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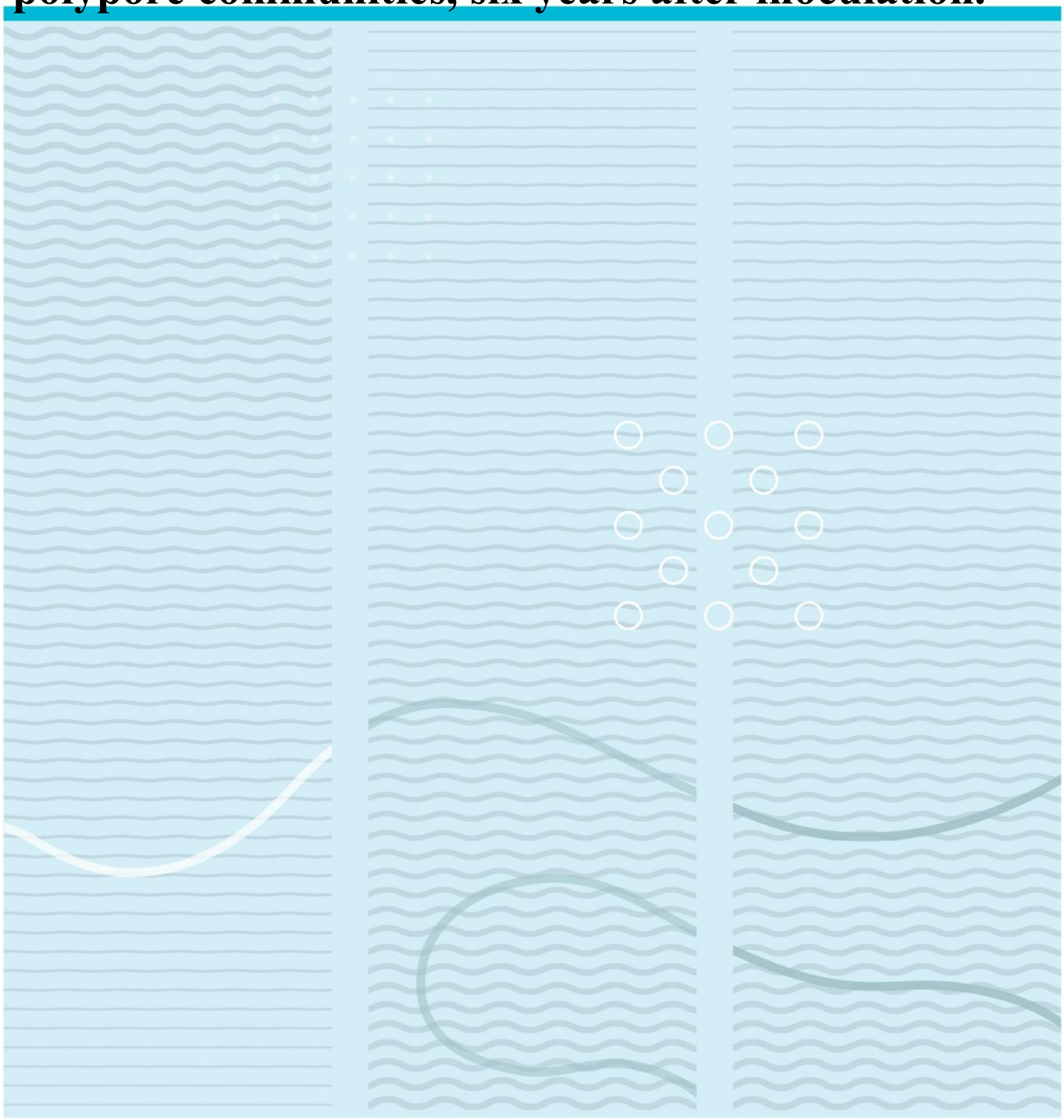
Using metabarcoding to study the differences in polypore communities, six years after inoculation.



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This thesis is worth 60 study points.

Abstract

The forests of Fennoscandia are essential habitats for polypores and wood-decaying fungi, but many rare polypores are experiencing declines due to habitat loss and fragmentation. DNA methods are enhancing our understanding of polypore ecology, providing valuable insights into their functional dynamics. This knowledge can greatly aid in polypore species conservation. In this study, polypores were monitored following the inoculation of *Fomitopsis rosea*, *Phellopilus nigrolimitatus*, and *Amylocystis lapponica*, carried out by Sundy Maurice in 2016. The 15 deadwood logs used for inoculation were situated in three Norway spruce (*Picea abies*) forests with different management histories: managed, semi-natural, and natural. This thesis studied the differences in polypore communities in these forests following inoculation, evaluated the effectiveness of metabarcoding in detecting rare polypore species, and explored the potential impacts of inoculation on community dynamics. The research was conducted with metabarcoding methods using tagged PCR techniques. A difference in community composition was observed between the managed forest and the other two sites, mainly due to the decay stages of the logs recorded in 2016. The semi-natural forest had the highest occurrence of red-listed species. The significance of the inoculations could not be tested due to the limited observations of the inoculated species, but potential interactions were seen in one deadwood log. There are few studies of this kind, and there is limited research on community assembly following inoculation. This study demonstrates the efficiency of metabarcoding in detecting community differences and rare species even when using a relatively small sample size. These findings emphasize the potential of metabarcoding as a powerful tool for ecological research and monitoring.

Contents

Foreword

I would like to give a special thanks to Sundy Maurice for letting me use her data from the inoculation project. I would also like to thank my supervisor Jørn Henrik Sønstebø, for his guidance and support throughout my master thesis. I am especially grateful for all the assistance in facilitating the logistics of my fieldwork. I was genuinely relieved and happy to say that we made it home safely from our field trips. I would like to express my appreciation for the support and expertise provided in bioinformatics and statistical analyses using Rstudio. The assistance greatly helped in obtaining and interpreting the results proficiently.

I would also like to acknowledge the valuable contributions of The Metabarcoding group meetings, which provided insightful academic discussions and information that influenced our research and enhanced our technical understanding. Thank you to Paulina Ropiak for helping with metabarcoding intricacies, and her collaboration to obtain the final samples during my fieldwork.

I would also like to express my appreciation to my supervisor and Sofie Geck for their guidance and support in using the lab equipment and setting up the Genestudio S5 platform for sequencing. Additionally, I am grateful to Melanie Schreier for her assistance with the qPCR procedures, which contributed to the reliability of my results.

Lastly, I would like to thank Erlend who always had my back during this whole journey and helped me keep my spirits lifted. For always having discussions about polypores whenever, and wherever. I appreciate your continuous support as my own personal academic writing center and English dictionary. I honestly believe that I wouldn't have been able to do this without your help. I’m glad we made it.

14.05.2023
Sofie Hagen

1 Introduction

1.1 Importance of wood decay fungi forest ecosystems:

Forest ecosystems host around 80% of all of the worlds terrestrial species (FAO, 2020), truly demonstrating the importance of forest ecosystems for terrestrial biodiversity. In Europe a lot of the forest cover is gone, and much of the remaining forests have undergone some degree of degradation due to human interference (Watson et al., 2018). Old growth forests provide a continuous environment that supports species with slow growth rates, relying on large deadwood in late successional stages (Esseen et al., 1992). There is currently no concrete measure of how much old growth forest is left in Europe, even though efforts have been made to generate an overview using new mapping techniques (Sabatini et al., 2021). Nevertheless, studies from Fennoscandia might indicate the current state of old growth forests in northern Europe. In Sweden, the old growth forests are quickly disappearing, and without any action Ahlström et al. (2022) estimated that most of it might be gone within 50 years and in Finland areas with old growth forest have gradually decreased (Kuuluvainen and Gauthier, 2018).

1.2 Differences between managed and natural forests:

The boreal forests of Fennoscandia contain the largest old growth forests in Europe. However, these old growth forests make up only a few percent of the total forest area in the southern parts of Fennoscandia (Junninen and Komonen, 2011), and most of the forests have some kind of recent management history. The main differences between intensely managed and unmanaged or less managed forests are that they often differ in species composition due to the different stand characteristics (Hottola et al., 2009).

How the forest is affected by forest management depends on the intensity and scale, but a common trait in managed forests is stand simplification (Penttilä, 2004). What is meant by stand simplification could be an establishment of a monoculture in tree species, a reduction in number and sizes of deadwood logs, and the diversity of trees in different decay stages. Furthermore, managed forests often lack large-diameter trees in varying decay stages, which leads to a reduction in the abundance of specialized species that depend on these habitats (Hottola et al., 2009). These species may be polypores, cortcioids, and other fungi, in addition to saproxylic beetles and other groups such as birds, amphibians, reptiles, mammals and plants (Birkemoe et al., 2018, Fred and Isabelle, 2010).

In contrast, the characteristics of more natural forests which usually have a higher level of stand heterogeneity, which can be seen in a variability of characteristics (tree species, size and decay stages) (Runnel et al., 2021). Additionally, natural forests often have an overall higher availability of CWD (coarse woody debris) compared to managed forests (Junninen and Komonen, 2011). These higher amounts of CWD create habitats of higher quality for populations of certain rare polypore species (Nordén et al., 2013), thus high amounts of CWD causes higher diversity. After a major human disturbance, it generally takes 200-300 years for an area to achieve optimal diversity of plants, animals, and fungi (Rayner, 1988). Yet, in managed Norway spruce forests, this desired state is often not reached as the forests are harvested prematurely at 60-80 years, preventing natural succession from fully unfolding.

1.3 Polypore threats and conservation:

Polypores are wood inhabiting fungi in the Basidiomycete phylum. Their main function in the biosphere is to decompose the complex structure of the dead wood (e.g., cellulose, hemicellulose, and lignin) and release the nutrients back to the soil. This makes polypores essential for recycling of nutrients and productivity in the forest ecosystem. Many polypore species in Fennoscandia use coniferous deadwood trees as their habitat, which are most abundant in the boreal forests. Historically, deadwood has been cleared from forests as a preventative measure against pests and disease, these measures in addition to intensive management after the industrial revolution might have reduced the amount of CWD in Fennoscandia by as much as 90-98% (Siitonen, 2001, Esseen et al., 1992).

The practice of clear-cutting and subsequent planting of monocultures became widespread in Fennoscandia around the 1940's, while selective logging was more prevalent previously (Esseen et al., 1992). Clearcutting, thinning and shelterwood logging are still practiced in Fennoscandia, and has led to a reduction of fungal biodiversity (Tomao et al., 2020). These logging practices have created large scale disturbances that have removed, fragmented, or isolated populations of polypores (Esseen et al., 1992, Määttänen et al., 2022). Since the mid-1990s there has been a shifting attitude towards the importance of deadwood in forest ecosystems, and the introduction of deadwood retention and retention trees has increased the volume of CWD both in Norway and Finland (Jonsson et al., 2016, Storaunet, 2015, Mäntyranta, 2019a, Gustafsson et al., 2010). However, at the present moment almost all of the productive forests in Fennoscandia are now under intensive management (Norberg et al., 2019). The historical and present management have led to many polypores being on the red

lists. Both in Finland and Sweden around 40% of polypores are threatened, and in Norway it is around 49% (based on 250 polypore species) (Hottola and Siitonen, 2008, ArtDatabanken, 2015, Brandrud, 2021, Hofton, 2011, Storaunet, 2015).

A considerable proportion of these polypores are classified as red-listed species due to their extended reproductive and dispersal timeframes. Rare polypores often have spore dispersal limitations and need large deadwood elements that are connected within a certain spatial configuration to colonize new substrates (Hottola and Siitonen, 2008, Ranius et al., 2019, Nordén et al., 2013). It has been suggested that a CWD volume of 20-50 m_3 ha^{-1} is needed to provide enough suitable substrates for the occurrence of red listed polypores (Nordén et al., 2020). To date, Norway's productive forest (11.1 m_3 ha^{-1}) and Finland's managed forests (2 to 10 m_3 ha^{-1}) do not uphold these qualities for deadwood volume (Storaunet, 2021, Siitonen, 2001). If a forest does not meet these needs, the populations of rare species within it risk further reduction. Thus, a lot remains to be done to meet the conservation requirements of red listed polypores in the current state of Fennoscandia's forests.

The study of rare polypore populations is complicated, since they can enter trees or logs in early successional stages, but only be visible to the naked eye during the time of fruitbody formation (Lõhmus, 2009). Some species of polypore also produce short-lived and annual fruitbodies, meaning that the timing of the survey is important. The merging of two compatible mycelia is needed to make a fruitbody, meaning that fruitbodies cannot always tell you the true content of each deadwood log (Abrego et al., 2016). This is why experimental studies on rare polypore populations can be useful to learn more about their establishment which can later be used for detection and conservation work. One type of experimental study design which might be useful in this regard is inoculation, where polypore mycelia is inoculated into deadwood.

1.4 Inoculation and aim of thesis:

Inoculation is the method of implanting polypore mycelia into deadwood by using dowels that are placed into holes in the log or stump of a fallen tree. Following a successful inoculation, the implanted mycelia will expand into the log, causing a colonization and establishment of the species, and hopefully resulting in the growth of a fertile fruiting body. This tool is not meant as a final solution to the issue of solving fungal biodiversity loss. However, inoculation

could become a vital supplementary tool to support emergence of threatened or isolated polypore populations.

