecular approaches often capture different components of fungal diversity. Culturing enables the recovery and functional characterisation of viable taxa, while metabarcoding provides broader detection of abundant and uncultivable fungi [35–37]. Combining these approaches therefore offers a comprehensive assessment of

132 fungal community structure and composition, linking the cultivable fraction to its wider
133 ecological context [38, 39].

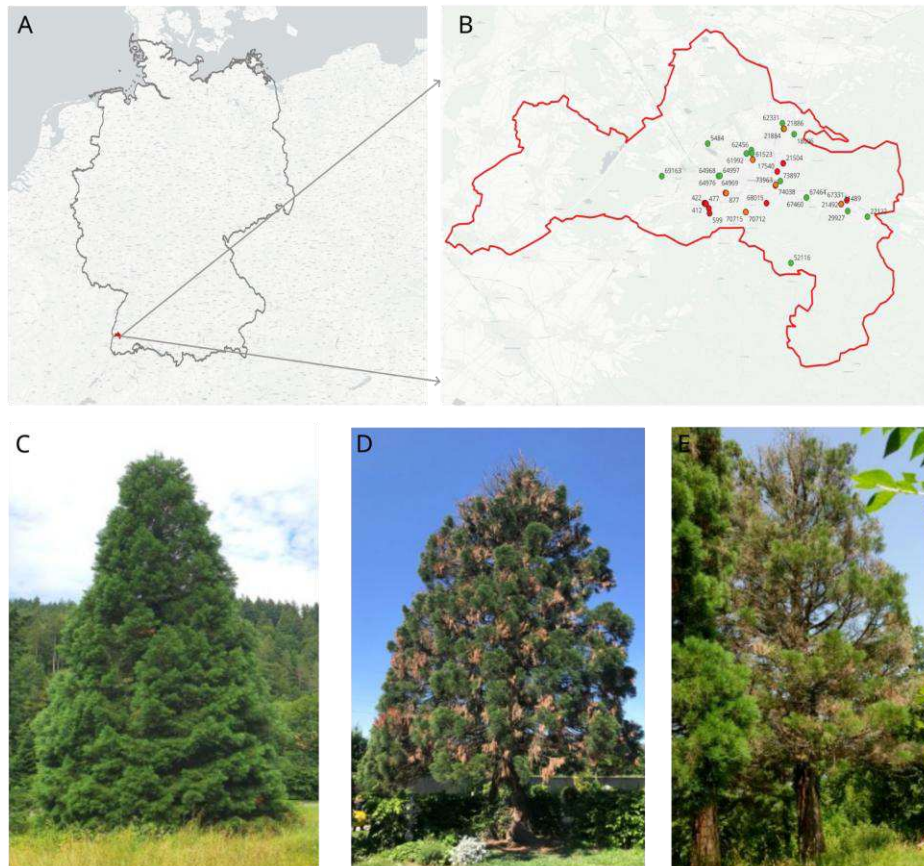
134 In this study, we examine the needle and twig-associated mycobiome of asymptomatic and
135 symptomatic *S. giganteum* trees. Using both culture-dependent isolation and ITS-based
136 metabarcoding, we specifically aimed to (i) characterise the fungal diversity and community
137 composition of *S. giganteum*, (ii) compare communities between asymptomatic and
138 symptomatic trees, (iii) assess the overlap between culturable and sequencing-detected taxa,
139 and (iv) explore patterns of fungal co-occurrence, with particular focus on the key
140 Botryosphaeriaceae pathogens *B. dothidea* and *N. parvum*, and their associations with
141 mycobiome structure and host health status. We hypothesised that the diversity and
142 composition of the fungal community would differ between symptomatic and asymptomatic
143 trees, reflecting differences in host-microbe interactions associated with host health status. We
144 further hypothesised that symptomatic trees would harbour a co-occurring assemblage of
145 potentially pathogenic fungi, consistent with a pathobiome.

Methods

Sampling of plant material

Sampling and vitality data of 209 *S. giganteum* trees used for this study were provided by the Tree Protection and Care department of the city of Freiburg. These trees were classified into five vitality categories ranging from 0 to 4. Vitality class 0 (VC0) represents healthy to slightly damaged trees; VC1, slightly to moderately damaged; VC2, moderately to severely damaged; VC3, severely to very severely damaged; and VC4, very severely damaged or dying trees. Additionally, we sampled 40 trees from various health classes across Freiburg, Germany (Fig. 1A, B). According to the municipality's inventory, the trees included two trees in vitality class 4 (VC4). However, during sampling, we found that one of these trees had already been removed, likely because it was dead. Consequently, the only tree in VC4 was combined with the trees in VC3 for statistical analysis. Therefore, the final dataset comprised 20 trees in VC0 (Fig. 1C), 8 in VC2 (Fig. 1D), and 12 in VC3 (Fig. 1E), resulting in 20 asymptomatic (VC0) and 20 symptomatic (VC2-VC3) trees. VC1 trees were not sampled, as the selected classes sufficiently captured the full range of health states.

From each tree, needles and twigs were collected at different heights and from three cardinal directions using telescopic scissors (FISKARS UP86) to ensure representative sampling within each tree. The samples were divided into two groups: one half was immediately stored in sterile tubes on ice and subsequently kept at -20°C until further processing (metabarcoding). The other half was stored in labelled paper bags and processed in the lab the same day, or refrigerated overnight and processed the following day using the classical isolation method.



167

168 Fig. 1 **A)** Location of the study area (Freiburg, Germany) **B)** Sampling locations of *S.*
 169 *giganteum* within the Freiburg region **C)** Sampled trees with vitality class 0 (VC0) - healthy to
 170 slightly damaged **D)** Vitality class 2 (VC2) - moderately to severely damaged trees **E)** Vitality
 171 class 3 (VC3) - severely to very severely damaged.

172 *DNA Extraction and Sequencing*

173 Samples collected from each tree were cut into smaller pieces and combined to ensure thorough
 174 mixing of the three subsamples per tree. The pooled material was first manually ground using
 175 a sterile mortar and pestle to obtain a homogeneous mixture. Subsequently, 50 mg of the
 176 homogenised material was transferred to lysis matrix tubes (Lysing Matrix A, MP Biomedicals,
 177 USA) and mechanically disrupted for 4 min at 4 M/S using a bead-beating homogeniser (MP
 178 Biomedicals FastPrep 24, USA). DNA was then extracted according to the manufacturer's

instructions using the Macherey-Nagel NucleoSpin Plant II kit. Amplicon-based high-throughput paired-end sequencing (2×250 bp) of the fungal internal transcribed spacer 2 (ITS2) region was performed for the 40 *S. giganteum* samples using the Illumina PE250 platform, generating approximately 50,000 paired-end reads per sample. The ITS2 region was amplified using the primer pair 5'-GCATCGATGAAGAACGCAGC-3' and 5'-TCCTCCGCTTATTGATATGC-3'. PCR and sequencing were carried out by Novogene GmbH (Planegg-Martinsried, Munich, Germany).

Raw data and bioinformatic processing

Analysis of mycobiome composition and diversity was carried out using QIIME2 version 2024.10 [40]. The paired-end sequences and the corresponding manifest file were imported into QIIME2. Forward reads showed higher quality scores than the reverse reads. To retain high-quality sequence data, forward and reverse reads were trimmed and truncated to 200 and 180 base pairs, respectively. The paired reads were denoised and merged using the DADA2 pipeline to produce the amplicon sequence variants (ASVs). The taxonomic assignment was performed against the UNITE fungal reference database (v10) [41], with species hypotheses defined based on a 97% sequence similarity threshold.

