Tromsø University Museum
Department of Natural Sciences

Faculty of Biosciences, Fisheries and Economics
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Diversity of marine wood-inhabiting fungi in North Norway

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Teppo Rämä
A dissertation for the degree of Philosophiae Doctor – spring 2014

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Supervisors

Assoc. Prof. **Geir H. Mathiassen**, Tromsø University Museum, University of Tromsø

Prof. **Inger G. Alsos**, Tromsø University Museum, University of Tromsø

Prof. **Håvard Kauserud**, Department of Biosciences, University of Oslo

Assoc. Prof. **Lennart Nilsen**, Department of Arctic and Marine Biology, University of Tromsø

PhD **Jenni Nordén**, Natural History Museum, University of Oslo

Prof. **Joseph W. Spatafora**, Department of Botany and Plant Pathology, Oregon State University

Cover picture: Spores of *Remispora stellata* in differential interference contrast, TRä3129H.

Preface

Fungi are our relatively close relatives in the Opisthokonta supergroup and in many ways meaningful, even indispensable in the human life. Despite their importance, fungi are not well known. This is my greatest motivation to study them. Marine fungi are even more poorly known than their land living relatives, diverse and beautiful to look at. This is what made my heart burn for them, this is the foundation of the thesis, and this is what the thesis is about.

To start studying marine fungi was in many ways a dive into the unknown for me. New fungi, new study environment and new molecular methods. However, I haven't regretted a day that I jumped for this salty dive, which I hope to last for the rest of my life. There are many to thank, and I hope to remember most of them here. First of all, Ove Eriksson, the father of this thesis, thank you for giving me the inspiration and guiding me to the fascinating world of marine fungi. The Kohlmeyers' offprints meant a lot to me, and I have read them all.

A driftwood log at 79° N, Nordenskiöldøya, Svalbard, 8.10.2011.

Geir, I am deeply thankful for having had you as the main supervisor. You always had time for me and thought for my best, made my working days as smooth as possible and arranged the best co-supervisors I could ever hope for. Håvard, thank you for introducing me to the world of molecular mycology and answering my big and many small questions swiftly and with patience. You had a major role in this thesis, and I do not know what this thesis would have been without you. You never stop amazing me with your ability to get everything arranged with ease in between everything else. Joey, you showed me how to think big and together with your lab took me the furthest away from my professional roots (I even assembled a fungal genome). I cannot thank you enough for showing me this dimension of mycology, which I definitely will revisit. Jenni, you always took a second look and I really appreciate that. I don't know any other researcher working as precisely as you do. Thank you especially for help with ordination analyses and for sharing the joyful and unpleasant parenthood experiences with me. Inger, you tried to keep me on track and showed a good example with the balance between home and work. Thank you also for paying for the pyrosequencing. Lennart, you could not have been a better official supervisor at the faculty. Marie Davey, you taught me a lot about many things, corrected my English writing and gave me hope when there was none – making bioinformatics seem as easy as tea drinking. Thank you all for your help, support and ideas.

I have been quite isolated from the active Finnish mycology "youth" during this thesis work, but the moments I have shared with it, discussing science and hobby in my own mother tongue have meant a lot. Thank you also for the mycological decade prior to the thesis, filled with courses, workshops, excursions and saunas. We really have sweat together. I am deeply grateful for Seppo Huhtinen, who has brought up this gang, organized most of the saunas and all the karaoke parties. Thank you for teaching me how to examine fungi under a microscope. I also want to express my gratitude to my other mentor, Lasse Kosonen, whom I leaned to through my first period of mycoenthusiasm. The second phase I spent mostly microscoping in the herbarium of the University of Oulu, learning skills still useful today. Esteri Ohenoja and all others at OULU are acknowledged for supporting me as budding mycologist. Thank you also Panu Halme, Hanna Tuovila, Timo Kosonen, Emilia Pippola, Juhani Ruotsalainen, Ilkka Kytövuori, Jukka Vauras, Tea von Bonsdorf, Olli Manninen and all other mycology students and teachers I have had pleasure to learn from and learn with.

During the thesis I have tried to keep my other foot dry, and work with terrestrial fungi. I appreciate being involved in the writing of the Finnish indicator fungus flora; it has been a good and pleasant experience. Thank you, Geir and Alfred Granmo, for letting me have a glimpse at your research and for inviting me to join your projects. Alfred, I am grateful for your help, Latin lessons and thoughtful discussions that always offered new aspects on life and mycology. All people at the "botanical station", research fellows and post docs (Inger Kristin, Per, Dilli and Sergei), are acknowledged for the good atmosphere, coffee talks and for all help you gave me. I am grateful to Nicolle Mode for checking the English of this thesis and Matti Blencke for comments on the synopsis. The Norwegian marine biobank (Marbank) is acknowledged for wonderful research cruises and resources for doing research, Kellfrid og Helge Jacobsens fond, Nordenskiöld samfundet, Tromsø University Museum and BFE faculty for financial support.

I express my greatest gratitude to my mother and grandparents for taking me to the forest from early on. There I found nature and mushrooms for the first time. I would have gone insane without my family who gave me a necessary break from research and always reminded me what really matters in life. I love you all and will always be there for you. Aarni and Unni, keep on spreading the joy of looking and finding, trying and succeeding. It means the world to me. Heini, thank you for giving me space, when I needed it – although you did not have it yourself.

Tromsø, March 2014

Teppo Rämä

Contents

List of original chapters

The thesis is based on the following chapters, which are referred to in the text by their Roman numerals:

I Halme P, Heilmann-Clausen J, Rämä T, Kosonen T & Kunttu P 2012. Monitoring fungal biodiversity – towards an integrated approach. *Fungal Ecology* 5: 750–758.

