Fungal Communities of Scots Pine Needles from a Marginal, Understudied Population in Türkiye

Funda Oskay,* Figen Çakır, and Meriç Çakır

Scots pine (*Pinus sylvestris* L.), a keystone species in Eurasian forests, supports diverse fungal communities and thus contributes to forest health and ecosystem functions. The southern marginal populations of *P. sylvestris* in Türkiye, situated in more arid and warm conditions, offer a unique environment to explore fungal biodiversity. In this study, the authors investigated the fungal assemblages in living (green) and dead (senescent, fallen, and litter) Scots pine needles in Türkiye. Using a culture-based approach coupled with DNA sequencing, distinct fungal communities were identified across different needle types. Frequent isolates included well-known *P. sylvestris* associates, including *Lophodermium pinastri*, *Sydowia polyspora*, and *Cyclaneusma minus*. Novel findings, such as unidentified Eurotiomycete and Basidiomycete taxa, suggest potentially new species and hidden fungal diversity in this biogeographically important region. Additionally, this study reports the first records of *Desmazierella acicola* and *Phacidium lacerum* in Türkiye, further underscoring the region's underexplored fungal biodiversity. Moreover, the biodiversity and community structure analysis revealed the intricate and complex nature of fungal colonization and succession, with significant variations between green, senescent, freshly fallen, and litter needles sampled concurrently. These findings provide new insights into the fungal communities of marginal, understudied Scots pine populations.

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INTRODUCTION

Scots pine (*Pinus sylvestris* L.), a keystone species across Eurasia, is the most widely distributed pine species globally. Its range extends across northern, central, and southern Europe to eastern Siberia, including Türkiye. Across its wide Eurasian distribution, encompassing boreal, temperate, and Mediterranean climates, its populations exhibit significant phenotypic and genetic variations (Jasińska *et al.* 2014; Durrant *et al.* 2016; Żukowska *et al.* 2023). The southernmost populations, such as those in Asia Minor, are particularly noteworthy as Tertiary relics, located in isolated, mountainous regions. The Turkish populations inhabit the warmest and most arid areas within the species' range and possess distinct adaptive traits, making them ecologically marginal populations (Dering *et al.* 2021). Scots pine is the third most widely distributed forest tree species in Türkiye and its forests extends from the Black Sea coast to the semiarid continental parts of Central Anatolia, covering approximately 2.1 million hectares representing about 6% of the country's total forested land.

Scots pine populations, particularly those at the dry edge of the species' range in the Mediterranean Basin, including Türkiye, are especially susceptible to climate change (Takolander *et al.* 2019; Dering *et al.* 2021). Studies demonstrated possible distribution range changes for various species, including Scots pine, in the face of climate change in Türkiye (*e.g.,* Bulut and Aytaş 2023; Acarer 2024; Acarer and Mert 2024). Marginal populations are crucial for understanding how species adapt to climate change and other stressors. Studies from such areas can enhance our understanding of species' resilience and biodiversity in response to changing environments. However, to fully comprehend species' resilience and biodiversity under climate change, it is essential to have comprehensive knowledge of the biodiversity of organisms associated with the species, obtained from a wide range of geographical areas with varying climatic conditions for detailed comparisons. While the Turkish Scots pine populations represent an important reference point for research on species' resilience and biodiversity, preliminary studies documenting the biodiversity in these marginal populations are needed to lay the groundwork for future ecological research.

Scots pine fosters diverse ecological interactions, particularly with fungi, which are essential for forest ecosystem health (Baldrian 2017; Baldrian *et al.* 2023; Terhonen *et al.* 2019). It is well known that the fungi play pivotal roles in nutrient cycling, decomposition, and maintaining tree vitality through symbiotic or parasitic relationships (Hyde and Soytong 2007; Peay *et al.* 2010; Hyde *et al.* 2020). These processes are enabled by the capacity of fungi to produce diverse enzymes and a broad range of bioactive compounds. Consequently, in addition to their ecological importance, fungi are of considerable value in biotechnology, with applications spanning from production of enzymes and biopesticides to bioremediation of contaminated sites (*e.g.,* Hyde *et al.* 2019; Alghonaim *et al.* 2023; Al-Rajhi *et al.* 2023a,b; Kılıçoğlu 2024). Nevertheless, despite extensive research on fungal communities in Scots pine ecosystems, most studies have focused on northern and, to some extent central and southern Europe (Zamora *et al.* 2008; Hui *et al.* 2011; Terhonen *et al.* 2011; Kipfer *et al.* 2012; Martínez-Álvarez *et al.* 2012; Rajala *et al.* 2012; Peršoh 2013; Põlme *et al.* 2013; Millberg *et al.* 2015; Sanz-Ros *et al.* 2015; Taylor *et al.* 2019; Lynikienė *et al.* 2020; Agan *et al.* 2021; Qu *et al.* 2021; Marčiulynienė *et al.* 2022; Schönrogge *et al.* 2022; Mishcherikova *et al.* 2023), leaving the fungal diversity in its isolated populations, including Türkiye, unexplored. The Turkish Scots pine populations, subject to Mediterranean and semi-arid climates, offer a distinct biogeographical context for studying fungal communities. Addressing these gaps would enhance our understanding of fungal communities in Scots pine ecosystems, both in Türkiye and globally. This knowledge is crucial for conservation efforts predicting environmental changes and exploring fungi's biotechnological potential.

Taken together, the aim of this study is to provide a preliminary understanding of the fungal communities associated with *P. sylvestris* needles in Türkiye. Rather than making a detailed comparison with fungal assemblages from other parts of the species' range, the focus was to document the fungal taxa present in these marginal populations. This foundational work sets the stage for future ecological studies that can explore how fungal communities in these populations differ from those in other regions and how they might influence the resilience of *P. sylvestris* under climate change. Additionally, this baseline could support future research into the complex ecological interactions in these understudied areas. The study area is located within a research forest, which facilitates multidisciplinary collaborations. The accumulated data from previous and ongoing long-

term studies in this area provide a valuable foundation for further investigation of these ecological interactions.

This study investigated the culturable fungal diversity associated with Scots pine needles, spanning the continuum from living (green) needles to those in early stages of decomposition (senescent, fallen, and litter). The authors specifically investigated how fungal communities vary across different needle types, with a focus on whether dead and litter-stage needles harbor more diverse and complex communities. Additionally, variations between green, senescent, freshly fallen, and litter needles sampled concurrently were evaluated to understand fungal successional patterns.

