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Pine species determine fungal microbiome composition in a common garden experiment

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ABSTRACT

The factors shaping the composition of microbial communities in trees remain poorly understood. We evaluated whether the core and satellite fungal communities in five pine species (*Pinus radiata, Pinus pinaster, Pinus sylvestris, Pinus nigra,* and *Pinus uncinata*) were shaped by the host species identity. Because the trees had earlier been inoculated with a fungal pathogen (*Fusarium circinatum*), we also explored the possibilities to detect its presence and potential co-occurrence networks. We found interspecific variation in the fungal community composition and abundance among the different tree species and the existence of a core microbiome that was independent of the host species. The presence of *F. circinatum* was confirmed in some samples through qPCR but the pathogen did not co-occur with a specific fungal community. The results highlight the importance of host species as a determinant of microbiome assembly in common environments.

1. Introduction

Plants are no longer considered as stand-alone entities but are rather seen as meta-organisms or holobionts, i.e., ecological units consisting of both the plant cells and tissues and their associated microorganisms (microbiota) (Sánchez-Cañizares et al., 2017; Berg et al., 2020). The associated microbiome of a plant is composed of endophytic (internal) and epiphytic (external) microbes, mainly bacteria and fungi (Christian et al., 2015). These microorganisms can significantly influence the phenotype of the plants, including plant growth, survival, and their resistance to biotic and abiotic stresses (Partida-Martinez and Heil 2011; Vandenkoornhuyse et al., 2015; Castrillo et al., 2017). Currently, the microbiome (i.e., the complete genetic content of the microbiota (Bordenstein and Theis, 2015) is understood as an extended phenotype of the plant genome, with the potential to strengthen the capacity of plants to cope with environmental stressors (Gehring et al., 2017). Especially in perennial plant parts (Dastogeer et al., 2020) and species such as the forest trees, the importance of the microbiome for the phenotypic traits such as stress and pest resistance may be pronounced (Witzell and Martín 2018). Therefore, in-depth knowledge about the diverse composition of the microbiome could support sustainable forestry in the future, when the trees are expected to be exposed to increased stress due to climate change and the growing threat of pests (Trumbore et al., 2015; Lehmann et al., 2020).

The structural and functional diversity of the plant-associated microbiome is likely to be modulated by multiple interacting drivers, including abiotic factors, such as soil properties, nutrient status, and climatic conditions but also host-plant specific, selective factors such as

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plant age, plant species, and physiological status (Berg et al., 2016; Dastogeer et al., 2020). For example, the features of the surface of the plant (stomata, trichomes, incident ultraviolet radiation, water availability, etc.) will determine the composition of the epiphytes while the internal, apoplast conditions like nutrient availability and especially plant defenses will define the endophyte community (Vorholt 2012; Hacquard et al., 2017). Several common garden experiments, often with fast-growing deciduous tree species such as poplars and aspen (*Populus* spp.), or birch (*Betula* spp.) have revealed the importance of host genotype as a determinant of endophytic communities (Elamo et al., 1999; Lamit et al., 2014; Albrectsen et al., 2018). Yet, the sensitivity of the microbiome to the genotypic traits of the hosts may be limited, e.g., Korkama-Rajala et al. (2007) found no difference in needle inhabiting endophytes among genotypes of Norway spruce (*Picea abies*). Recently, the concept of the core microbiome, i.e., the microbial consortia systematically associated with a given feature: similar habitats, or plant species or genotype regardless of the environment, has received increasing interest in research (Shade and Handelsman 2012; Toju et al., 2013; Berg et al., 2020). These consistently occurring organisms may profoundly influence the function of the host and changes in their composition can determine the community responses to perturbation (Shade and Handelsman 2012; Toju et al., 2018). The underlying assumption is that the composition of the microbial core community is established through evolutionary selection and carries genes essential for plant fitness (Toju et al., 2013, 2018). However, Lemanceau et al. (2017) have questioned the relevance of taxonomic core microbiota and instead emphasized the functional similarity as an important trait of the core microbiome.

Once the microbes have overcome the plant immune system, the interaction with the other microorganisms (co-occurrence patterns or microbial networks) shapes the structure of the endophytic microbial community (Cardinale et al., 2015). These interactions can involve different forms of competition or mutualism (Heydari and Pessarakli 2010). However, it has become increasingly clear that the order and timing of species arrival during community assembly have long-term effects on its composition, a phenomenon called priority effects (Fukami 2015; Carlström et al., 2019). For example, Hoffmann et al. (2021) found that the order of co-cultivation of *Fusarium, Alternaria* and *Pseudomonas* species on wheat plants influenced the outcome showing that the first inoculated species reduced the growth rate of the subsequent ones. While the established microbiome may act as a barrier or facilitator for colonization by an incoming microbe, the latter can also cause shifts in the dynamics and composition of the established microbiome and have consequences for the health of the host organisms (Pascale et al., 2020; Xu et al., 2020). Co-occurrence analyses (Barberán et al., 2012) provide a means to identify the taxa that may have an influential position in microbial networks (Hassani et al., 2018). An interesting experimental approach to gain insights into the nature of microbial interactions is also to study the response of microbial networks when the native microbiome is exposed to an introduced fungal pathogen.

