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Not seeing the tree for the Forest: Scattered trees can be unexpected hotspots of fungal diversity

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ABSTRACT

Scattered trees are hubs of biodiversity. Here, we present a study in which fungal communities in the caulosphere of scattered individual trees were compared to those of woodlands. We compared alpha and beta diversities of wood- and bark-inhabiting fungi from the stems of trees across seven vegetation types belonging to three species: *Pinus muricata*, *Quercus agrifolia* and *Notholithocarpus densiflorus*. Scattered pines were among the investigated vegetation types. Correlations between stand density, or other tree features, and alpha diversity matrices, were studied with six Generalized Linear Models (GLMs), while the effect of spatial dispersion of trees was studied by correlating Ripley's K values with diversity. Results show that scattered trees represent a hub for biodiversity of wood and bark fungi. We found that caulosphere fungal richness in scattered pines was higher than that of pines growing in stands and was as high as that in notoriously biodiverse oak woodlands. Beta diversity analyses showed that the high fungal diversity in scattered pines is explained by the large number of taxa unique to pines, by a significant overlap of fungi between scattered pines and other vegetation types, and by a significant number of fungi unique to scattered pines. The GLMs showed significant correlations between high species richness, Shannon's and Simpson's indices and low forest density. Finally, we show that the fungi in or on the stems of more dispersed vegetation types are more diverse. These surprising results suggest that preserving or planting scattered trees is a cost-effective strategy that could support as much, or even more, caulosphere fungal biodiversity than entire woodlands.

1. Introduction

Forest ecosystems are key components of the terrestrial biosphere. Besides providing important ecosystem services to sustain human life on earth, forests maintain life cycles, protect habitats and contribute to the preservation and fostering of terrestrial and aquatic biodiversity (Jenkins and Schaap, 2018). In fact, although forests make up <30 % of all terrestrial ecosystems, they contain over 80 % of the global terrestrial biodiversity (Bar-On et al., 2018). The major challenges that forests have been facing in the Anthropocene era (Swanson et al., 2021) will inevitably cause a dramatic reduction of the benefits these ecosystems provide (Edwards et al., 2019). Even if forests have evolved to resist biotic and abiotic stresses, human-mediated intensification of such stressors could impact their ability to resist and be resilient to climate change (Trumbore et al., 2015). Such anthropic stressors include the degradation of forest landscapes due to hunting and timber removal, fragmentation, pollution, land use changes (Foley et al., 2005; Song et al., 2018) and the introduction of nonnative alien species (Parker and Gilbert, 2007).

In order to maximize biodiversity preservation efforts, generally designed to counter climate- and human-driven habitat degradation processes, we need to know how biodiversity is structured across various landscapes. A significant body of literature indicates the valuable contribution that scattered trees bring to the biodiversity of terrestrial ecosystems. However, we need to point out that the definition of scattered trees is not univocal (Manning et al., 2006) and that the term "scattered trees" has been equivocally used to refer to isolated trees, trees in pastures, paddock trees, monumental trees, trees in small patches, and remnant trees (Dunn, 2000; Guevara et al., 1986; Law et al., 2020; Oktan and Atar, 2023; Otero-Arnaiz et al., 1999). The recent Global Forest Resources Assessment redacted by the Food and Agriculture Organization simply defines scattered trees as "trees outside

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forests" (Global Forest Resources Assessment, 2020). We are in general agreement with this definition, as long as it refers to individual trees or singletons growing outside of forests, woodlands, shrublands and savannahs. We will use the term "scattered tree" in the sense described above, while we will use the term scattered tree(s) sensu lato, when referring to the broader definition of scattered trees.

The important function of scattered trees sensu lato can be appreciated at local and landscape scales. Scattered trees can positively influence the local microclimate (Rudolph et al., 2018), the content of soil nutrients (Manning et al., 2006), and the structural complexity of terrestrial ecosystems, greatly increasing habitat availability for the fauna (Evans et al., 2019; Levin et al., 2009; Tölgyesi et al., 2023). These benefits are provided not only in natural environments, but also in monoculture plantations (Korol et al., 2021) and in urban landscapes (Mendonça-Santos et al., 2021). Scattered trees provide connectivity for forest populations and their inhabitants, and can be used as critical elements in large-scale restoration plans (Manning et al., 2006). Although it has been repeatedly shown that landscape-level benefits of scattered trees include increasing habitats for wildlife (Evans et al., 2019; Levin et al., 2009; Tölgyesi et al., 2023), the literature is relatively poor regarding studies aimed at understanding how scattered trees contribute to the overall microbial biodiversity, with published articles mostly focusing on soil or ectomycorrhizal fungi (Azul et al., 2010; Bennett et al., 2009). Likewise, little attention has been paid to the richness of fungal communities in the stems of scattered trees. The literature also lacks studies that specifically examine the diversity of fungi in the wood and bark of the stem of scattered trees compared to that of various sympatric vegetation groups belonging to multiple tree species. This gap in knowledge is noteworthy, given the recent discovery that communities of wood inhabiting fungi are hyperdiverse and even a single tree can contain hundreds of taxonomically distinct variants (Garbelotto and Johnson, 2023; Tedersoo et al., 2014), as opposed to tens of species per individual for mycorrhizal fungi (Peay et al., 2016).

The lack of information regarding the contribution of scattered trees towards the overall diversity of wood and bark fungal communities is particularly surprising, given that there is a large and growing body of literature describing the taxonomical and functional richness of the wood mycobiome. The technical advancements of Next Generation Sequencing have opened new perspectives on the understanding of fungal diversity (Nilsson et al., 2019). Nowadays, the study of environmental DNA (eDNA), including soil and wood eDNA, is a common practice in molecular ecology. When coupled with tools for taxonomic classification (Kõljalg et al., 2019) and functional annotations (Nguyen et al., 2016), the ecological significance that can be extracted from the data expands significantly. Studies about wood-inhabiting fungi have highlighted the richness of fungi, especially during wood decay processes (Fischer et al., 2012; Garbelotto and Johnson, 2023; Kebli et al., 2011; Kubartová et al., 2012). However, in-depth studies on the diversity of wood-inhabiting fungi in scattered trees and comparisons of that diversity with fungal diversity in woodland trees in the same region have yet to be conducted.

In this paper, we present a comparative analysis of the fungal richness of caulosphere fungi in scattered trees, in tree patches and in closed canopy woodlands all growing in the same geographic area. We compared alpha and beta diversity and evenness metrics of wood-inhabiting fungal communities from seven forest or vegetation types (VTs). Our sampled VTs included two types of California coast live oak woodlands, oak savannahs, tanoak woodlands, bishop pine woodlands, post fire bishop pine regeneration stands and scattered bishop pine trees.

Specifically, we investigate the following research questions:

a) Which vegetation types harbor the greatest alpha diversity of tree stem inhabiting fungi?
b) Are fungal communities from the stems of scattered trees as diverse as, less or more diverse than those from the stems of trees growing in woodlands?
c) Are fungal communities in the stems of juvenile populations less biodiverse than those from adult conspecific populations?
d) What is the structure of the stem mycobiome across various sympatric or neighboring vegetation types, and are spatially heterogeneous widespread vegetation types more diverse than homogeneous vegetation types with limited distribution over the landscape?
e) Is there a correlation between alpha diversity metrics of the caulosphere mycobiome and tree diameter, tree height, tree canopy thinning level, tree age, stand density and number of understory species?

2. Materials and methods

2.1. Field survey and sampling

Tree sampling and surveys were carried out in the Vandenberg Space Force Base (VSFB) hereafter also referred to simply as "the Base". The Base was chosen because of the coexistence of various diverse forest types in close proximity to one another. Table 1 summarizes the main characteristics of each VT studied, including the dominant tree species and the sampling effort for each VT. Table S1 lists, for each VT, the average tree diameter at breast height (DBH), tree height, plot density, plot understory plant species, estimated overall area, combined number of trees in all study plots, and estimated total number of trees in the region studied and their estimated total biomass. Table S2 and S3 display tree age classes and stem defects classes across VTs, respectively. Table S4 presents the number of occurrences of understory plant species per each VT.

Sampling consisted in the collection of wood samples from the stem and the root collar of trees and in the recording of geospatial and biometric variables using the Field Data Collection App Wildnote

Table 1
Description of the vegetation types (VTs) studied. Additional information is provided in Table S1-S3.

VTs	N	Dominant tree species	Description of each VT
CLOS (Coast live oak savannah)	40	*Quercus agrifolia* (California coast live oak)	Tees in small patches or rarely individually, growing in more limiting habitats that cannot support closed canopy woodlands
CLOW (Coast live oak woodland)	70	*Quercus agrifolia*	Dense and highly biodiverse ecosystems, hybridization among related taxa occurs.
MCLOW (Maritime coast live oak woodland)	38	*Quercus agrifolia*	Oak populations in these VTs are in the middle of their natural geographic range.
PINE SI (Pine singletons)	128	*Pinus muricata* (Bishop pine)	Scattered bishop pine trees, results of seedling plantation effort. Also referred to as pine singletons.
PINE ST (Pine stands)	54	*Pinus muricata*	Dense stands of mostly medium-sized bishop pine trees. Populations located in the middle of the range of bishop pine distribution.
PINE RE (Pine regeneration)	39	*Pinus muricata*	Populations located in the middle of the range of bishop pine distribution. Characterized by seedlings and small saplings, no adult trees are present.
TANOAK	50	*Notholithocarpus densiflorus* (Tanoak)	Denser woodlands at higher altitudes, growing on the top of two nearby mountains. The tanoaks populations studied are at the Southern edge of the natural range of this species.