EXPERIMENTAL

Description of Study Area and the Sampling Plot

The study area is situated in the Research Forest of the Forestry Faculty in north-central Türkiye, approximately 30 km northeast of Çankırı (latitudes 40°30'33.98"N to 40°30'32.00" N and longitudes 33°25'50.49"E to 33°26'17.49"E.). The area has a semi-arid climate with an average annual temperature of 11.2 °C and an annual precipitation of 450.3 mm. The forests in the area are dominated by Anatolian black pine (*Pinus nigra* J.F. Arnold subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*). In addition, there are also pure Scots pine stands found within the area surrounded by the Anatolian black pine stands. The area is located in the Irano-Turanian phytogeographical region, and the flora of the research forest was recently investigated by Tuttu and Ursavaş (2022). The Scots pine in the area has been identified as *Pinus sylvestris* L. var. *sylvestris* (Tuttu and Ursavaş 2022). However, due to uncertainties in taxonomic nomenclature, the Scots pine in the study area is referred to as *Pinus sylvestris* L. to prevent any potential misunderstandings in the future.

In the research forest, long-term sampling plots have been set up in various forest stands to investigate litterfall and pine needle decomposition rates (Çakır 2019; Çakır *et al.* 2020, 2023). Litterfall is being monitored in these sampling plots using litterfall traps, while the decomposition rates of pine needles are being studied *via* litterbags placed on the forest floor. Each sampling plot consists of three sub-plots, each measuring 15 m by 15 m and spaced approximately 150 m apart. Within each sub-plot, there are nine litterfall traps, each positioned at a specific distance from the center of the sub-plot.

Needle samples were collected from one of the long-term sampling plots established in a Scots pine stand in this research forest (40°30'11.4"N 33°27'13.3"E, 1634 m a.s.l.) (Fig. 1a, b). The average age of Scots pine trees in this stand was 72 with an average diameter of 25 cm and 15 m height. The amount of litter per square meter was 4096 g m^{-2}, and the litter layer consisting of OL, OF, and OH horizons reached up to 4 cm with a Eumacroamphi humus form (Çakır *et al.* 2020). The litterfall traps were set up in September 2018, and the and litterbags were prepared and laid on the forest floor in Nowember 2018 as described in previous studies (Çakır 2019; Çakır *et al.* 2023).

Collection of Needle Samples

To investigate the fungal communities of Scots pine needles at various life stages (living, senescent, dead, decomposing), the authors collected four types of needle materials: 1) healthy-looking green needles (GN); 2) senescent needles (SN) attached to twigs (same twigs as GN collected); 3) naturally fallen needles accumulated in litterfall traps (FN); and 4) decomposing litter needles in litterbags (LN). Litter needles (LN) and

litterfall needles (FN) were collected from a total of nine litterfall traps and litterbags (three traps and litterbags per sub-sampling plot). Litterfall traps contained fallen needles from the tree canopies accumulated over a 4-week period. Litterbags contained Scots pine needles laid on forest floor over an 8-weeks period. Living and senescent (GN and SN) needles were sampled from nine Scots pine trees (three trees per sub-sampling plot) (Fig. 1c, d). All needle samples were collected in same day (17.12.2018) in plastic bags and processed in the laboratory within 24 h.

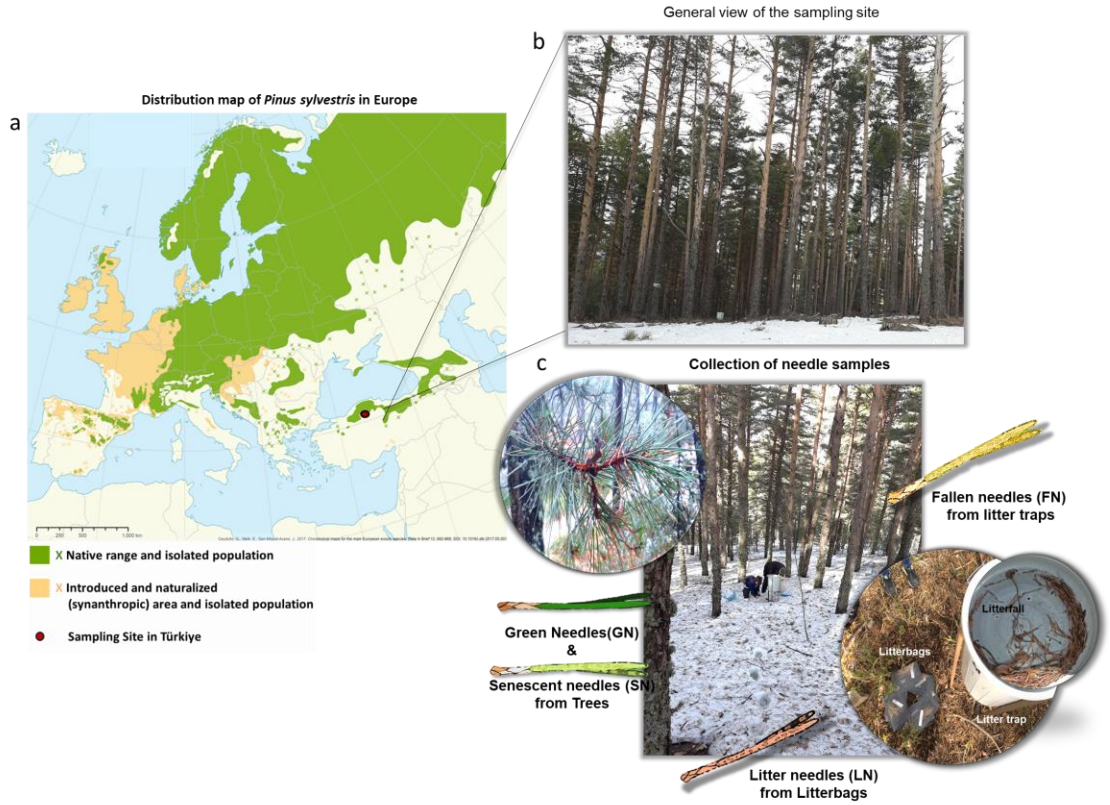


Fig. 1. Description of sampling location and sample collection for the study: (a) Map showing the native range and isolated populations (Green areas), and introduced and naturalized populations (Yellow areas) of *Pinus sylvestris* in Europe (Caudullo *et al.* 2018), and the location of the sampling site in Türkiye (red dot); (b) general view of the sampling site (c) collection of needle samples and visualization of needle sample types (GN: Green needles and SN: Senescent needles from trees; FN: Fallen needles from litter traps; LN: Litter needles from litterbags)

Isolation of Fungi

A total of 180 needle samples; 15 needles per needle type, per sub-sampling plot were selected to be used in fungal isolations (15 needle x 3 sub-sampling plot x 4 needle type). To determine the fungi colonizing inner tissues of needles (endophytic fungi), needles were subjected to surface sterilization prior to plating. Needles were surface sterilized by immersing first in 70% ethanol for 1 min, followed by 4% sodium hypochlorite solution for 5 min, then 70% ethanol for 30 s, and finally rinsing in sterile distilled water twice for 5 min. The surface-sterilized needles were dried on sterile filter papers in a laminar airflow cabinet. Needles were then cut into 0.5-cm-long fragments and plated onto Petri dishes containing 2% (w/v) malt extract agar (MEA, Fluka, Sigma Aldrich, Germany). For each needle, four fragments were plated. In total 1440 fragments (360 per needle type) were plated. Petri dishes were wrapped with parafilm and incubated

at room temperature in the dark. Emerging colonies from needle fragments were grouped into morphotypes according to morphological characteristics (*e.g.*, growth rate, color and structure of colonies, and sporulation) and representative colonies were transferred to new Petri dishes containing MEA. Reference isolates representing each morphotype group were selected and subjected to molecular identification. At least one isolate from each morphotype was selected as reference isolate and processed for DNA extraction and sequence analysis (Fig. A1).

