Endophytic fungi in living trees - are communities affected more by tree species, tree age, or geographic origin?

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Table of contents

1.1 Abstract

Endophytic fungi are ubiquitous in living vascular plants and have been identified in most terrestrial environments. The endophytes may have various ecological roles such as commensals, symbionts and latent invaders. Woody plants harbour a diverse array of different endophytic species that have been detected in various habitats, from leaves to roots. However, the knowledge and research about endophytic communities are scarce, especially in relation to conservation of biodiversity. One of the reasons is the difficulties in detecting and identifying the fungal species, which do not form fruitbodies. The project aims to investigate if the species density and species composition differ between tree species (*Acer platanoides, Fagus sylvatica, Quercus robur, Tilia cordata),* as a function of tree trunk circumference, and among three regions in southern Norway. The analyses were based on a dataset of environmental DNA samples from sapwood of 208 trees. The result show that *Q. robur* differs significantly from the other tree species in both species density and species composition and hosts considerably more red-listed species. Oak also harboured a substantial portion of species only detected in a single sample. There was no significant difference in species density among the three regions. Trunk circumference affected the fungal endophyte community composition. The results emphasize the importance of oak trees for fungal diversity and red-listed species.

1.2 Abstrakt

Endofytiska svampar är allmänt förekommande i levande kärlväxter och har blivit identifierade i flera olika miljöer. Endofyter kan ha många olika roller i ekosystem, från kommensalism till symbios. Vedartade växter agerar värd åt många olika arter av endofytiska svampar och har lokaliserats i värdväxtens alla delar, från löv till rötter. Dock är kunskap och forskning kring endofyter begränsad, speciellt inom bevarandebiologin. En anledning till att endofyter är relativt outforskade beror på att det är mycket komplicerat att lokalisera då de inte bildat fruktkroppar. Syftet med projektet är att undersöka om arttäthet och artsammansättning varierar mellan fyra trädslag (lönn, *Acer platanoides;* bok*, Fagus sylvatica;* skogsek*, Quercus robur;* lind*, Tilia cordata)* och mellan tre olika regioner i södra Norge. Projektet undersöker också om trädstorlek påverkar arttätheten och artsammansättningen hos endofyter. Analyserna var baserade på ett dataset med stickprover av miljö-DNA (e-DNA) från 208 träd. Resultatet visar att *Q. robur* skiljer sig signifikant från de andra trädslagen i både artsammansättning och arttäthet och har flest rödlistade arter. Ek agerade även värd åt ett stort antal av singletons, arter som endast identifierades i ett stickprov. Resultatet påvisade ingen skillnad i artsammansättning eller arttäthet mellan regionerna, dock påverkade stamomkretsen artsammansättningen men inte för alla trädslag. Detta indikerar att ek är en mycket betydande värdväxt åt den endofytiska svampdiversiteten och de rödlistade och sällsynta arterna.

2. Introduction

2.1 What are endophytic fungi?

Endophytes are fungi that lives inside various living plants, from leaves to roots (Rodriguez et al., 2008), and may have various ecological roles such as commensals, symbionts and latent invaders (Rodriguez et al., 2008). Most vascular plants harbour at least one endophytic species, but the more complex plant species, including trees, often carry several fungi species (Saikkonen, 2007). The endophytes usually belong to the phyla Ascomycota, Basidiomycota, or Glomeromycota (Terhonen et al., 2019)

The interest and research around endophytic fungi increased during the last few decades, especially among biologists with their primary research in ecology or evolution. The interest also expanded to other research fields, for example agriculture (Saikkonen, 2007). With increased interest and study on endophytes, the knowledge also increases, but endophytes living in trees are still very unknown (Pellietier et al., 2019) The research has mainly focused on investigating if relationships between the fungi and the host have developed, where investigations of possible symbiotic or pathogenic connections are the prioritised research questions (Saikkonen, 2007).

2.2 Classification

There are two major groups in the endophyte communities, the C-endophytes and the NC-endophytes. C-endophytes (*clavicipitaceous* species) are all phylogenetically similar. These endophytic species are selective and primarily detected in grasses. The C-endophytes origin implies having emerged from insect-pathogenic species and has then transferred through several phyla before being classified as endophytes and the respective phyla (Rodriguez et al., 2008).

NC-endophytes (*nonclavicipitaceous* species) operate on a larger scale, living in symbiosis with various vascular plants, and are identified in all terrestrial ecosystems (Rodriguez et al., 2008). Endophytes that are mutualists to their host supposedly has evolved from pathogenic fungi. However, the arguments indicating the opposite, that the mutualist fungi develop into pathogenic fungi and not the other way around is clear (Sieber, 2007).

Moricca & Ragazzi (2007) continue the argument discussing that the endophytic fungi in woody plants might not be dangerous or harmful to the tree initially, but if the plant is exposed to stress or harm, the endophytes could use the situation to their favour by initiating a saprobic process. However, it has been discovered that endophytes do benefit plants as well and affect the evolution of terrestrial flora. With woody plants', primarily trees, harbouring several different endophytic species compared to grasses. These findings may connect to the differentiation in tissue and composition during their long lifetimes in contrast to the grass endophytes (Moricca & Ragazzi, 2007).

NC-endophytes have proven beneficial for their host and often create a mutual symbiosis. The endophytes provide their host with valuable abilities, such as drought or stress tolerance. It has also been noted that some species provide their host with abilities to tolerate changes in, for example, pH and temperature (Rodriguez et al., 2008). It is also brought up that the endophytes harboured in tree bark, evidently has protected trees from the Dutch elm disease by inhabiting the bark tissue and minimising the available tissue for possible pathogens and insect attacks by creating biological compounds (Pellietier et al., 2019; Rodriguez et al., 2008). NC-endophytes also has been suggested to impact the ecophysiology of the hosts harbouring the fungi (Rodriguez et al., 2008).

2.2.1 Identification methods

The methods to discover and identify endophytes in woody plants are challenging and the number of endophytic fungal species identified depends on the methods detection (Sieber, 2007), with earlier methods such as pure culturing lately being replaced by various molecular methods.

Saikkonen (2007) mentioned that the concept of endophytes is misleading due to the various difficulties and complexity of detecting and researching the ecological field of the endophytic fungi lifecycles, especially for the species harboured by the more long-lived species such as trees and woody plants. Several characteristics within the woody plants might hinder the main methods of identification of endophytic species and the different ways the fungi interact and reproduce within and between hosting plant species (Saikkonen, 2007). The lack of research on endophytes in woody plants has also affected the classification of several fungi. Resulted in little knowledge about how geography and environment affect the endophytic species (Pellietier et al., 2019)

2.3 Conservation of fungi

As previously mentioned, the classification and identification of the fungi have been and continue to be complicated and the identification of the endophytic species in different ecological habitats has been affected as a result. Saikkonen (2007) discusses that the impact varies in different ecosystems and that the fungi could either benefit or disfavour of it. Furthermore, the endophytes in symbiosis with their hosts affect the fitness of the harbouring plant and the ecology and evolution of them. Which could lead to more extensive changes, such as the diversity within and between communities (Rodriguez et al., 2008).