(Wildnote, Inc. 793 E Foothill Blvd. STE A #11, San Luis Obispo, California, 93,405). Variables recorded included the date and time of sampling, latitude and longitude, VTs, tree species, location of sampling (stem vs. root collar), tree height, stem diameter, overall tree health, tree age, canopy thinning level, presence of signs or symptoms of pests or pathogens, tree density and the number and species' names of understory plants.

Woody and bark tissues were obtained by wiping the outer bark of each sample tree with 70 % Ethanol and by drilling perpendicularly into the stem using an electric drill fit with screw points spade drill bits 30 cm long and 0.3 cm in diameter. For each plant, wood sawdust was collected using this procedure at two different points, namely at 130 cm above the ground and at the root collar of the plant. Drilling stopped when the bit was 25 to 30 cm into the tree. Wood dust was collected immediately in a manila envelope as the bit was exiting the drill hole. The drill bits were cleaned with 70 % Ethanol between each drilling. Sampling involved three different tree species: *Notholithocarpus densiflorus* or Nd (tanoak), *Pinus muricata* or Pm (bishop pine), and *Quercus agrifolia* or Qa (California coast live oak). The location of sampled trees is shown in Fig. 1.

Samples were collected from seven different VTs, each from a separate plot (Fig. S1). Descriptions of environments, sample numbers, dominant species, and relevant metrics are summarized in Tables 1 and S1-S3. Two oak woodlands were sampled: California coast live oak woodland (CLOW, 70 samples) and maritime California coast live oak woodland (MCLOW, 38 samples), both dominated by *Quercus agrifolia*, a keystone species in California (Hauser et al., 2017) known for its high biodiversity (García-Guzmán et al., 2017; Swiecki et al., 1997). These populations are in the middle of their natural range, where hybridization with related oak species may contribute to biodiversity (Rushton, 1993). In savannahs (California coast live oak savannah or CLOS), 40 samples were collected from small tree clusters or occasionally individual trees in habitats unable to support closed-canopy woodlands.

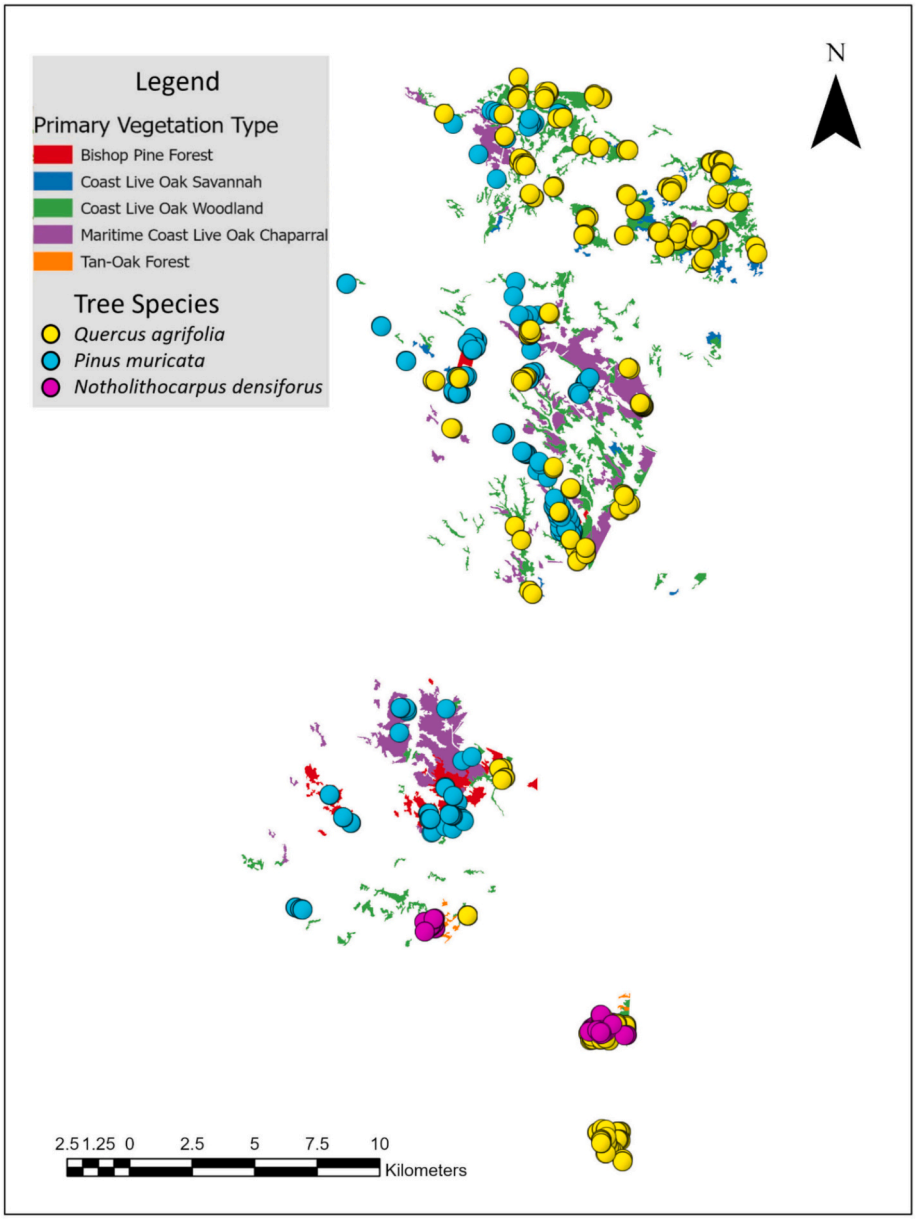

Fig. 1. Map showing sampling locations. For security reasons, the topographic layer has been removed, but vegetation types are shaded with different colors. Dots represent sampled sites for each of three tree species. Note that *Pinus muricata* and *Quercus agrifolia* trees were each growing in multiple vegetation types. Sampled plants in the "pine regeneration" and "pine singletons" vegetation types are identified by appropriately colored dots, but the underlying vegetation types were not available in the vegetation maps.

Savannahs represent a distinct and homogeneous VT with relatively high tree density in clusters (Table S1) and are also within the natural range of *Q. agrifolia*. Fifty samples were collected from tanoak woodlands (TANOAK) dominated by *Notholithocarpus densiflorus,* a species at the southernmost edge of its range and part of a monospecific genus that does not hybridize with other taxa. Three bishop pine (*Pinus muricata*) VTs were sampled: post-fire dense regeneration (PINE RE, 39 samples), monospecific stands (PINE ST, 54 samples), and scattered singletons (PINE SI, 120 samples). PINE SI trees originated from fortuitous recruitment or planting at the urban-wildland interface. The spatial distribution of pine VTs is shown in Fig. S2. Samples of sterile sawdust from autoclaved commercial dowels were used as negative controls, one per VT.

2.2. DNA extractions and ITS1 metabarcoding analyses

Genomic DNA was extracted with the Qiagen QiaAMP Fast DNA Stool Minikit according to the manufacturer's instructions, from 0.125 g of wood tissue mechanically disrupted using an MP Biomedicals FastPrep 24 Instrument Homogenizer. The polymerase chain reaction (PCR) amplification followed a two-step phased amplicon sequencing approach. In the initial step, two subsamples from each extraction were amplified using the ITS1F and ITS2 primer combination to target the ITS1 region (Gardes and Bruns, 1993). Primers included the published sequences along with one, two, three, four, or five random nucleotides as spacers, and complementary stubs for Illumina adapter pairing. This phased strategy effectively addresses the challenge of low diversity in the early cycles of Illumina sequencing by introducing a sequencing frame shift due to variable primer lengths, resulting in increased base diversity. This method enhances base read quality, boosts raw sequence output by approximately 15 % (eliminating the need to add PhiX to the sequencing run due to phasing), and reduces sequencing errors (Wu et al., 2015). The PCR cycling conditions consisted of an initial 2-min hot-start at 95 °C, followed by 35 cycles of 30 s at 95 °C for denaturation, 30 s at 50 °C (for ITS-1) or 55 °C (for ITS-2) for primer annealing, and 1 min at 72 °C for primer extension, concluding with a final extension of 5 min at 72 °C. The PCR products were validated using gel electrophoresis on 1 % agarose gels and visualized with UV light. In the subsequent step, Illumina adapters and barcodes were attached to the PCR products from the first phase. The Vincent J. Coates Genomics Sequencing Laboratory at the California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, supplied dual-indexed adapters from their collection of 960 compatible dual-index pairs. The PCR protocol for this step began with a 2-min hot-start at 95 °C, followed by 15 cycles of 30 s at 95 °C for denaturation, 30 s at 58 °C for primer annealing, and 50 s at 72 °C for primer extension, concluding with a final extension of 10 min at 72 °C. Clean-up after each PCR amplification was performed using AMPure XP beads in a 1.8 ratio. Quantification of PCR products was carried out using a NanoDrop 2000 spectrophotometer, and samples were normalized through dilution in PCR water. Fragment analysis of normalized pools was conducted using an Agilent Bioanalyzer. Ultimately, all samples were pooled and sequenced using the Illumina MiSeq platform with 2 × 300 cycles by QB3.