II Rämä T, Nordén J, Davey ML, Mathiassen GH, Spatafora JW & Kauserud H 2014. Fungi ahoy! Diversity on marine wooden substrata in the high North. *Fungal Ecology* 8: 46–58.

III Rämä T, Davey ML, Nordén J, Blaalid R, Mathiassen GH, Alsos IG & Kauserud H. Fungal communities in stranded North Atlantic driftwood as revealed by high throughput amplicon sequencing. Manuscript.

IV Davey ML, Rämä T, Bjørnsgaard Aas A, Mysterud A, Ohlson M, Vrålstad T & Kauserud H. Comparing culture dependent and culture independent methods for answering questions in fungal ecology. Manuscript.

V Rämä T, Mathiassen GH & Kauserud H. Marine fungi new to Norway, with an outlook to the overall diversity. Submitted manuscript.

The table shows the major contributions to the chapters.

	Chapter I	**Chapter II**	**Chapter III**	**Chapter IV**	**Chapter V**
Concept and idea	PH, PK, TK	TR, HK	HK, TR, JN	MD	TR, HK, GM
Study design and methods	-	TR, HK, JN, GM	TR, HK, JN, GM, RB	MD, HK, TR, AA, AM, TV	-
Data gathering and interpretation	TR, JHC, PH, TK, PK	TR, GM, HK, JN, MD, JS	TR, MD, JN, HK, RB, IA	MD, TR, AA, HK, AM, MO, TV	TR
Manuscript preparation	PH, JHC, TR, TK	TR, HK, JN, MD, JS	TR, JN, HK, MD	MD, TR, HK, AA, AM	TR, GM, HK

TR=Teppo Rämä, AA=Anders Bjørnsgaard Aas, IA= Inger G. Alsos, RB=Rakel Blaalid, MD=Marie L. Davey, PH=Panu Halme, JHC=Jacob Heilmann-Clausen, HK=Håvard Kauserud, TK=Timo Kosonen, PK=Panu Kunttu, GM=Geir H. Mathiassen, AM=Atle Mysterud, JN=Jenni Nordén, MO=Mikael Ohlson, TV=Trude Vrålstad, JS=Joseph W. Spatafora

Abstract

Marine fungi in arctic waters have rarely been studied. The main aim of this thesis was to explore the diversity of driftwood inhabiting fungal communities in the cold waters of North Norway.

As a prelude for the thesis, chapter **I** was aimed at evaluating the current tradition of fungal diversity data collection and storage, and sharing methodological ideas how this could be done in the future. An integrated approach was suggested when conducting fungal diversity surveys. Accordingly, two main methods were used for investigating the fungal diversity; culturing paired with Sanger sequencing, and high throughput sequencing of DNA extracted directly from wood. The latter was used for the first time to study wood-inhabiting marine fungi.

In chapters **II** and **III**, the diversity and community ecology of driftwood fungi was analysed using culturing and 454 amplicon pyrosequencing, respectively. With culturing, 143 operational taxonomic units (OTUs), based on clustering at 97% sequence similarity of the internal transcribed spacer region (ITS), were detected from 50 driftwood logs. Amplicon sequencing revealed 807 OTUs from the same logs and estimated the total diversity in the area to be 1,400 OTUs (97% ITS2 clustering). Approximately 75% of the OTUs had affinity to the phylum Ascomycota, and previously overlooked taxa were detected from the marine environment with both methods. Likewise, both methods indicated that one half of the OTUs were tentatively non-marine. The fungal communities were shown to be structured by many geographical and environmental variables, which were partly different between the methods.

Chapter **IV** had the specific aim of resolving how well the two main methods can be used to study the diversity of fungal communities, when applied separately. The taxonomic profiles were comparable at higher taxonomic levels, although less than 7% of the OTUs were shared between the study methods. This shows that the methods target different parts of the fungal community.

Finally, in chapter **V**, the results of the morphological and DNA barcoding work made for this thesis are summarized and the marine mycological efforts made in Norway reviewed. One undescribed and 16 new species to Norway were found on driftwood during the thesis work. Altogether, 61 species of marine fungi are registered in Norway since 1895, most of

them from wooden substrates. This chapter will form the backbone for future research on marine fungi in Norway, including work on DNA barcoding.

The rich and diverse mycota found in this thesis contributes to the global knowledge of wood-inhabiting marine fungi, and suggests that many overlooked and undescribed species exist in this habitat. The diversity was well characterized at higher taxonomic levels, but resolution should be increased towards the terminal branches of the fungal tree of life by means of more collecting, culturing and DNA barcoding of marine fungi. The role of many tentatively non-marine species found in driftwood will be scrutinized in the future using RNA-sequencing of environmental samples. The two main methods used in this thesis can be applied separately to study marine fungal diversity and community structure. However, the methods were complementary and hence the integrated approach will be continued in the future. Integration is also needed in the training of next-generation marine mycologists, since the main challenge in marine mycology is the need for researchers having both good knowledge on the biology of marine fungi and necessary skills for studying them using traditional and modern methods.

Introduction

Without Fungi, the world as we know it would not exist. They are and have been for a long time the primary decayers of lignocellulolytic substrates (Floudas *et al.* 2012), and the main keepers of great carbon storages in soil and dead organic material (Averill *et al.* 2014). Endophytic fungi help their hosts to persist in front of grazers, whereas others are parasites and pathogens of all kinds of organisms. From the human perspective, many fungi seem to have a negative influence: pests cause economic losses in forests and on cultivations, and pathogens cause troublesome diseases or even major famines (Padmanabhan 1973). On the other hand, fungi provide food and medicines, and can be harnessed for many kinds of useful purposes. Most of the existing fungi have not yet been found, let alone described for science (O'Brien *et al.* 2005). In addition, the ecology of many of the ca. 100,000 described fungi is poorly known. In other words, we know relatively little about how fungi really impact the biosphere and the biodiversity it hosts.

