Analysis of foliar fungi communities in Scots pine shelterwood regeneration

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Swedish University of Agricultural Sciences, SLU
Faculty of Forest Sciences
Southern Swedish Forest Research Centre & SLU Forest damage centre Forest and Landscape Program
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Abstract

This bachelor's thesis presents findings from the first year of a study by the SLU Forest Damage Centre on the effects of overstory density in shelterwood systems on the incidence of *Lophodermium* needle cast disease. Using needle samples collected for the two-year project, this work investigated how foliar fungi communities vary with shelterwood density and needle disease symptoms. Both *in vitro* culturing techniques and molecular identification were employed. Results showed that both average overstory density and needle symptom type had significant effects on fungal community composition. These findings contribute to a body of research on the foliar fungal communities of Scots pine in northern Europe, while highlighting the need for more comprehensive sampling.

Keywords: Pinus sylvestris, Sweden, shelterwood, clear cut-free forestry, fungal pathogen, endophyte, fungal community, Sanger sequencing, tallskärm, tallskytte

Acknowledgements

Thank you to: all forest owners for allowing us to sample their stands, Henrik for your herculean sample collection and documentation effort, Ira for your trust and guidance, Kuki for your collaboration, Mikolaj for your extra assistance, Emrik for your true support, Mom and Pop for keeping my stress low!

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Abbreviations

BLASTn	Basic local alignment search tool of nucleotide databases
DBH	Diameter at breast height
DNA	Deoxyribonucleic acid
GPS	Global positioning system
ID	Identifier
ITS	Internal transcribed spacer
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
SLU	Swedish University of Agricultural Sciences (*Sveriges lantbruksuniversitet*)
TAE	Tris-Acetate-EDTA
UNITE	User-friendly Nordic ITS Ectomycorrhiza database
WPGMA	Weighted pair group method with arithmetic mean

1. Introduction

1.1 Scots pine in Swedish silviculture & shelterwood systems

Swedish forestry is relatively uniform in terms of the species composition and silvicultural practices, with two conifer species—Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*)—accounting for almost 80% of the total standing timber volume (m^3sk) and even-aged rotation forestry used as the standard method (SLU Riksskogstaxeringen, 2024). In recent years, threats to forest ecosystems by climate change have highlighted the need to diversify approaches to forestry in Sweden. Altered growing conditions and abiotic disturbances like droughts and storms are stressors that are likely to become increasingly frequent in the near future. An already-stressed tree is less capable of resisting infections and pest infestations, which underscores the need for management diversification (Hirons and Thomas, 2018; Huo et al., 2025).

Shelterwood systems (*Fig. 1*) are a silvicultural method that have been gaining popularity in Sweden as an alternative to even-aged rotational forestry. In the past, there had been various opinions regarding the exact definition of a shelterwood system (Karlsson & Örlander 2004). Generally, the practice involves leaving behind some mature trees after harvesting a forest stand, then removing them once the regeneration reaches a certain height. In 2021, the Swedish Forestry Agency released an updated definition of continuous cover forestry, which included specific numeric parameters for a shelterwood system. By their definition, a shelterwood system requires:

- The number of retained trees per hectare must exceed a minimum threshold determined by a standardized curve (called the 5§ curve), which calculates acceptable density based on tree volume (m^3sk) and height. Mature trees should be spaced evenly across the stand, avoiding clusters that leave large gaps without canopy cover.
- Gradual thinning of the retained mature trees over time to 50% of the 5§ curve after the next generation has been established. The regeneration should be a certain density (seedlings per hectare) depending on the tree species and location in the country.
- Removal of the retained trees to 25 trees per hectare once the regeneration has reached an average height of 2.5 m.
- Remaining trees (except those retained for conservation) are allowed to be harvested once the regeneration exceeds 10m (which is where the 5§ curve begins) (Appelqvist et al. 2021).

Figure 1: Shelterwood of Pinus strobus in the US (Steven Katovich, Bugwood.org)

Pinus sylvestris is often the first choice in Swedish shelterwood systems because it makes up such a large percentage of forests, and mature trees are physiologically better adapted to grow in a shelterwood structure than spruce, largely because pine's deeper root system prevent windthrow damage (Karlsson & Örlander 2004). Considered a pioneer species, *P. sylvestris* tolerates a wide range of growing conditions, including low soil nutrients, sandy soils, and droughts (Durrant et al. 2016). It grows throughout Sweden and as of 2024 made up 40% of the standing volume in forests nationwide (SLU Riksskogstaxeringen 2024). Due to its ubiquity, pine is economically important (34% of total harvested volume in 2024 (SLU Riksskogstaxeringen 2024)) and ecologically vital within forest ecosystems (Huo et al. 2025). However, climate change-induced drought stress is increasingly afflicting *P. sylvestris* stands, heightening susceptibility to, among other problems, pathogenic fungi (Millberg 2015).

1.2 Foliar fungi

While the term "endophyte" technically refers to any organism living inside plant tissue, mycologists typically use the term "fungal endophyte" to describe fungi that colonize plants without producing visible disease symptoms (Schulz & Boyle 2005). Since this project examines pine needles with both symptomatic and asymptomatic fungal infections, the term "foliar fungi" will be used instead of "endophytes" to avoid implications about lifestyle.

Foliar fungi are extremely widespread and diverse with regards to their lifestyles, which can span from mutualism to parasitism (Rodriguez et al. 2009; Jain et al. 2019). A single fungal species may itself exhibit multiple lifestyles: enhancing host stress tolerance as a mutualist, persisting asymptomatically as an endophyte, or as a pathogen, causing varying levels of damage to the host plant (Schulz & Boyle 2005; Delaye et al. 2013). The outcome of the interaction depends on environmental factors (Delaye et al. 2013) and host genotype (Schönrogge et al. 2022). Pathogenic fungi affect their hosts through the secretion of various enzymes that release nutrients from the host tissue cells, thus creating visible symptoms of infection. Symptoms of disease can also be caused

by the host plant's immune response to the fungi, for example by destroying cells to deprive the pathogen of nutrients or releasing substances toxic to fungi (Sinclair & Lyon 2005).

The switch between lifestyles is increasingly observed in Swedish forests, where fungi that were previously endophytic such as *Sydowia polyspora*, *Lophodermella spp.,* and *Diplodia sapinea* are more frequently acting as serious pathogens due to increased abiotic stressors (Ridout & Newcombe 2018; Ata et al. 2022; Brodde et al. 2023).

One fungal genus of particular note is *Lophodermium* spp., which is a globally distributed group of conifer needle colonizers. Most species exist as asymptomatic endophytes, minor pathogens, or saprophytes, though their ecological roles vary by species and environment (Deckert et al. 2001; Sinclair & Lyon 2005). On the other hand, *L. seditiosum* has a pathogenic lifestyle that causes a serious needle cast disease (Håkansson 2000). The species has a one-year reproductive cycle that can be followed through the development of characteristic symptoms (Skogsstyrelsen 2025). Dark spots, often with yellow margins, develop on needles beginning in late autumn (*Fig. 2, left*), and by springtime the needles change from yellow to reddish brown. Infected needles are then prematurely cast from the branches, and fruiting bodies emerge from the dead needles on the forest floor (*Fig. 2, right*) (Staley & Nicholls 2004; Hanso & Drenkhan 2012).

Figure 2: Symptoms of Lophodermium spp. infection on living and dead pine (Pinus spp.) needles (Left: Darroll D. Skilling, USDA Forest Service; Right: Petr Kapitola, Central Institute for Supervising and Testing in Agriculture, Bugwood.org)

Lophodermium needle cast has plagued Swedish pine cultivation for over a century (Lagerberg 1915; Statens skogsförsöksanstalt 1936; Stefansson 1967). Historically, pine needle cast epidemics across northern Europe begin with mild winters followed by cool, wet summers (Sinclair & Lyon 2005; Hanso & Drenkhan 2012). Only the newest needles are affected and though rarely lethal, the loss of this needle cohort reduces the trees photosynthetic capacity and thus stunts the growth of young trees both upwards and outwards (Drenkhan et al. 2006). In Sweden, young pines in the south and center of the country (*Fig. 3*) along with nursery stock nationwide typically face the highest infection rates (Stenström 2008; Skogsstyrelsen 2025). It is also presumed that *Lophodermium* infections are higher within shelterwood systems because the canopy creates more favorable conditions for disease proliferation (Karlsson & Örlander 2004).