Molecular Analysis

Genomic DNA was isolated from approximately 50 to 100 mg of fresh mycelium harvested from the colony edges of three-week-old colonies of reference morphotype isolates grown in MEA. The mycelium was ground and homogenized in 2.0-mL microcentrifuge tubes with sterile metal beads (Ø 1.6 mm) using a Retsch MM400 homogenizer (Retsch GmbH). Genomic DNA was extracted using NucleoSpin® Plant II kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocols.

Amplification of the internal transcribed spacer (ITS) region was acquired using the fungus-specific primer pair ITS1F/ITS4 (White *et al.* 1990; Gardes and Bruns 1993). Polymerase chain reaction (PCR) was performed in 50 µL reactions and consisted of the following final concentrations, 5 ng.µL−1 template DNA, 200 µM of dNTPs; 750 µM of MgCl2; 0.025 µM polymerase (5 U/µ L) (FIREPol® DNA Polymerase, Solis Biodyne, Estonia), and 200 nM of each primer in 1 × buffer. The PCR program started with denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 45 s, annealing at 57 °C for 45 s and 72 °C for 60 s, followed by a final extension step at 72 °C for 5 min. Samples were sent for Sanger sequencing to BMlabosis, Ankara Türkiye. The retrieved raw sequence files were manually edited and aligned using BioEdit version 7.2.5 software.

Taxonomic assignments were made based on BLAST (Basic Local Alignment Search Tool, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on August 2024) queries against NCBI's rRNA_typestrains/ITS_RefSeq_Fungi (Internal transcribed spacer region (ITS) from Fungi type and reference material) (O'Leary *et al.* 2016) or nt (nucleotide collection) databases using BLASTN 2.14.1+ (Zhang *et al.* 2000). Uncultured/ environmental sample sequences were excluded from the searches. The results were double-checked to obtain a set of reliable reference sequences for each taxon with a focus on dependable culture collections, such as the Westerdijk Fungal Biodiversity Collection (CBS), particularly for those analyzed in CBS sequencing project (Vu *et al.* 2019) (Table 1). BLAST searches were repeated using the Blast 2 sequences program with the reference sequence sets as subject sequences (Zhang *et al.* 2000). Furthermore, taxonomic assignments at the species-level were reworked against the literature to confirm the association of taxa with *P. sylvestris* or other pines.

Functional groups of fungi identified to at least the genus level were assigned using the curated reference database of FungalTraits ver. 1.2 (Põlme *et al.* 2020) and FUNGuild [https://github.com/UMNFuN/FUNGuild (Nguyen *et al.* 2016)].

Data Analysis

The colonization frequencies of fungi were determined as the percentage of needle fragments colonized [Percentage frequency= (number of needle fragments from which the fungus was detected/total number of needle fragments) x 100].

Abundance (number of colonized needle fragments by a given fungus = number of fungi isolated) data were used to quantitatively infer the composition and diversity of the

fungi. Diversity measures, such as Species richness (S) and Shannon index (H′) were calculated using the software PAST (Hammer and Harper 2001). To determine the effect of needle type on the composition of fungal community, a permutational multivariate analysis of variance (PERMANOVA) was performed using PAST (Hammer and Harper 2001). Detrended Correspondence Analysis (DCA) was used in this study with CANOCO version 5.0 to determine compositional differences between fungal groups isolated from various types of needles (Šmilauer and Lepš 2014).

RESULTS

Identifications and Taxonomic Diversity

The isolations from 180 needles resulted in a total of 480 fungal isolates, which were classified into 49 different morphotypes. Upon microscopic examination, two of these morphotypes were identified as *Alternaria* spp. Molecular identification of twelve morphotypes was unsuccessful, and these isolates remained unidentified (UN). Based on sequence homologies obtained from BLAST results, seven taxa were identified to the species level (*Cenangium ferruginosum*, *Cyclaneusma minus sensu lato*, *Desmazierella acicola*, *Lophodermium pinastri*, *Microsphaeropsis olivacea*, *Phacidium lacerum*, and *Sydowia polyspora*), two to the genus level (*Cladosporium* sp. and *Epicoccum* sp.), and two were assigned a higher taxonomic rank (*Phaeomoniellaceae* sp. and *Basidiomycota* sp.). Including the unidentified morphotypes and the morphologically identified *Alternaria* spp., 25 taxa in total were determined (Table 1).

Alignments with reliable reference sequences (Table 1) enabled species-level identification *of C. ferruginosum, C. minus, D. acicola, L. pinastri, M. olivacea P. lacerum*, and *S. polyspora*. The *Cenangium* isolate yielded an amplicon of approximately 600 bp and showed 100% similarity to the reference sequences of *C. ferruginosum* (MH859846.1, MH854762, LT158467.1, LT158433.1, LT158434.1) including those from the type strains (CBS 556.70, CBS 111.24). On the contrary, 98.87% to *Cenangium japonicum* (LT158458.) and 85 % to the reference sequences of *Cenangium acuum* (LT158439.1, LT158436.1). *Cyclaneusma* isolates yielded amplicons of approximately 600 bp. This result agrees with previously reported ITS amplicon size for *C. minus* CBS 496.73 isolate (Hunter *et al.* 2016). Isolates showed 99.8 to 100% similarity to the reference sequences of *Cy. minus* (MH860757.1, KU170126.1, NR_153910.1) and 99.2% to *C. niveum* (KU170127.1). Despite the high sequence homology, the amplicon sizes of our *Cyclaneusma* isolates were shorter than the expected size for *C. niveum* CBS 495.73 isolate (KU170127.1) which is reported to be approximately 800 bp. Additionally, Hunter *et al*. (2016) reported *Cy. niveum* and *C. minus* "simile" strains to produce an amplicon of the same size. However, it is also important to note that there is no ITS DNA sequence data available in GenBank for delineating the *C. minus* morphotype. On the other hand, new species descriptions for the *C. minus* morphotypes (simile, verum and novus) is still in question. Taken together, all *Cyclaneusma* isolates obtained in this work were identified as *Cyclaneusma minus* sensu lato. *Lophodermium* isolates showed 99.68 to 100% similarity to the reference sequences of *L. pinastri* (MH856646.1, MH856647.1) from the type strains (CBS 323.50, CBS 324.50). In contrast, they were distantly related to other frequently occurring *Lophodermium* species on pines including *Lophodermium seditiosum* and *Lophodermium conigienum* (<90%). *Desmazierella* isolates yielded amplicons of approximately 600 bp with 99.7 to 100 % similarity to the reference sequences of *D.*

acicola (LN589932.1, LN589933.1;) from the type strains (CBS209.50, CBS302.81). For some of the Dothideomycet isolates [*Epicoccum* (Didymellaceae) and *Cladosporium* (Cladosporiaceae)], the sequence homologies showed 100% similarity to multiple species in BLAST searches against even sequences from type materials. Consequently, due to the ambiguity and overlap in genetic sequences, the authors refrained from assigning species-level identifications to the *Cladosporium* and *Epicoccum* isolates, as it would be unreliable.