The aim of our study was to increase our understanding of the drivers behind fungal communities in forest trees. Specifically, we hypothesized that the composition of fungal communities would vary among congeneric tree species growing in a shared environment, indicating that the species identity (that translates to substratum quality) is a strong determinant of the community composition. On the other hand, we expected that a limited number of taxa would form a stable core community shared by all species in the same environment. We focused on pines (*Pinus* spp.), a genus of trees with high economic and ecological importance worldwide, and approached the study of the fungal community through culture-independent next-generation sequencing (NGS), i.e. metabarcoding of the ITS region, which enables the detection of unculturable, slow-growing or rare species, which are underrepresented using traditional isolation techniques (Bullington and Larkin, 2015; Dissanayake et al., 2018; Glynou et al., 2018). Because the pines in the experimental area had been inoculated with an introduced fungal pathogen, *Fusarium circinatum*, we were also interested in checking whether this pathogen could be detected as a component of the communities. *Fusarium circinatum* causes Pine Pitch Canker (PPC) disease, which leads to reduced growth of adult trees in forest plantations, resinous bleeding cankers on trunks and large branches, and death of trees due to girdling (Martín-Rodrigues et al., 2013), also causing detrimental effects in nurseries (Wingfield et al., 2002).

2. Material and methods

2.1. Experimental site and sampling

The experimental area was located in northern Spain (Santibáñez, Cantabria, coordinates UTM 398672, 4792705). The site has a northern exposure, an altitude of 340 m a.s.l. and a 35% slope. The climatic features of the plot area are as follows: annual rainfall of 1133 mm, mean temperature of 13 °C, maximum mean temperature of 18 °C and minimum mean temperature of 8 °C (Ninyerola et al., 2005). The plot was part of a trial to test the susceptibility of 13 conifer species against *F. circinatum* (Martínez-Álvarez et al., 2014) in the field and to find an alternative species to replace Monterey pine (*Pinus radiata*) plantations. Twelve 2-year-old seedlings per species were manually planted in a grid with a 2 × 2 m planting distance in June of 2009. The plot was later fenced to prevent damage from animals. In November of 2010, the trees were inoculated using a local *F. circinatum* isolate obtained from the nearest diseased pine growing outside the plot to avoid the introduction of new fungal material. Trees were inoculated in the stems by wounding with a scalpel and pipetting 10 μl of a spore suspension (concentration 10^6 spores mL^{-1}) and then covering the wound with Parafilm© (Martínez-Álvarez et al., 2014). In May 2018, a total of 20 samples were collected from five different pine species (4 samples per tree species) (*Pinus nigra, Pinus pinaster, Pinus radiata, Pinus sylvestris,* and *Pinus uncinata*) which differed in their phenotypic susceptibility to the pathogen (Martínez-Álvarez et al., 2014) at 15 cm height from the ground (approx. the inoculation point). At the time of sampling, all the selected trees (10-year-old) showed clear, visible signs of pathogen infection, i.e., defoliation and presence of red twigs although individuals of *P. uncinata* exhibited fewer symptoms. The external part of the bark was manually removed to reduce the number of fungal propagules randomly present on the trunk surface. One sample (approx. 2 × 2 cm) per tree of phloem and xylem was collected using a blade. After each cut, the tool was disinfected with 2% w/v sodium hypochlorite to avoid contamination between the samples. The samples were immediately placed in a paper envelope and refrigerated at 4 °C until further processing.

2.2. Sample processing and bioinformatics pipeline

The samples were sliced, freeze-dried for 48 h, and then milled into a homogenous powder using tungsten beads in a MM301 ball mill (Retsch GmbH, Germany). Total DNA was extracted using the kit PowerSoil® DNA Powerlyzer (Qiagen, Germany) and purified by using Dneasy Powerlyzer power soil kit (Qiagen, Germany). The fungal internal transcribed spacer region (ITS) was amplified with several personalized WineSeq® primers (see Patent No.: WO2017096385; Becares and Fernández, 2017). The readings were generated using pairs of sequences of 2 × 301 bp (base pairs) with the Illumina MiSeq platform (Illumina Next Generation Sequencing, 2018). We used a customized bioinformatics pipeline to process the sequencing data (Becares and Fernández 2017) with the QIIME software (Caporaso et al., 2010). In brief, sequences were renamed to add sample labels and merged into a single FASTQ file, then we eliminate the adapters and chimeras. After that, the reads were quality-trimmed, and fungal internal transcribed spacer (ITS) sequences were clustered into non-singletons operational taxonomic units (OTUs) at a 97% sequence similarity level. Taxonomy assignation and abundance estimation were obtained by comparing the clusters of

the OTUs registered in the UNITE, GenBank, and WineSeq® (Patent WO2017096385) taxonomic databases.

2.3. Detection of Fusarium circinatum

To confirm the presence of the pathogen in the host tissue, quantitative real-time PCR (qPCR) reactions were performed with a QuantStudio 6 Flex Real-Time PCR System (Life Technologies, USA). Amplifications were carried out in 20-μl reaction volume using the FastGene Probe 2x No Rox qPCR Universal Mix (Nippon Genetics, Japan) and specific primers and probe for *F. circinatum* (FCIR-F, FCIR-R, FCIR-P, Ioos et al., 2009). For each sample, the reaction mix included 0.6 μl of respective forward and reverse primers (0.3 μM) (FCIR-F 5′-TCGATGTGTCGTCTCTGGAC-3′, FCIR-R 5′-CGATCCTCAAATCGAC CAAGA-3′), 0.2 μl of a dual-labeled probe in a concentration of 0.1 μM (FCIR-P, 5'-/56-FAM/CGAGTCTGGCGGGACTTTGTGC/3BHQ_1/-3′), 2 μl of template DNA, 10.0 μl of qPCR Universal Mix and 6.6 μl of sterile distilled water (SDW). DNA concentration was estimated by gel electrophoresis. Since bands were strong and further PCRs were not successful, we diluted the DNA. Therefore, quantitative PCR assays were carried out with samples in three different concentrations including original DNA, dilution 1/100, and 1/1000. In addition, to improve the possibilities of detecting *F. circinatum* a nested PCR with the same specific primers (FCIR-F, FCIR-R) was performed amplifying PCR products obtained in the conventional PCR (adapted protocol of Ioos et al. (2009)) in a qPCR assay. The quantitative PCR cycling conditions included an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation for 15 s at 95 °C and annealing-elongation for 55 s at 70 °C. The Ct value for each reaction was determined automatically by the software QuantStudioTM 6 Flex Real-Time PCR. Samples that presented a Ct value < 20 followed by an amplification curve with an exponential shape were judged as positive (named FC+) in relation to the presence of *F. circinatum* based on previous protocols (International Plant Protection Convention, 2017) otherwise considered negative (FC-).