Cline et al. (2017) describes the importance of the decay in wood composition among woody plants to have an essential role in the deadwood occurrence in forests and impact the carbon cycle. The endophytic fungi initiate the decomposition in trees while still living (Gilmartin et al., 2022) For example, knowing when and in which abundance of different fungi species inhabit the trees potentially affects the release of carbon rates (Cline et al., 2017). However, problem related to the detection and the small amount research is also highlighted by Cline et al. (2017) is that the possible changes between different areas and geographical origins are often incorrect or not included in the research because of not enough knowledge in these areas, even thou they could be important factors. It is suggested that the fungal community's contribution to wood decay could help decide the dynamics of the forest.

Saikkonen (2007) discusses the endophytes classified as specialists occur more frequently in habitats with a continuous mixed forest than in a managed homogenous forest. This statement suggests that the endophytic specialists, and perhaps all endophytic fungi, are influenced by the forests well-being, where succession patterns, management methods, and different environments and host species are important. The exposure of endophytic fungi varies among different environment, the younger trees seemingly have the highest exposure to fungal spores in mixed forests compared to managed forests with the lowest interaction with endophytes. It indicates that the natural forest positively impacts the diversity of the endophytic communities. The natural forest had around 70 % compared to the managed forests, which had approximately 20-30 % endophytic species. There have also been trends indicating that the endophytic fungi prefer older trees (Saikkonen, 2007).

Although, Petersson et al. (2019) mentions that several tree species endure difficulties with regeneration, caused by multiple different problems of disturbance and climate changes. The different changes possibly create harsher environments for multiple tree species and results in

decreasing abundance, especially for oak trees which has been mentioned as a tree species crucial for ecosystems diversity, being important for several other species both fungi and lichens (Petersson et al., 2019).

Errasti et al. (2010) mention that the endophytic fungi transmission between the fungi and the woody plant develops without wounding the bark. Then fungi, as mentioned earlier, interact with their host through symbiosis but can alter into mutualism, depending on several different factors. Therefore, the article describes the endophytes as critical for the development and welfare of forests. In addition, the fungi species possibly affect the dynamic, structure, and diversity of the environment their host lives in. Saikkonen (2007) suggests that with the previous information about transmission, fragmentation of habitats probably affects the endophytes in similar ways as their host. Furthermore, research has shown that fragmentation effects increase if the harbouring species is detected in foreign habitats.

3. Aim

Endophytes may have several essential connections and roles in the ecosystem. The interaction between the host and the fungi could be pivotal for the habitats and the harbouring plant populations welfare (Errasti et al., 2010). The fungi show signs of multiple areas where they are beneficial and vital for the survival of the host flora. The fungi have also proven important for the carbon cycle and the impact on the climate (Cline et al., 2017). Nevertheless, as mentioned earlier, research on particular the woody plants interactions with endophytic fungi are limited (Saikkonen, 2007; Pellietier et al., 2019). Therefore, any definitive conclusion is challenging to conclude.

The project aims to investigate if differences in tree species, tree age, and geographic origin are related to species composition and density among endophytic species associated with selected four tree species. Furthermore, what conditions may benefit the endophytes and do the endophytic fungi have preferred habitats or environments? The project also aims to investigate if different tree sizes affect the endophytes species density.

Learning about the influence and importance of different tree ages, species, and diversity in ecosystems is important for conservation strategies, the project will produce needed knowledge on the importance of the symbiotic relationship between fungi and flora and could improve conservation methods for the tree species and fungi communities.

3.1 Delimitations

The collected data were sampled from several different tissues of the trees, all areas exposed to endophytes. For the dataset, twigs, leaves, and stems were sampled over multiple sites in three different regions containing both fungi and non-fungi species samples. Due to the vast amount of collected data, the decision was made to only investigate the endophytic species sampled from the tree stems.

3.2 Research questions

- How do the endophyte communities and the red-listed endophyte communities vary in species composition and density between the tree species, *Acer platanoides*, *Fagus sylvatica*, *Tilia cordata*, and *Quercus robur*?
- Is tree size/tree age related to the species density and composition of endophytes and red-listed endophytes?

- Is the geographical origin of trees related to species composition or density?

4. Methods
4.1 Sampling
Sawdust samples were collected at 28 sites in three regions of Norway: Østlandet, Sørlandet, and Vestlandet (Fig 1), between 2019 and 2020. All sites were inside the natural distribution of the respective tree species (Nordén et al., 2015; Sverdrup-Thygeson et al., 2011). A total of 208 healthy looking trees of *Acer platanoides*, *Fagus sylvatica*, *Tilia cordata*, and *Quercus robur* were drilled with a 10 mm wood drill. In each of the trees, two 10 cm deep holes were drilled into the sapwood, one on the S, and one on the N side, each at 1 m above the ground and samples were pooled. In the field, trees were chosen as to represent four size classes (table 1; table 2).

Table 1: Size classes decided for the tree sampled. The largest size class for maple was excluded due to the lack of trees meeting the requirements for the size class.

Size class (CBH, cm)	
A	100–180
B	181–240
C	241–300
D	300+

Table 2: Number of samples collected from the different size classes for the different tree species.

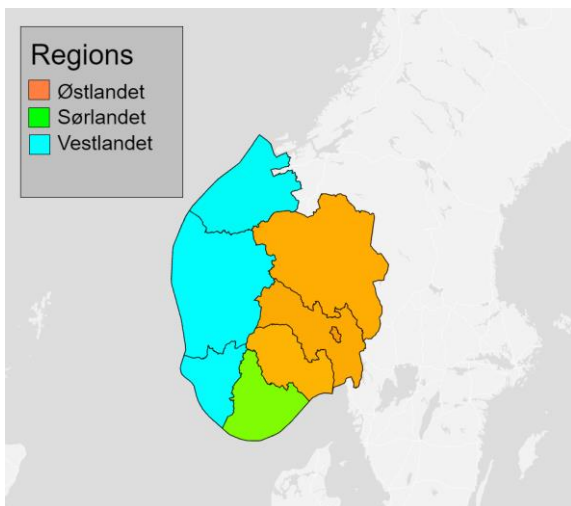| Samples/ size class within tree species					
Tree species	**A**	**B**	**C**	**D**	**Total**
Quercus robur	17	19	15	29	80
Fagus sylvatica	9	10	12	3	34
Tilia cordata	7	9	7	2	25
Acer platanoides	13	10	9	2	34

Figure 1: Map, visualising the tree regions where the trees sampled where located. Source: Natur I Norge – artsdatabanken.

4.2 DNA sampling
The method for the DNA sampling followed the methods used in Menkis et al. (2020). Sawdust from the samples was collected while drilling, resulting in separate samples for each tree. The

sawdust was collected purposely for DNA extraction. The sawdust was packed in filter paper with approximately 2 g of weight. Th packages were sealed and freeze-dried for 72 h in a VirTis SP Scientific Freeze Dryer (SP Industries Inc., Suffolk, UK.) with a temperature of – 105 ˚C. The sawdust not freeze-dried was stored at – 20 ˚C.

208 2 mL centrifugation screw cap tubes containing 0.4 g freeze-dried sawdust from the individual trees and a 3% CTAB solution were homogenised through metal bolts with a Precellys 24 homogeniser (Bertin Corp, Rockville, MD, USA). The resulting solution, after homogenisation, was then incubated at a temperature of 65 ˚C for 1.5 h and later cleaned with chloroform. The upper phase was cleansed using the Technum NucleoSpin soil kit (Macherey-Nagel, Düren, Germany), and the upper phase was cleaned following the recommendations from the company. An ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the DNA concentration were changed to 10 ng/mL resulting in an evaluation of the DNA content.