After more than 200 years of mycological research (Persoon 1801, Fries 1821), people have started to realize the importance of terrestrial fungi. However, after approximately 100 years of marine mycology since Cotton (1907) and Sutherland (1915), people still do not know much about marine fungi. Even many marine biologists are unaware of fungi dwelling in every marine habitat. It is true that marine fungi are more inconspicuous and fewer in number than their terrestrial relatives (O'Brien *et al.* 2005, Jones and Pang 2012). However, this does not mean that they are unimportant and can be overlooked. On the contrary, recent studies have shown that marine fungi are abundant actors in marine habitats and involved in various biogeochemical processes (Stoeck *et al.* 2007, Alexander *et al.* 2009, Stoeck *et al.* 2009, Edgcomb *et al.* 2011). Their home, the oceans, cover approximately 70% of world's surface with an average depth of almost 3,700 meters (Charette and Smith 2010). The oceans host an estimated 1.5 million species of organisms (Bouchet 2006). The marine environment seems to be of crucial importance in fungal evolutionary history, since the emergence and early diversification of fungi likely took place in the oceans (Le Calvez *et al.* 2009). Many marine organisms (if not nearly all) can be expected to support several species of fungi. When alive, they may be supported by endophytes or other symbiotic fungi including also host-specialists (Sakayaroj *et al.* 2012, Suryanarayanan 2012), or they may be plagued by parasites (Jones *et al.* 2012) and threatened by pathogens (Geiser *et al.* 1998, Hatai 2012). *Post-mortem* the material is decayed and recycled by saprotrophs (Jones *et al.* 2012), until the energy available and nutrients are used to support new life with new fungal partners and enemies. However, these ecological processes are poorly studied and insufficiently understood. More research on marine fungi is needed, since they can give us additional insights into the evolution of eukaryotes or prove to be of indispensable importance for humans.

Fungal diversity surveys (I, II, III, IV, V)

Biodiversity runs this world and sustains the excessive human population. That is one of the reasons why we need to study and conserve it. To optimize the study efforts, biodiversity surveys should be designed so that it is made clear why the survey is conducted, what is being monitored and how this is done (Yoccoz *et al.* 2001). Without taking time to answer these questions and sticking to the answers, researchers conducting surveys can easily end up trying to answer appropriate study questions with inappropriate methods or *vice versa*. In an optimal biodiversity survey, all species (or other taxa or entities surveyed) present in samples are detected and the distribution of samples in time and space is representative for the whole study area. In such a situation both the detection error and survey error are avoided, and solid conclusions can be drawn from the results (Yoccoz *et al.* 2001). It is well known that in most diversity surveys both sources of error are present – even if the target of the survey was some of the more easily detected and best known groups of organisms. Fungi are difficult to detect, their fruit bodies occur unpredictably and last usually for only a short time. Thus, most (if not all) fruit body surveys for monitoring fungal diversity are suboptimal. A second dimension of undetectability is brought in by non-fruiting fungi, the ones present in the studied substrates at the time of sampling only in their vegetative form. In fruit body surveys, all of the non-fruiting species, which represent the majority of the total diversity (e.g. Ovaskainen *et al.* 2013), are missed. With modern molecular environmental sequencing methods it is possible to survey also the non-fruiting fungi. With deep enough sequencing, all species present in a sample can be detected, but we are still lacking a lot of information to identify all this diversity. Given ca. 20,000 of the 100,000 described fungi are currently represented in public reference sequence databases, we are lacking molecular reference data for 99.6% of the estimated 5.1 million fungi (O'Brien *et al.* 2005, Nilsson *et al.* 2009). For many researchers, naming the entities of diversity is irrelevant, but as soon as the scientific data are used, for example in conservation management, (identified) species most often become the unit of diversity people focus on.

Identifying biodiversity has been vital in the survival of humans and that is why taxonomy is said to be the oldest profession of the world (Manktelow 2010). Biodiversity covers all levels of variation of life from genes through species to ecosystems. Undoubtedly, the species level is the most natural one for humans to deal with, and it was already used in 3000 BC when emperor Shen Nung in China wrote his pharmacopoeias (Manktelow 2010). The earliest proofs from the western world date back to ca. 1500 BC when medical plants were illustrated in Egyptian wall paintings. Since these times, studies based on morphological species identification have dominated the biodiversity studies, especially after Carl von Linné's stabilization of the naming system (Linnaeus 1753, Linnaeus 1758). The first molecular revolution in the late eighties with PCR and Sanger sequencing and the second with the implementation of high throughput sequencing approaches have resulted in increasing number of biodiversity studies being molecular based. These can be used to identify fungi to the species level, if one works with a well-sequenced and morphologically well-known group of fungi within a well-studied area. However, this is usually not the case, and most molecular diversity studies of fungi suffer from low resolution in species identification. The poor molecular species identification is due to a lack of molecular reference data. High throughput sequencing approaches are needed to explore the vast unknown diversity, including non-culturable fungi. But at the same time, morphological work and sequencing of voucher specimens should be continued, since this is the only way to provide reference material and improve species identification in molecular surveys. This is why molecular surveys targeting even the best-known groups of fungi should preferably be coupled with morphological studies, possibly culturing and barcoding of taxa.

