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Title: New saga in Finland: The rise of *Diplodia sapinea* in Scots pine

Year: 2025

Version: Published version

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Please cite the original version:

Eeva Terhonen, Tiina Ylioja, Tuija Hytönen, Katri Leino, Linda Mutanen, Markus Melin, Eeva Vaahtera, Suvi Sutela, New saga in Finland: The rise of *Diplodia sapinea* in Scots pine, *Fungal Genetics and Biology*, Volume 176, 2025, 103955, ISSN 1087-1845, <https://doi.org/10.1016/j.fgb.2024.103955> .

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New saga in Finland: The rise of *Diplodia sapinea* in Scots pine

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ARTICLE INFO

Keywords:

Pinus sylvestris
Emerging
Climate change
Forest pathogen

ABSTRACT

The intensity of fungal virulence is likely to increase in northern forests as climate change alters environmental conditions, favoring pathogen proliferation in existing ecosystems while also facilitating their expansion into new geographic areas. In Finland, *Diplodia sapinea*, the causal agent of disease called “Diplodia tip blight”, has emerged as a new pathogen within the past few years. To reveal the current distribution of the novel fungal pathogen, and the effect of temperature and rainfall on its distribution, we utilized citizen science for the detection and collection of symptomatic Scots pine (*Pinus sylvestris*) shoots. The Finnish culture collection of *D. sapinea* was initiated using in vitro cultured symptomatic samples, and selected strains were studied for their virulence and disease cycle. Furthermore, the mycobiome of selected symptomatic and asymptomatic Scots pine shoots was studied using amplicon sequencing and the presence of *D. sapinea* was confirmed with culturing, qPCR, and species-specific PCR. Based on over 500 Scots pine shoots testing positive for *D. sapinea*, the distribution of this fungal pathogen is concentrated along the coastal areas of Finland, extending up to 200 km inland from the coastline. The observed presence of *D. sapinea* followed the period of highest average temperatures recorded in Finland in 2023 and was also found to be related to less precipitation. The amplicon sequencing showed that abundance of *D. sapinea* was higher in the healthy tissues of symptomatic shoots compared to visually healthy shoots. Similarly, the abundance was higher in samples collected from coastal areas in South-western Finland, which are the most heavily impacted by this disease. Here, we show that the presence of *D. sapinea* is more extensive than previously assumed, and lastly illustrate the hypothesized disease cycle of the fungal pathogen in Finland based on observations made in the field from 2021 to 2024 and in vivo and in vitro studies.

1. Introduction

Diplodia sapinea is a new pathogen that causes Diplodia tip blight on Scots pines (*Pinus sylvestris*) and junipers (*Juniperus communis* subsp. *communis*) in Finland (Terhonen, 2023; Terhonen et al., 2023). The proliferation of *D. sapinea* is facilitated by warmer climates (Eldridge, 1961), with the optimal growth range defined between 25 and 30 °C (Bußkamp, 2018). *Diplodia sapinea* disseminates through the dispersal of asexual spores carried by wind and rain, which target the newest annual growth of pine trees or needles (Bachi and Peterson, 1985; Stanosz et al., 2001). For optimal spore production *D. sapinea* requires warm and humid conditions, which also facilitate the survival of the fungus. Studies indicate increased spore production during higher temperatures in spring (Brodde et al., 2019), summer (Caballol et al., 2022), and

winter (Bosso et al., 2017; Fabre et al., 2011). Mild winters contribute to the survival of the fungal hyphae in infected crowns of both trees and seedlings (Caballol et al., 2022). The optimum germination temperature for the fungal pathogen’s spores is identified as 24 °C, although germination is possible at significantly cooler or warmer temperatures (Brookhouser and Peterson, 1970). In Finland, *D. sapinea* was first documented in 2015 within Scots pine cones as saprophyte (Müller et al., 2019). Subsequently, in 2019, it was identified as an endophyte (Terhonen et al., 2021), ultimately reported in a pathogenic state in 2021 (Terhonen, 2022, 2023). In 2004, the first attempt to detect *D. sapinea* was made in a single area of approximately 30 ha in southern Finland, but without success (Müller et al., 2019). Between October 2015 and September 2016, surveys of fifteen mature Scots pine stands revealed the presence of *D. sapinea* in five stands (cones) along the

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<https://doi.org/10.1016/j.fgb.2024.103955>

Received 12 July 2024; Received in revised form 11 December 2024; Accepted 18 December 2024

Available online 19 December 2024

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southwestern coast of Finland (Müller et al., 2019). Since only one stand was examined in 2004 and no *D. sapinea* was found, its detection in 2015 in the same area suggests that it arrived in Finland sometime between 2004 and 2015, though the exact year remains unknown. Similarly, in 2019, visually healthy Scots pine shoots were collected in Finland, and *D. sapinea* was identified as an endophyte in two locations (Terhonen et al., 2019). No disease symptoms were observed at these locations in 2019. However, in 2021, it was found at the same location (Vantaa, southern Finland) causing disease as a pathogen (Terhonen, 2023). The appearance of this fungus first as a saprophyte (first confirmed case in 2015) (Müller et al., 2019), then as an endophyte (first confirmed case 2019) (Terhonen et al., 2019) and finally as pathogen (first confirmed case in 2021) (Terhonen, 2023; Terhonen et al., 2023) in Finland might indicate that environmental conditions have become more favourable for its establishment in new areas (Li et al., 2023).

Diplodia sapinea causes damage by killing annual shoots, and in extreme cases, it can lead to the mortality of mature trees within a single growth season (Bußkamp et al., 2020; Blumenstein et al., 2021b) or predispose trees to secondary disturbances (Ylioja et al., 2023; Ylioja et al., 2024). The disease manifests through symptoms such as tip blight (Fig. 1), stem canker, dieback of current-year shoots, and the development of blue staining in the sapwood (Piou et al., 1991, Brookhouser and Peterson, 1970, Luchi et al., 2014, Bußkamp et al., 2020). Although the symptoms of *D. sapinea* are well-known, its lifecycle in Scots pine has not been documented. The only described lifecycle, by Tattar, 1989, is for

Austrian pine (*Pinus nigra*).

Diplodia sapinea's capacity to transit from a latent to an opportunistic pathogen, adds complexity to understanding of its prevalence in apparently healthy trees. The inconspicuous spread facilitated by the endophytic life cycle raises concerns over detection and the extent of its establishment in asymptomatic trees in Finland remains unknown. Scots pine is the most common tree species in Finland's forests where it occupies 64 % of the productive forest area, with a total volume of 1244 million cubic meters that corresponds to 50 % of the total growing stock volume (Korhonen et al., 2021). Due to the predicted shifts in climate and the prolonged rotation periods of Scots pines in Finnish forestry, there is a concern that pines may struggle to adapt to these changes. A new threat is that they are likely to encounter drought stress and invasion by *D. sapinea*. The abundance of *D. sapinea* is currently increasing in European (e.g., Germany) forests as an endophyte (Bußkamp, 2018; Bußkamp et al., 2020; Blumenstein et al., 2021b). For these reasons, identifying *D. sapinea* in its endophytic stage is crucial, as is determining the most efficient method for doing so.

To reveal the unknown distribution of *D. sapinea*, we initiated a new study with the help of citizen science to gain an updated perspective on the current situation in Finland. After following the disease development in the field over three years, the disease development of *D. sapinea* in Scots pine is documented here for educational purposes. The objectives of this study are 1) to map the distribution of the recently emerged pathogen *D. sapinea* in Finland, utilizing citizen science as a tool for data



Fig. 1. *Diplodia* tip blight symptoms in Scots pine. (A) Shoot infected with *Diplodia sapinea* during the elongation phase in June 2023. (B) Progression of *D. sapinea* infection in older shoots in October 2023.

collection; 2) to assess the correlation between climatic factors, including average summer temperatures and precipitation, and the occurrence of *D. sapinea*; 3) to evaluate different detection methods for the pathogen, with a particular focus on distinguishing between symptomatic and asymptomatic infections; and 4) to elucidate the disease development due to *D. sapinea* on Scots pine in Finland.

2. Material and methods

The collection of Scots pine shoots.

Sample collection through citizen science.

