UiT The Arctic University of Norway

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Beneath the Surface: Fungal Community Associated with Brown Alga *Saccharina latissima*

A Molecular Characterization through DNA Extraction, PCR Amplification, Nanopore sequencing and BLAST Analysis

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Abstract

Fungi can be found in every marine habitat, but our knowledge about their diversity, function, and potential is still limited[1, 2]. Studies show that marine fungi play an important role in ecosystems by contributing to nutrient cycling and forming symbiotic relationships with marine organisms[3]. *Saccharina latissima*, also known as sugar kelp, is a brown algae with a circumpolar distribution in the Northern Hemisphere and has generated increased interest for cultivation in Europe and Norway[4].

The objective of this study was to identify the fungal communities on *S. latissima* through molecular characterization using DNA extraction, PCR amplification, Nanopore sequencing, and BLAST analysis. I aimed to investigate the fungal communities on *S. latissima*, the diversity of fungi in wild *S. latissima* compared to cultivated, and the diversity changes of fungi throughout the seasons. This research would expand the knowledge about marine fungi associated with *S. latissima*, laying the foundation for future research to ensure the sustainable cultivation of *S. latissima*.

I collected 12 *S. latissima* samples from a kelp forest outside the student diving club in Tromsø and 8 samples from a kelp cultivation facility at Krakens operated by Akvaplan-niva and Oceanfood. The samples were collected from February 2023 to October 2023.

I encountered difficulties in my methodology. The extraction of fungal DNA for the algal samples was not optimal, and I hypothesize that inhibitors were in the DNA extracts and did inhibit further analysis. These inhibitors could have inhibited DNA amplification or our choice of primers and blocking oligonucleotide were suboptimal and could limit the amplification of fungal DNA. Further, the purification of PCR products before sequencing resulted in a loss of DNA, making some samples unviable for sequencing due to low DNA concentration.

Nevertheless, I was able to document the presence of 10 different fungal taxa within an individual *S. latissima* sampled from the kelp forest. Future research should focus on optimizing DNA extraction and PCR amplification of fungal DNA from *S. latissima* and other kelp and brown algal species.

Key words: Marine Fungi, Fungal diversity, Kelp cultivation, *Saccharina latissima*, DNA extraction, PCR amplification, Inhibitors in DNA extraction

Acknowledgments

Most people are unaware of marine fungi. Going into this project, I knew they existed, but over the years, my fascination with mycology and marine mycology has grown a lot. Seeing how fungi, especially marine fungi, play such a vital role in our ecosystems and are studied so little is frustrating but has also inspired me.

I would like to thank my supervisor, Teppo Rämä. When I first approached him, he was as excited as I was to talk about fungi in an ecosystem perspective and look at marine fungi in their natural habitat and in a cultivated facility. We took a chance at each other and created a Master project together. Doing things that have not been done before, or at least with suboptimal methods, was a big challenge. I am forever grateful for all the time Teppo allocated to smash his head together with mine, against a brick wall to try to think of what the problem might be.

Chun Li, Hosea Masaki, Ole Christian Hagestad, Yannik Karl Heinz Schneider and Jeanette Hammer Andersen at Marbio and Marbank have also been a great help in the lab, in writing process, and troubleshooting different aspects of this master project. Thank you so much for the time and energy you have put in.

At Akvaplan-Niva, in collaboration with Oceanfood at Kraknes, I gathered cultivated algal material for this study. It's encouraging and motivating to be a part of research that can affect the industry, especially in the novel industry of kelp cultivation in Norway. I would like to especially thank Marianna Frantzen, Trond Ivarjord, and Laurids Enevoldsen from Akvaplan-Niva, and Renate Larsen from Oceanfood for allowing me to sample and join in the kelp cultivation.

In a speech at the graduation of my master's studies, a friend and fellow student, Sanne Marie L'Abée-Lund, said it takes a village to get a master's student finished, and that is very true. I want to thank my family, my partner, my friends, my fellow master's student in biology, my office mates, and the rest of Marbio and MarBank for holding my head above water as I dove deep into the kelp forest and the marine fungi.

I also want to thank Jan Fredrik Frantzen for collaborating in the making of a video and article that we published at Forskerhjørnet (UIT) and Forskersonen (forsking.no) about this study.

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Abbreviations

Abbreviation	Full Term
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
CTAB	Cetyltrimethylammonium Bromide
SSU	Small Subunit
18S	18S Ribosomal RNA (rRNA) gene
rDNA	Ribosomal DNA
gDNA	Genomic DNA
POM	Particulate Organic Matter
DOM	Dissolved Organic Matter
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
OTU	Operational Taxonomic Unit
SUT	The Tromsø Student Diving Club
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
SILVA	SILVA Ribosomal RNA Database Project
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance

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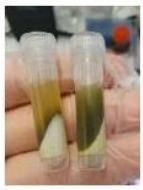


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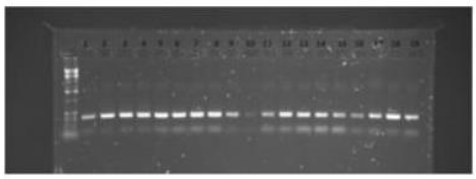

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1 Introduction

Marine fungi can be found in every habitat, but our knowledge about their diversity, function, and potential is still limited[1, 2]. Marine fungi can appear as parasites and pathogens of other organisms, mutualistic symbionts, saprotrophs, and play functional roles in nutrient cycling, biogeochemical processes, and food web dynamics in the oceans[3]. They are important not only in the ecosystems but also in human society. New metabolites found in marine fungi are currently being studied for their antibacterial, antiviral, and anticancer activities[2]. Today we have discovered around 2,000 marine fungal species. Conservative estimates suggest that there are 10,000 – 16,000 species, other studies suggest that there might be up to a million marine fungi species[5].

While we are still unraveling the potential of marine fungi and their role in ecosystems and medicine, we can also connect them to sustainable food production. As the human race increases in number, we threaten ecosystems and their function on a global scale [6-9]. Our increased numbers intensifies the exploration of new methods for agriculture, and low trophic species in the ocean are of special interest. In Norway and globally, one of the alternatives to terrestrial agriculture can be kelp. One of the species that is in focus in Norway, is sugar kelp (*Saccharina latissima*)[10]. However, introducing large kelp farms in the ocean, and fjord systems, might deplete nutrients, and introduce challenges with pathogenic species affecting the cultivated and wild kelp in the area. In Asia, commercial cultivation of kelp started in the 1920s, and in recent years due to higher intensification of aquaculture activity, an increased prevalence of diseases and pests has been observed [11-13]. To effectively cultivate kelp without harming the environment, there is a need for more knowledge about disease causing agents and the role of fungi in kelp. By better understanding marine fungi and their role in ecosystems and their importance for the kelp host, we can better ensure sustainable food production.

1.1 Fungi

Fungi are found in every ecosystem in the world. They differ greatly, from small, microscopic unicellular organisms to widely integrated huge organisms stretching over several kilometers[14]. Today, we estimate approximately 1-5 million different fungal species, but more precise estimates suggest 2.2-3.8 million species [15]. Compared to the 120,000 species we have described [15], many species remain undiscovered, underscoring the significant potential for future scientific discoveries.

The number of species in the fungal kingdom presents challenges in defining what is a fungus. Over the years, there have been several attempts to find synapomorphy in the kingdom of fungi. A common characteristic one can find in all fungi that connects them to a common ancestor and distinguishes them from other groups. However, no synapomorphy has been found characterizing all fungi, yet[16]. But they share some similarities. All fungi are eukaryotes and heterotrophic and rely on extracting nutrients from their environment[16]. Most multicellular fungi have hyphae that make up the mycelium network; this stretches out in the environments, increasing the surface area of the organism and secretes enzymes to break down molecules that the fungus and also other organisms, like plants, may absorb for nutrition and growth[17]. Many fungi interact with other organisms in symbiotic relationships[16]. Even though the reproductive strategies of fungi vary, including asexual and sexual reproduction, and contain vast and varying numbers of genders and reproduction strategies, there is a common strategy to produce spores for spreading their genes in environments[16, 18].

1.1.1 Fungi and their role in ecosystems

Fungi have an important role in the ecosystem. Fungi have a relationship with almost all known organisms, their versatility in making different nutrients bioavailable for other organisms is essential for life on Earth as we know it[19].

Fungi play an important role as a geoactive agent [20-22]. Fungi can both free minerals and work as mineralization-causing agents in soil, on stone, and on buildings. One of the better-known examples of this is the fungi-plant symbiotic relationship, where the fungal mycorrhiza works intra- or extracellular with the plant or plant roots, helping with uptake of minerals. Moreover, fungi can lower the concentration of toxic metals in soil and water, and decompose xenobiotic compounds like tar balls, and plastics [20, 23]. This can be useful for us; in a water reservoir in California, fungi and bacteria were used to mitigate high levels of selenium with good results[20].

Fungi play a vital role in the biocrust that consists of lichens, mosses, cyanobacteria, green algae, and microfungi [20]. Together, these organisms increase soil stability, keep moisture in the soil, and fixate nitrogen and carbon. The biocrust is vital for plants in nutrient-poor soils, making nutrients available. The biocrusts inhabit a large proportion of the earth's terrestrial areas, and in arid and semiarid areas, up to 70% of the living cover of the soil. Because they inhabit such large areas, they play a significant role in the global fixation of carbon and nitrogen.

Fungi symbionts in the lichens contribute to carbon capture and food, especially where plant growth is limited, and other food sources are scarce, like in the winter in caribou habitats. In caribou, as well as in most animals including humans, fungi play an important role in the gut microbiome and overall health [24-26]. The fungal microbiome works together with the rest of the microbiome to support digestion, bioremediation and host homeostasis of the gut. However, the fungal microbiome has potential to disrupt the gut stability, making them a two edged sword in the hosts gut ecosystem.

The pathogenic and parasitic relationships in organisms are also a driver for biodiversity[20, 27]. Through the coevolution of fungi and other organisms, fungal parasites impact individuals in a population who share similar genetics. This creates an opportunity for natural selection, where genetically diverse individuals have a higher fitness, ultimately leading to increased diversity within populations. As a result, this process generally contributes to greater biodiversity within ecosystems.

However, fungi are probably most known as decomposers [28], by breaking down organic matter and complex organic polymers and making organic matter available for other organisms. This is an immensely important function fungi have in the carbon cycle. This, in addition to the interaction fungi have with other organisms, makes them a contributing factor in all essential ecosystem services [19].

Fungi do play an important role for humans. By being a part of the ecosystems, contributing in maintaining ecosystem services, and directly as a source of food, food preparation, and conservation, and have at least been used in the fermentation of beverages for human consumption for 9000 years [29]. In more recent times fungi have been an important part of medical science since the discovery of penicillin in the 1920s[30]. Today new metabolites found in marine fungi are actively sought out and studied for their antibacterial, antiviral, and anticancer activities[2], that can result in new medicines in the near future. The drug Plinabulin, derived from a compound originally isolated from marine fungi, is currently in phase 3 of clinical trials for its cancer remission and antibacterial properties [31, 32].

1.1.2 Fungal diversity

A higher diversity of plant species in an ecosystem correlates with higher fungal diversity[33-38]. Evidence from various studies suggests that reduced plant diversity can result in a decline in fungal diversity, and overall fungal biomass[34-39]. The correlation with plant and fungi, could also apply to marine ecosystems.

The role fungi play in the ecosystem is also dependent on having a large diversity of fungi. A change in the fungal diversity in an ecosystem could change how the different fungi and other decomposers interact, affecting the carbon cycle[40]. Further experiments have shown that the decomposition of organic material is dependent on several species of fungi working together[40].

There is little knowledge about fungi in the kelp ecosystem. However, a study by Ward et al 2020 showed the importance of identifying the microbial communities within the kelp ecosystems[12]. Their study concludes that the continued advancement of kelp cultivation and in-depth exploration of various factors, including microbial communities, such as fungi, is crucial. Fungi play an important role as potential pathogenic agents and in maintaining the overall health of the kelp ecosystem.

1.2 Marine fungi

Fungi in the marine environment share ecological functions with their terrestrial relatives[20]. Just like terrestrial fungi live in symbiosis with plants and algae as lichens, fungi form symbiosis with algae, sea grasses and plants in the mangroves in the marine environment. The symbiosis includes mutualistic, communalistic, and parasitic relationships.

In all marine environments fungi play an important role in making organic matter available for other organisms [20]. Fungi break down particulate organic matter (POM) into dissolved organic matter (DOM), making it available to other organisms in the ecosystem. It is also hypothesized that fungi are food for detritivores and deep-sea macro and meiofauna. They are a nutrient-rich food source that provides vitamins and polyunsaturated fatty acids[41, 42]. Further, in hydrothermal vents fungi probably utilize the heavy metals dissolved in the surrounding waters. This bioremediation ability is a potential application for us to clean heavy metal pollutants from, for example, water sources[20].

Marine fungi also play a role in algae and animals. The marine fungus *Stigmidium ascophylli* is shown to have positive effects for the host alga *Ascophyllum nodosum* increasing the host´s tolerance for drought, increased reproduction, and better growth when the fungi is present[20, 43]. Further, there are also studies suggesting that among others, some *Chytridiomycota*, *Oomycota* and *Labyrinthulomycota* are mostly pathogenic for marine algae[20, 44, 45]. Moreover, in some fungi-algae interactions the fungi do not seem to affect the algae[20]. However, it is also observed that in situations with more stressors, the interactions can switch from little to no effect on the algae, to being heavily pathogenic. This can be a larger issue in places where nutrient depletion, increased temperature, and increased grazing can occur, like a cultivation facility. For animals' fungi can be directly feed, but also affect different aspects of the host animals. Fungi are observed in gills, gut, and on the surface of organisms, it is hypothesized that in the deep sea, they play an important role in the population dynamics[46]. Also, direct links to fungi and corals have been made, where different fungi can help the survival of the corals in bleaching events, but other fungi cause mass death and a shift in the ecosystem from coral-dominated to kelp-dominated[20]. Fungi do have an intricate role in the ocean, from making organic matter available to symbionts in algae and animals.

1.3 Sugar kelp (*Saccharina latissima*)

Figure 1: Sugar kelp field investigation by Guri Sogn Andersen (2023)

Saccharina latissima is a type of brown algae that has circumpolar distribution in the Northern Hemisphere. In Europe, its range extends from Portugal in the south to Nova Zemlya and Svalbard in the north[47-49]. *S. latissima* individuals can vary in how they look, but they usually have a wavy blade and a rough midrib and can be up to four meters long and up to 75 centimeters wide[49]. *S. latissima* is often divided into holdfast, stipe, and blade, this is shown in Figure 9. *S. latissima* grows as a sheet in both length and width directions at the start of the blade region near the stipe, making the blade's tip the oldest part[50]. *S. latissima* can be found on rocky shores but also in sandy habitats where they attach to small stones and shells[49]. They grow in the subtidal zone down to 30 meters, often in abundance in kelp forests mixed with other kelp species or in a kelp forest dominated by *S. latissima*.

The kelp forests are an essential structure in the marine ecosystem. Kelp forests work as a primary habitat for crustaceans, mollusks, and fish [51, 52]. Kelp being a nursery and food source for a vast number of species and also contributing to biomass export to terrestrial, intertidal, and deep-sea food webs, the kelp forest helps to sustain biodiversity[51-53]. Kelp forests are one of the most productive ecosystems in the world, with algae that increase their biomass by 2-4% each day in the growth season, thereby capturing large amounts of CO_2 and nutrients[51-53]. The nutrient uptake helps mitigate eutrophication and ensures clean water. Clean water and a healthy ecosystem also provide us with recreational activities like diving, birdwatching, and fishing[52, 53]. Further, the kelp forests also help mitigate erosion by protecting shorelines from waves[52].

1.4 Global kelp industry

Kelp and sugar kelp have been a food source for at least 12,000 years[54, 55]. Kelp has been used in a variety of ways, ranging from food and agar products, feed and feed supplements, cosmetics, and a potential biofuel source, to mention some[56-61]. Most kelp has traditionally been harvested from wild kelp sites, but in the last century, there has been an increased interest in kelp cultivation, especially in Asia[62]. In 2019, 36 million tons (wet weight) of kelp and seaweeds (cultivated and wild) were produced (Figure 2). Asia produced 97,4% of these and, Europe 0,8 %.

Figure 2: Kelp and seaweed production in 2019 (FAO, United Nations, 2022)

As the human population grows, our demand for resources such as materials, habitats, water, and food also increase. This demand leads us to exceed planetary boundaries, posing a significant threat to ecosystems and their functions on a global scale[6-9]. This threat intensifies the exploration of new methods for agriculture, and one of the focus areas is low trophic species in our oceans. Shifting food production to lower trophic levels, like kelp, will help mitigate climate change, and reduce the pressures on the planetary boundaries[9, 64, 65]. Given the history and growing interest in kelp production, cultivation, and harvest can play a vital role in a sustainable future and food security.

1.5 Norwegian kelp industry

In Norway, the kelp and macroalgae industry is mainly based on harvesting wild kelp, but in recent years several actors have started kelp cultivation projects on an industrial scale. The wild kelps being harvested are mostly *Laminaria hyperborea* and *Ascophyllum nodosum*, where *L. hyperborea* makes up ten times more of the harvest than *A. nodosum*. Approximately 130,000-180,000 tons of these kelps are harvested each year[66]. Whereas only 111 tons of cultivated *Saccarina latissima* and *Alaria esculenta* were harvested in 2019[66, 67].

In Norway, wild kelp has been harvested using mechanical tools since 1970. *L. hyperborea* and *A. nodosum* are being used in the production of alginate and algae flour. There are several regulations in place to ensure sustainable harvest. You cannot harvest under 20 meters, and each harvesting field is harvested only once per five years, giving the kelp time to regrow. In addition to this, some of the kelp forests in a harvesting field will not be harvested due to difficult terrain. The Norwegian Directorate of Fisheries estimates that the kelp harvest in Norway harvests approximately 0,3 % of the total kelp in Norway. However, estimates show that it takes 6-7 years to reestablish all flora and fauna in the harvested area. This opens up a discussion if the current practice of harvesting kelp is sustainable.[67]

Figure 3: Harvesting wild kelp on the coast of Norway [67]

Due to the grazing of sea urchins on kelp in mid and northern parts of Norway, the distribution of kelp forests has decreased[68, 69]. Kelp forests are taken over by sea urchin barrens, resulting in a massive loss of habitat, diversity, and production at different trophic levels[69-74]. This can pose a challenge for wild kelp harvesting.

Cultivated kelp farms can mitigate these challenges by working as an artificial habitat [4], in addition, they can meet the increasing need for food production[66]. In Norway scientists and industry have in recent years started working on optimizing the cultivation of macroalgae, and here the biggest focus is on sugar kelp (*Saccharina latissima*)[10, 66].

Introducing large kelp farms in our oceans, can work as an artificial habitat in areas where there is a loss of the natural kelp forest, but also impact the environment negatively by depleting nutrients, and introducing challenges with pathogenic species affecting both the cultivated and wild kelp in the area[4]. More knowledge is needed to ensure sustainability in the expansion of Norway's macroalgae production[66].