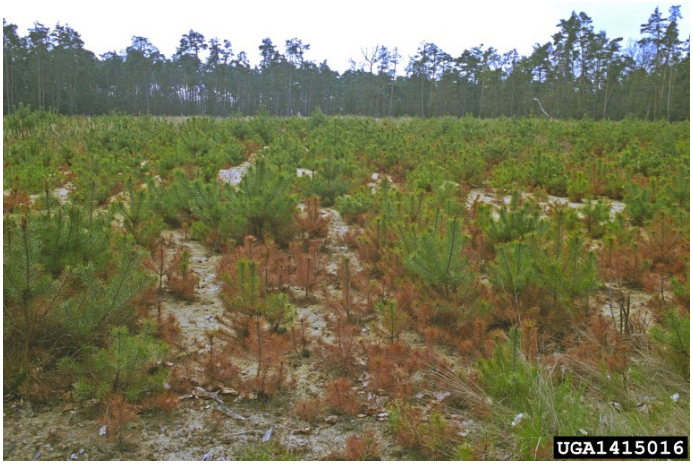

Figure 3: A recently regenerated P. sylvestris stand in which the seedlings have contracted a needle cast disease (Andrej Kunca, National Forest Centre - Slovakia, Bugwood.org)

1.3 Project background

This project connects the subjects of pine shelterwood systems in Sweden and the increasing threat of damage caused by *Lophodermium seditiosum* infection. Mikolaj Lula from the SLU Forest Damage Centre began the study to investigate the relationship between the overstory density in a shelterwood system and the presence of *Lophodermium spp.* or other pine needle pathogens (SLU 2023). The study begun in the summer of 2024, and I have been assisting as a laboratory technician, processing samples taken from the field since August.

As it is a two-year study, there will not be any results for the entire project yet, so the aim of this bachelor's thesis is to analyze the results of the work that I have performed thus far. During the process, I became interested in investigating a link between the symptoms present and the fungal communities in the needles that caused them.

1.4 Hypotheses

This study aims to assess the incidence and types of symptoms caused by fungi in pine needles. **Hypothesis 1**: The composition of foliar fungi communities in pine needles significantly differs among sites with varying average overstory densities. **Hypothesis 2**: Needles with different visible disease symptoms harbor significantly different fungal community compositions.

2. Method

2.1 Site selection and field sampling

Nine study sites (*Fig. 4*) were selected for the study based on the following criteria. All sites consisted of even-aged pine stands that had been harvested and replanted within the last 10 years. Sites 1–6 are located within production forests owned by Sveaskog, the Swedish state's forest company. Three additional sites (called T20CC, T20100, and T20200) were located in Tagel, an experimental forest owned by the Rappe von Schmiterlöwska foundation. This particular forest experiment is run by the SLU Unit for Field-Based Forest Research (Goude et al. 2022; Silvaboreal 2025). At eight of the nine study sites, mature trees were retained after final felling to create a shelterwood structure. The ninth site had been clear cut, and thus had no overstory.

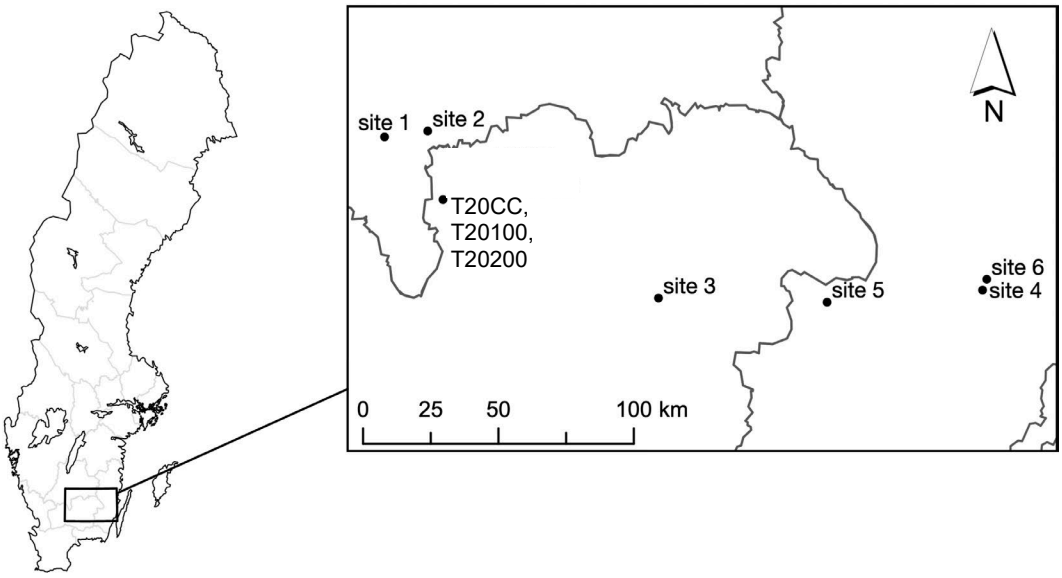

Figure 4: Map showing the location of the nine sampling sites in Southern Sweden, with their relative location in Sweden delineated. The three sites at Tagel are represented by one point because of close proximity but were three distinct stands.

Since the sites in Tagel were designed specifically to research shelterwoods, measurements of overstorey density were not taken as it was already known. Within each of the six production forest stands, a one-hectare area was designated as the sampling area. The overstorey was to be calculated using the density-adapted method (Jonsson et al. 1992). For taking the necessary measurements, five permanent circular plots (1.78 m radius) were established at each site (*Fig. 5*). The distribution of the plots was the same at every site, with a plot in each corner and in the center of the site. In each plot, the diameter at breast height (DBH) of the eight trees nearest to the plot center were measured using a caliper. The distance between the plot center and the furthest of the eight trees was recorded using a Vertex hypsometer, with the transmitter positioned at the plot center.

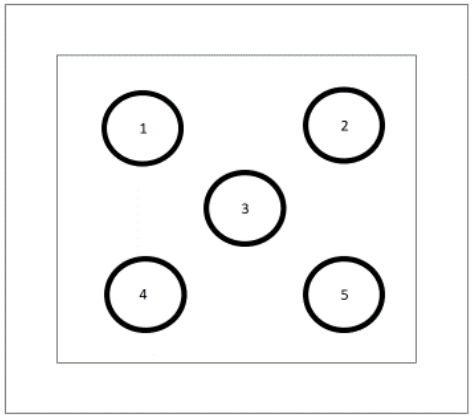

Figure 5: A visual representation of the five circular plots within the sampling area at each site, used for measuring overstory density

For the inventories and sample collection, 10 additional circular plots (1.78 m radius) were established within the sampling area at sites 1–6 while four circular plots were established in the sampling area of Tagel sites. To avoid sampling bias, the coordinates of the plot centers were chosen in advance. Fieldwork was conducted between mid- and late August 2024 (*Table 1*).

After the center of each plot was located, working clockwise, all pine regeneration taller than 15 cm was inventoried. Each individual was tagged with a unique number (ID), measured, and inspected for signs of fungal infection. For each type of damage present, three to six symptomatic needles were collected in paper bags labeled with site, plot, plant ID, and symptom type. The following data was recorded about the present infections: location on plant (upper/lower canopy), needle cohort of the sample taken (current/previous year), and infection severity.

Table 1: Sampling dates for each site

Date of field work	Sites sampled
13 August 2024	Site 6
24 August 2024	Site 2, 4, 5
29 August 2024	Site 1, 3, T20CC, T20100, T20200

2.2 Fungal isolation process

Samples were received and stored in a refrigerator at 4°C until they were processed. Over the next five months, samples were processed in batches of 60–70 samples at a time. Surface sterilization was performed in order to isolate only endophytic fungi and kill epiphytes which were not the target of this study (Sahu et al. 2022). The following order was used: 70% ethanol (30 sec), 2% sodium hypochlorite (2 min), 70% ethanol (30 sec), autoclaved deionized water (2 min). Samples were then shaken in 10 mL plastic tubes with autoclaved water for 2 more minutes.

For the first part of the isolation process, 9 cm Petri dishes were prepared with malt extract agar (malt extract, 30 g/L, VWR International, Belgium; agar, 15 g/L, Fisher Scientific, Belgium; deionized water) supplemented with chloramphenicol (0.15 g/L, PanReac AppliChem, Darmstadt, Germany**)** to suppress bacterial growth. After surface sterilization, needles were cut into 2–5 mm segments and placed on agar (8–12 segments per dish). If spots were present on the needles, extra attention was paid to bisect as many spots as possible. Dishes were sealed with Parafilm, stored at room temperature, and monitored regularly. Morphologically unique fungal colonies that emerged (as opposed to every single colony that emerged) were subcultured using scalpels onto 5 cm Petri dishes with malt extract agar without chloramphenicol. Only one colony was to be present on each 5 cm dish.