The project was disseminated through the official website and press channels of Natural Resources Institute Finland (hereafter referred to as Luke). Symptoms were described (Fig. 1), and citizens were requested to report pine trees with similar symptoms via electronic form or email. Upon receiving contact from citizens, we dispatched prepaid post letters, subsequently received information on tree locations, brief descriptions, and samples via mail during the years 2022 and 2023.

Targeted sample collection.

For more targeted collection, we focused separately on locations where *D. sapinea* could be detected as a pathogen, specifically areas where pine trees might be susceptible to drought stress. For this, we downloaded spatial data on the locations of rocky/rugged areas with minimal levels of mineral soils (areas with surfacing bedrock) from the topographic database of the National Land Survey of Finland (NLS, 2024). This was merged with nationwide raster data on forest structure based on the multi-source National Forest Inventory (ms-NFI). The ms-NFI data is also available on an open-access basis (Luke, 2024). These datasets allowed us to identify areas where pine-dominated forests were growing on drought-prone sites. Next, our area of interest (central and southern Finland) was delineated into 25 km grid cells and five locations of the identified rugged pine forests were randomly selected from each grid cell. Finally, field samples were collected in August 2023 from different locations to test for the presence of *D. sapinea*. All geospatial analysis and maps were created in QGIS with the software version 3.0 (www.qgis.org).

Diplodia sapinea identification, pathogenicity, and mapping.

All received shoot samples were defoliated and processed through the pipeline described in Terhonen (2023). The initial identification of *D. sapinea* isolates was based on morphology, followed by molecular methods (Terhonen, 2023). All the other outgrowing mycelia were discarded. Due to the large number of samples, no distinction was made between shoot samples with varying health statuses (diseased/dead). They were classified as either negative (no *D. sapinea* growth observed) or positive (*D. sapinea* observed) following culturing. The pathogenicity of selected Finnish *D. sapinea* strains (isolated as pathogens, ID: 67.4, 130.2, 138.1) was studied experimentally by inoculation of Scots pine seedlings. The side shoots of ten three-year-old Scots pines were inoculated (four side shoots per tree) with either *D. sapinea* (three genotypes) or with a mock control (agar plug) as described by Terhonen (2023). Similarly, the main shoots of two-year-old Scots pine seedlings (45 per treatment) were inoculated with two different genotypes or with a mock control (Terhonen, 2023). Necrosis was measured two weeks after the inoculation of three- and two-year-old Scots pines, and re-isolation of *D. sapinea* was performed (Terhonen, 2023). Strains are permanently stored in the collections of the Natural Resources Institute Finland (Luke). A distribution map was generated of the processed Scots pine and *D. sapinea* positive samples taking the forest owners' privacy into consideration (precise site locations (coordinates) are withheld). The distribution was aligned together in a map with weather data from 2023 (average temperature in °C and precipitation in mm from May to August) that was provided by the Finnish Meteorological Institute (FMI).

Mycobiome-sample collection.

We selected a site (Site 1) where *D. sapinea* was isolated in April 2022, and at two-month intervals (April, June, and August 2022) collected three shoots from each of the five healthy trees chosen for this

study (Table 1, Fig. 2). Sampling was done at different growth stages. In April, we collected only the 2021 growth, while in June and August, we also collected the 2022 growth, but sampled the 2021 growth for amplicon sequencing. The trees were situated on a rocky drought-prone area, encompassed by a Norway spruce forest (Myrtillus-site) and a Scots pine forest (Vaccinium-site). Shoots were collected from 1.3 m to 3 m, depending on the tree height (Table 1). All samples were collected and transported to -20 °C within one hour of cutting the shoots. Additionally, we gathered samples (5 or 10 shoots) from three distinct trees in the area (Site 2), identified as the most affected based on citizen observations in September 2022 (Table 1, Fig. 2). Two locations, denoted as tree numbers 6 and 7, were situated in a forestry site characterized by the predominant presence of Scots pine and classified as a Vaccinium-site. At the location of tree number 6, all the deceased trees, attributed to *Ips acuminatus* and *D. sapinea*, were removed during the summer 2022. For the Scots pine number 6 (asymptomatic), we collected 5 healthy shoots in upper crown (21 m) and 4 healthy shoots and one dead shoot from lower crown (12 m) (Fig. 2). Tree number 7 had few shoots showing the symptoms of Diplodia tip blight (symptomatic), but overall health status was still viable. Five diseased shoots were collected. The final tree, designated as number 8, was situated on a slope, categorized as non-forestry land, and exhibited characteristics typical of a Vaccinium-site. Similarly, to the tree no. 7, this tree had symptoms, but the overall status was viable. Five diseased shoots were collected for tree no. 8. The samples were defoliated, the bark was gently scraped with sterile scalpel, and samples with phloem, sapwood, and bark residues were placed in liquid nitrogen and transported to -20 °C within two days. The shoots had both 2021 and 2022 growth, and they were combined into a single sample to obtain sufficient plant material (sapwood, phloem, and bark residues) for DNA extraction (compare to site 1 where we used only 2021 growth). None of the sampled trees showed signs of additional pest infestations (*I. acuminatus* or *Heterobasidion annosum*). The health status of each sampled shoot, along with relevant information, is available in Supplementary File S1. From each collected branch, attempts were made to isolate *D. sapinea* using the traditional cultivation method (Terhonen, 2023), and subsequently, amplicon sequencing of fungal ITS1 region, qPCR (Luchi et al., 2005; Oliva et al., 2021), and PCR (Adamson et al., 2021; Terhonen, 2023).

DNA extraction from site 1 and 2 shoot samples.

The bark was gently scraped from collected shoot, and the phloem (comprising remnants of bark and a small amount of sapwood) was obtained from the healthy tissue. Samples were either prepared in laboratory (trees no 1 to 5) or in the field (trees no. 6 to 8). The plant material was homogenized with TissueLyser (Qiagen). DNA extraction, using 70 to 100 mg of plant material, was performed with the DNeasy Plant Pro Kit (Qiagen), following the manufacturer's instructions. The quality and quantity of DNAs was evaluated with Nanodrop OneC spectrophotometer (Thermo Fisher Scientific) (see Supplementary File S1).

Determination of the *D. sapinea* presence with PCR.

Species-specific primers DiSapi-F (3'-CCCTTATA-TATCAAATGCTTTGT-5') (Adamson et al., 2021) and Diplo-R (3'-TTACATAGAGGATTGCTTCG-5') (Adamson et al., 2021) were used to identify *D. sapinea* from unequilibrated shoot DNA. Original undiluted and tenfold diluted DNAs were used, in addition to different dilutions of shoot DNA further purified with DNA Clean & Concentrator-25 (Zymo Research). The protocol was based on one used in Terhonen (2023) with following modifications. Amplification was conducted with Phusion Plus Green PCR Master Mix (Thermo Scientific) with 0.4 μM primers in a reaction volume of 25 μl. PCR conditions were 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s, and 72 °C for 5 min. Sterile MQ H₂O were used as negative control.