2.3. Bioinformatic procedures and statistical analyses

Analyses of the ITS1 dataset were performed using the QIIME2 (q2cli V2021.8.0) pipeline (Estaki et al., 2020). Adapters were removed from the demultiplex paired-end sequences with Cutadapt (v4.6) (Martin, 2011). Paired-end sequence denoising, dereplication and chimeras filtering were performed with DADA2 (v1.6) (Callahan et al., 2016). A quality score-based denoising was performed, choosing four different quality score thresholds and selecting the one that gathered the most representative number of Amplicon Sequence Variants (ASVs) based on the post-denoising DADA2 statistical report and the number of ASVs obtained. We applied a quality score of 20 with a minimum overlap of 20 bp between paired-end reads. Prior to downstream analyses, ASVs with low overall relative abundance were filtered out applying a threshold of 0.005 % (Bokulich et al., 2013).

Taxonomic classification was performed with the QIIME2 release of the UNITE database (V9.0) (Abarenkov et al., 2023) containing all Eukaryotes sequences. Pre-training of the Naive Bayes classifier was performed following the QIIME2 protocol implemented in the pipeline. The taxonomic classification of representative sequences was obtained with the QIIME2 feature-classifier classify-sklearn plugin (Pedregosa et al., 2011). This method maximizes assembly of raw reads into ASVs and assignment of ASVs to a given taxonomic rank. However, the closest species match for any given ASVs could be under the standard 98 % homology threshold. Reliability of species assignment is quantified by the confidence values reported in the QIIME2 taxa table output. As confidence values drop below 0.90, species assignment becomes less reliable. After classifying the fungal ASVs, we further analyzed how many ASVs shared the same UNITE accession numbers. ASVs that shared the same UNITE accession were collapsed in the same operational taxonomic unit (OTU). Taxonomic rank of OTUs was at the species level, when possible, or above the species level for sequences missing a species-level homology in UNITE. All ASVs were used in the analyses independent of taxonomic assignment.

Functional annotations of ASVs were performed with FUNGuild (Nguyen et al., 2016). Results of functional annotation were used to produce a table of the most frequent trophic mode detected in the analyses (Table S5). In Table S5, we provide the most frequent twenty annotated ASVs, unannotated ASVs, phototrophs, saprotrophs, symbiotrophs, plant pathogens, and taxa present only in the PINE SI VT. Diversity metrics were computed using the QIIME2 core-metrics-phylogenetic method. Statistical analyses were performed on R (V4.3.0) with the phyloseq package (McMurdie and Holmes, 2013). Estimation of diversity indexes was performed with the R package Vegan (Dixon, 2003). The complete list of ASVs can be found in Table S6. The data used in this experiment are accessible trough the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1217464.

To handle unevenness of sampling intensity among VTs, accumulation curves were created for each vegetation group and for each of three alpha diversity metrics (species richness, Shannon's and Simpson's indices). Species accumulation curves were obtained estimating Renyi and Hill Diversities numbers (Hill, 1973; Mora and Walczak, 2016; Tóthmérész, 1995). These numbers represent a metric increasingly employed to quantify species or taxonomic diversity in molecular ecology studies. Diversity indexes were calculated using Hill's numbers and are represented on the y axes in Figs. 2 and 4 according to Rényi's scales of diversity. These scales are as follows: $\alpha = 0$ is the logarithm of species richness, $\alpha = 1$ equals the Shannon diversity index, $\alpha = 2$ is the logarithm of the reciprocal Simpson diversity index (Bromiley et al., 2004). Hill's numbers encompass three commonly used diversity measures: species richness ($q = 0$), Shannon diversity ($q = 1$), and Simpson diversity ($q = 2$) (Chao et al., 2014).

To handle a variable number of reads among VTs, rarefaction of ASVs by number of reads was performed using the R package iNEXT (Hsieh et al., 2016). The package provides a non-parametric method that combines rarefaction for samples exceeding a certain sequence threshold with extrapolation for those below the threshold (Chao et al., 2014). This method enables the comparison of alpha diversity indexes by accounting for unobserved species and incorporating variance estimates in measurement error models to compare diversity across ecosystems (Willis, 2019). These analyses were also conducted utilizing Hill's numbers.

Alpha diversity values were used to identify which VTs and tree species had the highest caulosphere fungal diversity. Differences among VTs and tree species were assessed using ANOVA, followed by post-hoc Tukey HSD tests to compare means. A Generalized Linear Model (GLM)

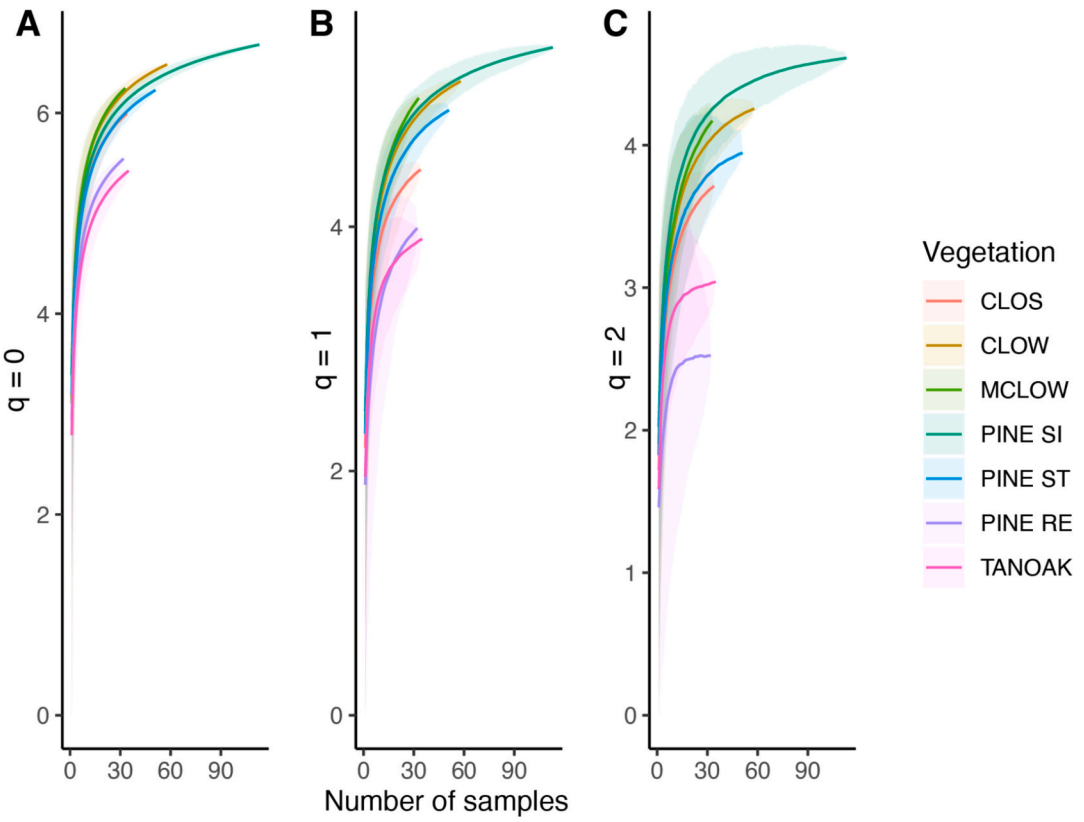

Fig. 2. Accumulation curves of ITS1- based amplicon sequence variants (ASVs) by number of samples. Shaded areas represent 95 % confidence intervals. Vegetation groups are represented separately by diversity order: Hills' q = 0 (species richness, Fig. 2A), Hill's q = 1 (Shannon diversity, Fig. 2B), Hill's q = 2 (Simpson diversity, Fig. 2C). Rényi's scales of diversity were used on the y axis and are as follows: α = 0 is the logarithm of species richness, α = 1 equals the Shannon diversity index, α = 2 is the logarithm of the reciprocal Simpson diversity index.

with a binomial error structure and logit link function was constructed to test correlations between alpha diversity indices (Species Richness, Shannon, Simpson) and explanatory variables, including understory species, tree density, DBH, tree height, tree age, and stem damage level. The best model for each alpha diversity index was selected based on the lowest Akaike Information Criterion (AIC). Model quality was checked using residual diagnostics, including Residuals vs Fitted, Quantile-Quantile, Scale-Location, and Residuals vs Leverage plots. All variables met the assumptions required for GLM analyses.

To test the effect of VTs and tree species on fungal community composition, we analyzed beta-diversity using two distance metrics: Bray-Curtis, which considers taxa abundance, and Jaccard, which focuses on presence/absence. These complementary metrics highlight differences in relative abundance and shared taxa, respectively. We visualized beta-diversity patterns using Redundancy Analysis (RDA) (Xia and Sun, 2023), a constrained ordination method combining linear regression and PCA and used to identify variation in fungal community composition explained by VTs. In RDA plots, the canonical axes of principal coordinates (CAP) on the x and y axes represent gradients of variation best explained by the VTs. CAP scores indicate the strength and direction of the relationship between fungal communities and explanatory variables, allowing us to interpret how communities vary across VTs and tree species in a reduced dimensional space.