2.4. Diversity and statistical analyses

All statistical analyses were performed in R environment v. 4.0.3 (R Development Core Team, 2020). Prior to the analysis, OTUs representing <0.005% of the data set and OTUs that only appeared once were removed to prevent inflated estimates of diversity as a result of erroneous reads (Bokulich and Mills 2013). In order to comprehensively evaluate the community, diversity was measured by taxonomic or ecological diversity (α- and β-diversity) and functional diversity (Moreno et al., 2018).

Taxonomic diversity of the fungal endophytic community of every tree was measured by the alfa diversity by the following indexes and estimators: observed taxonomic richness (S), Shannon diversity index (H), Simpson (Simp), Evenness (J) (i.e., species equitability or abundance of each species with Pielou) and diversity (invsimp) calculated with *vegan* package (v. 2.5.7; Oksanen et al., 2020). To evaluate the effect of the species on the ecological diversity indexes, an analysis of variance was calculated followed by a Tukey HSD multiple comparison test (residuals were checked for normality, homoscedasticity, and linearity). When the data did not meet these requirements, a robust ANOVA based on trimmed means using a percentile t bootstrap method (with 599 bootstrap samples) was calculated with *t1waybt* function from the *WRS2* package (v 1.1.0; Mair and Wilcox 2020) followed by a post hoc test with *mcppb20* function. The species richness values were employed to create sample-size-based rarefaction (interpolation) and extrapolation (prediction) curve (Colwell et al., 2012; Chao et al., 2014) with an endpoint of 30 individuals and 100 bootstrap repetitions. The curves were generated with *iNEXT* package (v 2.0.20; Hsieh et al., 2020) and visualized with *ggiNEXT,* the *ggplot2* extension for *iNEXT*. The relative abundances of the most abundant OTUs (present in at least 50% of the samples) were visualized using a heatmap in the *heatmap* function.

Beta diversity of the fungal community was explored by analysing differences in OTUs richness among plant species and were visualized by a robust unconstrained ordination (non-metric multidimensional scaling, NMDS) on the basis of Bray-Curtis distance (Anderson and Willis 2003). In order to evaluate the effect of plant species on the community composition of fungi, permutational multivariate analysis of variance (PerMANOVA) was carried out, using the *adonis* command in the *vegan* package, based on 999 permutations and Bray-Curtis distance. Besides the analysis of the data altogether, to separate core OTUs from satellite OTUs we used a persistence threshold methodology (Barnes et al., 2016; Unterseher et al., 2012). The persistence of each OTUs (i.e., the number of samples where it was found in) was plotted against relative abundance. Thus, the OTUs found in ≥75% of the samples were considered core taxa in our study, and those found in <75% were classified as satellite (low abundance and persistence).

The *eulerr* package (v 6.1.0; Larrson 2020) was used to visualize the shared and specific (indicator) species for the pine species with help of Euler and Venn diagrams. Furthermore, we aimed to identify specific taxonomic groups that were principally responsible for the significant differences in microbial community composition observed among pine species. Two statistical approaches were used for this purpose. The first one was to calculate the partition of the average Bray-Curtis dissimilarity between groups of samples, into components from different taxa, using SIMilarity PERcentage breakdown or SIMPER (Clarke 1993) using the *simper* function in *vegan* package. This allowed identification of taxa that were most important in creating observed patterns in dissimilarity. The second approach was the indicator value (IndVal) analysis (Dufrêne and Legendre 1997) that identifies species based on their specificity (i.e., uniqueness) to a particular habitat or factor and their frequency. The fungal OTUs with an indicator value > 0.25 and a significant P-value ($P < 0.05$) were considered indicator species. IndVal was performed using 999 permutations with the function *multipatt* from *indicspecies* package (v 1.7.9; De Caceres and Jansen 2016).

The functional diversity of the samples was evaluated by assigning a trophic mode of each OTUs based on available information on FUNGuild and checking the functional diversity of fungi. FUNGuild is a flat database hosted by GitHub that assigns a trophic mode to the fungal species investigated (Nguyen et al., 2016). The fungi are categorized as pathotroph, saprotroph, or symbiotroph based on the existing information present in the database (Nguyen et al., 2016). Fungal taxa belonging to putative contaminations from sample handling were removed from the original database in order to identify plant-associated fungi. Relative abundances of trophic modes were calculated for each sample and canonical correspondence analysis (CCA) was employed to analyse and visualize the data using *cca* function followed by *anova.cca* with 999 permutations in *vegan* package.

2.5. Microbial co-occurrence analysis

Co-occurrence analysis of fungal assembling by pine species was performed using the package *cooccur* (v 1.3; Griffith et al., 2016). Datasets were separated by pine species and converted in 0–1 binary matrices (i.e., 0 and 1 respectively mean absence or presence of a single taxon in the corresponding dataset). Co-occurrence matrices included *F. circinatum* as present whenever real-time PCRs resulted in positive amplification. The function *cooccur* was used to calculate the expected (Fexp) and observed (Fobs) number of samples where each pair of taxa co-occur. The effect of underrepresented taxa was controlled by filtering those pairs of taxa for which Fobs was less than 1 (threshold). Hypergeometric distribution was applied to calculate the probability of Fobs being lower or higher than Fexp (Plt and Pgt, respectively). The ecological meaning of each pairwise association was assigned as negative (Fobs < Fexp and Plt<0.05), positive (Fobs > Fexp and Pgt<0.05), or random in the rest of the cases. Maximal deviation of Fexp allowed for classifying any association as random was defined as rc: 0.25 * total

number of samples in the matrix (Veech 2013).