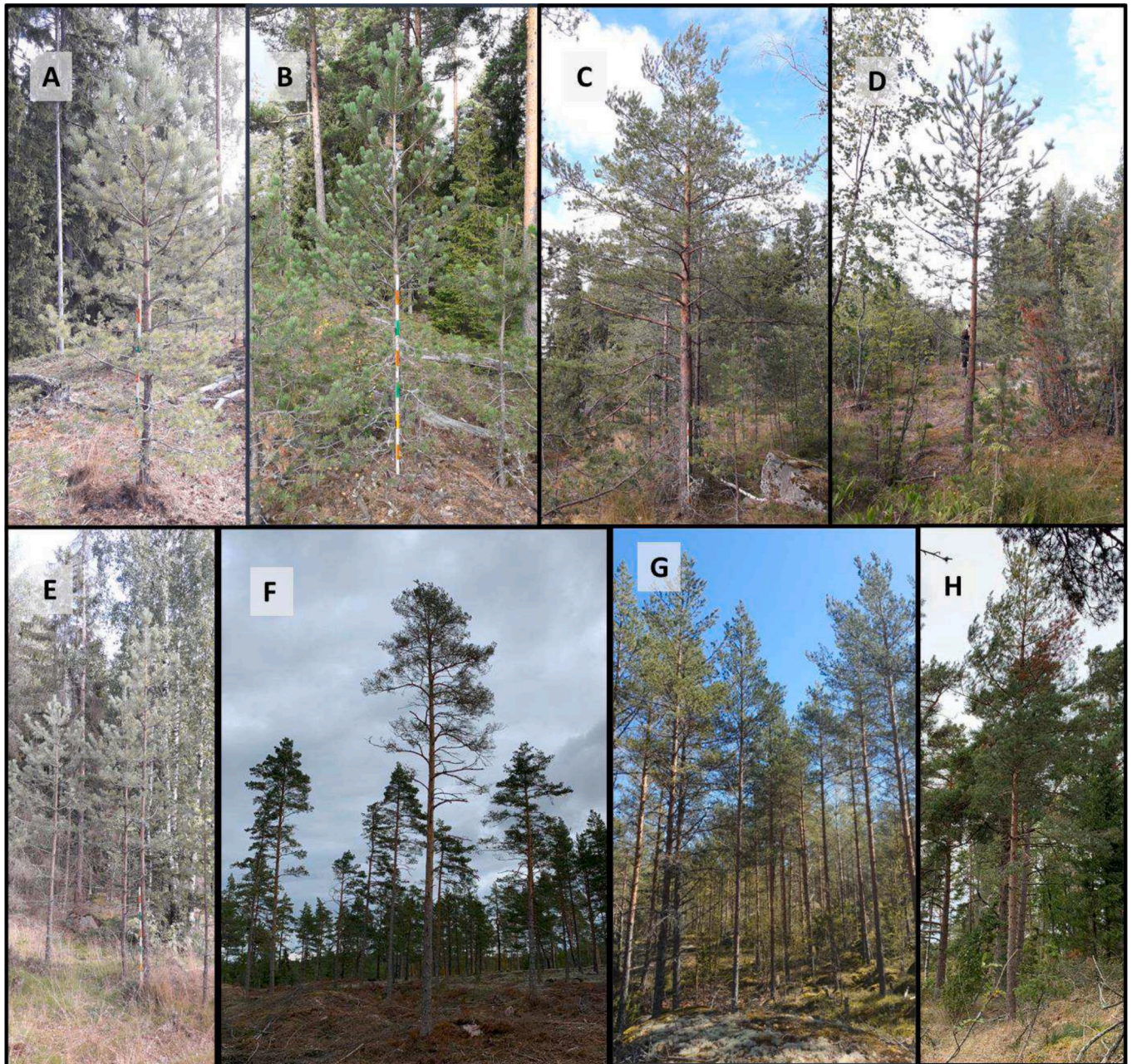
Determination of the *D. sapinea* presence with qPCR.

Primers and probe were based on Luchi et al. (2005): forward primer (5'-GTAAAACTGACGTTGAGGGAGC-3') and reverse primer (5'-CAT-AATTGTCTGCCCGGACTACT-3'). The internal probe (5'-

Table 1

Information (diameter, tree height, health status) on the sample trees and sampling (no. of shoots and time of sampling) of the shoots.

Tree no.	Site	Diameter (BH, cm)	Tree height (m)	Sampling (shoots) ¹	Time of sampling	Health status	Area / Region
1	1	4.2	2.9	3 (9)	April, June, Aug.	Healthy	Vantaa, South Finland
2	1	3.7	2.9	3 (9)	April, June, Aug.	Healthy	Vantaa, South Finland
3	1	22.2	10	3 (9)	April, June, Aug.	Healthy	Vantaa, South Finland
4	1	10	5	3 (9)	April, June, Aug.	Healthy	Vantaa, South Finland
5	1	5.3	4.2	3 (9)	April, June, Aug.	Healthy	Vantaa, South Finland
6	2	35.8	21.4	1 (10)	Sept.	Healthy ²	Nauvo, Southwest Finland
7	2	14.6	10.3	1 (5)	Sept.	Diseased	Nauvo, Southwest Finland
8	2	17	12	1 (5)	Sept.	Diseased	Parainen, Southwest Finland

¹ The number of sampling events and the total number of shoots collected.² Of the ten sampled shoots nine were healthy and one was dead.**Fig. 2.** Sampled trees numbered as 1–5 (A to E) originated from southern Finland (site 1), and trees 6–8 (F to H) from southwest Finland (site 2). Trees 6–8 were cut down for sampling.

AGGCTCGGGTAGCGAATAGGATTAGATACCC-3') hybridized within the region amplified by the PCR primers from base 710 to base 740. The length of the amplification product was 79 bp.

All DNA samples were assayed in Hard-Shell 384-well PCR plates (Bio-Rad) closed with Microseal B optical adhesive seals, using the CFX384 Touch Real-Time PCR System (Bio-Rad). The real-time PCR reaction was performed in a final volume of 10 µl. Each reaction contained: 300 nM forward primer (IDT Belgium); 300 nM reverse primer (IDT Belgium); 150 nM fluorogenic probe (IDT Belgium); 5 µl PerfeCTa qPCR ToughMix (Quantabio); 2 µl sample of original unequilized shoot DNA diluted tenfold. Each DNA sample was assayed in two replicates. Sterile water was used as negative control. The PCR protocol was 95 °C (3 min); 40 cycles of 95 °C (15 s); 65 °C (30 s) including plate read at 65 °C. The efficiency of the qPCR was evaluated by making tenfold dilutions of a DNA of Finnish *D. sapinea* isolate (ID138.1, isolated from southwest Finland). A set of shoot samples confirmed to be *D. sapinea* negative were used to determine the threshold for false positive and accordingly, samples with Cq of more than 35 were considered as negative.

Amplicon sequencing and bioinformatics.

The DNAs of trees 1–8 (Fig. 2, Table 1, Supplementary File S1) were used for amplicon sequencing. The internal transcribed spacer 1 (ITS1) region was amplified using the primer pair ITS1-1F (5'-CTTGGTCATTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS2 (5'-GCTGCGTCTTCATCGATGC-3') (White et al., 1990) via PCR. Subsequently, Illumina Sequencing platform was employed to sequence the amplicons, yielding paired-end raw reads of 250 bp length. Original unequilized shoot DNAs were sent to Novogene (Cambridge Science Park, United Kingdom) where at PCR, library preparation, and sequencing processes for 65 shoot samples was conducted. The raw data underwent processing through Novogene's in-house protocols. In brief, paired-end reads were initially sorted into samples based on their unique barcodes and then processed to remove the barcode and primer sequences. The merging of paired-end reads was accomplished utilizing FLASH (V1.2.7) (Magoč and Salzberg, 2011). Subsequently, stringent quality filtering was applied to the raw tags to retain only high-quality clean tags (Bokulich et al., 2013), adhering to the quality control procedures outlined in QIIME (V1.7.0) (Caporaso et al., 2010). The clean tags were subjected to chimera detection using the UCHIME algorithm (Edgar et al., 2011), and any identified chimera sequences were subsequently eliminated. For the characterization of mycobiome community composition within each sample, operational taxonomic units (OTUs) were generated through clustering at 97 % identity, with taxonomic assignments facilitated by comparison with the Unite database (Abarenkov et al., 2023).

The absolute number of sequences were used for further analyses. OTUs having five or less reads were removed from the analysis. One sample did not amplify and was removed from analysis (tree no 3, sampling time August). Raw data for trees 1–5 and 6–8 were processed separately, and all analyses were conducted independently for each dataset. The raw datasets generated and/or analyzed in this research are available on NCBI under BioProject PRJNA1125192 (Site 1) and PRJNA1125206 (Site 2), encompassing individual accessions for each shoot at Supplementary File S1.