1.6 DNA barcoding

"DNA barcoding is a powerful taxonomic tool to identify and discover species"[75]. Traditional taxonomy of species is often time-consuming and requires skilled and experienced taxonomists[75]. DNA barcoding is a standardized method, and does not require taxonomic expertise, can be applied to environmental samples with a mix of several species, is budget-friendly, and objective, especially when species lack clear synapomorphy, which is the case for fungi[75-77]. By utilizing genetic markers such as the internal transcribed spacer (ITS) in the ribosomal DNA (rDNA) of fungi, it is possible to amplify the DNA regions and use them in barcoding with fungi-specific primer pairs from small quantities of biological material[75, 78]. The process of DNA barcoding involves several steps, as shown in Figure 4. The first step is to collect and extract DNA, before the specific barcoding regions are amplified in polymerase chain reaction (PCR). The DNA sequences in barcoding regions vary between species but are identical or similar enough in different individuals of the same species, making them ideal for differentiating between species. The ITS is commonly used in fungal samples, providing high species resolution, and is recommended by the International Fungal Barcoding Consortium[79]. After the DNA is

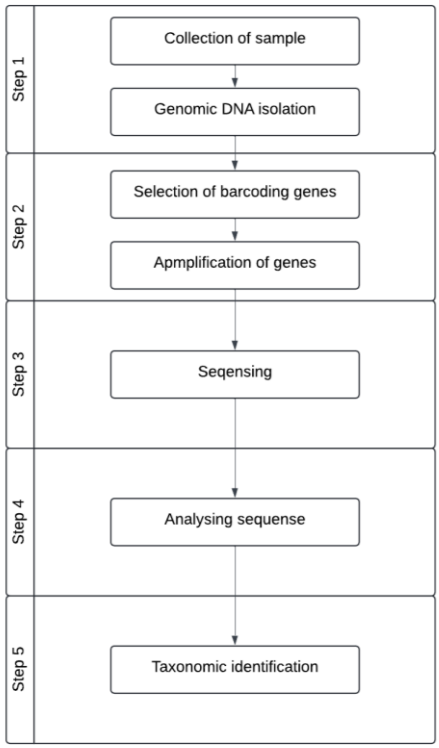

Figure 4: Schematic representation of the methodology for DNA barcoding. Made in Lucid.com

sequenced, the sequences are compared to other sequences in a reference database to identify the species[75].

In this study, I used a genomic region that is part of the SSU (small subunit or 18S rDNA), which contains eight variable regions (Figure 5). I followed the pipeline described by Yarza et al. (2017)[80], focusing on the 18S region. My selection of the 18S region was driven by its strong taxonomic resolution and the availability of blocking oligonucleotides for the primers in this region[81], which helped prevent the co-amplification of the host DNA.

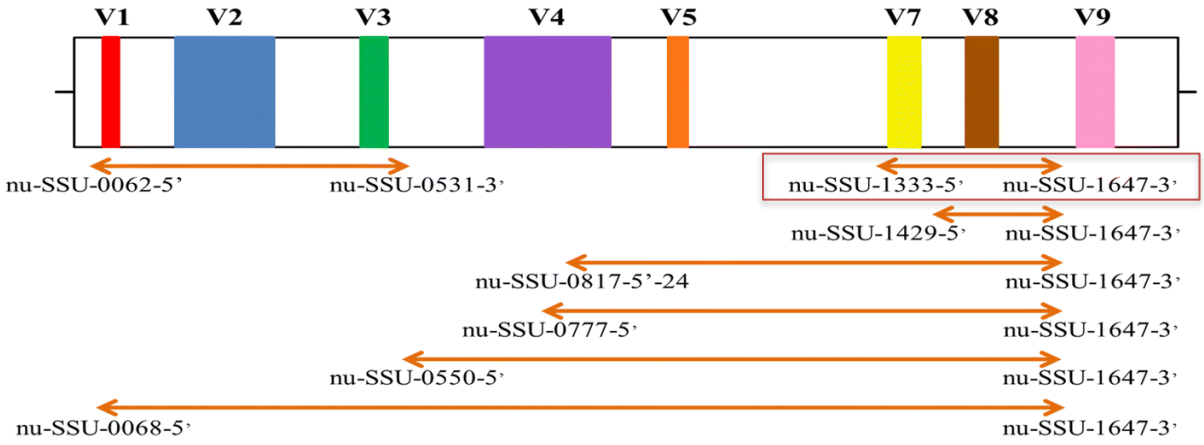

Figure 5: Primer pairs used in Banos et al. 2018 , covering the different variable regions of the fungal 18S rRNA gene sequence. The fungal 18S rRNA gene sequence possesses eight different variable regions, V1-V9 (V6 does not exist), colored differently. Primer names beside the arrow lines. In the red box are the primers FF390/FR1 (nu-SSU-1333-5´ /nu-SSU-1647-3). This figure is retrieved and modified from Banos et al. 2018.

While the ITS region is better for species-level identification, it requires comprehensive and accurate reference libraries to identify species accurately. Marine fungi are a relatively novel field of study, and the existing libraries are not sufficiently developed; in other words, they do not contain a sufficient amount of curated reference sequences from morphologically identified species.

1.6.1 DNA extraction

DNA extraction is a crucial step in DNA barcoding[75]. The goal is to obtain high-quality DNA from samples and remove contaminants like polysaccharides, proteins, and lipids from the DNA that might inhibit further modification of the DNA, like PCR[82]. Several methods are available, each with their advantages and limitations[82]. I used several DNA extraction methods in this study, and the CTAB (cetyltrimethylammonium bromide) method was used extensively.

CTAB was introduced by Doyle et al in 1990[82, 83], and is an efficient method in extracting DNA when high levels of polysaccharides are present[82]. *Saccharina latissima* consists of approximately 42% of water-solvable polysaccharides[84], making CTAB a good choice when conducting DNA extraction from *S. latissima*. However, CTAB extraction poses some challenges by being more time-consuming than using most DNA extraction kits, and in the CTAB extraction one uses hazardous chemicals, and CTAB can also degrade the DNA if not handled correctly[85, 86].

1.6.2 Polymerase chain reaction (PCR) and Primers

Polymerase chain reaction (PCR) is a method for rapidly amplifying specific DNA segments. By obtaining DNA and choosing specific synthetic DNA fragments and primers, one can choose the segment to be amplified. After amplification, one will have millions to billions of the desired genome segments and can more easily study the DNA further.[87]

The SSU is a smaller region on the genome, the sequence of the SSU show very limited variation within the organisms in the same lower taxonomic groups such as genera and family, but higher taxonomic groups can usually be separated from each other with high confidence[79, 80, 88, 89]. There are good fungal primers for this region that give a precise PCR amplification, hindering unwanted amplification of most other organism groups.

However, primers can co-amplify other organisms. In my study I used the FF390 and FR1 primers, these can co-amplify the clade Stramenopiles[81]. The described taxa in Stramenopiles consist of diatoms, brown alga, chrysophytes, and xanthophytes[90]. *Saccharina latissima* is a part of this clade. Therefore, the utilization of blocking oligonucleotides is an option and that has given good results in inhibiting co-amplification of stramenopiles in soil and aquatic environmental samples without losing fungal groups in earlier studies[81]. The blocking oligonucleotides hinder the elongation of the sequence in the amplification of dominant or unwanted DNA by overlapping with the primer binding sites[81, 91].

1.7 Research question and aim

In this project, I am examining the fungal community on *Saccarina latissima*, exploring its diversity and comparing two sites: a natural kelp forest and a kelp cultivation facility. Additionally, I will be studying seasonal changes in the fungal communities. The methods used to examine this are DNA extraction, PCR amplification, and BLAST analysis. The knowledge about the fungal diversity of wild and cultivated *S. latissima* can facilitate further research on

the role of fungi associated with the kelp hosts and encourage more research on marine fungi in northern coastal ecosystems.

1.8 Hypothesis

The hypotheses to be tested in this thesis are:

There are fungal communities on *S. latissima*.

There is a higher diversity of fungi in wild *S. latissima* individuals compared to cultivated ones.

The diversity community composition of fungi in *S. latissima* changes throughout the seasons.

2 Methods

2.1 Kelp sampling and description of the sampling locations

The sampling of *Saccharina latissima* was conducted from February to September 2023 at two sites. The location coordinates are shown in Table 1 with a map in Figure 6. Site one (SUT) is a wild kelp forest containing *S. latissima*, and the second site (Kraknes) is a cultivation facility for *S. latissima*. I sampled two individuals at each sampling site at least once each season. It was attempted to take one large (blade length over 1 meter) and one small (blade length under 1 meter) kelp individual at each site. However, this was impossible from the late spring to early summer and onwards due to the growth of the kelp at the sites, as most or all individuals were over 1 meter or of approximately the same size. After cutting the kelp at the top of the stipe, the individuals were transported to the lab at Marbio in a zip-lock bag with cooling elements in a styrofoam cooler. The individuals were placed in a -20°C freezer before further analyses.

Table 1: Coordinates over SUT and Kraknes , the sampling sites.

Site	Coordinates (N)	Coordinates (E)	Coordinate System
SUT	7743816.73	655988.4	EUREF89 UTM-zone 33
Kraknes	7743816.73	655988.4	EUREF89 UTM-zone 33

Figure 6: Map over the sampling sites.

2.1.1 Wild *Saccharina latissima*

The *Saccharina latissima* was sampled in a kelp forest by snorkeling. The snorkeling was conducted following the health and safety protocols of The Faculty of Biosciences, Fisheries, and Economics at the UiT, The Arctic University of Tromsø, when weather conditions were good. We cut *S. latissima* at the top of the stipe in the water with a knife to ensure regrowth. The knife was with or without an extender, depending on the tide. The sampling site chosen was outside of the student diving club in Tromsø (SUT) due to its thriving kelp forest. In the fjords surrounding Tromsø, many earlier known kelp forests have turned into sea urchin barrens[92]. However, outside SUT, the kelp restoration group "Tarevokterne" has been removing sea urchins for over two years, resulting in a diverse kelp forest. In addition, it is a convenient location, accessible by car, and has a diving center nearby. In the kelp forest at SUT, a large population of *S. latissima* grows shallow, making this an ideal site for collecting samples.

Before harvesting and choosing a site, I did several trial snorkels, investigating local knowledge about kelp forests in the area to try to find a kelp forest less impacted by humans. The site at SUT is heavily affected by humans in the sense that "Tarevokterne" removes sea urchins almost every month. However, the other locations I investigated had no persistent kelp forests or were inaccessible by car.

Figure 7: Foto from wild kelp site, above the surface. The sampling site is approx. 5 meters to the left of the lighthouse in this picture.

2.1.2 Cultivated *Saccharina latissima*

The cultivation of *Saccharina latissima* was conducted at Akvaplan NIVA's facility in Kraknes, where Oceanfood has a project on farming *S. latissima*. The mother plants for the kelp were harvested wild from the surrounding area of Kraknes. These mother plants are kept in the lab until they are ready to sporulate. Post-sporulation, the spores are sprayed onto ropes and grown in filtered and UV-treated seawater under a light regime. I sampled eight 5 cm rope stubs with juvenile kelp growing on them by cutting off a piece of the rope after 4 weeks of growth. After 6-8 weeks, the spores had grown to 0,5-1 cm in length. When the juvenile kelps were 0,5-1 cm, they were set out at an ocean rig. The batch of kelp examined in this study was set out at the ocean rig in November 2022 and scheduled for harvest in late summer to prevent overgrowth by epiphytes. In addition to the November 2022 batch, I also got samples from the mother plants used for juveniles scheduled to be set out at the ocean rig in November 2023.

Sampling from Kraknes was coordinated with the schedules of Akvaplan NIVA and Oceanfoods staff. Our ability to collect samples depended on their availability to conduct routine inspections of the ocean rig, underscoring the importance of the collaboration. Additionally, weather conditions needed to be good to access the offshore kelp facility. These factors impacted the frequency of the sampling.

2.1.3 Macroalgae species observed

During the *Saccharina latissima* sampling, I took video footage and noted some of the most prominent macroalgae species to get an idea of the algal community that may affect the composition of the fungal community at the sites. Observed macroalgae at the wild site where *Saccharina latissima, Chorda filum, Fucus serratus, Alaria esculenta, Ascophyllum nodosum, Fucus vesiculosus, Palmaria palmata, Ulva lactuca, Laminaria digitata,* and *Desmarestia aculeata*. These are only some of the most prominent species, I do not limit the species present to this list.

At the cultivated site I did not take video footage but did observe that the cultivated site mainly consisted of *S. latissima*, but some other algae were also present. I observed a few *Alaria esculenta* at the edges of the facility, and a thread-like brown algae on some of the *S. latissima* (Figure 8), I tentatively identified this to be *Laminarionema elsbetiae*[93].

Figure 8: Picture of cultivated S. latissima with Laminarionema elsbetiae.

2.2 Kelp sample preparation

At Marbio, I cut two 5 cm segments of the *Saccharina latissima* in the laminar flow hood, as shown in Figure 9. I then placed these pieces in falcon tubes for storage at -80 ℃.

Next, the algae material was crushed individually in the laminar flow hood, using liquid nitrogen and sterilized mortars and pestles to homogenize the samples. The crushed algae material was put in Erlenmeyer tubes and stored in a freezer at -80℃. All equipment was sterilized after each individual alga was cut and crushed using a bleach solution of approximately 1% hypochlorite. Some of the algae were close to 2 meters; this made it challenging to keep the algae in the laminar flow hood when cutting them. Due to this, the samples were exposed to a non-sterile area; the area was, however, cleaned before the samples were prepared.

Samples under 1 cm were sterilized using bleach containing approximately 1% hypochlorite before being directly used with the rope and the kelp in CTAB DNA extraction.

Figure 9: Schematic picture of S. latissima with blade, stipe, holdfast, and the 5 cm segment shown. This kelp is approximately 110 cm. Created with BioRender.com. Picture of the kelp: private.

2.2.1 Overview of kelp samples

I collected 20 samples through this study: 12 from the kelp forest and 8 from the cultivation facility. Table 2 show an overview of the sampling dates and sample lengths. These samples were not sequenced due to time limitations and challenges in the amplification and extraction of the fungal DNA. In addition, in yellow are samples that were depleted before using the second batch of CTAB as described in 2.4.2.3. The number in the sample ID represents the kelp specimen, S/K is the site, and a/b outermost segment (a), or (b) the second segment. The kelp blades were measured from where the stipe meets the blade to the tip of the blade.

Table 2: Overview of samples collected from February to November 2023. Marked in yellow are samples depleted before CTAB extraction due to errors in the preparation of CTAB. The number in the sample ID represents the kelp specimen, S/K is the site, and a/b is the segments.

Sample ID	Date sampled	Site	Blade length (cm)
1S_a	03.03.2023	SUT	20
1S_b	03.03.2023	SUT	20
2S_a	03.03.2023	SUT	100
2S_b	03.03.2023	SUT	100
3S_a	13.04.2023	SUT	20
3S_b	13.04.2023	SUT	20
4S_a	13.04.2023	SUT	85
4S_b	13.04.2023	SUT	85
5S_a	04.06.2023	SUT	90
5S_b	04.06.2023	SUT	90
6S_a	04.06.2023	SUT	210
6S_b	04.06.2023	SUT	210
7S_a	18.06.2023	SUT	100
7S_b	18.06.2023	SUT	100
8S_a	18.06.2023	SUT	110
8S_b	18.06.2023	SUT	110
9S_a	14.08.2023	SUT	65
9S_b	14.08.2023	SUT	65
10S_a	14.08.2023	SUT	85
10S_b	14.08.2023	SUT	85
11S_a	10.11.2023	SUT	45
11S_b	10.11.2023	SUT	45
12S_a	10.11.2023	SUT	115
12S_b	10.11.2023	SUT	115
13K_a	23.02.2023	Kraknes	>1
13K_b	23.02.2023	Kraknes	>1
14K_a	23.02.2023	Kraknes	>1
14K_b	23.02.2023	Kraknes	>1
15K_a	04.05.2023	Kraknes	50
15K_b	04.05.2023	Kraknes	50
16K_a	10.06.2023	Kraknes	100
16K_b	10.06.2023	Kraknes	100
17K_a	10.06.2023	Kraknes	145
17K_b	10.06.2023	Kraknes	145
18K_a	29.06.2023	Kraknes	150
18K_b	29.06.2023	Kraknes	150
19K_a	29.06.2023	Kraknes	110
19K_b	29.06.2023	Kraknes	110
20K_a	11.10.2023	Kraknes	Approx 100
20K_b	11.10.2023	Kraknes	Apporx 100

2.3 Chemicals, reagents and equipment

The products, equipment and primers used in this study are found in Table 3 and Table 4.

Table 3: The products and equipment used with specification and distributor.

Product/Equipment	Product ID/Equipment specifications	Distributor (Country)
UtraPure™ Agarose	15510-027	Thermo Fisher Scientific (Spain)
SYBR™ Safe DNA Gel Stain	S33102	Invitrogen™ (USA)
UtraPure™ TBE Buffer 10X	15581-044	Thermo Fisher Scientific, (USA)
UltraPure™ TAE Buffer, 10X	15558042	Thermo Fisher Scientific, (USA)
DreamTaq Green PCR Master Mix (2X)	K1081	Thermo Fisher Scientific, (USA)
repliQa HiFi ToughMix	95200-025	Quantabio, (USA)
1 kb Plus DNA Ladder	10787-018	Thermo Fisher Scientific, (USA)
6x DNA loding dye	R0611	Thermo Fisher Scientific, (Croatia)
BigDye® Terminator v1.1 & v.3.1 5X Sequence buffer	4226697	Thermo Fisher Scientific, (USA)
Methanol (MeOH)	34860	Sigma-Aldrich (USA)
Bleach	Store-bought	
Milli-Q Ultrapure water (ddH2O)		Merck Millipore
Ethanol	23H214013	VWR Chemicals
Isopropanol	A0398038	Acros Organics
CTAB	See protocol in appendix	
Ready-To-Go PCR beads		GE Healthcare (UK)
gDNA from 008cD	Isolated by Dr. Teppo Rämä	
Nuclease-free water		Thermo Fisher Scientific, (Croatia)
Betaine Enhancer Solution	733-1361	VWR life science (Denmark)
Mastercycler nexus gradient PCR machine		Eppendorf (Germany)
PTC-0200 DNA engine	91-PCS24	MJ Research (USA)
Precellys® 24, homogenization		MJ Research (France)
Analog Vortex mixer		VWR life science
Eppendorf® Centrifuge 5418 R G		Eppendorf® (Germany)
Agarose Gel electrophoreses system	Owl separation system, B2 model	Thermo Fisher Scientific, (USA)
GeneFLash Gel image system		SYNGENE Bioimaging, (UK)
Herasafe biological safety cabinet	Class 2	Thermo Fisher Scientific, (USA)
Heat block	QBD2	GRANT (UK)
Nanopore MinION Mk1B		Nanopore Technologies (UK)
QIAquick PCR Purification Kit	28104	QIAGEN

Table 4: Primers with sequences and distributors that were used in this study.

Primer	Sequense	Distributor (Country)
ITS4	5'-TCCTCCGCTTATTGATATGC	Sigma-Aldrich (USA)
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG	Sigma-Aldrich (USA)
FR1	5'-AICCATTCAATCGGTAIT	Eurofins Genomic
FF390	5'-CGATAACGAACGAGACCT	Eurofins Genomic
BON3	5'-TCGCACCTACCGATTGAA [Am C6]	Eurofins Genomic

2.4 Extraction and amplification of fungal DNA from kelp samples

To our knowledge, there is no generalized method for identifying the microfungal communities of *S. latissima*. The lab at Marbio has extensive expertise in extracting fungal DNA from environmental samples, wood-inhabiting fungi, and fungal cultures. We developed our method using thoroughly tested methods on similar material to identify the fungal DNA.

There were no extensive efforts made to observe fungal fruiting bodies of *S. latissima* samples, so I did not directly extract and cultivate fungi. Therefore, I extracted the fungal DNA, amplified it using PCR, and thereafter investigated bands in gel electrophoresis before PCR purification and sequencing. I used the 18S primers FR1/FF390 in addition to the complementary blocking oligonucleotide BON3 and the ITS primers ITS4/ITS5.

However, the methods for extracting fungal DNA, amplifying it, and sequencing it, were troublesome, and I did extensive troubleshooting and method optimization. The flow of this is shown in Figure 10.