To cross-validate UNITE assignments, representative ASV sequences were additionally queried against the NCBI core nucleotide (core_nt) database (version 2.16.0, 25 June, 2024) using BLASTn, retaining the top 10 hits per query ranked by bitscore. The taxonomic classification was assigned by the majority rule among the top 10 hits. Taxonomy was confidently assigned where both NCBI and UNITE provided the same classification. Discrepancies between database assignments were resolved by assigning taxonomy at the most specific rank at which both databases agreed, for example, at genus level when species-level assignments conflicted. Where only one database provided a classification, that assignment

was used. Taxa that could not be classified by either database were labelled 'unassigned'. Non-fungal sequences were removed, and only fungal sequences were retained for downstream analysis.

Culture-based fungal isolation and molecular identification

Fungal isolation was performed from the transition zone between the asymptomatic and symptomatic needle or twig tissues. Plant materials were surface-sterilised by sequential immersion in 70% ethanol for 45 sec, 5% sodium hypochlorite (NaOCl) for 3 min, and 70% ethanol for 20 sec, followed by two rinses in sterile autoclaved deionised water for 15 sec each. Sterilised tissues were air-dried on sterile filter paper under laminar airflow. Needles and twigs were then cut into small segments, and three segments per sample were placed onto malt-yeast-peptone agar (MYP) plates. Plates were incubated at room temperature until fungal growth was visible. Emerging colonies were subcultured by transferring individual hyphal tips into fresh MYP plates to obtain pure cultures. Isolates were initially grouped based on macroscopic and microscopic morphological characteristics and compared with reference cultures from an in-house fungal culture database.

The genomic DNA was additionally extracted for molecular identification. Fungal mycelium was scraped from actively growing cultures using a sterile scalpel and transferred into 1.5 mL microcentrifuge tubes. A 500 µL polyvinylpyrrolidone (PVP) extraction buffer was added to each tube, and samples were incubated at 65 °C for 15 min in a heat block with continuous mixing at 800 revolutions per minute (rpm). Following incubation, samples were vortexed for 20 s, and the cells were ultrasonically disrupted using two 2-s pulses with an ultrasonic homogeniser. Samples were incubated again at 65 °C for an additional 15 min (without mixing), and then vortexed for 20 s. The lysates were centrifuged at 5000 rpm for 10 min, after which 250 µL of the supernatant was carefully transferred to new 1.5 mL microcentrifuge

tubes. An equal volume (250 μ L) of pre-warmed sodium dodecyl sulfate (SDS) wash buffer was added, and the samples were mixed by vortexing for 20 s and centrifuged at 13,300 rpm for 15 min. After centrifugation, 300 μ L of the supernatant was transferred to fresh tubes and mixed with 255 μ L isopropanol to precipitate DNA. The tubes were gently inverted 20 times to ensure mixing before centrifugation at 13,300 rpm for 15 min. The resulting supernatant was discarded, and the DNA pellet was washed with 200 μ L of cold 70 % ethanol. After centrifugation at 13,300 rpm for 10 min, the ethanol was removed, and the pellets were dried on a heat block at 37 °C with open lids for 15 min. The DNA was resuspended in 50 μ L of nuclease-free water.

The ITS and LSU regions of the DNA were amplified using the corresponding primer pairs: ITSu1 (5'-GGA AGK ARA AGT CGT AAC AAG G-3') and ITSu4 (5'-RGT TTC TTT TCC TCC GCT TA-3'); LSU (5'-GAC CCG AAA GAT GGT GAA-3') and LR6 (5'-CGC CAG TTC TGC TTA CC-3'). The PCR was carried out using 7.5 μ L of DNA, 2.5 μ L of each primer pair (forward and reverse), and 12.5 μ L of FastGene Optima PCR HotStart ReadyMix Master Mix. PCR success was assessed using gel electrophoresis, and the PCR products were purified using the HighPrep PCR clean-up protocol (MagBio Genomics, Inc., USA). Sequencing was performed by the Microsynth Sequencing laboratory in Göttingen, Germany, and the obtained sequences were compared against reference sequences in the NCBI database using BLAST searches to assign taxonomic classification.

Statistical and network analyses

Amplicon sequence variants (ASVs) with five or fewer reads were filtered out, and the resulting data were used for downstream analysis using RStudio [42]. The QIIME2 feature table and taxonomy files (.qza) were imported into R and analysed together with the associated metadata. The ASV table, taxonomy table and sample metadata were processed into a phyloseq object

using the phyloseq package [43]. To standardise comparisons across all samples and mitigate biases introduced by varying sampling depths, read counts were normalised by the Scaling with Ranked Subsampling (SRS) method [44, 45]. Normalisation was performed with the minimum number of reads (Cmin; 45,242), retaining all samples in the analysis. The SRS curve was observed to determine whether the Cmin value was sufficient to capture species richness. SRS-normalised counts were used for relative abundance and diversity analyses (alpha and beta diversity), while raw counts were used for differential abundance testing and co-occurrence network analyses. For the relative abundance analysis, only the top 15 taxa and those identifiable at the genus level between samples were prioritised.

Alpha diversity was quantified using Observed richness, Shannon, and Simpson indices and compared between asymptomatic and symptomatic samples (n = 20 per group). Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test. In both sample categories, Shannon diversity met normality assumptions and showed no evidence of heteroscedasticity; therefore, differences were assessed using an independent two-sample t-test assuming equal variances ($\alpha = 0.05$). Observed richness and Simpson diversity deviated from normality in at least one group and were consequently compared using the Wilcoxon rank-sum test. P-values were adjusted using the Benjamini–Hochberg false discovery rate, and both raw p-values and adjusted q-values were reported. Data visualisation was performed using the ggplot2 package [46].

The ASV counts were transformed into relative abundances (per-sample proportions), and Bray-Curtis dissimilarities were computed using the phyloseq package. PCoA was used to visualise the Bray-Curtis dissimilarity. Community composition differences were assessed by PERMANOVA (999 permutations) using the adonis2 function in the vegan package [47]. The two explanatory factors investigated, i.e. health status (asymptomatic vs symptomatic) and

275 Vitality classes (VC0, VC2, VC3), were evaluated independently. For the Vitality class,
276 pairwise PERMANOVA tests were additionally performed between levels, with p-values
277 adjusted using the Benjamini-Hochberg (BH) correction across the three pairwise comparisons.
278 Multivariate dispersion was assessed using betadisper and permutest to check the homogeneity
279 of within-group distances.

280 To visualise genus-level intersections between culture-based isolation and metabarcoding, an
281 UpSet plot was created using the UpSetR package [48]. Data were stratified by health status
282 (asymptomatic vs symptomatic), resulting in four sets: culture-derived genera from
283 asymptomatic trees, culture-derived genera from symptomatic trees, and their metabarcoding
284 equivalents. Further comparisons were based on the presence or absence of genus-level species.
285 To avoid inflating apparent diversity with rare or unreliable detections, only genera recorded
286 in at least two trees within each health status group were considered.

287 Differential abundance analysis was performed using DESeq2 [49], and counts were modelled
288 using a generalised negative binomial linear model. Before analysis, low-prevalence ASVs
289 were filtered from the count table. Statistical significance was assessed using Wald tests, and
290 the p-values were adjusted using the Benjamini-Hochberg FDR. In addition to differential
291 abundance tests based on DESeq2, Linear Discriminant Analysis Effect Size (LEfSe) [50] was
292 applied to identify taxa with strong discriminatory power between health status groups.
293 Significance was determined using the Kruskal-Wallis test ($p < 0.01$), followed by linear
294 discriminant analysis (LDA) to estimate the effect size. Only taxa with LDA scores ≥ 4.0 and
295 $p < 0.01$ were considered potential biomarkers. Fungal taxa were functionally annotated using
296 the FUNGuild database implemented in R through the FUNGuildR package, based on ITS-
297 derived taxonomic assignments [51]. The relative abundances of the trophic modes and
298 functional guilds were calculated for each sample. The differences in guild abundances

299 between asymptomatic and symptomatic samples were assessed using the Wilcoxon rank-sum
300 test, with statistical significance set at $p < 0.05$.