2.3 Molecular analysis

All fungal colony isolates were morphotyped or categorized based on shared macro-morphological characteristics (form, size, elevation, margin, surface opacity, color). Distinct morphotypes were established through visual comparison with one another, and at least one single representative isolate was selected from each morphotype for DNA extraction and sequencing. All other isolates were stored at 6°C.

Fungal mycelia were scraped off of the subcultures and transferred to 2 ml plastic vials containing 2 mm glass beads. The samples were freeze dried, then mechanically pulverized using a MM200 Retsch ball mill (Retsch GmbH, Haan, Germany). DNA was then extracted from the homogenized tissue using the DNeasy Plant Kit (QIAGEN, Netherlands), following the manufacturer's protocol for dry samples. The quality of extracted DNA was inspected with the DS11 spectrophotometer (DeNovix).

Using ITS1F as the forward (3') primer and ITS4 as the reverse (5') primer, the highly variable internal transcribed spacer (ITS) region of fungal ribosomal DNA was amplified (Martin & Rygiewicz 2005). The process was carried out with the MiniAmp Thermal Cycler (ThermoFisher Scientific) using the following thermal cycling conditions: initial denaturation at 95°C for 2 minutes; 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 45 seconds, and extension at 72°C for 90 seconds; 72°C for 5 minutes post-amplification. Finally, we confirmed DNA presence in PCR products through electrophoresis on a 1.5% agarose gel in TAE (Tris-Acetate-EDTA) buffer (90V, 25 min), using an O'GeneRuler 1 kb DNA ladder (Thermo Scientific) for size comparison.

Extracted genomic DNA was submitted to Macrogen Europe (Amsterdam, Netherlands) for Sanger sequencing of the forward and backwards reads of the amplified ITS region. This means that each DNA sample was sequenced twice; because it is two-stranded we can get a result from each strand which can then be used to strengthen confidence of database matches. Macrogen returned our sequences with preliminary BLASTn analysis (Basic Local Alignment Search Tool of Nucleotide Databases) against the National Centre for Biotechnology Information (NCBI) GenBank database.

2.4 Data analysis

Information about the sites, plots, trees, and symptom type were imported into R Studio along with information about symptom incidence and disease severity, shelterwood density of the sites, morphotype IDs, and the BLASTn results. The sequences were also analyzed with the UNITE database, which is specifically designed for molecular analysis of fungal species (UNITE Community 2024). Ends of sequences were trimmed and bitscores were considered to choose the most likely match from the database between the forwards and backwards reads.

A Kruskal-Wallis test was used to statistically test for correlations between average overstory density and symptom severity. This type of test was chosen because it is non-parametric and does not assume normal distribution of the data (Lomuscio 2021), which is applicable to this dataset made up of community matrices, absence/presence data, and an uneven sampling. Spearman's rank correlation was used to assess relationships between seedling height (a continuous variable) and symptom severity as it similarly makes no distributional assumptions while handling ordinal data effectively (Bobbitt 2022). This approach ensured appropriate treatment of both categorical and continuous values without violating statistical assumptions.

Dendrograms were created in R to visualize the differences in fungal communities between the seedlings at different sites and among disease symptoms. First, fungal community dissimilarities were quantified using the Jaccard index because it accounts for presence/absence only, not abundance (Chung et al. 2019). The weighted pair group method with arithmetic mean (WPGMA) was used for producing the dendrograms, as it is better suited for sample groups of different sizes (Legendre & Legendre 2012).

Subsequently, a permutational multivariate analysis of variance (PERMANOVA) was performed using the “adonis2” function from the vegan package in R (Oksanen et al. 2025), and using method “jaccard”. PERMANOVA calculates statistical differences in communities across the same parameters that were set when making the dendrogram (Anderson 2001).

Finally, non-metric multidimensional scaling (NMDS) was performed to create two-dimensional visualizations of the complex, community-level relationships of our fungal samples. This was helpful because relational information is lost in the dendrograms, and the results of the PERMANOVA could be visualized. The analyses were performed using Jaccard dissimilarities, in four dimensions, and with 1000 minimum iterations.

3. Results

3.1 Field observations and measurements

Table 2: Column 2- Calculated average density of each site. Column 3- Number of pines per plot that exceeded the height threshold as per the inventory protocol. Column 4- Incidence (yes/no) of disease symptoms for pine seedlings above the height threshold.

Site name	Avg. overstory density (stems/ha)	Number of pines > 15 cm	Percent with symptomatic needles
T20CC	0	25	92%
S4	58	46	100%
S5	68	24	100%
S2	75	18	94%
S1	78	43	95%
S6	80	13	92%
T20100	100	21	81%
S3	151	4	75%
T20200	200	17	100%

The average overstory densities of the sites ranged between 0 and 200 stems per hectare, with most sites average calculated density falling between 50 and 100 stems per hectare. The total number of pine seedlings over 15 cm tall varied widely between 4 and 46 per site, indicating that the sites were at different stages in their regeneration. Disease incidence was high overall, with 75–100% of seedlings exhibiting symptomatic needles across sites (*Table 2*). Four symptom severity classes (0%, 1-9%, 10-20%, >20%) were used to approximate the percentage of needles on each seedling that were symptomatic.

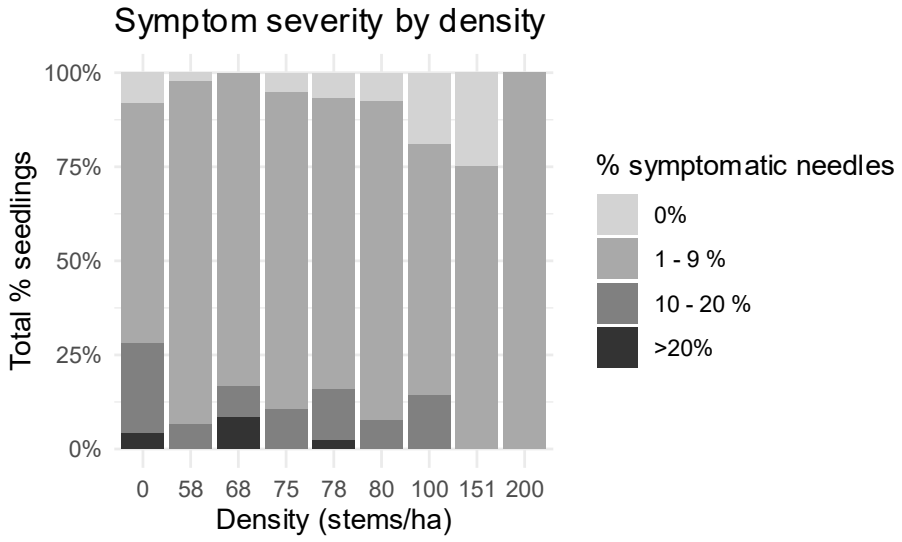

Figure 6: The distribution of seedlings (>15 cm) symptom severity at each site, ordered by the sites' average overstory density

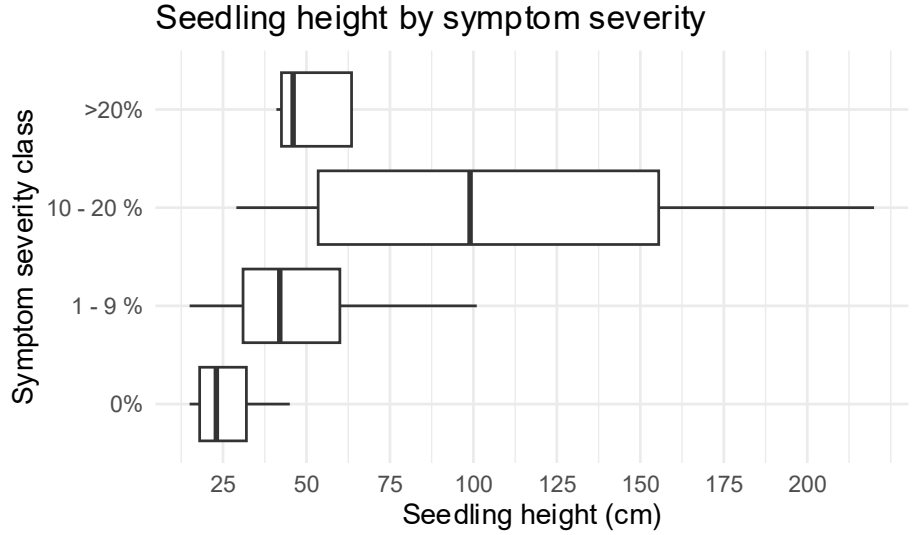

Figure 7: Relationship between pine seedling height and symptom severity classes. Boxplots show the distribution of heights (cm) for each severity class. Boxes represent interquartile ranges, and whiskers show 1.5× the interquartile range.