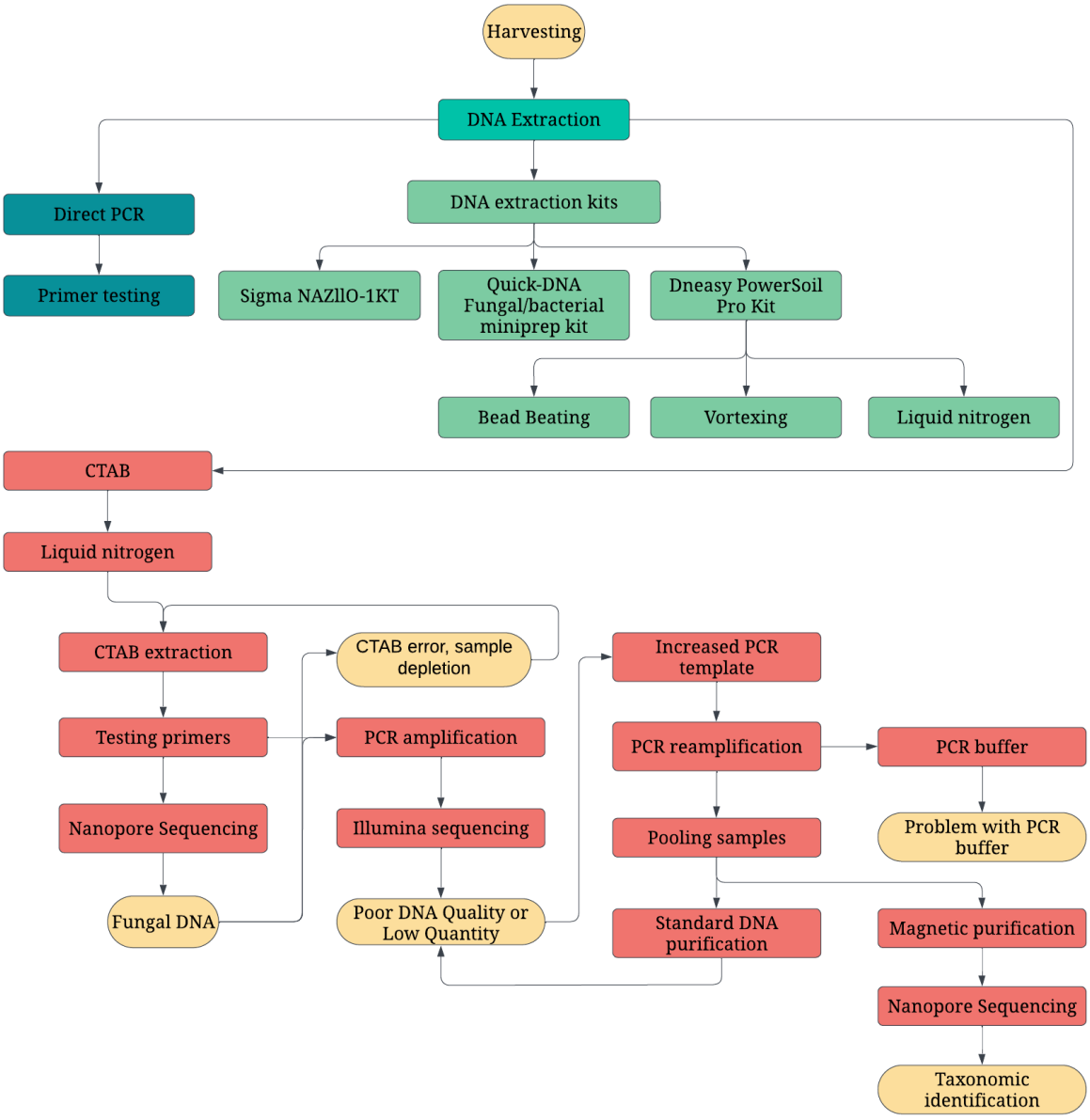

Figure 10: Flowchart with an overview of methods and approaches to optimize the methods. I used three methods to extract the fungal DNA from S. latissima samples: Direct PCR (dark blue), DNA extraction kits (green), and CTAB (read). The yellow circular boxes represent conclusions or challenges with the previous steps. The headings are referred to in the following text, and the steps are explained. The figure was made using Lucidchart.com.

2.4.1 Primers and blocking oligonucleotides for PCR amplification

The primers used in this study were the FF390 (5-CGATAACGAACGAGACCT-3) and FR1 (5-AICCATTCAATCGGTAIT-3), in addition to the primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC) and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG). The ITS4 and ITS5 were not extensively used throughout this study, but as the ITS region is a good genetic marker for fungi, it was used to confirm fungal DNA in our samples. The primer set FF390 and FR1 was developed by Vainio and Hantula 2000[94, 95]. These primers target the SSU rDNA (small subunit or 18S rDNA) region close to the ITS gene region.

The blocking oligonucleotide used in this study is BON3 (5'-TCGCACCTACCGATTGAA [Am C6]), hereby referred to as BON3. The BON3 is modified in the way that a six-carbon chain with an amino acid is attached to the last base, adenine, at the 3' prime end, this blocks the continuation of the elongation by the DNA polymerase on the RNA strand. This blocker is designed to block the forward primer FF390[81].

2.4.2 DNA extraction of fungal DNA from kelp samples

To extract the DNA from *Saccharina latissima*, I directly submerged and froze kelp samples before attempting direct PCR amplification, extraction using several kits, and cetyltrimethylammonium ammonium bromide (CTAB) DNA extraction.

2.4.2.1 Direct PCR of kelp with fungal primers

Directly freezing *Saccharina latissima* samples before using direct PCR amplification, I aimed to prevent contaminations, skip DNA extraction, and rapidly identify the fungal community. In addition, I aimed to check that the primers and blocking oligonucleotide did amplify the fungal DNA and inhibit the amplification of host algal DNA.

I sampled two brown algae with visible fungi to test up against sterilized *S. latissima* samples. I sampled *Fucus spiralis* with visible *Phoma sp.*, a ascomycete fungus, from Breivika havn and, *Ascophyllum nodosum* with visible *Calycina marina*, also a ascomycete fungus, found at Kvaløysletta fjærepark. I cut out a piece of alga material with visible fungi using sterile scalpels under a dissecting microscope, transferred it to an Eppendorf tube with 100 µl sterilized Milli-Q water, and froze it overnight in a -80°C freezer. The *S. latissima* individuals were sampled before the alga was moved to the ocean rig, making them between 0,5 and 1 cm in length on a 5 cm long thread. I cut off 1 cm of the thread and sterilized it using 1% hypochlorite solution first suspending the thread with *S. latissima* for 5 seconds in the solution, before rinsing it off

four times in separated Eppendorf tubes with sterilized Milli-Q water before adding it to a Eppendorf tube with 100 µl sterilized Milli-Q water, and froze it overnight in a -80°C freezer.

I let the samples thaw at room temperature, diluted them in Milli-Q water, and conducted PCR amplification. The PCR amplifications were conducted with and without blocking oligonucleotides in different dilutions of the algal-fungi samples in addition to a previously isolated fungal DNA from *Digitatispora marina* and the sterilized *S. latissima* samples. For the PCR amplifications without blocking oligonucleotides, I used a 25 µl reaction, with 1 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 10,5 µl of Nuclease-free water. For the PCR amplifications with blocking oligonucleotides, I used a 25 µl reaction, with 1 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 1,5 µl blocking oligonucleotides (10 µm), 9 µl of Nuclease-free water. The FF390/FR1 primer pair were used.

I used the following PCR regime: 95°C for 5 minutes, and 40 cycles with 95°C for 30 sec, 47°C for 30 sec, 72°C for 1 min, with a final 72°C for 10 min. I verified the PCR products using agarose gel electrophoresis; if there were positive bands, I purified the PCR products using the QIAquick PCR Purification Kit: QIAGEN, Cat no 28104, and then checked the concentration using NanoVue Plus (GE Healthcare, Great Britain) and in Qubit.

The gel was made using 1% agarose mixed with 100 mL 1xTBE buffer or 1xTAE buffer and heated in a microwave. When it was cooled down, 3 µl 100x gel red was added. The gel mixture was added to an Owl™ EasyCast™ B2 Mini gel electrophoresis system (Thermo Fisher Scientfic™, MA, USA). After about 20 minutes, 1xTBE or 1xTAE buffer was added and the following was added to the wells in the gel; 3 µl 1kb ladder and 5 µl sample mix. The gel was run for 20 min at 150-200 V. Pictures of the gels were taken using GeneFlash® (SYNGENE, Great Britain). This procedure was conducted for the following verifications of the PCR products presented through this study using agarose gel electrophoresis.

Chun Li and I sequenced the positive bands to ensure that they were amplicons of fungal DNA. Chun Li and I conducted the sequencing protocol as described in the attached *Protocol for Characterization of Fungal Strains (ITS)* in the appendix, steps 7 and 8.

2.4.2.2 DNA extraction kits

Table 5: DNA extraction kits used in this study.

Kit name	Supplier	Distributor	Product number
DNeasy PowerSoil Pro kit (250)	QIAGEN	MD, USA	Cat.no 47016 Lot 175012239
Quick-DNA Fugal/bacterial Miniprep Kit	Zymo research	California, USA	Cat.no D6005 Lot.no 211352
GenElute Bacterial Genomic DNA kit	Sigma-Aldrich	Darmstadt, Germany	NA2110

Table 5 Presents the DNA extraction kits used to extract fungal DNA from the *S. latissima* samples. I followed the protocol for the Quick-DNA and GenElute kits, but these kits did not yield satisfactory results. For the DNeasy kit, I tried different methods for homogenization: bead beating, vortexing, and homogenization using liquid nitrogen.

Several attempts were made to homogenize the sample using vortexing, but this approach was unsuccessful. Bead beating was attempted to homogenize the sample, but it made the sample too viscous for pipetting when using the recommended amount of sample material. By reducing the amount of sample material and bead beating time, I was able to conduct the rest of the extraction procedure, though PCR amplification did not yield bands. Subsequently, I homogenized the *S. latissima* samples using liquid nitrogen and a mortar, skipping the suggested homogenization steps in the protocol of the DNeasy kit.

PCR amplifications were conducted with and without blocking oligonucleotide. For the PCR amplifications without blocking oligonucleotide, I used a 25 µl reaction, with 1 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 10,5 µl of Nuclease-free water. For the PCR amplifications with blocking oligonucleotides, I used a 25 µl reaction, with 1 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 1,5 µl blocking oligonucleotide (10 µM), 9 µl of Nuclease-free water. FF390/FR1 and ITS4/ITS5 primer pairs were used.

I used the following PCR regime: 95℃ for 5 minutes and 40 cycles with 95℃ for 30 sec, 47℃ for 30 sec, 72℃ for 1 min, and a final 72℃ for 10 min. I verified the PCR products using agarose gel electrophoresis; if there were positive bands, I purified them using the QIAquick

PCR Purification Kit: QIAGEN, Cat no 28104, and then Ole Christian Hagestad (Marbank, Institute of Marine Research) checked the concentration using NanoVue Plus (GE Healthcare, Great Britain) and Qubit measurements.

2.4.2.3 CTAB extraction of fungal DNA from kelp samples

DNA from the *Saccharina latissima* samples was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol[3, 96]. As Murray and Thompson 1980 describe, I ground the kelp tissue into a fine powder using liquid nitrogen, prepared the CTAB buffer, and extracted the DNA following the protocol in the appendix (CTAB extraction modified for extraction of fungal DNA from *S. latissima* samples).

I conducted PCR amplifications on all *S. latissima* samples that were extracted using the CTAB method with and without BON3. For the PCR amplifications without BON3, I used a 25 µl reaction, with 1 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 10,5 µl of Nuclease-free water. For the PCR amplifications with blocking oligonucleotide, I used a 25 µl reaction, with 1 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 1,5 µl blocking oligonucleotide (10 µM), 9 µl of Nuclease-free water. Six samples of 5 µl Betaine Enhancer Solution were added; here, I reduced the amount of nuclease-free water accordingly. The FF390/FR1 primer pair was used.

I used the following PCR regime: 95°C for 5 minutes, and 40 cycles with 95°C for 30 sec, 44,5°C for 60 sec, 72°C for 1,5 min, with a final 72°C for 10 min. I verified the PCR products using agarose gel electrophoresis; if there were positive bands, I purified them using the QIAquick PCR Purification Kit: QIAGEN, Cat no 28104, and then checked the concentration using NanoVue Plus (GE Healthcare, Great Britain) and Qubit measurements.

Before performing the CTAB extraction on all samples, my supervisor and I ensured the successful amplification of fungal DNA using this method on five samples. The samples that yielded strong bands and the highest quality DNA in Qubit were also sequenced by Ole Christan Hagestad using Nanopore sequencing to ensure that the amplified DNA in the samples was fungal. Following successful results from Nanopore, a PhD student at Marbio, Hosea Masaki, and I proceeded to extract DNA from all 20 *S. latissima* individuals, two segments per individual, resulting in 40 samples, using a second batch of CTAB solution. However, the second batch of CTAB acted differently when adding isopropanol compared to the first batch

of CTAB solution. Therefore, I considered these samples not suitable for further analysis. Despite attempts to amplify the DNA from the second batch of CTAB solution, the results confirmed that these samples were not suitable for further use.

Due to the error in the second batch of CTAB solution we depleted eleven *S. latissima* segment samples. However, the remaining samples were extracted using the first batch of CTAB solution. After the extraction I amplified the samples using PCR.

The PCR product that yielded bands from the first batch of CTAB was purified and sent to Novogene for Illumina sequencing. However, the samples did not pass the quality control at Novogene due to poor DNA concentration and quality and no sequencing was done. Therefore, I aimed to improve the DNA quality and concentration and conduct Nanopore sequencing for all samples in-house.

2.4.2.4 Nanopore sequencing of PCR products from fungal DNA extracts from CTAB extraction

After attempts to amplify the DNA were inconsistent, I wanted to ensure I had fungal DNA in the first attempts to extract the DNA using the first batch of CTAB solution. Following the PCR regime described in section 2.4.1.2 using Ready-to-Go PCR beads to avoid the dye in DreamTaq and thereby skip purification before Nanopore. I conducted PCR amplification using the DNA template from one *S. latissima* individual, in nine PCR reactions. I used different amounts of BON3: three PCR reactions with 0 µl, three PCR reactions with 1,5 µl, and three PCR reactions with 3 µl. I used a 25 µl reaction, with 1 µl of DNA template, 0,5 µl of forward and reverse primer (10 µM), 23 µl of Nuclease-free water. For the PCR amplifications with BON3 I reduced the amount of Nuclease-free water so that the total reaction volume was 25 µl. I used the FF390/FR1 primers.

Each sample set of three, with the same parameters, was pooled together before Ole Christian Hagestad conducted Nanopore sequencing using the Rapid Barcoding Sequencing protocol from Nanopore (see appendix for full protocol).

The initial taxonomic alignment was generated using the SILVA database[97]. The operational taxonomic units (OTU) from SILVA were verified individually using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) reference database and local marine fungal reference sequence database consisting of marine fungal sequences from Dr. Ole C. Hagestad and Dr. Teppo Rämä[98].

2.4.2.4.1 PCR amplification with increased template volume

I attempted to change the PCR reaction's template volume to increase the PCR products' final DNA concentration.

I extracted DNA from *Saccharina latissima* using the first batch of the CTAB solution. For the PCR amplifications without BON3 I used a 25 µl reaction, with 11,5 µl of DNA template, 12,5 µl of DreamTaq Master mix and 0,5 µl of each forward and reverse primer (10 µM). For the PCR amplifications with BON3, I used a 25 µl reaction, with 10 µl of DNA template, 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM) and 1,5 µl BON3 (10 µM). FF390/FR-1 primer pairs were used. For a few samples, I had almost depleted the CTAB extracted DNA; therefore, some samples used between 8-11,5 µl DNA template, and the missing volume was displaced using Nuclease-free water.

I used the following PCR regime: 95℃ for 5 minutes, and 40 cycles with 95℃ for 30 sec, 44,5℃ for 60 sec, 72℃ for 1,5 min, with a final 72℃ for 10 min. I verified the PCR products using agarose gel electrophoresis; if there were positive bands, I purified them using the QIAquick PCR Purification Kit: QIAGEN, Cat no 28104, and then checked the concentration using NanoVue Plus (GE Healthcare, Great Britain) and Qubit.

2.4.2.4.2 PCR reamplification of PCR products with increased template volume

To increase the concentration further, I reamplified the PCR product from the CTAB extraction and amplification with the increased PCR template. I reamplified the samples that yielded bands.

For the PCR reamplification, I used a 25 µl reaction, with 1 µl of DNA template (amplified as described in 2.4.1.5), 12,5 µl of DreamTaq Master mix, 0,5 µl of each forward and reverse primer (10 µM), 10,5 µl of Nuclease-free water. The FF390/FR1 primer pair was used. In addition, I compared the use of two master mixes in the PCR amplification, repliQa® HiFi ToughMix® to DreamTaq.

I used the following PCR regime in the samples with DreamTaq: 95℃ for 5 minutes, and 30 cycles with 95℃ for 30 sec, 44,5℃ for 60 sec, 72℃ for 1,5 min, with a final 72℃ for 10 min.

For the samples using repliQa, I used the following PCR regime: 98℃ for 30 sec, 35 cycles with 98℃ for 15 sec, 44,5℃ for 10 sec, 68℃ for 20 sec, and a final extension of 68℃ for 2 min.

I verified the PCR products using agarose gel electrophoresis; if there were visible bands, I purified some of the PCR products using the QIAquick PCR Purification kit and checked the concentration using NanoVue Plus (GE Healthcare, Great Britain) and Qubit measurements.

The samples with repliQa® HiFi ToughMix® were purified, and sequenced with Nanopore, and taxonomic alignment was conducted following the same procedure as in 2.4.2.4.

2.5 Data analysis

The data obtained for the Nanopore sequencing was analyzed using Natrix2 pipeline[99]. Natrix is a user-friendly and reducible workflow solution for processing prokaryotic and eukaryotic environmental sequences (16S or 18S) coming from Illumina platforms. Natrix2 includes the handling of Nanopore reads[99]. Natrix2 removes the primers and poor-quality DNA strands, merging identical reads and overlaps and assigning the sequences to Operational Taxonomic Units (OTUs) using a 98% similarity threshold. The consensus sequences representing the OTUs are made using a mean of the 98% similarity threshold, where the quality of the sequences is checked using several algorithms. The OTUs from Natrix2 were verified individually using the Basic Local Alignment Search Tool (BLAST) using the National Center for Biotechnology Information (NCBI) reference database[98], and local fungal reference sequence database in Geneious, consisting of marine fungal sequences from Dr. Ole C. Hagestad and Dr. Teppo Rämä.

3 Results

The results are collected from the data acquired from analyzing the *Saccharina latissima* samples gathered, presented in Table 2: Overview of samples collected from February to November 2023.

3.1 Testing primers and blocking oligonucleotides on fungal DNA

To ensure that the primers, FF390/FR1, worked, I tested them and the BON3 on isolated fungal gDNA from *Digitatispora marina*. I observed that the primers worked on the isolated fungal gDNA, yielding bands in the gel electrophoresis and that the amount of blocking decreased the intensity of the band in the gel shown in Figure 11 and

Table 6. In Figure 11 You see, lane 1 is brighter than 2 and 3, and lane 4, with the highest concentration of blocking oligonucleotide, shows no clear band in the 400-500 base par region, which is expected for fungi using these primers.