301 Conditional dependence networks were inferred for the top 80 taxa at the genus level using
302 SPRING (NetCoMi v1.1.0) [52, 53]. For network construction, samples with fewer than 1,000
303 total reads were excluded. Network sparsification was performed using an association
304 magnitude cutoff of $|\text{weight}| \geq 0.3$. Network properties, including density, clustering,
305 modularity, and connectivity, were calculated using netAnalyze. For each health group, node
306 degree, closeness, betweenness, and eigenvector centrality were computed. Network properties
307 were compared between asymptomatic and symptomatic groups using netCompare with 1,000
308 permutations, and Jaccard indices were used to quantify overlap in sets of most central taxa
309 [53]. Network visualisation was performed using Gephi [54].

Results

Demultiplexing resulted in 4,191,059 paired-end reads across 40 samples. After processing, 5,295 unique amplicon sequence variants (ASVs), comprising 3,020,453 quality-filtered reads, were identified in all samples. The minimum sequencing depth per sample was 45,242 reads, and after normalisation, 1,859,280 reads (representing 62% of all reads) were retained across all samples. After removing low-abundance ASVs (≤ 5 reads) and non-fungal sequences, 942 ASVs remained and were used for downstream analyses. Ascomycota accounted for 75.1% of the fungal sequences, Basidiomycota for 24.2%, Mucoromycota for 0.4%, Zoopagomycota for 0.1% and unidentified phyla for 0.2%.

Relative abundance of taxa in symptomatic and asymptomatic samples (metabarcoding and culture)

Metabarcoding

Community composition varied between samples and health-status groups (Fig. 2). Asymptomatic samples were dominated by *Peltaster*, which occurred at very low abundance in symptomatic samples. In contrast, *Botryosphaeria* and *Neofusicoccum* showed higher relative abundance in symptomatic samples.

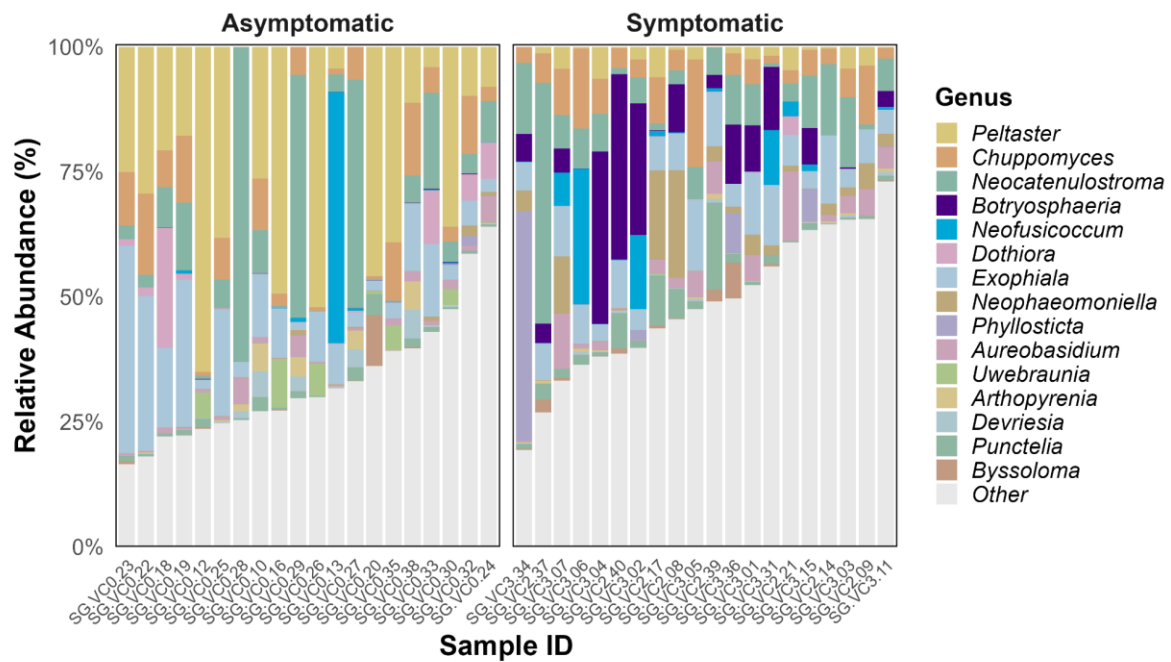


Fig. 2. Relative abundance of the 15 most abundant fungal genera in asymptomatic (n = 20) and symptomatic (n = 20) samples of *S. giganteum* based on ITS2 metabarcoding. Each bar represents an individual sample. Genera not included among the top 15 by mean relative abundance across all samples were grouped as Other.

Culture

Classical culture-based isolation revealed that *Diaporthe* species are the most frequently recovered, appearing in the highest number of trees in both health groups. *Botryosphaeria*, *Sordaria*, *Alternaria*, and *Sydowia* were more commonly isolated from symptomatic trees than from asymptomatic ones. In contrast, *Neofusicoccum*, *Epicoccum*, *Plenodomus*, *Pestalotiopsis*, and *Microspheopsis* were isolated exclusively from symptomatic trees (Fig. 3).

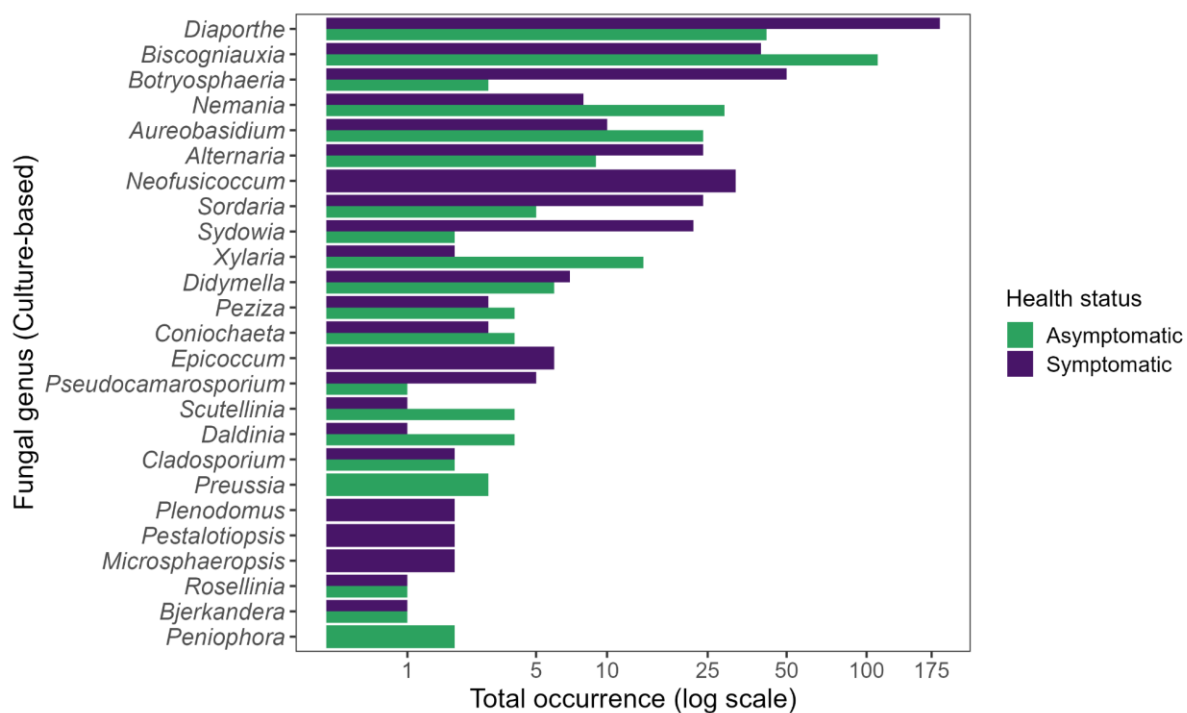


Fig. 3. The total number of isolates of each fungal species recovered from asymptomatic (n = 20) and symptomatic (n = 20) *S. giganteum* trees using culture-based isolation.