The relationship between symptom severity class and both average overstory density and seedling height was visualized using a stacked bar chart and a boxplot (*Fig. 6* and *Fig. 7*, respectively). Visually, there does not seem to be a connection between the average overstory density and symptom severity, with the majority of seedlings at each site falling in the 1-9% category (*Fig. 6*). Seedling height has a seemingly higher association with symptom severity. Only seedlings about 50 cm and smaller had no visible symptoms of disease. All inventoried seedlings that exceeded 50 cm in height displayed at least minor needle symptoms (*Fig. 7*).

Kruskal-Wallis testing confirms the patterns hinted at by Fig. 6; the result for overstory density ($\chi^2 = 3.9$, $D_f = 4$, p-value = 0.28) is not significant and suggests minimal differences between symptom severity classes at the different sites (Lomuscio 2021). Spearman's correlation test was used to test the relationship between seedling height and symptom severity since height is an ordinal variable (Bobbitt 2022). There was a statistically significant positive correlation between seedling height and symptom severity ($\rho = 0.39$, $p = 2.4\times10^{-9}$), indicating taller trees experienced more severe disease symptoms.

3.2 Needle samples: symptom types and counts

149 pine seedlings had needle samples taken for analysis of fungal communities. Every seedling had an asymptomatic control taken with additional symptomatic samples collected if present. Table 3 shows the count of each symptom type, with 458 needle samples collected in total. Representative photos of symptoms are included in the appendix (page 36).

Table 3: Summary of symptoms and their prevalence

# samples collected with symptoms		Total count: **458**	
Black discoloration	1	Spotting	135
Chlorotic tips	13	Spotting and banding	1
Control	149	Spotting and necrosis	6
Deformed and discolored	1	White banding	1
Deformed needles	2	White banding and necrosis	1
Discoloration	3	White spotting	33
Discoloration and spotting	1	White spotting and discoloration	1
Necrosis	105	Yellowing	2
Red discoloration	2	Yellowing and necrosis	1

3.3 Fungal isolation process

199 of the 458 collected needle samples have been prepared thus far (43% of the total), yielding approximately 1700 pure subcultures. Only 15 of the needle samples that were prepared yielded no fungal colonies, with 13 samples having no fungal growth at all and two samples where every subculture was discarded because of contamination.

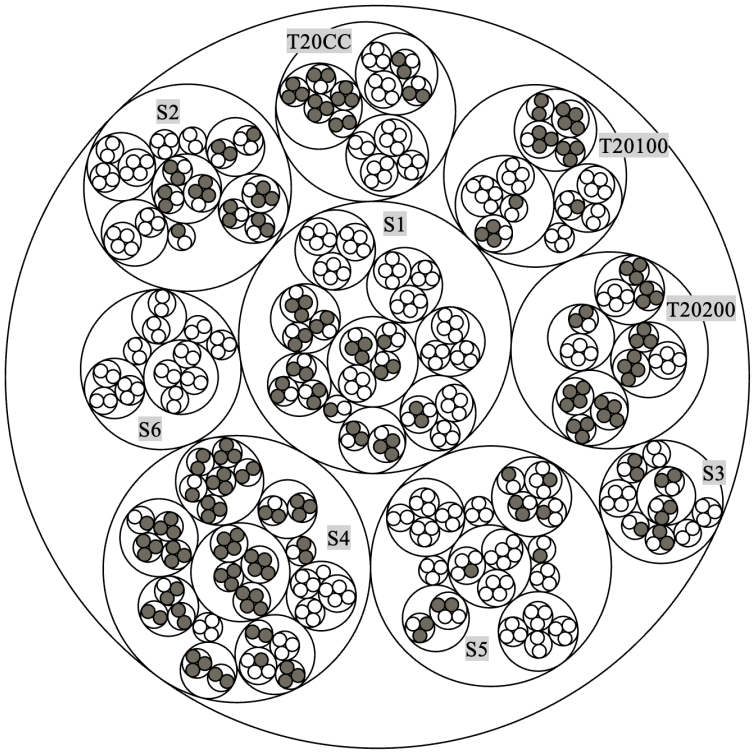

Figure 8: A visual representation of the sampling effort of the nine sites. The smallest circles represent all of the collected needle samples, which are grouped by tree, then plot, then site. Shaded circles represent samples that were prepared.

Due to the unexpectedly high number of subcultures as well as time constraints, not all needle samples were prepared, and those that were prepared were unevenly distributed (visualized in *Fig. 8*). Site 6 did not have any samples prepared at all. This is further represented in the species accumulation curve (*Fig. 9*), which plots the relationship between the number of trees sampled and the cumulative number of species found through DNA analysis. The curve does not reach its asymptote, indicating that the sampling effort was not exhaustive. More samples would need to be prepared to represent the full diversity of foliar fungi at the sampled sites (Gotelli & Colwell 2001).

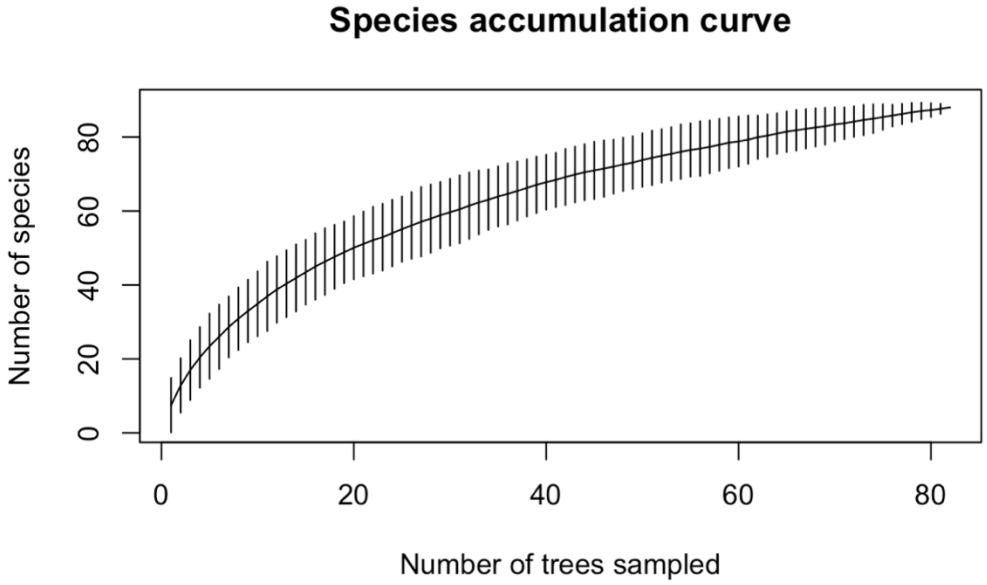

Figure 9: The curve represents the mean species richness based on random permutations of sampling order, with bars representing a 95% confidence interval. Results were similar with both forwards and reverse reads, and from both databases. UNITE results for forwards reads were used to make this figure.

From the morphotyping process in which morphology between samples was compared, we created 367 morphotype groups. At least one representative of each morphotype was selected for DNA analysis. However, 14 of these representative samples were not viable for DNA extraction and did not have a duplicate to use as a backup. This was due to samples desiccating because of holes in the Parafilm. So, of the original 367 morphotypes that we established, we were able to extract DNA from 355.

For seven of these morphotypes, we selected more than one sample for analysis, mostly driven by our curiosity about variation within specific morphotype groups. Five of the seven morphotype groups contained isolates that were different species after molecular analysis, meaning they were genetically different even though they were visually similar.

3.4 Database search results

Fungal identification was performed by searching ITS sequences (both forward and reverse reads) in the UNITE and NCBI GenBank databases. This approach resulted in four taxonomic identifications per sample. UNITE returned identifications to at least family level for 96% of samples, with the remaining 4% yielding either no result ("NA") or the designation "Fungal sp." Consistency between read directions was relatively higher with UNITE results; 86% of forward/reverse read pairs matched at genus level and 82% at species level. The BLAST results showed lower resolution, with 85% of forward reads and 84% of reverse reads achieving family-level identification; unclear reads returned designations including "BLAST search," "Fungal sp.," "Foliar endophyte," or "Uncultured fungus." 64% of BLAST results matched between forward and reverse reads at family level, decreasing to 56% at species level.