Table 1. Fungal Taxa Identified from The Scots Pine Needles Based on BLAST Searches (*Reference sequences used for BLAST searches using the Blast 2 sequences program)

	Closest GenBank Match			
Identification	ID	%	Accession No	Culture Collection Strain
Cenangium ferruginosum	*C. ferruginosum*	100	MH85984*	CBS 556.70
Cladosporium sp.	*Cl. antarcticum*	100	NR_121332	-
Cyclaneusma minus s.l.	*Cy. minus*	99.8 to 100	MH860757*/ KU170126. */ NR_153910*	CBS 496.73
Desmazierella acicola	*D. acicola*	100	LN589932*	CBS209.50
Epicoccum sp.	*E. layuense*	100	NR_158265	
Lophodermium pinastri	*L. pinastri*	100	MH856647*	CBS 324.50
Microsphaeropsis olivacea	*M. olivacea*	100	MT790320	
Phacidium lacerum	*P. lacerum*	100	MH856297*	CBS 540.70
(Unidentified Eurotiomycete) *Phaeomoniellaceae* sp.	*Aequabiliella effusa*	99.45	NR_132005.1	
Sydowia polyspora	*Hormonema macrosporum*	100	NR_145340.1	
(Unidentified Agaricomycete) *Basidiomycota* sp.	*Athelia* sp.	99.69	OR751421.1	
Alternaria sp1.	-	-	-	
Alternaria sp2	-	-	-	
Unidentified fungi (12 morphotypes)	-	-	-	

The isolation frequency of unidentified fungi was 15%, the communities of culturable fungi identified in Scots pine needles at their different life stages were dominated by ascomycetes (12 taxa), while basidiomycetes were represented by a single unidentified taxon (unidentified *Basidiomycota* sp.) that occurred on 4% of needle fragments (Fig. A2a). The majority of isolates belong to the Ascomycota phylum (81%), with noticeable contributions from the classes Leotiomycetes (42.5%) and Dothideomycetes (32.5%). Unidentified fungi, Basidiomycota and Pezizomycetes (Ascomycota) occurred mainly in litter needles (Fig. A2b).

Fungal Community Composition and Biodiversity Across Needle Types

In total, 33.3% of needle fragments were colonized by fungi. Fungal colonization was higher on litter needles (10.8 %) followed by senescent needles (36.7%). Only 5.4% of green needles were colonized by fungi. *L. pinastri*, *Cy. minus*, *S. polyspora*, *Alternaria*

sp1., *M. olivacea*, *D. acicola*, and *Basidiomycota* sp. were the dominant fungal taxa of the studied needle communities with frequencies of 5% or more on at least one needle type (Table A1). Regardless of the life stage or type of needle, the most frequently isolated fungal taxon from Scots pine needles was *L. pinastri* (117 out of 480 isolates; 24.38%). The *L. pinastri* colonized 8.1% of needle fragments. This fungus showed noticeable presence, particularly in senescent needles (SN), where it dominated with 17.5%. The overall colonization frequency of *L. pinastri* on SN was four times higher than in GN. Its colonization frequency decreased in FN and LN to 2.1% and to 0.6%, respectively. The second most common fungus *Cy. minus* colonized 5.4% of needle fragments. It was most prevalent in green needles (GN), making up 69.2% of the fungi found there (54 out of 78 isolates from GN) and 15% of fungi colonizing the needle fragments (Table A1).

A one-way PERMANOVA analysis using Bray-Curtis dissimilarity (Permutation N = 9999) was performed to compare fungal communities among different needle types (GN, SN, FN, and LN). The results indicated significant differences among the needle types ($F = 4.66$, $p = 0.0001$). Pairwise comparisons between needle types, using both Bonferroni-corrected and uncorrected p-values, did not show any significant differences ($p > 0.05$).

The NMS plot (Fig. 2) demonstrates distinct clustering of fungal communities associated with different needle types, suggesting clear differentiation in fungal community structure among the different needle types.

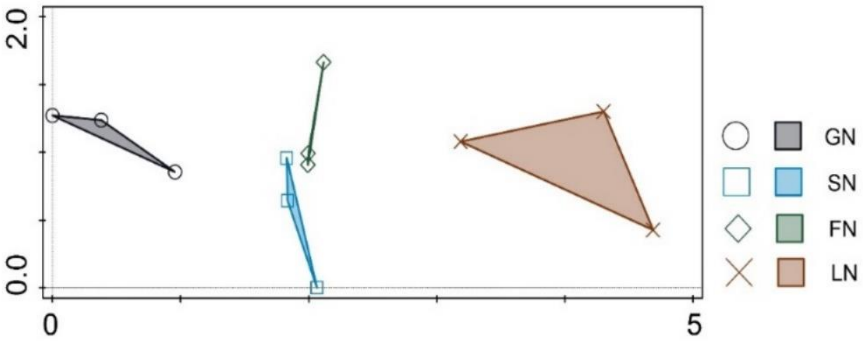


Fig. 2. NMS plot visualizing the relationships between the fungal communities of different needle types. Samples that are closer together in the plot are more similar in their fungal community composition, while samples that are further apart are more dissimilar.

In accordance with NMS analysis, the DCA analysis showed clear differences in fungal community structure among the different types of pine needles, with distinct clusters for each needle type (Fig. 3). Axis 1, Axis 2, and their combination explained 28.41%, 6.4%, and 42.46% of the total variance in density of the fungi community, respectively. The ordination plot displays a clear gradient along Axis 1 (28.41% of the variance), from living needles (GN) on the left to decomposing needles (LN) on the right. This gradient reflects the progression from living tissue-associated fungi (*e.g.*, Cyl: *Cy. minus*) to decomposer fungi (*e.g.*, Des: *D. acicola*, Phace: *P. lacearum* and various unidentified taxa).

