Foliar fungal communities of naturally regenerated and nursery-produced Scots pine (*Pinus sylvestris*) seedlings

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Abstract

Endophytic fungal communities living in leaves of trees can have a significant impact on their hosts, showing both beneficial and detrimental effects. Some mutualistic fungal species protect the host against pathogens or herbivores, while pathogenic species can increase the vulnerability of the host against biotic and abiotic stresses. The Swedish forestry model consists of planting nursery-produced seedlings after final felling. In the nurseries, the seedlings are grown densely in containers under greenhouse conditions and for disease prevention the Integrated Pest Management strategy is followed, where fungicides are applied to protect the plants. It is known that the intensive nursery production system affect the fungal communities present in the roots of seedlings by making them less diverse, but the effect in the foliar fungi is still unknown. The main objective of this study was to investigate the diversity of foliar fungal communities in Scots pine (*Pinus sylvestris*) seedlings produced in the nursery after their transplantation to the forest clear-cuts and to compare them with naturally regenerated seedlings. Needle samples of both types of seedlings were collected from three different forest clear-cut sites in Uppland, Sweden. The time scale and the exposure time of the needle were taken into account by sampling at 8-time points of the seasonal period and taking one-year old needles and current-year needles. Fungal communities were characterised by high-throughput sequencing of the ITS2 rRNA region of healthy-looking needles. Sequences were filtered and grouped into operational taxonomic units (OTU). The taxonomic classification of each OTU was determined using the PROTAX-fungi and massBLASTer databases and fungal lifestyle was assigned using the FungalTraits database. Results show that exposure time and needle age are the main factors affecting fungal diversity in needles, irrespective of whether seedlings are from nurseries or natural regeneration. Overall, fungal communities on needles of Scots pine are similar at different sites. The needle mycobiome was dominated by a fungal species belonging to the family *Pseudeurotiaceae* (10%), a species of the genus *Cladosporium* (9%), the species *Sydowia polyspora* (4%) and a species of the family *Herpotrichiellaceae* (2%). Further studies should focus on how colonisation of new needles develops in nursery-produced seedlings and compare this with naturally regenerated seedlings.

Keywords: Endophytes, high-throughput sequencing, foliar fungi, forest nursery, fungal community, fungal pathogens, metabarcoding, mycobiome, *Pinus sylvestris*, pine seedlings, Scots pine needles

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Abbreviations

CA	Correspondence Analysis
G	Gåvstahagen
IPM	Integrated Pest Management
M1	Marielund 1
M2	Marielund 2
N	Naturally regenerated seedling
OTU	Operational Taxonomic Unit
P	Nursery-produced seedling
PT	Pair Treatment
PY	Pair Year
Y21	Year 2021
Y22	Year 2022

1. Introduction

1.1 Swedish forest and forestry

Scots pine (*Pinus sylvestris*) is an evergreen conifer that has a wide distribution in Eurasia, reaching the northernmost latitudes. This species is one of the dominant trees found in the Fennoscandian forest, and it can thrive in various types of soil. In Sweden, Scots pine is primarily found in drier soils that have coarse, sandy soil, whereas the other main tree species in Sweden, Norway spruce (*Picea abies*), tends to grow on well-drained soil. However, the current distribution of Scots pine in this region has expanded compared to historical times due to human management of the forest, driven by the high market value of pine wood products (Richardson, 2000). Around 80% of the standing volume in Sweden are conifers, consisting of 40% spruce and 38% Scots pine (SLU, 2018).

Sweden, despite harbouring only 1% of the world's forest area (SFIF, 2018), is the third largest exporter of paper, pulp, and sawn timber globally. Although, the land has a relatively low productive capacity and vegetation growth can be slow (KSLA, 2009), the Swedish forestry model is highly efficient in harvesting large part of the productive forest for timber (SLU, 2018). This approach typically involves a rotational clear-cutting regime of even-aged and mono-specific stands, followed by regeneration, which yields the desired results.

Before planting, the soil is typically prepared through a process called scarification, which has two main methods: trenching and mounding. Trenching involves digging continuous trenches in the soil and inverting the organic soil layer, while the mounding method uses an excavator to invert the organic soil layer intermittently. This allows the roots of the planted seedlings to have contact with the mineral soil, which promotes fertilization. Furthermore, it reduces competition

from other vegetation, providing a good start of the planted seedlings (Jonsson, 2020).

The methods of regeneration have changed significantly over time. Originally, natural regeneration based on seed-trees was the trend, but in the mid-1990s, this technique was surpassed by an increase in seedling plantation (SLU, 2012). In the 2000s, seed sowing got an increase in usage, but it still accounts for only a small fraction of the total regeneration area. Presently, the most common method of forest regeneration involves planting of nursery-produced seedlings, which accounts for 75% of the total regeneration area, followed by natural regeneration (25%) and seeding (Ibid.).

The yearly production of nursery seedlings was 422 million in 2022, with *P. sylvestris* and *P. abies* being the primarily species grown (www.skogsstyrelsen.com). The production strategy in nurseries involves growing the large number of seedlings in monoculture using plastic trays under greenhouse conditions, followed by transferring them to outdoor settings (Lilja *et al.*, 2010). Moreover, it is applied the integrated pest management strategy (IPM), which established the potential pest and its critical threshold (Larsson *et al.*, 2023). This involves preventive measures to avoid outbreaks, focus on selecting a healthy environment with optimal soil drainage, water, temperature, and crop density. Additionally, sanitation is increased throughout the removal of symptomatic individuals, as well as biological and fungicide controls are used to protect the seedlings (Dumroese, 2012). Nevertheless, these intensive management and production practices can result in seedling stress, increasing their susceptibility to disease (Menkis *et al.*, 2016).

1.2 Foliar fungi community

Fungi play various roles in ecosystems. There are lichenised fungi can be the primary colonizers of extreme habitats or new niches, saprophytes specialized in breaking down dead plant material, and mycorrhizal fungi that form symbiosis with plant roots and enhance plant nutrition (McLaughlin & Spatafora, 2014; Peay *et al.*, 2016). Besides, there is a diverse group of foliar fungi, that can be composed of

epiphytes, which colonize the surface of the host tissue, and/or endophytes, which live within plant tissues. The endophytic species can be asymptomatic for the host, acting as mutualistic, latent saprotroph or commensal, or pathogenic in a latent state and whose development can be induced by plant stress (Sieber, 2007). This foliar mycobiome is mainly composed of members of the phylum Ascomycota, although basidiomycete yeasts and spores of ectomycorrhizal fungi can also be present on leaves (Peay *et al.*, 2016).

As a result of this great fungal diversity, there are foliar fungal species beneficial to plants that promote plant fitness by protecting against pathogens and herbivores, as well as increasing plant tolerance to environmental stress factors (Arnold *et al.*, 2003; Miller *et al.*, 2008; Bae *et al.*, 2009; Illescas *et al.*, 2022).

Furthermore, foliar fungi pathogens can affect trees by reducing leaf vitality and photosynthetic capacity, ultimately stunting growth (Hanso & Drenkhan 2012; Manter *et al.*, 2003). These pathogens can also lead to the death of the trees or increased vulnerability to biotic and abiotic stresses, such as drought or storms (Millberg *et al.*, 2015, Oliva *et al.*, 2014). Therefore, fungi diseases are one of the most damaging pests in forestry (Lilja *et al.*, 2010; Põlme *et al.*, 2020). Despite their significant role in tree health and fitness, it is still unknown which are the main factors of foliar colonisation or how foliar fungi community develop over time.

1.2.1 Factors of foliar mycobiome

Foliar fungal communities are affected by abiotic and biotic factors that can determine the species composition. The effect of abiotic factors such as temperature and humidity (Arnold, 2007), which are related to latitude, altitude and geographic location (Millberg *et al.,* 2015; Peay *et al.,* 2016), has been proven. Additionally, the impact of climate on fungal species diversity is of special interest due to the ongoing climate change.

Biotic factors have been less studied, because of the complex fungi-plant relationships involved. Nonetheless, it is known that the foliar mycobiome is different between broadleaf and conifer host species (Ricks & Koide, 2019; Tanunchai *et al.,* 2023), and that the host genetics also matter (Redondo *et al.*, 2022). Specifically, mycobiome diversity is closely linked to the spores available

from the surrounding vegetation (Redondo *et al.*, 2020) where diversity is defined by species richness and evenness.

The age of needles and their health status can also impact the composition of the fungal community. For example, research by Legaults *et al.* (1989) has shown that fungal composition can vary depending on needle age, while Millberg *et al.* (2015) found a greater fungal diversity in needles with disease symptoms compared to healthy needle without symptoms. However, the frequency and length of new needles produced each year are influenced by environmental conditions during bud formation in the previous year (Salminen & Jalkanen, 2005).

Anthropogenic activities such as forest nursery production may potentially influencing the fungal community composition (Menkis *et al.*, 2016). It is known that forest nursery practices have an impact on the fungal communities present in seedling roots, affecting both species diversity and community structure (Menkis *et al.*, 2005; Okorski *et al.*, 2019). However, how the mycobiomes perform above ground is less well understood, but it is expected that the intense nursery production will also have an effect on the needle fungal community. Thus, the planted material from nurseries may harbour a different fungal community compared to naturally regenerated seedlings.

1.2.2 Foliar mycobiome formation

The ecological succession theory can be applied to fungal communities in both growing young needles as well as in old ones, where new tissues are new niches that will be colonised by those primary colonising species, allowing for the establishment of other species (Chang & Turner 2019). Scots pine is a conifer, that retain their needles for several years (Reich *et al.*, 1996), with new needles being produced each summer during the growing season and the release of old needle cohorts in autumn (Jalkanen, 1998; Millberg *et al.*, 2015). The age of needles can impact the endophyte infection and colonization, as needles that are retained for longer periods provide more time for fungi to establish (Arnold & Herre, 2003; Elfstrand *et al.*, 2020). This highlights the importance of needle age when studying the composition and diversity of foliar fungal communities in Scots pine.

Foliar fungi are horizontally transmitted to new tissues (Arnold & Herre, 2003) through spore dispersal from other individuals, meaning that new needles do not somatically acquire fungal species from other host tissues (Yan *et al.*, 2015). Therefore, the dispersal capacity of fungal species will determine the order of species arrival and therefore the assemblage of the fungal community. Moreover, the first arriving fungal species can influence the overall composition of the community as well as its diversity (Peay *et al.*, 2016). Afterwards, the community will be determined by the competition with species gains and losses as well as the dominance of environmental favoured species.

1.2.3 Diversity: some concepts

The assessment of diversity depends on the geographical scale studied and can be measured in terms of three different aspects (Fig. 1). Alpha diversity refers to the diversity of species within a single habitat or forest stand and is a local scale measure. Beta diversity is defined as the difference in species composition between multiple habitats or environments and is a measure at a regional scale. Meanwhile, gamma diversity is the total diversity of species across large geographic scales.

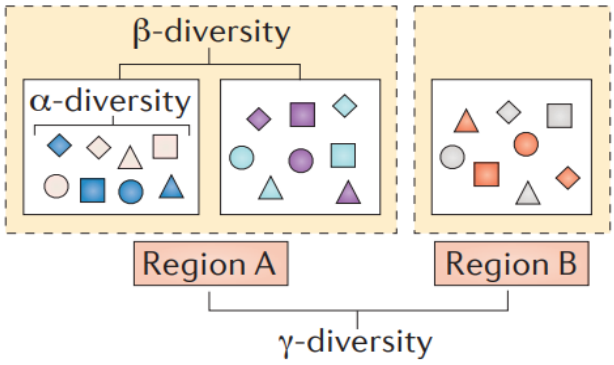

Figure 1. Diagram of alpha, beta, and gamma diversity taken and adapted from the study by Peay et al., 2016.

It is widely accepted that fungal communities at local scales are significantly heterogeneous in terms of the overall composition and the number of species present (Peay *et al.*, 2016). This suggests that local factors play an important role in shaping fungal communities while the underlying mechanisms driving this variation are not fully understood.