After obtaining the results, one should not start collecting more samples before the data are curated and appropriately stored. Careful curating, storage and sharing of the published data with the scientific community are too often neglected. Physical samples should be stored in maintained scientific collections, molecular data and preferably also data on study samples and sites in public databases such as GenBank, EMBL, UNITE, DataDryad.org or Figshare (Kanz *et al.* 2005, Kõljalg *et al.* 2013, Benson *et al.* 2014). Storage forms that are public or at least accessible by other researchers should be preferred in order to enable other researchers to check control data or conduct meta-analyses. If not stored properly, data might be lost due to unintentional reasons such as breakdown of a hard drive, before they were even properly used by the scientific community. Just as studies should be repeatable, data should be accessible.

Marine fungi (II, III, IV, V)

Marine fungi live in marine, intertidal and estuarine habitats. Obligate species are restricted to marine habitats in every stage of their life cycle, whereas facultative marine fungi can grow (and possibly also sporulate) in marine habitats, but are also encountered in terrestrial or freshwater habitats (Kohlmeyer 1974). The habitat is the main ecological feature defining marine fungi which, on the other hand, include species with highly differing ecological niches (Kohlmeyer and Kohlmeyer 1979). The most studied phylum containing marine species is Ascomycota. Together with Basidiomycota, it forms the Dikarya, fungi which possess dikaryotic hyphae in parts of their life-cycle (Hibbett *et al.* 2007). Dikaryan marine species are secondary colonizers of the marine environment, whereas the more basal groups such as Chytridiomycota and Cryptomycota have likely diversified in the marine realm (Spatafora *et al.* 1998, Kohlmeyer *et al.* 2000, Le Calvez *et al.* 2009, Jones *et al.* 2011). Dikarya has also been called the 'higher fungi' and is the main target group of this thesis. The term 'marine fungi' is used hereafter to refer to dikaryan fungi, excluding species occurring predominantly as yeast forms, unless otherwise specified. Currently, only 530 species of obligate marine fungi are known world-wide (Jones *et al.* 2009). However, it is estimated that the true number (including yeast, non-culturable and marine-derived species, and a small number of other than higher fungi) is 12,000 (Jones and Pang 2012). Most of the yet undiscovered diversity is suspected to be found among algicolous fungi and among the Chytridiomycota (Jones and Pang 2012, Richards *et al.* 2012).

Marine fungi are typically small. Most species form perithecial ascomata (fruit bodies) that are ≤0.5 mm in diameter (Jones *et al.* 2009). Typical microscopic features include unitunicate and deliquescent asci and a diversity of different kinds of spore appendages (Jones and Moss 1978, Kohlmeyer and Kohlmeyer 1979). These characteristic features are evolutionary adaptations arisen in response to the environmental conditions of the marine milieu, such as mechanical stress caused by waves, sand scour, and water as dispersion matrix for propagules. The small size and perithecial fruit bodies, often fully or partially seated inside the substrate, enable the fruit bodies to stay attached without being washed away.

The spores are passively dispersed, washed out from the ascomatal cavity by sea water. Sheaths and appendages on spores help them to float enabling longer dispersal distances and efficient attachment to surfaces (Hyde *et al.* 1993). The marine environment also possesses several physiological stress factors, including high salinity, sodium levels and pH, as well as low water potential. Marine fungi cope with these challenges by, for example, maintaining a suitable internal potassium concentration important for growth in a saline environment (Jennings 1983). The toxic effects of sodium are avoided by sequestering it into vacuoles or pumping it out (Jennings 1983, Benito *et al.* 2002). Osmolytes, such as glycerol and mannitol, are accumulated to make the water potential in the fungal cells lower than in the surrounding sea water enabling water uptake (Blomberg and Adler 1992). However, it should be kept in mind that obligate marine and terrestrial fungi also share many physiological features. For example, based on *in vitro* experiments it seems that marine fungi have similar carbon, nitrogen and vitamin nutrition with their terrestrial counterparts (Jennings 1983). In addition, some fungi occurring in the terrestrial environment also seem to tolerate salinity during their vegetative growth, with the exception of species belonging to Basidiomycotina which are sensitive to salinity (Jennings 1983, Blomberg and Adler 1992).

Despite their interesting ecophysiological adaptations, the focus of marine mycological studies has been on the taxonomy of these organisms. Wood-inhabiting species are among the best studied group, but the environmental factors influencing these communities are insufficiently studied. It has been shown that temperature and salinity are the main drivers behind biogeographical patterns of marine fungi (Hughes 1974, Booth and Kenkel 1986). Communities are also structured by habitat, substrate, vertical zonation and length of submersion (Kohlmeyer and Kohlmeyer 1979, Tan *et al.* 1989, Petersen and Koch 1997, Jones 2000). However, earlier studies have mainly focused on the morphologically identified fruiting community and few environmental factors influencing their structure. Hence, the community ecology of these fungi is poorly understood. Molecular tools have been applied since the 1990s, but the number of studies is relatively low due to few researchers operating in the field. Many aspects remain unknown. For example, the first phylogeographic study of a marine fungus using molecular tools was just published (Pang *et al.* 2013). On the other hand, many recent publications about marine fungi, perhaps even the majority, focus on applied science aspects and bioprospecting. Marine fungi are considered to be an excellent source of novel substances with more than 1,100 new natural substances discovered from them (Ebel 2012).