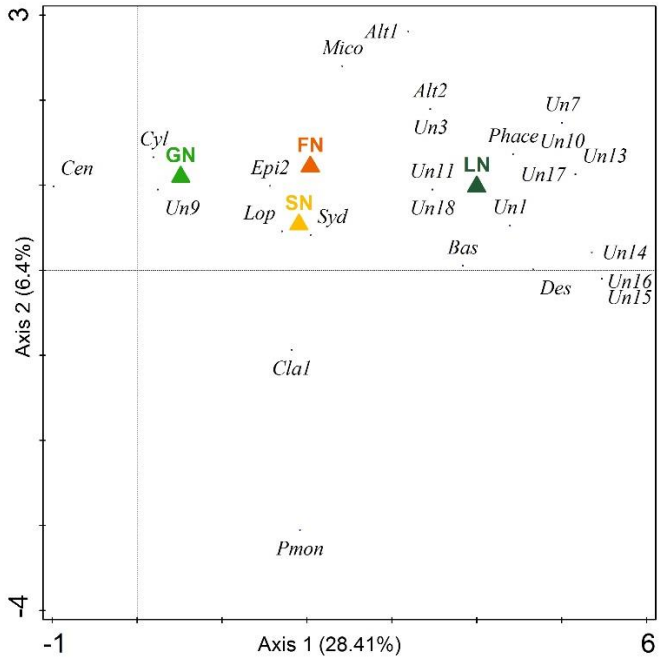


Fig. 3. Detrended Correspondence Analysis (DCA) of fungal communities isolated from different types of pine needles

The fungal diversity analyzed using various diversity indices across different types of pine needles was significantly different between the four needle types. The LN exhibited the highest species richness and diversity, with the highest Chao1, Shannon index, and Simpson's Index of Diversity (1 - λ). The GN showed the lowest values in all indices, indicating limited richness and diversity. Both SN and FN exhibited intermediate values, with FN generally showing slightly higher diversity than SN (species richness and Chao 1: $F = 100{,}10$, $p < 0.01$; H': $F = 87.739$, $p < 0.01$; $1 - \lambda$: $F = 43.87$, $p < 0.01$) (Fig. 4).

The functional roles assigned using the databases FUNGuild and FungalTraits aligned with the observed patterns of community composition explored *via* biodiversity indices and multivariate analyses. Early stages (GN, SN) were dominated by endophytes and weak pathogens, whereas later stages (FN, LN) showed a shift to saprotrophic fungi. The trophic modes representing the functional roles that fungi play in their ecosystem also revealed that the Pathotroph-Saprotroph-Symbiotroph mode is the most dominant in senescent (SN) and fallen needles (FN). Saprotrophs become more prominent in litter needles (LN), as expected in the final stages of decomposition (Fig. 5). The primary lifestyle of fungi changed significantly across the different needle types. Green needles were primarily inhabited by plant pathogens. As needles age and fall, unspecified pathotrophs and litter saprotrophs increase. The secondary lifestyle distribution showed a similar trend, with a significant presence of plant pathogens in green and senescent needles and a dominance of unspecified pathotrophs and litter saprotrophs in fallen and litter needles. Litter saprotrophs and wood saprotrophs increase in abundance as the needles transition from senescence to litter.

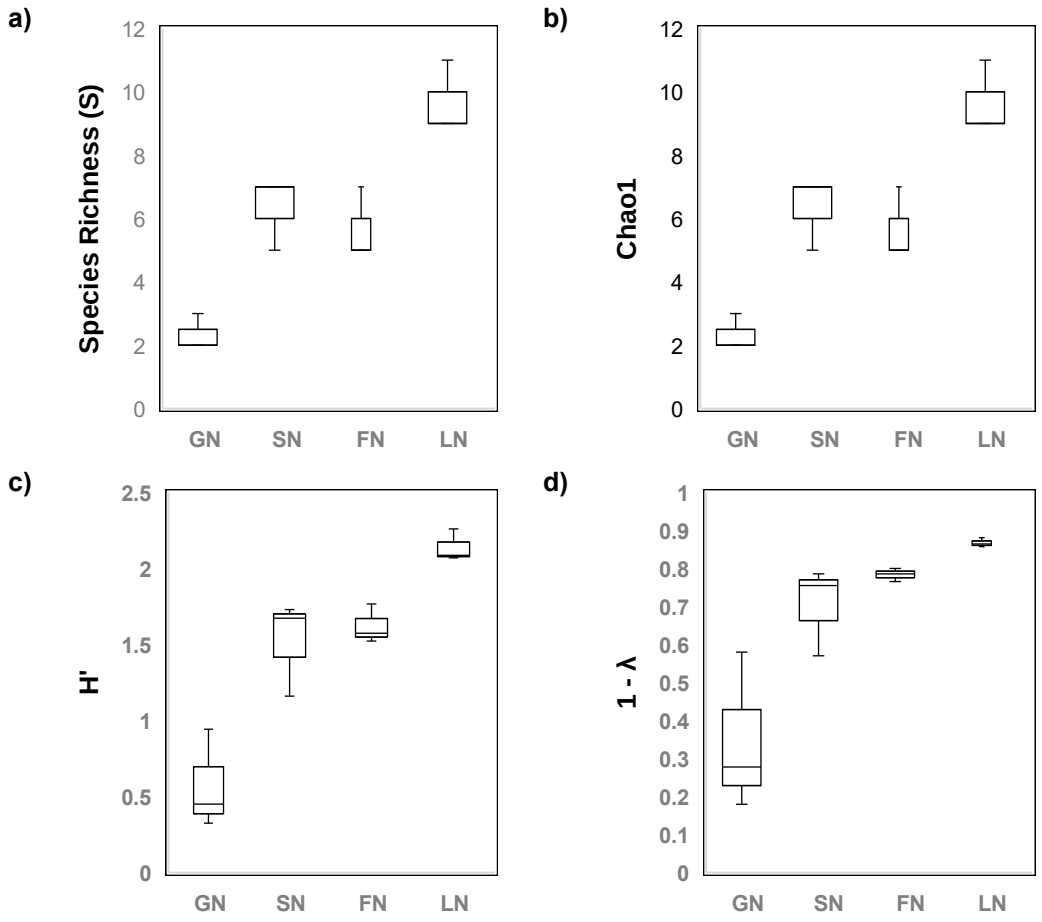
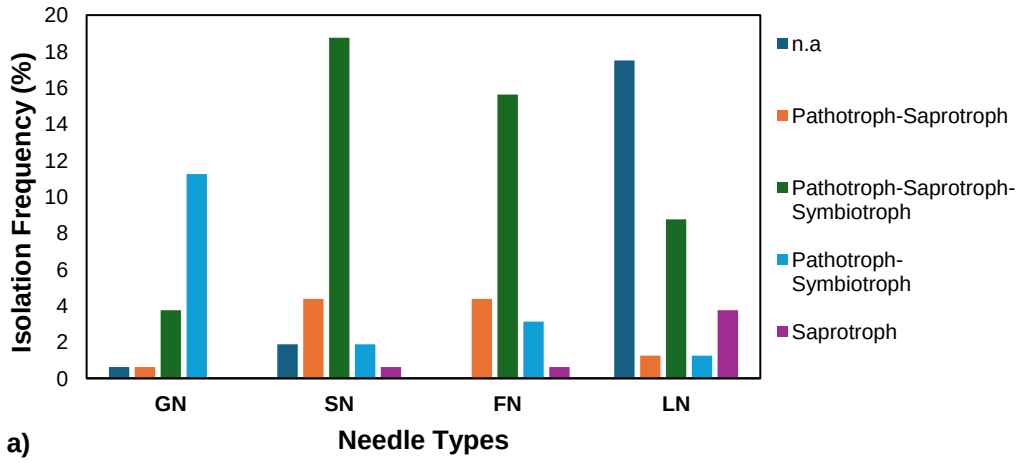


Fig. 4. The fungal diversity across different types of pine needles (Green Needles (GN), Senescent Needles (SN), Fallen Needles (FN), and Litter Needles (LN): (a) species richness (S), (b) Chao 1 estimator, (c) Shannon-Wiener index (H'), and (d) Simpson's diversity index (1 - λ)



a)

Fig. 5a.