2.1. Statistical analysis

All analysis was run with R version 4.2.2 (R Core Team, 2023). Statistical analyses were conducted separately for each site. Normality of the data was assessed using the Shapiro-Wilks normality test (Royston, 1982). Alpha diversity was determined using Simpson (Simpson, 1949) and Shannon-Wiener (Shannon, 1948) indexes. For normally distributed data, the standard *t*-test was employed for factors with two variables, while ANOVA was utilized for factors with more than two variables. Post hoc analyses were conducted using Tukey's HSD test when factors exhibited significant variation. Non-normally

distributed data were analyzed using the non-parametric Kruskal-Wallis test for factors with more than two variables, followed by post hoc analysis using Dunn's Bonferroni correction. The non-parametric Wilcoxon rank sum test was applied for factors with two variables. *P*-values were adjusted using the Bonferroni method. The differences in abundance (as the number of reads) were compared among *D. sapinea* in different trees/upper and lower crown/diseased versus healthy twigs. The dead shoot (no. 9, Supplementary File S1) was removed from *D. sapinea* abundance analysis in healthy versus diseased shoot analysis. The presence (positive/negative) of *D. sapinea* was compared to the averages of weather data from the sampled locations in 2022 and 2023 (average temperature in °C and precipitation in mm from May to August), which was provided by the Finnish Meteorological Institute (FMI).

The permutational analysis of variance (PERMANOVA) based on Bray-Curtis with 999 permutations were performed with using the *adonis* function in *vegan* (Oksanen et al., 2022) to identify differences in mycobiome between trees/sampling time/health status within individual sites (with no inter-site comparisons). Principal coordinate analysis (PCoA) was used to visualize the fungal community structure. This was performed using *vegan* (*bray*) (Oksanen et al., 2022) and *ggplot2* (Wickham, 2016) packages in R. A correlation (Spearman's rank correlation) analysis was carried for the *D. sapinea* or sampling time/health status and the most abundant identified fungal OTUs in each site. Indicator species for sampling time were identified with the R package *indicspecies* (Cáceres et al., 2010). A *p*-value below 0.05 was considered statistically significant in this study.

Schematic presentation of *Diplodia sapinea* disease symptoms.

The disease development was monitored year-round at two different locations in Finland (Terhonen, 2023; Terhonen et al., 2023). The presence of the pathogen was confirmed using the cultural methods described above. The inoculated seedlings used above (side-shoots of three-year old or main shoots of two-year-old) were followed year-round, and the development of fruiting structures was confirmed through microscopy and spore plating.

3. Results

The distribution of *D. sapinea*.

Altogether we processed 2603 shoot samples, of which 21 % were positive for *D. sapinea* based on species identification of isolated endophytes. To safeguard citizens' privacy, we aggregated multiple samples from the same area to represent a single positive spot on the distribution map. This resulted in 164 sample locations, with 64 positive observations (Fig. 3). All the strains of *D. sapinea* chosen to examine the pathogenicity of the Finnish strains caused necrosis for the three- and two-year-old Scots pine seedlings, confirming the pathogenic status (Supplementary File S2), and the pathogen was re-isolated from the borderline between living and dead phloem and sapwood. The inoculation of representative pathogenic strains (ID 67.4, 130.2, 138.1) into Scots pine seedlings resulted in typical symptoms of *Diplodia* tip blight (Fig. 1). Koch's Postulates 2–4 were fulfilled here for the putative causal agent, *D. sapinea*, as follows: *D. sapinea* was the most commonly isolated fungus from diseased Scots pine, inoculation of healthy Scots pine with *D. sapinea* caused the disease; and *D. sapinea* was re-isolated from the inoculated, diseased Scots pine. However, the length of necrosis varied between *D. sapinea* strains when inoculated into side shoots. In the main shoot, there was no difference between the two strains used.

The distribution of *D. sapinea* in Finland corresponds to areas with the highest average temperatures, as shown for the year 2023 (Fig. 4). Similarly, most observations are along the coastline, which represents areas with lower precipitation. The presence (positive/negative) was statistically supported ($p < 0.001$) (Fig. 5), as the positive observations of *D. sapinea* were associated with lower precipitation (mm) and higher average temperatures (°C) in the years 2022 and 2023.

Mycobiome composition in South Finland (Site 1).

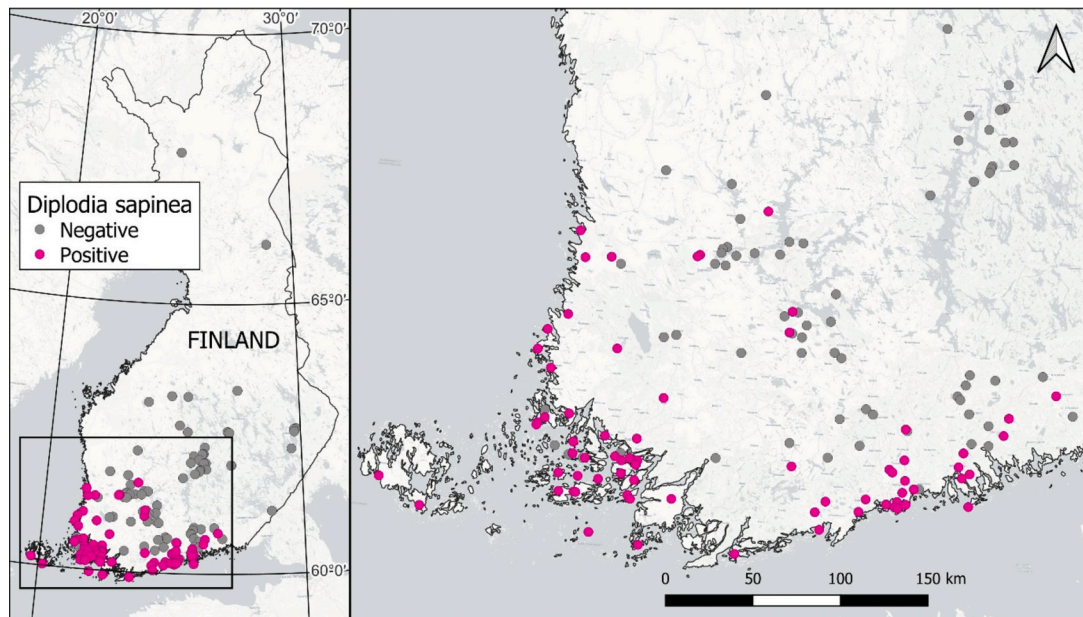


Fig. 3. The locations of scots pine trees of which shoot samples were processed and the presence (purple) or absence (gray) of *Diplodia sapinea* was examined. Basemap: ESRI light gray via QGIS software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Altogether 1030 OTUs were generated, of which 13 % could be assigned only to the kingdom Fungi level. 680 OTUs were assigned to Ascomycota, including Archaeorhizomycetes (1 %), Arthoniomycetes (0.4 %), Dothideomycetes (23 %), Eurotiomycetes (11.6 %) Lecanoromycetes (3.6 %), Leotiomycetes (10 %), Lichinomycetes (0.1 %), Orbiliomycetes (0.7 %), Pezizomycetes (1.3 %), Saccharomycetes (0.2 %), Sordariomycetes (6.5 %), Taphrinomycetes (1 %) of all OTUs. Basidiomycota were 186 OTUs, from which Agaricomycetes (12.6 %), Agaricostilbomycetes (0.5 %), Cystobasidiomycetes (0.2 %), Exobasidiomycetes (0.7 %), Microbotryomycetes (0.6 %), Pucciniomycetes (0.2 %), Tremellomycetes (2.8 %), Ustilaginomycetes (0.1 %) of all OTUs. Eleven OTUs were assigned to Chytridiomycota, one to Glomeromycota, 13 to Mortierellomycota, one to Rozellomycota and one to Zoopagomycota. The most common fungus was *Sydowia polyspora* (OTU 1, 687,697 reads). *Diplodia sapinea* was observed from Site 1 with only 26 reads (Supplementary File S1). The mycobiome (OTU composition) did not differ between the sampled trees ($p = 0.1367$) (Fig. 6), but it changed during the growth season ($p < 0.001$) (Fig. 6).

The diversity indexes (Shannon-Wiener and Simpson) were not meaningfully different between trees. Simpson index did differ between sampling seasons ($p = 0.003985$), but the Shannon-Wiener indexes were not different ($p = 0.7766$). Post hoc test showed that the difference was between sampling times June and August. Indicator species for the month of April included 26 OTUs, mainly considered rare (read no. < 850). More relevant indicator species with a read number greater than 850 were *Lapidomyces* sp. (OTU 5) and *Rhytismatales* sp. (OTU 16) and *Alternaria alternata* (OTU 103). For June, 75 indicator species were found. *Sydowia polyspora* was identified as indicator species for June ($p = 0.0005$). Similarly, *Herpotrichiellaceae* sp. (OTU 7), *Perusta inaequalis* (OTU 8), *Dermateaceae* (OTU 17), *Peziza succosa* (OTU 28), *Dothideomycetes* sp. (OTU 102) were found as indicator species with reads greater than 850. Spearman's rank correlation analysis showed only strong correlation between sampling time and OTU 10 (Fungi sp., $r = 0.74$) (Supplementary File S2).