3. Results

3.1. Overview of fungal diversity and detection of F. circinatum

A total of 541 OTUs representing six phyla, 27 classes, and 273 orders were recovered from the samples from different tree species. The sequence datasets generated during the current study are available at NCBI Sequence Read Archive (SRA) Bioproject ID PRJNA704936. The fungal OTUs belonged to phyla *Ascomycota* (71.96%), *Basidiomycota* (23.48%), *Mucoromycota* (1.70%), *Mortierellomycota* (1.89%), *Glomeromycota* (0.75%) and *Kickxellomycota* (0.18%). At class level, fungal OTUs were dominated by *Dothideomycetes* (18%), *Sordariomycetes* (18%), *Eurotiomycetes* (17%), *Agaricomycetes* (11%) and *Leotiomycetes* (6%) (Fig. 1). The NGS analysis did not detect *F. circinatum* but the other six species of *Fusarium* (*F. acutatum, F. oxysporum, F. keratoplasticum, F. delphinoides, F. equiseti,* and *F. pseudensiforme.*) and *Fusarium* sp. were present in all pine species (Supplementary Table 1). According to qPCR, however, 11 samples were considered positive for *F. circinatum* presence (Ct < 20 and exponential-shape of the amplification curve) as seen in Table 1. The best amplification curves resulted from the nested PCR (Supplementary Fig. 1). With the exception of *P. pinaster, F. circinatum* was amplified from tissues of all pine species.

3.2. Evaluation of alfa diversity and OTUs richness

Results from alpha diversity indexes are shown in Fig. 2. The Shannon diversity index values did not differ between the samples from the different host species (ANOVA, F = 1.38, $p = 0.286$). Simpson index (robust ANOVA) was significantly different between *P. pinaster* and all the other species: *P. radiata* ($p = 0.016$), *P. nigra* ($p = 0.000$) and *P. uncinata* ($p = 0.000$). Species richness was significantly higher (robust ANOVA) in *P. uncinata* than in *P. nigra* ($p = 0.000$), *P. radiata* ($p = 0.003$) and *P. sylvestris* ($p = 0.043$), whereas *P. nigra* was different than *P. pinaster* ($p = 0.000$) and *P. radiata* ($p = 0.026$). Evenness was significantly different (robust ANOVA) among *P. uncinata* and *P. pinaster* ($p = 0.01$). Lastly, diversity measured by invsimp index showed significant differences (robust ANOVA) among *P. pinaster* and *P. radiata* ($p = 0.023$), *P. nigra* ($p = 0.006$) and *P. uncinata* ($p = 0.000$). Individual-based rarefaction curves calculated for each of the tested pine species (Supplementary Fig. 2) showed that the OTUs abundance was not saturated in the samples and did not reach asymptotes, suggesting that further sampling would provide more information about missing OTUs.

The heatmap showed that 23 OTUs, (present in at least 50% of the samples) represented 46% of the relative abundance of the total OTUs. The heatmap revealed that the occurrence of some relatively abundant fungal OTUs was very similar among pine species (Fig. 3a). The core fungal microbiome (Supplementary Fig. 3) based on the threshold of abundance (found in ≥75% of the biological samples) was composed of

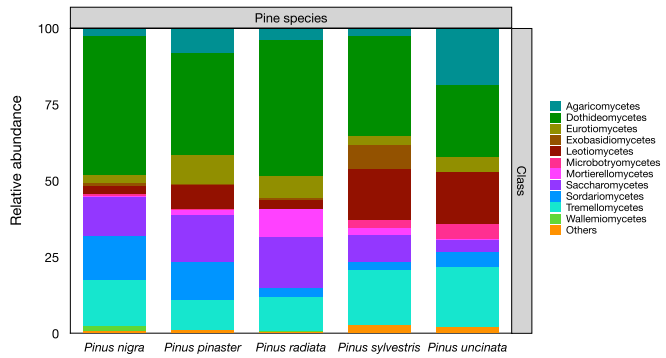

Fig. 1. Relative abundance of fungi at class level in the different pine species.

Table 1
Cycle threshold values obtained in a quantitative polymerase chain reaction (qPCR). * Samples were considered as positive with Ct < 20 (International Plant Protection Convention, 2017). n.a.: no amplification.

Species	Ct value				Presence of *F. circinatum*
	no dilution*	1/100*	1/1000*	Nested PCR	
P. radiata	n.a.	–	–	n.a.	absent
P. radiata	>20	>20	–	29.633	absent
P. radiata	n.a.	–	–	6.344	**present**
P. radiata	7.638	>20	n.a.	9.025	**present**
P. pinaster	>20	>20	–	n.a.	absent
P. pinaster	n.a.	–	–	>20	absent
P. pinaster	15.026	>20	n.a.	n.a.	absent
P. pinaster	>20	n.a.	n.a.	n.a.	absent
P. sylvestris	n.a.	–	–	n.a.	absent
P. sylvestris	>20	n.a.	–	n.a.	absent
P. sylvestris	15.636	n.a.	n.a.	11.023	**present**
P. sylvestris	>20	n.a.	–	13.405	**present**
P. nigra	16.083	n.a.	n.a.	17.857	**present**
P. nigra	16.599	17.623	n.a.	>20	absent
P. nigra	>20	n.a.	–	4.629	**present**
P. nigra	n.a.	–	–	13.407	**present**
P. uncinata	n.a.	–	–	>20	absent
P. uncinata	n.a.	–	–	6.777	**present**
P. uncinata	n.a.	–	–	>20	absent
P. uncinata	14.653	n.a.	–	6.256	**present**
Positive control	24.148	>20	>20	<4 (n.a.)	**present**
Positive control	25.622	18.691	–	<4 (n.a.)	**present**