Applying high throughput sequencing on fungi (III, IV)

Until the 1990s, mycological diversity studies were largely based on fruit bodies. The wide application of first-generation sequencing techniques based on Sanger sequencing introduced new possibilities. By pairing Sanger sequencing with cloning, it became possible to survey the non-fruiting and even non-culturable members of the communities under scrutiny (Rondon *et al.* 2000). However, this method is very laborious and time-consuming and a large part of the diversity remained undiscovered due to a shallow sequencing depth. The second molecular revolution in fungal ecology took place during the mid-2000s, when new high throughput sequencing techniques were introduced. High throughput sequencing may produce thousands of sequences (reads) per sample in a single run. How this is done depends on the sequencing platform and the approach chosen.

The first available platform used the 454 pyrosequencing (Margulies *et al.* 2005), which for several years produced the longest sequence reads, long enough to cover, for example, the fungal ITS1 or ITS2 region. The two main approaches applying the 454 pyrosequencing are shotgun sequencing and amplicon sequencing. Using the former, the extracted DNA (or RNA) pool is chopped into shorter templates, sequenced and the resulting sequences assembled into contigs using bioinformatic tools. This approach is often applied in functional profiling of communities or genome sequencing (Tringe *et al.* 2005, Bushley *et al.* 2013). In amplicon sequencing, a certain marker region is chosen, amplified within and across the samples using PCR, and the amplicons are sequenced. Hence, each of the derived sequences belongs to a certain species in the sample. If amplicons were tagged and samples multiplexed prior to the sequencing event, sequences can be traced to original samples. This is a cost-efficient way of studying fungal communities, despite the high one-off cost of the sequencing. If samples are handled correctly, they may give a good representation of the communities *in situ*. Depending on the study set up, amplicon sequencing data can be used to answer different types of questions. Typically, the resulting sequences are clustered into operational taxonomic units (OTUs) using a certain cut-off value, with the idea that each OTU represents a certain species. However, this is often not the case (Blaalid *et al.* 2013).

Sequencing phylogenetic marker genes allows phylogenetic profiling and taxonomic annotation of the OTUs. This is often referred to as DNA metabarcoding (Taberlet *et al.* 2012).

In 454 amplicon pyrosequencing, DNA samples are first subjected to multiple parallel emulsion PCRs, in which millions of copies of each template are produced, all attached to the same microscopic bead. The sequencing takes place on a picotiter plate, where single nucleotides are flown in a specific order over the wells of the plate, where each well includes one bead with a specific template. Each new nucleotide is incorporated only if a complementary base is the next available on the template strand. Pyrophosphates are released at every successful incorporation of nucleotides and these are converted to light signals by the luciferase enzyme. If the same bases follow each other in the template (=homopolymer), the light signal becomes stronger. The plate is photographed following every addition of nucleotides and these so-called flowgrams are used to create the sequence files.

The DNA extraction, initial amplification of the target locus in the PCR and the sequencing are the critical steps of the amplicon pyrosequencing approach (Huse *et al.* 2007, Lindahl *et al.* 2013). During extraction, samples may be contaminated if not handled under sterile conditions. Different types of fungi may yield different amounts of DNA during DNA extraction which may result in quantitative biases. During PCR, various fungi may be amplified to different extents due to primer mismatches (Bellemain *et al.* 2010). Moreover, during PCR, artificial mutations (roughly 1 per 1000 base pairs) are introduced because the polymerase enzyme does not work perfectly and chimeric sequences may also be produced, merging two original templates into one sequence. When it comes to the sequencing itself, most errors in 454 sequencing are related to homopolymer overcalls or undercalls, for example, the sequence "…TCAAATC…" including the homopolymer "AAA" is sequenced as "…TCAAAATC…" or "…TCAATC…". Biases should be evaluated and minimized when planning the study, and taken into account in the bioinformatics and data analyses. The above mentioned errors, introducing noise in the raw sequence data, can be corrected to a certain extent. In other words, the raw sequence data needs to be filtered, trimmed, denoised and chimera checked before analysing it further. In the filtering steps, individual reads are inspected whether they correspond to a certain read length range and quality parameter setting. If not, they are removed. In trimming, error-prone regions, typically at the terminal ends of reads (Balzer *et al.* 2010), are removed. In the denoising step, flowgrams created during the sequencing are used to correct noisy reads against tentative high quality reads.

Typically, a relatively small number of reads contain the majority of errors (Huse *et al.* 2007, Quince *et al.* 2011), so quality checking the raw data means getting a slightly smaller, but better quality dataset with which to work.

The aims of the study

The main aim of this thesis was to explore the diversity of marine wood-inhabiting fungi in cold, northern waters. In order to gain as comprehensive view as possible, different methodological approaches were applied on the same study substrates, and the recovered fungal communities were analysed for taxonomic, phylogenetic and ecological aspects of diversity.

The specific aims were:

1. To evaluate the tradition of data collection and storage and share new methodological ideas for fungal biodiversity surveys (**I**)
2. To study the community structure and ecology of fungi on driftwood (**II**, **III**)
3. To assess the pros and cons of high throughput sequencing vs. culturing studies for studying fungal diversity (**IV**)
4. To summarize the marine mycological efforts made in Norway and compile their results in the form of a checklist (**V**)

Material and methods

Study area and sampling (II, III, IV, V)