Mycobiome composition in Southwest Finland (Site 2).

Altogether 712 OTUs were generated, of which 89 OTUs could be assigned only to the fungal kingdom (12.5 %). Ascomycota were assigned to 446 OTUs, including Archaeorhizomycetes (0.4 %), Arthoniomycetes (0.3 %), Dothideomycetes (21.6 %), Eurotiomycetes (9.5 %)

Lecanoromycetes (2.8 %), Leotiomycetes (13 %), Lichinomycetes (0.1 %), Orbiliomycetes (0.7 %), Pezizomycetes (0.7 %), Saccharomycetes (0.7 %), Sordariomycetes (5.8 %), Taphrinomycetes (1.1 %) of all OTUs. Basidiomycota were 162 OTUs, from which Agaricomycetes (12.6 %), Agaricostilbomycetes (1 %), Cystobasidiomycetes (0.1 %), Exobasidiomycetes (0.8 %), Malasseziomycetes (0.1 %) Microbotryomycetes (2 %), Pucciniomycetes (0.1 %), Tremellomycetes (5.3 %) of all OTUs. Four OTUs were assigned to Chytridiomycota, 7 to Mortierellomycota, one each to Mucoromycota, Neocallimastigomycota and to Olpidiomycota.

The prevalent fungus observed was *Sydowia polyspora* (OTU 1, 453,356 reads), followed by *Diplodia sapinea* (OTU 2, 236,503 reads). Analysis revealed differences in mycobiome composition (OTU composition) between sampled trees ($p = 0.0024$, Fig. 7) and among different health statuses ($p = 0.0202$, Fig. 7). However, no significant difference was observed in the mycobiome between the upper and lower crowns of tree no. 6 (Fig. 7). The Shannon-Wiener and Simpson indexes did not differ between trees or healthy and diseased trees.

The abundance of *D. sapinea*, measured in terms of sequence reads, exhibited variability among the sampled trees, yet this difference did not reach statistical significance. Similarly, although the upper crown of tree no. 6 tended to harbour more *D. sapinea* compared to the lower crown, this difference lacked statistical significance. Notably, while the abundance of *D. sapinea* was higher in healthy tissue of diseased shoots compared to healthy ones, this difference did not attain statistical significance.

According to the Spearman's rank correlation analysis, a strong correlation was observed between *D. sapinea* and certain microbial taxa (Supplementary File S2). Specifically, the family Teloschistaceae (OTU 6) exhibited a strong negative correlation ($r = -0.61$), while *Pleurophoma ossicola* (OTU 29) showed a strong positive correlation ($r = 0.65$) with *D. sapinea*. Additionally, a moderate negative correlation ($r = -0.46$) was observed between *D. sapinea* and *S. polyspora*.

The dead shoot from tree no. 6 was removed from the data analysis, and the correlation between tree health and the most common OTUs showed a strong negative correlation with Dothideomycetes ($r = -0.65$), *Mycosphaerella tassiana* ($r = -0.71$), *Cladosporium tenuissimum* ($r = -0.67$), *Dermateaceae* ($r = -0.62$), Fungi (OTU17) ($r = -0.65$), *Dermateaceae* ($r = -0.65$), *Perusta* sp. ($r = -0.69$).

Detection of Diplodia sapinea symptomatic and healthy Scots pine

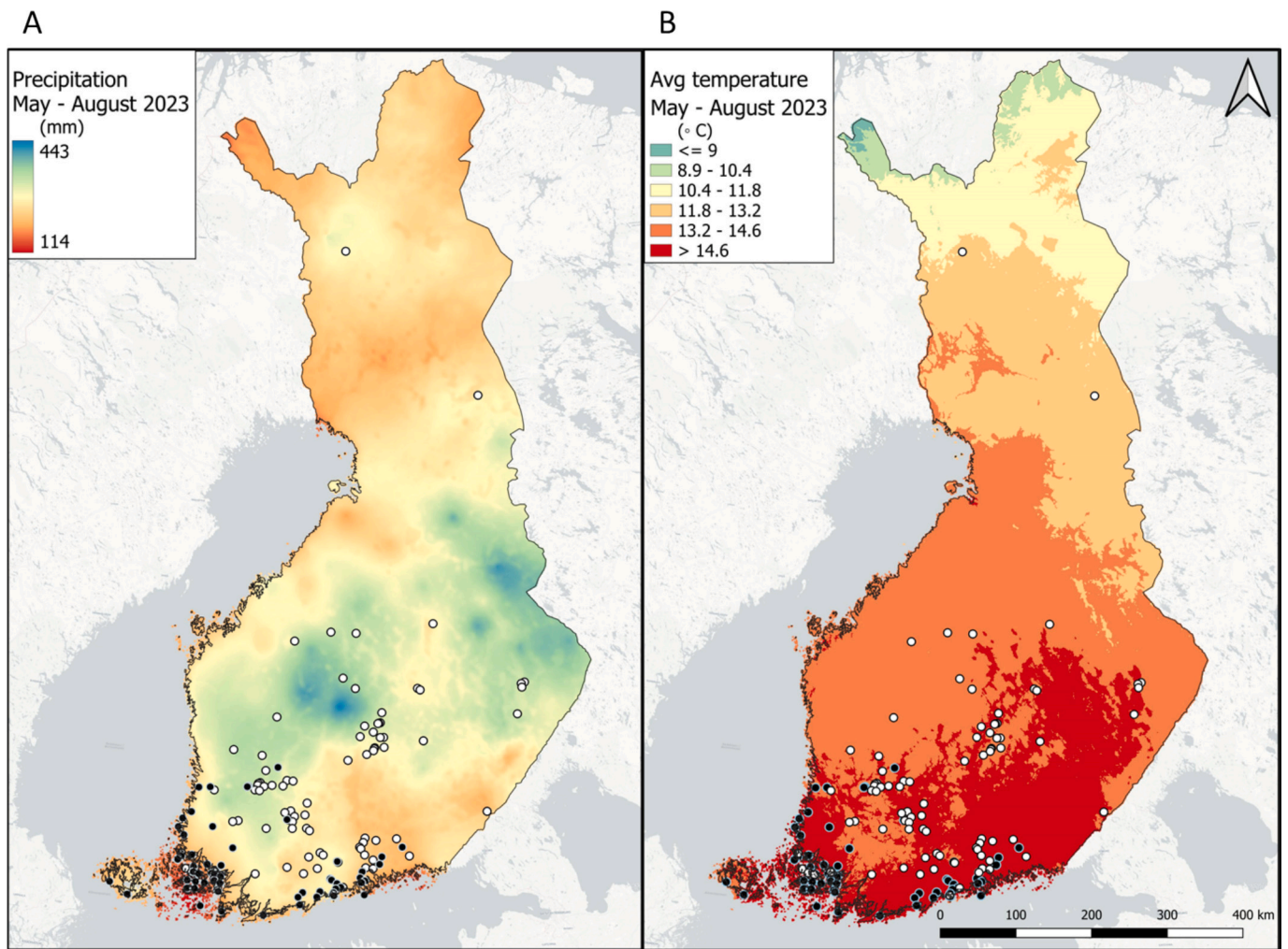


Fig. 4. The presence of *Diplodia sapinea* aligned with the weather data (FMI). Black dots represent positive samples, while white dots represent negative samples. A) Precipitation in mm, with positive (black dots) and negative (white dots) observations of *D. sapinea*. B) The average temperature from may to august 2023 in mm, with positive (black dots) and negative (white dots) observations of *D. sapinea*. Basemap: ESRI light gray via QGIS software.