9 OTUs: *Alternaria alternata, Aureobasidium pullulans, Botrytis cinerea, Cladosporium cladosporioides, Cladosporium halotolerans, Cladosporium herbarum, Cladosporium sphaerospermum, Cryptococcus terreus,* and *Pleospora herbarum*. The core microbiome based on 100% of occupancy (shared among all samples) was composed by *Alternaria alternata* and *Pleospora herbarum.* All pine species hosted similar numbers of unique OTUs except *P. uncinata* that had the highest number of OTUs (Supplementary Fig. 4). Results from the PERMANOVA showed that the core fungal microbiome was stable across the samples, and was not dependent on the pine species (R = 0.09, $p = 0.097$). However, the satellite microbiome was dependent on pine species (R = 0.214, $p = 0.01$). We found dissimilarities between the satellite community composition of *P. pinaster* and *P. sylvestris* ($p = 0.021$) and *P. pinaster* and *P. uncinata* ($p = 0.033$) (Supplementary Fig. 5).

3.3. Beta diversity of fungal communities in different pine species

The relative abundance of fungal communities was significantly different among pine species ($p = 0.035$) (Fig. 4). The pairwise comparison showed that significant differences were found between fungal communities from *P. pinaster* vs *P. sylvestris* ($p = 0.028$), *P. pinaster* vs. *P nigra* ($p = 0.033$), and between *P. sylvestris* vs *P. nigra* ($p = 0.036$). The SIMPER analyses identified the OTUs that explained most of the dissimilarities within pine species. For example, the fungal taxa *Alternaria alternata, Cryptococcus terreus, Lachnum virgineum, Pleospora herbarum,* and *Cladosporium ramotenellum* accounted for 35% of the dissimilarities between *P. sylvestris* and *P. nigra*. Complementary, IndVal analyses were conducted to detect OTUs significantly associated with the different pine species (Table 2). A total of 13 OTUs were identified, 3 OTUs as indicators for *P. nigra*, 5 for *P. pinaster*, 4 for *P. uncinata,* and 1 was shared among *P. nigra* and *P. sylvestris*.

3.4. Co-occurrence analysis and functional guilds

The co-occurrence analysis revealed that all association in the analyzed fungal communities were random with no specific co-occurrences between *F. circinatum* and other fungal OTUs. More

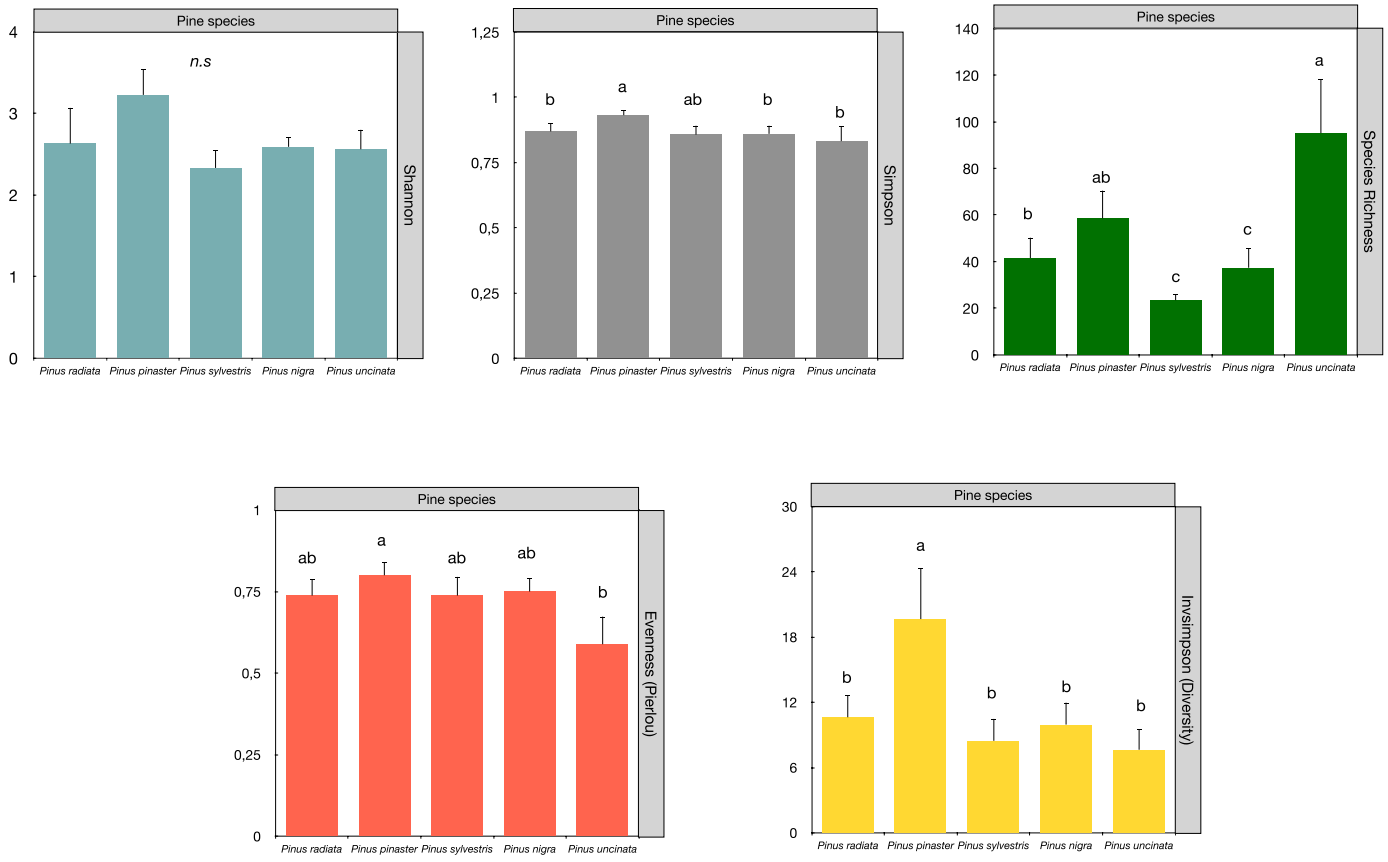

Fig. 2. Alpha diversity indexes (mean value ± standard error) of samples from different pine species. Means with different letters show values significantly different at $P < 0.05$ (robust ANOVA). n.s = non significant.