Full consistency across all results (both read directions in both databases) occurred for 42% of samples, where identical species-level matches were returned from all four queries. The majority of these results were from the genus *Lophodermium*, accounting for 82% of matches (*L. pinastri*: 59, *L. seditiosum*: 35, *L. conigenum*: 36). The remaining 18% comprised 12 other species, including *Cenangium ferruginosum* (11) and *Hypoxylon rubiginosum* (6) (*Table 4*).

Table 4: Fungal species with fully consistent identifications across all search parameters (forward/reverse reads in both UNITE and NCBI GenBank databases), showing total counts per species among concordant samples (n = 159)

Species	***Count***
Lophodermium pinastri	59
Lophodermium conigenum	36
Lophodermium seditiosum	35
Cenangium ferruginosum	11
Hypoxylon rubiginosum	6
Hypoxylon fragiforme	3
Xylaria hypoxylon	2
Biscogniauxia nummularia	1
Cenangium acuum	1
Desmazierella acicola	1
Peniophora cinerea	1
Rosellinia thelena	1
Sordaria fimicola	1
Xylaria ellisii	1
Xylaria xylarioides	1

However, we intentionally retained less precise identifications (genus-level, family-level, and even "Fungal sp.") for further multivariate analyses. For samples without full consensus across databases and read directions, we prioritized UNITE annotations due to its fungi-specific curation and threshold-based species hypotheses. This approach was to maintain representation of community patterns

that could be obscured by overly strict filtering. While most conflicts were resolvable with UNITE's validated species hypotheses, remaining cases resolved by considering sequence quality (read coverage and bit scores).

3.5 Instances of *Lophodermium* spp.

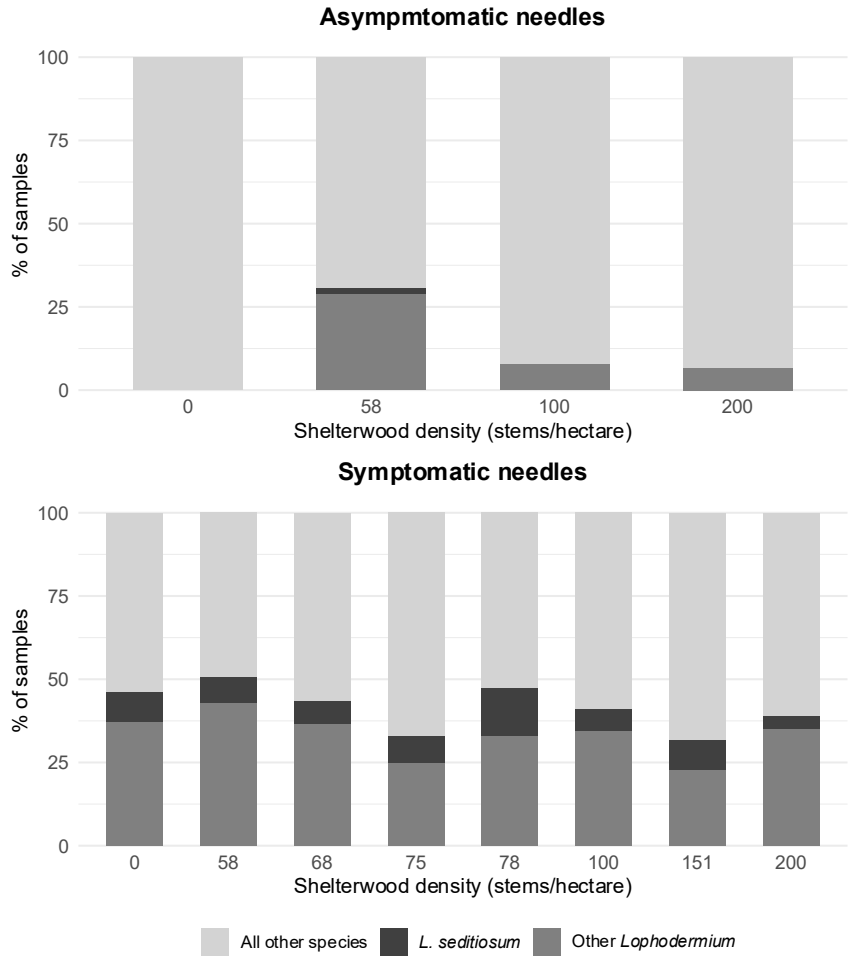

Figure 10: Relative abundance of Lophodermium spp. and other fungal taxa in needle samples across sites, grouped by their average shelterwood density (stems/ha).

A stacked bar chart (*Fig. 10*) was created to compare the relative abundance of *Lophodermium* spp. across sites, arranged by their average shelterwood density. The x axis shows shelterwood density (stems/ha), and the y axis shows the percentage of samples where: *L. seditiosum* (dark gray), other *Lophodermium* species (medium gray), and all other fungal taxa (light gray) occurred.

A distinction was made between instances of *Lophodermium* isolated from needles with and without symptoms, though not all trees had paired control samples prepared. Although different number of samples were prepared from each site (*Fig. 8*), some interesting patterns emerge through the visualization of the DNA sequencing results. The genus as a whole made up between ~30-50% of the

isolates from symptomatic needles and between 0 and ~30% of isolates from asymptomatic needle samples. The pathogenic fungus which causes the detrimental needle cast disease was only found in one isolate in asymptomatic needles, and the rest of the instances were in symptomatic needles.

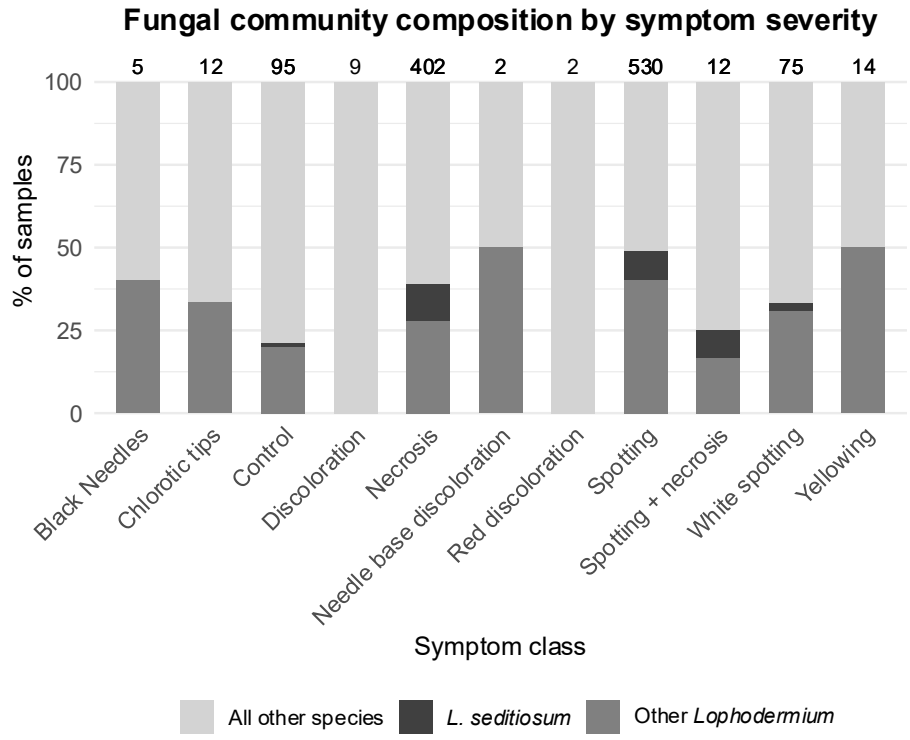

Figure 11: Relative abundance of Lophodermium spp. in needle samples grouped by symptom type.

A stacked bar chart (*Fig. 11*) compared the relative abundance of *Lophodermium* spp. across needle samples categorized by symptom type. The numbers at the top of each bar are a count of the total number of fungal isolates obtained from each symptom group. For instance, five isolates were obtained from samples with the symptom “black needles”.

The isolate counts are indicative of both the sample preparation effort and how many (morphologically) unique fungi emerged from each sample. Symptoms necrosis and spotting were the most commonly found in the field (*Table 3*) and thus most commonly prepared. The counts above the bars in the above figure also shows they yielded the most unique fungal isolates.