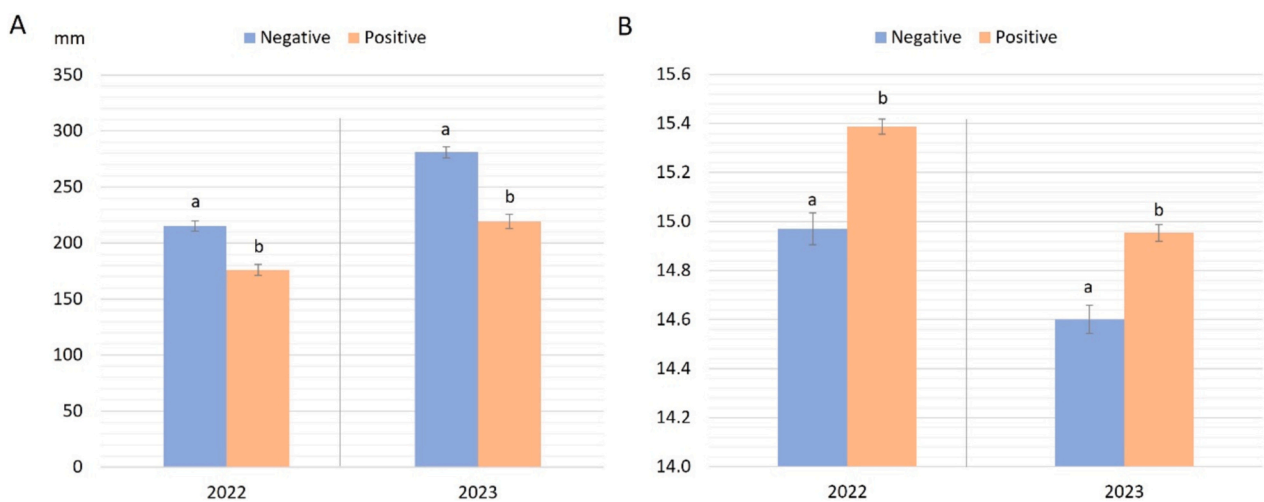


Fig. 5. The sampled locations for the presence of *Diplodia sapinea* (positive/negative) up until 2023 were compared to weather data (FMI) from the same locations for the period of may to august in 2022 and 2023. A) Precipitation in mm; B) average temperature. The presence of *D. sapinea* is statistically associated with lower precipitation and higher temperatures.

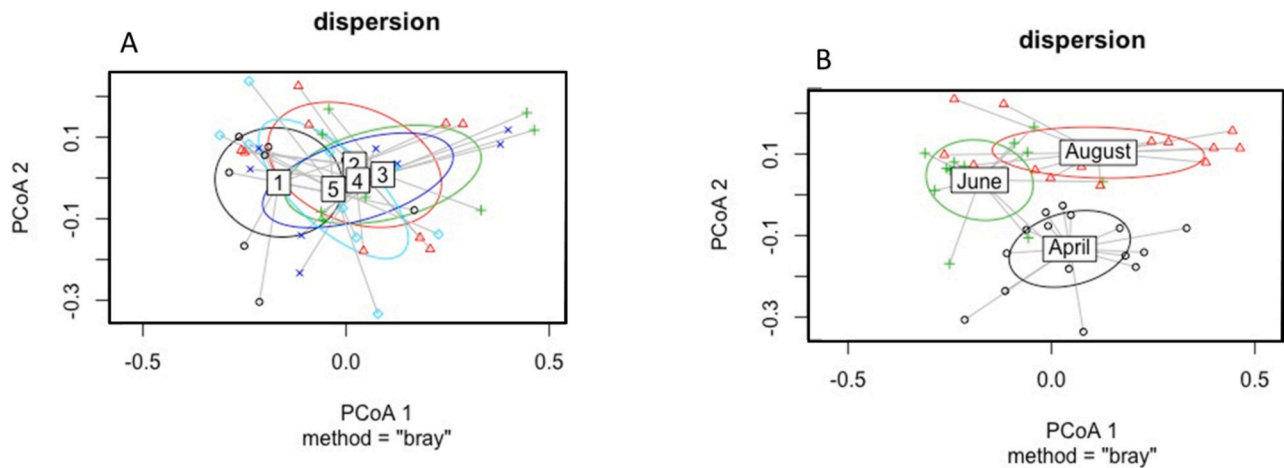


Fig. 6. A) Dispersion of mycobiome (OTU composition) among sampled healthy trees (no. 1 to 5) based on bray-curtis. B) Dispersion of mycobiome (OTU composition) between sampling months in site 1 (south Finland).

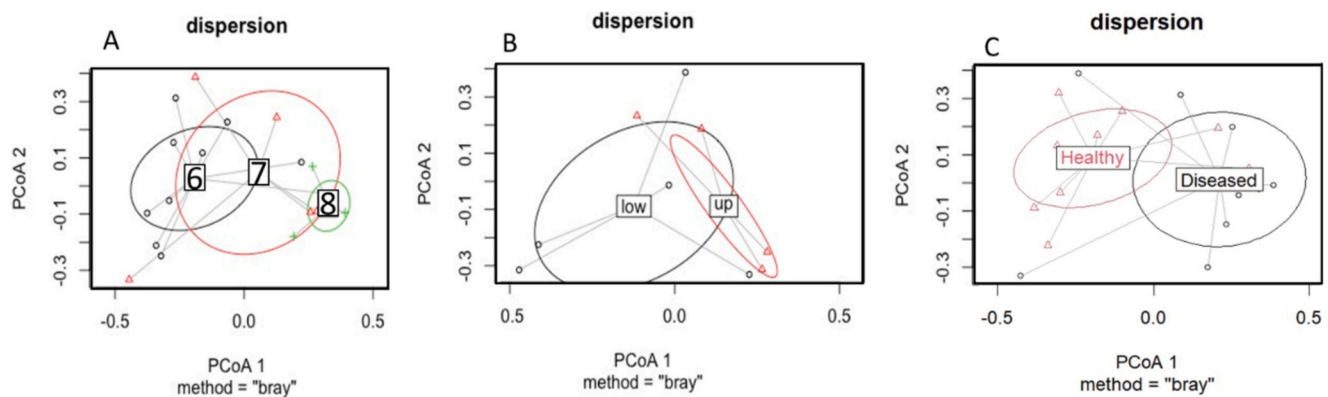


Fig. 7. Fungal communities' dispersion based on bray-curtis distances. A) Among sampled trees; B) among the upper and lower crown of tree no. 6; C) among healthy and diseased shoots in site 2.

shoots.

Basic PCR and qPCR were utilized for the detection of *D. sapinea* in shoots collected from healthy and diseased trees of Sites 1 and 2. Both

Table 2

The detection success (-,+) of *D. sapinea* from three scots pine trees (6, 7, and 8) of study site 2.

Tree no.	Shoot ID	Shoot status	Number of reads	Success of isolation	PCR result	qPCR result
6	1	Healthy	5491	-	-	-
6	2	Healthy	177	-	-	-
6	3	Healthy	5628	-	-	-
6	4	Healthy	1977	-	-	-
6	5	Healthy	6274	-	-	-
6	6	Healthy	859	-	-	-
6	7	Healthy	152	-	-	-
6	8	Healthy	7774	-	-	-
6	9	Dead	12	-	-	-
6	10	Healthy	16	-	-	-
7	16	Diseased	30	-	-	-
7	17	Diseased	541	-	-	-
7	18	Diseased	68,984	-	+	+
7	19	Diseased	545	ND	+	-
7	20	Diseased	17,122	+	+	+
8	21	Diseased	18,048	-	+	+
8	22	Diseased	76,836	+	+	+
8	23	Diseased	9772	-	+	+
8	24	Diseased	335	-	+	+
8	25	Diseased	15,930	+	+	+

methods detected *D. sapinea* in shoots showing symptoms of Diplodia tip blight (Table 2, Supplementary File S1). The detection of *D. sapinea* was achieved using less than one nanogram of Scots pine shoot DNA.

Schematic presentation of Diplodia sapinea disease cycle in Scots pine in Finland.