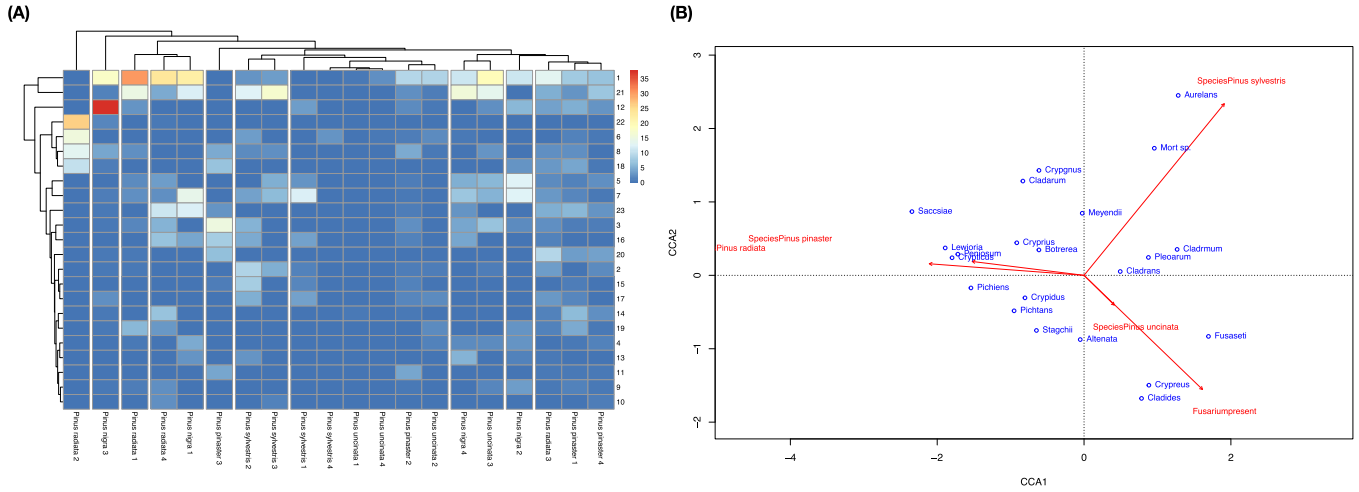

Fig. 3. (**A**) Heatmap of the OTUs present in at least 50% of the samples (23 OTUs). Numbers correspond to the following OTUs: (1) Altenata, *Alternaria alternata*; (2) Aurelans, *Aureobasidium pullulans*; (3) Botrerea, *Botrytis cinerea*; (4) Cladides, *Cladosporium cladosporioides;* (5) Cladrans, *Cladosporium halotolerans*; (6) Cladarum, *Cladosporium herbarum;* (7) Cladrmum, *Cladosporium sphaerospermum;* (8) Cryprius, *Cryptococcus aerius;* (9) Crypidus, *Cryptococcus albidus;* (10) Crypgnus, *Cryptococcus magnus;* (11) Crypticus, *Cryptococcus podzolicus,* (12) Crypreus, *Cryptococcus terreus;* (13) Fusaseti, *Fusarium equiseti* (14) Lewioria, *Lewia infectoria;* (15) Metsrima, *Metschnikowia pulcherrima,* (16) Meyendii, *Meyerozyma guilliermondii;* (17) Mort sp. *Mortierella* sp., (18) Peniosum, *Penicillium lapidosum;* (19) Pichtans, *Pichia fermentans;* (20) Pichiens, *Pichia membranifaciens;* (21) Pleoarum, *Pleospora herbarum*; (22) Saccsiae, *Saccharomyces cerevisiae* (23) Stagchii, *Stagonosporopsis dorenboschii.* (**B**) Variation in core fungal OTUs (50% threshold) visualized by Canonical Correspondence Analysis (CCA) ordination biplot (axes 1 and 2) based on the pine species.

specifically, 76.95% species co-occurrences were discarded by threshold use in *P. nigra* samples resulting in 4586 analyzed co-occurrences. The total number of analyzed co-occurrences in *P. pinaster*, *P. radiata, P. sylvestris,* and *P. uncinata* datasets were 6691 (66.38%), 4942 (70.32%), 3125 (76.62%), and 23511 (64.61%), respectively (numbers in brackets denote the percentage of pairs removed after threshold use). The analysis was run also without threshold use showing complete random associations in each dataset.

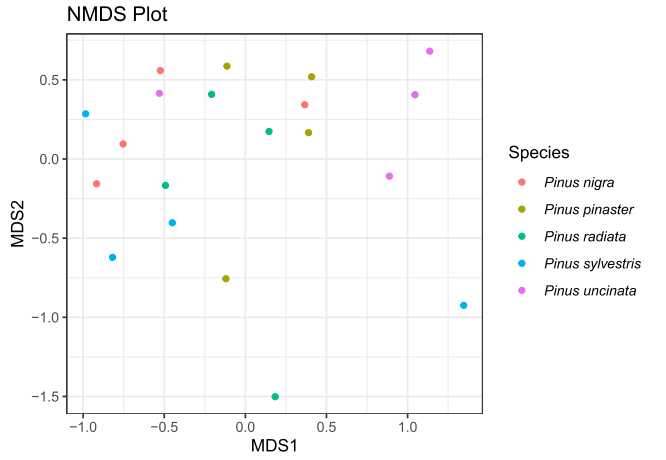

Fig. 4. Non-metric multidimensional scaling (NMDS) ordination plots of fungal communities based on Bray–Curtis dissimilarity matrix representing different pine species.