Testing inoculation in the field is important, as deadwood is affected by many different biotic and abiotic conditions, which might determine the success of establishment. The terrain, precipitation, age composition of trees, visibility through the canopy, and tree species composition set the microclimatic conditions. These conditions affect temperature, moisture, pH, aeriation, and determine the resource quality, and habitat selection of polypore species (Rayner, 1988). Conducting inoculation experiments in different forest types might be useful to determine the efficiency of the method and possible uses.

In addition to the abiotic conditions of the forest, biotic interactions can also affect the species composition of a polypore community. Therefore, these conditions are also important in determining the efficiency and use of inoculation. Interaction in fungal communities can be either intraspecific or interspecific, and the types of interactions can be either antagonistic, mutualistic, or parasitic (Boddy, 2000). There is also a hierarchical element of colonization in the successional stages of deadwood, where species have a predecessor-successor relationship. The predecessor is needed to physically and chemically alter the deadwood elements before colonization of a successor species (Renvall, 1995). It is also indicated that the location and species pools can affect community development through priority effects, meaning that the first colonizers as well as the unique abiotic conditions of the location work to shape the community assembly (Hiscox et al., 2016).

The ecological strategies of each species (r- or k- selection) affect temporal colonization (Hiscox et al., 2018). Typically, common polypores have r-strategy, and rare polypores use a k-strategy. Though there are suggestions that other strategies might also affect assembly such as stress-, disturbance-, and combat- selection (s- , d- , and c- selection) (Rayner, 1988). These strategies produce different interactions in a deadwood community, and by studying community interactions, the scientific field will gain more knowledge about the ecology of wood-inhabiting fungi to make better conservation measures and possibly restore populations.

Advancements in DNA technology have made it possible to profile fungal communities in deadwood in their microscopic vegetative states, without relying on the presence of fruit bodies. This enables further insight into the ecology of wood-inhabiting fungi. By using metabarcoding, the mycelia of different fungal species can be identified by using fungal

specific primers that amplify the ITS region of their genome with polymerase chain reaction (PCR) (Peay et al., 2008). Since little is known about the factors which influence community assembly, inoculation of wood living fungi has become one important tool for filling this knowledge gap (Fukami et al., 2010). Only a few studies have used inoculation of polypores and used metabarcoding methods to study its effect on the fungal community (Lindner et al., 2011, Abrego et al., 2016, Fukami et al., 2010). There are also ongoing studies about inoculation (Nordén et al., 2020), one being a larger project in Finland focusing on population reintroduction (Mäntyranta, 2019b).

This thesis will explore whether metabarcoding can be used to classify the state of a forest based on the community of wood living fungal species. Key questions that this thesis will attempt to answer are:

- What are the main differences between the forests with different management history?
- What factors determine the community assembly? E.g., does the fungal community vary in each location?
- Can metabarcoding be used in monitoring of rare species?
- Further, this study will explore if the inoculation of the three red listed fungal species was successful, and how inoculation affects fungal community dynamics.

2 Methods

2.1 Groundwork for the study:

This study is based on the groundwork of the unpublished study by Maurice (*Pers. comm.*). Where Norway spruce (*Picea abies*) logs were selected within three different forests with different degrees of human disturbance: managed forest, semi-natural forest, and near natural protected forest. Five logs were selected from each site, and all the logs were mostly in early decay stage 1a-2, and one log in an intermediate decay stage 3. The logs were inoculated with three red listed polypore species *Fomitopsis rosea*, *Amylocystis lapponica* and *Phellopilus nigrolimitatus*. The inoculations of each of the species followed the same order, but each log had different start-species at the basal part of the log. There were six drill holes using a 10 mm electric drill in each log, and the dowels were placed evenly ca.1 m from the base of each log. All the species with fruitbodies on the logs preceding inoculation were described, in addition to other trunk characteristics e.g., epiphyte and bark cover, diameter, and length of the logs.

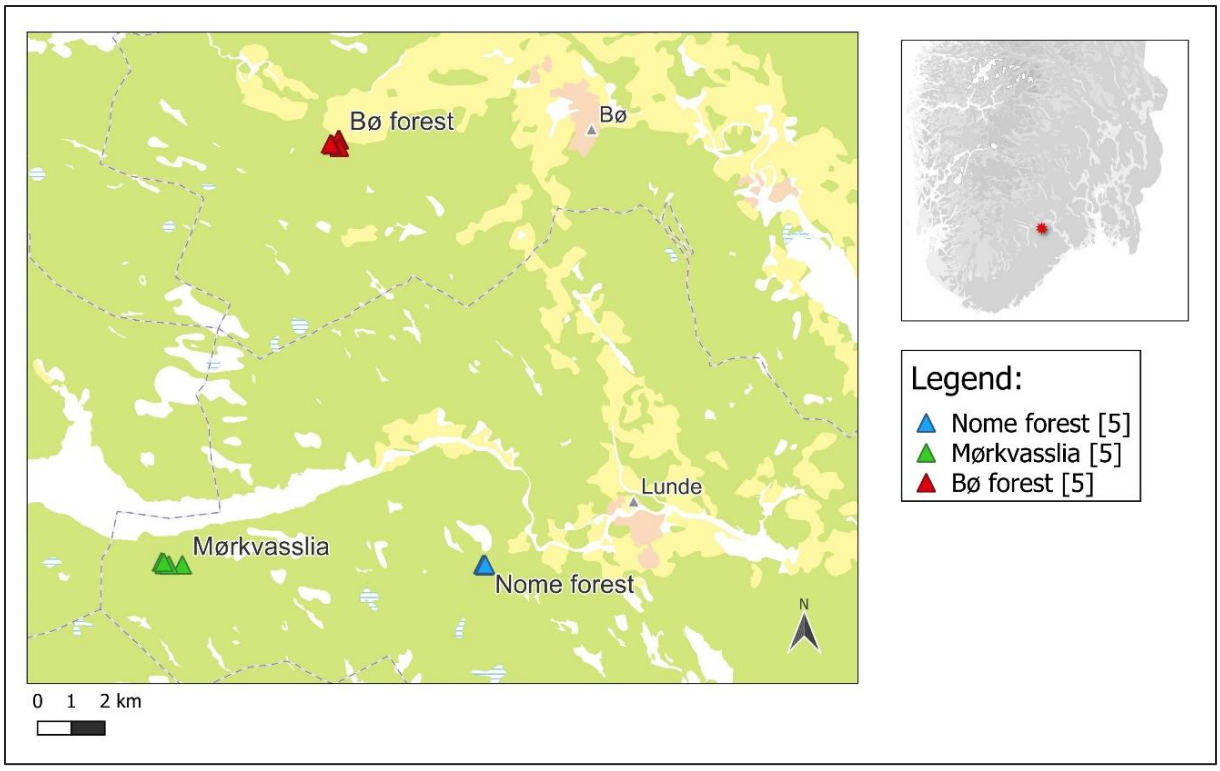

Figure 1 A local map (1:10 000) with municipality borders made in QGIS 3.30.0, showing the three study areas: Nome forest (blue), Mørkvasslia (green), & Bø forest (red). There are 5 logs in each study area. A regional map shows the position of the area (red, dot) in Norway.

2.2 Descriptions of the study areas:

The study area is in the Norwegian county Vestfold & Telemark within the municipalities of Nome and Midt-Telemark, which can be seen on a map in figure 1. In 2022, the municipalities of Midt-Telemark and Nome collectively boasted a forest area spanning 710 km^2, representing a forest cover of approximately 75% (SSB, 2022). The study area therefore hosts an abundant potential of habitats for wood decaying fungi. The tree species composition of the study area was similar to most forests in the region: dominated by Norway spruce, pine trees (*Pinus sylvestris*), and with some appearances of deciduous trees like birch (*Betula pubescens*) and/or oak (*Quercus robur*).

The managed forest was in Nome municipality. Some of the study area is sloped and has a homogenous stand structure with closely planted Norway spruce trees, in addition to some pine and birch. The area has been recently logged and planted with Norway spruce. There are also plans to do logging nearby the study area, and to possibly protect some lying deadwood according to a MiS-mapping by NIBIO found in naturbase.no (Miljødirektoratet, 2023). The logs in this area exhibited a higher level of decay and were often covered by moss, likely attributed to the presence of a nearby stream in the study area.

The semi-natural forest was close to Bø village in the municipality of Midt-Telemark. This forest had an especially high occurrence of *F. rosea*, which is typical of Vestfold & Telemark County, compared to the rest of Norway where the polypore is relatively rare (Kauserud (*pers. comm.*). The forest structure was relatively mixed with variability in stand age. Some areas have been left untouched because of the difficult terrain and due to rockfall, while areas outside the study area have been recently cut and planted. The forest in this area has historically undergone various degrees of management, including selective logging, as evident from the presence of an old horse and carry road within the study area.

The most natural forest is Mørkvasslia. It is situated in a nature reserve called Mørkvassjuvet in Nome municipality. The area was first officially protected in 2002 and is around 99km^2 large, at 72 – 847m above sea level (Miljødirektoratet, 2010). However, the site seems to have maintained a low level of disturbance before protection. This might be due to its canyon shape, and many streams, which makes the forest very inaccessible, and has been historically hard to manage. There has been some selective logging near some accessible sites near the old

horse and carry road. Many red listed polypores have been registered through Artsobservasjoner.no (n.d.), to name a few: *P. nigrolimitatus, F. rosea, A. lapponica, Antrodiella citrinella (synonym: Flaviporus citrinellus), Leptoporus mollis* and *Steccherinum collabens.* The area is stated to have been protected due to its great scientific value, both because of its large size, and recordings of many lichens and fungi that are vulnerable and rare (Miljødirektoratet, 2010).

2.3 Fieldwork and sampling methods:

The field work for this thesis was conducted between Sept. - Des. of 2022 mostly by myself and my supervisor. To find the sample logs we used the coordinates of Maurice (*Pers. comm.*). When collecting the sawdust samples, in order to prevent sequencing of surface species, moss and bark was removed from the log surface, and surface wood pieces were avoided during collection. Five holes were drilled for my sampling at approx. 0.5 m in between each inoculation dowel, using a 10 mm electric drill (figure 2). The samples were marked with the log number acquired from the metal plaque together with a letter. The first sample from each log was marked with the letter A to represent the basal part of the log, alphabetically ordered to the letter E, corresponding to the upper parts of the trunk. To prevent cross contamination, the drill was washed with 50% chlorine solution, or residual sawdust was burned using a handheld butane gas burner. Sawdust samples from all the 15 logs were collected and came out to a total of 75 samples. All samples were collected in individual and marked falcon tubes and stored in the lab freezer at –20°C to prevent degradation.

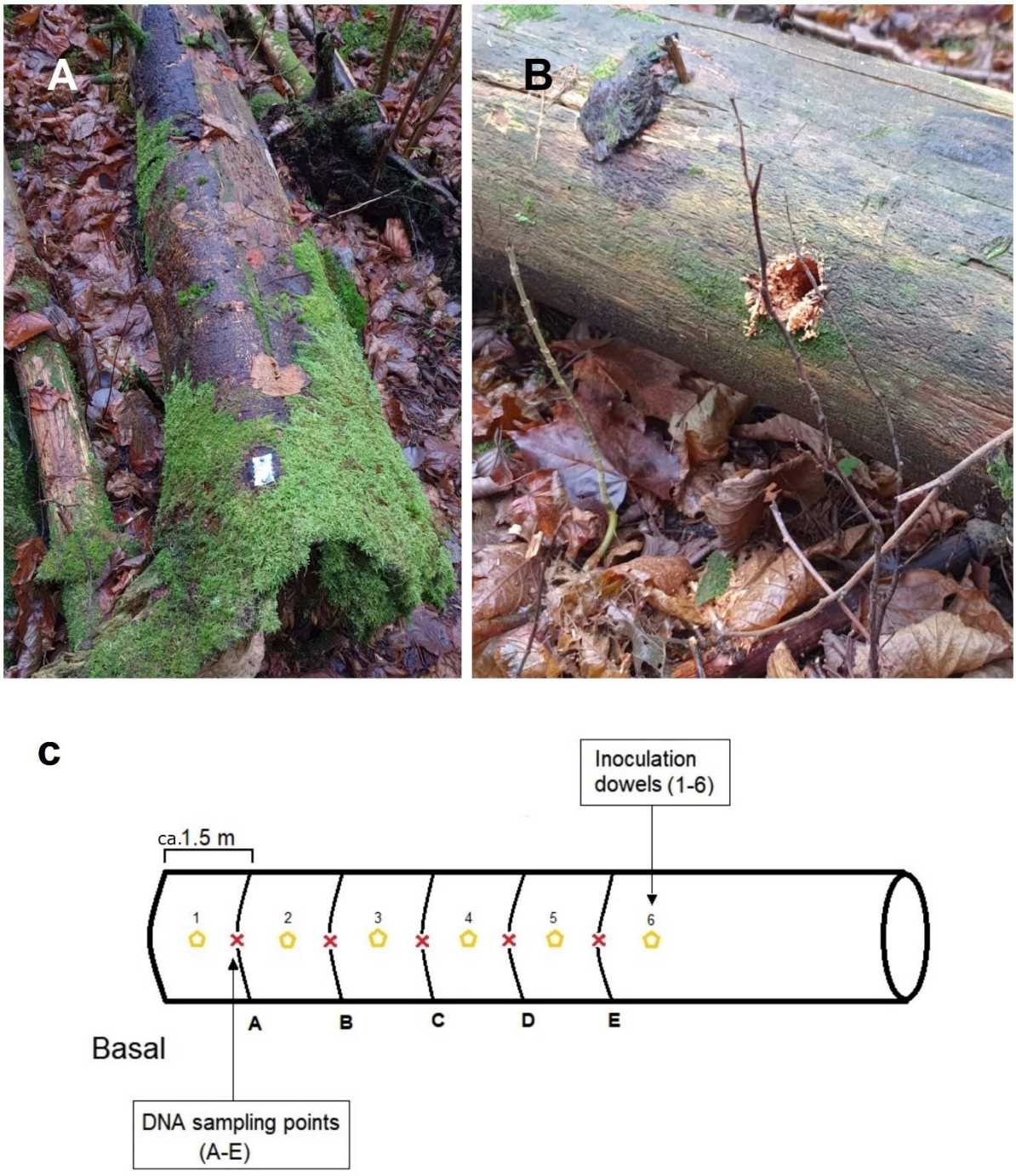

Figure 2 A photograph showing one of the logs in the field (A). A representation of a sample point next to an inoculation dowel covered by blackened beeswax (B). Lastly, a schematic drawing (C) of the sampling process, showing a figurative placement of inoculation dowels (yellow) and where the sawdust samples were taken (red) at approximately 0.5 m in between the inoculated sites.