The DNA samples were amplified using a primer pair gITS7, and ITS4 to individual amplify each sample. The two primers contained sample identification barcodes and resulted in 208 barcode samples. 15 µl reactions containing an amount of 1.2 µl DNA, 1 % Taq polymerase (5 u/µl), (DreamTaq Green, Thermo Scientific, Waltham, USA), 11 % of 10 x buffer, 11% dNTPs (10mM), 1 % $MgCl_2$ (25 nM), 2 % each primer (200 mM) and 72 % Milli-Q water were used to run PCR. The reactions were put through Applied biosystems 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) and performed 35 cycles. The process steps for the amplification consisted of initial denaturation at a temperature of 95 ˚C for 2 min followed by with 30 s denaturation at 95 ˚C, 30 s annealing at 56 ˚C, continued with further annealing for 1 min at 72 ˚C which than extended to 7 more min.

The amplified samples from the PCR were placed on a 1.5 % agarose gel (Agarose D1, Conda, Madrid, Spain) with an electrophoresis scan performed for 30 min with 300 V using QuantityOne software (Piovanelli, 2006). The resulting DNA concentration from the electrophoresis was analysed/evaluated with a NanoDrop 3300 flurospectrometer (Thermo Fisher Scientific, Waltham, MA, USA). After evaluation, the 208 equimolar PCR samples were produced and cleansed using EZN.A cycle pure kit (Omega Bio-tek, Norcross, GA, USA). The quality was controlled with NanoDrop 3300 flurospectrometer and an Invitrogen QUBIT fluorometer (Fisher Scientific, Loughborough, UK). The sequencing was completed at SciLifeLab (Uppsala Genome Centre, NGI Uppsala, Sweden) using the Pacific Biosciences (PacBio RSII) platform.

The bioinformatic sequences generated were controlled and clustered in the SCATA NGS sequencing pipeline. Filtering the sequences resulted in removing sequences shorter than 200 bp, primer dimer, homopolymers, and sequences difficult to read caused by low quality. The cause of the removal was collapsing to 3 bp during sequencing. Also, sequences with missing barcodes or primers were removed. The barcodes and primers were removed but stored as meta-data to keep the information. The sequences clustered together in different taxa with single-linkage clustering as a method that is based on 98% similarity. The most commonly occurring taxa were chosen to represent the taxon.

The identification of fungi species relied on the GenBank (NCBI) database and the BLASTN algorithm. During identification, the criteria used were sequence coverage >80 %, genus-level 94-97 %, and species-level ≥ 98 %. If the criteria were not achieved, the samples were unidentified. Taxa identified as Lecanoromycetes were excluded because the majority of the species belonging to the taxa represent lichen mycobionts. Moreover, it could result from contamination from the bark during sampling. The result from the samplings and analysis should be cautiously interpreted due to further contamination with different fungi.

4.3 Analysis
4.3.1 Preparing dataset
Columns with no species names (unidentified) were excluded. The remaining columns were then arranged, and species epitomes were added to the columns with only genus names while columns with the same species were combined. Singleton species (occurring only in one samples) were removed for the test PERMANOVA, PERMDISP, and Principal component analysis (PCO) to run the analysis, as well as samples containing no fungi species. The sample with the tag Endophytes_3_tag_81_tag_81 was removed after the singletons were excluded, containing 0 species.

The data necessary for the PCO contained the individual tree samples and the different fungi species identified. Meanwhile, the general linear mixed model (GLMM) data included the data for the individual trees, the different tree species, regions, sites, habitats, and the fungi species. The same data were then used for the general linear model for the red-listed species.

3.3.2 Statistical tests
Statistical analyses for species density for both the endophytic and red-listed species were performed in RStudio v. 4.2.0, with GLMM using packages DHARMa, tidyverse and glmmTMB. For the red-listed endophytes the analyses were run with a general linear model (GLM) using function glm () in R-base and the package MASS for the confidence intervals, as the initially run GLMM had zero variance associated with the random effect. For the GLMM negative binomial distribution with log -link functions were used due to high overdispersion when using the Poisson distribution. The references levels chosen for the categorical variables were *Q. robur* for tree species and Østlandet for regions. Site were used as random factor for the GLMM. Residual plots were inspected and overdispersion tests were done using the performance R package.

The accumulation curves of the four tree species were made with EstimateS (Version 9.1.0) and drawn using ggplot2 R packages. These perform a comparison of samples with different sample sizes, using rarefaction. Accumulation curves were also extrapolated to twice the samples size through the Chao2 asymptotic species density estimator.

Primer 7 with the PERMANOVA+ addon. was used to perform a PCO on the sequenced data, using the number of sequences ran as presence/absence. Sørensen similarity index were used for the PCO to visualise the species composition and for permutational MANOVA (PERMANOVA) main test and pair-wise tests for species density between the different regions and tree species. A

distance-based test for homogeneity of multivariate dispersions (PERMDISP) was run performed to analyse beta-diversity in the four tree species.

5. Results

The environmental sequencing gave 105 000 sequences which were automatically blasted against UNITEs database and resulted in 1842 species hypothesises. After DNA extraction and amplification, a total of 2181 OTUs were detected in 366 tree samples. Excluding the data collected from the non-fungal species, a total of 1837 fungal species remained for the fungi sampled. After the quality filtering, the results from 21 trees had no high-quality sequences and were excluded, which resulted in 187 samples analysed. The trees sampled were distributed over the three regions with 145 samples from Østlandet, 25 samples from Vestlandet and 17 from Sørlandet. 11 red-listed species were detected in the samples.

5.1 Species density of endophytes

The general linear mixed model (GLMM) result for all endophyte species indicates that Norway maple *Acer platanoides,* beech *Fagus sylvatica*, and linden *Tilia cordata* all harbour significantly fewer species per sample than oak *Quercus robur* ($p < 2 \times 10^{-15}$) (table 3, fig. 2). Norway maple had 14.9 % species density compared to oak*,* linden had 14.5 %, and beech 21 % of oak. The model also indicated that the overall effect of trunk size (CBH, cm) was small and not statistically significant.

Table 3: Table of results from General linear mixed model for endophytes for tree species, regions, and size.