Table 2
Indicator Value (IndVal) analyses, relative abundance of each fungal taxon (%), Indval value and p-values.

Species	Indicator fungal taxa	Relative abundance (%)	Indval value	P value
Pinus nigra	*Pichia kluyveri*	5.54	0.707	0.0149
	Cladosporium cladosporioides	2.40	0.656	0.0229
	Fusarium equiseti	2.44	0.644	0.0203
Pinus pinaster	*Mortierella clonocystis*	0.42	0.754	0.0181
	Cryptococcus podzolicus	2.42	0.738	0.0026
	Aspergillus piperis	1.02	0.692	0.0124
	Metschnikowia sp.	0.98	0.611	0.0183
Pinus radiata	*Trechispora cohaerens*	2.79	0.654	0.0479
Pinus uncinata	*Pilidium concavum*	10.7	0.586	0.0348
	Chrysosporium lobatum	0.07	0.554	0.0171
	Nakazawaea ernobii	0.27	0.503	0.0171
	Acremonium alcalophilum	0.31	0.467	0.0177
Pinus nigra + *Pinus sylvestris*	*Cladosporium sphaerospermum*	7.26	0.634	0.0412

Pine species and *Fusarium* re-isolation (Fc+ and Fc-samples) exhibited no effects on the relative abundance of each fungal functional guild (Fig. 5) based on the permutational test (cca.anova). The canonical correspondence analysis produced eigenvalues (i.e. the amount of the original variance explained by each of the axes) of 0.087, 0.078, 0.036, and 0.003 for axes 1,2,3, and 4 respectively from total inertia of 0.68, (i.e. total variance explained) indicating that no association was found between our variables. However, based on graphical visualization, *Pinus uncinata* showed a positive effect on pathotroph-saprotroph and *P. sylvestris* a negative one on pathotroph-saprotroph-symbiotroph (Supplementary Fig. 6).

4. Discussion

Our results indicate that the fungal community of pine trees growing in a common environment shows host species-specific structures. The finding is in agreement with several earlier studies, in which host species has been identified as one of the major factors shaping the mycobiome of plants (Schulz and Boyle 2005; Stone et al., 2000) and providing selective pressure during community assembly (Lebeis 2014). The high dissimilarity of communities between *P. pinaster*, *P. sylvestris,* and *P. nigra* may reflect the differential quality of the pine species as a substratum for fungi (Wang et al., 2019). The species used in the study belong also to different pine clades: *P. sylvestris, P. nigra,* and *P. uncinata* corresponding to section *Pinus*, subsection *Pinus*; *P. pinaster* to subsection *Pinaster* and *P. radiata* to section *Trifoliae*, subsection *Australes* (Eckert and Hall, 2006; Gernandt et al., 2005). These species differ in several features, such as chemical composition (e.g. secondary metabolites) and anatomical characters (Camarero et al., 1998; Ferreira-Santos et al., 2020) as a result of an evolutionary adaptation to different habitats. Species-specific traits such as leaf length, water, and nitrogen content or stomatal conductance (among others) have been found to shape the phyllosphere microbial community among *Picea* species in a common garden experiment (Li et al., 2018). Variability in microbial communities has also been related to genotypes (Cregger et al., 2018; Terhonen et al., 2019; Leopold and Busby, 2020) and to the developmental stage of the host (Skaltsas et al., 2019). More detailed studies would, however, be needed to unravel the importance of the specific host traits and microbial community composition in the studied pines.

We examined the partitioning of fungal communities into core (common, stable, and well-adapted) and satellite (more rare and stochastically present) fractions. The *core-satellite* hypothesis predicts that core taxa have the largest impact on the ecosystem (Unterseher et al., 2012; Berg et al., 2020). In our study, the fungal core microbiome was composed of nine OTUs with high persistence (≥75%), mainly ubiquitous fungi, and not genus-specific. The functional role of the core community in pines is therefore difficult to evaluate. Some of the identified core species such as *Cladosporium* spp. could be beneficial for the host since they have been described as plant growth-promoting endophytes (Hamayun et al., 2009), or inducers of systemic resistance in plants (Naznin et al., 2014). Other fungi have been shown to be antagonists (*Alternaria* spp.) of *F. circinatum* on infected pine seedlings (Martínez-Álvarez et al., 2016) or pathogens on other plant species, such as *Botrytis* spp. (Li et al., 2018). Nevertheless, the interpretation of the

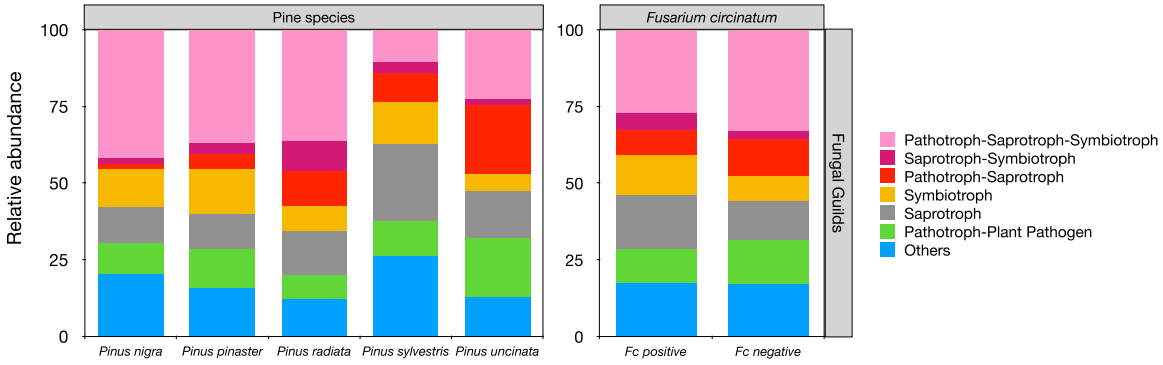

Fig. 5. Relative abundance of each trophic mode in different pine species in positive/negative samples of *F. circinatum*.