2.4 DNA extraction:

All the lab work was conducted at the University of South-Eastern Norway (USN) DNA-lab at Bø campus. The samples were thawed and mixed in their falcon tubes before extraction. The kit used for extraction was DNeasy powersoil (QIAGEN N.V., 2017). All of the instructions in the protocol were followed, except for sample weight, which was reduced from 250 mg to 150 mg. At first the recommended weight was used, but quality and concentration measurements seemed unstable in most of the samples, and it seems as if the porosity of sawdust was making the lysis process less effective. This is the main reason why the sample weight was reduced. Other studies also either recommended or used a lower sample weight (Tedersoo et al., 2022, Abrego et al., 2016).

All samples had a different texture, some were finely ground, while other samples were coarser, with larger intact wooden pieces. As well as a difference in moisture content. This then affected how much lysate was extracted during the first steps of the DNA extraction process. Both texture and moisture content might have affected the total volume of lysate extracted after vortexing with the PowerBead Tube. The samples that amounted to the lowest volume of lysate were those that were finely ground and dry, and the samples containing coarser wood pieces were still largely intact after vortexing. A possible way of reducing this bias could have been to use liquid nitrogen and grounding the sawdust into fine powder. However, this was not available at the USN lab. After extraction, the concentration and purity were measured with a NanoDrop™ Lite Spectrophotometer. The concentrations of the samples, as well as A60/A80 ratios can be found in appendix 1. The final DNA lysates were then stored frozen at -20 °C.

2.5 PCR amplification:

The ITS2 region was amplified using the primer pairs ITS4 and fiTS7, additional information can be seen in table 1. The fragment length amplified for each primer ITS4 is approx. 350-400 bp, and for fiTS7 about 200-300 bp. The primers were diluted to a working solution of 10µM in the lab.

Table 1 The primers used for amplifying the extracted DNA from the sawdust samples. The primer pairs consist of a forward primer (fiTS7 F) and a reverse primer (ITS4 R).

Region	Primer name	Primer Sequence 5′ to 3′	Source
ITS2	fiTS7 F	GTGARTCATCGAATCTTTG	White et al. (1990)
ITS2	ITS4 R	TCCTCCGCTTATTGATATGC	Ihrmark et al. (2012)

I used the method of PCR and library preparation similar to the method called "Tagged PCR and library build on amplicon pool" as shown in figure 2e) of Bohmann et al. (2022). In this method, unique primers containing a small tag before the target sequence are used for each sample, enabling demultiplexing of samples after sequencing. This means that many samples can be sequenced together at relatively high depth, and there is no need for developing individual libraries. The method also only contains one step of PCR, which is one of the reasons why this method is both cost- and labor efficient.

The ITS2 region of each sample was amplified using a PTC-200 Peltier Thermalcycler. The PCR mix contained 12.5 µl of Phusion plus PCR master mix 2X (Thermo Fisher Scientific Inc., 2021) and 5 µl of GC enhancer was added, with a primer mix (10.0 uM) of 1.5 µl, and 1.5 µl nuclease free water, together with 5 µl of template DNA (15ng/µl).

The settings for the PCR reaction cycle are described in this paragraph: the initial denaturation process used 98 °C for 30 sec for a single cycle, followed by a denaturation of the same temperature, but at 10 sec. The annealing took place at 58 °C for 10 sec each 35 of the cycles. Followed by an extension at 72 °C for 15 sec. The final extension was performed at 72 °C for 5 min in a single cycle. All PCR products were then stored in the lab freezer at -20 °C to prevent degradation.

2.6 Quality testing and normalization:

After amplification, 38 of the PCR products were quality tested on an electrophoresis device Invitrogen E-gel Power snap using the gel E-gel EX Invitrogen Agarose 1% (Thermo Fisher Scientific Inc., 2020). For the rest of the samples (37) the quantity was calculated using the high sensitivity Qbit for double stranded DNA. There was a second run of PCR for samples that did not appear in the gels, either randomly or because of low concentration, and samples that had a low Qbit concentration (0.1 – 2 ng/µl). Only two samples improved their Qbit concentrations after reamplification. To prepare the PCR products for sequencing the amplicons had to be normalized according to their concentrations. Normalization is important

to accomplish an even yield of all fragments and give accurate results, and was done by analyzing the electrophoresis gels, and Qbit measurements.

2.7 Library preparation:

The normalized samples were pooled, and the final concentration of 18.4 ng/µl was found by using the Qbit. Library preparation was done using the Ion Plus Fragment Library Kit following the protocol Prepare Amplicon Libraries without Fragmentation (p.10) (Thermo Fisher Scientific Inc., 2016b). First the amplicons were end repaired, then the pooled sample of 6 µl was diluted with 73 µl nuclease free water. Lastly, end repair buffer and enzyme were added according to the protocol, and subsequent purification with the Agencourt AMPure XP kit using freshly prepared 70% ethanol.

Adaptors were ligated (Ion Xpress™ Barcode) together with nick repair. The barcode has a length of 6 nt. and is used for indexing PCR's which allows for demultiplexing after sequencing. Nick repair is used for repairing nicks or gaps in the DNA fragments, which might affect adaptor ligation, and helps improve sequencing quality and reduce library bias. After this, purification was done with the volume of 100 µl AMPure XP Reagent, based on the library size of 400-base-read.

qPCR (quantitative real-time PCR) was used to quantify the library by using the quantitation kit together with the Ion Library TaqMan® protocol (p.10) (Thermo Fisher Scientific Inc., 2016a). The qPCR was conducted using the StepOnePlus™ Real-Time PCR System by Applied Biosystems™. The formula (1) for determining the undiluted library concentration is shown together with the calculated results. qPCR results decided that the sample was to be diluted x17 before proceeding to sequencing.

$$concentration\ determined\ by\ qPCR\ \times library\ dilution = undiluted\ library\ concentration \quad (1)$$

$$1.259\ \times 800 = 1007.2\ \text{pM}\ (1.0072\ \text{nM})$$

2.8 Sequencing:

Template preparation and loading of the final library on an Ion 530 chip was done using the protocol for the Ion 510™ & Ion 520™ & Ion 530™ Kit with an Ion Chef (Thermo Fisher Scientific Inc., 2022). The sequencing was done using an Ion Genestudio S5 platform which is a type of NGS (Next generation sequencing) that uses sequencing-by-synthesis method called ion semiconductor sequencing (Merriman et al., 2012). During the emulsion PCR,

DNA fragments are attached to beads where they are amplified and sequenced on the semiconductor chip. During the polymerization reaction of the fragments, H+ protons are released which generate a change in pH after being flushed over the semi-conductor chip, and the signal for each nucleotide is processed to determine the DNA sequence.

2.9 Bioinformatics:

The raw data was downloaded from the Torrent server as fastq files. Demultiplexing and initial quality filtering was done using Cutadapt v. 4.1 (Marcel, 2011, Wood and Salzberg, 2014). Further filtering of low-quality reads, identification of amplified sequence variants (ASV's) and removing chimeras were done using the dada2 pipeline (Callahan et al., 2016). Taxonomic inference was done using the taxonomy assignment in dada2 with the reference database UNITE (Nilsson et al., 2018).

2.10 Statistical tests:

The downstream analyses of the ASV's with taxonomic identifications were done using Rstudio with the packages "biobase" (Huber et al., 2015), "phyloseq" (McMurdie and Holmes, 2013) and "tidyverse" (Wickham et al., 2019). Since several ASV's were identified to the same species I combined these into a single accession using the tax_glom function in the phyloseq package. For testing and analysis of the data I used the features for alpha diversity, beta diversity, boxplot, ANOVA, and PERMANOVA from the R-package "vegan" (Oksanen et al., 2015) and ggplot2 for visualization of alpha- and beta diversity (Wickham, 2016).

Alpha diversity was calculated as observed richness, ACE (Abundance-based coverage estimator) and Shannon diversity, which are common to use when analyzing microbial diversity. Observed and ACE are measures of species richness in a sample, while Shannon index is an estimate of the diversity within the samples. What separates these different tests is that observed richness measures the number of species detected, and ACE estimates the species richness. Shannon index applies evenness to the diversity measurement, meaning that it also measures the proportions of different ASV's in each sample. If a sample has a high diversity and an even distribution of different ASV's, the sample gets a higher score. A boxplot and ANOVA test were also deployed to visualize and test the significance of difference in observed richness between the locations.

A principal coordinate analysis (PCoA) was done to study the structure of the communities. The ordination was done on a distance matrix constructed using Bray-Curtis distances. The first two PCoA axes were plotted to visualize the differences between samples in the community. Location of the log was visualized with different color and decay stage in 2016 as different shapes. The decay stage (2016) variable represents the decay stage of each log measured by Maurice (*Pers. comm.)* at the start of the inoculation experiment. The beta diversity plot was followed up with a PERMANOVA of location, decay stage (2016), middle trunk diameter, sample placement and trunk length to test which factors might have significantly affected the fungal community.

2.11 Comparison to the Norwegian red list of 2021:

To record how many rare species each location possessed, there was made a comparison to the Norwegian red list 2021 to the ASV's (Artsdatabanken, 2021). This was done by exporting all names of the fungal species with their IUCN categories from artsobservasjoner.no, and then comparing the exported species names to the names of the ASV's that were assigned by the UNITE database. Then giving the appropriate category to each ASV. A list of this data can be found in appendix 2, with total number of reads of every species from each location. The data from this appendix was used to calculate the percentage of each IUCN category and can be found in figure 10. Additionally, this data was used to make the short species lists found in table 4, 5, & 6. This was done to test whether metabarcoding could be used to record how many red listed species could be found in each location.

A drawback of this method arose due to discrepancies between the species names in the Norwegian red list 2021 and the UNITE database. The names in the UNITE database are constantly being updated because of phylogenetic research. Because of this, some species might have been overlooked in the comparison. Two species were accounted for, such as *A. citrinella (F. citrinellus)* and *Spongiporus undosus (Postia undosa).* For these two species I used the names from UNITE, since they are more up to date. Although a more comprehensive search could have potentially prevented the error, it was discovered late in the writing process.

2.12 Polypore community interactions:

To study possible interactions, the presence of *Fomitopsis pinicola, P. nigrolimitatus* and *F. rosea* as a fruitbody in 2016, and as a fruitbody, or DNA in 2022 were compared. The reason behind this comparison is that *F. pinicola* and *P. nigrolimitatus* are known to have a negative relationship (Ottosson et al., 2014). While *F. rosea* is known to have a positive relationship with *F. pinicola*. The method of comparison is simply observational and not possible to test for significance due to low sample size.

2.13 Sources of error:

The absence of controls has introduced some potential sources of error and noise in this thesis. This means that there is no guarantee that some of the results might be false positives stemming from cross contamination, which could have occurred either during the fieldwork or in the lab. There is also a high risk of spillover when handling PCR products and barcodes, and controls can help to filter out these flaws from the final results.

3 Results

After the sequencing, the samples with reads <5000 were filtered out. Figure 3 shows the sequencing depth of each sample after filtration. This resulted in a total of 1054927 reads from 54 samples (out of 75 total). These samples include 14 from Mørkvasslia, 19 from Nome forest, and 19 from Bø forest. The reads were then clustered into a total of 370 ASV's. The samples were dominated by Basidiomycota at 63%, and 35% belonged to Ascomycota. The average number of polypore ASV's (*Hymenochaetales* & *Polyporales*) per sample was 2.85 (±1.6 sd), while the average of polypore ASV's per trunk were 10.3 (±4.1 sd). Figure 4 shows the taxonomy of polypores for all samples within each location.