North Norwegian mainland coast and the archipelago of Svalbard forms a large area covering 15 degrees in latitude (ca. 65°30′–80°50′ N) and 25 in longitude (ca. 11°50′–36°47′ E) (Figure 1). The highly indented coastlines with diverse marine habitats and rich flora and fauna (Narayanaswamy *et al.* 2010) is a captivating study area for any biologist interested in arctic biodiversity. The terrestrial vegetation in the mainland is boreal, except for the alpine mountain areas and easternmost part of the outer coast which are arctic (Moen 1998, Walker *et al.* 2005). The marine environment belongs to the Arctic biogeographical zone of marine fungi with surface temperatures of the warmest calendar month <10° (Hughes 1974, Locarnini *et al.* 2010). Salinity is <35‰ (Antonov *et al.* 2010). These waters are characterized by two sea currents: Norwegian Atlantic Current (NWAC) which is the northeastern branch of the North Atlantic Current (the Gulf Stream) transporting warm and saline water northwards, and the Norwegian Coastal Current having its origin in the Baltic Sea (Sætre and Mork 1981) (Figure 1). The variation in regional hydrography is influenced mostly by fluctuations in temperature and salinity of NWAC and large-scale wind fields (Sætre and Mork 1981, Wassmann *et al.* 1996). In the truly arctic Svalbard, warmest waters are found in the West Spitsbergen Current (WSC) which is the northwestern extension of the NWAC and carries relatively warm and salty water along the west coast of Svalbard. There it meets and mixes with less saline and cold water masses coming from the North Pole.

Samples for this thesis were mostly collected in Troms and Finnmark counties along the Norwegian mainland coast (Figure 1). The main source of drift wood along this coastline is in central parts of Siberia (Johansen and Hytteborn 2001, Hellmann *et al.* 2013), but in the western parts mostly local wood can be found in the marine environment. Substrate units (hereafter logs) showing signs of long-term and recent submersion in the sea, as indicated by the presence of marine organisms (Kohlmeyer and Kohlmeyer 1979), were looked for in the intertidal zone. If no logs were found there, logs in the breaker zone were included. Some sea-bottom logs were also examined. Position, site and substrate level parameters were systematically measured in the field for each log. For chapters **II** (**IV, V**) and **III** (**IV, V**) 50 and 49 logs were sampled, respectively. In order to get rid of inactive propagules resting on the wood surface, as thin as possible a slice was removed with a sterilized knife at each sampling point, and sterilized instruments were used to obtain samples: wooden cubes for incubating on Agar plates (**II, IV, V**) and drilled wood samples for next generation sequencing (**III, IV, V**). For chapter **V,** logs were inspected in the field with an illuminated hand lens and samples with fungal structures collected for later examination and DNA barcoding. Smaller pieces of wood were collected entirely. Approximately 150 samples of the 800 collected were examined morphologically with a dissecting and light microscope. The remaining samples are still unexamined.

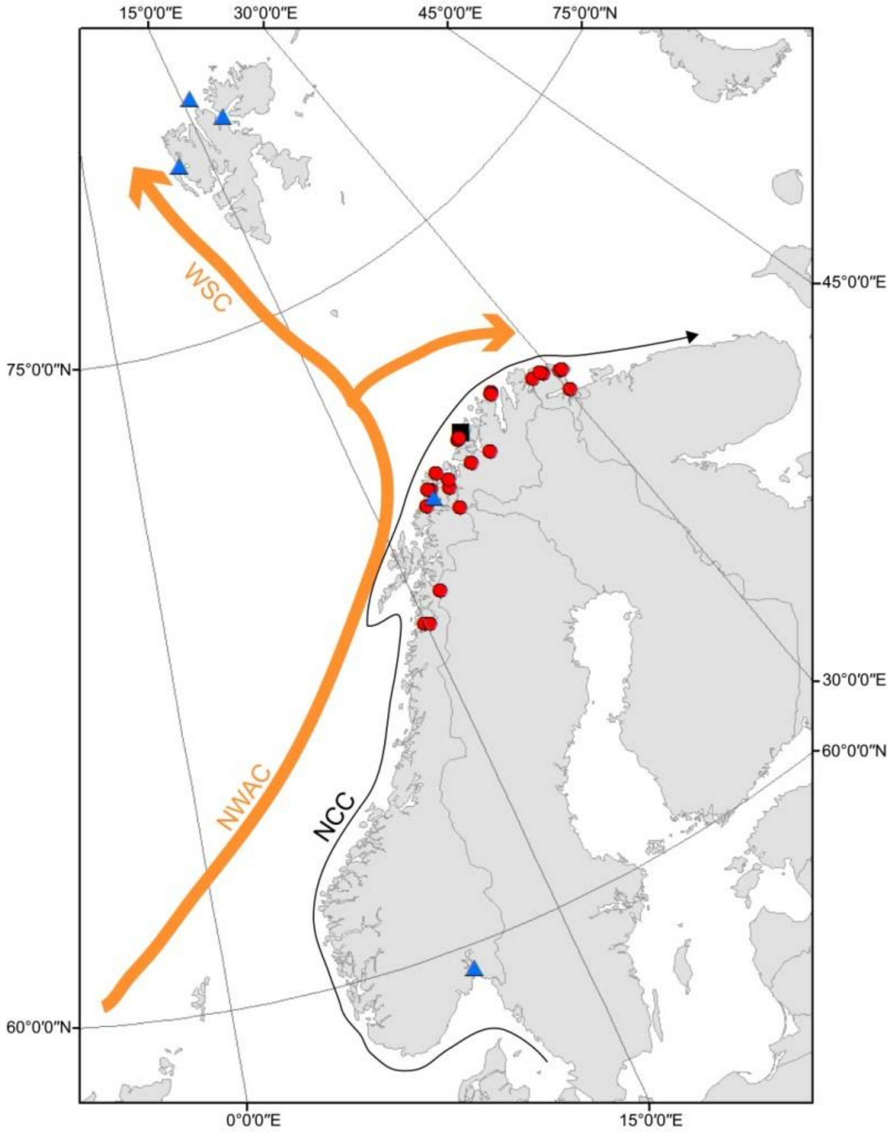

Figure 1. The study sites in North Norway. Samples included in the different chapters are marked with the symbols: red dot=**II–V**, black square= **II** and **IV,** and blue triangle=**V**. A simplified presentation of the sea currents is drawn after Asplin and Dahl (2003): NWAC=Norwegian Atlantic Current, WSC=West Spitsbergen Current and NCC=Norwegian Coastal Current.