The illustrated disease cycle of *D. sapinea* in Finland (Fig. 8) is based on three years of field observations (2021–2023), followed by the identification of pathogenic *D. sapinea* in plant tissue. Similarly, we infected two- and three-year-old Scots pine seedlings and monitored disease progress year-round. Asexual spores were produced in dead plant material, primarily in needles. Infections in nature occur through stomata or in the current-year shoots before lignification (Bachi and Peterson, 1985; Stanosz et al., 2001) (spring to summer), and the infection may remain latent. The pathogenic stage is triggered by additional stress experienced by the host (such as lower precipitation) or higher temperatures. If the infection occurred in the previous year (e.g., during 2021), the pathogen may begin killing the shoot in early spring (2022), leading to shoot death before new growth. Current-year shoots may die during the summer (May to July), resulting in short needles. In autumn, the dead shoots are easily identified by their short, brown, attached needles.

4. Discussion

In the present study, *D. sapinea* was found in Finland in locations with higher average summer temperatures (May to August) and lower precipitation. Based on our results on symptomatic Scots pines,

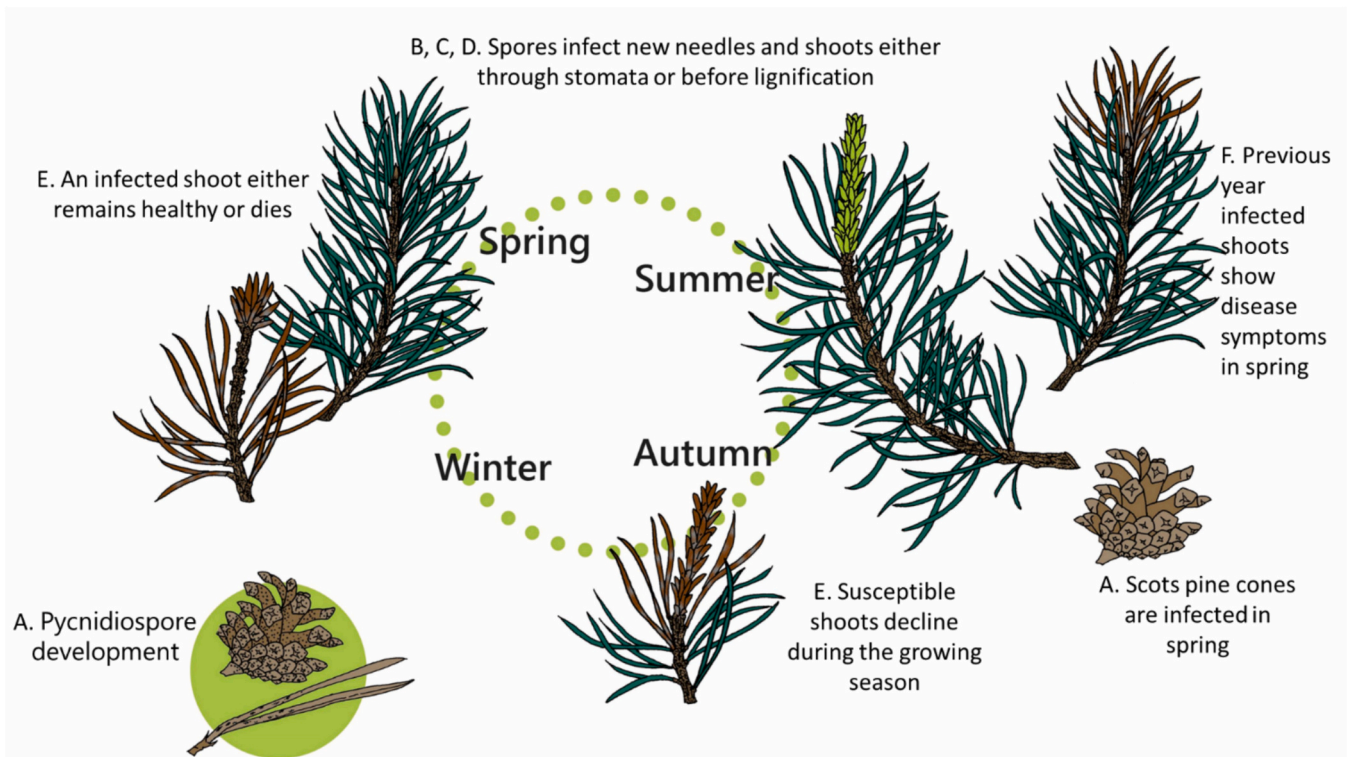


Fig. 8. The disease cycle of *Diplodia sapinea* in Finland. A) Pycnidia develop at the base of needles and on cone scales in autumn of the infection year, where they produce asexual pycnidiospores (Brookhouser and Peterson, 1970; Peterson, 1977; Phillips et al., 2013). B) The spores have shown to be produced from April to November (Brookhouser and Peterson, 1970; Brodde et al., 2019). C) The needles are susceptible to infection throughout the growing season (after needle emergence) (Brookhouser and Peterson, 1970) and spores enter through stomata (Li et al., 2019). D) *D. sapinea* can also infect elongating shoots before they lignify (Flowers et al., 2001; Oostlander et al., 2023). E) The infection can remain asymptomatic, or the shoot may start to decline in the summer after infection, with needles remaining short. F) The infected, visually healthy shoots begin to show symptoms of the disease in the spring.

D. sapinea is currently heavily infecting trees in the coastal areas of Southwest Finland, covering areas where *D. sapinea* was previously found as a saprophyte (Müller et al., 2019; Terhonen et al., 2021). Overall, the previous findings of *D. sapinea* as saprophyte or endophyte are observed in the current distribution area. Given the endophytic nature of this pathogen, it is likely that the actual disturbance region extends further. The northernmost disease spot observed in this study was in Ylöjärvi, Tampere region, representing the most northern limit of *D. sapinea* (between parallel 61–62 latitude) in Finland. As temperature sums are predicted to rise and droughts to increase (Mikkonen et al., 2015; Rantanen et al., 2022) the distribution of *D. sapinea* can be assumed to extend in Finland. Based on observations made in Sweden (Brodde et al., 2019), this can happen unnoticed, resulting in sudden disease outbreaks (Brodde et al., 2023). The exact timing and location of *D. sapinea* arrival in Finland remain uncertain. In 2004, it was not detected at a surveyed site, but it was present there by 2015 (Müller et al., 2019). The pathogen clearly follows the coastline, suggesting its introduction occurred through southern and southwestern Finland. Moreover, there must be several points of introduction for *D. sapinea*. It is known that spores can be transported in seeds (Cleary et al., 2019), or the pathogen may be introduced through diseased yet visually healthy seedlings (Stanosz et al., 2007). It has also been shown that *Ips acuminatus* can carry *D. sapinea* (Davydenko and Baturkin, 2020). As the main vector, timing, and entry point of *D. sapinea*'s arrival in Finland remain uncertain, we will focus on the clear fact that it can establish itself in Finland, reproducing, infecting, and ultimately causing disease in Scots pine (Terhonen, 2023) and junipers (Terhonen et al., 2023).

Climate change seems to contribute to the establishment of *Diplodia sapinea* in northern regions. Increased drought, driven by climate change, poses a significant threat to the health of forest ecosystems (Seidl et al., 2017). This threat originates from the complex and dynamic

interactions within forest pathosystems, wherein prolonged periods of drought-induced stress in trees can disrupt the symmetry between fungal pathogens and their hosts. Under such conditions, there is an increased risk that fungal pathogens gain an advantage over their hosts, which potentially leads to detrimental consequences for increased disease outbreaks. While climate change can intensify fungal virulence by creating more favourable conditions for pathogen proliferation within existing ecosystems (Desprez-Loustau et al., 2006; Terhonen et al., 2019), it also facilitates the expansion of pathogens into new geographic areas (Desprez-Loustau et al., 2007). As climate patterns shift and previously inhospitable regions become more hospitable to these pathogens, they may spread into previously unaffected areas and become established there. Consequently, comprehensive strategies for forest management and conservation must address not only the impacts of climate change on existing pathosystems, but also the potential for the emergence of novel pathogen-host interactions in previously unaffected regions.

Utilizing education alongside citizen science proved to be highly beneficial in this study, enabling us to accurately delineate the current distribution of this pathogen. Citizen observations play a crucial role in monitoring tree health, particularly in urban environments, highlighting the importance of science education for all.