This study aimed to analyse the fungal community present in the Scots pine needles from naturally regenerated and nursery-produced seedlings, considering the temporal scale. In doing so, this thesis contributes to increasing our knowledge about the foliar fungal community composition, covering three distinct aspects: the effect of the origin and history of seedlings, the development of colonization in new needles, and the seasonal change of the fungal community.

2. Aims of the study

The objective of this study was to determine the diversity, composition and dynamics of the endophytic fungal communities present in the needles of both nursery-produced and naturally regenerated Scots pine (*P. sylvestris*) seedlings. The study also considers the growth year of the needles (2021 and 2022), the exposure time of the nursery-produced seedling in the forest after planting, and the site effect sampling three difference stands. With the aim to answer the following questions:

- What happens to the endophytic fungal communities present on the nursery-produced seedlings once they are planted in the clear-cut? Will they change over time?
- Will the fungal communities present on the current year's needles be more similar to the community of the one-year-old needle or will they be more like the communities present on naturally regenerating seedlings?

The following hypotheses will be tested:

1. Foliar fungal communities in the needles of nursery-produced seedlings will be different to the communities present in the needles of naturally regenerated seedlings.
2. Foliar fungal community from the nursery-produced seedling will shift and over time become more similar to the fungal communities of the naturally regenerated seedlings.

3. Material and Methods

To test the study's hypotheses, pine needles were collected from nursery-produced (P) and naturally regenerated (N) seedlings at three clear-cut sites in the county of Uppland (Fig. 2): Gåvstahagen (G), Marielund 1 (M1), and Marielund 2 (M2). The seedling needles were collected every week over the growing season, i.e., from the 1st of June to the 29th of September 2022.

Figure 2. Map of the three sites respectively from Uppland.

3.1 Stand management and site ecology

The study sites (Gåvstahagen, Marielund 1, and Marielund 2) are productive coniferous forests with occasional deciduous trees, but they have distinct ecological and silvicultural characteristics:

Gåvstahagen (17.85906º, 59.93655º) is situated on wet clay soil with a north-east orientation (App. 1). The forest in this area was previously dominated by Norway spruce (*Picea abies*), and retention trees have been left in the wet areas. The surrounding forest is a productive mixed stand with pine and spruce of approximately 40 years old. After the clear cut, the soil preparation involved mounding the planting spots.

Marielund 1 (17.84833º, 59.82927º) is situated in a downhill area with a north-west orientation (App. 1). The soil is sandy and rocky with high drainage capacity. The surrounding forest is a productive pine forest of approximately 30-40 years old. The previous stand was a pine forest and soil trenching were performed followed by planting.

Marielund 2 (17.84477º, 59.82356º) is located in a flat and open area with a north-south orientation. Therefore, it is likely to be more exposed to weather conditions such as rain, wind and snow. The site is a transition zone between sandy soils and humid areas (App. 1), surrounded by a productive pine forest like Marielund 1. However, there is also a productive spruce forest in the valley area. Soil preparation involved trenching prior to planting.

All nursery seedlings used in the study were 2 years old and produced in a container culture system. They were supplied by the nursery Lugnets, Svenska skogplantor (Sveaskog). However, the seed orchard sources differed between the sites. The seedlings used in Gåvstahagen were from Lycksta seed orchard, while those used in the Marielund sites were supplied from the Mosås seed orchard (Table 1).

Table 1. Planted material´s information per sites

Site	Province	Area	Origin	Certificate	Pot size (cm^3)
G	Gåvstahagen	3.5 ha	Lycksta FP-603	S20/070	50
M1	Marielund	12 ha	Mosås FP-602	S20/038	50
M2	Marielund	16 ha	Mosås FP-602	S20/038	50

3.2 Sampling

From each seedling, two needle samples were taken from one-year-old cohort (Y21) and from the current-year cohort (Y22). This was done for a total of 20 nursery-produced seedlings per site, spaced at least 10 metres apart. The estimated sampling area per site was 0.8 hectares. Naturally regenerated needles were sampled from seedlings of different age (2-4 years) at the forest edges surrounding each site. Plastic gloves were used for sample picking to prevent contamination between seedling types.

A key principle used for selecting needles was their appearance of health. Healthy needles were identified by their green colour and lack of visible signs of fungal infection, such as yellow-brown spots or bands. As the sampling progressed, it became increasingly difficult to find needles that were free of symptoms. Moreover, there was a large variation between sites in both the size and number of needles of nursery-grown seedlings.

The needles from each year (Y21 or Y22) and type of seedling (N or P) were pooled in a falcon tub (15 ml). To preserve the fungal community, the tubs were stored in an icebox in the field and then transferred to a freezer at -20ºC until further processing. A total of 96 samples (8-time points x 3 sites x 2 seedling types x 2 needle ages) were used for the further analysis (Table 2).

Table 2. Samples used for the study from each site

Site	Y	Week 1 (15/06)	2 (22/06)	3 (29/06)	4 (06/07)	5 (13/07)	6 (20/07)	7 (27/07)	8 (03/08)	9 (10/08)	10 (17/08)	11 (01/09)	12 (29/09)	14	16	17	18
M1P	21	X	X	X	X	…	X	…	X	….	…	X	X				
	22	NS	X	X	X	X	…	X	…	X	…	X	X				
M1N	21	X	X	X	X	…	X	…	X	…	…	X	X				
	22	NS	X	X	X	X	…	X	…	X	….	X	X				
M2P	21	X	X	X	X	…	X	…	X	…	…	X	X				
	22	NS	NS	X	X	X	X	….	X	…	X	X	X				
M2N	21	X	X	X	X	…	X	…	X	…	…	X	X				
	22	NS	X	X	X	X	…	X	…	X	….	X	X				

Site	Y	01/06	07/06	15/06	22/06	29/06	06/07		20/07	03/08	17/08	01/09	29/09
GP	21	X	X	X	X	…	X		X	….	X	X	X
	22	X	X	X	X	…	X		X	…	X	X	X
GN	21	X	X	X	X	…	X		X	…	X	X	X
	22	NS	NS	X	X	X	X		X	X	…	X	X

X: Selected samples. G: Gåvstahagen. M1: Marielund 1. M2: Marielund 2. P: nursery produced seedlings. N: naturally regenerated seedlings. 21: needle age from 2021. 22: needle age from 2022. NS: no sample available. ...: sample available but not used in the study.

3.3 DNA extraction and library preparation

The samples were sterilized in 70% ethanol for 30 s, then in 2% sodium hypochlorite (NaOCl) for 30 s and washed in sterile water for 3 mins (Oono *et al.*, 2015). Subsequently, they were freeze-dried for 65 hours. The samples were homogenized using a ball mill (Retsch MM400). DNA was extracted from each sample (15-20 mg) and from controls using the Macherey-Nagel™ kit (NucleoMag™ Plant/384 Plant). To each sample, 500 µl of MC1 Buffer and 10 µl of RNAase A were added, before shaken and incubated for 30 minutes at 65°C. After centrifugation, the supernatant was placed on a pre-loaded DNA plate according to the instructions of the extraction robot (Maelstrom 4800). The DNA concentration was measured by the NanoDropTM (Thermo Scientific) and samples were diluted 0.5 ng/µl.

PCR was performed using the Taq polymerase (DreamTaq 5 U/µL), dNTPs (2 mM) and the internal transcribed spacer (ITS) primer pair fITS7 and ITS4 with

barcodes (Clemmensen & Ihrmark, 2014). First, all the samples were run at 25 cycles with an annealing temperature of 57ºC. The products were verified on a 1% agarose gel. The samples without visual products were run at 30 and 35 cycles to optimize the products, using the same PCR program (Lindahl *et al.*, 2013). Negative controls were included in all PCRs and gels as well as duplicates were performed for each sample. All the products were cleaned with an AM-Pure®magnetic bead kit (Agencourt) and their DNA concentrations measured using the Promega QuantusTM Fluorometer (Fisher Scientific). An equimolar proportion of the PCR product from each sample (20 ng) and the negative controls was pooled. DNA concentration of the pool was measured in a NanoDropTM and the DNA 1000 bioanalyzer (Agilent Technologies Inc.). Then, the pool was sequenced using PacBio RSII at SciLifeLab facilities National Genomics Infrastructure (NGI), Uppsala.

3.4 Sequence analysis and classification of taxa

The derived sequences were quality filtered and clustered using the SCATA pipeline (http://scata.mykopat.slu.se). Sequences with a minimum mean quality of bases in a read < 20, minimum allowed base quality <10 or those that missed the primer sites (Primer match value = 0.9) were deleted. The quality screening was done using the amplicon quality alternative. The sequences that passed it were clustered into operational taxonomic units (OTUs) using a threshold of 1.5% similarity for the clustering distance as well as one mismatch and one gap extension penalty. Then, each OUT is a fungal individual, grouped by similarity. For quality control, singletons and clusters with less than 12 sequence reads were removed. Samples with less than 42 sequence reads were also discarded. Moreover, samples with missing or mixed tags for the forward and reverse primer sequences were removed.

The taxonomic classification of each OTU was determined using the PROTAX-fungi and massBLASTer (UNITE/INSD fungi) databases implemented with PlutoF biodiversity platform (https://plutof.ut.ee). The threshold value for PROTAX-fungi was 0.5 for a confidence classification. For the identification in massBLASTer, the

criteria were set to a sequence coverage of at least 80%, more than 94% of matching for the genus level and more than 98% similarity for the species identification. Each OTU corresponds to a fungal species, but the databases lack information on all of them. If species level is not achieved but another taxonomic level is reached, e.g., family, this means that the OTU is a species with a sequence similar to that family.

For the top 100 most abundant OTUs, PROTAX outputs were manually compared with the identities defined by massBLASTer analysis in PlutoF. For OTUs with no matching identities, GenBank (NCBI) database was consulted to determine the most accurate taxonomic level for the OTU using the Blastn algorithm. The top 500 most abundant OTUs were compared using only the PROTAX and massBLASTer outputs, while the remaining OTUs were only classified using PROTAX. However, for those OTUs classified as "Fungi" double checks were conducted with massBLASTer.

FungalTraits database (Põlme *et al.*, 2020) was used to assign a fungal lifestyle to each OTU that were identified to the genus level. The output of this analysis resulted in 17 distinct primary lifestyles that were further categorized into nine groups: Ectomycorrhizal, Endophyte (foliar/root), Epiphyte, Lichenized, Mycoparasite, Parasite (animal/lichen), Plant pathogen, Saprotrophs (dung/litter/nectar/soil/sooty mould/wood/unspecified), and Unidentified.

3.5 Statistical analysis

The data analysis and visualization were perform using R v 4.2.0 (R Core Team, 2022). To estimate the effectiveness of the sampling effort per sample and site (G, M1, M2) rarefaction curves were performed using the R package “vegan” (Oksanen *et al.*, 2022). The bar plots, that shows the relative abundance of sequence reads and OTUs over weeks, were constructed through the R package “ggplot2”.

Venn Diagrams were done using the “vennCounts” and “vennDiagram” functions from the R package “limma” (Ritchie *et al.*, 2015) to illustrate the number of shared operational taxonomic units (OTU) among seedling types (P, N), sites (G, M1, M2) and needle age (Y21, Y22). To visualize the relationships between samples and OTUs, ordination plots based on Correspondence Analysis (CA) were

constructed using the "fviz_ca_row" and "fviz_ca_col" functions from the R package "factoextra"(Kassambara & Mundt, 2020). These functions allowed for the visualization of the CA results for the rows (samples) and columns (OTUs) of the contingency table, respectively.

The normality of the data and the homoscedasticity of the residuals were assessed using statistical tests including the Shapiro-Wilks test, Fligner-Killen test (Conover *et al.*, 1981) and histograms. The methodology described in Redondo *et al.* (2020) was used to perform statistical analyses of biodiversity. A non-rarefied OTU table was utilized, and the square root of the total number of sequences reads per sample was included as the first factor in all models. This approach was chosen to account for potential biases resulting from differences in the total number of reads among samples (Hong *et al.*, 2022; Laporte *et al.*, 2021).