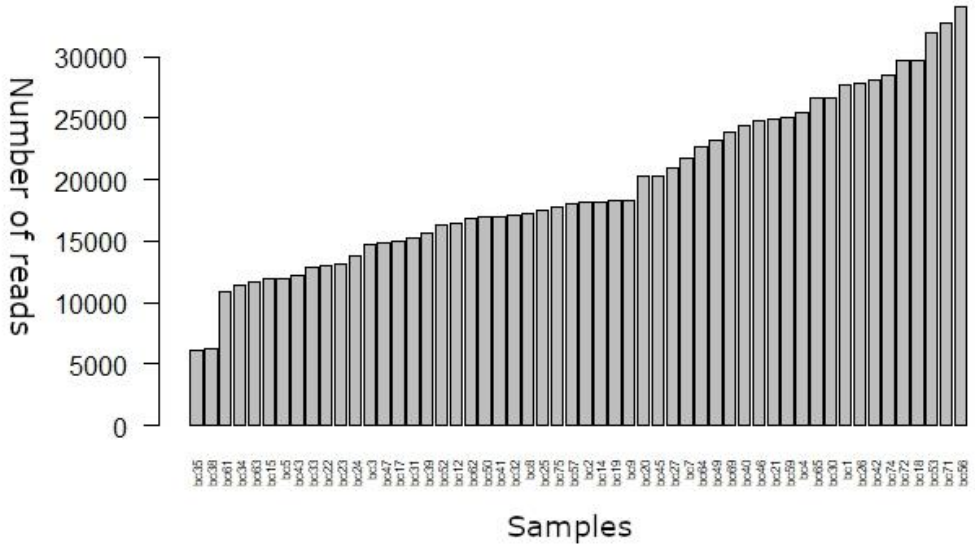

Figure 3 Bar plot showing the sequencing depth of the samples (x-axis) and number of reads (y-axis).

The species with the highest sum of reads for each location was calculated, and the top three species can be found in table 2. In Bø forest *Xylodon asperus* has the most reads, though it only appears in three samples (bc71, bc72 & bc75). The opposite can be said about *Antrodia Serialis* in Mørkvasslia, where the number of reads were spread out into 8 different samples. In Nome forest *F. pinicola* had the highest number of reads, and appeared in 6 samples.

Table 2 The top three fungus species with the highest sum of reads in each location.

Rank	Bø forest	Mørkvasslia	Nome forest
1.	*Xylodon asperus*	*Antrodia serialis*	*Fomitopsis pinicola*
2.	*Tubulicrinis borealis*	*Resinicium bicolor*	*Dacrymyces stillatus*
3.	*Postia leucomallella*	*Phellinus viticola*	*Tubulicrinis borealis*

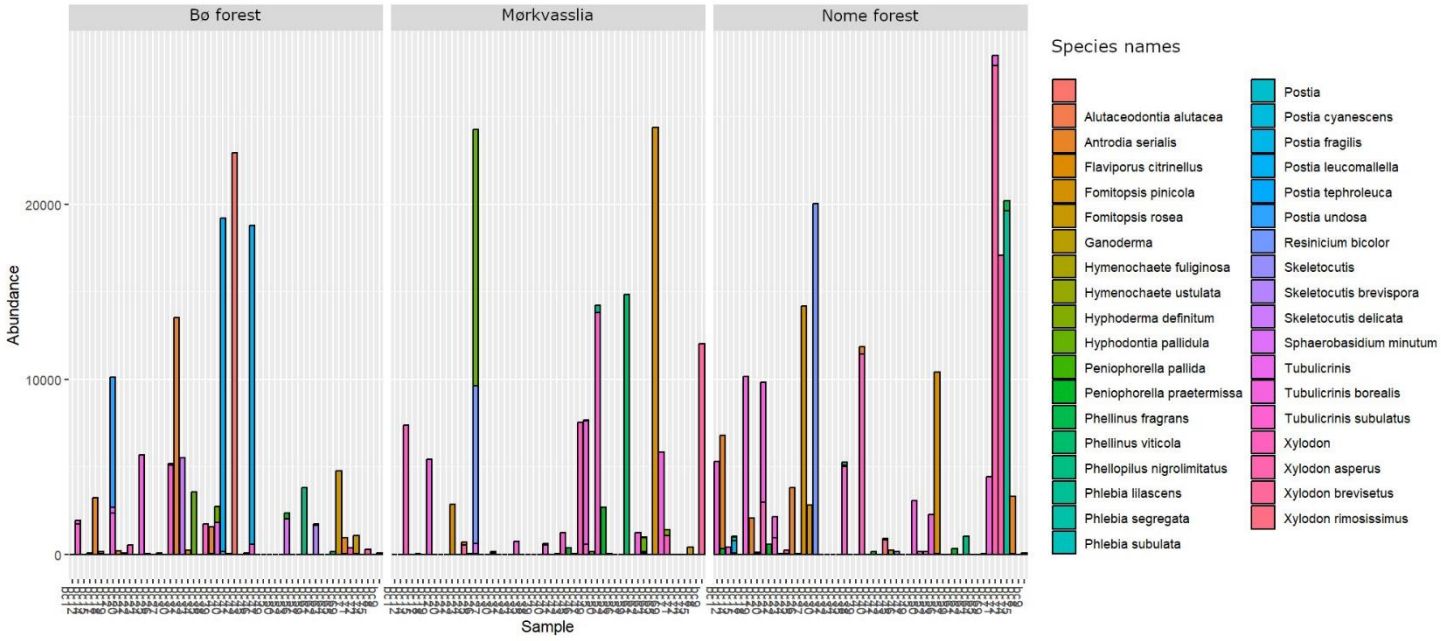

Figure 4 Barplots showing the abundance of reads of polypore species (y-axis) with samples of each location (x-axis), Bø forest on the left, Mørkvasslia in the middle, and Nome forest on the right. Species names can be found in the legend.

3.1 Alpha- and beta diversity:

Alpha diversity tests were completed to study the diversity within each sample. In figure 4 the alpha diversity of each sample paired with location is displayed with different types of alpha diversity: observed, ACE and Shannon diversity. The diversity estimates of the individual samples (figure 5) shows a large variation within the different forests, although in the observed species richness on the left it seems that the samples from Mørkvasslia in general are slightly higher than samples from Nome forest. Bø forest's samples seem to be intermediate and overlap with samples of both forests. Secondly, the plot for ACE richness in the middle of figure 4 looks quite similar to the observations plot. However, it seems that it is more compacted and less definite which forest has the lowest richness, but still Mørkvasslia seems to be the forest with the highest number of rare ASV's. Lastly, the plot for Shannon diversity on the right of figure 4, seems even less conclusive than the previous two plots, and

it is not possible to tell which forest scores the highest or lowest. Though some samples from Mørkvasslia and Bø forest have a high Shannon score of >2.

The difference in alpha diversity of each forest is visualized in a boxplot (figure 6). Where the boxplot Mørkvasslia clearly has the highest median above 40, while the medians of Bø forest and Nome forest are both around 30. However, the upper and lower quartiles, as well as the min-max whiskers are very different for the two lower boxplots. An ANOVA test was performed to compare the alpha diversity variations for each location and suggested that the difference was significant ($p=<0.001$).

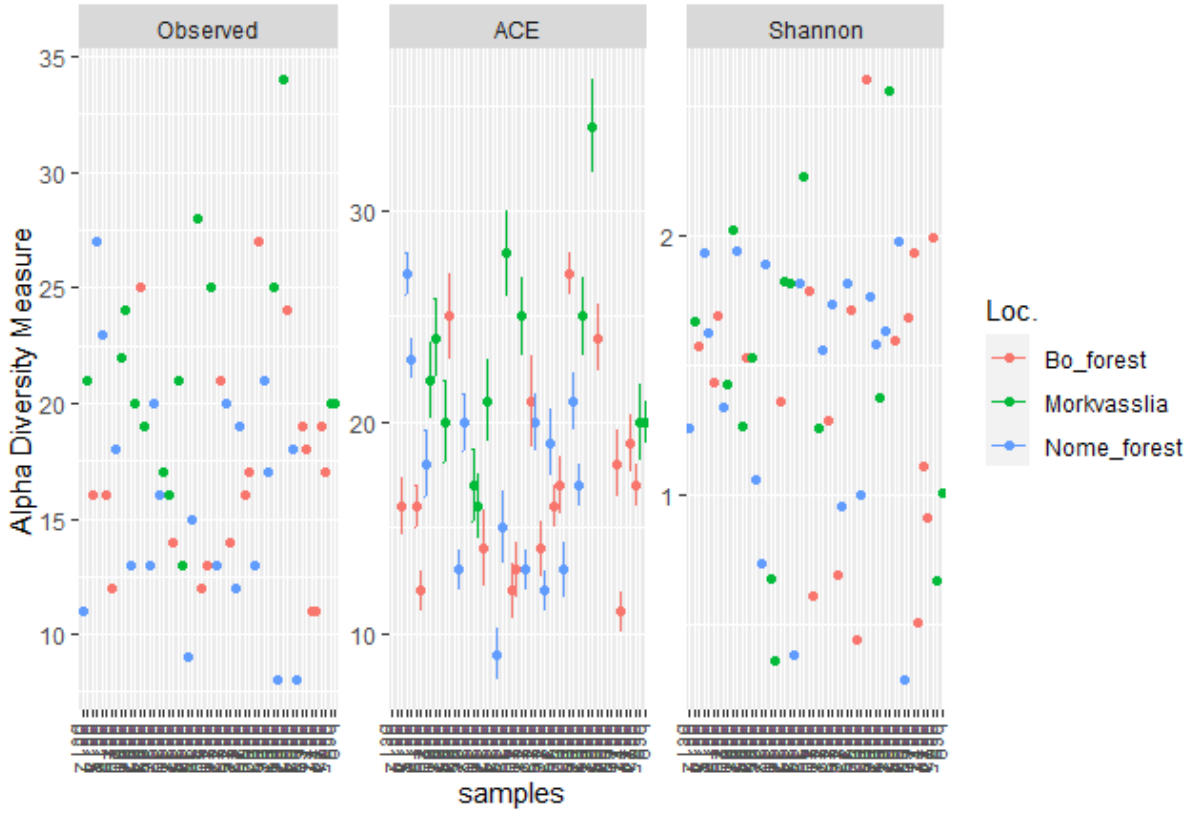

Figure 5 Three scatterplots showing alpha diversity measure (y-axis) using three different tests (observed, ACE & Shannon) with the sample barcodes (x-axis), and colored according to their location: Bø forest (green), Mørkvasslia (green) and Nome forest (blue).

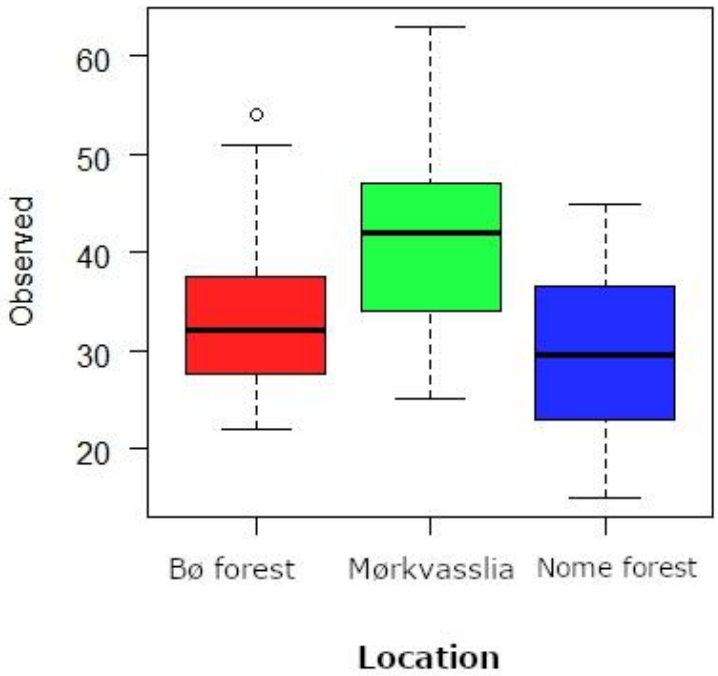

Figure 6 Boxplot showing the differences in mean alpha diversity measure (y-axis) and locations: Bø forest, Mørkvasslia & Nome forest (x-axis).

Beta diversity was plotted to study the differences between communities of the three locations in addition to decay stage (2016). Figure 7 shows the complete scatterplot in 1st and 2nd dimension, where the 1st dimension explains 11.1%, and 2nd dimension explains 8.8% of the difference in species composition between samples. In this plot there seems to be a slight clustering of samples from each location. Nome forest samples tend to cluster away from the other locations, while Mørkvasslia and Bø forest generally cluster together. Additionally, the samples from Nome forest in decay stage 3 seem to cluster with the samples from Mørkvasslia and Bø forest in decay stage 1-2. The second figure 8, shows the samples in a scatterplot of the 3rd dimension explaining 7.4%, and 4th dimensions explaining 5.7% of the differences. Here the clustering is less obvious, although there is a tendency that Mørkvasslia and Nome is separated on the fourth axis, with Bø forest as intermediate.