Culture versus Metabarcoding

Using a prevalence threshold requiring genera to be detected in at least two trees, metabarcoding detected 237 genera in symptomatic trees compared to 19 detected by culture-based methods. Of these, 13 genera were shared between both approaches, whereas 224 and six genera were exclusive to metabarcoding and culture respectively (Fig. 4a). In asymptomatic trees, metabarcoding detected 306 genera compared to 17 by culture, with 11 shared genera and 295 and six genera exclusive to metabarcoding and culture, respectively (Fig. 4b). In particular, irrespective of the sequencing method, the following 13 genera were consistently identified in the symptomatic trees across both approaches: *Alternaria*, *Aureobasidium*, *Biscogniauxia*, *Botryosphaeria*, *Cladosporium*, *Diaporthe*, *Didymella*, *Epicoccum*, *Microsphaeropsis*, *Neofusicoccum*, *Pestalotiopsis*, *Plenodomus*, *Pseudocamarosporium*.

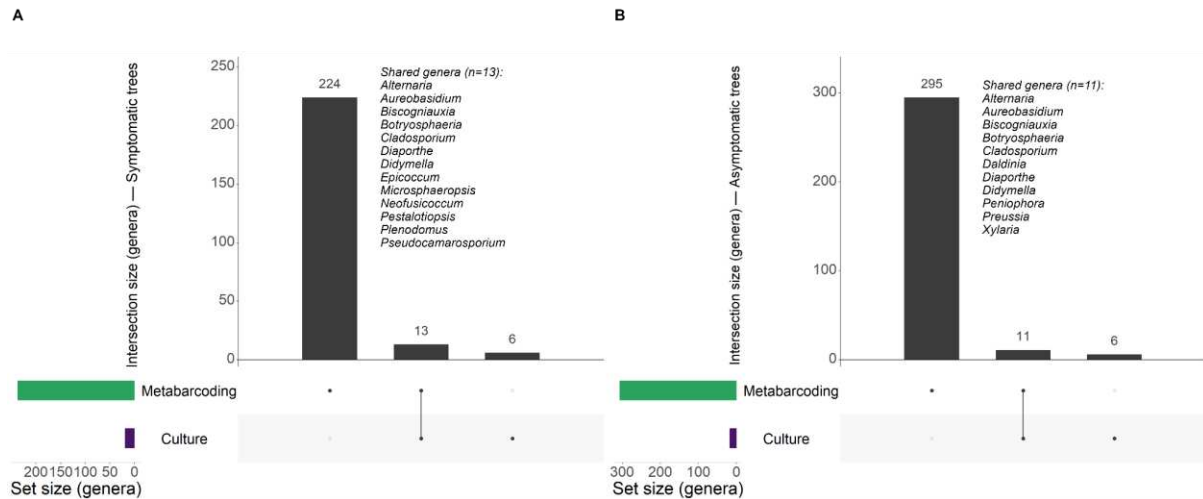


Fig. 4. Unique and shared genera between metabarcoding and culture-based isolation in (A) symptomatic (n = 20) and (B) asymptomatic samples (n = 20) of *Sequoiadendron giganteum* trees, after applying a prevalence filter of genera detected in at least two trees per group.

Diversity and functional analyses

Alpha Diversity assessment

Alpha diversity analysis showed no significant difference between health status groups following Benjamini-Hochberg correction across all diversity indices ($q \geq 0.119$). Shannon diversity showed a nominal difference (t-test: $p = 0.048$), with higher values in the symptomatic group (mean = 3.14) than in the asymptomatic group (mean = 2.94) (Additional file 1: Fig. S1); however, this effect was not significant after correction ($q = 0.119$). Observed richness was higher in asymptomatic trees (mean = 182) than in symptomatic trees (mean = 163), although the difference was not statistically significant (Wilcoxon rank-sum test: $p = 0.081$; $q = 0.119$). Simpson diversity also indicated no statistically significant difference between the groups (Wilcoxon rank-sum test: $p = 0.140$; $q = 0.140$).

Beta Diversity assessment

Health status explained 15.8% of the variation in fungal community composition (PERMANOVA: $R^2 = 0.158$, $F = 7.13$, $p = 0.001$; Additional file 1: Table S1), with homogeneous within-group dispersion (betadisper: $F = 0.019$, $p = 0.892$). Similarly, Vitality class accounted for 17.8% of the variation (PERMANOVA: $R^2 = 0.178$, $F = 4.01$, $p = 0.001$; Additional file 1: Table S1). Pairwise comparisons revealed that VC0 differed significantly from both VC2 and VC3 ($p = 0.001$; q (BH) = 0.002), whereas VC2 and VC3 exhibited no significant difference ($p = 0.525$; q (BH) = 0.525) (Additional file 1: Table S2; Fig. 5). Dispersion was homogeneous across vitality classes (betadisper: $F = 0.135$, $p = 0.869$), indicating that compositional differences were not driven by unequal within-group variability.

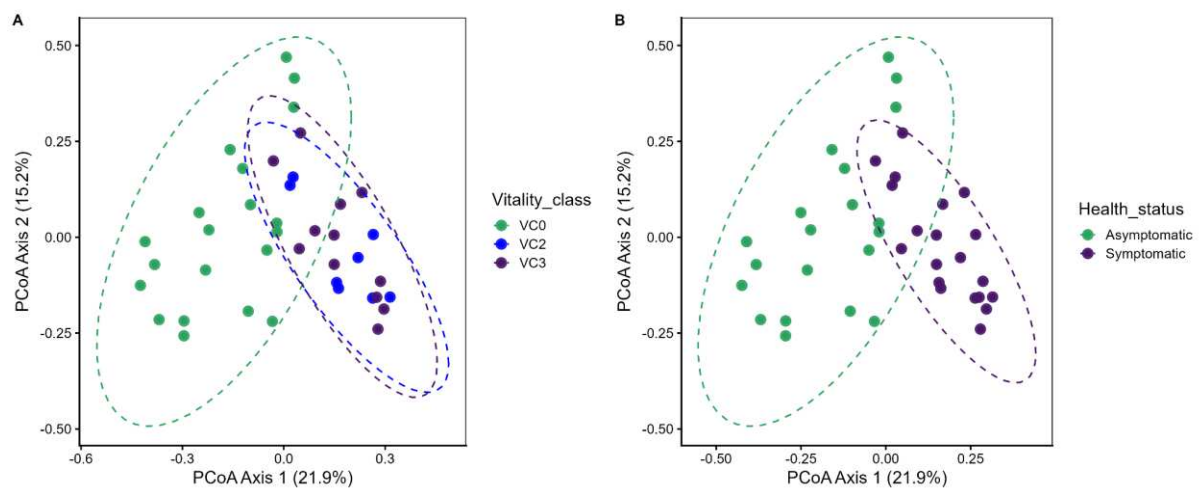


Fig. 5. Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarities. Points represent samples, and ellipses show 95% confidence intervals. (A) Samples colored by vitality class (VC0, VC2, VC3). (B) Samples colored by health status (asymptomatic, symptomatic). Axis labels indicate the percentage of variance explained.