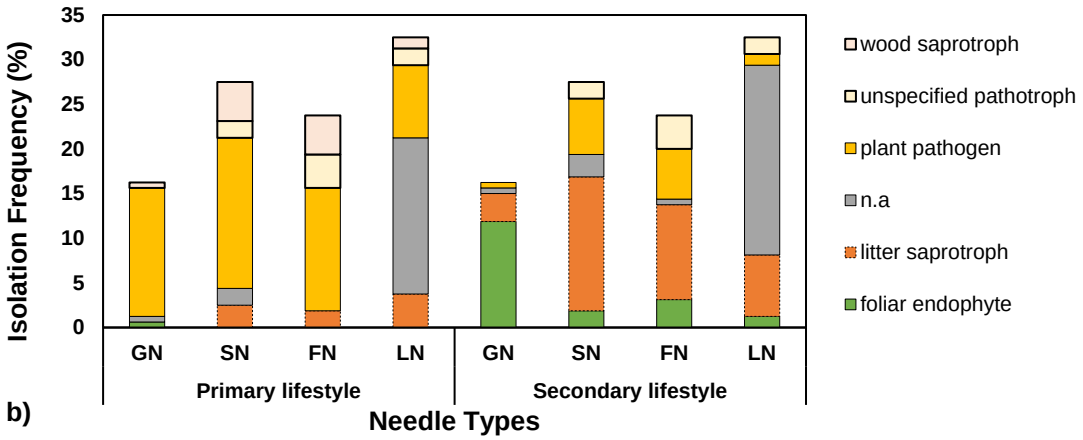


Fig. 5b. The distribution of functional groups of fungi in different types of pine needles (green needles (GN), senescent needles (SN), fallen needles (FN), and litter needles (LN)): a) different fungal trophic modes assigned using the database of FUNGuild, and b) fungal lifestyles classified by primary and secondary lifestyles assigned using database of FungalTraits. The lifestyles include wood saprotrophs, unspecified pathotrophs, plant pathogens, litter saprotrophs, foliar endophytes, and unidentified fungi (n.a).

DISCUSSION

Across four types of needles—green, senescent, fallen, and litter—representing a transition from living to early stages of decomposition, 11 fungal taxa were identified through DNA sequencing. These taxa include *Cenangium ferruginosum*, *Cladosporium* sp., *Cyclaneusma minus* sensu lato, *Desmazierella acicola*, *Epicoccum* sp., *Lophodermium pinastri*, *Microsphaeropsis olivacea*, *Phacidium lacerum*, *Sydowia polyspora*, *Phaeomoniellaceae* sp. (unidentified Eurotiomycete), and *Basidiomycota* sp. (unidentified Basidiomycete). The fungal community isolated from these needles also included two taxa morphologically assigned to the genus *Alternaria* and twelve unidentified fungal morphotypes. Excluding the unidentified basidiomycete fungus (*Basidiomycota* sp.) and the unidentified Eurotiomycete (*Phaeomoniellaceae* sp.), *L. pinastri*, *Cy. minus*, *S. polyspora*, *Alternaria* sp1., *M. olivacea*, *D. acicola*, and *Basidiomycota* sp. were the dominant fungal taxa of the studied needle communities with frequencies of 5% or more on at least one needle type. While *L. pinastri*, *Cy. minus* and *S. polyspora* were the most frequently encountered species in this study and this finding is consistent with many studies investigating fungal assemblages of *P. sylvestris* needles conducted across the natural distribution range of this host, especially in Europe (*e.g.*, Kowalski 1982, 1993; Helander *et al.* 1994; Millberg *et al.* 2015; Behnke-Borowczyk *et al.* 2019; Taylor *et al.* 2019; Schönrogge *et al.* 2022). Indeed, *L. pinastri*, *Cy. minus*, *D. acicola* and *P. lacerum*, and *C. cenangium*, have commonly been found as relevant fungi associated with Scots pine needles at their various stages from living to decomposition since the earlier investigations on needle fungal communities of this host, yet with differences in frequencies or occurrences based various factors such as needle life stage or age and geography (*e.g.*, Kendrick and Burges 1962; Hayes 1965; Gremmen 1977; Minter and Millar 1980; Kowalski 1982, 1993; Ponge 1991; Helander *et al*. 1994; van Maanen and Gourbière 2000; van Maanen *et al.* 2000; Gourbière *et al*. 2001; Boberg *et al.* 2011; Koukol 2011; Osono and Hirose 2011). On the other hand, only a few of these fungi are also known to occur on

pine needles globally (*e.g.*, Ata *et al.* 2024). For instance, *d*espite its frequent detection from healthy living needles, *Cy. minus* is considered as a notable widespread needle pathogen causing *Cyclaneusma* needle cast on various pine hosts globally, especially in *Pinus radiata* in New Zealand (Ismael *et al.* 2020). However, it is notable that in DNA based studies, detection of *L. pinastri* on needles from non-Eurasian pine species in for instance, North America or Japan is either very rare, uncertain, or absent in contrast to other *Lophodermium* species such as *L. nitens, L. australe* (*e.g.*, Oono *et al.* 2014; Broders *et al.* 2015; Jeewon *et al.* 2018; Salas-Lizana and Oono 2018). The relatively high proportion of unidentified fungi (15%) further mirrors findings from previous culture-based studies of Scots pine needles, where a few dominant species were identified alongside a long tail of less frequent species and unidentified fungi (Kowalski 1982, 1993; Helander *et al.* 1994; Taylor *et al.* 2019; Schönrogge *et al.* 2022).

Although this study relied on culture-based techniques, which have known limitations (Koukol 2011; Jeewon *et al.* 2018), it provided valuable insights into the dominant fungal assemblages associated with *P. sylvestris* needles in Türkiye. The results of some studies provide reassurance that culturing and metabarcoding provide a degree of congruence in descriptions of the composition of the endophyte community of *P. sylvestris* (Taylor *et al.* 2016; Schönrogge *et al.* 2022).