Furthermore, in the first figure there are five samples from Mørkvasslia that appear in the upper left corner. They are the samples bc33, bc21, bc41, bc8 & bc23, and the genera of each sample is shown in figure 8. The samples bc33, bc21, bc41 & bc8 contain species of *Heterobasidon* genus, a type of root rot polypore that may be pathogenic to trees. This species also appears in bc52 and bc45, but it is not in the cluster. One sample with a different species composition (bc23) seems to cluster with these samples even though it does not have *Heterobasidon* reads. This sample contains most reads of *Lentomitella which are said to be* phaeoisaria-like anamorphs, *and Meliniomyces* a group of fungi associated with roots (Hambleton and Sigler, 2005, Réblová and Seifert, 2007). The sample within closest proximity (bc33) does not seem to have many reads in common, except for the two genera previously mentioned, in addition to *Helicogloea* which is another anamorph fungus. The divergent sample bc23 does not seem faulty when studying the matrix in Rstudio.

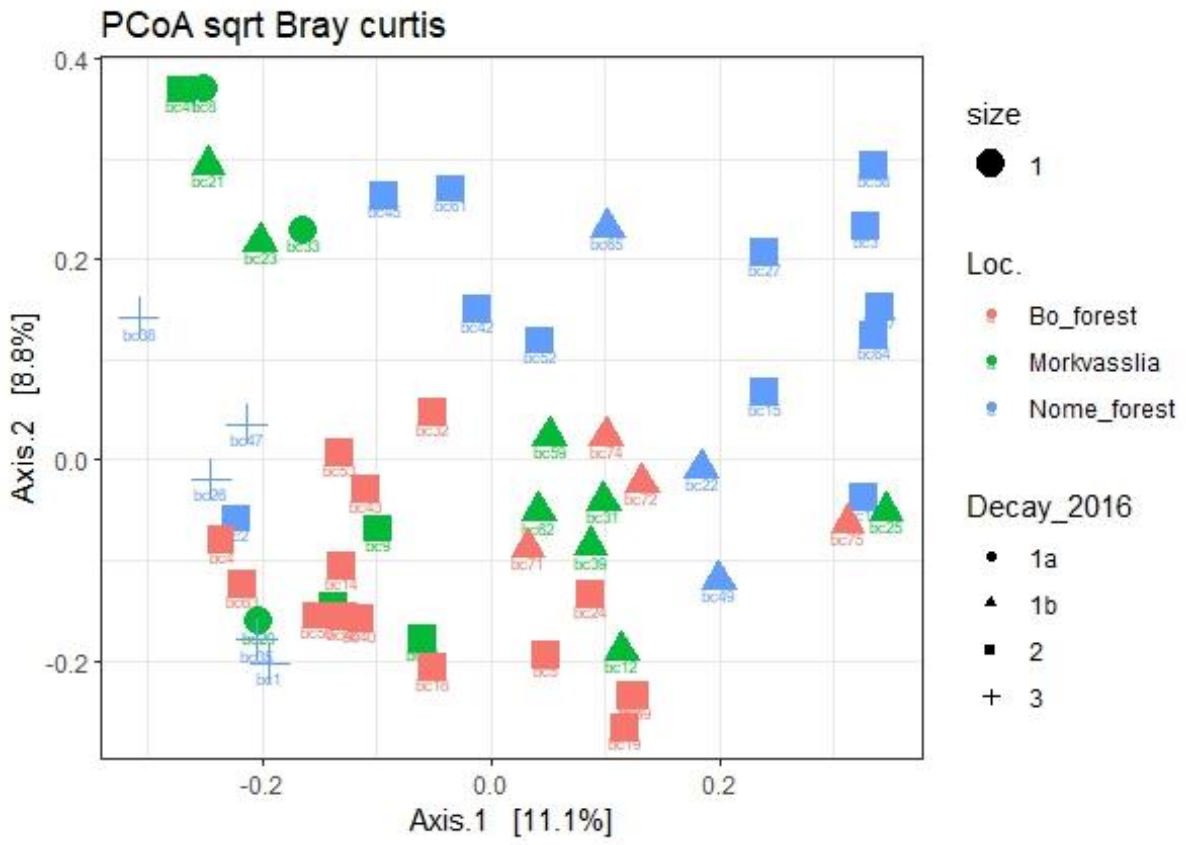

Figure 7 A Principal Coordinate Analysis (PCoA) of Bray-Curtis distance matrix in the 1st (x-axis) and 2nd dimension (y-axis), where each sample is represented with distance and two variables. The variable "Location" is represented by color: Bø forest (red), Mørkvasslia (green) & Nome forest (blue). And the variable "Decay stage (2016)" is represented by geometric shape: 1a (circle), 1b (triangle), 2 (square) & 3 (cross).

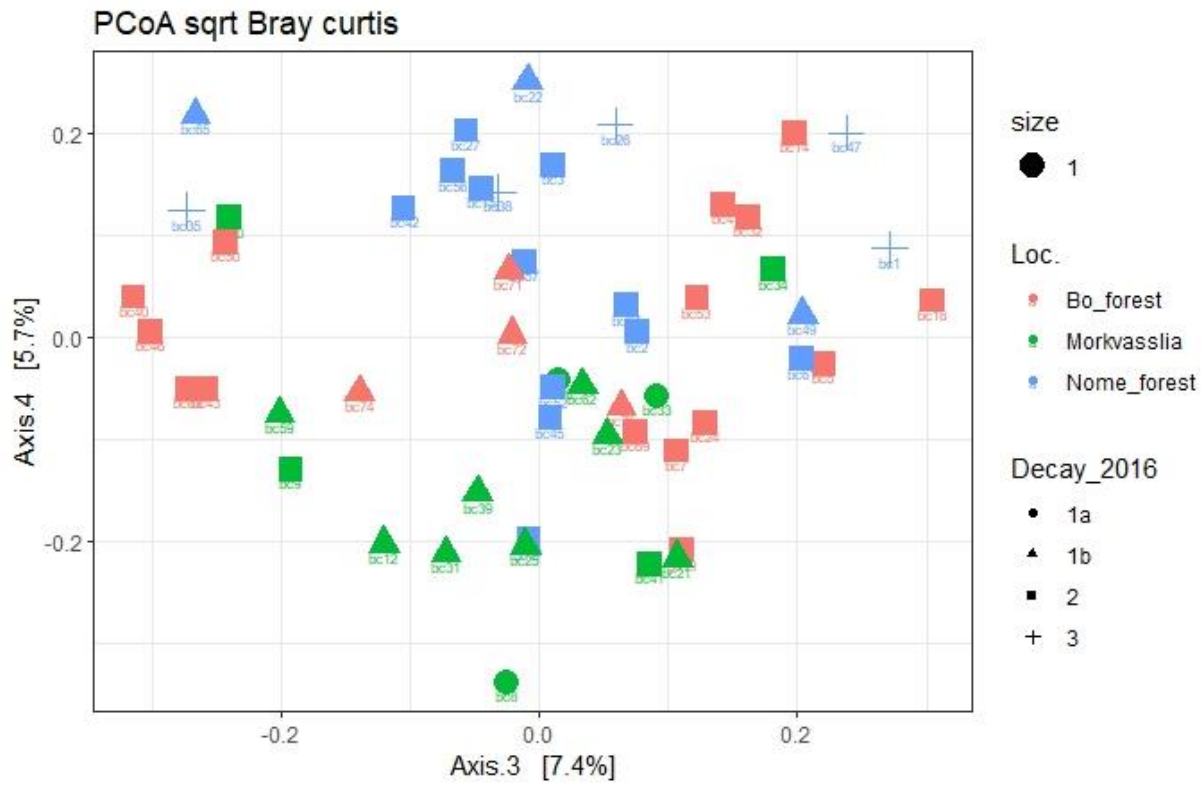

Figure 8 A Principal Coordinate Analysis (PCoA) of Bray-Curtis distance matrix in the 3rd (x-axis) and 4th dimension (y-axis), where each sample is represented with distance and two variables. The variable "Location" is represented by color: Bø forest (red), Mørkvasslia (green) & Nome forest (blue). And the variable "Decay stage (2016)" is represented by geometric shape: 1a (circle), 1b (triangle), 2 (square) & 3 (cross).

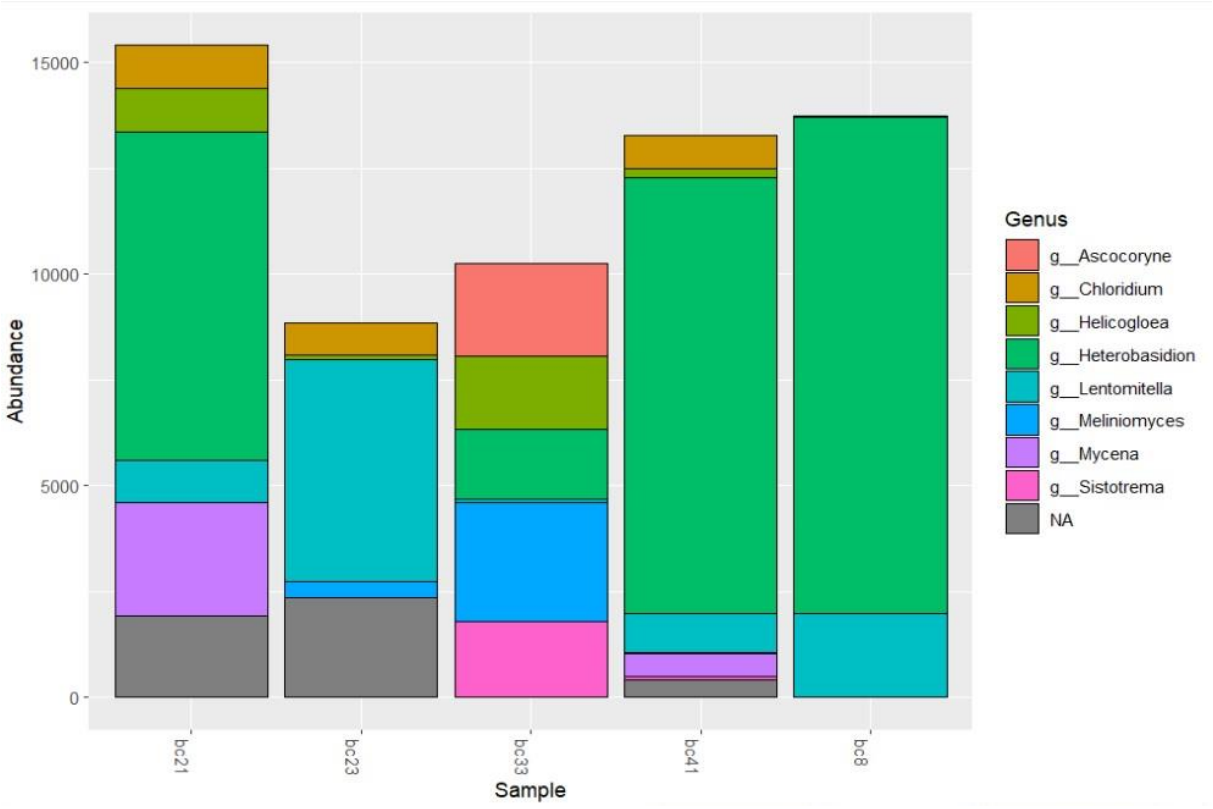

Figure 9 Genera found in samples bc21, bc23, bc33, bc41 & bc8.

In order to identify potential factors explaining the difference in fungal composition we performed a PERMANOVA analysis. In this test the response variable was the Bray-Curtis distance between samples, and the explanatory variables were location, decay stage (2016), and mid trunk dm in 2016. The results can be seen in table 3 where both location (p=<0.001), decay stage (2016) (p=<0.05), in addition to mid trunk dm in 2016 (p=<0.05) were significant environmental factors contributing to community differences. However, measured trunk length in 2016 and sample placement on the log (A-E), were not significant in explaining the differences (p=>0.05). The significant test variables explained around 20% of the variation in the community, while around 80% could not be explained.

Table 3 PERMANOVA test explaining the differences between response variable bray-curtis distance and explanatory variables: decay stage (2016), trunk diameter & length.

	r^2	**df**	**SS**	**Pseudo-F**	**P (perm)**
Location	0.064	2	1.41	1.82	0.001
Decay stage (2016)	0.079	3	1.75	1.50	0.003
Mid Trunk diameter	0.032	1	0.71	1.84	0.003
Sample placement	0.073	4	1.61	1.04	0.310
Trunk length	0.021	1	0.46	1.20	0.185
Residuals	0.731	42	16.22		
Total	1.0	53	22.16		

3.2 Observations of red listed species:

When comparing presence/absence of the ASV reads to the red list, a total of 19 species were found (figure 10), in addition to reads from 60 species with sustainable populations. The total number of species was 79. Note that this figure is based on read length, and not presence/absence.

A short summary of the percentages for each IUCN category at the different locations is shown in figure 10. Bø forest had 83% of species with LC, 3% of VU, 4% of NT and 11% of NE. This forest recorded the highest percentage of red listed species, and the largest number species in NE category. While Mørkvasslia had 94% LC, with 1% VU, 3% NT and 1% NE. This forest had the second highest percentage of red listed species. While Nome forest had

98% LC, and only 2% NE. This forest had the highest number of common species, and least number of red listed species. When only counting presence/absence of red listed species Bø forest had 14 species, while Mørkvasslia and Nome forest both had 11 species.