Differential Abundance

Analysis of the differential abundance of identified taxa using DESeq2 revealed 62 genera that were significantly differentially abundant (adjusted $p < 0.05$), of which 32 were enriched in symptomatic and 30 in asymptomatic samples. The top 40 genera are shown in Fig. 6. The strongest enrichment in symptomatic samples was observed for *Botryosphaeria* ($\log_2FC = 7.82$, $padj < 0.001$), *Phyllosticta* ($\log_2FC = 5.88$, $padj = 0.006$) and *Bannoa* ($\log_2FC = 5.81$, $padj < 0.001$), with *Cytospora*, *Diaporthe*, and *Neofusicoccum* also identified among the significantly enriched. In asymptomatic samples, *Uwebraunia* ($\log_2FC = -9.67$, $padj < 0.001$), *Dissoconium* ($\log_2FC = -6.45$, $padj < 0.001$), and *Naevula* ($\log_2FC = -6.41$, $padj < 0.001$) showed the strongest enrichment. *Heterobasidion*, *Peltaster*, and *Peniophora* were also significantly enriched.

The LEfSe analysis ($LDA \geq 4.0$; $p = 0.01$; Additional file 1: Fig. S2) revealed 30 fungal taxa significantly enriched in asymptomatic samples, notably *Dothiora*, *Peltaster cerophilus*, and *Taphrina*, together with several unclassified taxa. In contrast, 22 fungal taxa were significantly associated with symptomatic samples, including *Botryosphaeria dothidea*, *Cytospora*, *Diaporthe*, *Trichomerium*, and Dothideales.

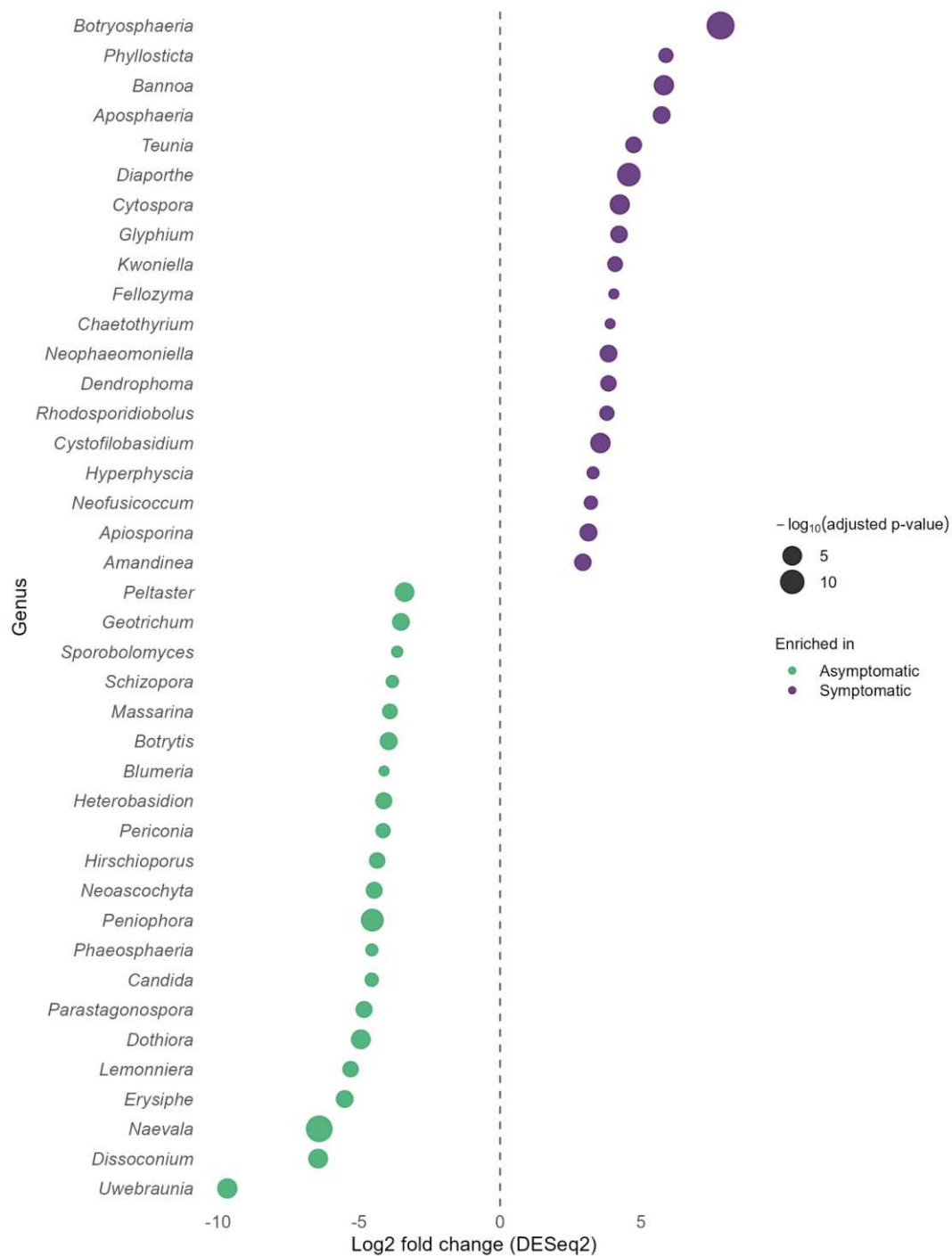


Fig. 6. Differentially abundant fungal taxa between symptomatic and asymptomatic samples identified by DESeq2. A positive $\log_2$ fold change indicates enrichment in symptomatic samples; negative values indicate enrichment in asymptomatic samples. The point size reflects statistical significance ($-\log_{10}$ adjusted p-value). The top 40 taxa with adjusted $p < 0.05$ are shown.

The functional guild analysis (Fig. 7) revealed pathotrophs and saprotrophs as the dominant abundant trophic modes, with no significant differences between asymptomatic and symptomatic samples ($p > 0.05$). In contrast, symbiotrophs and a few lower-abundance guilds, including fungal parasites and leaf saprotrophs, were significantly more abundant ($p < 0.05$) in symptomatic than in asymptomatic samples.