This study revealed distinct fungal community compositions across different needle types, demonstrating a clear successional pattern. Using non-metric multidimensional scaling (NMDS) and detrended correspondence analysis (DCA), significant shifts were observed from endophytic and pathogenic taxa in living needles to saprotrophic taxa in decomposing needles. Species richness and diversity increased as needles transitioned from living to decomposing stages. Functional guild assignments through FUNGuild and FungalTraits further supported these changes, with early colonizers identified as endophytes or weak pathogens, and later colonizers predominantly saprotrophs. These findings align with previous research (Kendrick and Burges 1962; Gremmen 1977; Ponge 1991; Müller *et al.* 2001; Osono 2006; Schneider *et al.* 2012; Voříšková and Baldrian 2013; Yuan and Chen 2014), emphasizing the plasticity of fungal functional roles. As needles age and decompose, the community shifts to predominantly saprotrophic fungi, which play a crucial role in nutrient cycling and litter decomposition (Osono and Hirose 2011). For instance, in this study *L. pinastri* and *S. polyspora* were detected across all needle stages, underscoring their persistence and role as pioneer decomposers (Osono and Hirose 2011; Boberg *et al.* 2011). While *S. polyspora* has been shown to inhibit *Diplodia sapinea*, a serious pine pathogen (Beram and Bıkzad 2024), it lacks the ability to decompose pine needles (Boberg *et al.* 2011). Nevertheless, not only substrate utilization, but also life cycle and antagonistic interactions are also known to reflect the performance of endophytes in litter and many endophytic fungi could have antagonistic interactions against other fungi or herbivores (Osono and Hirose 2009; Baldrian 2017; Terhonen *et al.* 2019). Nevertheless, the interpretation of the functional role of the fungi could be challenging because many fungal species can change from several types of symbiosis with the host, depending on the different factors.

This study reveals important contributions to the funga of Türkiye by documenting both known and previously unreported fungal taxa associated with pines. While *Alternaria* sp., *C. ferruginosum, Cladosporium* sp., *Cy. minus, L. pinastri*, and *S. polyspora* have been noted in earlier studies from Türkiye (Sesli *et al.* 2020; Oskay and Karataş 2021; Aday Kaya *et al.* 2023; Aday Kaya and Karaman 2023; Beram and Demiröz 2024; Beram and Bıkzad 2024), *D. acicola* and *P. lacerum* emerge as new records for the country, and *M.*

olivacea is reported on a pine host in Türkiye for the first time (Sesli *et al.* 2020). These findings underscore the underexplored fungal diversity of the region. Furthermore, the detection of previously unidentified fungi, including the Eurotiomycete (Eurotiomycetes, Phaeomoniellaceae) and the Basidiomycete taxa, highlights the potential for discovering novel fungal taxa on a global scale.

Notably, this study represents the first exploration of fungi in ecosystem processes in Türkiye, filling a crucial gap in ecological studies on fungi in the country. This study highlights Türkiye's importance as a region of interest for fungal ecology, given its unique biogeographic location and understudied status. Through documenting fungal diversity in Scots pine needles, this preliminary study provides baseline data for future ecological and biogeographical studies including for research on species' resilience and biodiversity. Future research using next-generation sequencing (NGS) techniques and investigating advanced decomposition stages could further reveal Türkiye's fungal biodiversity.

CONCLUSIONS

1. This study provides the first exploration of fungal communities and their succession in *Pinus sylvestris* needles from Türkiye.
2. The fungal assemblages showed similarities to those in Europe, with common species such as *Lophodermium pinastri*, *Sydowia polyspora*, and *Cyclaneusma minus*.
3. Novel discoveries, including unidentified Eurotiomycete and Basidiomycete taxa, indicated hidden biodiversity and potential for new species. Additionally, this study presents the first records of *Desmazierella acicola* and *Phacidium lacerum* in Türkiye, underscoring the underexplored fungal diversity in this region.

   The analysis of fungal community structure across different needle types revealed significant variations, highlighting the intricate and complex nature of fungal colonization and decomposition processes. Moreover, the observed differences between green, senescent, freshly fallen, and litter needles suggest a clear successional pattern, with distinct shifts in fungal community composition as needles progress through their life cycle.

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APPENDIX

Supplementary Materials

Table A1. Colonization Frequencies of Fungi Determined as the Percentage of Needle Fragments Colonized

	Overall Colonization Frequencies (%)					Colonization Frequencies by Needle Types (%)			
	Total	GN	SN	FN	LN	GN	SN	FN	LN
Dominant Fungi*									
Lophodermium pinastri	8.13	1.04	4.38	2.08	0.63	4.17	**17.50**	**8.33**	2.50
Cyclaneusma minus	**5.42**	3.75	0.63	1.04	-	**15.00**	2.50	4.17	-
Sydowia polyspora	3.54	0.21	1.46	1.46	0.42	0.83	**5.83**	**5.83**	1.67
***Alternaria* sp1.**	2.92	-	-	1.46	1.46	-	-	**5.83**	**5.83**
Microsphaeropsis olivacea	2.50	-	0.63	1.25	0.63	-	2.50	**5.00**	2.50
Desmazierella acicola	1.67	-	0.21	0.21	1.25	-	0.83	0.83	**5.00**
***Basidiomycota* sp.**	1.46	-	0.21	-	1.25	-	0.83	-	**5.00**
Fungi of Partial Distribution									
Cladosporium sp1.	1.04	-	0.63	0.42	-	-	2.50	1.67	-
UnM14	0.83	-	-	-	0.83	-	-	-	3.33
UnM1	0.63	-	-	-	0.63	-	-	-	2.50
UnM13	0.63	-	-	-	0.63	-	-	-	2.50
UnM17	0.63	-	-	-	0.63	-	-	-	2.50
Alternaria sp2.	0.42	-	0.21	-	0.21	-	0.83	-	0.83
Epicoccum sp2.	0.42	-	0.42	-	-	-	1.67	-	-
Phacidium lacerum	0.42	-	-	-	0.42	-	-	-	1.67
UnM3	0.42	-	0.21	-	0.21	-	0.83	-	0.83
UnM15	0.42	-	-	-	0.42	-	-	-	1.67
UnM18	0.42	-	-	-	0.42	-	-	-	1.67
Cenangium ferruginosum	0.21	0.21	-	-	-	0.83	-	-	-
Phaeomoniellaceae sp.	0.21	-	0.21	-	-	-	0.83	-	-
UnM7	0.21	-	-	-	0.21	-	-	-	0.83
UnM9	0.21	0.21	-	-	-	0.83	-	-	-
UnM10	0.21	-	-	-	0.21	-	-	-	0.83
UnM11	0.21	-	-	-	0.21	-	-	-	0.83
UnM16	0.21	-	-	-	0.21	-	-	-	0.83
TOTAL	33.33	5.42	9.17	7.92	10.83	21.67	36.67	31.67	43.33

[Green Needles (GN), Recently death Senescent Needles (SN), Freshly Fallen Needles in Littertraps (FN), Decomposing Litter Needles in litterbags (LN))
*Dominant fungal taxa of the studied needle communities with frequencies of 5% or more on at least one needle type.