Molecular methods (II, III, IV, V)

For the chapters **II, IV** and **V**, DNA was extracted using a cetyltrimethyl ammonium bromide extraction protocol (Murray and Thompson 1980, Mysterud *et al.* 2007). Natural markers of choice were the barcoding locus, internal transcribed spacer (ITS) and large subunit (LSU) of the nuclear rDNA which has been the standard phylogenetic marker in recent phylogenetic studies of marine fungi (Suetrong *et al.* 2009, Sakayaroj *et al.* 2011, Schoch *et al.* 2012). These loci were amplified using the primer pairs ITS5-ITS4 (White *et al.* 1990) and LR0R-LR5 (Vilgalys and Hester 1990, Rehner and Samuels 1994). PCR products were cleaned and sequencing reactions performed on an Applied Biosystems 3730 DNA analyzer using PCR primers as sequencing primers and the BigDye Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, California, USA).

For the chapters **III** and **IV**, sub-samples of approximately 25 ml were taken from grinded wood samples, and one ml of each was used for DNA extraction performed using Nucleospin Soil DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Samples were prepared for 454 pyrosequencing using a nested PCR approach, in which primer dimerization due to long primer constructions is avoided (Wallander *et al.* 2010). The nested PCR produces amplicons having the same orientation, and the work-load and costs are decreased compared to approaches where tags and/or sequencing adaptors are ligated to the amplicons after the PCR (Lindahl *et al.* 2013). In the first PCR the primer pair ITS1F-ITS4 (White *et al.* 1990, Gardes and Bruns 1993) was used. The second PCR was performed using fusion primers including ITS3 and ITS4 (White *et al.* 1990) and eight unique 10 base pair tags. The 454 pyrosequencing was performed on a Genome Sequencer FLX (Roche, Basel, Switzerland).

Data handling and analyses (II, III, IV, V)

Sanger sequenced forward and reverse strands (**II, IV, V**) were assembled and manually checked in Geneious (Biomatters Ltd.). Consensus sequences were used in OTU clustering and alignments (**II**) built with MUSCLE and/or MAFFT (Edgar 2004, Katoh and Standley 2013) and corrected by eye. The 454 pyrosequencing raw data (**III, IV**) consisting of ca. 180,000 reads was quality checked in the Qiime pipeline (Caporaso *et al.* 2010), and clustered into OTUs based on 97% ITS2 sequence similarity. The most abundant reads were picked as representative sequences of the OTUs. Single read OTUs (singletons) were removed and the dataset checked for chimeric sequences (Schloss *et al.* 2009).

OTU accumulation curves, total diversity estimate and extrapolation were calculated in EstimateS and Qiime (Raaijmakers 1987, Colwell *et al.* 2004, Colwell 2009, Caporaso *et al.* 2010, Colwell *et al.* 2012, Colwell 2013). The taxonomy of the OTUs was annotated manually based on ITS and LSU BLAST matches and constructed phylogenies. In the conservative ecology annotation, the World Register of Marine Species (Appeltans *et al.* 2012) and marine mycological literature was utilized. An OTU was considered marine if the taxon (**II**) or genus (**III**) had been reported from the marine environment. Phylogenies based on maximum likelihood were constructed in RAxML with the rapid hill climbing algorithm (Stamatakis 2006, Stamatakis *et al.* 2007), whereas Bayesian analyses were run with MrBayes (Ronquist *et al.* 2012), preceded by substitution model selection in Mrmodeltest (Nylander 2004).

Multivariate analyses exploring the community structure against geographical and ecological variables was studied with non-metric multidimensional scaling (NMDS; **II**, **III, IV**). The effect of rare OTUs (occurring on a single log) on the ordination was evaluated (cf. Poos and Jackson 2012, **III**), or they were directly excluded from the analyses (**II**). Dissimilarity indices were compared, and the most suitable was used in the community matrix. NMDS is an unconstrained ordination method which does not assume a specified form of regression and is not dependent on the Gaussian distribution. Thus it is appropriate for non-linear species responses to multiple environmental variables and detecting underlying ecological gradients (Manjarrés-Martínez *et al.* 2012). It is a robust method working with rank-order relations between community dissimilarity and distance in the ordination space. For these reasons, it was a natural choice to use for the multivariate datasets. MEtaGenome ANalyzer (MEGAN) was used to compare the taxonomic profiles between marine and terrestrial wood-inhabiting communities (**III**), or communities derived with culturing and pyrosequencing (**IV**). The advantages of this method are that it enables quantitative comparison at each node of the backbone phylogeny and provides simple visualization of the results.

In this thesis, the OTUs are generalized to represent species. The author is well aware that some fungi have >3% intraspecific variation of the ITS region, meaning one species would consist of two or more OTUs as defined here. However, for all fungi and for the phylum Ascomycota the average intraspecific variation of the ITS is ≤3% which most likely results in underestimating species richness based on our OTUs (Nilsson *et al.* 2008).

Results and discussion

Fungal richness in driftwood (II, III, IV, V)

Altogether 925 OTUs and species were found in the wooden substrates in North Norway, and different study methods detected different levels of diversity (Figure 2). In the morphological examinations, 26 wood-inhabiting species were identified. Sixteen of these represented new records to Norway. Seven have so far been DNA barcoded and were detectable with other methods. The culturing combined with molecular characterization of the isolates revealed 143 OTUs, while the amplicon pyrosequencing revealed 807 OTUs.