Despite uneven sampling, sequencing results revealed some patterns. *Lophodermium* spp. were detected in all symptom categories except "discoloration" and "red discoloration." The pathogen *L. seditiosum* was rare in asymptomatic needles (1 isolate) but prevalent in symptomatic material—present in 44 necrotic samples, 46 with spotting, two with white spotting, and one with combined "spotting and necrosis."

3.6 Analysis of fungal communities

3.6.1 Fungal community across sites (dendrogram)

When analyzing the following dendrograms, the vertical order of the different factors can be ignored. What matters for analysis is the length of the horizontal lines and the positions in which they branch off from one another, when reading from left to right (Drout & Smith 2012). As the distance increases from 0, groups containing different objects start to form until all objects become part of the same group (Legendre & Legendre 2012). The further to the left the branching, the closer in composition the fungal communities.

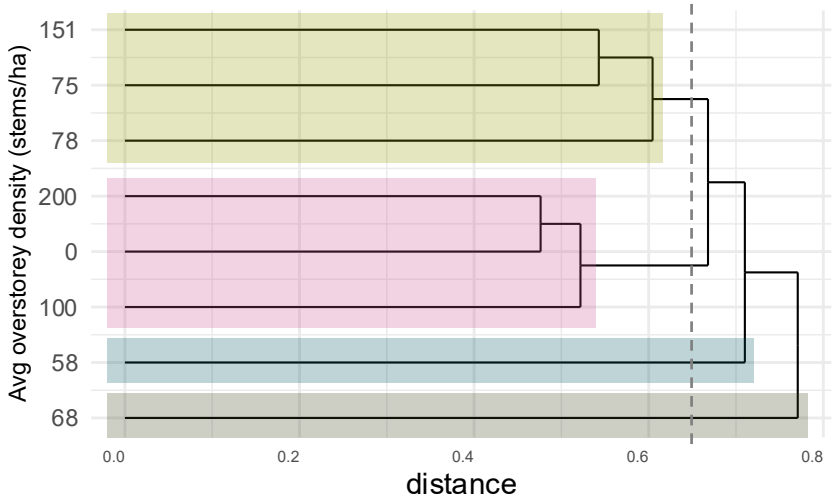

Figure 12: Dendrogram of fungal communities across sites with varying average overstory density.

Hierarchical clustering of fungal communities across shelterwood density gradients (*Fig. 12*) revealed two somewhat distinct clades and two single leaves (note the dotted line drawn at 0.65). Clade 1 is made up of average densities 151, 75, and 78 stems per hectare. Clade 2 contains 0, 100, and 200 average stems per hectare (the Tagel sites). The two leaves are sites with average density 58 and 68 stems per hectare.

If there was a connection between shelterwood density and fungal community, we might expect to see 0 stems/ha as a single leaf and some clades forming with similar densities. However, no gradient forms along the average densities. Almost the opposite effect is seen where the clear cut and the densest shelterwood actually have the most similarities relative to all other groups.

3.6.2 Fungal community across sites (PERMANOVA)

Table 5: PERMANOVA for fungal community by site

	D_f	Sum of squares	R^2	F	p-value
Avg. density	7	4.9783	0.1935	2.5364	0.001 ***
Residual	74	20.7487	0.8065		
Total	81	25.7270	1.0000		

The results of the PERMANOVA (*Table 5*) show that there are significant compositional differences in fungal communities across the different sites ($F = 2.54$, p-value < 0.001). Degrees of freedom (n–1) reflect 82 trees sampled for this study across 8 sites. The sum of squares for the treatment (avg. density) was about four times lower than the residual, meaning that there was some variation between sites, but variation within sites was much higher. The R^2 shows that 19% of variance can be attributed to the shelterwood density and the remaining 81% of variation is due to other factors that were not tested.

3.6.3 NMDS ordination of fungal community across sites

NMDS ordinations are used in this section and in 3.4.6 for visualizing similarities and differences in fungal community composition by site and by symptom. By definition, NMDS is a non-parametric method, which is reflected in the axes lacking specific units or fixed directions. Instead, they represent the points' relative distances from one another. Each point in the ordination corresponds to a needle sample, and the proximity of points indicates similarity in community composition.

The "spider legs" connect points to the centroid, or calculated average position, of their respective groups (e.g., site or symptom category). Thus, both within-group and between-group differences can be visualized. Tighter clusters suggest more similar fungal communities and more dispersed or distant clusters indicate greater compositional differences. Accompanying stress values from the tests indicate the level of distortion between the ordination and actual community configurations (Clarke 1993).

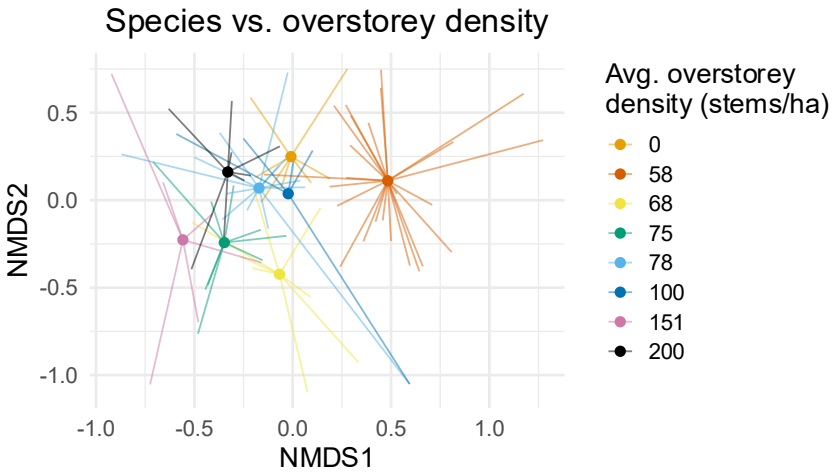

Figure 13: NMDS ordination of fungal communities across sites with varying average overstory density (stress = 0.130, a "usable picture" (Clarke 1993))

This ordination (*Fig. 13*) does not seem to reflect the same grouping as seen in the dendrogram (*Fig. 12*). The site with density 58 stems/ha is the most distinct, but 68 stems/ha seems to have some overlap with the majority of the sites. It's unclear why this would form the most distinct single leaf in the dendrogram if this is the case. Sites 0, 78, 100, and 200 stems/ha have centroids near each other.

Sites 75, 68, and 151 stems/ha also have nearby centroids. These six sites have quite a bit of overlap, possibly a visual indication of the high residual sum of squares.

3.6.4 Fungal community across symptoms (dendrogram)

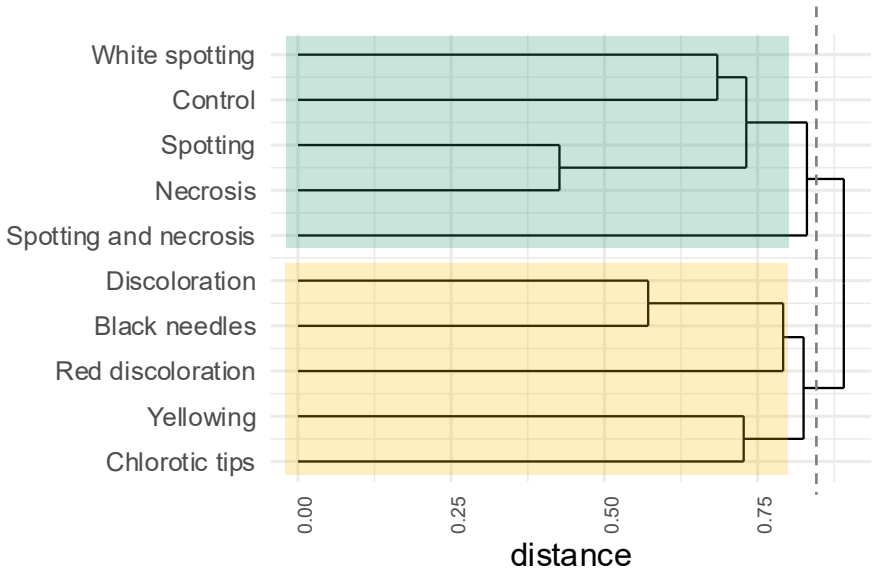

Figure 14: Dendrogram of fungal communities across symptoms, aggregated by site

Hierarchical clusterings of fungal communities across symptoms (*Fig. 14*) are more difficult to conclusively interpret. The first split creates two major clades, but the dashed line does not need to be shifted much to the left for other configurations to form. This ambiguity suggests that although there is relatively high distance between the two clades, communities across various symptoms may not be so distinct. Clade 1 is made up of the symptoms white spotting, control, spotting, necrosis and "spotting and necrosis". Clade 2 is made up of discoloration, black needles, red discoloration, yellowing and chlorotic tips.