The endophytic nature of the pathogen poses challenges for its identification in new locations. All tested methods (culturing, amplicon sequencing, species-specific PCR, qPCR) can be used in the detection of the pathogen in diseased shoots. Based on the amplicon sequencing, *D. sapinea* was also found present in the healthy shoots of diseased Scots pines in Southwest Finland. However, the presence of pathogenic fungus could not be confirmed from healthy shoots using PCR or qPCR. This was surprising, since the shoot DNA used in species-specific PCR and qPCR with fluorescent probe was the same used in amplicon sequencing.

Based on the Bray-Curtis distances the mycobiome of healthy and diseased Scots pines was statistically different. Thus, is it possible that the DNA extracted from healthy shoots contained other microbes, causing competition between target sequences. Here, we did not distinguish how much of the extracted shoot DNA originated from Scots pine, fungi, or bacteria, nor did we equalize the quantity of DNA utilized in *D. sapinea* detection. The DNA concentration of healthy shoots was higher than the DNA concentrations of diseased shoots, probably due to greater quantity of host DNA. However, consistent results were generated using further purified shoot DNA and/or dilutions, indicating that inhibition caused by impurities in DNA was not causing false negatives. Hence, there is still a need for a reliable and sensitive detection method for endophytic *D. sapinea*. The choice of identification method depends on the study question and available funds. As the sample amount for typical DNA extraction is quite low, several extractions (and assays thereafter) would be needed to screen a given shoot or tree. For instance, here, only 70 to 100 mg per shoot was used. With the conventional culturing method (Terhonen, 2023) a lot of plant material can be assayed reliably and relative fast.

In the west coast area (Site 2, Southwest Finland), *D. sapinea* was the second most common fungus following *Sydowia polyspora*. The presence of *D. sapinea* in asymptomatic shoots of a completely healthy tree in the heavily infected area confirms its proliferation as an endophyte (Blumenstein et al., 2021b). At Site 1 (South Finland), where *D. sapinea* was isolated but most trees appeared healthy, the number of reads in the amplicon data was low or non-existing, and the presence of pathogen could not be confirmed with PCR or qPCR from healthy shoots. Based on these results, it seems *D. sapinea* was not able to accumulate at Site 1, even though the fungus was isolated from a diseased Scots pine shoot earlier within close distance of sampled healthy trees. This raises the question: what restricts the establishment of *D. sapinea* in South Finland? A rough comparison of climate data for the past ten years reveals that the monthly rainfall (mm) at Site 1 (South Finland) averaged 61, compared to 44 at Site 2 (Southwest Finland). The average monthly temperature (°C) over the same period was 6.5 at Site 1 and 7.6 at Site 2. Desprez-Loustau et al. (2007) simulated *D. sapinea* using the CLIMEX model, employing past climatic data, and showed a high potential for the development of the disease in the southern part of France. Similarly, they highlight that in most southwestern European regions, climate change will be characterized by warming and decreased precipitation during the vegetative season, favoring the establishment of *D. sapinea* (Desprez-Loustau et al., 2006, 2007). A rough analysis here of climate data over the past 10 years shows that Site 2 experienced higher temperatures and lower rainfall every month compared to Site 1. Perhaps the higher temperatures are the necessary factor to the better establishment and accumulation of *D. sapinea*. Another explanation could be the introduction of new, more aggressive strains. Adamson et al. (2021) showed that the first observed *D. sapinea* strains in Finland had relatively high genetic diversity (11 strains analyzed, 6 different haplotypes defined), resembling similar levels of genetic diversity as the older subpopulations found in central and southern Europe. As Finland shares the same haplotypes as other European countries (Adamson et al., 2021) combined with only recent observations of the pathogen (Müller et al., 2019; Terhonen et al., 2021; Terhonen, 2023), it is most likely that some *D. sapinea* strains have been introduced to Finland. The time, place, and substrate of the introduction remain a mystery. However, we hypothesize that the more critical factor is the environment. *Diplodia sapinea* has been able to establish itself better in Finland and will most likely continue emerging to more northern latitudes. The factors leading to the establishment of *D. sapinea* as latent opportunistic pathogen should be studied further. The data would be needed for future risk assessment for disease development.

The mycobiome of Scots pine shoots changed during the growing season, with fungal diversity and quantity increasing towards the end of the season, in accordance with previous studies (Terhonen et al., 2011; Blumenstein et al., 2021a). The mycobiome in Finland included the

same fungal genera and orders as previously observed in Europe from Scots pine (Blumenstein et al., 2021b), e.g., *Perista*, *Capnodiales*, *Chaetothiales*, and *Exophiala*, just to name a few. However, the most common ones observed in Germany (*Truncatella conorum-piceae* and *Microspphaeropsis olivacea*) were not found at the Finnish study sites. *Sydowia polyspora* was the most common fungus in both of our datasets in accordance with Blumenstein et al. (2021b) and Terhonen et al. (2011) datasets of Scots pine shoots and needles, respectively. The occurrence of *S. polyspora* has been shown to negatively correlate with *D. sapinea* by Oliva et al. (2021), a finding somewhat supported by the data from the present study. Oliva et al. (2021) hypothesized that symptoms of Diplodia tip blight do not develop, and trees stay asymptomatic when other endophytes, such as *S. polyspora*, can use the stress-related metabolite proline as a food source instead of *D. sapinea*. This could explain the negative correlation observed between *S. polyspora* and *D. sapinea* in trees. In theory, other fungal species could prevent the growth or transition of *D. sapinea* to a pathogen in the same niche (Oliva et al., 2021). Given the foliar fungal compositions of Scots pine across geographic latitudes (Millberg et al., 2015), combined with niche competition theory (Blumenstein et al., 2015), this could explain why *D. sapinea* is unable to establish itself in certain areas. However, this would require in vitro studies to demonstrate the fungal utilization of available resources (Blumenstein et al., 2015) as well as the identification of the different competitive strains (Blumenstein et al., 2021b; Oliva et al., 2021). Surprisingly, the absence of *D. sapinea* (in terms of number of reads) at Site 1 and from the seasonal data means we could not provide accumulation data for it during the growing season. Blumenstein et al. (2021b) found that *S. polyspora* did not exhibit seasonal variation when compared in June and September. However, our data showed that the abundance of *S. polyspora* in healthy tissue of Scots pine (Site 1) changed between seasons, being statistically higher in June compared to August.

The goal of this work was to determine the distribution and validate the virulence of *D. sapinea* in Finland, compare different identification methods, increase awareness of *D. sapinea* impact on pine trees in Finland, and promote further research into sustainable management strategies to combat this fungal pathogen. There is a clear need for improvement in diagnostic tools and predictive modelling methodologies to assess the prevalence of *D. sapinea*. Utilizing education alongside citizen science proved to be highly beneficial in this study, enabling us to accurately delineate the current distribution of this pathogen. Citizen observations play a crucial role in monitoring tree health, particularly in urban environments, highlighting the importance of science education for everyone. The projected climate change will most likely promote the spread of *D. sapinea* to new areas in Finland. We urgently need forest management guidelines for this disease. These guidelines should include protocols for the entire lifespan of a tree, from seed orchard, seed extraction, nursery, and regeneration to the tree growth period and mature tree.

Funding sources

This work was funded by Alfred Kordelin Foundation and Natural Research Institute Finland (Luke).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used OpenAI's ChatGPT in order to improve the accuracy and clarity of the English language. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

Availability of data and materials

The amplicon datasets produced and/or examined in the present

research are accessible at NCBI under BioProject PRJNA1125192 (Site 1) and PRJNA1125206 (Site 2). Other research material is available from the corresponding author on reasonable request. The *D. sapinea* strains are stored in Natural Resources Institute Finland, Helsinki, and are available for other researchers.

CRedit authorship contribution statement

Eeva Terhonen: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Tiina Ylioja:** Writing – review & editing, Investigation, Data curation. **Tuija Hytönen:** Methodology, Data curation. **Katri Leino:** Methodology, Data curation. **Linda Mutanen:** Methodology, Data curation. **Markus Melin:** Writing – review & editing, Visualization, Methodology, Data curation. **Eeva Vaahtera:** Visualization, Methodology. **Suvi Sutela:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We acknowledge and thank all Finnish citizens who contributed samples for this study. We would like to thank Prof. Dr. Jarkko Hantula, Prof. Dr. Matti Koivula, Elisa Vuorimaa and Aliisa Pöllänen for collecting additional samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fgb.2024.103955>.

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