Alpha diversity was evaluated using the Shannon index, which was computed with the "diversity" function from the “vegan” R package. Linear models were used to explore the relationships between alpha diversity and factors such as seedling type (N, P), needle age (Y21, Y22), sites (G, M1, M2), and weeks. Differences in alpha diversity between samples were tested using ANOVA, and the Tukey HSD test was used as a post-hoc test. Boxplots were used to represent alpha diversity, which were generated using the function “boxplot” from the “graphics” package.

Beta diversity was calculated by computing the relative abundance of OTUs using the Hellinger transformation method, which was implemented using the "hellinger" function in the “labdsv” package. This approach controlled for differences in the total number of reads among sites and other potential biases (Laporte *et al.*, 2021). To analyse differences in beta diversity between samples, a permutational multivariate analysis of variance (PERMANOVA) on a Bray-Curtis dissimilarity matrix with 999 permutations was performed using the "adonis2" function in the “vegan” package. For beta diversity, a heatmap was constructed using the “vegdist” function available in the “vegan” package, and it was plotted using the “heatmap.2” function from the “gplot” package.

Furthermore, a paired sample design of the dataset was performed following Karlsson *et al.* (2017) to determine the effect of the seedling type (N, P) or the needle age (Y21, Y22) in the fungal alfa diversity (Shannon index). The samples were grouped in pairs based on the seedling type (N, P) or the needle age (Y21, Y22), resulting in two news variables: Pair Treatment (Pair T) and Pair Year (Pair Y), respectively. A linear model with the Shannon index as response variable and the square root of the total number of sequences reads per sample as first factor was used. Then, a paired t-test for unequal variance was performed.

The relative abundance was calculated for each OTU and was visualized over time for all samples as well as for the subsets of Pair Treatment (PT) and Pair Year (PY) using the R packages “reshape2” and “ggplot2”. To provide a clearer representation, only the top 20 most abundant OTUs were identified with their taxa name, while the remaining OTUs were aggregated and labelled as "Others". The same packages were used to visualise the relative abundance of each primary lifestyle over time.

4. Results

4.1 Sequencing output

A total of 72 pine needle samples were retained, after sequencing and quality control (see the Material and Methods section). These comprised 34 samples from naturally regenerated seedlings (N) and 38 samples from nursery-produced seedlings (P). The distribution of the samples among the sites was 24, 26, and 22 for Gåvstahagen (G), Marielund 1 (M1), and Marielund 2 (M2), respectively. Furthermore, there were 38 samples for one-year-old needles (Y21) and 34 samples for current-year needles (Y22).

From all the needle samples, 322,704 sequence reads were generated using the PacBio platform out of which 173,022 (54%) sequence reads passed the quality control. In SCATA, the high-quality reads were grouped in 2,143 clusters and 1,768 singletons, which were removed. After quality control (see 3.4 section) and removing the non-fungal operational taxonomic units (OTU) a total of 821 OTU remained, which covered 97% of the total number of reads.

The average number of sequence reads per sample for naturally regenerated seedlings and nursery-produced seedlings were 2,261 and 2,295, respectively. The average number of sequence reads per sample in Gåvstahagen was 2,226, in Marielund 1 was 2,275, and in Marielund 2 was 2,332. One-year-old needles had an average of 2,415 sequence reads per sample, while current-year needles had an average of 2,123 reads per sample. Table 3 shows the maximum and minimum values of sequence reads for seedling type, needle age and site.

The average number of OTUs per sample was 143 and 154 in naturally regenerated and nursery-produced seedlings, respectively. The mean of OTUs per sample was 148 in Gåvstahagen, 147 in Marielund 1 and 150 in Marielund 2. One-year-old needles had an average of 145 OTUs compared to the average of 151 in

current-year needles. The maximum and minimum values of the number of OTUs for each variable are shown in Table 3.

Table 3. Summary of the maximum and minimum values of the number of reads and OTUs for Seedling type, Site and Year.

		Seedling type		Site			Needle age	
		N	**P**	**G**	**M1**	**M2**	**Y21**	**Y22**
Reads	Max	4381	4453	3823	4453	4381	4453	4381
	Min	773	1057	1057	773	910	1382	773
OTUs	Max	248	299	299	248	245	299	245
	Min	18	36	18	33	36	18	33

The rarefaction curves showed that in all three sampling sites (G, M1, M2) (Fig. 3) and in several samples (App. 2), the OTU asymptote was almost reached.

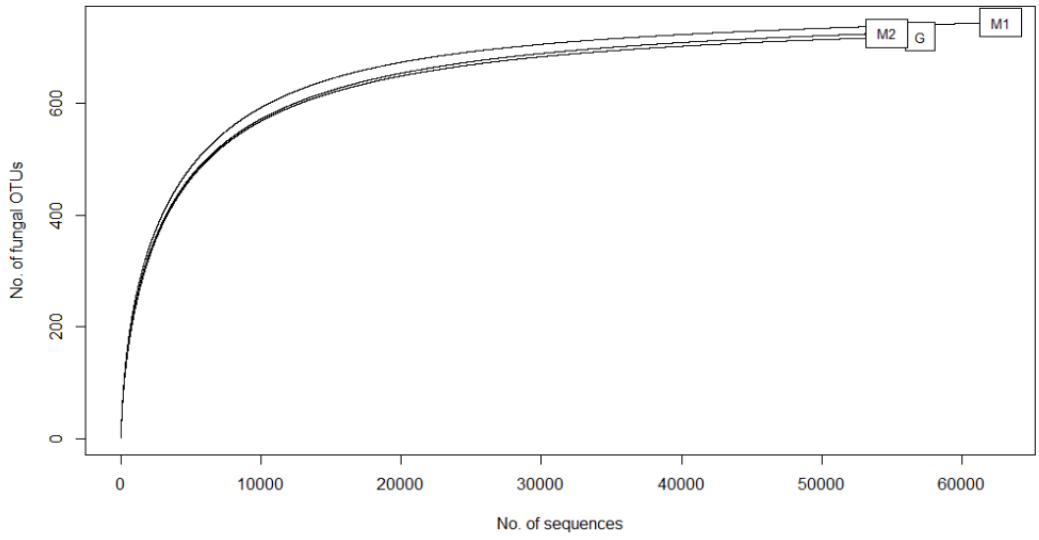

Figure 3. Rarefaction curves for each sampling site, Gåvstahagen (G), Marielund 1 (M1) and Marielund 2 (M2)

4.2 Fungi sequences and OTUs distribution

In general, the relative abundance of sequence reads (blue bars) corresponds with the relative abundance of OTUs (red line) detected throughout the weeks (Fig. 4). When the relative abundance of sequence reads decreases, usually the relative abundance of OTUs follows the same pattern (decrease). Nonetheless, exceptions

are observed during weeks 3, 4, and 5 where the relative abundance of sequence reads decreased while the relative abundance of OTUs increases. Moreover, during week 16, there was a reduction in the OTUs abundance while the abundance of sequence reads remained constant. The same pattern was observed when the data was sorted by site (M1, M2, G) and seedling type (naturally regenerated and nursery-produced seedlings) (App. 3).

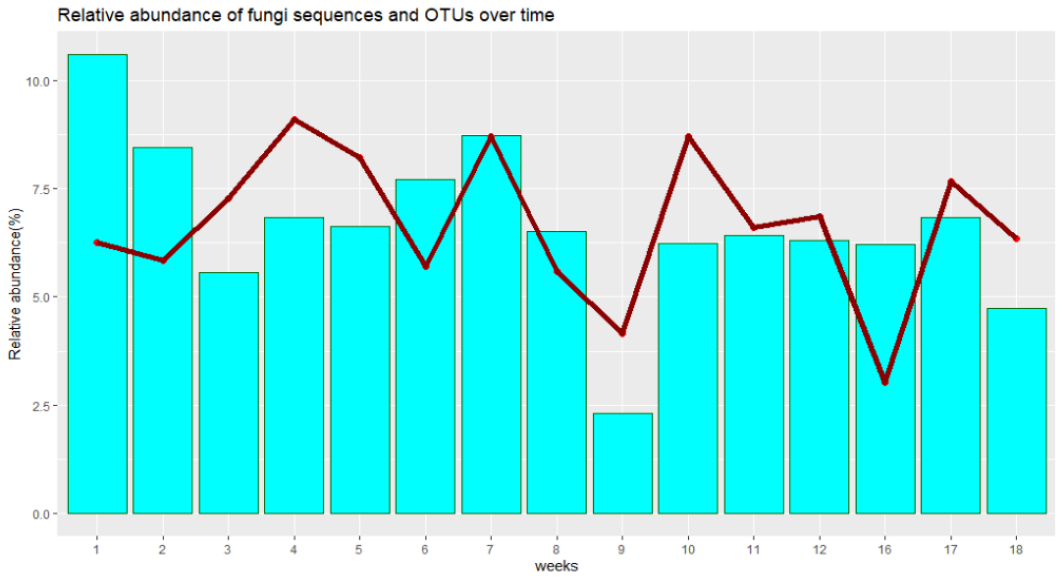

Figure 4. Relative abundance of sequence reads (blue bars) and OTUs (red line) through the weeks for all samples

The number of shared OTUs between the two-seedling types was 720 (Fig. 5). Naturally regenerated seedlings (N) had 62 unique OTUs, whereas nursery-produced seedlings (P) had 40 unique OTUs. Needles from one-year-old (Y21) and current-year needles (Y22) shared 720 OTUs (Fig. 5). One-year-old needles displayed 64 unique OTUs, whereas current-year leaves presented 38 unique OTUs. The three sites shared a total of 614 OTUs (Fig. 5). Moreover, Marielund 1 (M1) and 2 (M2) shared an additional 51 OTUs, and Marielund 1 displayed more unique OTUs (32) than Marielund 2 (20). Gåvstahagen (G) shared 47 additional OTUs with Marielund 1 and 40 with Marielund 2, and this site had 18 unique OTUs.