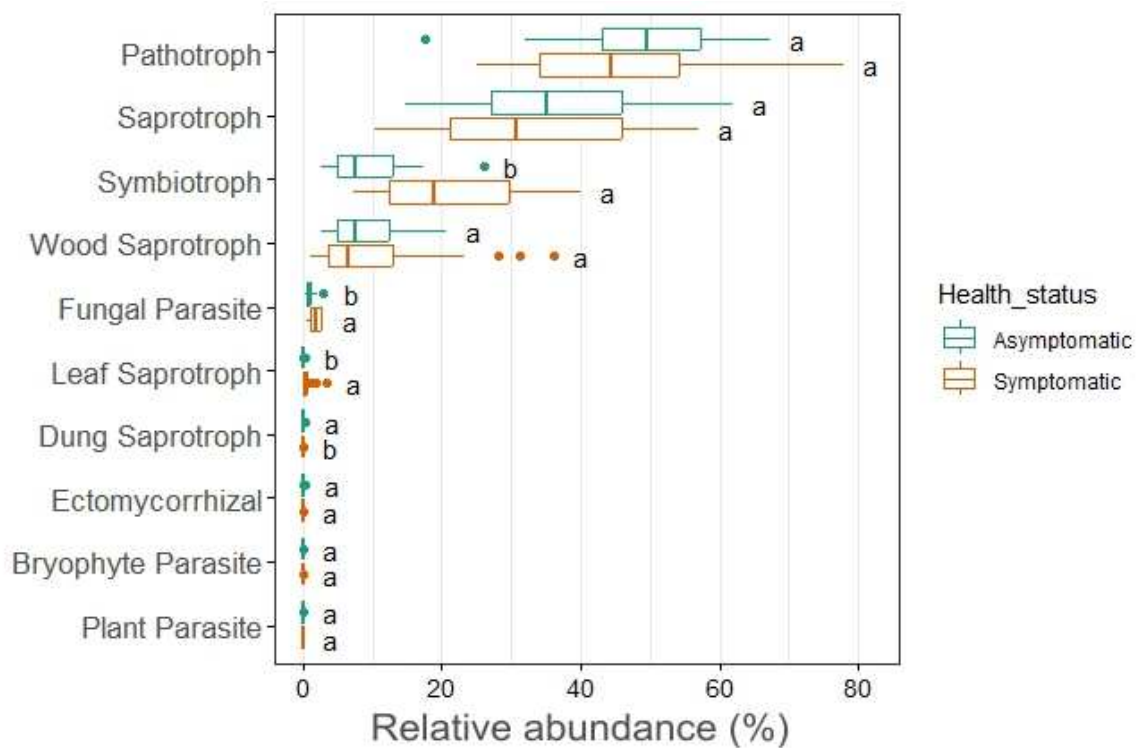
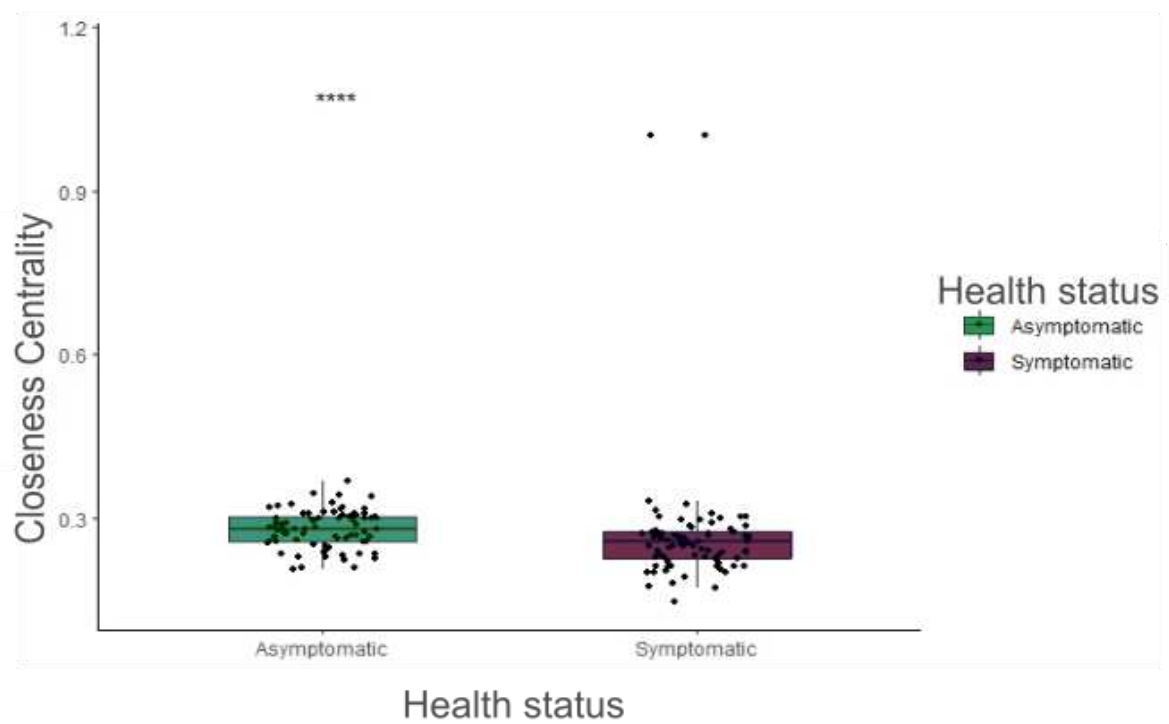


Fig. 7. Relative abundance of fungal functional guilds in asymptomatic ($n = 20$) and symptomatic ($n = 20$) *S. giganteum* samples based on FUNGuild annotation of ITS2 metabarcoding data. Different letters (a, b) indicate significant differences between groups (Wilcoxon rank-sum test, $p < 0.05$).

Co-occurrence network analysis

The SPRING networks contained 141 edges in the asymptomatic group and 126 edges in the symptomatic group. The asymptomatic network formed a single connected component,

427 whereas the symptomatic network was fragmented into four components (Additional file 1:
428 Table S3). Both networks were highly modular, with modularity values of 0.53 and 0.60 for
429 the asymptomatic and symptomatic networks, respectively (Additional file 1: Table S3). The
430 most central taxa differed between health groups. Overlap was significantly lower than
431 expected by chance for closeness centrality (Jaccard index = 0.143, $P(\leq J) = 0.009$; Fig. 8),
432 eigenvector centrality (Jaccard index = 0.143, $P(\leq J) = 0.009$), and hub taxa (Jaccard index =
433 0.000, $P(\leq J) = 0.039$). In contrast, overlap based on degree and betweenness centrality did not
434 deviate significantly from random expectation (Additional file 1: Table S3). The asymptomatic
435 network showed greater overall connectivity and less fragmentation than the symptomatic
436 network (Fig. 9A1, B1). *Neofusicoccum* showed positive associations with *Ramularia* and
437 *Meniscomyces* in symptomatic samples (Fig. 9B2), while in asymptomatic samples it was
438 positively associated with *Dendrophoma* and negatively with *Paracladophialophora* (Fig.
439 9A2). Similarly, *Botryosphaeria* was negatively associated with *Cytospora* in symptomatic
440 samples (Fig. 9B3), but positively associated with *Diplodia* and *Arthrocatena* in asymptomatic
441 samples (Fig. 9A3).



442

443 Fig. 8. Closeness centrality of fungal genera in asymptomatic and symptomatic networks. The

444 asterisks indicate statistically significant differences ($p < 0.05$).