For statistical evaluation of beta diversity, we performed a permutational multivariate analysis of variance (PERMANOVA) for both Bray-Curtis and Jaccard distances. This method tests whether the variation in community composition among groups (VTs or tree species) is significantly greater than that expected by chance (Anderson, 2014). Bray-Curtis and Jaccard distances were calculated from the fungal community data. PERMANOVA was conducted with 999 permutations, using VTs as the explanatory variable. To further investigate beta-diversity differences among vegetation types or among tree species, pairwise PERMANOVA analyses were performed for both distance metrics (Bray-Curtis and Jaccard). By combining overall and pairwise PERMANOVA analyses, we were able to comprehensively assess how fungal communities varied across vegetation types and tree species. Venn diagrams were used to represent the level of shared ASVs among select VTs, with ASVs pooled at the species level or at the best taxonomic level possible.

To explore the relationships between fungal richness and the spatial distribution of trees across the various VTs, we created maps illustrating the distribution of tree species and pine VTs. Furthermore, we calculated Ripley's K function at various scales, starting from zero and ending with the calculation of a final observed K value using a rectangle containing all GPS points as our final scale, i.e. the one of interest to us (Ripley, 1977). In order to determine whether trees in each VT may be clustered or dispersed, we calculated the observed K function (K_obs(r)) representing the actual distribution of trees and compared it to the theoretical K function (K_theo(r)), representing the expectation under Complete Spatial Randomness (CSR). Range of variability in the theoretical K distribution under CSR was obtained through 999 Monte Carlo simulations. To test our hypothesis that VTs with a more dispersed distribution may exhibit higher alpha diversity, we performed linear regressions between Ripley's final observed K value for each VT and Species Richness, Simpson's, and Shannon's indices of diversity.

3. Results

Our demultiplexed sequence count summary yielded a total number of 40,355,818 forward reads and 40,355,818 reverse reads. Statistics of DADA2 denoising showed that, on average, 52.25 % of processed reads passed the quality filter. On average, 30,155.12 reads per sample were denoised and 21,323.56 reads per sample were merged. On average, a

total of 10,153.34 paired-end reads per sample were non-chimeric. Negative controls, one for each VT, were all negative. After the taxonomic classification of representative sequences, 2697 ASVs were classified as "Fungi" at Kingdom level, and a total number of 1920 ASVs were classified at the species level (Table S6). We note however that not all 1920 ASVs were classified at the species level with high confidence (e.g. > 0.90). Although we opted to assign taxonomy rank at the maximum level possible, the reliability of each assignment depends on the confidence level. For instance, the ASV classified as *Antarctolichenia onofrii* was the nineth most abundant in the "Top 20 annotated ASVs" (Table S5), however the 0.7 confidence level suggests that this assignment, although the best possible, is not reliable. In fact, when we blasted this ASV using the NCBI nucleotide blast tool, the nucleotide homology with *A. onofrii* was only 84 % (data not shown), suggesting it is not only a different species, but possibly a different genus. Unfortunately, a sequence with a better match was not deposited in the databases at the time we performed our analyses (see below). Nevertheless, our diversity analyses do not take taxonomic assignments into account but are based on presence/absence or abundance of unique ASVs, independent of taxonomic classification.

It is important to consider that taxonomic identification of species based on DNA barcode sequences relies strongly on the quality of the reference databases that link genetic sequences to taxonomic names and on the level of taxonomic resolution offered by the selected barcode (Keck et al., 2023). Taxonomic identification performed by our pipeline had an average confidence of 0.92 (SD = 0.09), indicating the list of taxa we generated was sufficiently accurate to perform a functional analysis. However and additionally, while ITS is regarded as one of the major DNA barcodes for the fungi and it has been broadly used to identify taxa at the species level (Osmundson et al., 2013; Schoch et al., 2012), it often cannot differentiate between closely related species. This intrinsic limitation of ITS, thus, can result in a conservative underestimation of diversity and at the same time in an incorrect species assignment. ITS data can thus be used to conservatively calculate biodiversity, but additional sequence data is often needed to provide reliable species identification. Notwithstanding the limitations of ITS, the overall functional annotation indicated that saprotrophic species and fungal species that alternate between saprotrophic and pathogenic trophic modes were the two best represented groups. Latent pathogens, i.e. fungi that can alternate between symbiotrophic, pathogenic and saprotrophic ranked as a very close third in abundance. Purely pathotroph or symbiotroph were far less represented, and even less represented were fungi that could be both pathotroph and symbiotroph (Fig. S3).

3.1. Alpha diversity values

Table 2 summarizes the estimated values of alpha diversity. MCLOW (3.84 ± 0.81) and PINE SI (3.66 ± 0.72) had the highest estimated values for the Shannon's index. The same result was observed for Pielou evenness, with MCLOW (0.74 ± 0.11) and PINE SI (0.73 ± 0.11) ranking first and second, respectively. Finally, the highest values of Simpson diversity were those of the PINE SI (0.85 ± 0.10) vegetation type (VT), with PINE ST (0.80 ± 0.15) and CLOW (0.80 ± 0.15) VTs tied in second place. Conversely, PINE REGEN was the vegetation group that always displayed the lowest Shannon diversity (2.73 ± 1.28), Pielou evenness (0.60 ± 0.23) and Simpson diversity (0.69 ± 0.26).

Table 2
Values of Alpha diversity indices or of evenness including standard deviations, for each vegetation type.

VTs	Observed Species Richness	Shannon's Diversity Index	Simpson's Diversity index	Pielou's Evenness Index
CLOS	401	3.16 ± 0.97	0.78 ± 1.15	0.68 ± 0.10
CLOW	655	3.51 ± 0.93	0.80 ± 0.15	0.70 ± 0.13
MCLOW	517	3.84 ± 0.81	0.79 ± 0.21	0.74 ± 0.11
PINE SI	797	3.66 ± 0.72	0.85 ± 0.10	0.73 ± 0.11
PINE ST	507	3.50 ± 0.85	0.80 ± 0.15	0.71 ± 0.13
PINE RE	256	2.73 ± 1.28	0.69 ± 0.26	0.60 ± 0.23
TANOAK	227	3.00 ± 0.85	0.73 ± 0.23	0.69 ± 0.10

Fig. 2 illustrates the results from the ASV accumulation curves with increasing sample size. Every VT is represented by a curve and a color in the figure. For each curve, the interval of confidence is represented as a colored shade under the line. We note that saturation, as indicated by a flattening of the line, was approached for all VTs when looking at Species Richness, while all VTs but MCLOW approached saturation with regards to the other two diversity indices, suggesting that MCLOW may have been under sampled. However, when considering the number of ASVs as the number of DNA reads increases (see explanation below), saturation was reached for all VTs and all indices. Hence, our analyses can be deemed overall reliable.

Fig. 2 compares diversity indices across VTs. In the first panel (Fig. 2A), which represents species richness (Hill's number = 0), PINE SI shows the highest value, followed by CLOW and MCLOW, while PINE RE and TANOAK exhibit the lowest. At a standardized sampling size of 38 samples per VT, PINE SI ranks third, close to CLOW and MCLOW, and exceeds all other VTs, including PINE ST. The second panel (Fig. 2B) depicts Shannon's index (Hill's number = 1), with PINE SI again ranking highest, followed by CLOW and MCLOW. CLOS shows intermediate diversity, while PINE RE and TANOAK have the lowest values. At 38 samples, PINE SI ranks second, close to MCLOW. The third panel (Fig. 2C) shows Simpson's index (Hill's number = 2), with PINE SI maintaining the highest value, while CLOW, MCLOW, CLOS, and PINE ST are intermediate, and PINE RE and TANOAK the lowest. At 38 samples, PINE SI ranks first. Overall, PINE SI consistently exhibits the highest or near-highest diversity across all indices, while TANOAK and PINE RE show the lowest.

The ASV diversity curves with increasing number of reads and estimated using the non-parametric iNEXT method are shown in Fig. 3. As for the accumulation curves, the diversity indices are represented as Hill's numbers. Each curve corresponds to a different VT, each distinguished by a unique color. For each curve, the sample-size-based rarefaction is represented by a solid line, while the extrapolation is shown as a dotted line. The X-axis represents the number of reads. These curves show that ASV saturation was reached for each and all VTs, suggesting our description of the wood fungal community is complete. The first panel (q = 0) shows species richness, the second (q = 1) shows Shannon's diversity index, and the third (q = 2) shows Simpson's diversity index. The trend remains consistent across all three panels: PINE SI consistently exhibits the highest values for species richness, Shannon's, and Simpson's diversity. CLOW follows with slightly lower values, then MCLOW and PINE ST. CLOS shows a further drop in diversity, while PINE RE and TANOAK consistently record the lowest diversity values. The results observed with this method mirror those from the accumulation curves in Fig. 2. They confirm that pine singletons consistently contain the most diverse stem mycobiome, regardless of whether we examine species richness, Shannon, or Simpson diversity indices.