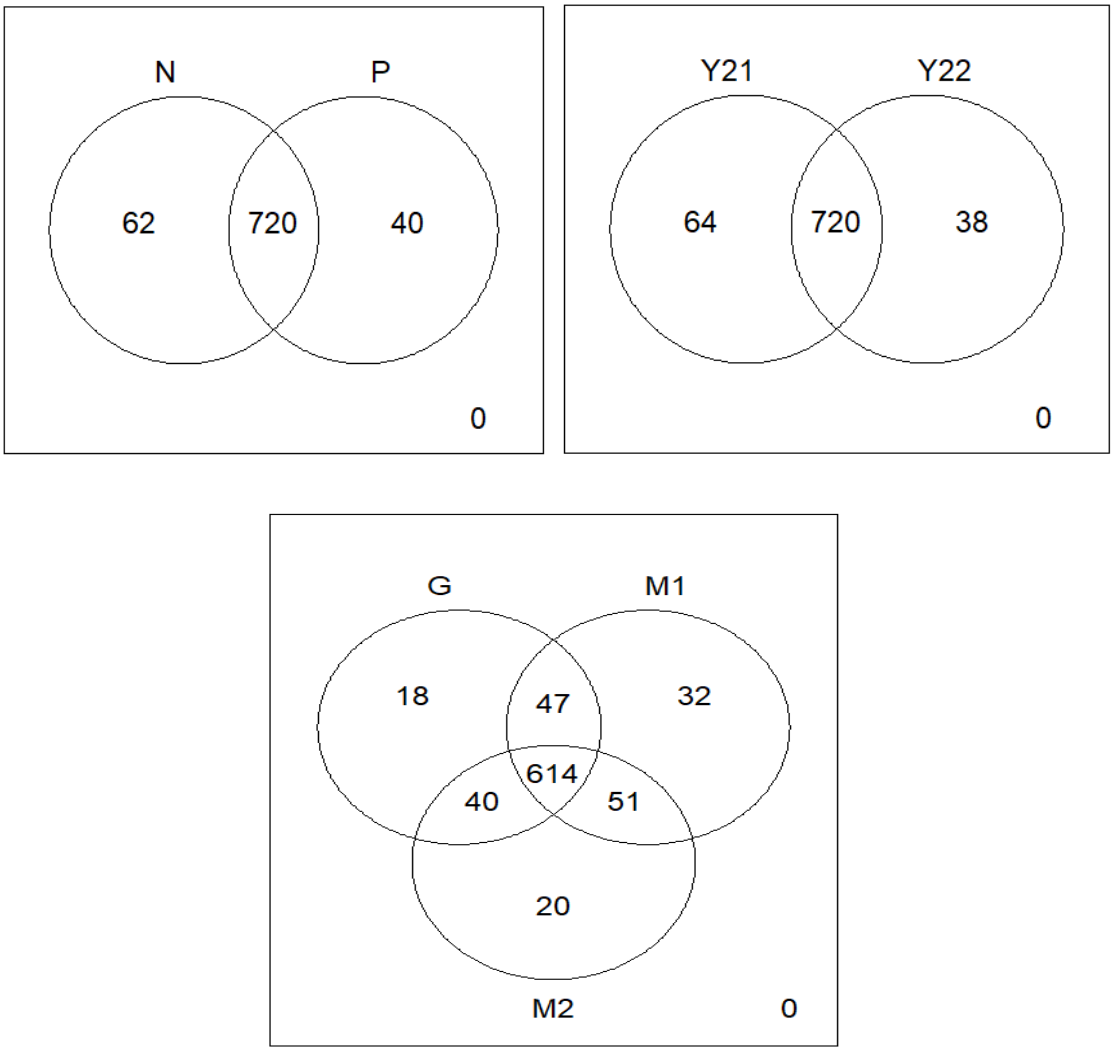

Figure 5. Venn diagram of OTUs distribution for the factors: seedling type, needle-age and site

N: naturally regenerated seedlings. P: nursery produced seedlings. Y21: needle age from 2021. Y22: needle age from 2022. G: Gåvstahagen. M1: Marielund 1. M2: Marielund 2.

By conducting Correspondence Analysis (CA), we identified two outlier samples (47 and 59) that were significantly distant from the rest of the data (App. 4). These outlier samples were associated with their own unique OTUs. However, due to their deviation from the rest of the samples, they were excluded from the subsequent statistical analysis.

After removing the outliers, we observed another outlier (sample 1) in the resulting CA, which were kept in the subsequent analysis (App. 4). Moreover, the

distribution of the remaining samples was mainly explained by the first dimension of the correspondence analysis, which had an inertia value of >6%. The distribution of the OTUs was explained by both the first and second dimensions of the correspondence analysis.

4.3 Local and across samples diversity

The data had a normal distribution with homoscedastic residuals (App. 5). The alpha diversity measured with the Shannon index showed significant differences for both the number of sequence reads and weeks, with p-values of <0.05 and <0.01, respectively (Table 4). After conducting the post hoc analysis (the Tukey HSD test), it was found that there are significant differences between the week 16 and the weeks 3, 4, 5, 7, 11, 12, and 18 (Fig. 6). The beta diversity showed a significant difference in the number of reads among samples (Table 5) and the heat map shows two main groups of samples (Fig. 7), but none of the variables studied explain this clustering.

Table 4. Alpha diversity with Shannon index

Shannon index	df	F	P
Read.counts	1	5.134	0.02782 *
Treatment	1	0.709	0.40373
Site	2	2.376	0.10330
Year	1	0.349	0.55730
Week	14	2.768	0.00415 **
Residuals	50	-	-

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 5. Beta-diversity summary

Beta diversity	df	F	P
Read.counts	1	2.0645	0.012 *
Treatment	1	1.4847	0.076 .
Site	2	1.4123	0.069 .
Year	1	1.3326	0.161
Week	14	1.0620	0.161
Residuals	50	-	-
Total	69	-	-

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

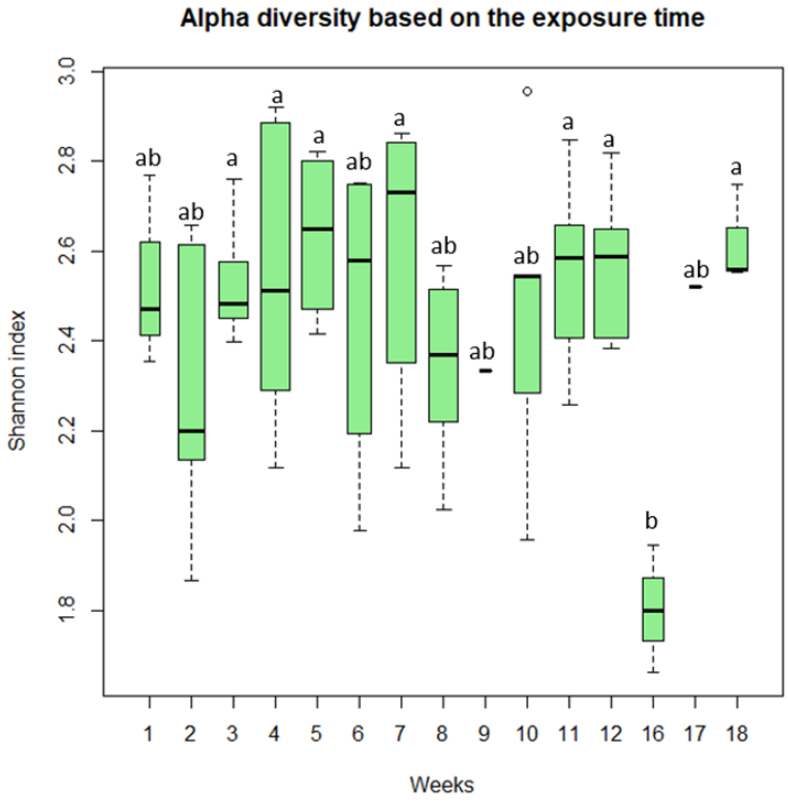

Figure 6. Box plot of alpha diversity of all samples over time. Significant differences in the post-hoc test (Tukey HSD) are indicated by different letters.

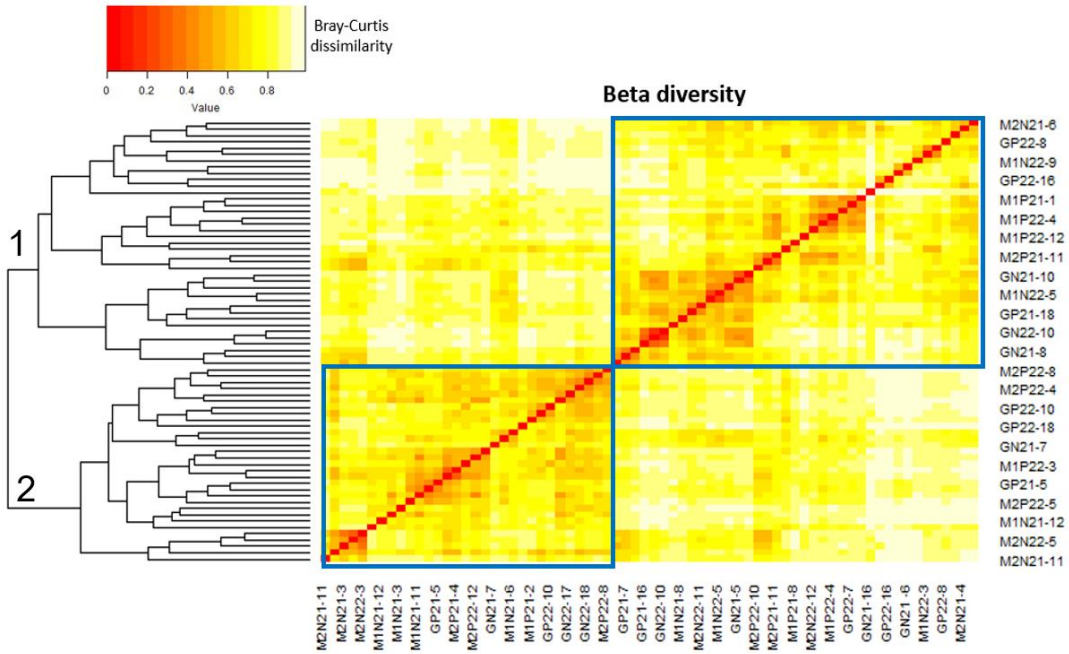

Figure 7. Heat map of the beta diversity of all samples based on Bray-Curtis dissimilarity, yellow (high) to red (low). The two clades are visible with the blue boxes

Regarding the paired sample design, the total of pairs for the variable Pair Treatment were 22 pairs and for the variable Pair Year were 19 pairs. The t-test was not significant for the treatment pair (Pair T) (Table 6) but it was significant for the needle age pair (Pair Y) variable (Table 7) with a p-value < 0.05.

Table 6. Permutation table for paired sample based on the seedling type (Pair T)

	df	F	P
Read.counts	1	3.100	0.0929 .
Pair T	21	1.205	0.3366
Residuals	21	-	-

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 7. Permutation table for paired sample based on the needle age (Pair Y)

	df	F	P
Read.counts	1	2.689	0.1184
Pair Year	18	2.901	0.0146 *
Residuals	18	-	-

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.4 Species distribution over time and between pairs

Over time

After taxonomic classification of the 821 operational taxonomic units (OTUs), 488 OTUs were identified at a genus level and 113 at a species level. By joining the 7 taxonomic levels (kingdom, phylum, class, order, family, genus and species), 475 different taxa were identified. Among the 20 most abundant species, the needle mycobiome was dominated by a fungal species belonging to the family *Pseudeurotiaceae* (10%), a species of the genus *Cladosporium* (9%), the species *Sydowia polyspora* (4%) and a species of the family *Herpotrichiellaceae* (2%) (Fig. 8). Furthermore, the relative abundance of certain species fluctuates over time. For instance, the abundance of the fungal species belong to the family *Pseudourotiaceae* decreases over time, whereas the species belonging to *Cladosporium* increases. Additionally, some fungal species appear later in the season, such as the fungal species belonging to the genus *Phodosphaera*. Moreover, the relative abundance of *Sydowia polyspora* and *Hypogymia physodes* remains relatively constant throughout the observed period.

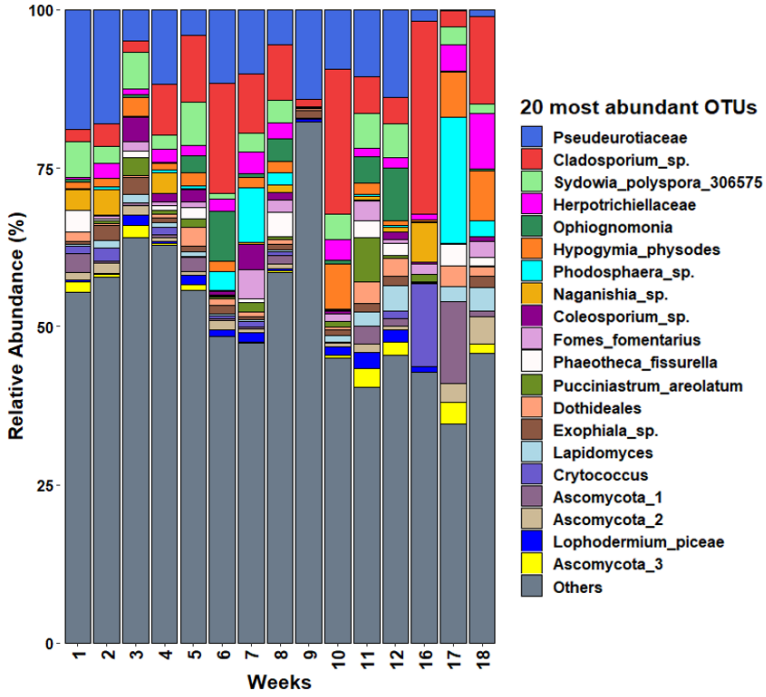

Figure 8. Relative abundance of all the samples, showing the 20 most abundant operational taxonomic units (OTUs)

Distribution between paired samples based on the needle age

At Gåvstahagen (Fig. 9), it was observed that the relative abundance of the 20 most dominant OTUs varied among needles from different years that come from seedlings produced in the nursery (P). This pattern persisted over time, as demonstrated by the year-pairs of weeks 5, 7, 16, and 18. However, for needles from naturally regenerated plants (N), the pattern was less clear. In the week 10, the relative abundance of each fungal species was similar between the one-year-old (21) and current-year needles (22), while in the week 7 the relative abundance of species in the N varied between years.