Species density endophytes					
Term	**Estimates**	**Std. error**	**P-value**	**CI 2.5 %**	**CI 97.5 %**
*Quercus robur**					
Fagus sylvatica	0.215	1.203	$<2 \times 10^{-15}$ ***	0.149	0.310
Tilia cordata	0.145	1.211	$<2 \times 10^{-15}$ ***	0.099	0.210
Acer platanoides	0.149	1.252	$<2 \times 10^{-15}$ ***	0.096	0.231
Østlandet*					
Sørlandet	0.876	1.328	0.64	0.484	1.509
Vestlandet	0.680	1.307	0.15	0.389	1.170
Size CBH (cm)	1.001	1.001	0.336	0.999	1.002
Random factor	**Variance**	**Std.Dev**			
Site	0.1792	0.4233			

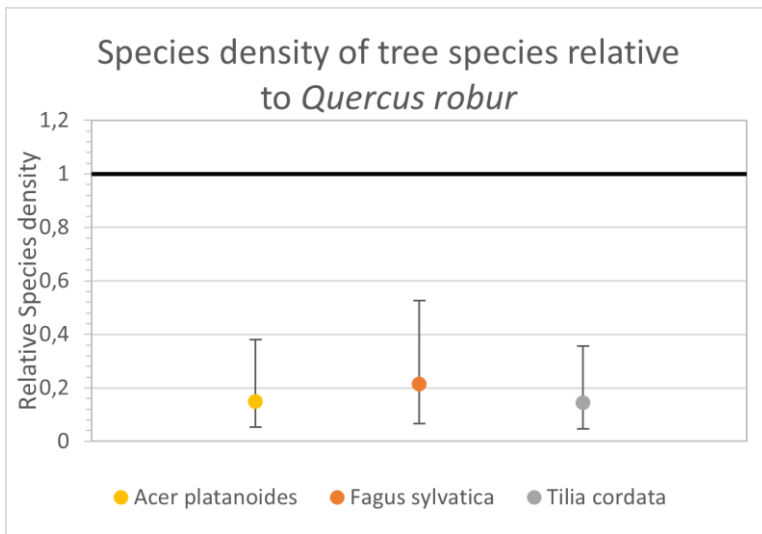**Note:** * = Reference term, *** = Significant value

Figure 2: A visualisation of the result of the GLMM for tree species, where the x-axis is located at the value 1 at the y-axis representing the reference tree species *Quercus robur*. The error bars are based on 95 % confidence intervals (table 3).

Both the regions Sørlandet and Vestlandet had a lower species density than Østlandet, where Sørlandet had about 88% of the Østlandet species density, and Vestlandet had around 68%, but the difference was not statistically significant (table 3, fig. 3).

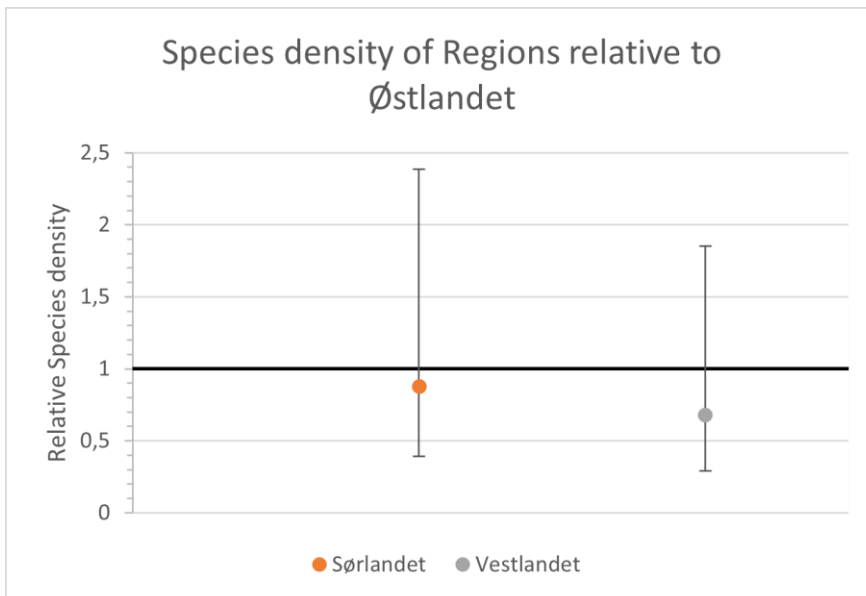**Figure 3:** Visualizing of the result from the GLMM for the regions, where the X-axis represents the reference region Østlandet located at the value 1 on the Y-axis. The error bars are based on 95 % confidence intervals (table 3).

5.1.1 Species density of red-listed species

Of the 11 red-listed species detected in the samples, the GLM run for them indicates that oak harboured almost exclusively the red-listed species (table 4).

Table 4: Result of the General linear model analysis for red-listed endophytes.

				CI	CI
Term	**Estimate**	**Std. error**	**P-value**	**2.5%**	**97.5%**
*Quercus robur**					
Fagus sylvatica	0.052	2.766	0.004	0.003	0.245
Tilia cordata	0.289	1.710	0.021	0.085	0.739
Acer platanoides	0.092	2.102	0.001	0.015	0.316
Østlandet*					
Sørlandet	0.558	1.650	0.244	0.185	1.376
Vestlandet	0.745	1.529	0.489	0.299	1.620
Size CBH (cm)	0.997	1.002	0.086	0.993	1.000

Note: * = Reference term

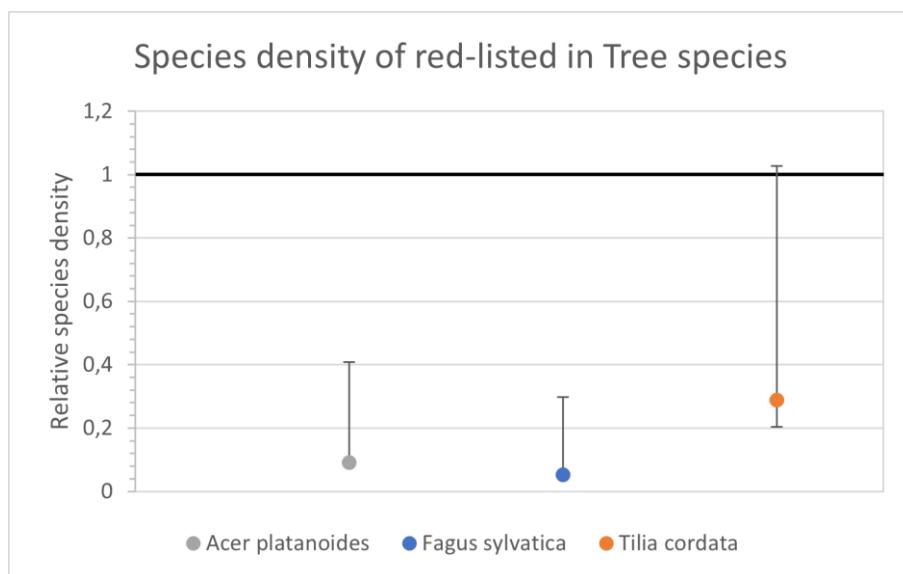**Figure 4:** Visualization of the species density between the tree species for the red-listed species identified and analysed with GLM. Only *T. cordata* has confidence intervals crossing the x-axis at 1 on the y-axis representing the reference tree species *Q. robur*, indicating that the lindens species density is not significantly lower than *Q. robur*. The error bars are based on 95 % confidence intervals (table 4).
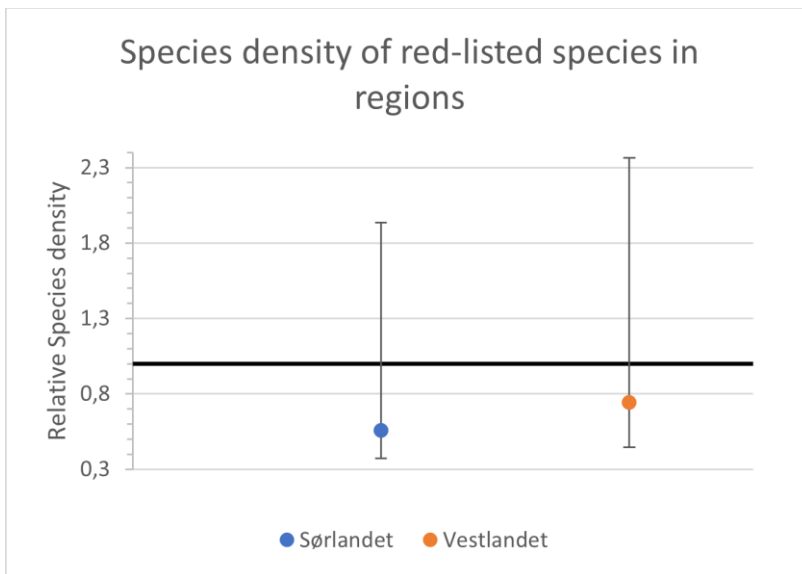
Figure 5: Visualization of the result of the GLM for the red-listed species from the sampled data for regions, with both areas having a lower species density but not significantly lower than the reference region Østlandet located as the highlighted x-axis at the value 1 on the y-axis. The error bars are based on 95 % confidence intervals (table 4).