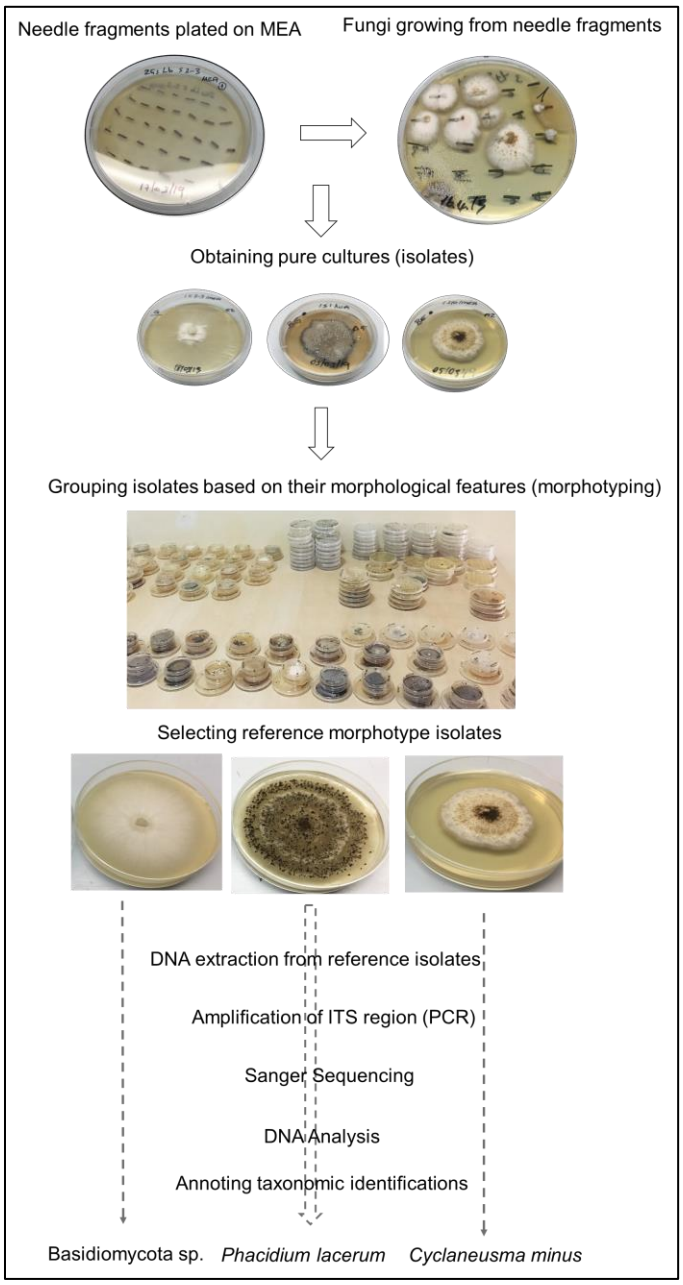

Fig. A1. Schematic representation of fungal isolations and molecular identification of isolates

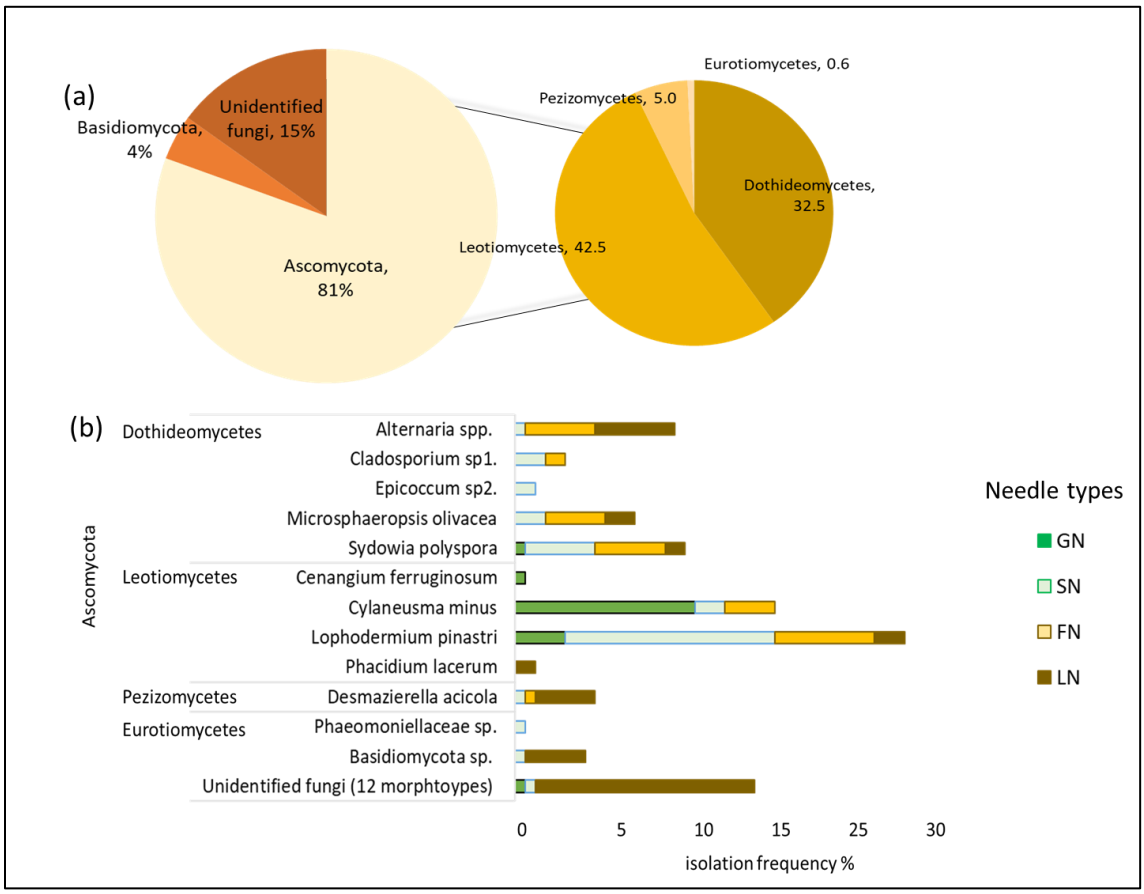

Fig. A2. Taxonomic distribution of fungal taxa across different needle types (GN: Green needles, SN: Senescent Needles; FN: Fallen needles; LN: litter needles): (a) Pie chart representing the overall composition of fungal phyla and classes within the Ascomycota, (b) Horizontal bar chart comparing relative abundance of each fungal taxa across different needle types