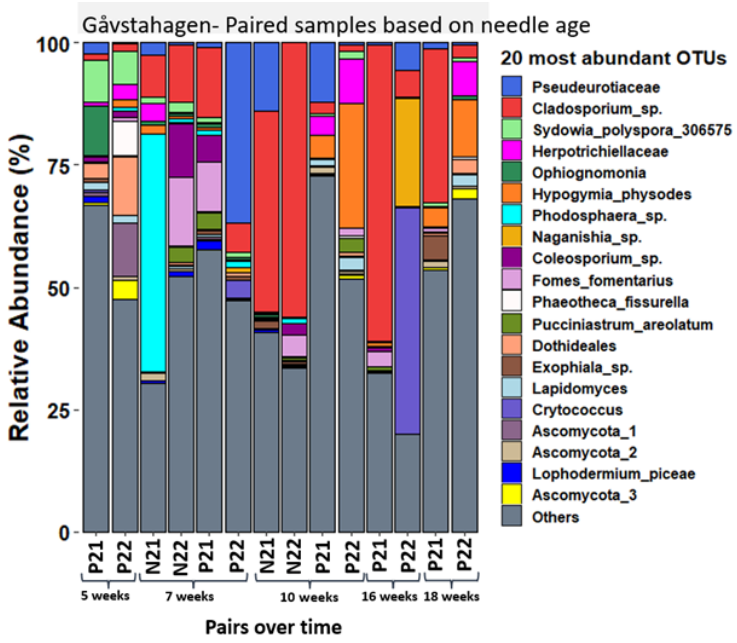

Figure 9. Relative abundance of fungal species for each pared sample based on the needle age (Pair Y) at Marielund 1 site.

In the first weeks (week 2, 3 and 4), the needle mycobiome of Marielund 1 shows that the relative abundance of species had a different pattern between the needles of one year (21) and those of the current year (22), irrespective of the type of seedling from which they come (Fig. 10).

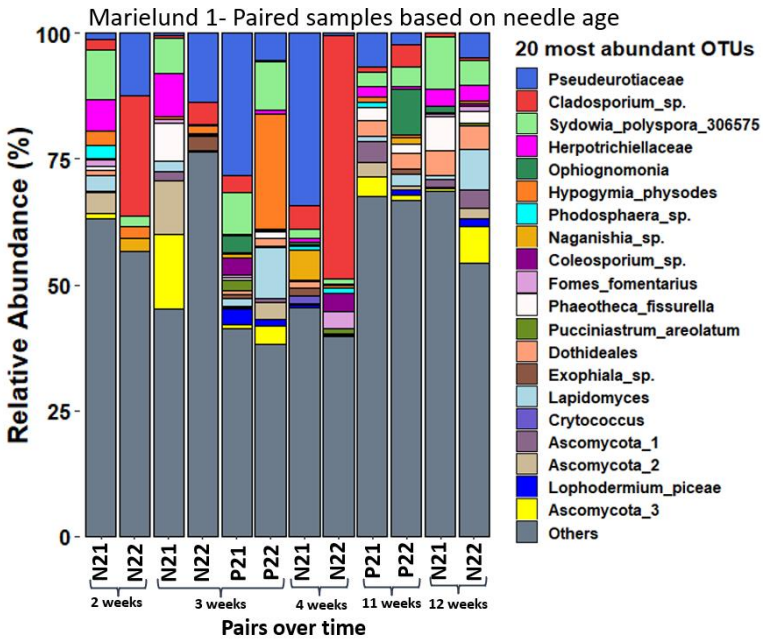

Figure 10. Relative abundance of fungal species for each pared sample based on the needle age (Pair Y) at Gåvstahagen site.

In Marielund 2, the needle pairs from nursery-produced seedlings (P) show that the relative abundance of species varies between one-year-old (21) and current-year needles (22) for samples taken in weeks 4 and 8. In week 12, the needle samples from P seedlings exhibited a similar community between pairs. As for the samples from naturally regenerated seedlings (N), their fungal composition varied being similar at the beginning of the timeline (week 3), while the fungal community was different for weeks 11 and 12 (Fig. 11).

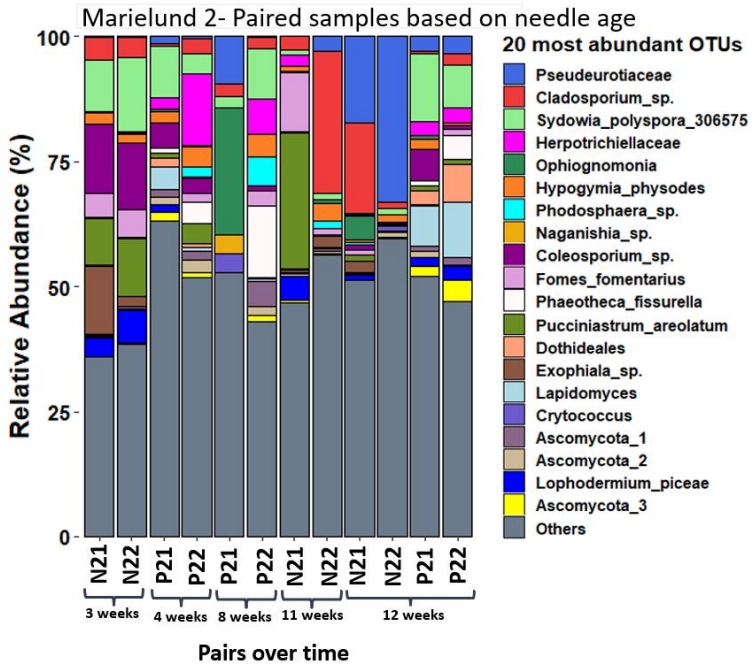

Figure 11. Relative abundance of fungal species for each pared sample based on the needle age (Pair Y) at Marielund 2 site.

4.5 Fungal primary lifestyles

Regarding dominant fungal traits and trophic modes, saprotrophs, ectomycorrhiza, lichenized fungi and molds represented 28.7%, 3%, 1.6%, and 0.6% of the total number of fungal reads, respectively. Additionally, there were 20% of plant pathogens, 2.7% of animal parasites, 1.6% of mycoparasites, and 62% of unidentified lifestyles. The relative abundance graph of the primary lifestyles (Fig. 12) showed that the proportion of each trait remained similar over time, with the prevalence of saprotrophs and plant pathogens.

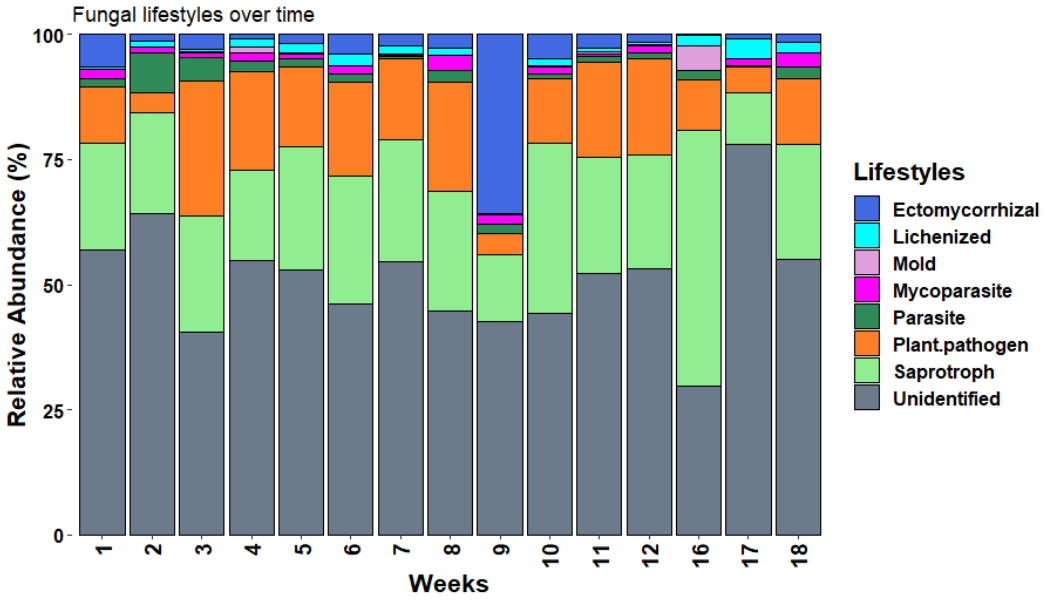

Figure 12. Relative abundance of fungal functional traits over time from all the samples of pine needles

5. Discussion

The aim of this study was to analyse the diversity, composition and dynamics of the fungal community present in the Scots pine needles from nursery-produced seedlings and naturally regenerated seedlings, considering the temporal scale. Specifically, we aimed to assess whether the fungal community of needle from nursery-produced seedlings differs from that present in naturally regenerated seedlings. Our results showed that the foliar mycobiome from Scots pine seedlings are similar at different sites and the seedling type (naturally regenerated and nursery-produced seedlings) does not affect the fungal diversity in the needles, but the time of exposure and the age of the needles are the main factors driving the community.

5.1 Foliar fungal communities' composition and OTUs distribution

The richness in fungal OTU presented in the Venn diagrams of this study (Fig. 3) shows the variation in sampling depth among our variables. Sequencing depth is related with the number of times that a sequence has been read, so it gives information about the sampling (Persson, 2020). The lack of overlap in the OTU richness between Gåvstahagen (G) and both Marielund sites may be attributed to the historical forest cover of each site, which can significantly influence the composition and diversity of endophytic fungal communities (Redondo *et al.*, 2020; Ricks *et al.*, 2019). Gåvstahagen had previously been dominated by Norway spruce, which may result in a fungal community adapted to Norway spruce as a host.

The rarefaction curves (Fig. 3) generated from the number of OTUs, and the number of reads reached an asymptote in all sites and most samples, suggesting that the sampling effort was sufficient and representative of the fungal community in Scots pine needles at these sites.

Nevertheless, the component analyses (CA) of the samples (App. 3) did not reveal any distinct clusters, indicating that the fungal communities are not different from one another. This finding may be due to the host tree is the same, and the selected sites are relatively close to each other, which limits the variation of fungal species available and specific to this tree host.

5.2 Factors for fungal community assembly in needles

The first hypothesis of this study postulated that the foliar fungal communities in the needles of nursery-produced seedlings will be different to the communities present in the needles of naturally regenerated seedlings. The results show that whether the seedlings come from nurseries or from natural regeneration does not affect the diversity of the fungal community present in Scots pine needles, both in terms of alpha and beta diversity (Table 4 and 5). Therefore, this rejects the first hypothesis and contrasts with findings described in similar studies but exclusively with roots (Southam *et al.,* 2022), which found a higher ectomycorrhizal richness in naturally regenerated seedlings compared to planted seedlings of Whitebark Pine (*Pinus albicaulis*). Besides, other research has determined that nursery production affects the diversity and composition of the fungal community in roots of nursery-grown seedlings (Menkis *et al.,* 2005; Okorski *et al.,* 2019).