The species density between the tree species indicates that beech and Norway maple had very low species density compared to oak with 5.2 % respectively 9.2 %. linden seems to have 28.9 % of the species density of oak. The density for Norway maple (p=0.001), linden (p=0.021) and beech (p= 0.004) are shown to be significantly lower than oak (table 4, fig.4).

Both Sørlandet and Vestlandet had a lower species density than Østlandet (Sørlandet 55 % of the species density of red-listed species compared to Østlandet, Vestlandet 74 %, table 4, fig. 5). However, the difference in species density was not significant. The trunk sizes didn´t have a significant effect on red-listed endophytic fungal species density.

5.1.2 Species accumulation curves

The species accumulation curves (fig. 6) show that oak is separate from the remaining three species, with a much higher number of species, controlling for an increased sample size. Norway maple, beech, and linden are all similar and show an overlap indicating a similar total number of endophytic species. Norway maple, beech, and linden seem to have reached close to their total number of species in the sample, indicated by their curves flattening, while the accumulation curve for oak is still rising, indicating that there is still a substantial number of unsampled species present in the species pool.
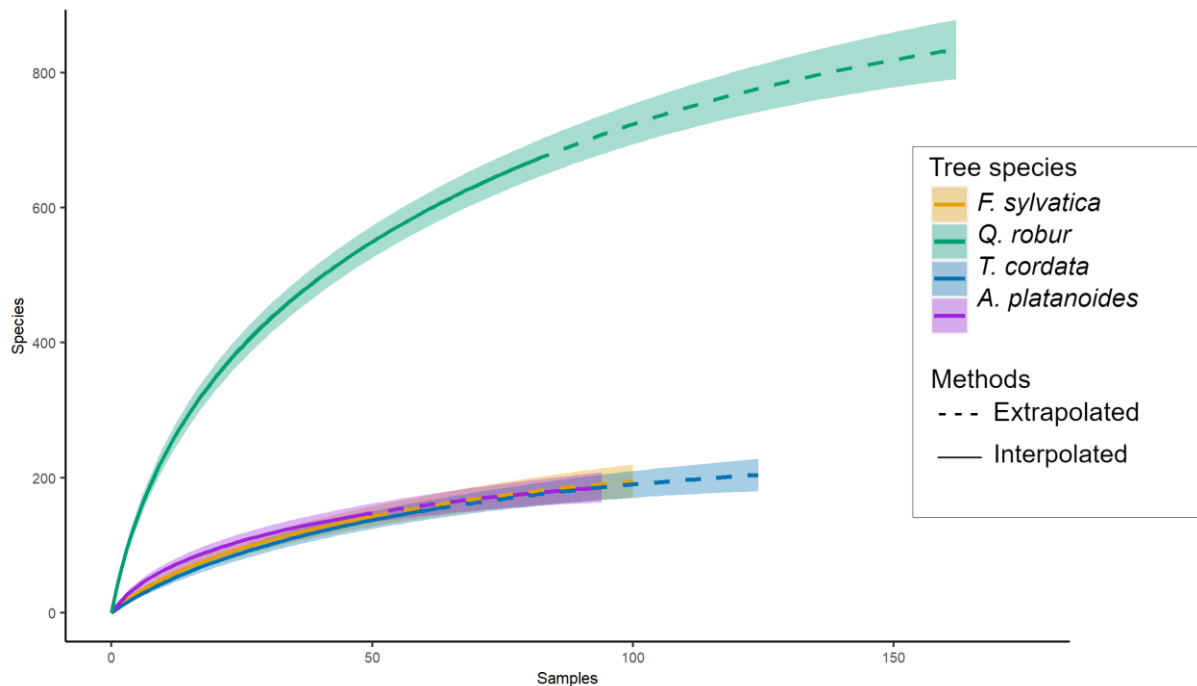
Figure 6: Accumulation curves for the *Q. robur*, *A. platanoides*, *F. sylvatica*, and *T. cordata* demonstrating the total number of endophyte species with both rarefaction (interpolation) and the extrapolation to twice the samples size. The error bars are based on 95 % confidence intervals.

5.3 Species composition of endophytes

271 singletons were identified out of 750 species. oak had a substantially higher proportions of singleton species than the other tree species (32%, see table 5).

Table 5: Singletons identified in the four tree species, *F. sylvatica*, *T. cordata*, *A. platanoides*, and *Q. robur*.

Singletons among the tree species			
Tree species	**Total species**	**Singletons**	**Percentage singletons**
Fagus sylvatica	143	16	11%
Tilia cordata	154	13	8%
Acer platanoides	144	25	17%
Quercus robur	670	217	32%
Total	750	271	36%

The principal coordinates analysis (PCO) showed that oak was clearly separated in terms of species composition, while the three other tree species were largely overlapping (fig. 7). However, a relatively low amount of the sample variation is explained in the PCO; axis 1, 8.5% and axis 2, 6.2 %, respectively. The PERMANOVA test supported the PCO visualisation, demonstrating that there is a significant effect of the tree species on endophytes species composition (Sums of squares = 25 756, pseudo-F (3) = 2.26, p =0.0001).

There was also a lower but significant effect of trunk size on species composition (SS=12 963, pseudo-F (3) = 1.3, p= 0.0143). The PERMANOVA also showed a significant interaction

between tree species and the size of the trees (SS = 35911, pseudo-F (9) = 1.2, P= 0.005), indicating that the effect of tree species on endophyte species composition is dependent on trunk size and vice versa.
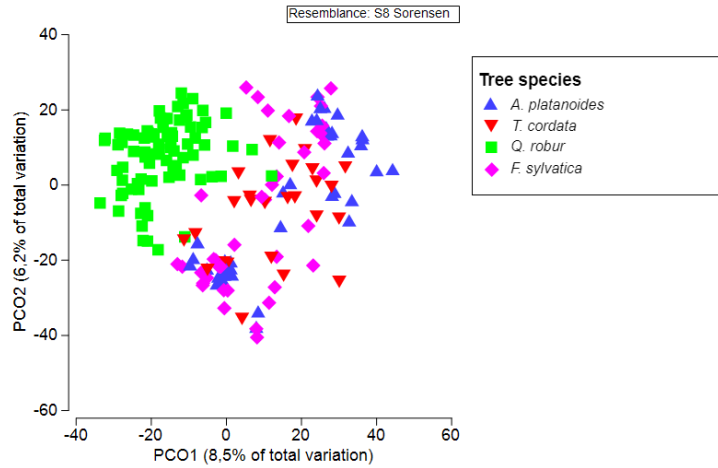

Figure 7: PCO visualising the relationship between the four tree species *A. platanoides*, *T. cordata*, *Q. robur*, and *F. sylvatica* in terms of fungal species composition. Axis 1 represents 8.5% of the total variation, and axis 2 represents 6.2 % of the total variation of the total samples. Each point represents a tree sample.