3.6.5 Fungal community across symptoms (PERMANOVA)

Table 6: PERMANOVA for needle symptoms, aggregated by site

	D_f	Sum of squares	R^2	F	p-value
Symptoms	9	3.9603	0.37813	1.5539	0.001 ***
Residual	23	6.5130	0.62187		
Total	32	10.4732	1.00000		

According to the PERMANOVA results (*Table 6*), there is a significant difference between the fungal communities in needles with different symptoms ($F = 1.55$, p-value < 0.001). Degrees of freedom ($n - 1$) reflect 10 symptoms and 33 symptom-by-site combinations, where each row in the community matrix aggregates all needles with a given symptom collected from a specific site. The

sum of squares for the treatment (symptoms) was approximately 1.6 times lower than the residual, indicating that while there is some differentiation between symptom groups, most variation still occurs within them. About 37.8% of the variation in fungal communities can be attributed to symptoms, while the remaining 62.2% is due to other untested factors.

3.6.6 NMDS ordination of fungal community across symptoms

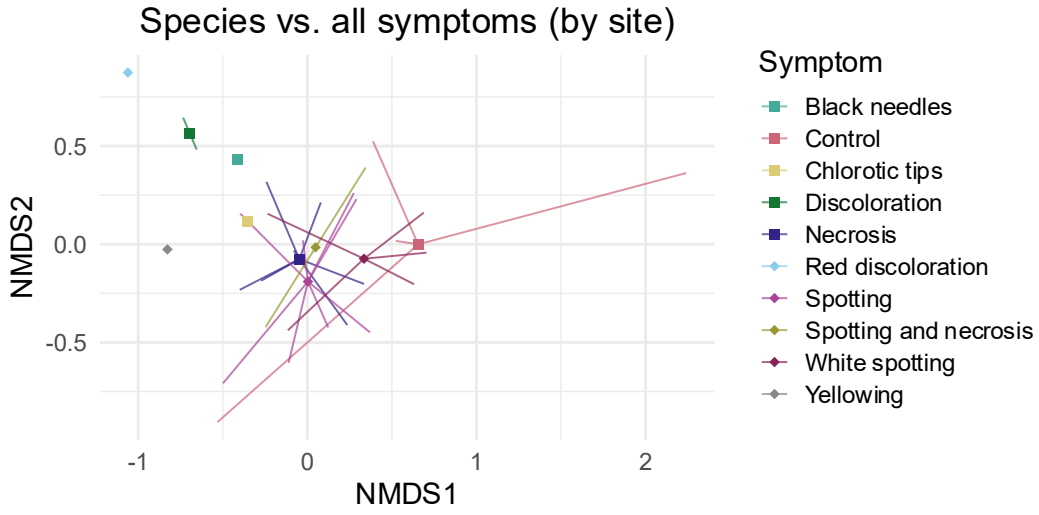

Figure 15: NMDS ordination of fungal communities across symptoms with site-level aggregation (stress = 0.095, a "good ordination" (Clarke 1993))

This NMDS ordination (*Fig. 15*) visualizes overlaps in community composition between the different symptoms, relationships which are obscured in the dendrogram (*Fig. 14*). The clades that formed within the dendrogram are somewhat reflected in the above ordination. The isolated symptoms spotting and necrosis seem to overlap with the cooccurring symptom "spotting and necrosis", hinting that they have similar fungal community makeup. Control does not seem to have much overlap with the previously mentioned symptoms, and white spotting has overlaps with both groups like the center of a Venn diagram.

The low incidence of symptoms from clade 2 is reflected in the points having only centroids and few to no "spider legs". It is therefore more difficult to understand their relationships to one another from this ordination. Their distant centroids, though, suggest that the community composition is not similar. When referring to the dendrogram, the grouping of clades 2 still seem logical. Discoloration and black needles have the least distance in the dendrogram as well as in the NMDS ordinations.

4. Discussion

4.1 Hypotheses

4.1.1 Fungal community vs. shelterwood density

Hypothesis 1 proposed that fungal community composition would differ across sites with varying overstory densities. As per the study design, one value for overstory density was used– a calculated average from five sample plots at each site. Therefore site and average overstory density have been used interchangeably throughout this paper. The first hypothesis was supported by the PERMANOVA results, which showed a statistically significant difference in fungal communities between sites ($p<0.001$), with shelterwood density accounting for 19.4% of the observed variation. However, the relatively high residual variance (80.6%) suggests that other unmeasured factors also influence community composition.

One such unmeasured factor is the microclimate surrounding each individual tree. Overstory density was calculated as the average of five sample plots per site, and this approach may have masked small-scale variability in light availability, humidity, and temperature. Sites 1-6, located in production forests, were not managed to maintain uniform overstory densities, unlike the Tagel experimental stands. The method of using site-level averages instead of measuring overstory density at each sampling plot flattens site-level variations that also might exist due to these different management strategies.

Another factor that was not considered in the analysis is seedling age. All stands had been regenerated in the last 10 years, but there can be pronounced developmental dissimilarities between seedlings planted at both ends of that time frame. Older seedlings have had more time to be exposed to fungal spores and have more surface area for colonization, but also have more developed defenses against pathogens (Millberg 2015). Spearman's test showed that height was a highly significant factor for the severity of disease observed on the seedlings. If taller seedlings are significantly more likely to have greater disease symptoms, and we assume that older seedlings are taller on average, seedling age should be a controlled factor in future iterations of the study to further isolate the effects of shelterwood density.

The dendrogram and NMDS did not reveal any grouping among sites with similar average overstory densities. In fact, the two sites with least dissimilarity in fungal community were the lowest and highest density sites (0 and 200 stems/ha) from the Tagel experimental forest. All three Tagel sites were grouped in clade 2, possibly because they are nearest in proximity to each other, located in the same forest. All other sites were in different forests, so physical proximity cannot explain grouping in clade 1.

As shown in Figure 8, the sample preparation effort across sites was not exhaustive. Some sites (S5, T20CC) had far fewer processed needle samples than others (S4), which may have biased the observed community patterns. Due to the low number of samples from the clear-cut site, it could not serve as a control for the stand overstory. This uneven effort limits comparability between sites and

may underrepresent rare taxa present at under-sampled locations. In future studies, including multiple clear-cut sites would provide a more robust source of control data and improve confidence in differences caused by overstory density.

4.1.2 Fungal community vs. needle symptoms

Hypothesis 2, that different needle symptoms would correlate with distinct fungal communities, was also supported through PERMANOVA results. The test showed a significant effect of symptom type ($p < 0.001$) that accounted for 37.8% of the variation in fungal composition. This is a stronger effect than observed for average overstory density, but again, we cannot draw definitive conclusions because of the uneven sample preparation effort.

Two noteworthy patterns emerged from the dendrogram and NMDS visualizations. First, the most frequently collected symptoms (white spotting, spotting, necrosis and "spotting and necrosis") and the asymptomatic control clustered together within clade 1 of the dendrogram. That is, higher incidence and sampling effort resulted in greater overlap in fungal community composition. Spotting and necrosis (the two most frequently collected symptoms) had the most similarity according to the dendrogram, but needle samples that had both symptoms at once ("spotting and necrosis") have a relatively distant composition. Again, this could be because of sampling bias since there appears to be high overlap in the NMDS ordination.

Second, needles with rare symptoms (discoloration, black needles red discoloration, yellowing, chlorotic tips) were clearly separated from the more commonly sampled categories in the ordination, with distant centroids indicating distinct fungal communities. Although these rare symptoms were only represented by one to three samples each, their clear separation suggests the presence of unique fungal communities. If more samples with these rare symptoms were analyzed, it is possible that some degree of overlap with other categories would emerge. However, their current separation raises the possibility that these symptoms are associated with fungi not commonly found in more prevalent symptom types or asymptomatic tissue.

4.2 Methodology considerations

4.2.1 What worked?

An in-depth sampling effort was undertaken in the field, with a wide range of detailed information collected in each sample plot. This dataset could be useful for future studies into effects of seedling size on fungal communities, pine competition with other species, or needle cohort infections, for example. Although the samples prepared were not evenly distributed, by following the lab methodology we were able to produce hundreds of pure fungal cultures that were both morphologically and genetically diverse. Though definitive conclusions cannot be drawn, this project will hopefully help to create a more streamlined methodology and achieve more conclusive results in future iterations of the study.