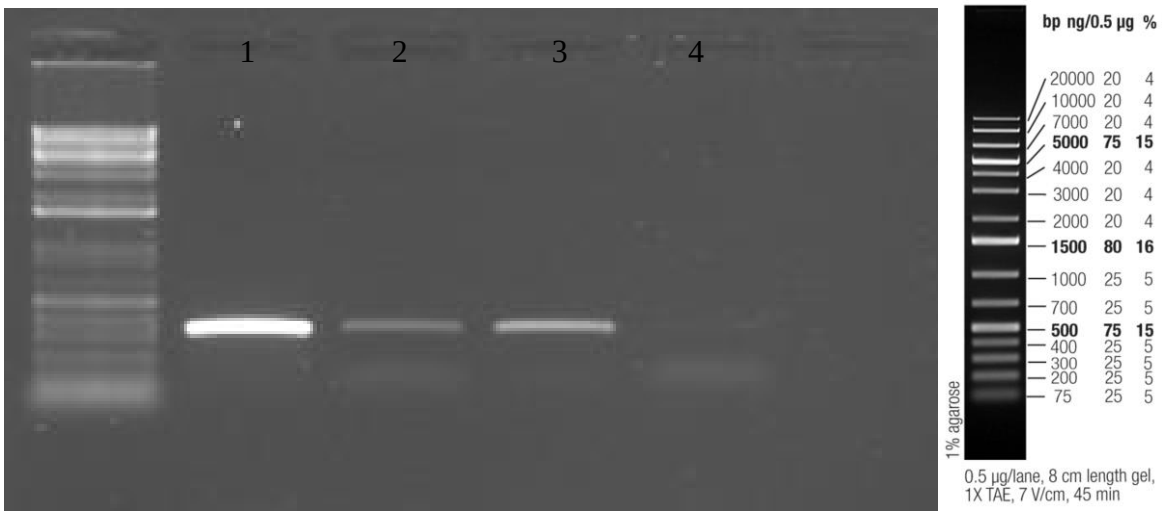

Figure 11: Gel electrophoresis picture of PCR product using FF390/FR1 primers, and BON3 blocking oligonucleotides on isolated fungal DNA. The 1kb+ DNA ladder was used. The ladder and the base pair length of the DNA are shown to the right (figure from Thermo Scientific™). Numbers 1-4 correspond to the numbers in Table 6

Table 6: Template, blocking oligonucleotides and amount, and primers used in testing using isolated fungal DNA.

No:	Template	Blocking	Primers
1	gDNA from 008cD1.1 (0,06ng/µl)		FF390/FR1
2	gDNA from 008cD1.1 (0,06ng/µl)	BON3 (1,5 ul)	FF390/FR1
3	gDNA from 008cD1.1 (0,06ng/µl)	BON3 (3 ul)	FF390/FR1
4	gDNA from 008cD1.1 (0.06ng/µl)	BON3 (5 ul)	FF390/FR1

3.2 Direct PCR of brown algae with visible fungi

In the initial phase of my study, I began by testing PCR amplification on *Fucus spiralis* samples with visible *Phoma sp*. using direct PCR methods. However, these attempts did not yield consistent PCR products, indicating a need for alternative approaches or sample preparations.

I then shifted my focus to *Ascophyllum nodosum* samples that exhibited clearly visible fruiting bodies of *Calycina marina*. My initial PCR attempts remained unsuccessful, as I did not observe consistent bands on the gel electrophoresis.

To enhance the likelihood of successful amplification, I proceeded to dilute the DNA extracts further. This modification did yield visible bands as seen in lanes 2 and 6, indicating that the initial DNA concentration might have been too high or that inhibitors were present in the extract shown in Figure 12 and in the bold letters in Table 7.

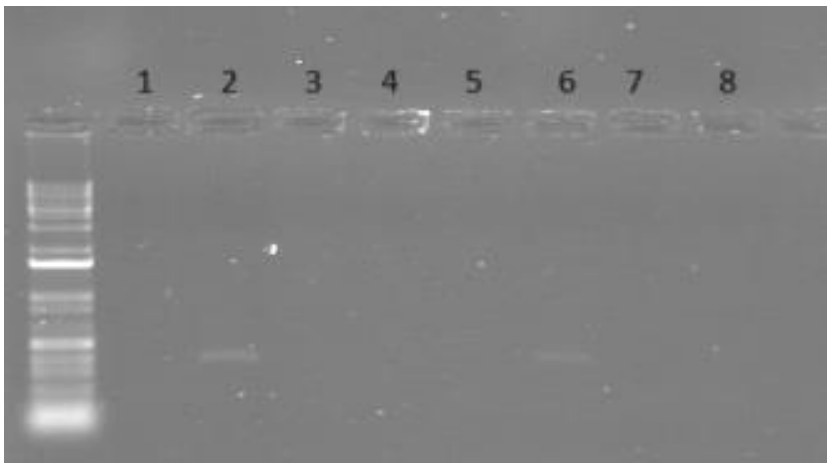

Figure 12: Gel electrophoresis of Direct PCR products from A. nodosum and C. marina samples, and D. marina isolates. Lanes 1-8 correspond to numbers table 7. The first lane (not numbered) is the ladder 1Kb+.

Table 7: Samples and primers used in PCR amplification of A. nodosum and C. marina samples, and D. marina isolates. The samples that showed visible bands are in bold.

No:	Template	Primers
1	*A. nodusum* and *C. marina* 1 Not diluted	FF390/FR1
2	***A. nodusum* and *C. marina*, 1:100 from 02.10.2023**	**FF390/FR1**
3	*A. nodusum* and *C. marina*, 1:1000 from 02.10.2023	FF390/FR1
4	*A. nodusum* and *C. marina* 2, not diluted	FF390/FR1
5	*A. nodusum* and *C. marina* 2, 1:100 from 02.10.2023	FF390/FR1
6	***A. nodusum* and *C. marina* 2, 1:1000 from 02.10.2023**	**FF390/FR1**
7	*A. nodusum* and *C. marina* 2, 1:1000 old	FF390/FR1
8	*Digitatispora marina* 1.1 (0.06ng/µl)	FF390/FR1

The dilutions of the DNA extracts that showed bands in Figure 12, "*A. nodusum* and *C. marina*, 1:100 from 02.10.2023" and "*A. nodusum* and *C. marina* 2, 1:1000 from 02.10.2023" were tested with the blocking oligonucleotides. In addition, I included DNA extracts from sterilized small (less than 1 cm) *Saccharina latissima* from the cultivated site, and isolated *Digitatispora marina* DNA extracts, as shown in Figure 13. Bands are visible in lanes 1, 2, 4, 5, 6, 11, 16, 17, and 18.

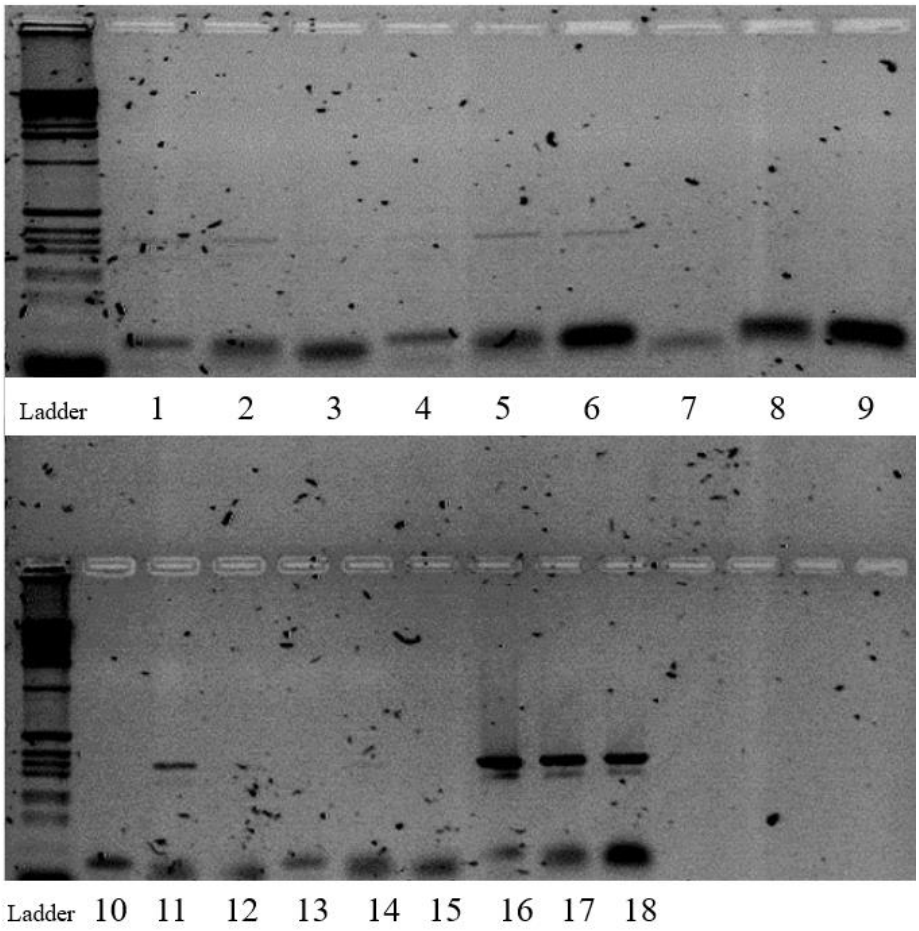

Figure 13: Gel electrophoresis of PCR products from Table 8. The 1kb+ DNA ladder was used. The samples were successfully amplified using PCR products indicated in bold letters in Table 8

Table 8: Samples, primers, and blocking oligonucleotides used PCR amplification of A. nodosum and C. marina samples, and D. marina isolates The samples that had visible bands are in bold.

No:	Template	Blocking	Primers
1	***A. nodusum* and *C. marina* 1, 1:100 from 02.10.2023**		**FF390/FR1**
2	***A. nodusum* and *C. marina* 1, 1:100 from 02.10.2023**	**BON3 1,5 µl**	**FF390/FR1**
3	*A. nodusum* and *C. marina* 1, 1:100 from 02.10.2023	BON3 5µL	FF390/FR1
4	*A. nodusum* and *C. marina* 2, 1:1000 from 02.10.2023		FF390/FR1
5	***A. nodusum* and *C. marina* + C.marina 2, 1:1000 from 02.10.2023**	**BON3 1,5 µl**	**FF390/FR1**
6	***A. nodusum* and *C. marina* 2, 1:1000 from 02.10.2023**	**BON3 5 µl**	**FF390/FR1**
7	Sterilized *S. latissima* from 11.10.2023		FF390/FR1
8	Sterilized *S. latissima* from 11.10.2023	BON3 1,5 µl	FF390/FR1
9	Sterilized *S. latissima* from 11.10.2023	BON3 5µl	FF390/FR1
10	Sterilized *S. latissima* from 11.10.2023 1:100		FF390/FR1
11	**Sterilized *S. latissima* from 11.10.2023 1:100**	**BON3 1,5 µl**	**FF390/FR1**
12	Sterilized *S. latissima* from 11.10.2023 1:100	BON3 5µl	FF390/FR1
13	Sterilized *S. latissima* from 11.10.2023 1:1000		FF390/FR1
14	Sterilized *S. latissima* from 11.10.2023 1:1000	BON3 1,5 µl	FF390/FR1
15	Sterilized *S. latissima* from 11.10.2023 1:1000	BON3 5µl	FF390/FR1
16	**Isolated *D. marina***		**FF390/FR1**
17	**Isolated *D. marina***	**BON3 1,5 µl**	**FF390/FR1**
18	**Isolated *D. marina***	**BON3 5µl**	**FF390/FR1**

To further ensure the presence of fungi in my amplifications, Chun Li and I conducted Sanger sequencing on the PCR products from samples 1, 2, 5, 6, and 11. These samples were chosen because they yielded visible bands in the gel. The sequences were identified using the Basic Local Alignment Search Tool (BLAST) using the National Center for Biotechnology Information (NCBI) reference database in addition to local databases. The blast result did show that I had the Basidiomycota fungus *D. marina* in all samples sequenced. However, this should not be the case in *A. nodusum* with *C. marina* samples, as these are Ascomycota, or in the sterilized *S. latissima* samples. This suggests that these samples were contaminated with the isolated *D. marina*.

3.3 DNA extraction using several commercially available DNA extraction kits

Using Quick-DNA Fungal/bacterial Miniprep Kit and GenElute Bacterial Genomic DNA kit, I was not able to extract or amplify the fungal DNA from *Saccharina latissima*. Vortexing did not successfully homogenize the sample. Bead beating, although effective for homogenization, resulted in samples that were too viscous for pipetting. Adjusting the amount of sample material and bead beating time allowed us to proceed with the protocol, but PCR amplification did not produce bands. Using liquid nitrogen and a mortar for homogenization resolved the viscosity and homogenization issues, but still did not yield bands in the gel after attempts to amplify the DNA using PCR amplification. The Quick-DNA Fungal/bacterial Miniprep Kit and GenElute Bacterial Genomic DNA kit were discarded as options after these attempts and after a recommendation to use the DNeasy PowerSoil Pro kit by Detmer Sipkema and Federica Schanz at Wageningen University & Research. The protocol in DNeasy PowerSoil Pro kit suggested different methods for homogenization. I attempted the methods mentioned in the kit in addition to the homogenization using liquid nitrogen. This resulted in low DNA quality and quantity, and yielded no bands after PCR amplification (data not shown).

3.4 CTAB extraction of fungal DNA from *Saccharina latissima*

The CTAB extraction method was successful, and I did amplify the fungal DNA. However, the different batches of the CTAB solution caused the depletion of eleven kelp samples, marked in yellow in Table 2. In addition, due to lack of time, I did not manage to sequence all samples.

My supervisor and I did utilize the first batch of the CTAB solution to extract fungal DNA of five *Saccharina latissima* samples, amplifying it to make sure our methods worked. The five extractions were made from the same individual, the extraction with highest DNA concentration measured in Qubit was analyzed further, this was sample "CTAB extracted *S. latissima* (4) diluted 1:50", this had a concentration of 22,8 ng/µl measured in Qubit. Table 9 and Figure 14 show the amplification of the extracted DNA, "CTAB extracted *S. latissima* (4) diluted 1:50", in addition, I included samples; "CTAB extracted *S. latissima* (5) diluted 1:50", isolated *D. marina*, and two negative controls using different primer sets. The *S. latissima* samples and isolated fungal samples show strong bands in all samples. We also see a band in the samples with FF390/FR1 primers. It was confirmed that there was fungal DNA amplified in these samples by using Nanopore sequencing (see section 3.5 for the results from Nanopore) before continuing extraction using CTAB for the rest of the *S. latissima* samples.

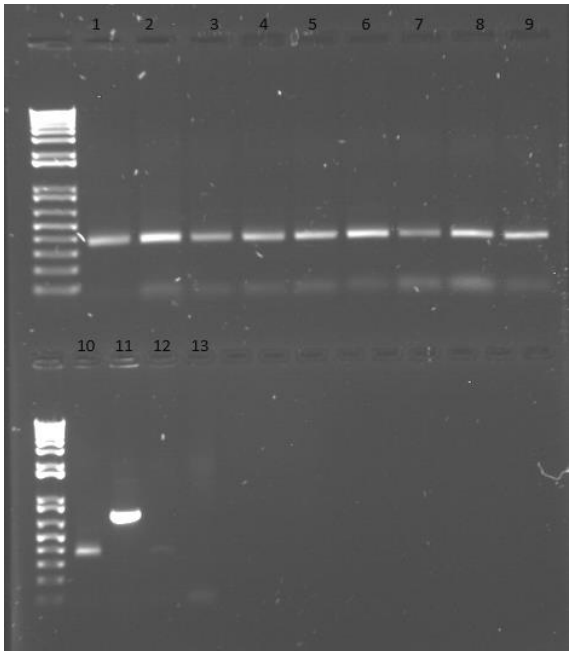

Figure 14: Picture of the gel electrophoresis of the PCR products from CTAB extracted fungal DNA from Saccharina latissima that yielded the highest concentration in Qubit after the first extraction of fungal DNA from S. latissima. The sample IDs correspond to sample the IDs table 10. The ladder used is the 1Kb+.

Table 9: Template for PCR analysis of CTAB extracted fungal DNA from Saccharina latissima.

No:	Template	BON3	Primers
1	CTAB extracted *S. latissima* (4) diluted 1:50		FF390/FR1
2	CTAB extracted *S. latissima* (4) diluted 1:50		FF390/FR1
3	CTAB extracted *S. latissima* (4) diluted 1:50		FF390/FR1
4	CTAB extracted *S. latissima* (4) diluted 1:50	1,5 µl	FF390/FR1
5	CTAB extracted *S. latissima* (4) diluted 1:50	1,5 µl	FF390/FR1
6	CTAB extracted *S. latissima* (4) diluted 1:50	1,5 µl	FF390/FR1
7	CTAB extracted *S. latissima* (4) diluted 1:50	5 µl	FF390/FR1
8	CTAB extracted *S. latissima* (4) diluted 1:50	5 µl	FF390/FR1
9	CTAB extracted *S. latissima* (4) diluted 1:50	5 µl	FF390/FR1
10	CTAB extracted *S. latissima* (5) diluted 1:50		FF390/FR1
11	Isolated *D. marina*		ITS4 and ITS 5
12	Negative		FF390/FR1
13	Negative		ITS4 and ITS5

After extracting all samples using the second batch of CTAB, I saw samples with heavy precipitation after adding the isopropanol (Figure 16). These samples did show DNA concentrations ranging from 3-66 ng/µl measured in Qubit. Therefore, I attempted to amplify the DNA from the second batch of CTAB solution, the results, Figure 15, confirmed that these samples, thus high concentrations measured in Qubit, were not suitable for further use.

Due to the error in the second batch of CTAB solution, I depleted eleven *S. latissima* segment samples.

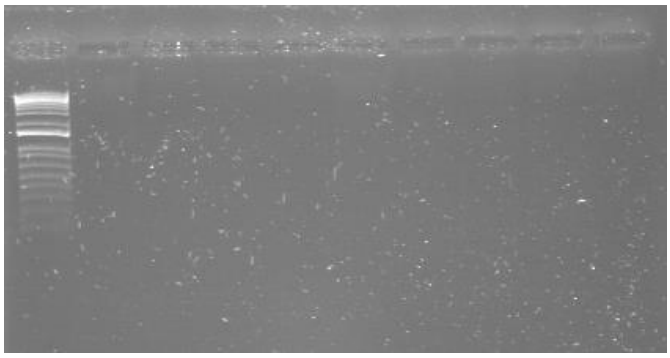

Figure 15: Picture of the gel electrophoresis of the PCR products from three S. latissima samples from the second batch of CTAB solution , first three wells are without BON3, the three next are with 1,5 µl BON3. I used samples that had higher that 50 ng/µl DNA measured in Qubit in the extracted template. We see no bands. The ladder used is the 1Kb+.

Figure 16: Samples extracted using the second batch of CTAB after adding isopropanol.

The remaining 29 segment samples were extracted using the first batch of CTAB and amplified. I did see bands in several samples, but not in every sample, see Figure 17 and Figure 18. In addition, I attempted to use Betaine Enhancer Solution from VWR life science (Denmark) in some samples, but I saw no increased consistent yield using the Enhancer. Further, I saw bands in most samples but fewer bands when BON3 was in the reaction. I investigated the concentration after purification in NanoVue, with the concentration varying from -1,6 to 78 ng/µl (see appendix: "NanoVue of samples, the first batch of CTAB"). I sent the samples that yielded bands to Illumina sequencing, but the quality or quantity of DNA was too low for sequencing.