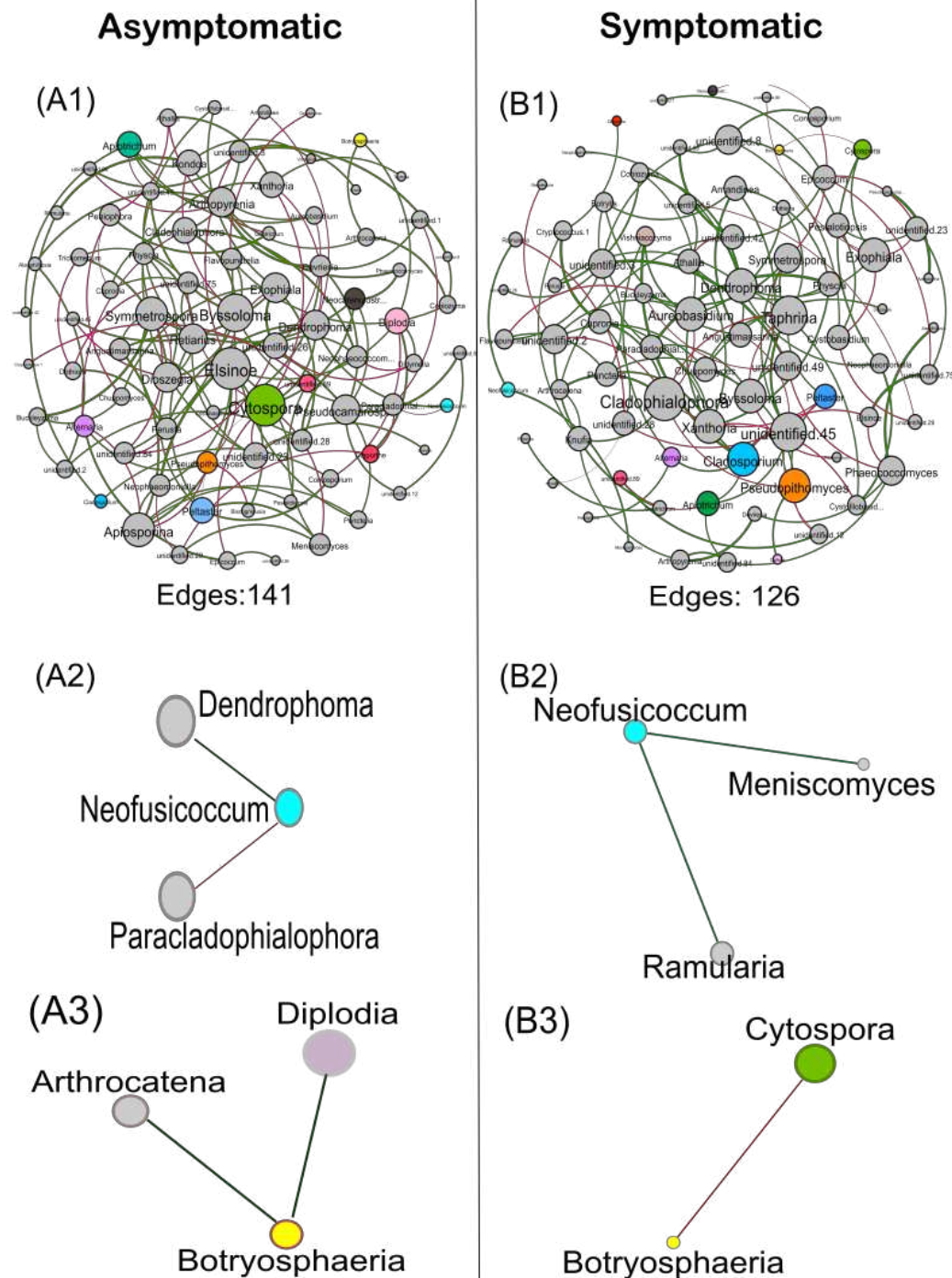


Fig. 9. Co-occurrence networks of the top 80 fungal genera in asymptomatic (A1–A3) and symptomatic (B1–B3) samples. The complete networks are shown in (A1) and (B1), with subnets highlighting the direct neighbourhood of *Neofusicoccum* (A2, B2) and *Botryosphaeria* (A3, B3). Nodes represent fungal genera, and edges indicate significant co-occurrence associations, with green and red edges representing positive and negative associations, respectively. Edge thickness corresponds to interaction strength.

Discussion

The fungal community structure differed significantly between asymptomatic and symptomatic *S. giganteum* trees, with symptomatic trees showing enrichment of several fungal taxa and altered co-occurrence patterns consistent with disease-associated mycobiome restructuring. To our knowledge, this study provides the first high-throughput sequencing-based characterisation of the foliar fungal mycobiome of giant sequoia. By integrating culture-dependent isolation and ITS metabarcoding, we characterised fungal communities associated with needles and twigs, identified taxa associated with contrasting health states, assessed the overlap between culturable and sequence-detected taxa, and explored patterns of fungal co-occurrence among key community members.

A central finding of this study is that declining tree health was associated with restructuring of the fungal community rather than the emergence of a single dominant pathogen. Similar disease-associated restructuring of host-associated microbial communities has been reported in other plant pathosystems, including citrus affected by huanglongbing [55] and oil palm leaf blight [56]. In our study, symptomatic trees differed from asymptomatic trees in community composition, taxonomic enrichment patterns, and network structure, collectively indicating that disease is accompanied by larger changes in the fungal assemblage. The enrichment of specific taxa, alongside shifts in community composition and association patterns, in symptomatic samples is consistent with a disruption of microbial equilibrium in which opportunistic or pathogenic microorganisms gain dominance over a previously stable assemblage [57, 58]. Such shifts are closely related to disease processes, through their influence on host susceptibility and pathogen establishment [59–61]. These observations align with the pathobiome concept, which proposes that disease results from alterations in the structure and function of entire microbial communities rather than solely from the activity of a single pathogen. More broadly, our findings support the view that plant health is shaped by dynamic

interactions among host traits, microbial communities, pathogens, and environmental conditions, with disease-associated mycobiome restructuring representing one component of a larger host-pathogen-microbe-environment network that ultimately determines disease outcomes [62].

Although observed richness showed a non-significant trend toward higher values in asymptomatic trees, alpha diversity indices did not consistently differentiate health groups. In contrast, beta-diversity analyses revealed significant differences in community composition associated with both health status and vitality class, suggesting that fungal assemblages respond to host decline along a continuum rather than through abrupt shifts. The absence of compositional differences between VC2 and VC3 further suggests that major mycobiome restructuring may occur during the transition from healthy to declining trees, after which community composition remains comparatively stable across later stages of decline. This pattern indicates that microbial community changes may accompany disease onset rather than accumulate progressively with increasing symptom severity. Similar observations have been reported in other plant pathosystems, where disease-associated microbial restructuring occurred despite limited changes in overall diversity metrics [63, 64]. Together, these patterns are consistent with early dysbiosis, in which fungal community composition reorganises around disease-associated taxa without an overall collapse in diversity, reflecting a gradual transition in microbial lifestyle rather than an abrupt community shift.

Differential abundance analyses further highlighted taxa associated with contrasting health states. Symptomatic trees were enriched in *Botryosphaeria*, *Neofusicoccum*, *Cytospora*, *Diaporthe*, and *Phyllosticta*, genera that include endophytic and opportunistically pathogenic species frequently associated with cankers, dieback, and tissue decline in woody hosts [65, 66]. Their enrichment further supports the association between disease symptoms and fungal

assemblages characteristic of stressed or declining tissues. In contrast, asymptomatic trees were enriched in taxa that included *Peltaster*, *Dothiora*, *Taphrina*, *Dissoconium*, and *Uwebraunia*. Although the ecological roles of these fungi in giant sequoia remain poorly understood, their preferential association with asymptomatic trees suggests that they may represent stable members of the healthy mycobiome. The concordance between the DESeq2 and LEfSe analyses, particularly for *Botryosphaeria* and *Peltaster*, strengthens confidence in the biological relevance of these associations and supports their potential as candidate biomarkers of host health status.