4.2.2 Shelterwood availability and future research potential

The Swedish Forestry Agency's new definition of shelterwood systems has only been in use since 2021. Because of this, the number of sites that met the new definition's specific parameters was limited and introduced challenges in assembling a representative gradient of overstory densities with comparable regeneration. In contrast to the established system of even-aged rotation forestry in Sweden, shelterwood management remains relatively uncommon (especially under the new definition). The scarcity of suitable sites constrained both replication and the generalizability of results.

This limitation also reflects a broader issue in forest management. Shelterwood systems are often viewed as a riskier management choice due to the perception that they increase the likelihood of fungal pathogen spread, yet this view is not strongly supported by empirical evidence (Karlsson & Örlander 2004; Roberts et al. 2020). Without further studies, forest owners may continue to rely on clear-cutting and other homogeneous regeneration methods simply because they are better studied and perceived as lower-risk.

Diversification of forest management methods is necessary in Sweden to meet national goals for sustainable forestry (Appelqvist et al. 2021), and methods like shelterwood systems could play an increasingly important role. Continued research will be essential in assessing both the ecological trade-offs and long-term disease risks associated with this approach. Over time, a stronger evidence base may increase the adoption of shelterwood systems, potentially creating a positive feedback loop where broader use enables more research, and more research builds confidence in the method.

4.2.3 Sample preparation process

In the beginning of the study, it was expected that all of the needle samples would be processed, but the time constraints and abundance of fungal isolates slowed the progression. The result was an uneven sampling effort that hinders our ability to draw definitive conclusions about the fungal communities that were found to be present. In the future of this study, as well as in similar studies, one should expect a large number of fungal communities to emerge from sample tissue during culturing and choose the samples accordingly. Each site should be represented, at least one sample per plot if possible, and each tree with a sample prepared should have both the asymptomatic and symptomatic needles prepared together. This way, asymptomatic needles could be used as a control for needles with symptoms of disease.

Some isolates were lost during the culturing step when cut needles were placed on a 9 cm Petri dish. Fast-growing fungi sometimes grew on top of slow-growing fungi, making it impossible to isolate a pure colony of the slow-growing individual. This could be avoided to some extent by placing fewer needle fragments on each dish to allow more room for growth.

4.2.4 Morphotyping

It is not possible to extract DNA from every sub-cultured isolate due to time and money constraints. Morphotyping was necessary to simplify our group of

subcultures, but the process is problematic in its own way. The morphology of various fungal isolates could look the same, but in reality, they are distantly related taxonomically. This is why we also use molecular methods and not only morphological in our community analysis, but still some information is lost at the morphotyping step (Guo et al. 2003).

4.2.5 DNA extraction, sequencing, and analysis

Not all fungi were identified to species level, either due to database gaps (e.g., missing reference sequences) or low-quality DNA extractions. 17% of the total sample set were not identified to species level, which leads to a potential underestimation of community diversity across samples. Family- or genus-level identification may artificially increase perceived similarity between sites or symptom types.

Different species within the same fungal genus can have prominently different lifestyles, ranging from endophytic to pathogenic (*Lophodermium sp.*, for instance) (Sinclair & Lyon 2005). As a result, less granular family- or genus-level identification may obscure important functional differences between communities. Future iterations of the study could prioritize combining a more thorough sample preparation effort with stricter quality thresholds during sequence analysis to achieve this.

5. Conclusion

This project used samples collected for a two-year study on *Lophodermium* needle cast in Scots pine shelterwood stands to analyze overall fungal community composition at different sites and across needle disease symptoms. Using *in vitro* culturing techniques, we isolated over 1,700 pure fungal cultures from pine needles collected from 82 seedlings across eight sites in southern Sweden. Morphological analysis and Sanger sequencing identified 87 unique fungal taxa, with resolution ranging from family to species level depending on results from database searches.

Statistical analyses showed that fungal communities differed significantly between sites, with geographically closer stands tending to share more similar communities. Symptom type, including the asymptomatic control, also had a significant effect on community composition. However, the uneven sampling effort limits the strength of any definitive conclusions past that distinct community patterns appear to be associated with both site and symptom differences.

Several opportunities exist for improving and expanding this research in future studies. Controlling for seedling age or size could help isolate the effects of shelterwood density, especially given the observed relationship between height and disease severity. To strengthen the role of clear-cut stands as a control, future studies could include multiple clear-cut sites and ensure more balanced sample preparation across all locations. Increasing the number of samples from rare symptom types may also provide a clearer understanding of their unique fungal communities.

Methodological refinements such as anticipating high fungal diversity during culturing, reducing competition between isolates on culture plates, and improving DNA extraction quality could increase both data quality and community resolution. Continued research is also needed to evaluate the long-term disease risks and ecological trade-offs associated with shelterwood systems. Together, these improvements could build a stronger foundation for understanding fungal community dynamics and informing sustainable forest management strategies.

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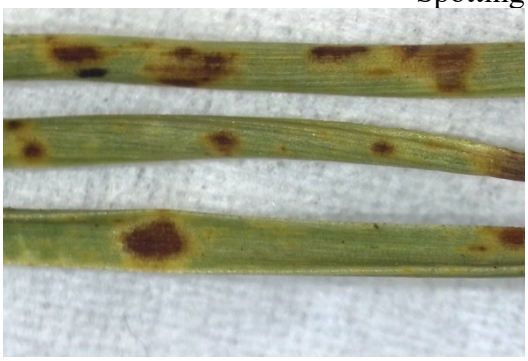
Appendix

Representative photos of symptoms, 10x magnification

Control

Necrosis

Spotting



White spotting

Deformed

Chlorotic tips (bottom needle)

Deformed + necrosis

Necrotic tips

Discoloration

Spotting + necrosis + yellowing 1/4

Spotting + necrosis + yellowing 2/4

Spotting + necrosis + yellowing 3/4

Spotting + necrosis + yellowing 4/4

Representative photos of symptoms, no magnification

Control

Necrosis

Spotting

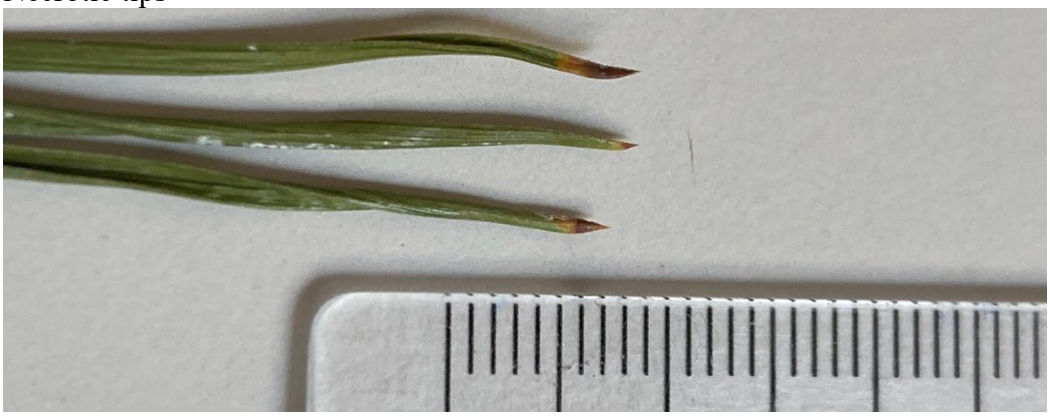
White spotting

Deformed

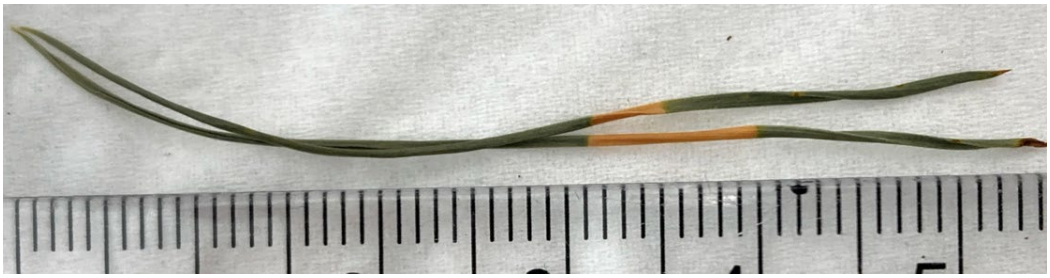
Chlorotic tips

Deformed + necrosis

Necrotic tips



Discoloration



Spotting + necrosis + yellowing

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