Alpha diversity values were also estimated for each tree species. We calculated species richness, Shannon's index, Simpson's diversity, and Pielou's evenness across the three tree species (*Pinus muricata*, *Quercus agrifolia*, and *Notholithocarpus densiflorus*)\. ANOVA results revealed significant differences in all indices except for Pielou's evenness (p-value >0.05). Species richness showed the most pronounced differences among the tree species (F = 9.70, p-value <0.001). *Q. agrifolia* had the highest richness (35.01 ± 22.91), followed by *P. muricata* (29.95 ± 13.57) and *N. densiflorus* (20.74 ± 13.40), with all pairwise comparisons being statistically significant (Tukey, p-value <0.05). For Shannon's index, significant differences were also observed (F = 3.67, p-value = 0.0266). Post-hoc Tukey tests indicated that *P. muricata* (3.47 ± 0.92) and *Q. agrifolia* (3.49 ± 0.94) exhibited the highest Shannon diversity, with no significant difference between them, while *N. densiflorus* (3.01 ± 0.85) showed significantly lower diversity. Simpson's diversity index

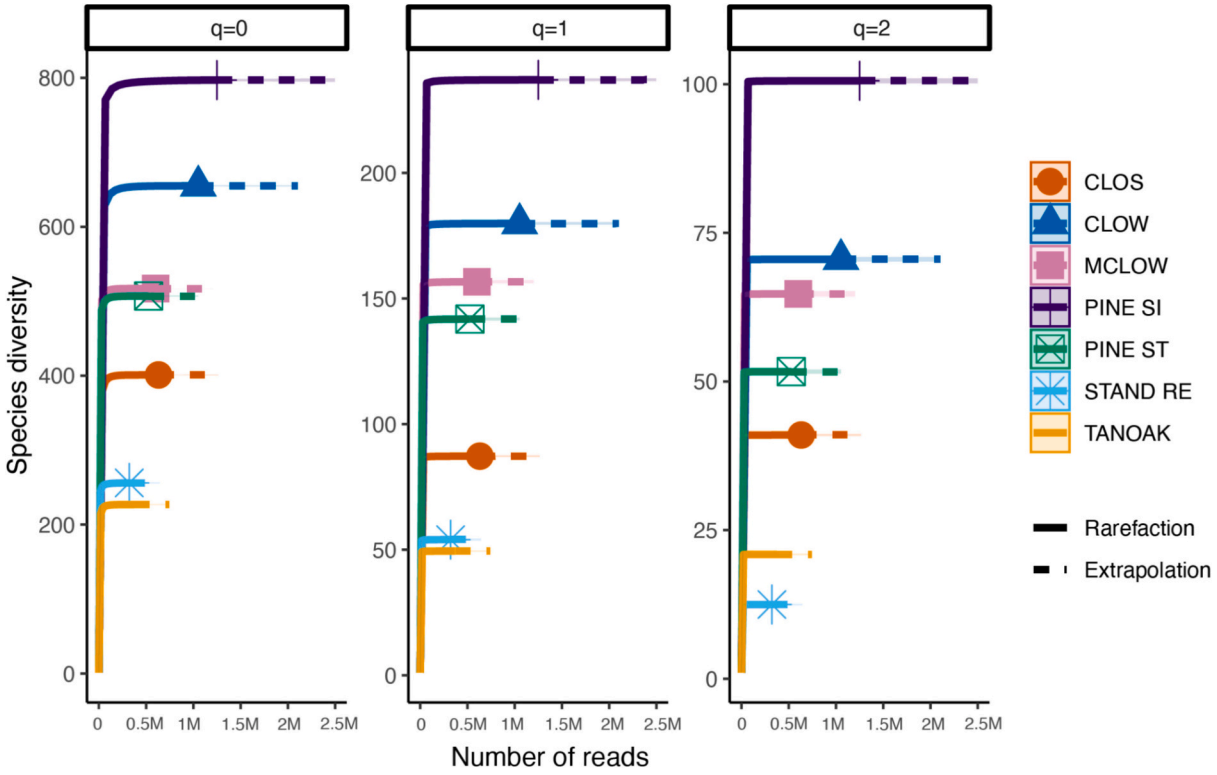

Fig. 3. Rarefaction curves iNEXT showing diversity of fungal amplicon sequence variants (ASVs) with increasing number of DNA ITS1 sequence reads, based on Species Richness (Hill's q = 0), Shannon's diversity (Hill's q = 1) and Simpson's index (Hill's q = 2). Rényi's scales of diversity were used on the y axis and are as follows: α = 0 is the logarithm of species richness, α = 1 equals the Shannon diversity index, α = 2 is the logarithm of the reciprocal Simpson diversity index. Rarefaction is based on actual data, while extrapolation is a projection based on the trend of the actual data.

also differed significantly among species (F = 3.41, *p*-value = 0.0343). *P. muricata* exhibited the highest value (0.81 ± 0.16), significantly different from *N. densiflorus* (0.73 ± 0.23). *Q. agrifolia* had an intermediate value (0.79 ± 0.17), not significantly different from either of the other two species. Pielou's evenness did not differ significantly among the tree species (*p*-value = 0.766). Evenness values were similar for *P. muricata* (0.71 ± 0.15), *Q. agrifolia* (0.70 ± 0.12), and *N. densiflorus* (0.69 ± 0.10), suggesting a relatively uniform distribution of fungal taxa across these host species.

Statistical analyses identified differences when comparing the alpha diversity indices values of the various VTs. One-way ANOVAs showed that differences occur among VTs for all three diversity indices as shown below: Species richness (p-value = 2.77e-05), Shannon diversity index (p-value = 6.54e-07), Pielou's evenness (p-value = 0.0002) and Simpson index (p-value = 0.0001). Tukey-HSD post hoc tests identified those VTs that have statistically different levels of alpha diversity indexes (Fig. 4). Homogeneous groups were identical for Species Richness, Shannon's and Peliou's index, with PINE SI, CLOW and MCLOW making up the most diverse, or most even in the case of Peliou's index, group of VTs. An intermediate group included the CLOS and PINE ST VTs, while the least diverse/even group included TANOAK and PINE RE. In the case of the Simpson's index, results were slightly different. PINE SI was the most diverse VT, followed by PINE ST, by a group including all three oak VTs (CLOS, CLOW, MCLOW), by TANOAK and ending with the PINE RE VT.

3.2. Beta diversity analyses

The similarity among caulosphere fungal communities of the seven VTs was investigated factoring in the abundance of taxa or simply utilizing presence/absence of each taxon. The first analysis was performed using a Bray-Curtis distance matrix, while the second was performed using a Jaccard distance matrix. The results of both analyses were visualized through a canonical analysis of principal coordinates (CAP) (Fig. 5). Permutational pairwise PERMANOVAs performed on the Bray-Curtis distance matrix indicated that all pairwise comparisons of caulosphere fungal community composition of the various VTs were different from one another (p-value = 0.021), except for the PINE ST vs. PINE SI (p-value = 0.168) and the CLOW vs. MCLOC (p-value = 0.357) comparisons (Table S7). When the same analysis was performed on the Jaccard distance matrix, all comparisons were different (p-value = 0.021–0.03), except for the CLOW VT vs MCLOC VT comparison (p-value = 0.357) (Table S8). When the caulosphere fungal composition was compared by tree species, pairwise PERMANOVAs executed using both distance matrices indicated that each tree species was different from the other two (p-value = 0.003) (Tables S7-S8).

When visualizing beta diversity through CAP graphs, analyses based on both distance matrices identified: a) a significant separation of the three pine VTs from the four oak/tanoak VTs along the horizontal axis, b) a separation of the PINE RE VT from the other two pine VTs along the y axis with minimal boundary overlap, c) a separation of the TANOAK VT on the x axis with a partial overlap with the CLOW and CLOS VTs, d) a significant overlap of MCLOW and CLOW VTs, e) a moderate overlap between CLOW and CLOS VTs, and, f) a moderate overlap among the PINE ST, PINE SI, MCLOW and CLOW VTs. However, when comparing the PINE ST and PINE SI VTs, we observed greater dispersion of points and greater overlap with the MCLOW and CLOW VTs in the latter. This difference was more noticeable in the Jaccard dataset analysis, indicating that an increased separation of the caulosphere fungal community of scattered pines from that of pine stands is driven by fungal taxa with lower abundance. This result agrees with the pairwise PERMANOVA results, which identified a significant difference between PINE SI and PINE ST VTs only in the analysis based on the Jaccard distance matrix.