Species composition between the three regions seem to overlap in a higher degree compared to the tree species. The region Østlandet do indicate to be slightly more separate from the two other regions, which seem to have a more similar species composition. The species found at the region Østlandet do seem to interrogate the other regions in a higher degree compared to the other, however Sørlandet shown to have the largest number of species identified from the two other regions compared to Vestlandet and Østlandet which seem to have more species composition less intertwined with the other two. Although, the variation presented in the ordination diagram is low with only 8.5 % of the total sample represented on the axis 1 and 6.2% represented on the axis 2 (fig 8).
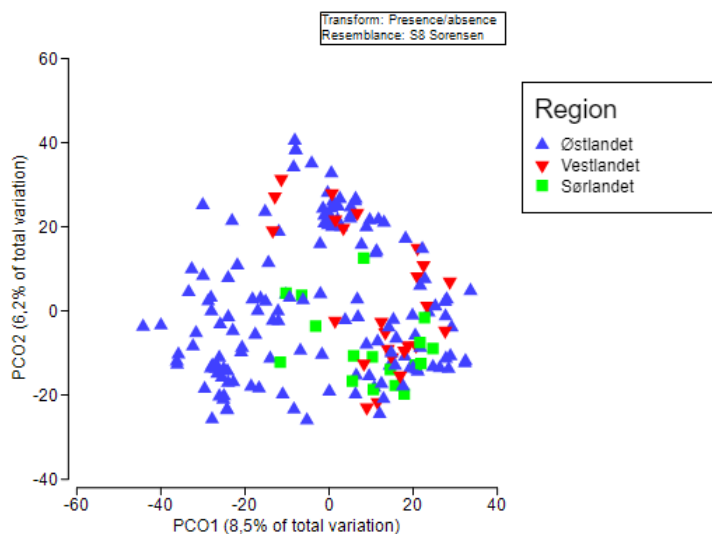

Figure 8: Visualisation of the species composition for regions in an ordination diagram. The variation of the total sample on axis 2, 6.2 % and 8.5 % on the axis 1. Each point represents a tree sample.

Table 6: Result for species composition for the tree species within the different size classes. The interaction where the analysis couldn't be run, due to lack of samples isn´t shown.

PERMANOVA pair-wise test
Tree species within size class

Size class A		Size class B	
Groups	**P(perm)**	**Groups**	**P(perm)**
oak, maple	0.394	oak, maple	0.106
oak, beech	0.185	oak, beech	0.127
oak, linden	0.059	oak, linden	0.019
maple, beech	0.085	maple, linden	0.149
maple, linden	0.569		
Size class C		**Size class D**	
Groups	**P(perm)**	**Groups**	**P(perm)**
oak, maple	0.051	oak, beech	0.005
oak, beech	0.045	oak, linden	0.039
		beech, linden	0.547

Table 7: Result from species composition for the size classes within the different tree species. The interactions where the analysis couldn't be performed, due to lack of samples isn´t shown.

PERMANOVA pair-wise test
Size class within tree species

Quercus robur		*Fagus sylvatica*	
Groups	**P(perm)**	**Groups**	**P(perm)**
A, B	0. 441	A, B	0.321
A, D	0.622	A, D	0.519
A, C	0.795	A, C	0.348
B, D	0.087	B, D	0.502
B, C	0.311	B, C	0.488
D, C	0.273	D, C	0.846
Acer platanoides		*Tilia cordata*	
Groups	**P(perm)**	**Groups**	**P(perm)**
A, B	0.022	A, B	0.734
A, C	0.163	A, D	0.059
B, D	0.571	B, C	0.243
B, C	0.248		
D, C	0.367		

PERMDISP was run for the four tree species for a total number of 185 samples demonstrating that the average multivariate dispersion (beta-diversity) decreases with larger size classes indicating that the species composition becomes more similar among the tree species when the tree sizes grow. Although the result only differs significantly for the largest class (D) compared to the other classes, class D has the significantly lowest beta-diversity of the four size classes.

PERMADISP also shows that oak differs from the different tree species with the lowest beta-diversity but only significantly lower than *T. cordata* (p=0.008) and *F. sylvatica* (p=0.001). Note however that for this analysis, singletons were removed.

6. Discussion
6.1 Species composition and species density
6.1.1 Tree species and trunk diameter
The result demonstrated that the species composition of endophytic fungi in oak was almost totally separated from the other tree species. In contrast, living trees of beech, linden, and Norway maple all harboured relatively similar fungal communities (fig. 7). Seemingly harbouring no or only a few host-specific fungi.

The very distinct funga of oak was surprising considering earlier results where species usually located in other trees also were detected in oak, and only a few host-specific species were collected (Kowalski & Kehr, 1992). Although oak harboured multiple fungi species and several were detected in deadwood (Kowalski & Kehr, 1992). It is worth noting that the results of the PERMDISP however indicate that oak has a lower beta-diversity than the other tree species, indicating oak trees' species composition is more similar to each other, especially compared to linden and beech. However as previously mentioned, all singleton species were excluded from the analysis of species composition. Out of 271 fungal singleton species, most of the singletons were identified in oak (table 5), showing that oaks diversity contains many rare species, only identified in one sample. Accounting for these singletons, the beta diversity for oaks would undoubtedly be higher.

The result indicates that oak has a favourable wood composition, preferred by multiply fungal species. Moricca & Ragazzi (2007) discuss that woody plants, especially trees, have a longer life cycle than other hosting plants and could differentiate in wood tissue with increased tree age. With the result indicating that species composition is significantly affected by tree size (table 5), but to a lower degree than the different tree species on the species composition, perhaps the wood composition differentiates with trunk size as well. Also, the interactions between the tree species and the size classes are dependent on each other. The interactions for the size classes had a substantial impact on the two smallest trunk sizes, A and B, but only for maple. Meanwhile, oak deviated from the other tree species for the higher size classes of the tree species. Possibly the effect of the changes in trunk size impact the wood composition similar as to Moricca & Raggazi (2007) mentions for the long lifespans of trees which could imply that the number of species supposedly increases to a more preferred wood composition.

Gange et al. (2010) mention that the fungi living in the woody parts of the trees choose their host both dependent on external factors, influencing the distribution of suitable hosts as previously discussed and based on what the individual flora offers in tissue composition and chemistry. A possible problem with the oak being significantly separated from the other tree species and with minimal overlapping of the communities could be that if the oak populations begin to decrease or disappear. Possibly as Abrego et al. (2017) mention, due to human disturbance or Petersson et al. (2019) proposes, increased herbivory could negatively affect the endophyte communities. It could mean that endophytic fungal species exclusively inhabiting oak trees could be severely reduced

eventually when their habitat and preferred host disappear, with connectivity issues and fragmentation possibly increasing as a cause of continued human disturbance (Abrego et al., 2017).