This lack of significant difference may be due to the leaf mycobiome of nursery-grown seedlings has shift very soon after transplanting them into the forest (1-2 weeks), but this study cannot prove this. However, other factors such as plant age, soil type, light exposure, water, and nutrient availability, etc. (Hata *et al.*, 1998; Juniper, 1991; Millberg, 2015), may have also influenced the composition of the fungal community and obscured possible similarities or differences between the two types of seedlings. Additionally, some of these factors may be the reason for the two different clades in the heat map (Fig. 7). Nevertheless, there are no studies that compare foliar fungal communities of nursery-produced seedlings with those of naturally regenerated seedlings, which make this research an exploratory study. Therefore, greater efforts are needed to draw conclusions on this topic, which is relevant for forest resilience and productivity.

Regarding the second hypothesis stating that the foliar fungal community present in the nursery-produced seedlings will shift and over time become more like those of naturally regenerating seedlings. The results showed that the exposure time of the needles does affect the alpha diversity of the foliar mycobiome (Table 4). These findings are consistent with the second hypothesis that there is a change in the foliar fungal community over time, which has also been observed in previous studies (Arnold & Herre, 2003; Larsson *et al.*, 2023).

However, it should be noted that only the week 16 shows lower diversity compared to most of the other weeks (Fig. 6). This is because the OTU identified as *Cladosporium* sp. was the dominant species in the samples during that week (Fig. 8). As the Shannon index gives more weight to species richness than evenness, an increase in abundance of that species contributes less to the diversity value than a decrease in richness. Therefore, the use of other diversity indices with less weight on species richness may give a different result.

Moreover, the lack of differences from the beginning between seedling types does not allow to prove whether the fungal community of nursery-grown seedlings tends to be more similar to the communities of naturally regenerated seedlings. Therefore, the second hypothesis cannot be totally verified.

The analysis from the paired samples design based on the age of the needle revealed significant differences, indicating that the fungal diversity of the needle varies according to its age. This finding is consistent with previous studies that have shown the influence of needle age on fungal diversity (Agan *et al.*, 2021; Terhonen *et al.*, 2011).

The fungal diversity variation due to needle age can be related with several factors, including changes in needle physiology, nutrient availability, and the composition of the surrounding fungal community (Hata *et al.*, 1998; Juniper, 1991; Redondo *et al.*, 2020). Particularly, as needles age, they experience changes in their chemical composition that may affect the colonisation of certain fungal species (De Marco *et al.*, 2012). This is supported by the observation that fungal species richness and abundance vary depending on needle age, with older needles typically exhibiting higher fungal diversity (Agan *et al.*, 2021; Millberg, 2015).

5.3 Fungal species assembly: time and needle age effects

In this study, species belonging to the family *Pseudeurotiaceae*, the genus *Cladosporium* sp., the species *Sydowia polyspora*, and the family *Herpotrichiellaceae* were most frequently found in the fungal community (Fig. 8).

Pseudeurotiaceae is a new family where some of its members are not monophyletic, and some genera included in this family are *Xyleria* sp., *Claviceps* spp. (Suh & Blackwell, 1999). Additionally, *Herpotrichiellaceae* is a family belonging to the group of "black yeasts", where some species are known to be plant and animal pathogens (Sterflinger, 2006). It is a versatile fungal family that can thrive in various environmental conditions (Haase *et al.*, 1999).

The genus *Cladosporium* spp. has a cosmopolitan distribution and includes numerous plant pathogenic species (Bensch *et al.*, 2012). Its presence has also been described in nurseries in previous studies (Okorski *et al.*, 2019; Martin *et al.*, 2004), and recently an outbreak of disease in nurseries caused by *Cladosporium* spp. pathogens was reported in Sweden (Larsson *et al.*, 2023).

Regarding *Sydowia polyspora*, it is a common latent pathogen found in both needles and seeds of various conifer genera such as *Pinus* sp. and *Abies* sp. (Ridout & Newcombe, 2018). It can sometimes act as a weak and localized pathogen causing defoliation in seedlings, but it can also be more harmful and cause shoot and tip dieback as well as lesions in various *Pinaceae* species (Smerlis, 1970). Furthermore, it can be present in the fungal community of these plant genera without tissue symptoms (Ridout & Newcombe, 2015). Thus, it is a species with a significant impact on successful seedling recruitment in both field and nursery settings.

The OTUs belonging to the previously described taxonomic groups exhibit different abundance over time in this study (Fig. 8). The relative abundance of the *Pseudeurotiaceae* species decreases as the abundance of *Cladosporium* sp. increases over time. On the other hand, the pathogenic species *S. polyspora* and the species belonging to the *Herpotrichiellaceae* maintain a constant relative abundance over time. Therefore, the fungal community diversity becomes more homogenous as the proportion of 'Other' decreases, and potentially pathogenic

species become more dominant over time. Larsson *et al.* (2023) study showed a similar trend in the fungal community towards homogenization. Through the analysis of the relative abundance of the 20 most dominant species (Figs. 9-11), no clear pattern is observed, and the proportion of fungal species differs among the samples.

5.4 Fungal traits development

The results show that the community is dominated by saprotrophs (28%) and plant pathogens (20%). Their relative abundance remains constant over time, except for week 9 where ectomycorrhizal increase. Rajala *et al.* (2014) discovered that one-third of the endophytic species comprising the needle community may be facultative saprotrophs, because these species were present in fresh and decomposing tissues. This finding may explain the high proportion of saprotrophs observed in the fungal community of this study. Additionally, a high abundance of plant pathogenic fungi has been previously demonstrated in studies of fungal communities on healthy looking nursery seedlings (Larsson *et al.*, 2023).

5.5 Limitations of this study

During the needle collection, we specifically selected those that appeared healthy, but towards the end of the sampling period, it became challenging to find needles without disease symptoms. As demonstrated by Millberg (2015), this could have influenced the fungal community present in the later stages of the study. Moreover, the different size and frequency of needles between nursery-grown seedlings from each site may influence the development of fungal diversity (Gamboa *et al.,* 2003). As is often the case in this type of study, not all samples could be successfully sequenced (Hong *et al.,* 2022), resulting in the inability to make comparisons for certain time points. Then, it may be beneficial to reduce the number of time points and increase the number of replicates per time point in future studies.

In this study, the lack of differences between the foliar mycobiome of nursery-grown seedlings and the community of naturally regenerated seedlings may be

because we only measured the fungal community after transplanting. Future studies might also examine the community before transport the seedlings and planting them on the clear-cut sites.

There are several limitations associated with the methodology of metabarcoding in this study. The taxonomic identification of fungi relies on the internal transcribed spacer (ITS) region of the nuclear rRNA operon, which allows for species-level resolution. However, the amplification of the ITS region introduces biases that can lead to the underestimation of certain fungal taxonomic groups (Peay *et al.,* 2016). This is due to taxonomic marker sequence does not align perfectly with the biological concept of species in fungi. Instead, operational taxonomic units (OTU) are defined based on a threshold of sequence similarity, causing sequences from ecologically distinct species to be grouped together within the same OTU. Additionally, bioinformatic pipelines are not optimised to deal with some specific features of ITS sequences and different bioinformatic pipelines may give different results from the same data. (Siegwald *et al.,* 2019).

Furthermore, depending on the diversity index that it used the results and conclusions may differ. This is due to each index have different assumptions or consider more relevant different ecological aspects. For example, the Shannon index gives more weight to species richness while Simpson's index cares more about relative abundances (Oksanen *et al.,* 2022).

6. Conclusion and future prospects

The fungal communities inhabiting trees affect their hosts, but factors influencing fungal community composition in seedlings remain largely unknown. This study aimed to compare the fungal community present in the Scots pine needles from nursery-produced seedlings and naturally regenerated seedlings, considering the temporal scale. The results show that the seedling origin does not have an effect in the foliar mycobiome. Instead, exposure time of the needle and needle age are the main variables affecting fungal diversity in the needles. Moreover, these results suggest that the endophytic fungal communities in Scots pine needles are relatively similar across different sites, with little variation in species composition. Additionally, the most abundant taxa in the needle mycobiome were a fungal species belonging to the family *Pseudeurotiaceae* (10%), a species of the genus *Cladosporium* (9%), the species *Sydowia polyspora* (4%) and a species of the family *Herpotrichiellaceae* (2%). Furthermore, saprotrophs (28%) and plant pathogens (20%) were the main fungi traits. This research enhances our understanding of the mycobiome composition found in needles, focusing on the influence of seedling origin and history, the progression of colonization in new needles, and the fluctuation of the fungal community across seasons. Further studies should consider analyse the foliar mycobiome present in nursery-produced seedlings before transporting them to the forest stand and use seedlings from several nurseries and different latitudes. In that way, the knowledge about how the forest nursery system impact the foliar mycobiome will improve.

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Popular science summary

Sweden's forestry model has made it one of the world's leading exporters of paper, pulp and sawn timber. This is due to its efficient approach, based on a system of rotational felling and planting of nursery stock. In fact, Sweden produced a staggering 430 million nursery seedlings in 2022. These seedlings are grown in nurseries under intensive management practices, including the use of fungicides. As a result, the seedlings experience markedly different environmental conditions compared to naturally regenerated seedlings in the forest.

When we think of fungi, we often envision the visible mushrooms that sporadically emerge in forests. However, the world of fungi is much more diverse. From the intricate mycelial networks connecting tree roots to the fungi that gradually develop on bread, fungi thrive in various environments. Among them are foliar fungi, which inhabit plant leaves. While some of these fungal species can be beneficial, protecting plants against herbivores, pathogens, and drought conditions, there are also pathogenic species that can harm plants and even cause their demise.

Recent discoveries have shown that these pathogens coexist without causing immediate damage. Instead, they remain dormant until provoked by stressful circumstances, eventually resulting in visible symptoms on the host plant. However, the specific factors that trigger this shift are still not fully understood. This study aimed to clarify what happens to the foliar fungal communities present on nursery-produced pine seedlings once they are planted in the forest stand. Would these communities undergo transformations over time, and would they differ from the fungal communities found on naturally regenerated seedlings?

The answers are that there are no differences in foliar fungal communities between nursery-grown and naturally regenerated seedlings. Instead, the observed disparities were attributed to the age and exposition time of the leaves themselves. By shedding light on the actors of leaf fungal communities, this study contributes

to our understanding of the complex interactions between seedlings and their fungal partners.

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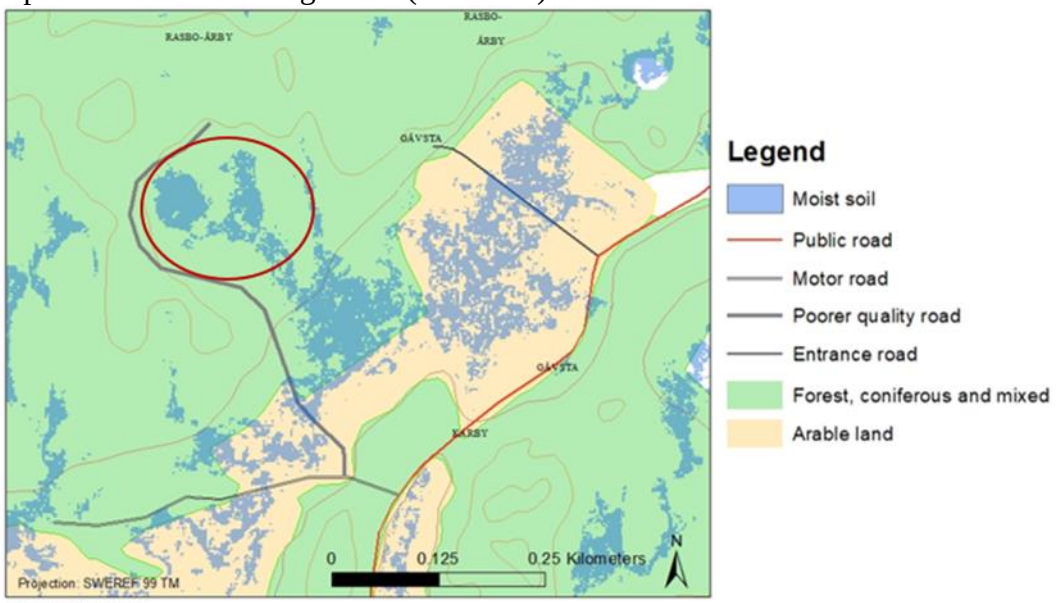
Last but not least, I would like to give my warmest thanks to all my loved ones who constantly motive and support me, even if they don't fully understand where I am or what I am doing. Thanks a lot.