Finally, we used Venn diagrams to assess the overlap of fungal

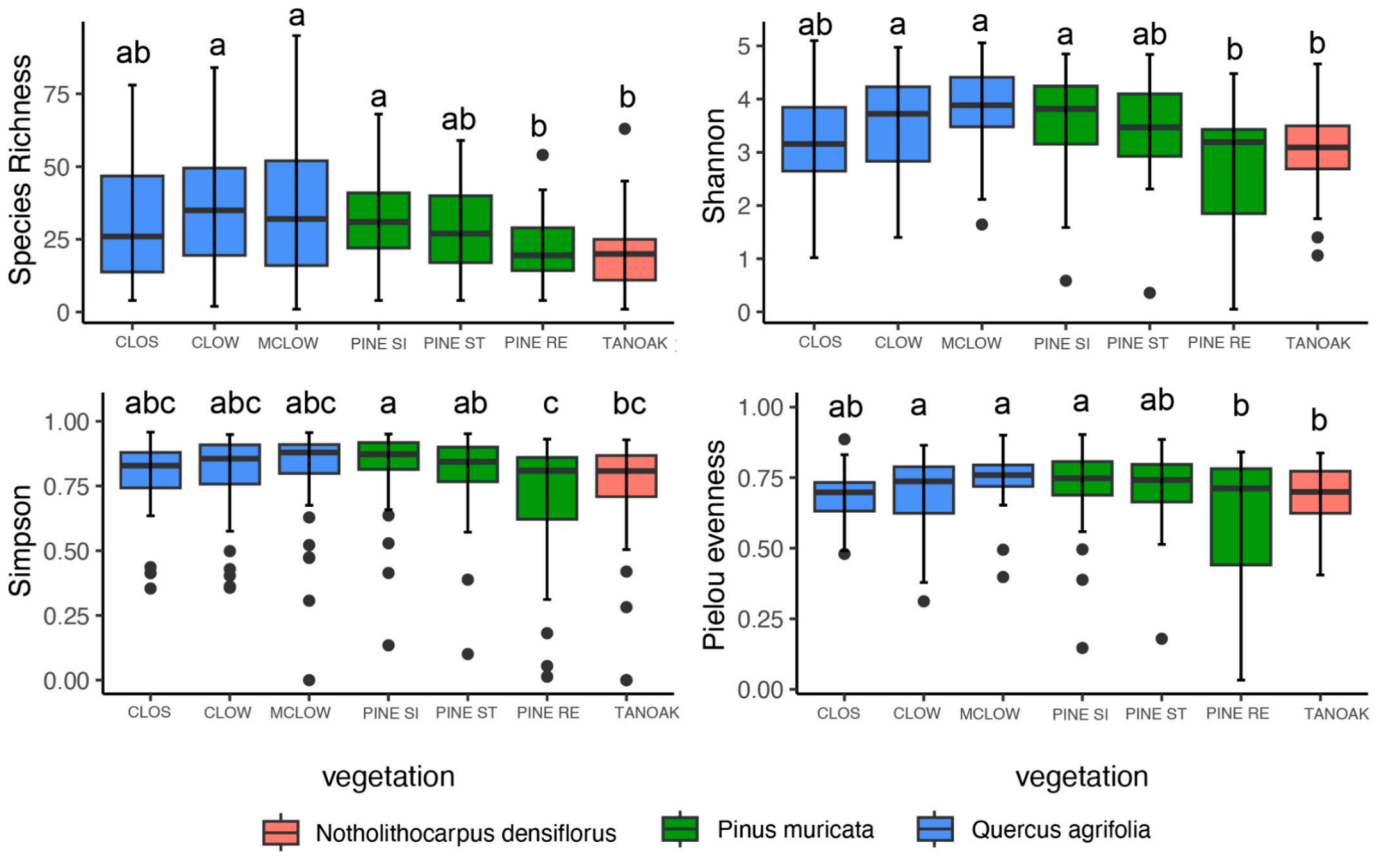

Fig. 4. Distribution of diversity indices values of the seven vegetation types studied. Compact letters indicate statistically homogeneous groups based on the results of the Tukey-HSD post hoc test conducted in a one-way ANOVA, with a confidence level of 95 %.

amplicon sequence variants (ASVs) among different VTs after taxonomic classification to the best possible level (Fig. 6). The analysis highlights how tree species and VTs within species shares fungal taxa. The first Venn diagram (Fig. 6A) shows the overlap of fungal species among oak VTs (MCLOW, CLOW, CLOS), tanoak (TANOAK), and pine singletons (PINE SI). Oaks and tanoak share 39.3 % of ASVs, while 24.3 % of ASVs in PINE SI overlap with these two hosts, despite their phylogenetic distance. Few ASVs ($n = 8$) are shared exclusively between TANOAK and PINE SI, which are not spatially adjacent (Figs. 1, S1). Notably, PINE SI has 117 private ASVs, a high number compared to 154 for combined oak VTs and only 52 for tanoak. The second diagram (Fig. 6B) examines the three pine VTs (PINE ST, PINE RE, PINE SI), showing that 51.7 % of ASVs are shared among them, as expected for the same species. However, only 1.3 % of ASVs are shared between PINE ST and PINE RE, which are not spatially adjacent (Fig. S2). Pine singletons (PINE SI) share 8.2 % of ASVs with PINE RE and 26 % with PINE ST, consistent with their spatial proximity to both (Fig. S2). Interestingly, 26.3 % of ASVs are exclusive to PINE RE, even compared to other conspecific VTs.

3.3. GLM results

We developed six GLMs (Table 3) to test correlations between alpha diversity and selected variables (see Tables S1-S3 for details). The first GLM showed no significant correlation between DBH and any diversity metrics. The second revealed a significant inverse relationship between tree height and both species richness and Shannon's index, but no correlation with the Simpson index, suggesting lower fungal diversity in taller trees. The third GLM showed that higher stem damage levels are associated with increased species richness and Shannon entropy, but not the Simpson index. The fourth demonstrated a significant positive correlation between tree age and all diversity metrics, with mature and overmature trees showing higher fungal diversity. The fifth GLM identified a significant negative impact of tree density on species richness, Shannon entropy, and the Simpson index, with lower density categories supporting higher fungal diversity. Finally, the sixth GLM found no significant correlation between the number of understory species and any alpha diversity indices.

3.4. Spatial analyses

The maps (Figs. 1, S1-S2) indicate that most VTs spatially overlapped to some extent, however TANOAK only overlapped with CLOW and bordered CLOS, while PINE ST never overlapped with PINE RE. However, PINE ST overlapped with oak VTs and the PINE SI VT. Ripley's K analysis (Fig. S4) indicated that all VTs were clustered when compared to random distributions (p-value <0.05). However, at the final scale analyzed, the one encompassing the entire study area, the level of clustering varied and VTs clustered in the following order, starting from the most clustered VT: TANOAK (final K_obs = 0.04830207), PINE RE (final K_obs = 0.03560702), PINE ST (final K_obs = 0.03420743), CLOS (final K_obs = 0.025061), MCLOW (final K_obs = 0.02079044), CLOW (final K_obs = 0.01589921), PINE SI (final K_obs = 0.01409425). Linear regressions indicated a significant negative correlation between clustering expressed as final K_obs and Species Richness (Species Richness = 883.65071–14,567.613 * Final K_obs; p-value = 0.01, R Squared = 0.76) and Simpson's index (Simpson's I = 0.8646538–3.1582395 * Final K_obs; p-value = 0.05, R Squared = 0.55), indicating more dispersed VTs were more diverse. A similar trend was observed for Shannon's index, however the linear regression, in this case, was only significant at alpha = 0.08 (Shannon's I = 3.9567829–22.156368*Final K_obs; p-value = 0.08, R Squared = 0.48).

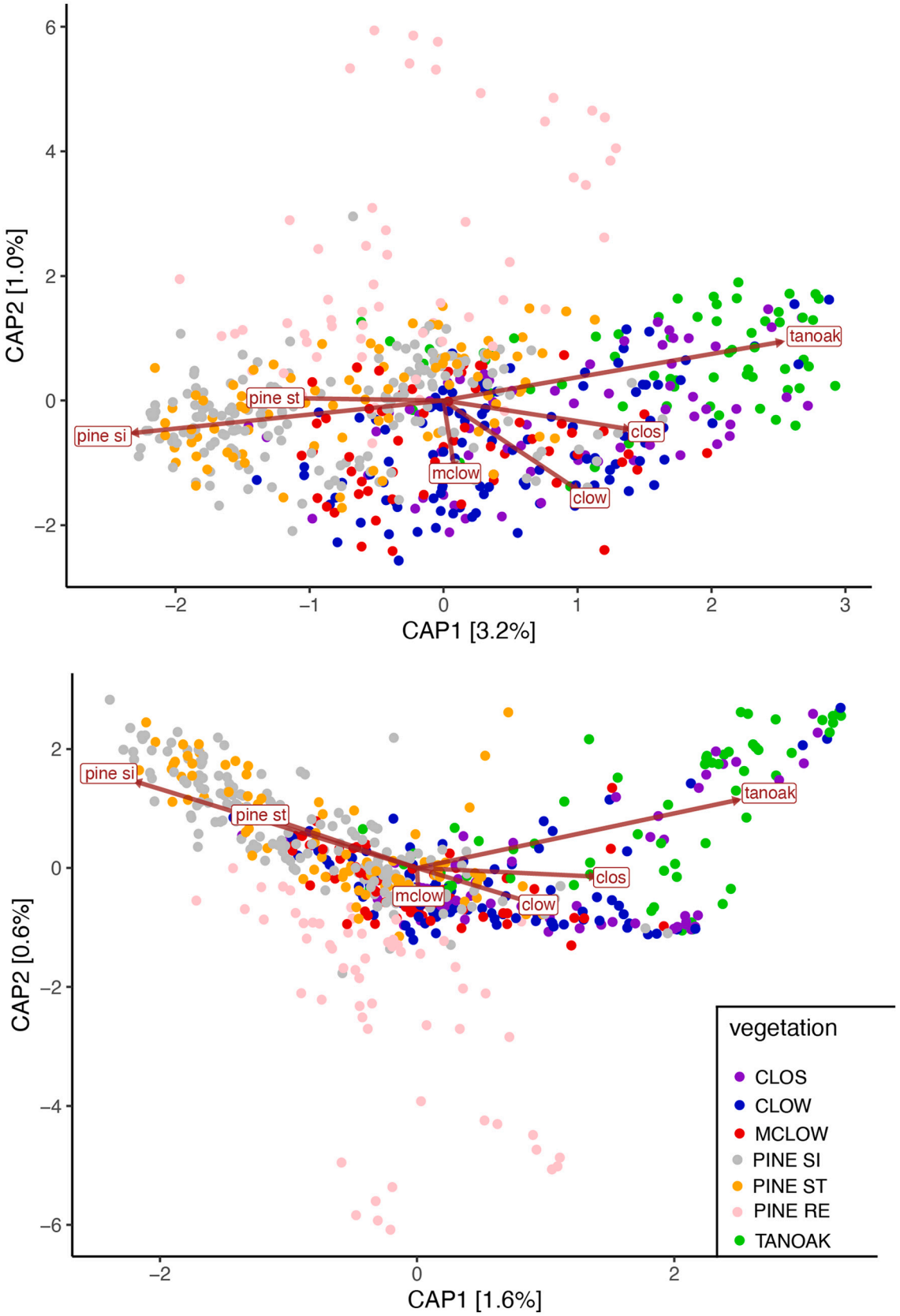

Fig. 5. Canonical analysis of principal coordinates (CAP) based on a Bray-Curtis distance matrix (panel A) and a Jaccard distance matrix (panel B). Each color represents a vegetation type included in the representation of beta diversity. Labeled arrows indicate the direction of the distribution for each vegetation type's cluster of points.