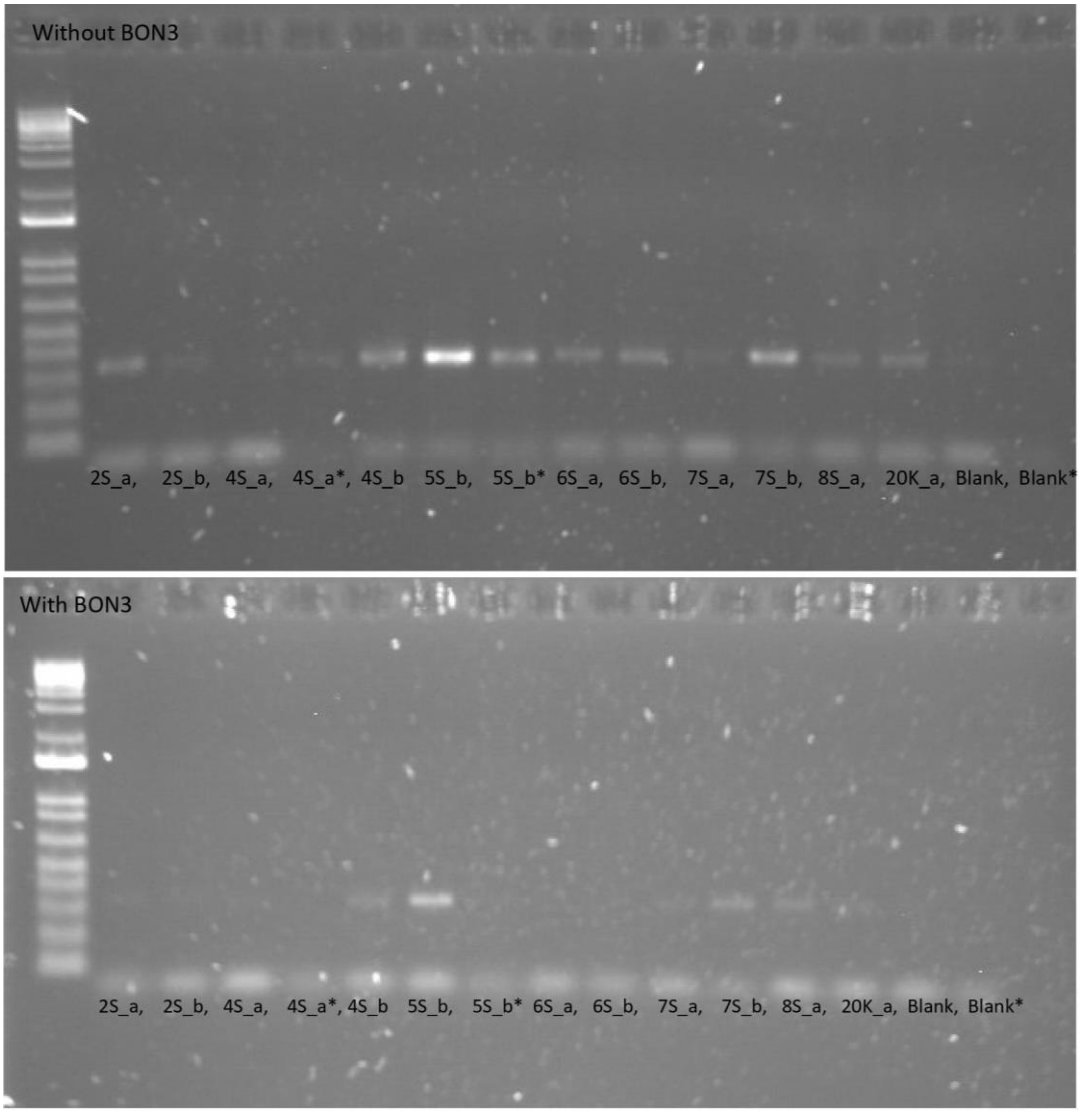

Figure 17: Picture of the gel electrophoresis of the PCR products from eleven S. latissima samples with and without BON3 from the first batch on CTAB , along with two samples treated with a DNA enhancer. The sample IDs correspond to the sample IDs in Table 2. Two blank (control) samples were included: one treated with the DNA enhancer and one without. The ladder used is the 1Kb+. The gel displays PCR products amplified both without and with BON3. Samples treated with the DNA enhancer are indicated with an asterisk (*).

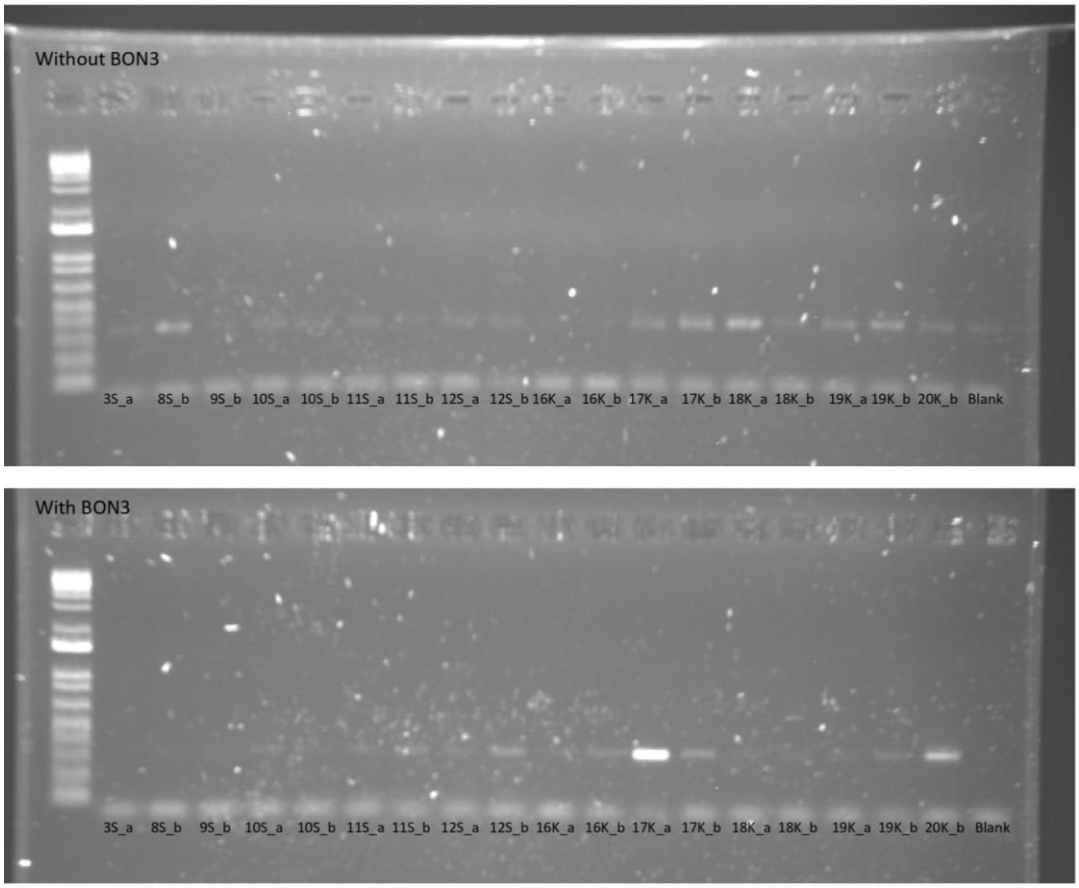

Figure 18: Picture of the gel electrophoresis of the PCR products from eighteen S. latissima samples from the first batch of CTAB with and without BON3. The amplified DNA was extracted using the first batch of CTAB solution. One blank (control) sample was included. The ladder used is the 1Kb+. The sample IDs correspond to the sample IDs in Table 2.

To increase the DNA yield, I attempted to increase the DNA template in the PCR reaction. I increased the template for the PCR reaction for samples without BNO3 from 1 µl to 11,5 µl, and for samples with BON3, I increased it from 1 µl to 10 µl. I did not see bands in all samples, nor were the bands stronger in Figure 19 and Figure 20 compared to the earlier PCR amplifications.

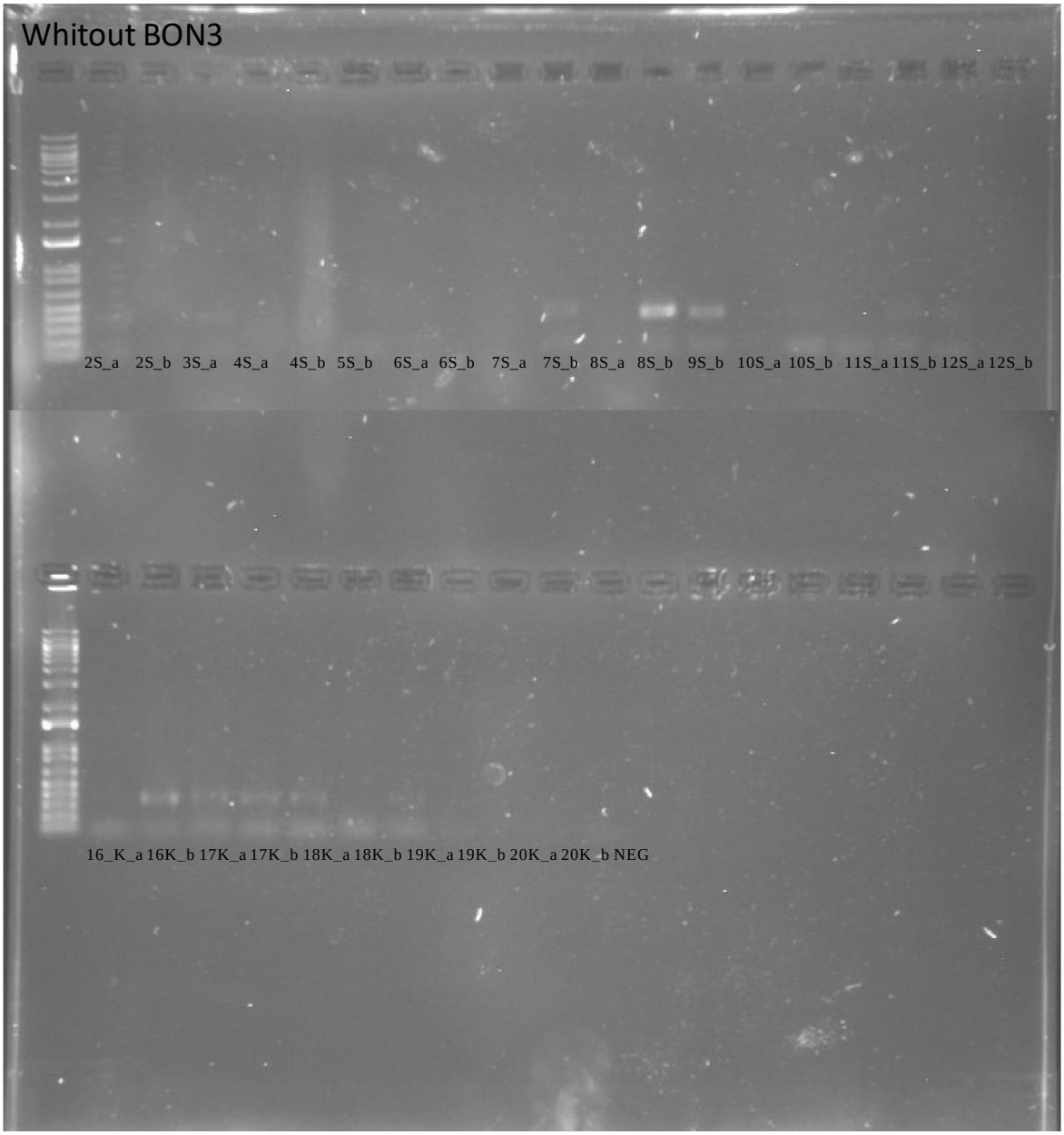

Figure 19: Picture of the gel electrophoresis of the PCR products from 29 S. latissima segment samples without BON3 and increased DNA template. The amplified DNA was extracted using the first batch of CTAB solution. The DNA template in the PCR reaction was increased to 11,5 µl DNA template. One control sample was included. The ladder used is the 1Kb+. The gel displays PCR products amplified without BON3. The sample IDs correspond to

the sample IDs in Table 2.

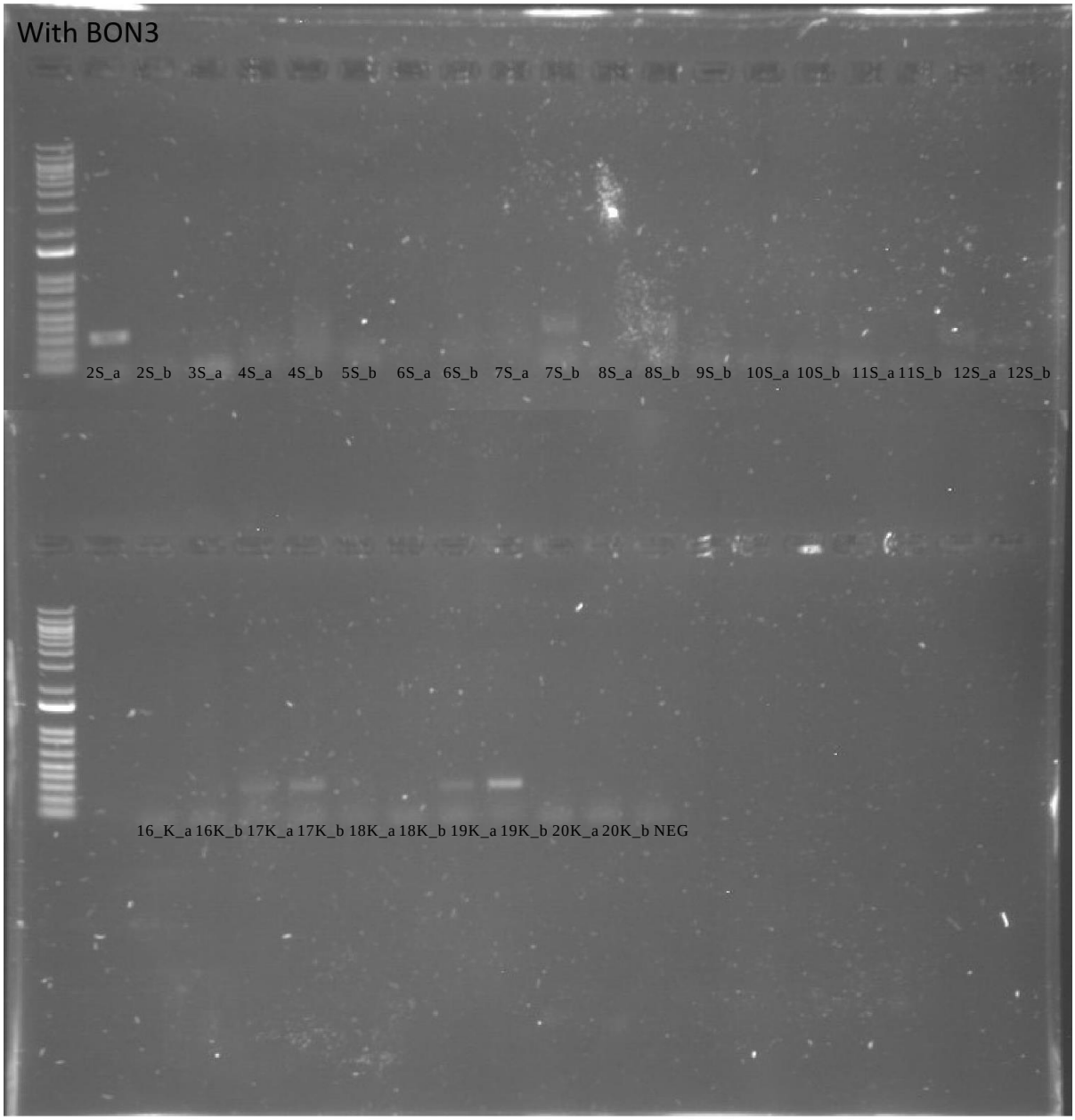

Figure 20: Picture of the gel electrophoresis of the PCR products from 29 S. latissima segment samples with BON3 and increased DNA template. The amplified DNA was extracted using the first batch of CTAB solution. The DNA template in the PCR reaction was increased to 10 µl DNA template. One control sample was included. The ladder used is the 1Kb+. The gel displays PCR products amplified with BON3. The sample IDs correspond to the sample IDs in Table 2.

I attempted to reamplify the PCR products that yielded positive bands in the increased template PCR reaction. This yielded stronger bands, Figure 21, and a bit higher concentration. I checked the concentrations for 4 samples in NanoVue, they ranged from 3,7-8,5 ng/µl.

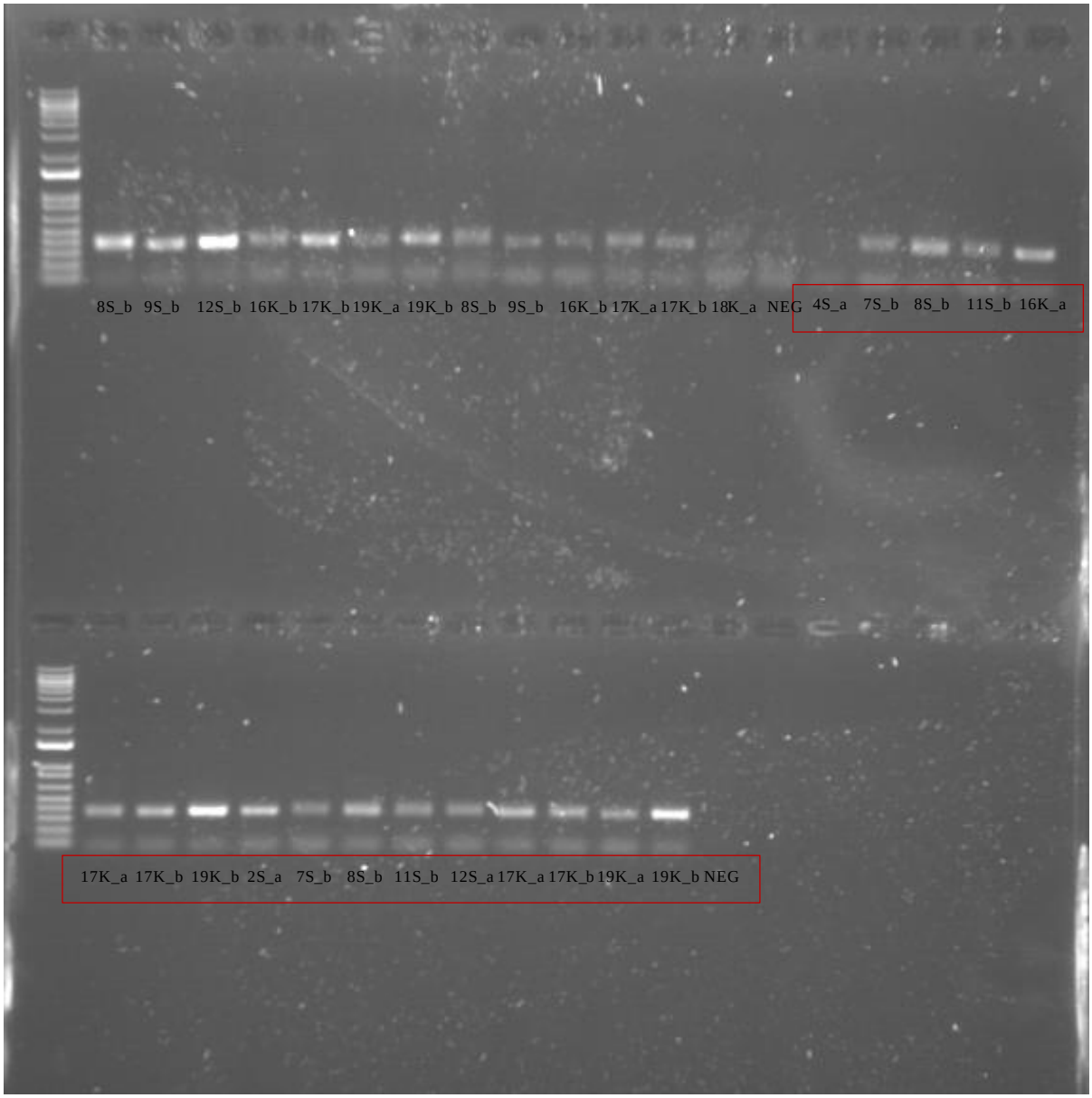

Figure 21: Picture of the gel electrophoresis of the PCR products from the reamplfication of the increased template PCR amplification (Figure 19 and Figure 20). Samples not outlined did not have BON3 in them when amplified with the increased template, samples in the red boxes are samples that had BON3. The ladder used is the 1Kb+.