The significantly higher species density for oak compared to the other tree species is not surprising. With species accumulation curves also demonstrating oak trees harbouring a much higher number of species and indicate that the total endophytic fungal species isn´t reached (fig. 6; fig. 2). Petersson et al. (2019) mention that the amount of dead wood is high for oak. Kowalski & Kehr (1992) show that the fungi occurred mainly in dead bark for the tree species, compared to living branches with very little colonisation. Several different fungal species occurred in the dead bark of the trees.

As previously mentioned, a substantial portion of the endophytic species in oak are potential rare species which could be classified as specialists (table 5), implying that oak is their preferred habitat. Furthermore, oak harbouring the most substantial number of fungal species. It is interesting that the specialists benefit from continuously mixed forests and occur at higher frequencies than in the managed forest with trees of the same tree size and species (Saikkonen, 2007). The presumably longer lifespan for oak trees, could also be a cause of why the oak had the highest species density among the tree species as Moricca & Raggazi (2007) discusses. Oak had the highest abundance of sampled trees distributed over the four classes (table 2). The different fungal density presumably results from the species-habitat relationship, implying that the abundance of endophytic fungal species follows the abundance of their hosting trees and a possible reason to oak differentiating from the other three tree species sampled (Stokland et al., 2012). The high diversity of fungal species indicates that oak trees are important habitats and have been mentioned as foundation species due to their presumably long lifetimes and a high abundance of deadwood (Petersson et al., 2019). Although, the number of rare species identified could perhaps concern the survival of the species if the oak trees continue to struggle with regeneration problems and decrease in density. Stokland et al. (2012) argue that specialist species are more affected by human disturbance and habitat changes, as for fragmentation. The species-habitat relationship between fungal species and oak trees may be an imminent factor in the interactions. Where oak trees' ongoing struggle contributes to decreasing important habitats could lead to decreasing or extinction of several fungal species in advance due to habitat loss (Stokland et al., 2012).

Perhaps other tree species are unable to replace the oak population in these areas and mirror the habitats which endophytic fungi prefer. Petersson et al. (2019) discuss that oak regeneration is difficult, referring to several ongoing environmental changes, where the abundance of herbivory has increased, especially on younger trees. This could become a problem for the endophytes harboured in oak trees and could be critical for the supposedly rare species. Saikkonen (2007) discusses that the endophytic specialists are affected by different environments, including succession patterns and management methods. Implying that the managed forest suffers in species composition and density compared to the mixed forests. With the significantly higher species density and different composition of oak, the sites where oak is located might be a priority to protect because, as Lindbladh & Foster (2010) refers to oak as essential habitats for fungi. Stokland et al. (2012) discuss that the abundance of deadwood has proven to be higher in

natural forests. Oak presumably containing a high amount of deadwood could be a reason for seemingly being the preferred tree species among the four sampled.

Interestingly, Errasti et al. (2010) continue the discussion of the importance of the environmental impact on the endophytes. As mentioned earlier, Petersson et al. (2019) refer to herbivory as one reasons for the difficulties of regeneration of oak trees. Which could disfavour the endophytes' way of living due to possibly wounding the host and inducing the endophytic fungi to act harmful instead of living in symbiosis. With an increased risk of wounding the host, the density of endophytic fungi harboured might negatively affect the host. The risk for several endophytes to show saprophytic tendencies would increase. The significantly higher species density in oak trees could become a concern. Whether the endophytes benefit or harm the harbouring plants could affect the forest dynamic, structure, and diversity. That the abundance of species differentiates significantly among the different tree species does not necessarily mean that a larger part of the wood tissue in the hosting plant differs. The tree species could have a similar number of endophytic fungi living inside them. Oak does have multiple different species meanwhile, the other trees could have just as many species but of the same type, inhibiting the same size of tissue.

6.1.1.2 Wood-decay endophytes and human disturbance

As Cline et al. (2017) mentioned, the wood-decay fungi are essential for the carbon cycle, and Gilmartin et al. (2022) continue the argument by discussing the importance of wood decomposition endophytes in the standing and living trees without causing any wounding of the bark. The endophytic fungi's capacity to decompose wood is a vital component of the species density and the well-being of the forests, but also for the carbon cycle (Cline et al., 2017). Therefore, the essential component for the fungi communities when choosing a host is the tree species and size of the deadwood for the wood-decay species. Gilmartin et al. (2022) present several wood-decay fungi species: taxon *Fusarium*, *Penicillium*, *Xylaria*, and Phallaceae. This could be a possible reason for the significant species density in oak. Furthermore, the dead wood on standing living trees is considered high, attracting multiple endophytic fungi to inhabit oak trees.

The endophytes harboured in the woody parts of the flora are affected by different forest management. The managed forests negatively impact the fungal communities and contribute to fragmentation and decreased abundance of deadwood due to thinning and clear-cutting methods used in the process (Abrego et al., 2017). It is important to mention that human impact on the habitat does benefit some endophytes. However, human disturbance is not solely negative, resulting in the different species often classified as wood-decay endophytes do not respond the same to environmental changes and external factors. There is no general response, either negative or positive, from endophytes when facing changes in their substrates. The effect of variation of connectivity also influences the welfare of the endophytes (Abrego et al., 2017), showing that the fungal species could be affected by different factors. Although multiple factors could impact the abundance of endophytes, further visualising the complexity of the fungal communities and the external factors entangled (Abrego et al., 2017). Abrego et al. (2017) also discuss the wood decomposition fungi and the rate the wood decays affect the species density of endophytic fungi.

Still, the relations are complicated and dependent on external factors. The discussion continues with the amount of, and relevance of the resources available in the different habitats for the

endophytic fungi varies between different sites. The result showing that oak trees harbouring the highest amount of endophytic fungal species and multiply different species could be a concern with oaks continues problems with for example regeneration, where oak trees as previously mentioned contain large amount of deadwood and possibly harbouring several wood-decay species (Petersson et al. 2019).

6.1.2 Regions

The species composition for the three regions are similar to each other compared to the species composition for the tree species. Implying that the geographic origin and the distance between the three regions do not influence the species composition of the endophytic communities to the same degree as tree species does. This result is surprising because Kowalski & Kehr (1992) mention that the species composition is dependent on the locations. The species composition seemingly varies the most for Østlandet, were fungal species identified in the region also occur in the other regions at a higher rate than the other regions (fig. 8). In Gilmartin et al. (2022) study, the fungi communities did differ between different sites for all trees endophytes identified. Gilmartin et al. (2022) continue to discuss that the differentiation between the sites did depend on the geographical distances among the sites, to a higher degree than the climate influence different locations. It may imply that the distances between the three regions are not far enough to significantly impact the species composition.