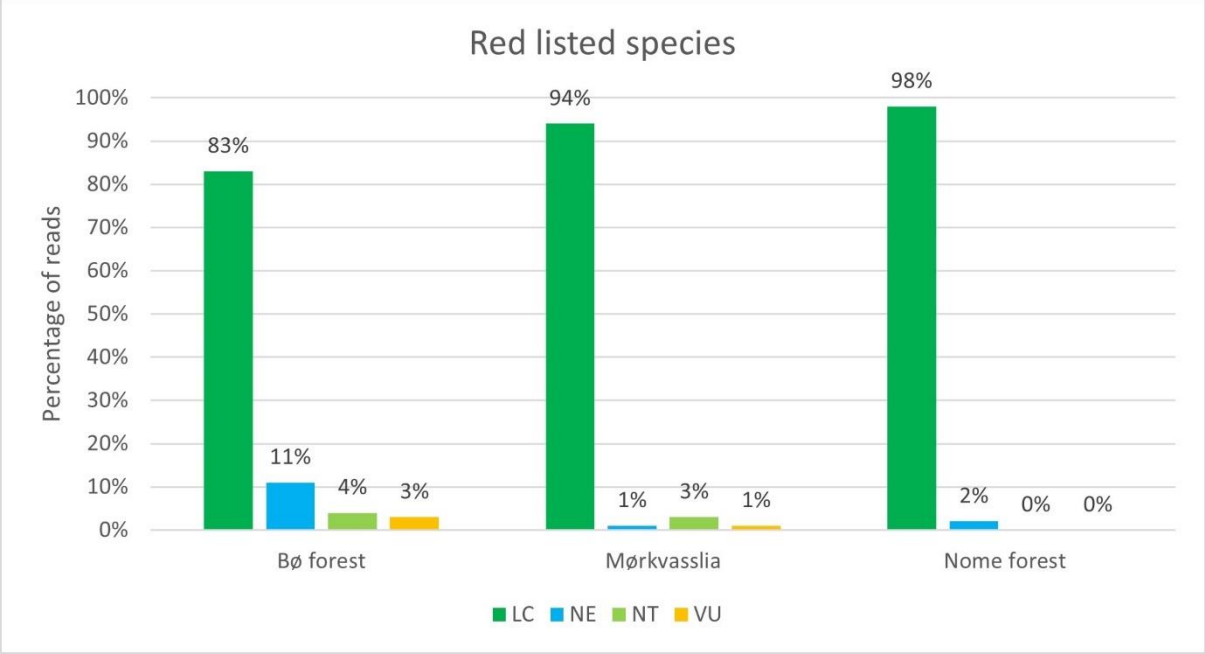

Figure 10 Barplot showing the percentages of species reads and their IUCN categories of Bø forest, Mørkvasslia and Nome forest. The abbreviations and label of the categories: LC – least concern (green), NE – not evaluated (blue), NT – near threatened (light green) & VU - vulnerable (yellow). There are most red listed species in Bø forest (left) and Mørkvasslia (middle). Bø forest has the most species in the NE category. Nome forest (right) has the least species in the red list.

In Norway, only a small number of polypore species (7) have yet to be assessed for the red list (Brandrud, 2021). Meaning that there are few polypores in the NE category, and since so many species in the NE category were not polypores, they were not discussed or added into the tables below. Except for *Phlebia lilascens* (NE) which is a rare corticioid. Other species that were included in this list, but are not polypores were: *Mucronella bresadolae* which is an aphyllophoroid wood inhabiting fungus, and *Botryobasidium medium* which is also a corticioid fungus (Kunttu et al., 2015). Other fungal species worth mentioning since they are indicator species, but not in the red list (LC), were *Phellinus viticola*, *P. undosa* and *Pseudographis pinicola* (Nitare and Skogsstyrelsen, 2019).

The total list of these red listed species from Bø forest can be seen in table 4, which includes number of reads. In Bø forest *Phlebia subtulata* was found on one log (01795), together with *M. bresadolae* and *F. rosea*. *Skeletocutis delicata* was found on log one log (01794) together with *F. citrinellus*. *B. medium* was found on one log (01792) together with *P. nigrolimitatus.*

P. liascens was found on one log (01791) with *F. rosea*. The logs 01791, 01793 and 01795 had fruitbodies of *F. rosea* (Lien, 2023).

Table 2 Reads from the location of Bø forest showing fungal species with the IUCN categories VU and NT, in addition to one corticioid fungus in the NE category.

Sp.	**IUCN Category**	**No. of reads**
Phlebia subulata	VU	40
Skeletocutis delicata	VU	5510
Botryobasidium medium	NT	229
Fomitopsis rosea	NT	6266
Mucronella bresadolae	NT	29
Phellopilus nigrolimitatus	NT	1462
Flaviporus citrinellus	NT	161
Phlebia liascens	NE	19634

Mørkvasslia had less species in these categories, and a total list including the number of reads which can be found in table 5. *Skeletocutis brevispora* was found in one log (01783), and *S. delicata* was found in a single log as well (01781). *P. nigrolimitatus* was found in both log no. 01782 and 01785, in the log last mentioned *P. viticola* (LC) was also present. Another indicator species, *P. pinicola* (LC), was found in log no. 01784. Even though *F. rosea* did not appear as DNA, it was found as a fruitbodies on logs no. 01782 and 01785 (Lien, 2023).

Table 3 Reads from the location of Mørkvasslia showing fungal species with the IUCN categories VU and NT, in addition to one corticioid fungus in the NE category.

Sp.	**IUCN Category**	**No. of reads**
Skeletocutis brevispora	VU	1643
Skeletocutis delicata	VU	22
Phellopilus nigrolimitatus	NT	3922

Lastly, Nome forest had the least number of species in these red list categories which are shown in table 6. There were no species in the VU category, and only one NE species, *P. nigrolimitauts* which was found in log no. 01789. *P. liascens* was found on a different, log no. 01790. The indicator species, *P. undosa* (LC), was found in log no. 01788.

Table 4 Reads from the location of Nome forest showing one fungal species in the IUCN category NT, in addition to one corticioid fungus in the NE category.

Sp.	**IUCN Category**	**No. of reads**
Phellopilus nigrolimitatus	NT	172
Phlebia liascens	NE	244

3.3 Inoculation monitoring:

In terms of the results related to monitoring of the inoculation experiment, the number of ASV's were not adequate for further statistical analyses. *A. lapponica* was not found at all, neither as a fruitbody nor DNA. *F. rosea* were found in three samples (bc69, bc72 & bc75) and *P. nigrolimitatus* in six samples (bc9, bc30, bc35, bc50, bc59 & bc63), still there is no evidence that the species might already have been present in the log prior to inoculation. In 2016, Maurice (*pers. comm.*) recorded the fruit bodies of each log, and Figure 11 illustrates the presence or absence of F. pinicola and F. rosea. The second figure 12 displays the presence/absence in 2022 of both fruitbody occurrence surveyed by Lien (2023), or ASV of *F. pinicola, F. rosea* and *P. nigrolimitatus* in each log.

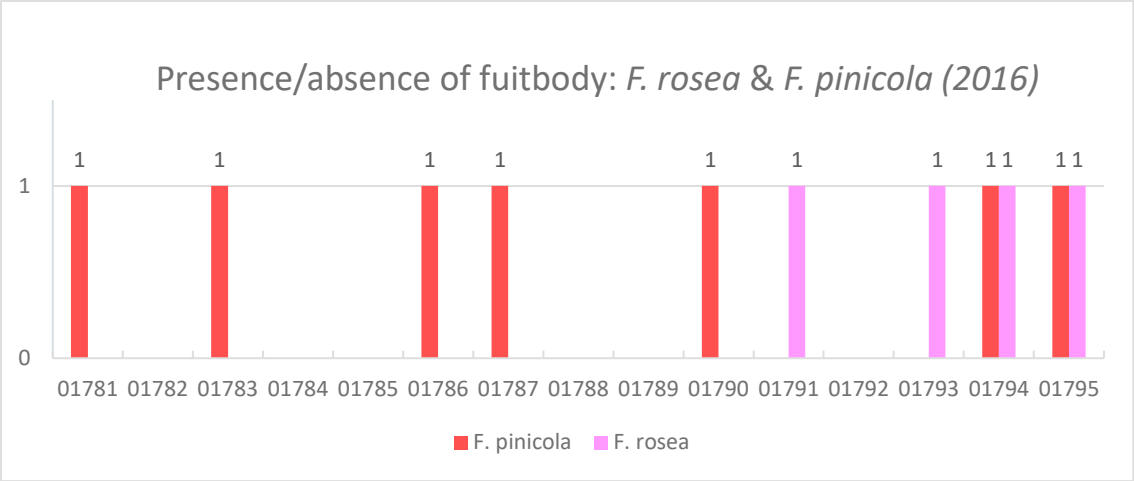

Figure 11 Barplot showing presence (1) and absence (0) of F. rosea, F. pinicola (y-axis), in the sample logs with tree ID (x-axis). Their presence is recorded as fruitbody in 2016. Mørkvasslia: 01781-01785, Nome forest: 01786-01790 & Bø forest: 01791-01795.

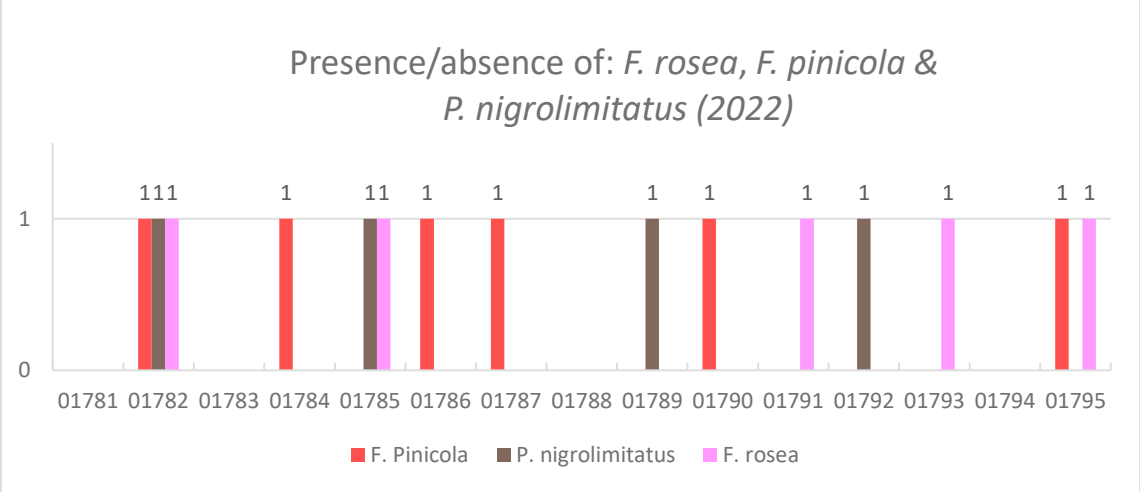

Figure 12 Barplot showing presence (1) and absence (0) of F. rosea, F. pinicola and P. nigrolimitatus (y-axis), in the sample logs with tree ID (x-axis). Their presence is either noted as fruitbody in 2022 or as ASV's. Mørkvasslia: 01781-01785, Nome forest: 01786-01790 & Bø forest: 01791-01795.

4 Discussion

4.1 Differences in community assembly and richness:

The fungal community in Mørkvasslia and Bø forest clustered together in the principal coordinate analysis, while most samples from Nome forest formed a separate cluster. This clustering indicates that Mørkvasslia and Bø forest have some habitat characteristics in common, while Nome forest is somewhat different. The importance of location in shaping the fungal community was supported by the Permanova analysis, which also found that decay stage and log diameter were important factors. The importance of decay stage may be exemplified by the samples from Nome forest which showed similarities to the other two areas. These samples were all taken from log no. 01789, which was in decay stage 3 at the time of inoculation, but was almost completely decomposed in 2022 (Lien, 2023). This deadwood log was the only sampled log that was in a moderate state of decay in 2016, while all the other samples were in early decay stages (1a-2) at the time.

The similarities observed in the community of this late decay stage deadwood log in Nome forest and logs from the other two sites, could potentially be attributed to the process of succession. As the log of Nome forest enters late stages of decay, it creates favorable conditions for a wider array of fungal species to establish. Norberg et al. (2019) states that polypore communities tend to become more similar along succession. Therefore, a possible explanation for log no. 01789 being similar to logs from Bø forest and Mørkvasslia might be due to its more advanced state of decay having allowed for a similar community.

In this study, the decay stage of 2016 was identified as one of the factors that had the greatest impact on community assembly, which is similar to the findings of Runnel et al. (2021). The species present in early decay stages of deadwood has been documented to influence the later composition of successor species through what is called priority effects (Ottosson, 2013). Therefore, it is likely through priority effects that the decay stages recorded in 2016 are still significant in 2022. The assembly is also determined by the number of successor species available at the site (Hiscox et al., 2016). Thus, priority effects may explain why the community assembly in Nome forest is different from the rest. For a site to host many successor species, especially rare polypores, there must be a low degree of fragmentation (Nordén et al., 2013). Consequently, for the rare successor polypores to disperse effectively, it

is necessary for the sites to have forest connectivity. Thus, the results may indicate a lower degree of connectivity in Nome forest.