The reamplification was also attempted using repliQa® HiFi ToughMix® to increase the DNA yield. I have an additional band in the gel or smear, as shown in Figure 22, compared to the other PCR amplifications and did therefore not proceed with repliQa. We did sequence the samples reamplified with repliQa® HiFi ToughMix® using Nanopore, but this gave no sequences. This suggests that the extra bands do not contain any relevant sequence information or potentially are a mix of sequences that could not have been resolved by Nanopore sequencing.

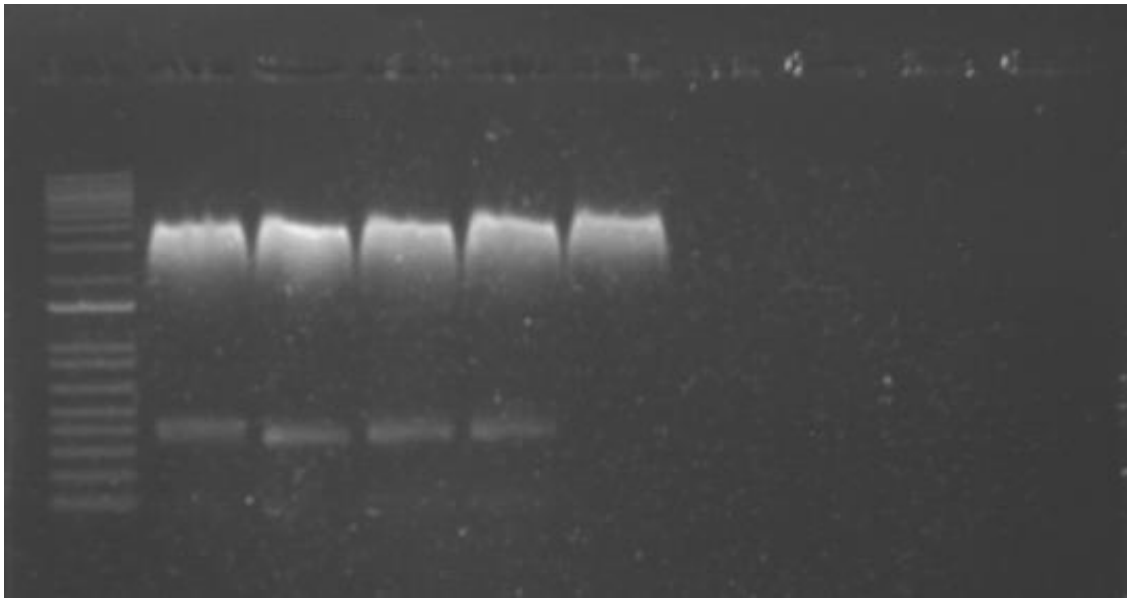

Figure 22: Picture of the gel electrophoresis of the PCR products from the reamplification using repliQa® HiFi ToughMix . The ladder used is the 1Kb+.

3.5 Nanopore sequencing of PCR products from algae

We conducted Nanopore sequencing to investigate the presence of fungi in the five samples on five PCR products, one isolated fungal DNA from *Digitataspora Marina* sample that was amplified with ITS primers, three liquid nitrogen homogenized and CTAB extracted *S. latissima* samples with 0 µl, 1.5 µl, and 3 µl of BON3 that were amplified with 18S primers, and one a negative control sample amplified with 18S primers. The test was made on PCR products derived from DNA extracts that were extracted using the first batch of CTAB solution early in the project. It was meant as a control to see if there were fungi in the *S. latissima* samples, but remained the only sequence results and are presented here as our main results due to failure in getting other samples amplified and prepared for sequencing.

The nanopore sequencing yielded 10 fungal taxonomic groups, shown in Table 10, in addition to other eukaryotic taxa (Table 10 and Figure 22). The fungal DNA is dominant in all samples sequenced. The sample of fungal gDNA 008cD1.1 that was amplified with ITS primers was used to make sure the PCR reaction worked. I did see sequence reads from the isolated fungal gDNA sample 008cD1.1, and I did see that this was the only species in the sample. In samples with BON3, we can see fewer reads in general, and with a higher volume of BON3, we see fewer reads and fewer other taxa than fungi. In the negative samples, I got, in general, few reads, but I have reads from two fungal taxa in addition to Bryozoa and Cercozoa.

Table 10: Taxonomic identification on fungal taxa in the S. latissima samples with the number of reads per taxa and total reads.

Region	*S. latissima* (18S)	*S. latissima* (18S and BON3 (1.5 µl))	*S. latissima* (18S and BON3 (3 µl))	Negative control	Taxon
18S	672	339	190	11	*Digitatispora marina*
18S	14	23	63	0	*Burgella sp.*
18S	53	15	8	0	*Malassezia sp.*
18S	54	0	0	11	*Meyerozyma sp.*
18S	4	52	3	0	*Clavispora sp.*
18S	19	29	10	0	*Halocyphina sp.*
18S	16	8	0	0	*Cladosporium sp.*
18S	16	6	0	0	*Taphrina sp.*
18S	17	0	0	0	*Metschnikowia sp.*
18S	0	4	0	0	*Rhodotorula sp.*
Total fungal reads	865	476	274	22	

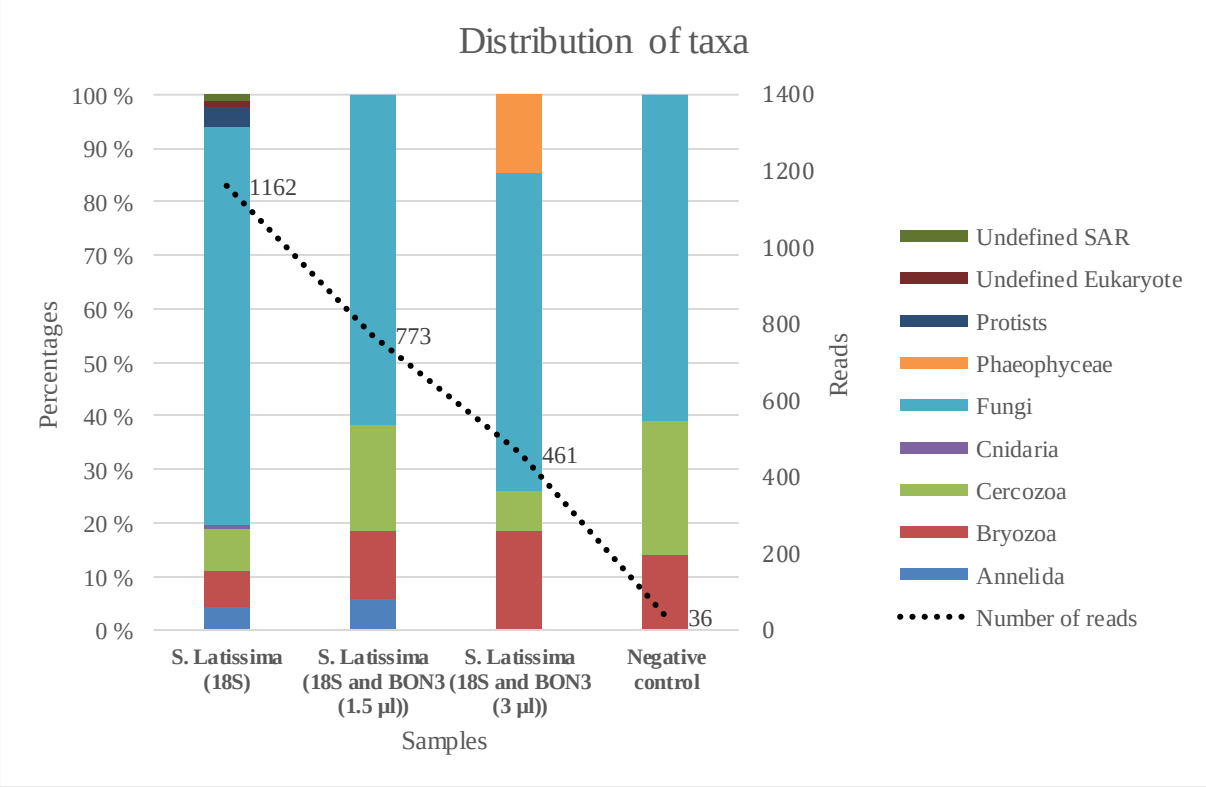

Figure 23: Graphical presentation of the distribution of the higher taxa in the S. latissima samples. The number of reads per sample is also shown using the dotted line. Mark that the number of reads is different for each sample.

4 Discussion

The aim of this study was to investigate the fungal community on *Saccharina latissima*, the diversity of the fungal community, and how this differs between a natural kelp forest and a kelp cultivation facility, in addition to seasonal variation. The results of this study show that there are fungal communities on *Saccharina latissima*. However, due to the extensive troubleshooting of DNA extraction and amplification to get genomic data for the fungal communities on *S. latissima*, I cannot draw conclusions about the seasonality or differences between the cultivated and wild *S. latissima*.

4.1 Methods

4.1.1 Kelp sampling

The sampling methods were successful but had some limitations. Following the health and safety protocols, I was unable to dive underwater and was dependent on having a knife with an extender that made harvesting kelp material difficult, especially at high tide. The harvesting of the samples was attempted during low tide and in good weather conditions. However, this also needed to fit me and my snorkeling partner's schedule for us to be able to sample. Furthermore, as I transported the samples from the water to land, it was impossible to keep the samples from each other if I took several individuals at a time. In addition, the samples were attached to me as I came up from the water in a collection bag that was in contact with the surrounding environment. This could potentially provide some contaminants from the surrounding area. However, this is probably not a big problem, as I expected the fungi that are present in the water and surrounding the *S. latissima* probably also be present on the beach and surrounding area vice versa.

For the cultivated facility, time scheduling was the biggest threshold for sampling. As I was reliant on the workers from Akvaplan-NIVA and Oceanfood for sampling at the cultivated site, it had to fit with our and their schedules. This limited the number of samples I was able to get. However, this was easier to collect as we could lift the rope at the ocean kelp cultivation rig and easily cut off some individuals. However, this was also not done sterile, and the kelp was in contact with the surrounding area and potentially contaminated.

However, with 20 samples, I did get samples from every season, with samples from both the cultivated and kelp forest. If the DNA extraction and amplification methods had worked, I think that these samples would have given a representative overview of the fungal communities.

Furthermore, general observations comparing the cultivated and kelp forest sites show a big difference in species inhabiting the kelp and surrounding area. It's natural that we see more species of macroalgae in a kelp forest than in a cultivation facility. In addition, I do see fewer epiphytes on the *S. latissima* in the cultivation facility compared to the wild individuals.

4.1.2 Challenges and improvements in fungal DNA extraction from *Saccharina latissima*

The methods for extracting fungal DNA from *S. latissima* need improvement.

The commercial kits used previously in the lab at Marbio did not yield satisfactory results when extracting my kelp samples for DNA. I attempted using several kits, as shown in Table 5, but the DNA extracts were of poor quality, and the PCR amplification using FF390 and FR-1 and ITS4 and ITS5 primers did not produce bands in the gel electrophoresis for the *S. latissima* samples.

In addition, the homogenization method in all kits did not work. By vortexing the kelp, it did not get homogenized, and the bead beating turned the samples to a viscous, slime-like texture, making it hard to pipet. The viscosity problem and amplification problem can be due to abundant polysaccharide compounds in *S. latissima*. Kelp and *S. latissima* contain a substantial number of polysaccharides and alginate. Polysaccharides and alginate are used in several products for their thickener effects, this ability might also have affected my method. In addition, polysaccharides are a known inhibitors in PCR amplification [100].

To resolve the viscosity problem and improve the DNA yield, I abandoned the bead-beating and vortexing stage and homogenized the kelp samples using liquid nitrogen and a mortar. This method adjustment resolved the viscosity problem but did not improve the PCR amplification after DNA extractions, nor did it result in a high concentration of DNA after purification. After several trials using all three kits, I concluded that the kits were unsuitable for our samples. Our hypothesis is that the DNA extraction kits do not clean the DNA well enough.

However, our colleagues at Wageningen University & Research, Detmer Sipkema, Federica Schanz and Thomas Weisbeek, did proceed using the DNeasy Powersoil kit and bead beating homogenization before following the protocol from the producer and successfully using it to amplify bacterial DNA from *S. latissima*[101]. They did not meet the viscosity problem as I did. However, when amplifying fungal DNA using the FF390/FR1 primers, Andrea Molina Almeida, Detmer Sipkema and Federica Schanz shared in personal communication that they

also encountered that the PCR amplification did not yield sufficient or good enough quality DNA to sequence.

Given the difficulties with the kits, I decided to try the cetyltrimethylammonium bromide (CTAB) DNA extraction. My supervisor, Teppo Rämä, used this method in his Ph.D. for next-generation 454 pyrosequencing of wood-inhabiting fungi with good results[3]. Our hypothesis is that we had several difficulties extracting the DNA using the kits because of the high concentration of polysaccharides that inhibited PCR amplification and homogenization. Using the CTAB method would mitigate this problem [102].

Two batches of CTAB were prepared. The first batch yielded bands when I conducted gel electrophoresis after PCR amplification, but the second batch did not; in addition, it acted differently when adding isopropanol compared to the first batch. As seen in Figure 16 with the precipitation of a white material. This could have been DNA, but after the full protocol, the Qubit measurement was not as high as I would expect if the precipitation had been DNA, and there were no bands in the gel after PCR. Therefore, I think that this might have been some kind of contaminant or content from the CTAB solution. Yannik Karl Heinz Schneider at Marbio did conduct mass-spectrometry (MS) to investigate if it was polysaccharides in these samples, but did not detect that, the result from the MS analysis is attached in the appendix.

The CTAB extraction method using the first batch of CTAB solution yielded a higher DNA concentration in the extracts and bands in gel electrophoresis compared to the kits, but it did not give us consistent results or a good enough quality or concentration to send to sequencing at a commercial company, Novogene.

Nevertheless, I did send our samples to Novogene for Illumina sequencing. This did not yield results, as the samples did not pass their quality control. I hoped that the sequencing might work on some samples, and even though the samples were under the company´s limiting values, I had hoped that some results would be provided. However, all samples showed a low concentration or too low a quality of DNA for it to be sequenced.

I attempted to increase the amount of template material in the PCR reactions. Too much DNA may inhibit the PCR amplification, but I do not think this is a problem in my study. The DNA concentration must be over 500-1000 ng/µl to inhibit the PCR reaction[103], the qubit measurements for the extracts did not yield higher than 70 ng/µl for any samples. The measurements taken after amplification with more DNA template did yield a slightly higher

concentration in NanoVue; however, the quality remained low. The quality was assessed by looking at the ratio between the A260/A280 spectra and the ratio between the A260/A230 spectra in NanoVue[104]. The ratio of A260/A280 is a purity measurement where 1,8 is the optimum, and the A260/230 is also a purity measurement where 2-2,2 is considered pure DNA[105]. In my measurement, there are no consistent trends other than my samples generally being under over the values of what is considered pure DNA, making it impossible to determine from these results what might be the contaminant or problem.

The extractions using the kits and the CTAB method exhausted some of our samples. In addition, I was still struggling to get a high-quality DNA concentration after purification. I, therefore, decided to try to reamplify the already amplified PCR products. I followed the suggestions in the protocol from UC Davis labs for PCR reamplification (attached in the appendix). The results from the reamplification did yield higher concentration, but this increase was not significant, nor was the quality good.

In addition, PCR reamplification can be done, but it is prone to some risk. Reamplification of PCR products can increase the chances of nonspecific primer binding, chimeric sequences, DNA degradation, increased risk of contaminants or increased primer biases[106].

To further increase the DNA concentration, I wanted to try to repeat the amplification of the same samples and pool them together to increase the DNA concentration after purification. This was done for the PCR products from algae that we conducted the Nanopore sequencing on, described in section 3.5. However, these were not reamplified due to time limitations; I aimed to do this on all samples yielding bands in the increased PCR template amplification. Another problem was the purification of the DNA using the QIAquick PCR Purification Kit, which also caused us to lose some DNA. I planned to use magnetic bead purification to prevent DNA loss, with an optimized protocol that should yield up to 80% DNA after purification of the samples. Repeating the reamplification and optimizing the DNA for magnetic bead purification took some time, and I did not manage to do this in time for this thesis.

Circling back a bit, in the nanopore sequencing we did use samples amplified with Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK). I used the PCR beads instead of DreamTaq to skip purification needed to remove the dye in Dreamtaq before Nanopore sequencing. The purification would lead to loss of DNA lowering the DNA concentration in the samples. The skipping of the purification could result in some potential

errors in the Nanopore sequencings, but the aim was to confirm the presence of fungal DNA in the samples, and the problems with low DNA concentration were bigger than the concern of errors in nanopore sequencing. I did get sequences read from these samples but did not use the PCR beads further.

I did not use PCR beads further because I did not observe a big difference in the bands in the gel electrophoresis for samples amplified with PCR beads and DreamTaq. Therefore, I concluded to continue to use DreamTaq instead of using the PCR beads. In hindsight, I did get the fungal DNA from these samples amplified using PCR beads, and was able to assign OTUs to the sequences. I was not able to sequence the samples using DreamTaq. I should have experimented more using PCR beads.

4.1.3 Primers and PCR conditions

I did use the SSU (small subunit ribosomal RNA or 18S) gene instead of the ITS (internal transcribed spacer) region. The SSU is more effective because there is a broader selection of sequences available, increasing confidence in the sequencing results when aligned with the reference library[80]. While the ITS region is better for species-level identification, it requires comprehensive and accurate reference libraries to identify species accurately. Marine fungi are a relatively novel field of study, and the existing libraries contain a limited number of reference sequences. Therefore, our goal was not to describe the fungal DNA found down to the species level, but rather to identify higher taxonomic groups to describe the fungal diversity.

A further advantage over ITS is that the 18S primers I used in the amplification of the SSU region, FR1/FF390, have complementary blocking oligonucleotides available that block unwanted amplification of non-fungal organisms[81]. The blocking oligonucleotide I used was BON3. BON3 was designed to block stramenopiles. As *S. latissima* is a stramenopile, I chose this blocking oligonucleotide to block the amplification of host DNA. However, Banos et al., 2018, include 4 blocking oligonucleotides that each inhibit the amplification of different groups. Their paper does, however, investigate the DNA in environmental and soil samples, and as our sample materials were not as complex, the need for several blocking oligonucleotides might not be necessary. However, we obtained sequence reads from some organism groups that were blocked by the other oligonucleotides used by Banos et al. 2018, such as the SARs group, from which we obtained reads in our Nanopore sequencing. If I had used several blocking oligonucleotides, I might have gotten a cleaner sequencing result with fewer other organisms than fungi. Primer bias is another crucial factor to consider, as certain primers may amplify

particular sequences better than others, skewing the representation of the amplified DNA. Indeed, the results do show that the amount of BON3 does change the sequencing results by decreasing the number of overall reads, but also decreasing the number of detected taxa as expected, as detected in Banos et al. 2018[81].

The PCR conditions using the 18S primers were also optimized during the project. At the start of my PCR amplification testing, I used a higher annealing temperature than recommended for the primers. I used 47 ℃ as an annealing temperature instead of the recommended 44,5 ℃ as recommended by Banos et al. 2018. All results here are presented after we changed to 44,5 ℃. The higher temperature might have caused the primers to not bind to the DNA template optimally, and the unoptimized temperature PCR testing did cost us a lot of time at the start of the study.

A high annealing temperature increases amplification specificity but might result in reduced PCR products, whereas a lower annealing temperature might lead to nonspecific amplification. Ideally, a gradient PCR should be performed with a range of temperatures to determine the optimal annealing temperature for maximum efficiency.

4.1.4 Sequencing data

We performed nanopore sequencing on three pooled samples. When I encountered difficulty in amplifying the fungal DNA in my samples, I began to question the presence of fungal DNA in the samples. As a result, we conducted Nanopore sequencing on some samples to verify the presence of fungal DNA before proceeding with the remaining DNA extractions and amplification. The sequencing provided positive results, confirming the presence of fungi. This demonstrated that the CTAB extraction and PCR amplification using the PCR regime successfully amplified the fungal DNA in *S. latissima*.