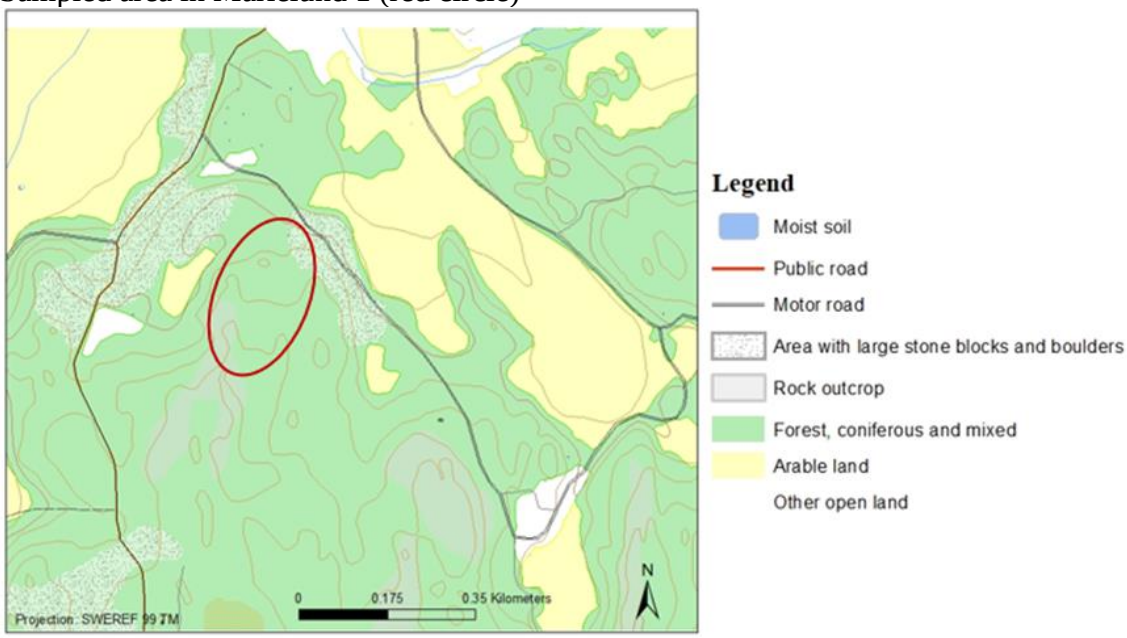
Appendix 1

Sampled area in Gåvstahagen site (red circle)



Source: Lantmäteriet ©
Autor: Sara López Fernández, 2023

Sampled area in Marielund 1 (red circle)



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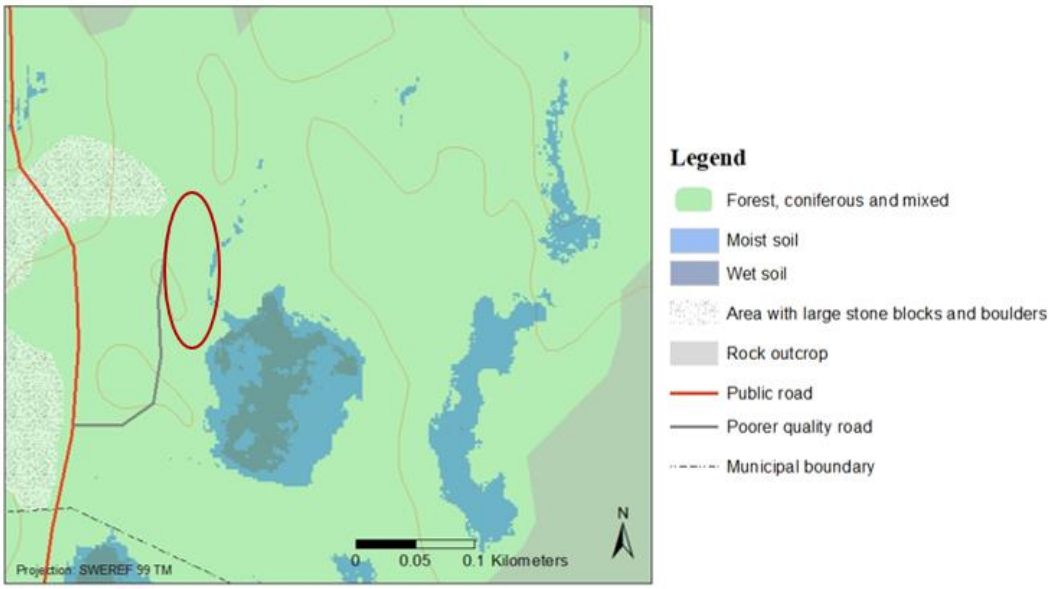
Sampled area in Marienlund 2 site (red circle)



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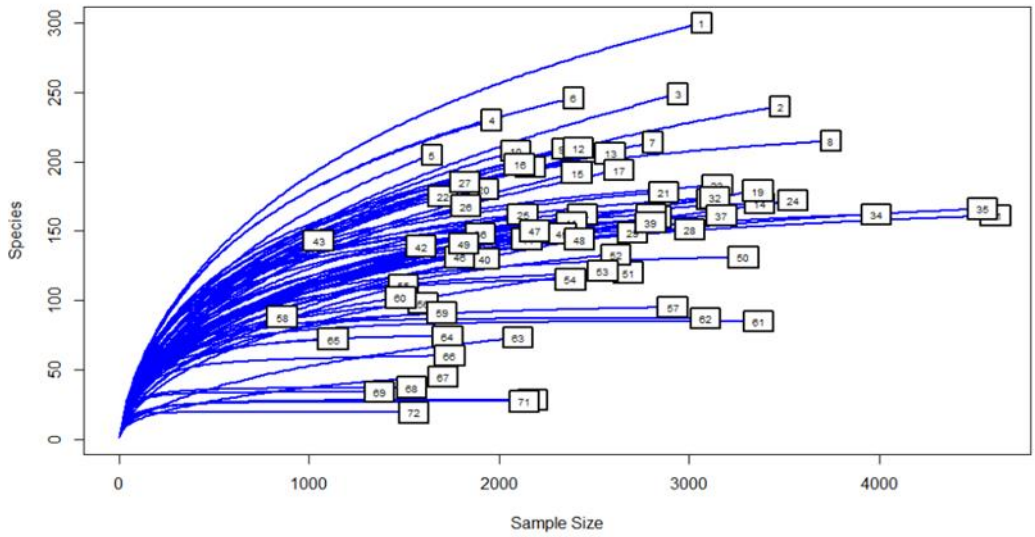
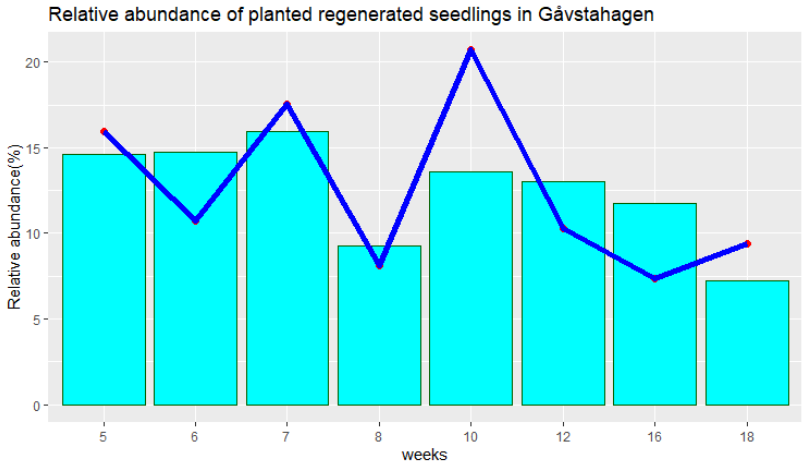
Appendix 2

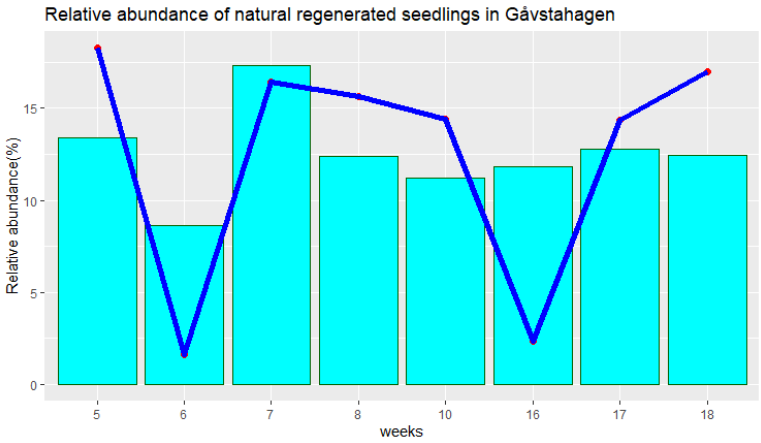
Rarefaction curves for each sample; species is the nº of OTUs explained by the nº of reads.

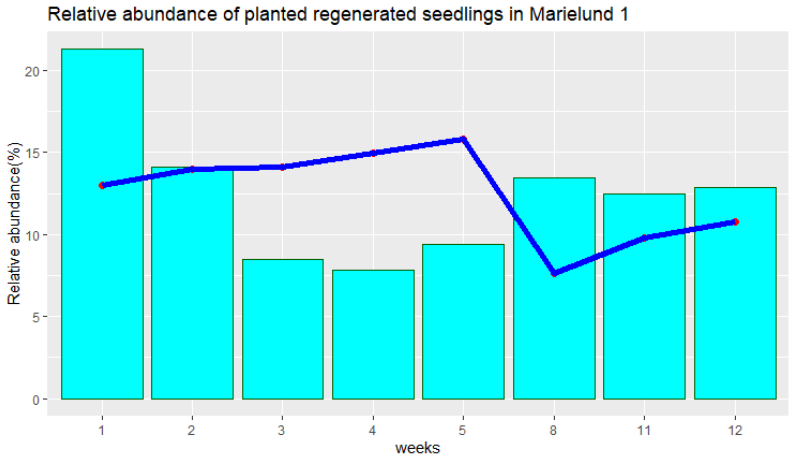


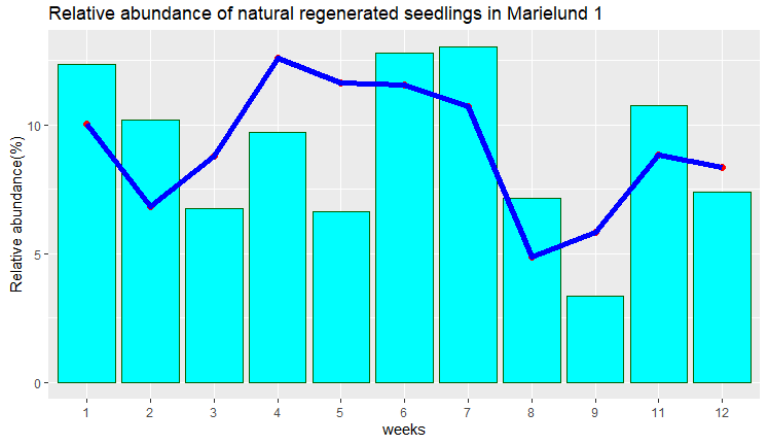
Appendix 3

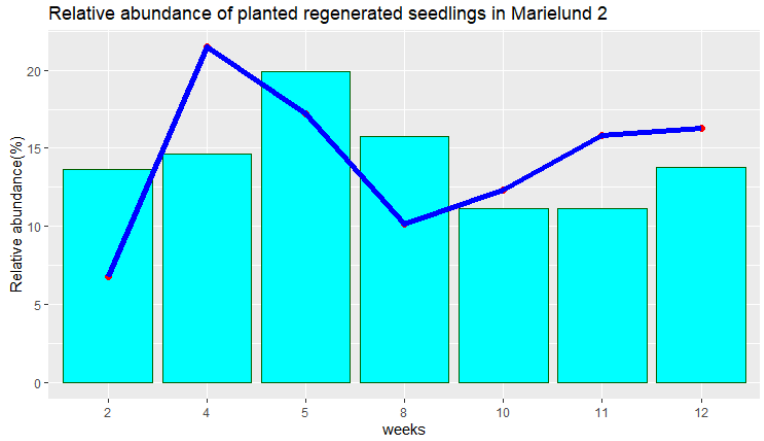
Relative abundance of sequence reads and OTUs soft by sites and seedling type.