The species density did not differ significantly for the different regions, but both Sørlandet and Vestlandet had a lower species density than Østlandet. Both Sørlandet and Vestlandet have over 50 % of the species density of Østlandet. Sørlandet and Østlandet seem to be closer, with Sørlandet having a higher percentage of the species density of Østlandet than Vestlandet (table 3). The three regions did not differ in geographical origin. The endophytic fungi are possibly sheltered from external factors, such as climate changes, by living inside the trees. However, it is possible that the distance between the three regions was not significant far to affect the species density, which was previously mentioned as a possible cause for the statistically non-significant difference in species composition. Also, the distribution over the tree regions isn´t evenly distributed, with Østlandet having a noticeable larger sample size compared to the other regions. Furthermore, the different tree species occurrence varies. Norway maple is mainly distributed in southern Norway, commonly occurring in Østlandet and Sørlandet, while Norway maple is only sparsely located in Vestlandet. Both linden and beech occur in Norway, but beech has a relatively small population, linden has mainly been located in the southern areas. Oak are the most common oak species detected in Norway and has been observed along the coastal areas in all three south regions (Nordén et al., 2015; Sverdrup-Thygeson et al., 2011). The different distributions could influence the species density for the three regions, due to the tree species occurrence.

Plants are described as being able to adapt to different environments and hosts. For example, applying the symbiotic interactions where the endophytes manage drought or pH to adapt to the environment (Rodriguez et al., 2008; Vandenkoornhuyse et al., 2015). Abrego et al. (2017) mention that the fungi harbouring in woody plants also get affected by the abundance of deadwood in the area and the locations climate, the landscape, and the status of the forests (managed or natural). Following the discussion, Abrego et al. (2017) also mentions that some fungi species are seemingly present in specific geographical regions. Possibly further research could investigate several areas in perhaps Norway, including the northern regions, to see if the

species density differs significantly between the two northern regions compared to the southern, which did not show any significant differences among them.

6.1.3 Red-listed species

Out of the species identified from the samples, only 11 endophytic species were classified on the red list (SLU SSIC, 2020). In general, the number of red-listed species identified was not substantially high. The species density for the red-listed species demonstrates that oak harbouring almost exclusively the red-listed species, both beech and Norway maple only having a few percentages of the species density of oak. Linden did have approximately a fifth of oak (table 4). As previously discussed, oak hosts the largest number of singleton species among the four tree species. This implies that oak could be the preferred tree species and habitat for the rare endophytic fungal species and specialists (Saikkonen, 2007), which could increase the need to protect oak in all regions to prevent rare species from disappearing. Also, for the red-listed species, the regions did not significantly differ in species density between the regions (table 4, fig. 5).

6.2 Improvement and future studies

To improve the knowledge of the species composition, the sampling of trees should be more evenly distributed over the different size classes. It could significantly affect the result of the PERMANOVA pair-wise test, where oak was the only tree species that differed significantly in species composition from the other tree species for the larger size classes. As previously discussed, there was a lack of samples for the larger size classes for the different tree species, especially maple, and the test was not able to run for the size classes C and D. The distribution of the tree samples over the three regions is very uneven, for future research a more even distribution should be in consideration.

Oak differs significantly compared to the other tree species in both species' density and composition. Further research questions appear: Are the oak trees sampled older than the trees for the other tree species, implying that the endophytes prefer the older trees as hosts? Furthermore, could the woody tissue in the oak tree be a vital factor in the endophytic fungi choice of a host? As Abrego et al. (2017) previously pointed out, it seems necessary, especially for the wood decomposition fungi. Species density might be higher in sites with a higher quality of the resources important for the fungal species. Petersson et al. (2019) mention that endophytic fungi presumably preferred oaks' long lifespans for trees which could be a contributing factor. Further research might be to collect tree samples, investigating if the four tree species do differ in age and if it could possibly impact the endophytic species density.

The DNA sequences were automatically BLASTed against UNITE and conjured up the endophytic fungal species, which should be considered when analysing the dataset because there is a possibility that when the species sequences are BLASTed against UNITE or GenBank, it could result in different species matching the sequences than the automatically BLASTed did.

The result showing the most occurring fungal species in the dataset is uncertain. Also, several species conjured up during the automatically BLASTed were classified as lichens, which could result from contamination during sampling. While sampling, there is a chance of contamination while collecting data from inside the trees. There are several possible ways contamination can occur, and the different methods of isolating the samples could be difficult and risky (Sieber, 2007). Gilmartin et al. (2022) point out that part of the fungal structure could be lost, resulting in

an incomplete sampling and inability to identify. As previously mentioned by Sieber (2007), detecting, and identifying endophytic fungi is difficult. It is very dependent on which stage in the fungal life cycle they are at.

7. Conclusions

The species composition and density differ between the four tree species. Endophytic fungi seemingly preferring oak as host, having the highest species density. Oak also harbours mainly different fungi communities compared to the other tree species, indicating that oak has a very distinct funga with a significantly higher abundance of species. With oak almost entirely harbouring the red-listed species and large portions of singleton species, it demonstrates that the oak is an important host for many rare species as well.

The endophytic fungal communities did not change with different regions, indicating that habitat is the primarily factor when choosing a host. The distance between the regions was not enough to cause differences in the species composition. Although, trunk size does have a small effect on the species composition, which shows oak statistically significantly different from the other tree species, especially for the larger size classes. The results emphasise the importance of oak trees for the endophytic communities for both species composition and density. The oaks give the species a dependable host for the endophytic fungi compared to other tree species. Including the analysis for the rare and red-listed species strengthen the argument of protecting oak trees to prevent endophytic fungal species from decreasing in abundance.

8. Acknowledgment

9. References

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Appendix 1: Endophytes on a search for the perfect host.

What are endophytes?

Endophytes are fungi that live inside plants. The endophytes have been found in different kinds of plants, from grasses to the largest trees. The fungi usually live together with their host without harming the plant. The endophytes have lived inside plants since life became terrestrial but the knowledge about these fungi is scarce, with the difficulties of finding them as one of the causes.

How do endophytic fungi impact conservation biology?

Endophytes can benefit their host in various ways by sharing their abilities to adapt to changes in the environments their hosts. For example, the endophytes have been shown to help their host plants deal with drought and different tree diseases, by occupying the wood tissue inside the trees, creating a kind of shield. Endophytes also have a vital role in the carbon cycle with wood-decaying fungi decomposing deadwood in the environment they live.

The relationship between the fungi and the trees harbouring them play an important role. Endophytes are seemingly sensitive to human disturbance, and managed forests contain fewer endophytes compared to natural forests. And with their hostplants negatively influenced by for example fragmentation the endophytes might also get affected poorly.

Which is the preferred host tree?

The different regions didn´t seem to affect the endophytes' choice of host drastically, implying that the tree species present in the area is more important/relevant for the fungi. Oak trees were shown to be the tree species offering the best abilities and environments for the endophytes to live in. Oaks harbour many different species inside them, and several rare and red-listed species, compared to the other tree species. The endophytes seem to prefer oak trees, which show the importance of them for the endophytes., displaying the importance of oaks when trying to prevent species from decreasing or disappearing. To protect the endophytic species, the host plants need to be protected too.

Conservations actions to protect the trees and the endophytes.

The trees harbouring the endophytes, especially the ones hosting the rare and red-listed species should be protected from human disturbance such as fragmentation and monocultural forestry with same tree size and tree species to be able to create a successful and stable environment for endophytes, containing a large number of the preferred tree species and trees in different sizes. Continuing to study different factors as to why endophytes choose their hosts will contribute to increased knowledge about the relations between the endophytes and the trees and highlighting the importance of the symbiosis between the plant and the fungi for the wellbeing of both participants.