functional role of the core community could be challenging because many fungal species can change from several types of symbiosis with the host depending on the different factors. For instance, a reduced defensive capacity of a tree can trigger a shift of an endophyte to a pathogenic phase (Sieber 2007). In our study, the fungal satellite species composition in *P. pinaster* seemed to be different from that in *P. sylvestris* and *P. uncinata,* but whether this finding has a link to the disease susceptibility of the species is unclear. Both SIMPER and Indicator species analysis indicated that only a few species were responsible for the dissimilarities between species.

The use of NGS in fungal community studies has been discussed previously since these studies give a detailed picture in terms of diversity and community composition (Tedersoo et al., 2019; Lucaciu et al., 2019). A major advantage of the amplicon-NGS culture-independent approach is the ability to detect slow-growing microbes and microorganisms that cannot grow on artificial culture media (Blumenstein et al., 2021). However, both active and inactive fungal taxa are detected by amplicon sequencing since they do not differentiate live from dead cells (Lebeis, 2014). Therefore, analyzing microbial communities based on rRNA instead of their rRNA genes is assumed to reflect the physiologically active microbiota in a sample (Knief, 2014). However, for a functional description of a microbial community, the Illumina sequencing platform is considered a good choice that allows sequencing to high depth in order to gain as much information as possible, especially from less-dominant microorganisms that may nevertheless play important roles for ecosystem functioning (Knief, 2014).

We are also aware that the use of relative abundance data can lead to misinterpretations of microbial community structures, as the increase of one taxon leads to the concurrent decrease of the other(s) (Jian et al., 2020). A putative solution for this could be combining real-time PCR and next-generation DNA sequencing to provide absolute concentration and quantitative comparisons of fungal populations as previously seen accurate estimation of absolute taxon abundances from NGS data (Dannemiller et al., 2014; Jian et al., 2020).

In our study, rarefaction curves did not reach the plateau, suggesting that more sampling effort could provide a more accurate description of the fungal biota. This is a common problem in microbiome studies and emphasizes the general need for a larger sampling effort in these kinds of studies (Bullington et al., 2021). However, this approach is a good proxy for identifying the members of microbial communities or comparing their composition in different samples. Diversity studies are usually based on the ITS region (Knief, 2014), which is used as a standard solution in many recent publications and has been described as a universal marker of fungi (Schoch et al., 2012; Kõljalg et al., 2013). In spite of this, the data from the high-throughput sequencing using ITS primers showed the absence of *F. circinatum* in the samples. The use of alternative markers with proven specificity at the species level in the genus *Fusarium* (e.g. region of the translation elongation factor 1-alpha (*EF-1alpha*) gene, International Plant Protection Convention, 2017) could have clarified the identification of the pathogen. However, there were some technical limitations in Illumina that make the use of such markers difficult because the amplicon sizes of these markers do not fit with the requirements of the sequencing platform (Blumenstein et al., 2021). Therefore, while the universal barcode ITS is very useful for fungi in general, for *Fusarium* genus it is not sufficiently polymorphic for species that are closely related (International Plant Protection Convention, 2017). Thus, both quantitative and nested PCR appear as good tools to detect *F. circinatum* in plant samples.

Plant infection by pathogenic microbes often correlates with microbial community shifts (Bullington et al., 2018; Hassani et al., 2018), but contrary to our expectations, the detection of *F. circinatum* in the samples did not seem to be accompanied by a different co-occurrence of fungal assemblage. The presence of the pathogen was not related either to the relative abundance or any trophic mode. This may reflect the difficulty of capturing the spatial and temporal patterns of fungal networks through a sampling effort that is limited due to practical reasons (Bullington et al., 2021). Based on earlier studies (Iturritxa et al., 2012, 2013), *P. radiata* and *P. uncinata* were expected to be the most susceptible of the studied species and *P. sylvestris* the lowest (Martínez-Álvarez et al., 2014). However, the analyses did not suggest either a higher presence of *F. circinatum* or a higher presence of symbiotic mutualists in the samples collected from these trees. The patchiness of spatial variation in colonization is a problem in most tree microbiome studies, and thus a specific target taxon could escape detection that is based on a small amount of biomass. Nevertheless, we standardized our sampling procedure in order to acquire comparable data. In our study, the samples were collected 8 y after the inoculation and the trees were growing in the field; thus, the impact of the environment and the sampling time may have modified the outcome of the tree-pathogen interaction. Isolate-specific differences in resistance (Iturritxa et al., 2013) or differences in growth conditions, inoculation method, or timing of the sampling may also explain this discrepancy. Further studies in controlled conditions are recommended to overcome these limitations.

5. Conclusions

Our study provided evidence for the relative importance of the host species as a selective determinant of the endophytic communities in pines. The capacity of host species to drive the microbial community was evidenced and explained by the fact that they provide different habitats and resources. Further analyses examining the host phenotypic characteristics and their impacts on the microbiome are still needed to explain the species-specific patterns. Similar to many other microbiome studies our sampling effort covered only a small spatial fraction of the plants, which limits broader generalizations based on the collected data. Further studies are warranted to evaluate possibilities to unravel the growth and health of the trees using a holobiome approach.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2021.101137.

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