In particular, *Botryosphaeria* and *Neofusicoccum* were detected in both asymptomatic and symptomatic samples, suggesting that they are likely stable members of the giant sequoia mycobiome rather than strictly disease-associated organisms. However, their significantly higher abundance in symptomatic samples suggests that host decline may favour their proliferation. Such patterns are consistent with the well-documented ability of many *Botryosphaeriaceae* species to persist as latent endophytes and transition to pathogenic lifestyles when hosts become physiologically stressed [25]. Significant enrichment of *Neofusicoccum parvum* in symptomatic trees, together with its exclusive recovery from symptomatic tissues by culture-based methods, supports the hypothesis that this species behaves as a stress-associated opportunistic pathogen in giant sequoia. *Cytospora* has been documented as a canker and dieback pathogen of coniferous trees [66], and its negative association with *Botryosphaeria* in symptomatic samples may reflect competitive interactions or niche partition between two pathogenic genera in diseased tissue. In asymptomatic samples, *Botryosphaeria* co-occurred positively with *Diplodia* and *Arthrocatena*, and *Neofusicoccum* with *Dendrophoma*. This is further supported by the known endophytic lifestyle of *Diplodia*, which may move towards pathogenicity under stress conditions [26, 67, 68]. Our findings complement recent reports of *N. parvum* from diseased giant sequoia in Germany [32] and

Switzerland [30], suggesting that this species may represent a recurring threat across the European introduced range of this iconic tree species.

The enrichment of specific fungal taxa in symptomatic trees was accompanied by changes in microbial association patterns. Symptomatic networks showed lower connectivity, greater fragmentation, and a distinct hub composition compared with asymptomatic networks. Based on network metrics, these patterns suggest that asymptomatic communities are more globally integrated, with greater connectivity and fewer isolated modules. The higher connectivity and centrality observed in asymptomatic networks may reflect a more cohesive community structure that can maintain ecological stability through a dense web of microbial associations. In contrast, the fragmented structure observed in symptomatic trees may indicate a disruption of these interactions and a reduced community cohesion under disease conditions. Similar disease-associated alterations have been reported in microbial interaction networks in other pathosystems, suggesting that network reorganisation may represent a common feature of host decline in diverse plant systems. A comparable pattern was reported in a study of pathogen-driven tree decline, in which reduced connectivity and altered microbial network structure were associated with disease development [69]. Similar network-level changes were reported in oil palm, where disease development was associated with the reorganisation of microbial interaction networks and the emergence of distinct keystone taxa, despite no significant differences in diversity between health states [56]. Similarly, disease-associated changes in microbial interaction networks were identified during huanglongbing progression [55], while notable changes in microbial network structure reflecting shifts in host-mycobiome interactions were reported in Chinese hickory trees affected by rot diseases [70]. Together, these studies suggest that disease development often involves alterations in microbial interaction patterns and community organisation.

Network analysis also revealed altered associations involving key members of the *Botryosphaeriaceae*. In symptomatic trees, *Botryosphaeria* was negatively associated with *Cytospora*, while *Neofusicoccum* showed positive associations with *Ramularia* and *Meniscomyces*. In contrast, asymptomatic trees exhibited positive associations between *Botryosphaeria* and *Diplodia* and between *Neofusicoccum* and *Dendrophoma*. Although co-occurrence patterns do not necessarily imply direct ecological interactions [71], these observations suggest that disease development may involve reorganisation of fungal interaction networks along with changes in taxonomic composition. Importantly, some taxa enriched in symptomatic trees also showed altered co-occurrence patterns, indicating that disease-associated changes involve not only shifts in abundance but also changes in the ecological context in which these fungi occur.

The combined use of culture-dependent and metabarcoding approaches revealed both shared taxa and method-specific differences in the detected communities. Metabarcoding recovered a broader taxonomic spectrum, reflecting well-documented methodological biases of culture-based methods towards fast-growing and readily cultivable fungi [37, 38]. However, several taxa were consistently detected by both methods, supporting their ecological relevance within the mycobiome of *S. giganteum*. In all samples, the fungal community of these trees was dominated by members of the Ascomycota and Basidiomycota, a pattern widely reported in plant and conifer tissues [24, 72, 73]. The dominance of these phyla suggests that the giant sequoia mycobiome shares broad structural characteristics with fungal communities associated with other long-lived woody hosts, despite this species' unique ecological and evolutionary history.

Nevertheless, this study focused exclusively on the fungal component of the broader pathobiome through ITS metabarcoding, so it could not assess potential interactions involving

bacteria, archaea, viruses, and other microbial groups. Future studies integrating host physiological traits, pathogen biology, multi-kingdom microbiome composition, and environmental variables would provide a more comprehensive understanding of disease development in giant sequoia and evaluate how microbial interactions contribute to host decline within the broader host-pathogen-microbiome-environment paradigm.

Conclusions

This study provides the first integrated characterisation of the foliar fungal mycobiome associated with asymptomatic and symptomatic *S. giganteum* trees using a combined culture-dependent approach and ITS metabarcoding. Although alpha diversity did not differ significantly between health states, symptomatic trees exhibited pronounced shifts in community composition, distinct taxonomic enrichment patterns, and substantial reorganisation of co-occurrence network architecture. In particular, the increased abundance of *Botryosphaeria*, *Neofusicoccum*, *Cytospora*, and *Diaporthe* in symptomatic trees, coupled with reduced network connectivity and greater fragmentation, suggests that disease emergence is driven by ecosystem-level restructuring of fungal communities rather than the dominance of a single primary pathogen. The consistent detection of Botryosphaeriaceae members across both asymptomatic and symptomatic trees further supports the presence of latent, stress-responsive taxa that can transition from endophytic to pathogenic lifestyles under conditions of host decline. Collectively, these results reinforce a pathobiome framework for understanding giant sequoia health, emphasising that disease development in long-lived forest trees is best interpreted as an emerging property of shifting microbial interactions rather than isolated host-pathogen relationships.

598 **List of abbreviations**

599 **ASV** - Amplicon sequence variants

600 **ITS** - Internal transcribed spacer

601 **LEfSe** - Linear discriminant analysis Effect Size

602 **LSU** - Large subunit ribosomal RNA

603 **PERMANOVA** - Permutational multivariate analysis of variance

604 **SRS** - Scaling with Ranked Subsampling

605 **VC** - Vitality class

606

607 **Ethics approval and consent to participate**

608 Not applicable.

609

610 **Consent for publication**

611 Not applicable.

612

613 **Availability of data and material**

614 The datasets and raw paired-end fungal ITS amplicon sequencing reads generated in this study
615 are available in the NCBI Sequence Read Archive under BioProject accession PRJNA1483848
616 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1483848>), with SRA run accession numbers
617 SRR39385120-SRR39385159. The sequences generated from the cultured fungal isolates are
618 available in GenBank under accession numbers PZ934814-PZ934830 and PZ894910-
619 PZ894912. The R scripts used for data analysis are available in the GitHub repository
620 (<https://github.com/blessingdurodola-cpu/sequoia-foliar-mycobiome-analysis>).

621

622 **Competing interests**

623 The authors declare that they have no competing interests.

624

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632 **Authors' contributions**

633 BA designed the study, supervised the experimental work, performed bioinformatic and
634 statistical analyses, constructed co-occurrence networks, and wrote the first draft. EL and FS
635 performed sampling, DNA extraction, culturing, and PCR. AAA performed FUNGuild and
636 LEfSe analyses, contributed to co-occurrence network visualisation, manuscript writing and
637 revision. KB contributed to the writing and reviewed the manuscript. All authors read and
638 approved the final manuscript.

639

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645

646 **Authors' information**

647 **Chair of Pathology of Trees, Institute of Forestry, Faculty of Environment and Natural**

648 **Resources, University of Freiburg, Bertoldstr. 17, 79098 Freiburg, Germany**

649 Blessing Alabi, Emma Lühns, Frederik Siebert & Kathrin Blumenstein

650 **Department of Forest Sciences, University of Helsinki, Latokartanonkaari 5, P. O. Box**

651 **27, 00014, Helsinki.**

652 Abiodun Abieb Azeez

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