However, performing the CTAB extraction on all 29 *S. latissima* samples using the two batches of CTAB solution did not yield satisfactory results and was time-consuming. Consequently, I did not have the time to sequence the rest of the samples, nor did I have the opportunity to sequence all samples as some were depleted or did not yield bands in gel electrophoresis.

The results obtained for the main results were from the Nanopore sequencing of three PCR products from the extract of one *S. latissima* sample. These results revealed the presence of 10 taxa of fungi in *S. latissima*. It's important to note that these results are somewhat limited as I used one *S. latissima* individual, and, therefore, these results might not be representative of the

kelp forest. The samples were not carefully prepared as they were part of a test to ensure the presence of fungi and were not intended to be included as our main results. The Nanopore sequencing yielded just over 1000 reads; however, for more robust results, one should aim for a range of 500,000 to 1,000,000 reads. Additionally, misreads in barcoding can lead to incorrect taxa assignment, and the use of data processing cutoffs may limit accuracy.

Furthermore, the presence of *Digitatispora marina* might be due to contamination in the sample preparation, particularly since it was also found in the negative control. Other factors that might impact sequencing data include the quality and concentration of the extracted DNA, which was not satisfactory in my case.

The absence of a comprehensive database for marine fungal DNA sequences can hinder the accuracy of identifying the taxa in the results. A lack of reference sequences can lead to underrepresentation or misidentification of the taxa in our samples. This underscores the importance of addressing the knowledge gaps in marine fungal databases.

4.1.5 Fungal taxa identified

Taxa identified in this study are linked to various marine fungi. ***Digitatispora marina (Basidiomycota)***, although it may be a contaminant, is found in the wood logs and branches in the surrounding area of Tromsø [107]. ***Malassezia* spp.** are associated with skin diseases in humans and animals, as well as marine animals, such as deep-sea sponges [108]. ***Meyerozyma* spp**. are yeasts isolated from a wide range of environmental sources, including fresh and salt water, soil, sand, amphibians, birds, and humans. They are also a known source of infections [109]. ***Halocyphina* spp**. specifically *Halocyphina villosa*, are a *Basidiomycota* associated with mangroves [110]. ***Cladosporium* spp**. are associated with molds and plants, mangroves, and diseases in corals, and are also found in brown algae washed up on beaches in Norway [111, 112]. ***Taphrina* spp.** is parasitic on vascular plants but has also been isolated from Antarctica where there are no vascular plants [113, 114]. ***Metschnikowia* spp.** are yeasts commonly found in nectar and fruits, where they play a role in fermentation[115], but are also commonly found in marine animals, including fish, and arthropods, as well as deep-sea sediments [112, 116]. ***Rhodotorula* spp**. are yeasts found in fresh, brackish, and marine waters, mangroves, and macroalgae in Antarctica [112].

It is also important to note that *D. marina* and *Halocyphina* spp. are close relatives and have similar 18S sequences. Therefore, there is a possibility that the detected *Halocyphina* spp. in this study are representing *D. marina*.

Nevertheless, this shows that even though I have limited reads and several limitations to my study and results, several of the fungal taxa found in the sequencing data are associated with the marine environment.

4.2 Expected results

I expected to find a wide variety of fungi on *S. latissima*, and I anticipated that this would vary with the seasons. Different types of fungi would be more prevalent during different seasons[117]. Additionally, I believed that the kelp forest sampling site with more macroalgal species would exhibit a higher diversity of fungi compared to the cultivated site, similar as shown in terrestrial studies where more species rich habitats contain more fungi[34-39].

If our results had confirmed these expectations, it would have been an important step in the research related to kelp cultivation in Norway. It would have provided new insights into the fungal communities associated with *S. latissima* in Northern Norway. This is particularly important as kelp cultivation is still in its early stages in Norway, knowledge about marine fungi is limited, and there is much that we do not yet understand.

In Asia, kelp and macroalgae cultivation facilities have faced challenges with pathogenic fungi that have diminished yields[12]. Having a better understanding of the fungal communities associated with *S. latissima* is crucial for the knowledge and monitoring of fungal communities in cultivation facilities. Comparing wild and cultivated sites gives us insight into the differences in diversity between a natural, diverse kelp forest and a more uniform cultivation facility. Understanding these differences can help us mitigate diversity differences and have a health ecosystem.

Biodiversity plays a major role in the resilience of an ecosystem [33-38, 118]. A cultivation facility is essentially a part of the ecosystem, impacting the sea floor by providing nutrients and absorbing nutrients from the upper waters[4]. However, pathogens in the cultivation facility can potentially spread to nearby natural kelp forests. By identifying the fungi present in the kelp forest and comparing them to those in the cultivated facility, we can mitigate the spread of potential pathogens. Additionally, cultivation facilities could encourage higher fungal diversity, leading to greater resilience in the kelp and reducing the potential need for pesticides.

Previous studies have shown that brown algae are in symbiotic relationship with fungi. One example of such symbiosis is the one between the brown alga A*scophyllum nodosum* and the fungus *Stigmidium ascophylli*. This symbiosis increases the resistance of the host alga to drought, enhances its reproduction, and promotes the general growth of the algae [43]. Similar symbiotic relationships likely exist among kelp and fungi in the marine environment[44]. Such potential symbiosis could be used to enhance the yield and health of the kelp. By mapping out the fungi associated with *S. latissima,* we would be one step closer to understanding this.

The expected results would also have significant implications for natural kelp forests, particularly considering the changing environment and the impact of climate change in the north. Many natural kelp forests in mid to northern Norway have been grazed down by sea urchins, and efforts are underway to restore them. Understanding the fungal communities associated with healthy kelp forests would enhance monitoring and restoration efforts.

Furthermore, looking at the bigger picture the sequencing data would have contributed to broader ecological studies. Comparing fungi across different regions and environmental conditions would provide insights into the drivers of fungal diversity and distribution. This knowledge could be valuable in understanding how fungi adapt to changes in the environment, such as increased temperature or acidification. Our data could have been used as a foundation for comparing fungi associated with *Saccharina latissima* in northern Norway and elsewhere.

In conclusion, the expected results would advance our understanding of the fungal communities associated with *S. latissima*, and they would also have implications, or at least lay the foundation, for improving kelp cultivation, ecosystem resilience, and contributing to broader ecological research.

5 Conclusion and future research

The study's aim of examining the fungal communities on *S. latissima* was not met to the extent I wanted. I amplified the fungal DNA using PCR and conducted a DNA analysis; the methods did work but need substantial improvements in several steps. I did not manage to amplify the fungal DNA from all samples, making us unable to compare seasonal differences and site differences in the samples. However, I could document the presence of 10 different fungal taxa within one *S. latissima* individual. Therefore, I conclude and verify our hypothesis that there are fungal communities in *S. latissima*.

But due to us not being able to amplify all samples I cannot verify or falsify, our other hypothesis; "There is a higher diversity of fungi in wild *S. latissima* compared to cultivated ones" and "The diversity community composition of fungi in *S. latissima* changes throughout the seasons". Nevertheless, previous studies discussed here suggest that these hypotheses may be correct, as increased plant diversity has been shown to correlate with higher fungal diversity, supporting our initial assumptions about kelp ecosystems[33-38].

Further research should focus on optimizing the DNA extraction and PCR amplification of fungal DNA from *S. latissima* and other kelp and brown algal species. A focus for optimization should be i) DNA extraction as the algal material can inhibit further analysis, ii) DNA amplification with optimal primers and blocking oligonucleotides, and iii) purification that limit loss of DNA. We hope that this study will provide some insights and help in the optimization process.

We still aim to sequence the remaining samples we were unable to sequence through this thesis.

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AI disclosure statement

During the preparation of this work, ChatGTP and Grammarly were used to improve the writing style and check grammar and spelling. After using these tools, all content has been reviewed and edited as needed, and I, Simmen Karoliussen, take full responsibility for the content of this thesis.

Appendix

Appendix 1: CTAB extraction modified for extraction of fungal DNA form *S. latissima* samples (first batch)

Cetyltrimethylammonium bromide (CTAB) DNA extraction protocol modified for extraction of fungal DNA from *S. latissima* samples

1. Select the sample material for extraction and bring it into the Lamina flow hood for homogenization using liquid nitrogen.

2. Grind the tissue using an autoclaved mortar and pestle, adding liquid nitrogen, until you obtain a powdered sample.

3. Use a sterile spatula to place the sample into labeled Eppendorf tubes for future storage. Several Eppendorf tubes should be used for large amounts of ground tissue.

4. Add 200±10 µg of the homogenized kelp in a autoclaved Eppendorf tube.

5. Add 600 µL of preheated (65°C) autoclaved CTAB extraction buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, CTAB [2% w/v]) to each Eppendorf tube with 200 µg of kelp.

6. Freeze the sample at -80 °C overnight.

7. Take out the sample and incubate it for 30-40 min at 65°C on a heating block with occasional mixing.

Note: From steps 8-10, this part should strictly be carried out in a fume hood

8. Add 600 µL (Equal volume to CTAB buffer) of chloroform-isoamyl alcohol (24:1) to each sample and invert twice (a few times is OK) to mix.

9. Centrifuge at max g for 10 min

10. Transfer 400 µL of the aqueous upper layer into tubes containing 400 µL of cold isopropanol that were stored at –20°C. Invert the tubes and let the DNA precipitate for 10 min.

11. Centrifuge the tubes at max g for 5 min at 4°C. Place all the tubes the same way so the pellet is easier to locate.

12. Carefully decant supernatant from each sample (make sure you do not lose your DNA pellet); if a lot of the supernatant is still in the tube, remove using a pipet. Add 300µl 80% EtOH.

13. Centrifuge at max g for 5 min at 4°C.

14. Decant supernatant from each sample (if a lot of the supernatant is still in the tube, remove using pipet) and place the tubes in room temp for a couple of hours until dry.

15. Resuspend the pellet in 60 µL of sterile milli-Q water at 4°C. Put it on the heating block over night at 50 C. Try to mix and dissolve the pellet, a pipet can be used to carefully scrape the pellet to more easily dissolve it.

16. Test the concentration using Qubit and Nanoview before storing them at -20°C. The samples are now ready for future analyses.

Appendix 2: NanoVue of samples, first batch of CTAB

Table 11: Summary of purified PCR products and DNA Concentrations of 29 S. latissima segment samples using the first batch of CTAB solution.

Sample ID	Band	Nano viue				
			A values		Ratio	
2S_a	Yes	4,1	A230	0,41	A260/A280	0,828
			A260	0,165		
			A280	0,182	A260/A320	0,251
			A320	0,083		
2S_b	Weak	34	A230	9,8	A260/A280	2,491
			A260	9,41		
			A280	9	A260/A320	0,636
			A320	8,73		
3S_a	Weak	15,9	A230	0,318	A260/A280	1,554
			A260	0,54		
			A280	0,42	A260/A320	3,17
			A320	0,218		
4S_a	No	31	A230	9,43	A260/A280	310
			A260	9,71		
			A280	9,09	A260/A320	1,824
			A320	9,09		
4S_b	Yes	7,8	A230	-0,017	A260/A280	1,49
			A260	0,18		
			A280	0,129	A260/A320	-3,69
			A320	0,025		
5S_b	Yes	9,6	A230	-0,052	A260/A280	1,297
			A260	0,214		
			A280	0,17	A260/A320	-2,595
			A320	0,022		
6S_a	Yes	7,6	A230	-0,12	A260/A280	1,831
			A260	0,223		
			A280	0,154	A260/A320	-0,796
			A320	0,071		
6S_b	Yes	4,8	A230	0,195	A260/A280	0,865
			A260	0,26		
			A280	0,275	A260/A320	3,097
			A320	0,164		
7S_a	Yes	9,4	A230	-0,069	A260/A280	1,79
			A260	0,204		
			A280	0,121	A260/A320	-2,212
			A320	0,016		
7S_b	Yes	6,8	A230	-0,04	A260/A280	0,913
			A260	0,168		

			A280	0,181	A260/A320	-1,889
			A320	0,032		
8S_a	Yes	4,7	A230	-0,117	A260/A280	1,621
			A260	0,198		
			A280	0,162	A260/A320	-0,425
			A320	0,104		
8S_b	Yes	15,6	A230	10,63	A260/A280	-1,205
			A260	10,6		
			A280	10,03	A260/A320	0,915
			A320	10,29		
9S_b	Weak	9,9	A230	0,071	A260/A280	1,387
			A260	0,39		
			A280	0,33	A260/A320	-1,628
			A320	0,192		
10S_a	Weak	10,6	A230	-0,003	A260/A280	1,153
			A260	0,316		
			A280	0,288	A260/A320	-1,954
			A320	0,105		
10S_b	Weak	10,1	A230	0,165	A260/A280	1,683
			A260	0,324		
			A280	0,242	A260/A320	4,699
			A320	0,122		
11S_a	Weak	7,5	A230	0,254	A260/A280	1,25
			A260	0,329		
			A280	0,299	A260/A320	2
			A320	0,179		
11S_b	Weak	8,7	A230	0,072	A260/A280	1,395
			A260	0,308		
			A280	0,259	A260/A320	-2,746
			A320	0,135		
12S_a	Weak	13,4	A230	0,222	A260/A280	1,644
			A260	0,47		
			A280	0,37	A260/A320	15,8
			A320	0,25		
12S_b	Weak	9,2	A230	0,275	A260/A280	1,713
			A260	0,51		
			A280	0,44	A260/A320	-3,491
			A320	0,328		
16K_a	Weak	8,5	A230	0,189	A260/A280	1,259
			A260	0,328		
			A280	0,293	A260/A320	5,484
			A320	0,158		
16K_b	Weak	2,9	A230	0,065	A260/A280	0,58

			A260	0,273		
			A280	0,279	A260/A320	-0,509
			A320	0,179		
17K_a	Yes	13,6	A230	0,249	A260/A280	2,256
			A260	0,55		
			A280	0,4	A260/A320	-10,5
			A320	0,275		
17K_b	yes	7,7	A230	-0,012	A260/A280	1,07
			A260	0,278		
			A280	0,268	A260/A320	-1,117
			A320	0,125		
18K_a	yes	3,2	A230	0,005	A260/A280	0,587
			A260	0,194		
			A280	0,239	A260/A320	-0,512
			A320	0,13		
18K_b	yes	8,7	A230	0,271	A260/A280	2,086
			A260	0,37		
			A280	0,275	A260/A320	2,203
			A320	0,192		
19K_a	yes	8,7	A230	0,082	A260/A280	1,45
			A260	0,34		
			A280	0,285	A260/A320	-2,096
			A320	0,165		
19K_b	yes	17,5	A230	0,63	A260/A280	1,613
			A260	0,8		
			A280	0,67	A260/A320	1,802
			A320	0,45		
20K_a	Yes	11,1	A230	0,097	A260/A280	1,057
			A260	0,42		
			A280	0,41	A260/A320	-2,242
			A320	0,196		
20K_b	Yes	6	A230	-0,202	A260/A280	1,072
			A260	0,247		
			A280	0,239	A260/A320	-0,361
			A320	0,128		
Blank	Weak	6,7	A230	-0,255	A260/A280	0,943
			A260	0,192		
			A280	0,2	A260/A320	-0,424
			A320	0,05		
2S_a BON3	Weak	5,9	A230	0,003	A260/A280	0,893
			A260	0,241		
			A280	0,255	A260/A320	-0,967
			A320	0,124		

2S_b BON3	no	4,7	A230	0,012	A260/A280	1,576
			A260	0,327		
			A280	0,293	A260/A320	-0,419
			A320	0,234		
3S_a BON3		6,7	A230	-0,189	A260/A280	1,602
			A260	0,2		
			A280	0,15	A260/A320	-0,52
			A320	0,067		
4S_a BON3	No	40,5	A230	9,48	A260/A280	7,57
			A260	9,7		
			A280	9,01	A260/A320	1,397
			A320	8,9		
4S_b BON3	Yes	61,5	A230	7,83	A260/A280	3,739
			A260	7,99		
			A280	7,09	A260/A320	1,15
			A320	6,76		
5S_b BON3	Yes	6	A230	-0,38	A260/A280	1,308
			A260	0,183		
			A280	0,16	A260/A320	-0,264
			A320	0,068		
6S_a BON3	No	4	A230	-0,141	A260/A280	1,27
			A260	0,221		
			A280	0,204	A260/A320	-0,284
			A320	0,141		
6S_b BON3	No	8	A230	0,039	A260/A280	1,169
			A260	0,308		
			A280	0,285	A260/A320	-1,445
			A320	0,149		
7S_a BON3	No	4	A230	-0,216	A260/A280	0,738
			A260	0,203		
			A280	0,231	A260/A320	-0,232
			A320	0,124		
7S_b BON3	Weak	4,1	A230	-0,54	A260/A280	0,88
			A260	0,129		
			A280	0,14	A260/A320	-0,137
			A320	0,048		
8S_a BON3	Weak	8,9	A230	-0,006	A260/A280	1,171
			A260	0,36		
			A280	0,33	A260/A320	-0,967
			A320	0,178		
8S_b BON3	No	4,3	A230	-0,42	A260/A280	0,782
			A260	0,157		
			A280	0,181	A260/A320	-0,176

			A320	0,071		
9S_b BON3	No	1	A230	-0,48	A260/A280	0,328
			A260	0,152		
			A280	0,191	A260/A320	-0,031
			A320	0,133		
10S_a BON3	Weak	5,2	A230	-0,212	A260/A280	1,364
			A260	0,263		
			A280	0,235	A260/A320	-0,284
			A320	0,158		
10S_b BON3	No	0,5	A230	-0,122	A260/A280	0,141
			A260	0,175		
			A280	0,236	A260/A320	-0,035
			A320	0,165		
11S_a BON3	Weak	42	A230	4,13	A260/A280	1,909
			A260	4,71		
			A280	4,31	A260/A320	2,593
			A320	3,87		
11S_b BON3	Yes	48,5	A230	7,45	A260/A280	1,764
			A260	7,85		
			A280	7,43	A260/A320	1,702
			A320	6,88		
12S_a BON3	Weak	3,7	A230	-0,215	A260/A280	2,517
			A260	0,268		
			A280	0,224	A260/A320	-0,178
			A320	0,195		
12S_b BON3	Yes	6,2	A230	-0,264	A260/A280	2,841
			A260	0,291		
			A280	0,21	A260/A320	-0,291
			A320	0,166		
16K_a BON3	Weak	-1,6	A230	-0,215	A260/A280	-8,25
			A260	0,142		
			A280	0,179	A260/A320	0,0858
			A320	0,175		
16K_b + 17K_a BON3	Yes/ strong	78	A230	6,27	A260/A280	3,545
			A260	5,42		
			A280	4,3	A260/A320	0,647
			A320	3,86		
17K_b BON3	Yes	0,8	A230	-0,4	A260/A280	0,357
			A260	0,137		
			A280	0,164	A260/A320	-0,029
			A320	0,122		
18K_a BON3	No	1,2	A230	-0,34	A260/A280	0,243
			A260	0,145		

			A280	0,227	A260/A320	-0,54
			A320	0,12		
18K_b BON3	Weak	1,2	A230	-0,02	A260/A280	0,694
			A260	0,44		
			A280	0,45	A260/A320	-0,058
			A320	0,41		
19K_a BON3	Weak	2,2	A230	0,01	A260/A280	0,512
			A260	0,39		
			A280	0,44	A260/A320	-0,129
			A320	0,35		
19K_b BON3	Weak	0,6	A230	-0,129	A260/A280	0,113
			A260	0,143		
			A280	0,237	A260/A320	-0,046
			A320	0,131		
20K_a BON3	Weak	3,7	A230	-0,121	A260/A280	0,88
			A260	0,244		
			A280	0,254	A260/A320	-0,25
			A320	0,171		
20K_b BON3	Yes	0,1	A230	0,17	A260/A280	-0,033
			A260	0,86		
			A280	0,83	A260/A320	-0,001
			A320	0,86		
Blank BON3	No	2,6	A230	-0,222	A260/A280	0,718
			A260	0,179		
			A280	0,199	A260/A320	-0,146
			A320	0,128		

Appendix 3: Mass-spectrometry results

To investigate the composition of the second batch of CTAB solution used for DNA extraction, we performed mass spectrometry on the DNA samples extracted with this batch. We expected to detect polysaccharides. Given the slimy texture after adding isopropanol. However, the results do not show presence of polysaccharides. The analyze did indicate the preces of compounds in the CTAB solution (Table 12, Figure 24, Figure 25 and Figure 26), this was the only peeks of interest in the MS analysis. The second batch of CTAB had higher counts than the first batch of CTAB.