Lien (2023) found a wider range of decay stages in Mørkvasslia and Bø forest, whereas deadwood in Nome forest consisted of predominantly early decay. The abundance of deadwood in early decay stages in Nome creates favorable conditions for species with ruderal strategies to thrive and establish themselves. While the wider range of decay in Mørkvasslia and Bø forest promotes higher abundance and variety of species within those ecosystems. Abrego et al. (2017) discovered that species exhibiting tolerance to or benefiting from management practices were more abundant in managed forests, particularly early decay species referred to as r-selected or pioneer species. Therefore, the low species richness and low occurrence of rare species in Nome forest may be attributable to consisting of mostly r-selected species, and may have caused the distinct community assembly in this site.

The diameter of the logs also affected community assembly. This matches with results from earlier studies, which document the importance of trunk diameter, especially for rare polypores (Nordén et al., 2013, Junninen and Komonen, 2011, Penttilä, 2004). This makes sense, as most r-selected polypores do not necessarily need large volumes of deadwood, while k-selected often do (Rayner, 1988). Therefore, volume of deadwood can serve as a limiting factor for k-selected species. To sustain populations of species employing this strategy, it is imperative to maintain a diverse range of sizes in the available CWD.

It was further observed that there was a greater difference in community assembly between sites, than in diversity within samples. However, there was a significant difference in species richness between locations. Many factors have been found to influence species richness of wood decaying fungi when comparing managed and natural forests (Runnel et al., 2021, Abrego and Salcedo, 2013, Tomao et al., 2020). The high richness observed in Mørkvasslia can likely be explained by its protection and low level of human interference. While Bø forest had an intermediate species richness, which indicates that it might be heading toward a more natural state, if the forest is left without major human disturbances.

Nome forest was the site with the lowest overall richness. This site was also the area with the highest number of reads of the combative *F. pinicola*. According to Lindner et al. (2011), they found a simpler community structure and greater mass loss in samples where *F. pinicola* became dominant. This could mean that the low richness in Nome forest might be explained

by the dominance of *F. pinicola*. In this forest, some of the logs were found in piles of deadwood where this combative species seemed to have targeted large amounts of resources. This is a behavior commonly observed for this species (Norberg et al., 2019). This study highlights the ability of *F. pinicola* to target large volumes of deadwood and effectively hinder the colonization of other species.

In contrast to *F. pinicola*, Lindner et al. (2011) also found *R. bicolor* to decompose deadwood at a slower rate, which was found to promote species richness. This matches with observations from Mørkvasslia, which had the highest species-richness, and *R. bicolor* as the second-most abundant species.

4.2 Monitoring of rare species:

Metabarcoding is an emerging field of study which can be a cost and time-efficient tool for surveying forest ecosystems. Rare species are especially important for determining habitat quality, and many of these can be found on red lists. Traditional surveying has long been the established method of mapping polypore communities; however this method requires a high level of specialized knowledge and skills, which only a few people possess. Furthermore, species that do not have distinct or special features, or demand microscopy are the ones most likely to be overlooked or misidentified by untrained identifiers. Knowledge about characteristics is not the only thing that is needed to be a good surveyor, knowledge about the ecology such as habitat niche e.g., growing placement on the trunk, and preferred decay stage is also needed. In comparison, metabarcoding requires less specialized training.

Nevertheless, metabarcoding does not always give the same complete overview of species composition as a professional mapping. This is demonstrated in my data. When comparing my ASV's with data from Mørkvasslia from previous surveys, I only captured 3 of the 14 rare spruce-living polypores that have been observed in Mørkvassjuvet (Artsobservasjoner.no, n.d.). One of them, *P. nigrolimitatus* could have entered through inoculation. This means that very few species were captured, but it was also hard to estimate the exact accuracy of metabarcoding when only a few logs were compared to the entire nature reserve. Additionally, *F. rosea* did not appear as an ASV, but was observed as fruitbodies on some logs. The fact that this rare species was not recorded as an ASV in Mørkvasslia at all, points to a possible disadvantage with the metabarcoding method. However, the benefit of the

metabarcoding method is that it reduces time spent in the field and can still give an important description of the communities within the deadwood.

However, the two rare species *S. brevispora* and *S. delicata* were discovered using metabarcoding in this study. Only *S. brevispora* has been recorded in Mørkvassjuvet by other surveyors (Artsobservasjoner.no, n.d.). This might be because the taxonomy of *Skeletocutis spp*. was recently changed in 2018 (Miettinen and Niemelä, 2018), and *S. brevispora* and *S. delicata* might have been recorded as the same species. Recent taxonomical changes like these might be harder for a non-specialist surveyor to identify differences based on small color nuances or fruitbody characteristics, especially if the surveyor has no training in microscopy which might be needed to separate these two *Skeletocutis spp*. Therefore, metabarcoding can be used to identify rare species with characteristics that require a certain level of expertise in polypore morphology.

Bø forest contained a larger list of rare species (table 4) compared to Mørkvasslia. This is noteworthy because there have not been any direct surveys of this study area. The knowledge obtained from metabarcoding can be used for a later traditional surveying of fruitbodies. By using metabarcoding as a snapshot of the potential species one might find, this can give some hints to what species the surveyor might be on the lookout for when in the field.

Combining traditional mapping with metabarcoding would be the best option, since both methods have their own limitations. This is also the conclusion which Ottosson et al. (2014), Ovaskainen et al. (2013) and Saine et al. (2020) came to. Fruitbody surveying is important to identify which polypores are reproducing, and thereby estimating the population, while metabarcoding can be used to record whether rare or recently described species are present in the area. DNA samples also give more leeway for renaming species after a survey, by simply redoing the taxonomic inference to a reference database.

4.3 Inoculation monitoring and community interactions:

The project has been ongoing since 2016, with monitoring of fruitbody development for many years. In 2018 the logs were surveyed for fruit bodies of the inoculated species, but few were recorded. According to the documentation of Maurice (*Pers. comm.),* there seemed to be more visible mycelia in the logs of Mørkvasslia compared to the other sites. There has also been some sporadic surveying of some logs before this study, but no fruitbodies of special interest have been sighted. Lien (2023) surveyed 11/15 logs and found visible fruitbodies on all of

these, but of the inoculated species only *F. rosea* was observed. *P. nigrolimitatus* was only found as an ASV, while *F. rosea* was found as fruitbodies and ASV, and *A. lapponica* was not found at all. Although two out of three inoculated species were detected, the number of reads for these were too low for statistical testing. Therefore, the inoculation could not be deemed successful.

However, inoculation has been proven to be viable, as Abrego et al. (2016) documents a successful inoculation. They state that the inoculation was more successful in deadwood of early decay stages possibly due to competitive interactions with the species already established in the deadwood element. They did not use *P. nigrolimitatus*, but *A. lapponica* and *F. rosea* were included in their study. The inoculation in 2016 was mostly done in early decay stages, meaning that another factor might possibly have influenced the result of this inoculation. Additional research is necessary to investigate and comprehend the factors that may have led to the limited effectiveness of this inoculation.

The absence of *A. lapponica* in the study could indeed be attributed to the sampling effort. Alfredsen et al. (2014) mentions that *A. lapponica* without a fruitbody, tended to have its mycelia in the core of less decayed logs. During the sampling process of this thesis, many of the logs were quite decayed and often had a rotten core. When sampling the less rotten logs, the drilling depth was more inconsistent which might have led to an avoidance of the possible mycelia.

In my study, I had fewer logs in addition to fewer drill points, compared to other studies that have successfully used metabarcoding to detect the species content of deadwood (Ottosson et al., 2014, Ovaskainen et al., 2013, Abrego et al., 2016, Kubartová et al., 2012). For example the study of Kubartová et al. (2012) found sampling placement on the log varied significantly, but my study did not find this to be significant. Whether this is due to the sampling effort, or some other factor cannot be verified.

The additional workload of increasing sampling points from six to ten samples would have been too time-consuming for this thesis, given that the number of samples for this thesis is 75, which is a high number of samples. One potential method to increase the sample size would be to merge multiple samples into a single sample for DNA extraction. A practical approach would be to combine two sawdust samples into a single composite sample, effectively

doubling the number of samples obtained from each log. This can be suggested method for future studies, in addition to using liquid nitrogen before grounding the samples into powder.

While the inoculation did not yield significant results, there is potential to further investigate the log content based on presence/absence using studies that have established significant relationships between polypore species. Although beyond the scope of this thesis, the observations from this study can inform future research, as additional testing is needed to determine the significance and validity of these potential interactions.

Many different interactions can be produced in deadwood, and most changes in community structures are driven by antagonistic interactions (Hiscox et al., 2016). Nonetheless, certain species can occur together without their coexistence necessarily involving interaction, as it could be that the species simply prefer the same habitat niches (Alfredsen et al., 2014). However, some relationships between polypores can be either be significantly positive or negative (Ottosson et al., 2014, Ovaskainen et al., 2013).

One of the most combative and common species of polypore is *F. pinicola*. It is an r-selected opportunist which can quickly decay large elements of deadwood (Norberg et al., 2019). A comparison between *F. pinicola* and the two inoculated species was made in figures 11 and 12. Ottosson et al. (2014) found that *F. rosea* does not have that many antagonistic relationships, but a positive relationship with *F. Pinicola*. Furthermore, they document a negative relationship between *P. nigrolimitatus* and *F. Pinicola*. Both of which, Ottosson et al. (2014) suggests to have negative relationship with *Hetrobasidion spp*.

The presumed negative relationship between *F. pinicola* and *P. nigrolimitatus* matches with the fact that these two species were almost never recorded together. Except for one log in Mørkvaslia (no. 01782) where *F. pinicola* was found together with of *P. nigrolimitatus.* In this log *F. pinicola* was only recorded as a fruitbody by Lien (2023), and not as an ASV. *F. rosea* was also present in this log as a fruitbody. In addition to these two combative species, *Hetrobasidion spp.* was also found with a high number of reads. Suggesting a potential ongoing interaction.

For future studies on this deadwood community, it would be interesting to monitor the interactions further along succession. As Ottosson et al. (2014) highlights, there is a limited understanding about the impact of *Hetrobasidion spp*. on fungal wood-decay communities. It

might also be of interest to monitor the fruitbody size of *P. nigrolimitatus*, considering the previously discussed interactions. Ovaskainen et al. (2013) found that *P. nigrolimitatus* produces large fruitbodies when it establishes dominant mycelial positions. A comparison of fruitbody sizes of *P. nigrolimitatus* between this log and other logs would have been interesting to measure, to record whether the interactions could possibly have had an impact on fruitbody size. Still, it is important to acknowledge the influence of other factors that might impact fruitbody production and community dynamics. To get a comprehensive understanding of the community structure in a detailed study, it would be essential to also consider abiotic factors like soil pH, moisture levels, and nutrient availability, as these factors been recognized to also influence community dynamics (Norberg et al., 2019, Hiscox et al., 2016, Fukami et al., 2010).

Conclusion

The main discovery from this study is that metabarcoding has an advantage in detecting variations in community composition even when using a limited number of samples. Metabarcoding methods serve as a valuable tool to obtain a snapshot of species present in a given area, while also enabling the detection of recently described rare polypore species. By first assessing the richness and rarity of species through metabarcoding, informed decisions can be made regarding the need for more extensive traditional surveys and conservation efforts in specific areas.

Metabarcoding provides us with the advantage of studying real-time community changes, allowing us to observe the mechanisms involved in the formation of a fungal community rather than solely relying on the end result of fruitbody production. Through metabarcoding-based monitoring of polypores, one can capture the assembly of the current community and over time, potentially observe interactions between polypores and other wood-decaying fungi as the community undergoes changes due to succession provided by interactions and other influencing factors.

Overview of Figures and Tables

		represented by color: Bø forest (red), Mørkvasslia (green) & Nome forest (blue). And the variable "Decay stage (2016)" is represented by geometric shape: 1a (circle), 1b (triangle), 2 (square) & 3 (cross).
9	27	Genera found in samples bc21, bc23, bc33, bc41 & bc8.
10	29	Barplot showing the percentages of species reads and their IUCN categories of Bø forest, Mørkvasslia and Nome forest. The abbreviations and label of the categories: LC – least concern (green), NE – not evaluated (blue), NT – near threatened (light green) & VU - vulnerable (yellow). There are most red listed species in Bø forest (left) and Mørkvasslia (middle). Bø forest has the most species in the NE category. Nome forest (right) has the least species in the red list.
11	32	Barplot showing presence (1) and absence (0) of F. rosea, F. pinicola (y-axis), in the sample logs with tree ID (x-axis). Their presence is recorded as fruitbody in 2016. Mørkvasslia: 01781-01785, Nome forest: 01786-01790 & Bø forest: 01791-01795.
12	32	Barplot showing presence (1) and absence (0) of F. rosea, F. pinicola and P. nigrolimitatus (y-axis), in the sample logs with tree ID (x-axis). Their presence is either noted as fruitbody in 2022 or as ASV's. Mørkvasslia: 01781-01785, Nome forest: 01786-01790 & Bø forest: 01791-01795.