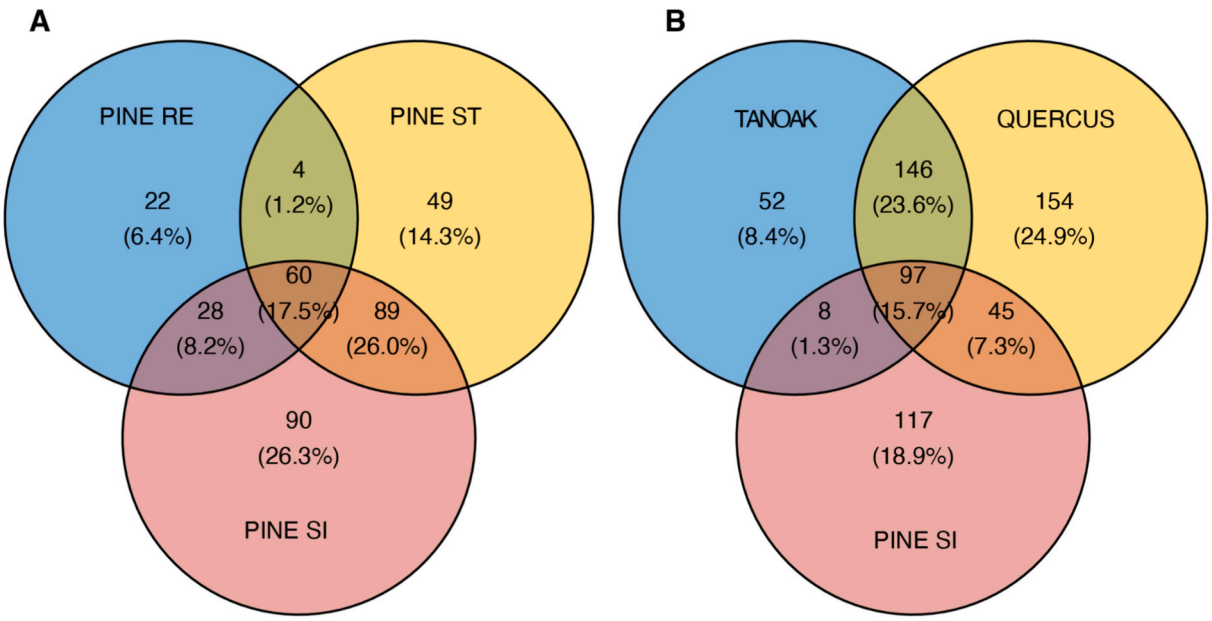

Fig. 6. The Venn diagrams in this figure display the number of fungal species shared between pine singletons and the other two pine vegetation types (Fig. 6A) or between pine singletons, tanoaks and all oak vegetation types (Fig. 6B). The number of shared species is shown as both counts and percentages. Fungal species were defined based on identical UNITE accession numbers.

Table 3
Results of five Generalized Linear Models (GLMs), each investigating the correlation between tree or stand variables and fungal diversity, based on three distinct diversity metrics.

	Species Richness			Shannon's Index			Simpson's Index		
	β	SE	p-value	β	SE	p-value	β	SE	p-value
Model 1: DBH									
Intercept	30.062	1.596	< 2e-16	3.406	0.088	< 2e-16	0.000	0.009	1.000
DBH	0.029	0.048	0.552	0.001	0.003	0.712	0.001	0.000	0.158
Model 2: Tree height									
Intercept	34.448	1.555	< 2e-16	3.581	0.084	< 2e-16	0.000	0.009	1.000
tree height	−0.120	0.041	0.004	−0.004	0.002	0.026	0.000	0.000	0.868
Model 3: Canopy thinning level									
Intercept	28.780	0.966	< 2e-16	3.383	0.056	< 2e-16	−0.005	0.009	0.561
0–05 %	11.720	12.048	0.331	0.140	0.651	0.830	0.019	0.109	0.861
6–10 %	1.220	12.048	0.919	−0.721	0.651	0.269	−0.140	0.109	0.198
11–25 %	27.220	12.048	0.024	0.692	0.651	0.289	0.082	0.109	0.453
26–60 %	52.220	17.011	0.002	1.505	0.919	0.103	0.142	0.153	0.357
61–100 %	15.562	2.920	0.001	0.393	0.165	0.018	0.047	0.027	0.090
Model 4: Tree age									
Intercept	25.955	2.680	< 2e-16	2.939	0.146	< 2e-16	−0.100	0.024	0.001
Immature	4.302	3.432	0.782	0.473	0.179	0.267	0.113	0.028	0.165
Mature	5.196	2.879	0.072	0.558	0.156	0.001	0.114	0.026	0.001
Overmature	9.920	4.511	0.029	0.621	0.243	0.011	0.116	0.040	0.004
Model 5: Density									
Intercept	20.316	4.014	0.001	2.828	0.234	< 2e-16	−0.099	0.039	0.011
zero	12.945	4.207	0.002	0.729	0.244	0.003	0.116	0.041	0.005
small	11.722	4.465	0.009	0.664	0.257	0.010	0.111	0.043	0.010
medium	4.716	4.579	0.304	0.321	0.263	0.223	0.061	0.044	0.161
Model 6: Number of understory species									
Intercept	31.588	1.471	< 2e-16	3.416	0.081	< 2e-16	0.000	0.009	1.000
num of understory species	−0.230	0.317	0.467	0.003	0.018	0.869	0.001	0.000	0.727

4. Discussion

Biodiversity is recognized as a key aspect of a healthy ecosystem. Likewise, achieving an accurate assessment of biodiversity levels serves as a valuable tool for ecosystem management (Laurila-Pant et al., 2015). The application of next-generation sequencing for environmental

biodiversity assessment is a powerful way (Taberlet et al., 2012) to achieve such a goal. Numerous examples exist that illustrate how this technology is applied to understand the state of biodiversity in various environments and using various organisms as indicators of biodiversity. Notable applications include, among others, metabarcoding of arthropods (Yu et al., 2012), eDNA metabarcoding of generalist predators a (Nørgaard et al., 2021), and the evaluation of fish biodiversity in estuaries using eDNA metabarcoding (Ahn et al., 2020). While acknowledging the pitfalls of metabarcoding (Fonseca, 2018), it is clear that this technique enables us to capture and describe the diversity of various life forms.

Fungi are important and meaningful biodiversity indicators. They are ubiquitous organisms essential to the functioning of most ecosystems. The examples in this regard are numerous and span across multiple organisms and ecosystem types, where fungi can play very different roles. While the examples are too numerous to list exhaustively, fungi, for instance, play a role as mycorrhizal plant symbionts (Brundrett, 1991), play multiple roles in marine ecosystems (Hyde et al., 1998), serve as mediators linking organisms and ecosystems (Bahram and Netherway, 2022), and contribute to soil health (Frąc et al., 2018). They can survive and be functional even in extreme environments, such as deserts (Sterflinger et al., 2012), and are major players in the global carbon cycle (Stark, 1972) and in the weathering and bioremediation of soils (Finlay et al., 2009; Ceci et al., 2019).

In this study, we chose caulosphere fungi as indicators of biodiversity. We focused specifically on fungal communities that have been less studied, namely those residing within the woody trunks and on the stem bark of tree species. The reason for this choice lies in the recent discovery that fungal communities in the wood of trees are surprisingly hyper-diverse and play crucial roles in determining plant health and in the cycling of carbon (Ekblad et al., 2013; Garbelotto and Johnson, 2023; Slippers and Wingfield, 2007; Zhu, 2003). Our goals were to compare various adjacent forests/vegetation types (VTs) to assess their biodiversity from a caulosphere fungal perspective. In particular, we were interested in determining whether oak woodlands are in fact the most biodiverse and whether scattered pine trees may significantly contribute to fungal biodiversity when compared to sympatric woodlands and savannahs.