Table 12: Compounds found in MS of the first and second batch of CTAB solution used for DNA extraction.

M/z	RT (min)	Compound	%	In CTAB
312,36378	9,86	C12H45N	100	Yes
340,34256	10,53	C23H44N	100	Yes
368,42442	11,75	C25H53N	100	Yes
284,24559	11,98	C18H37NO	100	Yes

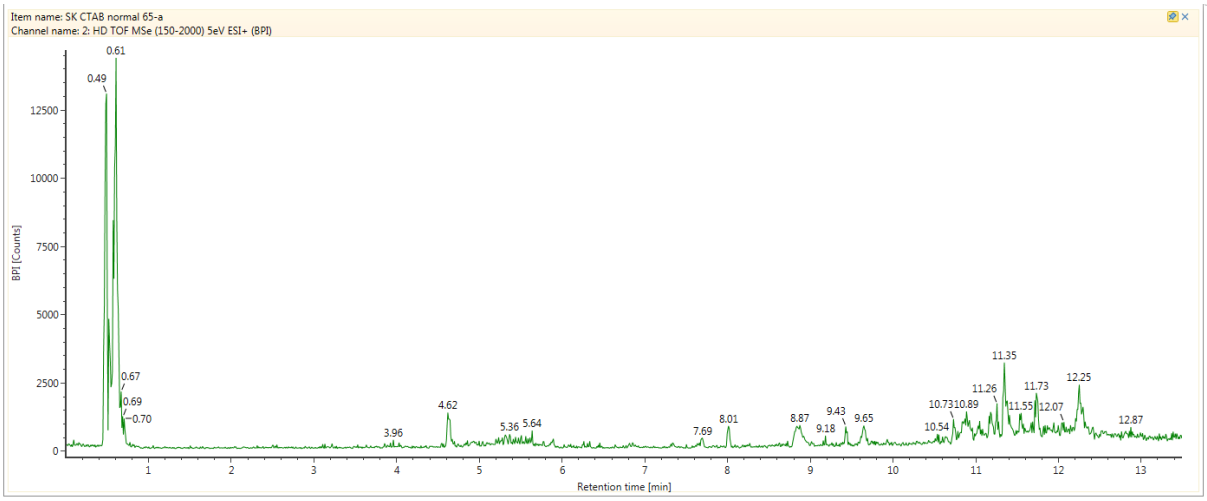

Figure 24: Full MS results from samples extracted using the first batch of CTAB solution.

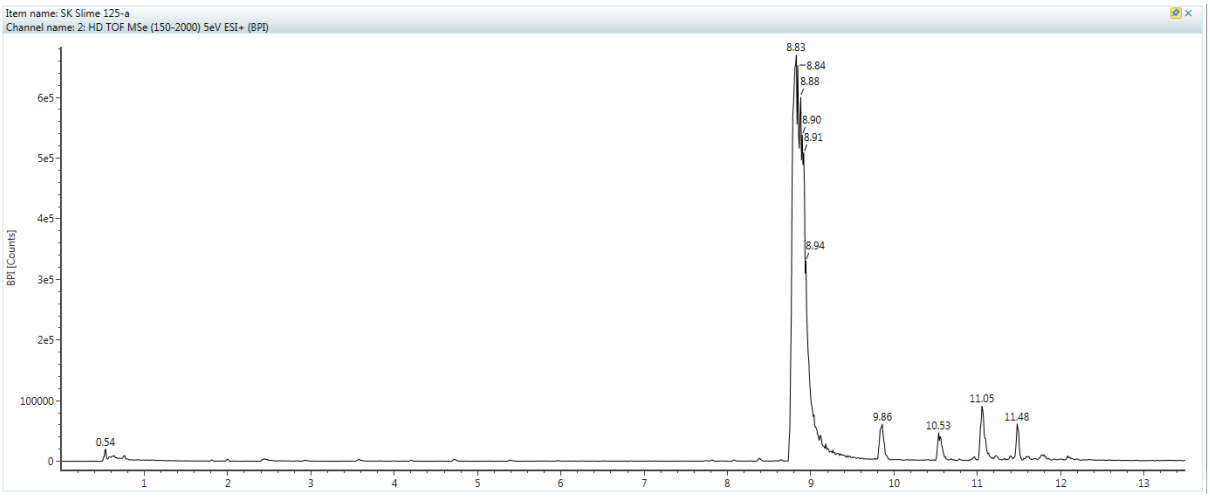

Figure 25: MS result segment of interest from samples extracted using the second batch of CTAB solution.

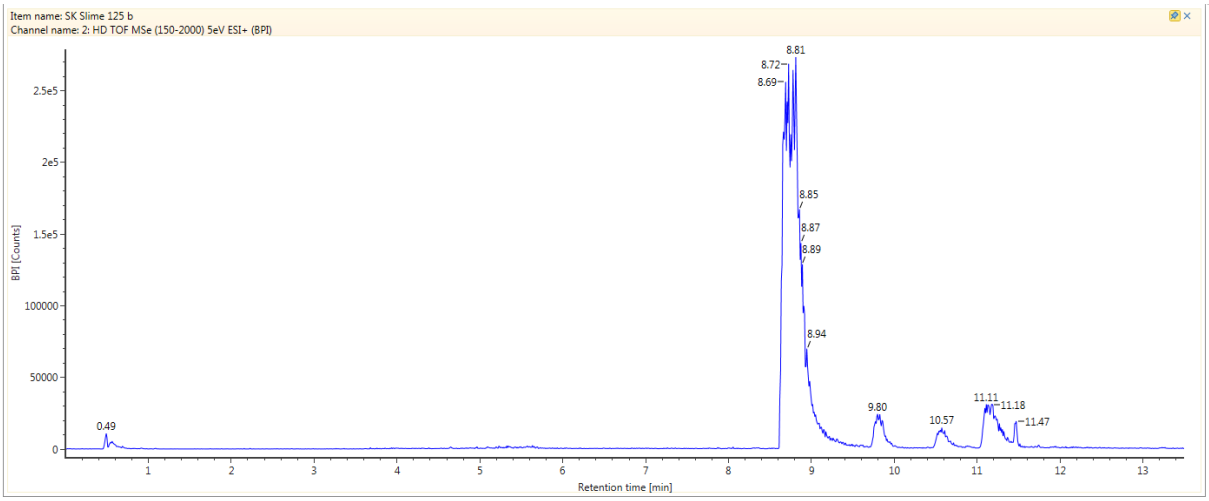

Figure 26: MS result segment of interest from samples extracted using the second batch of CTAB solution.

Appendix 4: Protocol for Rapid Barcoding Sequencing

Rapid Barcoding Sequencing (SQK-RBK004)

Version: RBK_9054_v2_revZ_14Aug2019
Last update: 28/03/2022

Oxford NANOPORE Technologies

Flow Cell Number: ..... DNA Samples: .....

Before start checklist

Materials	Consumables	Equipment
☐ 200 ng high molecular weight genomic DNA	☐ Flongle device - flow cell and adapter	☐ Ice bucket with ice
☐ Rapid Barcoding Sequencing Kit (SQK-RBK004)	☐ 1.5 ml Eppendorf DNA LoBind tubes	☐ Microfuge
☐ Flongle Sequencing Expansion (EXP-FSE001)	☐ 0.2 ml thin-walled PCR tubes	☐ Timer
☐ Flow Cell Priming Kit (EXP-FLP002)	☐ Nuclease-free water (e.g. ThermoFisher, cat # AM9937)	☐ Thermal cycler or heat block at 30°C and 80°C
	☐ Agencourt AMPure XP beads	☐ Pipettes and pipette tips P2, P20, P100, P200, P1000
	☐ Freshly prepared 70% ethanol in nuclease-free water	
	☐ 10 mM Tris-HCl pH 8.0 with 50 mM NaCl	

INSTRUCTIONS	NOTES/OBSERVATIONS
Library preparation	
IMPORTANT ☐ Flow cell deterioration/saturation	RB 01
IMPORTANT ☐ Flongle Sequencing Expansion	
Thaw kit components at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below: ☐ Fragmentation Mix RB01-12: not frozen, briefly spin down, mix well by pipetting ☐ Rapid Adapter (RAP): not frozen, briefly spin down, mix well by pipetting ☐ Sequencing Buffer (SQB): thaw at RT, briefly spin down, mix well by pipetting* ☐ Loading Beads (LB): thaw at RT, briefly spin down, mix by pipetting or vortexing immediately before use ☐ Sequencing Tether (SQT): thaw at RT, briefly spin down, mix well by pipetting Prepare the DNA in Nuclease-free water ☐ Transfer ~200 ng genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube ☐ Adjust the volume to 3.75 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 3.75 µl 200 ng template DNA ☐ 1.25 µl Fragmentation Mix RB01-12 (one for each sample)	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the tube at 30°C for 1 minute and then at 80°C for 1 minute. Briefly put the tube on ice to cool it down. ☐ Pool the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube. ☐ Resuspend the AMPure XP beads by vortexing. ☐ To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry. ☐ Remove the tube from the magnetic rack and resuspend pellet in 5 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. Remove and retain 5 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads ☐ Add 0.5 µl of RAP to the tube. ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 5 minutes at RT.	
IMPORTANT ☐ Following standard input recommendations, the protocol should produce enough final library (adapted DNA in EB) to load at least two Flongle flow cells. We recommend reserving enough library to load a second Flongle flow cell. We recommend loading 3-20 fmol of this final prepared library onto the flow cell. Loading more than 50 fmol can have a detrimental effect on throughput. Dilute the library in EB or Nuclease-free water up to a final volume of 5 µl.	
The prepared library is used for loading into the Flongle flow cell. Store the library on ice until ready to load.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
Loading the Flongle flow cell	
IMPORTANT ☐ Flow cell deterioration/saturation	
IMPORTANT ☐ Flongle Sequencing Expansion	
IMPORTANT ☐ Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.	
IMPORTANT ☐ Do NOT touch the reverse side of the Flongle flow cell array or the contact pads on the Flongle adapter. ALWAYS wear gloves when handling Flongle flow cells and adapters to avoid damage to the flow cell or adapter.	
The diagram below shows the components of the Flongle flow cell: ☐ Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) and Flush Buffer (FB) from the Flongle Sequencing Expansion and Flush Tether (FLT) from your sequencing kit at RT. ☐ Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at RT. ☐ Place the Flongle adapter into the MinION or one of the five GridION positions.	
IMPORTANT ☐ The adapter needs to be plugged into your device, and the device should be plugged in and powered on before inserting the Flongle flow cell.	
☐ Place the flow cell into the Flongle adapter, and press the flow cell down until you hear a click. ☐ In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flush Buffer (FB) with 3 µl of Flush Tether (FLT) and mix by pipetting. Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed, as follows: ☐ Lift up the seal tab: ☐ Pull the seal tab to open access to the sample port: ☐ Hold the seal tab open by using adhesive on the tab to stick to the MinION Mk 1B lid: ☐ To prime your flow cell with the mix of Flush Buffer (FB) and Flush Tether (FLT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow cell. To avoid flushing the flow cell too vigorously, load the priming mix by twisting the pipette plunger down. Vortex the vial of Loading Beads II (LBII). Note that the beads settle quickly, so immediately prepare the Sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows: ☐ 15 µl Sequencing Buffer II (SBII) ☐ 10 µl Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using. LS can be used instead of LBII when preparing libraries with the Ligation Sequencing Kit (SQK-LSK110) ☐ 5 µl DNA library	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ To add the Sequencing Mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 tip inside the sample port and slowly dispense the Sequencing Mix into the flow cell by twisting the pipette plunger down. Seal the Flongle flow cell using the adhesive on the seal tab, as follows: ☐ Stick the transparent adhesive tape to the sample port. ☐ Replace the top (Wheel icon section) of the seal tab to its original position. ☐ Replace the sequencing platform lid.	

Appendix 5: Protocol for Characterization of Fungal Strains (ITS):

Disclaimer: Mark that this protocol is for ITS primers and was used as a template for 18S primers. The PCR conditions have been modified in this study and are found in the methods.

Introduction

The internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit is by far the most commonly sequenced region for queries of systematics and taxonomy at and below the genus level of fungi. Unlike traditional procedure to isolate the whole genomic DNA, amplify a marker region (e.g. ITS) and then sequence to characterize fungal species, this protocol is a culture-independent method that enables analysis of the ITS within a tiny amount of fungal sample. Microbiology researchers can use this protocol to analysis of ITS sequence as a cost-effective technique to identify strains. Briefly, inoculate as little as possible of fungi (from culture, colonies, or pellets) for PCR template, the amplicon (500-900bp) will be purified and then used as template for sequencing. In this protocol, three steps are needed: PCR, purification of amplicon, and sequencing.

Important Notes:

- Avoid too much fungi in the PCR reaction, it is recommended to run two PCR reactions for different template dilutions.

Reagents

- DreamTaq Green PCR Master Mix (2X), Thermo Scientific, Cat no K1081/82
- Forward and Reverse primer: (eg. ITS4 and ITS5)
- Gel Red (10,000 x): BioTium, Cat no 41003
- Agarose: Life technologies, UltraPure™ Agarose Cat no 15510-027
- 10 x TBE: Life technologies, Cat no 15581-044
- DNA ladder (1 µl DNA ladder + 1 µl of 6x gel loading dye + 4 µl of water): Life technologies, Cat no 10787-018.
- Agarose gel loading dye (6x): Amresco®, Cat no E190-5 ml.
- PCR purification:

QIAquick PCR Purification Kit: QIAGEN, Cat no 28104.

ExoSAP-IT (PN: 78200 - for free from ArticZymes)

- BigDye 3.1version and 5x sequencing buffer are purchased from MH sequencing lab

Sample: Fungus

Instruments:

- Agarose Gel electrophorese system: OWI separation system Inc, B2 model
- Gel image system: GeneFlash®, SYNGENE Bio imaging
- NanoVue Plus™: GE healthcare (or NanoDrop®)
- PCR machine

Procedure:

1. Inoculate fungi from culture, colonies on the plate, or pellets in the storage as template of PCR reaction.

2. Amplification PCR reaction (25ul):

Fungal template	X µl
2x DreamTaq	12.5 µl
Forward primer	1 µl (10 µm)
Reverse primer	1 µl (10 µm)
ddH_2O	10.5 – X µl

Table 1 - Cycle scheme of amplification PCR

Initial Denaturation		95 °C	5min
Cycle x35	Denature	95 °C	30sec
	Annealing	47 °C*	30sec
	Elongation	72 °C	1min (1min <2kb products)
Final Extension		72 °C	10min
Hold		4 °C	∞

*Primer dependent: see table at the end of document

3. Determine the PCR product by gel electrophoresis:
4.
    1. Prepare a 1 % solution of agarose by melting 1 g of agarose in 100 mL of 1x TBE buffer.
    2. Allow to cool for a couple of minutes or cool with cold water until you can hold it without problems then add 10 µl of 10.000x GelRed, stir to mix.
    3. Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 15-20 min at room temperature on a flat surface.
    4. Load the following into separate wells: 6 µL of 1kb ladder; 6 µL of sample (no need for loading dye if DreamTaq is used).
    5. Run the gel for 15 min at 150-200 V depending on desired separation speed.
    6. Expose the gel to UV light and photograph.
5. PCR product purification:

QIAquick PCR Purification Kit: following manufacture's instruction.

6. Use NanoVue® to measure the concentration and quality of purified PCR products. (Only when purified using QIAquick)
7. Sequencing PCR: (PCR template: 100-200bp, 1-3ng**; 200-500bp, 3-10ng; 500-1000bp**, **5-20ng**; **1000bp-2000bp, 10-40ng**; >2000bp, 20-50ng)

Fungal template	x µl (depends on concentration from NanoVue)
BigDye 3.1	1 µl (use up to 1 µl per 1kb of template)
5x sequencing buffer	2 µl
Forward or reverse primer	1 µl (1 µm)
ddH_2O	y µl (Total volume of 10 µl)

Table 2 - Cycle scheme of sequencing PCR

Initial Denaturation		96 °C	1min
Cycle x30	Denature	96 °C	10sec
	Annealing	47 °C*	5sec
	Elongation	60 °C	2min (45 sec for <700bp)
Hold		4 °C	∞

*Primer dependent: see end of document

8. Deliver the product to the fridge outside MH L6.205 for sequencing. Remember to fill in form.

Trouble shooting

No PCR	**R**educe the amount of fungal as template. Too much fungi will have negative impact on PCR reaction.
Little PCR products	**R**un several parallel PCR reactions to pool them together for purification. Do not increase PCR cycles which might introduce mutations.
Bad sequencing signal	**U**sing less amount of sequencing primer.
Loss of signal strength	**T**oo much template or too little BigDye.

Safety Information:

- Wear gloves during the procedure.

Primer overview:

Primer Pair		**Annealing (ºC)**	**Product**
ITS5 ITS4	5'-GGAAGTAAAAGTCGTAACAAGG-3' 5'-TCCTCCGCTTATTGATATGC-3'	47 °C	~500-1100bp
NS1-NS4 (SSU)		42 °C	~1200bp
LR0R-LR5 (LSU)		47 °C	~900-1200bp

Remember to check primer compatibility! Especially if running multiplex PCR (multiple primer pairs).

Appendix 6: Protocol for PCR Reamplification

This protocol is from the UC Davis labs:

(http://genome-lab.ucdavis.edu/Protocols/pcr_tips/pcr_reamplification.htm)

PCR Reamplification for Inadequate or Failed Amplifications

Change your standard PCR protocol as follows:

- decrease the number of cycles by 10
- raise the annealing temperature by 2°C
- lower the final primer concentration by 0.1 uM
- lower the final MgCl2 concentration by 0.5 mM (but not lower than 1.0 mM)
- add 1.0 µl of the previous PCR reaction (cannot contain loading dye)
- in addition, attempt reamplification of the negative control(s) from the previous reaction to be sure the reamplification product is not contaminated

Other Notes from Various Sources

The only issue is not overloading a PCR reaction with too much of the original PCR product---make a dilution of 1/50 (30-35 PCR) and even 1/1,000,000 for a 40-45 cycle PCR. May need to titrate this out to find the proper dilution of the original PCR product to use in a re-PCR.

An even easier approach is to stick a sterile toothpick or needle into the gel at the site of the banded fragment, and to swish this through a new PCR reaction mix in order to inoculate it with a tiny amount of specific DNA. (Kadokami, Y. and Lewis, R. V. (1994) BioTechniques 17, 438)

The sub-standard PCR product can also be resolved on a normal 1% agarose gel. In this case, however, the agarose plug should not be allowed to dissolve in the 50 µl of ddH_2O since agarose in higher quantities inhibits PCR. The agarose plug should be warmed at 55°C for 10-15 minutes to facilitate the elution of the PCR product, and then the Eppendorf tube should be centrifuged to compact the agarose on the bottom of the Eppendorf. When I reamplifying the PCR product, the annealing temperature should be raised to 55°C or higher (depending on the annealing temperature of the primers), remember that this time the match between the priming sites and the primer is perfect. (http://evoamazon.net/legal/index.php/protocols/laboratory/pcr/pcr-reamplification)