Tables		
Number	Pg.	Description
1	16	The primers used for amplifying the extracted DNA from the sawdust samples. The primer pairs consist of a forward primer (fiTS7 F) and a reverse primer (ITS4 R).
2	22	The top three fungus species with the highest sum of reads in each location
3	28	PERMANOVA test explaining the differences between response variable bray-curtis distance and explanatory variables: decay stage (2016), trunk diameter & length.

4	30	Reads from the location of Bø forest showing fungal species with the IUCN categories VU and NT, in addition to one corticioid fungus in the NE category.
5	30	Reads from the location of Mørkvasslia showing fungal species with the IUCN categories VU and NT, in addition to one corticioid fungus in the NE category.
6	31	Reads from the location of Nome forest showing one fungal species in the IUCN category NT, in addition to one corticioid fungus in the NE category.

Appendix 1

The table contains an overview of the samples' concentrations and quality of the DNA after extraction. In addition to where the sample was taken on the logs, assigned barcodes with log ID, location. Decay stage, middle diameter and length measured by Maurice (pers. comm.) in 2016. Decay stage 2022 was measured by Lien (2023). The samples with grey background were not sequenced.

Abbreviations: Sap. Pl. = sample placement, seq. = sequenced, cons. (ng/µl) = DNA concentration (ng/µl) & mid-dm (cm) = middle diameter (cm).

Sam. Pl.	Sq.	Bc.	Log ID	Cons. (ng/ul)	A60/A80 ratio	Decay 2016	Decay 2022	Location	Mid-dm (cm)	Length (cm)
C	No	bc11	01781	2.3	1.53	1b		Mørkvasslia	16	20.58
E	Yes	bc12	01781	1.5	1.52	1b		Mørkvasslia	16	20.58
A	Yes	bc23	01781	0.1	1.52	1b		Mørkvasslia	16	20.58
D	Yes	bc25	01781	0.8	1.45	1b		Mørkvasslia	16	20.58
B	No	bc37	01781	4.5	1.51	1b		Mørkvasslia	16	20.58
C	Yes	bc9	01782	7.7	1.81	2	3	Mørkvasslia	16.2	14.7
A	Yes	bc30	01782	1.9	4.83	2	3	Mørkvasslia	16.2	14.7
D	Yes	bc34	01782	2	2.53	2	3	Mørkvasslia	16.2	14.7
B	Yes	bc41	01782	2	1.68	2	3	Mørkvasslia	16.2	14.7
E	No	bc54	01782	1.1	1.47	2	3	Mørkvasslia	16.2	14.7
C	Yes	bc21	01783	1.4	1.39	1b		Mørkvasslia	15.2	10.55
D	Yes	bc31	01783	1.4	2.01	1b		Mørkvasslia	15.2	10.55
B	No	bc51	01783	3.6	1.56	1b		Mørkvasslia	15.2	10.55
E	No	bc62	01783	1	1.47	1b		Mørkvasslia	15.2	10.55
A	No	bc70	01783	0.1	0.06	1b		Mørkvasslia	15.2	10.55
A	Yes	bc8	01784	1.4	1.32	1a	2	Mørkvasslia	7	18.2
C	No	bc13	01784	1.2	1.39	1a	2	Mørkvasslia	7	18.2
B	Yes	bc20	01784	4.6	1.87	1a	2	Mørkvasslia	7	18.2
D	Yes	bc33	01784	3.9	1.82	1a	2	Mørkvasslia	7	18.2
E	No	bc66	01784	0.4	1.59	1a	2	Mørkvasslia	7	18.2
A	No	bc10	01785	1.7	1.47	1b	2	Mørkvasslia	16	16.1
D	Yes	bc39	01785	1.2	1.74	1b	2	Mørkvasslia	16	16.1
E	Yes	bc59	01785	0.8	1.12	1b	2	Mørkvasslia	16	16.1
B	No	bc67	01785	1.9	1.73	1b	2	Mørkvasslia	16	16.1
C	No	bc68	01785	1.4	10.08	1b	2	Mørkvasslia	16	16.1
D	No	bc16	01786	2.4	1.57	1b		Nome forest	21	22.9
C	Yes	bc22	01786	4.2	1.57	1b		Nome forest	21	22.9

B	No	bc48	01786	3	1.75	1b		Nome forest	21	22.9
E	Yes	bc49	01786	3.8	1.52	1b		Nome forest	21	22.9
A	Yes	bc65	01786	2.5	1.67	1b		Nome forest	21	22.9
B	Yes	bc3	01787	1.8	2.12	2	2	Nome forest	12	17.5
E	Yes	bc17	01787	2.6	1.89	2	2	Nome forest	12	17.5
A	Yes	bc56	01787	2.7	1.72	2	2	Nome forest	12	17.5
D	No	bc57	01787	3	1.77	2	2	Nome forest	12	17.5
C	No	bc60	01787	5.5	1.77	2	2	Nome forest	12	17.5
E	Yes	bc2	01788	3	1.7	2	2	Nome forest	23	15.9
A	Yes	bc45	01788	4.3	1.74	2	2	Nome forest	23	15.9
C	Yes	bc52	01788	0.5	1.86	2	2	Nome forest	23	15.9
D	No	bc55	01788	1.2	1.57	2	2	Nome forest	23	15.9
B	Yes	bc61	01788	3.4	1.61	2	2	Nome forest	23	15.9
D	Yes	bc1	01789	6.3	1.7	3	3	Nome forest	15	9.8
E	Yes	bc26	01789	5	1.58	3	3	Nome forest	15	9.8
A	Yes	bc35	01789	5.3	1.63	3	3	Nome forest	15	9.8
C	Yes	bc38	01789	5.1	1.68	3	3	Nome forest	15	9.8
B	Yes	bc47	01789	5.6	1.56	3	3	Nome forest	15	9.8
D	Yes	bc15	01790	4.1	1.57	2	3	Nome forest	15	11.4
C	Yes	bc27	01790	4	1.49	2	3	Nome forest	15	11.4
A	No	bc36	01790	3.1	1.48	2	3	Nome forest	15	11.4
E	Yes	bc42	01790	2.1	1.74	2	3	Nome forest	15	11.4
B	Yes	bc64	01790	8.1	1.61	2	3	Nome forest	15	11.4
A	Yes	bc71	01791	5.7	1.75	1b	4	Bø forest	21	10.1
B	Yes	bc72	01791	3.6	1.78	1b	4	Bø forest	21	10.1
C	No	bc73	01791	2.6	1.71	1b	4	Bø forest	21	10.1
D	Yes	bc74	01791	4.1	1.78	1b	4	Bø forest	21	10.1
E	Yes	bc75	01791	3.5	1.74	1b	4	Bø forest	21	10.1
B	Yes	bc40	01792	1.1	1.02	2		Bø forest	23	9.1
E	Yes	bc43	01792	0.9	1.1	2		Bø forest	23	9.1
D	Yes	bc50	01792	3.5	2.08	2		Bø forest	23	9.1
C	Yes	bc53	01792	2.7	1.84	2		Bø forest	23	9.1
A	Yes	bc63	01792	1.6	1.24	2		Bø forest	23	9.1
C	Yes	bc4	01793	3.1	1.53	2	4	Bø forest	20	14.4
A	Yes	bc5	01793	2	1.6	2	4	Bø forest	20	14.4
D	Yes	bc14	01793	2.5	1.5	2	4	Bø forest	20	14.4
E	No	bc28	01793	2.1	1.9	2	4	Bø forest	20	14.4
B	Yes	bc46	01793	2	1.78	2	4	Bø forest	20	14.4
B	Yes	bc24	01794	2	1.92	2		Bø forest	22	16.6
A	No	bc29	01794	2.1	1.42	2		Bø forest	22	16.6
E	Yes	bc32	01794	1.1	1.29	2		Bø forest	22	16.6
C	No	bc44	01794	1.8	1.99	2		Bø forest	22	16.6
D	No	bc58	01794	2.1	1.29	2		Bø forest	22	16.6

A	No	bc6	01795	1.4	1.49	2	3	Bø forest	19	13.3
D	Yes	bc7	01795	0.7	1.12	2	3	Bø forest	19	13.3
E	No	bc18	01795	1.2	1.39	2	3	Bø forest	19	13.3
B	Yes	bc19	01795	2.8	1.96	2	3	Bø forest	19	13.3
C	Yes	bc69	01795	0.6	1.02	2	3	Bø forest	19	13.3

Appendix 2

The table provides a comprehensive list of species found in each forest, including the number of reads and their corresponding IUCN category from the Norwegian Red List 2021.

Bø forest	Mørkvasslia	Nome forest	Species	IUCN category
40	0	0	*Phlebia subulata*	VU
0	1643	0	*Skeletocutis brevispora*	VU
5510	22	0	*Skeletocutis delicata*	VU
229	0	0	*Botryobasidium medium*	NT
6266	0	0	*Fomitopsis rosea*	NT
29	0	0	*Mucronella bresadolae*	NT
1462	3922	172	*Phellopilus nigrolimitatus*	NT
161	0	0	*Flaviporus citrinellus*	NT
66	318	235	*Carcinomyces polyporinus*	NE
290	42	328	*Claussenomyces prasinulus*	NE
0	217	40	*Colacogloea peniophorae*	NE
695	11	0	*Dacrymyces fennicus*	NE
0	72	8	*Dacrymyces lacrymalis*	NE
180	30	7	*Dacrymyces ovisporus*	NE
3407	797	1487	*Hyaloscypha aureliella*	NE
17	175	551	*Hyphodiscus hymeniophilus*	NE
0	0	23	*Orbilia xanthostigma*	NE
19634	0	244	*Phlebia lilascens*	NE
0	0	20	*Syzygospora effibulata*	NE
1670	23	2750	*Amyloxenasma allantosporum*	LC
6711	25719	3229	*Antrodia serialis*	LC
2563	689	1031	*Athelia decipiens*	LC
9	0	24	*Athelia epiphylla*	LC
0	0	5	*Athelopsis glaucina*	LC
47	0	3918	*Basidiodendron caesiocinereum*	LC
0	0	38	*Boidinia furfuracea*	LC
186	0	23	*Botryobasidium conspersum*	LC
139	1654	0	*Botryobasidium intertextum*	LC
72	2236	8198	*Botryobasidium laeve*	LC
2396	0	1795	*Botryobasidium subcoronatum*	LC
0	50	78	*Calocera furcata*	LC
270	681	49	*Calocera viscosa*	LC
5557	1800	0	*Chlorencoelia versiformis*	LC
215	0	0	*Chlorociboria aeruginosa*	LC
1196	105	21067	*Dacrymyces stillatus*	LC
0	227	54707	*Fomitopsis pinicola*	LC
0	367	0	*Galerina marginata*	LC
0	17	0	*Galzinia incrustans*	LC
0	194	0	*Gyromitra esculenta*	LC

0	0	65	*Gyromitra infula*	LC
0	0	249	*Hymenochaete fuliginosa*	LC
363	168	72	*Hyphoderma definitum*	LC
1705	3549	14667	*Hyphodontia pallidula*	LC
49	0	0	*Lactarius cyathuliformis*	LC
1056	0	0	*Lobulicium occultum*	LC
86	2621	40	*Mollisia cinerea*	LC
0	87	0	*Mycena epipterygia*	LC
0	0	64	*Mycena leptocephala*	LC
1104	0	0	*Mycena maculata*	LC
383	4066	8	*Mycena stipata*	LC
5893	0	22	*Mycena zephirus*	LC
14	0	0	*Peniophora pithya*	LC
170	0	0	*Peniophorella pallida*	LC
351	1037	3546	*Peniophorella praetermissa*	LC
0	14863	0	*Phellinus viticola*	LC
0	9	0	*Phlebia segregata*	LC
0	44	87	*Postia cyanescens*	LC
0	0	706	*Postia fragilis*	LC
37219	0	0	*Postia leucomallella*	LC
0	0	20	*Postia tephroleuca*	LC
0	0	7408	*Postia undosa*	LC
0	6	0	*Pseudographis pinicola*	LC
0	20036	8973	*Resinicium bicolor*	LC
0	3	0	*Rhytisma acerinum*	LC
0	30	0	*Sistotrema autumnale*	LC
0	1844	0	*Sistotrema brinkmannii*	LC
2079	0	955	*Sphaerobasidium minutum*	LC
0	0	328	*Trechispora farinacea*	LC
9	0	0	*Trechispora hymenocystis*	LC
39252	9117	16262	*Tubulicrinis borealis*	LC
15155	6906	6559	*Tubulicrinis subulatus*	LC
0	0	82	*Tylospora asterophora*	LC
0	0	72	*Tylospora fibrillosa*	LC
0	1198	0	*Veluticeps abietina*	LC
280	0	0	*Xenasmatella tulasnelloidea*	LC
7177	603	0	*Xeromphalina campanella*	LC
45325	20	20	*Xylodon asperus*	LC
567	12012	0	*Xylodon brevisetus*	LC
830	0	0	*Xylodon rimosissimus*	LC

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