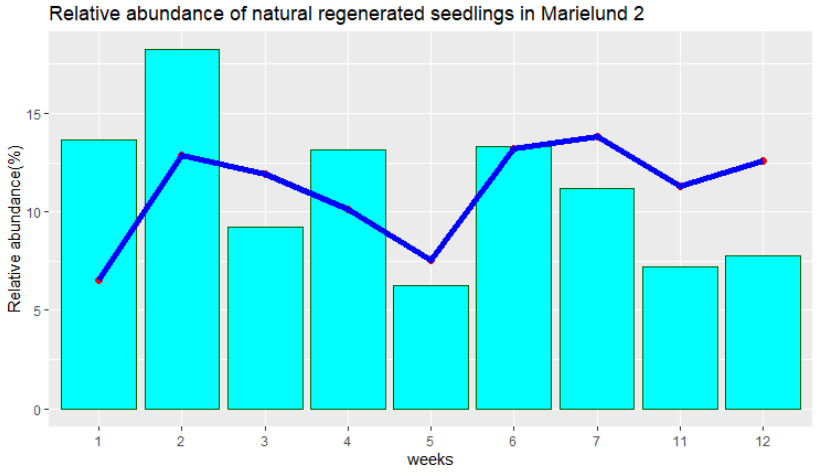






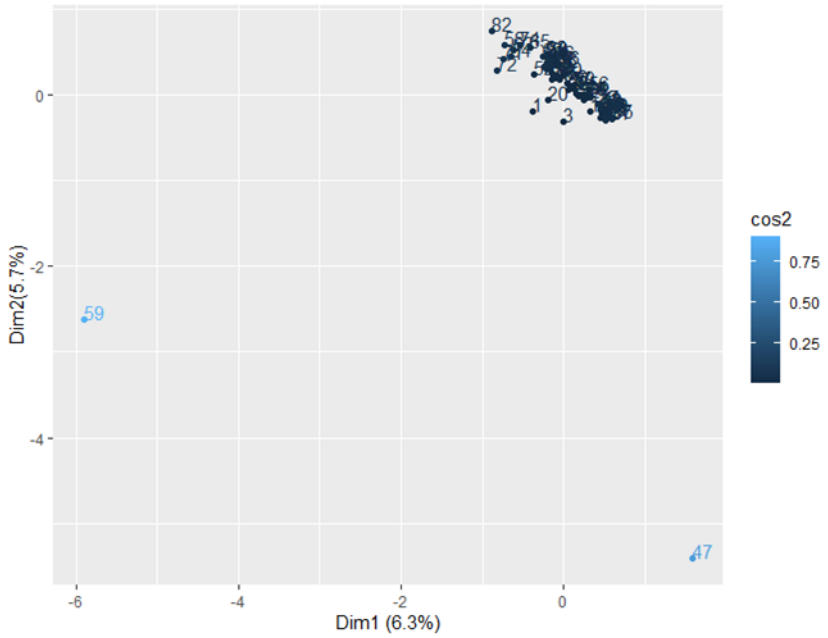

Relative abundance of natural regenerated seedlings in Marielund 1
Relative abundance(%)
0
5
10
1
2
3
4
5
6
7
8
9
11
12
weeks


Relative abundance of planted regenerated seedlings in Marielund 2
Relative abundance(%)
0
5
10
15
20
2
4
5
8
10
11
12
weeks

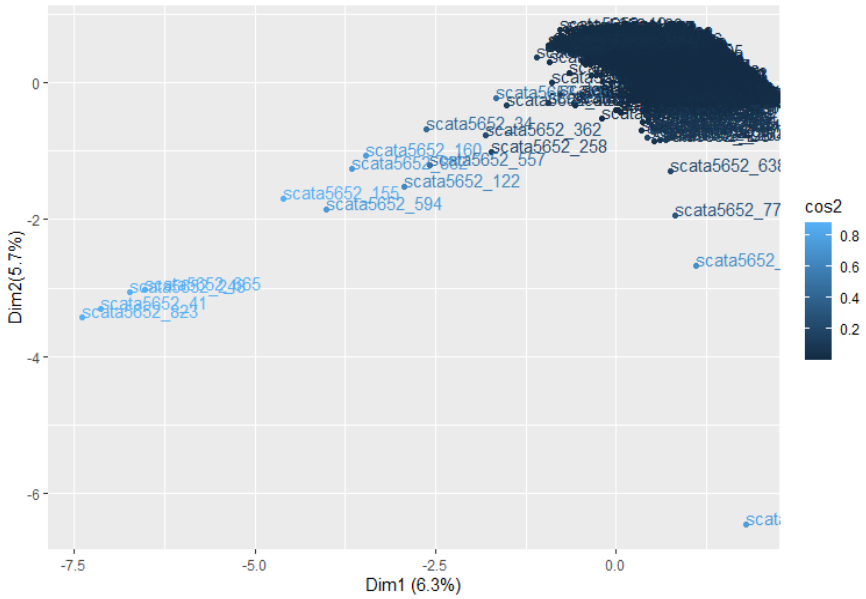

Relative abundance of natural regenerated seedlings in Marielund 2
Relative abundance(%)
0
5
10
15
1
2
3
4
5
6
7
11
12
weeks

Appendix 4

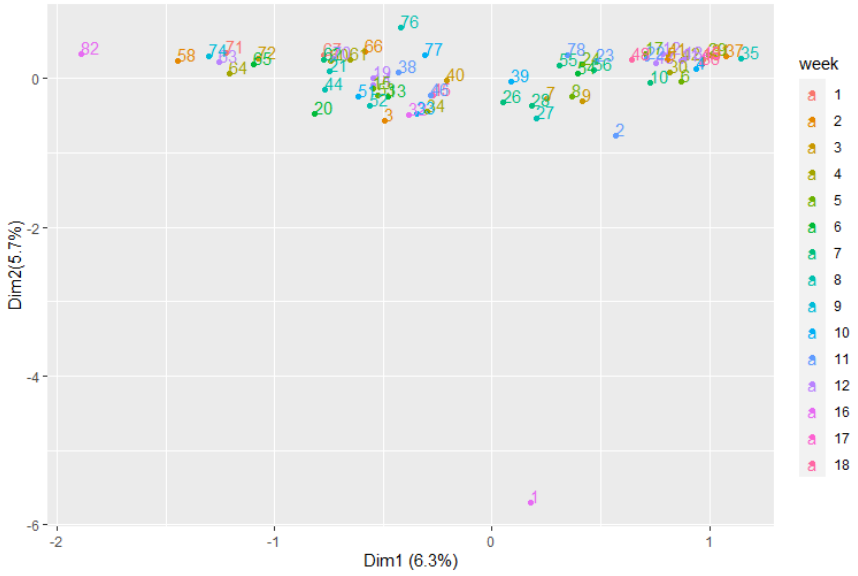
Correspondence Analysis (CA) with the samples



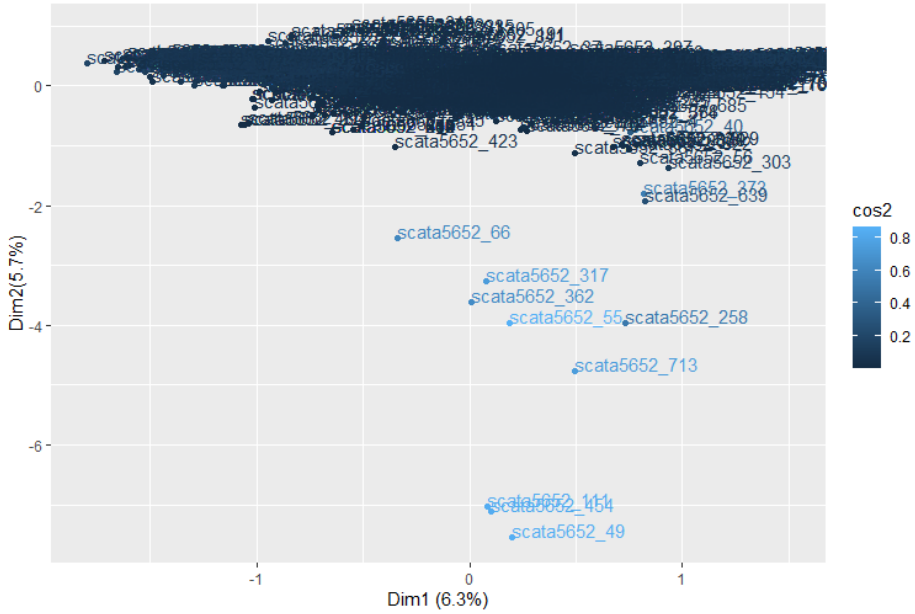
Correspondence Analysis (CA) with the operational taxonomic units (OTU)



Correspondence Analysis (CA) with the samples and the week's number (colors)

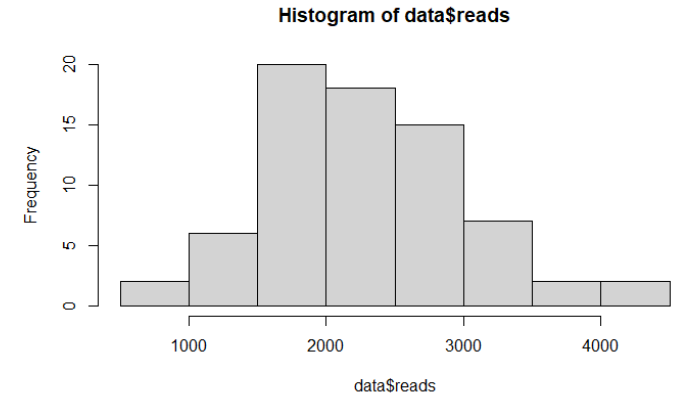


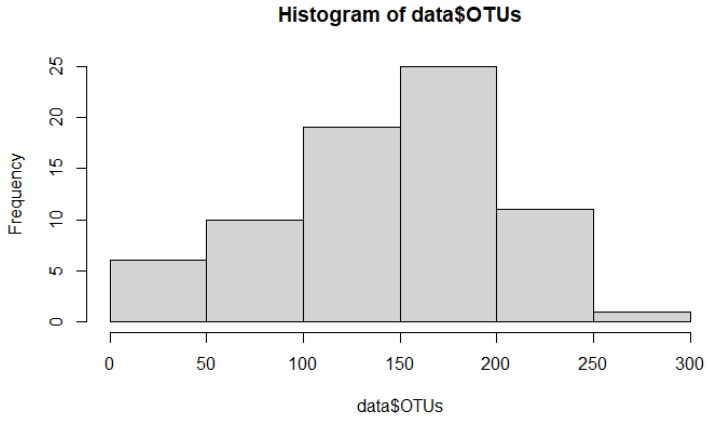
Correspondence Analysis (CA) with the operational taxonomic units (OTU)



Appendix 5

Histograms of sequence reads and OTUs to assess the normality of the data.





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