Our findings indicate that the mycobiome of the stem and root collar of scattered pine trees, some planted in anthropized settings and some the result of natural recruitment, exhibits a high diversity comparable to that of oak woodlands and surpassing that of pine woodlands, oak savannahs, tanoak woodlands and post-fire pine recruitment. These results are in part unexpected, based on previous reports that a) ectomycorrhizal communities from woodlands are more diverse than those from individual trees or clusters of trees (Peay et al., 2007) and more diverse than those from disturbed sites (Karpati et al., 2011), b) biodiversity is positively correlated with ecological habitat continuity (Nordén et al., 2014), and, c) natural forests are more diverse than anthropogenic ones (Da Silva et al., 2019).

The higher diversity of the caulosphere mycobiome of scattered pine trees compared to that of pine stands, though, is in part consistent with an extensive literature that associates higher levels of biodiversity either with the structural complexity of mature semi open multi layered forests (Gao et al., 2014) and/or with the presence of ecotones (Horak et al., 2014; Kark, 2013), including those represented by Mediterranean savannahs (Marañón et al., 2009). In agreement with the above papers which deal with biodiversity from various non-fungal angles, we also found that trees in closed-canopy woodlands with the clear dominance of a single species, support lower overall or comparable fungal diversity than singletons, in spite of their biomass being two (e.g. for PINE ST) to four orders (e.g. for CLOW) of magnitude larger than that of singleton pines (Table S1). Given that both the sampling effort and the number of reads had approached or reached saturation for CLOW, PINE ST and PINE SI, we believe the diversity indices presented in this study for these three VTs to be truly representative of the three habitats.

Our functional analyses provides new evidence that fungi that alternate between symbiotic and other lifestyle are the majority of the fungi identified in the stem: these are the fungi normally referred to as latent pathogens and/or endophytes, often little understood, but obviously, as we show here (Fig. S3), massively present in trees (Rodriguez et al., 2009; Slippers and Wingfield, 2007). Monospecific woodlands can promote the spread of fungal endophytes or pathogens adapted to narrow environmental conditions and host-specific to the dominant tree species (Gilbert et al., 2002). In contrast, scattered trees create sharp ecotones, acting as the dominant vegetation layer alongside woody shrubs and herbaceous plants. The greater range of environmental conditions found in these small ecotones is likely to facilitate a greater diversity of fungi infecting and colonizing the wood of scattered trees possibly for two reasons: a) the variation in temperature, shading, relative humidity and wood bark wetness will affect different fungal species differently (Giauque and Hawkes, 2013), and, b) open spaces such as shrublands and grasslands normally have significantly larger loads of fungal infectious propagules (i.e. airborne spores) than closed canopy forests (Sesartic and Dallafior, 2011). The effect of larger spore loads will be particularly notable for that large number of wood endophytic fungi and latent pathogens that are known to be generalists (Rodriguez et al., 2009).

Our analysis of fungal beta diversity across tree species revealed that a large proportion of fungal amplicon sequence variants (ASVs) is shared among VTs, as shown by both the CAP analyses and Venn diagrams. These findings suggest that many of the detected fungi are generalists, capable of horizontal transmission within and among plant species. However, the CAP analysis also highlights the significant role of tree species in shaping fungal community composition, with a clear distinction between pine and oak/tanoak VTs. This conclusion is further supported by pairwise PERMANOVA analyses, which demonstrate significant differences in caulosphere fungal communities among the three tree species (Tables S7-S8). Notably, the CAP diagrams show greater dispersal of PINE SI points compared to PINE ST, indicating that a portion of the fungal community in pine singletons is unique to this VT. This uniqueness is more pronounced when using the Jaccard distance matrix, suggesting that less abundant taxa, that is taxa present in some trees but absent in trees located elsewhere, contribute substantially to the higher fungal diversity in pine singletons. Pairwise PERMANOVA results further confirm that fungal communities in the stems of pine singletons differ significantly from those in pine stands, reinforcing the distinct ecological role of scattered pine trees in supporting diverse fungal communities (Tables S7-S8).

Our results support the hypothesis that stems of scattered trees are hotspots of caulosphere fungal diversity. We found a negative relationship between tree density and fungal diversity, as well as a positive association between diversity and tree maturity. Although no correlation was observed with stem diameter size, taller trees were associated with lower fungal diversity, likely because tree height is greater in denser stands (Fayolle et al., 2016), whereas mature and overmature trees are more common in less dense forests (Peet and Christensen, 1987). This suggests that fungal biodiversity is promoted in open or semi-open settings, especially when trees reach maturity. Conversely, regeneration through post-fire recruitment, characterized by high-density and monospecific stands of small trees (1.6 ± 1 cm DBH; Table S1), showed the lowest fungal diversity and evenness metrics, indicating dominance by a few fungal species. Smaller trees may have limited woody biomass for fungal colonization, while mature trees accumulate fungal species over time, aided by the persistence of endophytes (Genevieve et al., 2019) and the progression of wood decay processes (Fukasawa and Matsukura, 2021; Garbelotto and Johnson, 2023). Additionally, our GLM analysis revealed a positive correlation between fungal diversity and stem defects. Damaged trunks may create microhabitats that support different fungal species, with some fungi contributing to the damage and others benefiting from it.

Landscape homogeneity in closed-canopy monospecific woodlands

and the spatially limited distribution of the PINE ST VT compared to that of pine singletons, as indicated by the distribution map of pine VTs (Fig. S1) and by Ripley's K analysis (Fig. S4), may be responsible for limiting fungal diversity, in spite of the presence of two orders of magnitude greater biomass in pine stands compared to that of scattered pines (Table S1). The presence of a negative correlation between spatial clustering of VTs and the diversity of caulosphere fungi strongly supports the hypothesis that more dispersed VTs support more diverse fungal communities in their stems. By the same token, the high fungal diversity in the wood of the stem of scattered trees may be in part explained by the heterogeneous habitats in which such trees are found, as evidenced by the fact that singleton pines were growing in sites across the entire base (Fig. S2) and in habitats as diverse as urban areas, landscaped sites and the edges of natural woodlands (data not shown). We note that the oak savannahs studied here mostly comprised clusters of trees and very few scattered singletons. In these savannahs, trees were always growing in grassland settings, hence oak savannahs were homogeneous when compared to the highly diverse habitats in which pine singletons were growing.

Based on the spatial limitations in fungal spore dispersal (Peay et al., 2010), we conclude that fungal airborne inoculum composition varies across spatially distinct and ecologically diverse sites, such as those with scattered pines. Planting individual trees in multiple locations is likely to promote higher fungal diversity than clustering trees in a single site. This is supported by our CAP analyses, which show minimal overlap of ASVs between tanoaks and scattered pines or between pine stands in urban areas and post-fire pine recruitment zones (Figs. 5–6 and S1-S2). In contrast, adjoining VTs exhibited significant ASV overlap (Figs. 5–6 and S1), further highlighting the influence of spatial and ecological heterogeneity on fungal community composition.

We recognize that fungal diversity may vary depending on tree species and the study site's location relative to the trees' natural range. In our study, tanoaks showed less diverse fungal communities in the stem, while pines and oaks had similarly high caulosphere fungal diversity, with oaks being more diverse for some indices. This suggests that the diversity in scattered pines is influenced more by the unique distribution and ecological setting of singleton trees than by tree species alone. Our data also indicate that native trees within the core of their natural range (e.g., bishop pine and California coast live oak) support higher fungal diversity than trees at the edges of their range (e.g., tanoaks). This aligns with previous findings on ectomycorrhizal fungi (Lankau and Keymer, 2016) and is consistent with our observation of reduced wood mycobiome richness in tanoaks, a species at the southernmost edge of its range.

5. Conclusions

Overall, our results indicate that planting or conserving individual (mature) pine trees across the landscape may contribute significantly to fungal biodiversity, at least with regards to caulosphere fungi, potentially surpassing the fungal diversity of pine woodlands and even rivaling the fungal diversity of entire oak woodlands, notorious for being hotspots of biodiversity in general. Single trees may act as ecotones. Fostering considerable variability in fungal diversity. As observed in a broad body of literature (Crandall et al., 2020; Grilli et al., 2017; Rudolph et al., 2018; Su et al., 2022), local mosaic diversity creates structural heterogeneity that supports a greater number of fungal species. Therefore, we hypothesize that this environmental diversity positively contributes to higher fungal species richness. Our results support this hypothesis in the context of scattered trees, making their stems a hotspot of fungal biodiversity and an indispensable element in the restoration and management plans aimed at preserving or increasing biodiversity and ecosystem services. We recognize that this study does not address fungal viability and that spores are also detected using the experimental approach employed in this study. Nonetheless, given that this limitation would affect all vegetation types studied, we believe the results of this study do provide an excellent comparative analysis of overall fungal diversity associated with the stems of trees in various habitats. Obviously, the preservation and restoration of woodlands remain fundamental, but our results show that the preservation or restoration of individual trees may also be a valuable tool, one that is bound to cost far less and to provide unexpected large benefits in terms of fungal diversity.

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CRediT authorship contribution statement

Edoardo Scali: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Michael Johnson:** Supervision, Methodology, Investigation, Data curation, Conceptualization. **Giovanni Emiliani:** Formal analysis. **Douglas Schmidt:** Writing – review & editing, Supervision, Data curation. **Tina Popenuck:** Writing – review & editing, Investigation. **Matteo Garbelotto:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data are available upon request and sequence data generated in this study are available through the NCBI Sequence Read Archive (SRA), under the BioProject accession number PRJNA1217464.

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