Species or OTU counts are often hard to understand, unless they are placed in a broader context. Twenty-six morphologically identified species represent almost half of the 61 species recorded in Norway since 1895 (**V**). The number of recovered OTUs is higher than that of obligate marine fungi world-wide (Jones *et al.* 2009, Jones and Pang 2012). Although the 899 molecularly identified OTUs likely include some genuinely terrestrial taxa, the recovered total richness strongly supports the estimate of at least 12,000 marine fungal species (Jones and Pang 2012). The OTUs found represent 7% of the estimated number of marine fungi (Jones and Pang 2012). It is hard to believe that almost one tenth of the global diversity would be found on wood in North Norway, especially if one takes into account that the wood samples originate from relatively few logs, which were not sampled intensively (cf. Ovaskainen *et al.* 2010). Consequently, it seems that Jones and Pang (2012) underestimate the number of marine fungi. Based on their estimate, only 0.2% of the total fungal diversity of 5.1 million species would be living in marine habitats (O'Brien *et al.* 2005).

In chapter **III**, we compare the wood-inhabiting fungal diversity detected with amplicon pyrosequencing on land and in the sea. Using conifer logs sampled in both habitats, we demonstrate that approximately one and a half times as many OTUs are found in the terrestrial milieu. A similar ratio is found when comparing the marine data with logs sampled in Sweden (Kubartová *et al.* 2012). These rough ratio estimates also signal for the existence of a more species-rich mycota in marine wooden substrates than currently estimated. However, it needs to be kept in mind that 454 pyrosequencing can also detect dormant and dead undegraded DNA (Baldrian *et al.* 2012, van der Linde and Haller 2013), which both can inflate the number of marine-derived terrestrial species detected.

Amplicon pyrosequencing clearly detected most OTUs, and approximately 5% of the OTUs between it and the culturing method are shared (Figure 2). The low frequency of shared OTUs was expected, since it is known that different methods have different biases and detect different fungi (Ovaskainen *et al.* 2010, Lindner *et al.* 2011). In chapter **IV**, the method biases between culturing studies and high throughput sequencing studies of fungi are scrutinized for the first time using multiple datasets. It is shown that the approaches are complementary and target different parts of the fungal community. For marine wood-inhabiting fungi the number of shared OTUs is relatively low, but the taxonomic profiles are comparable between the study approaches at higher taxonomic levels. However, the correspondence between the approaches might be method or context dependent, and does not necessarily apply to wood-inhabiting marine fungi elsewhere or marine fungi on other substrates.

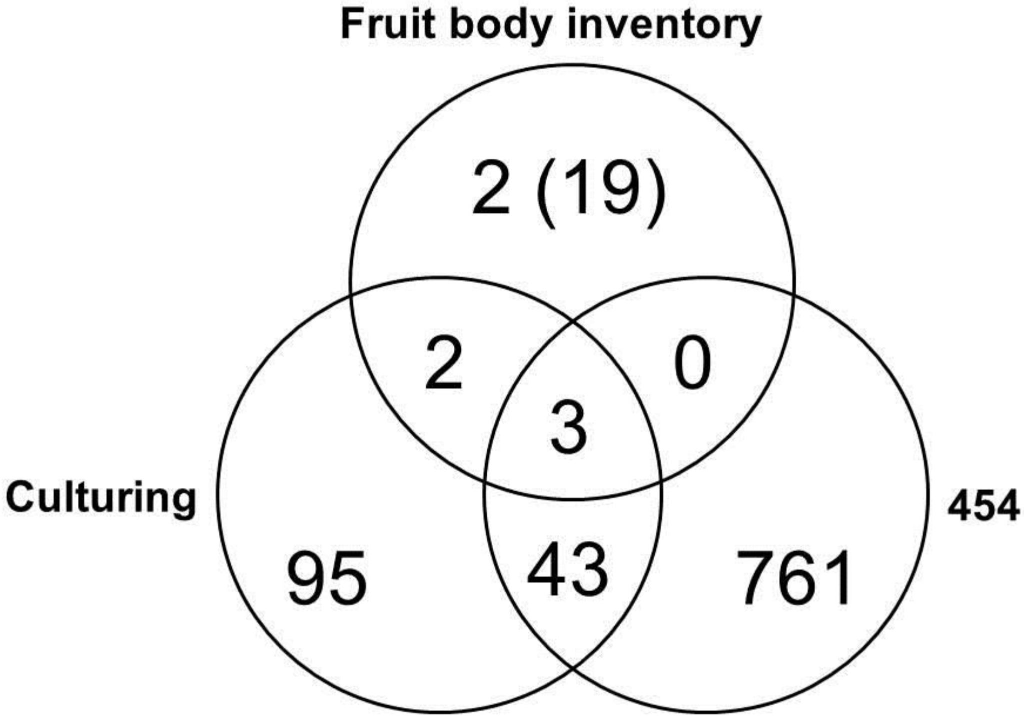

Figure 2. Numbers of fungal OTUs and species detected with different study methods and combinations of these. The number of species that are not sequenced, and could not be identified with the other methods, is given in parentheses. Three culturing OTUs were assimilated to other OTUs as identical, and the corrected OTU number for **II** is presented here. Data for the figure were produced by extracting ITS2 of all sequences using the ITS extractor of Nilsson *et al.* (2010) and clustering the reads with BLASTclust (http://toolkit.tuebingen.mpg.de/blastclust).

Taxonomic and phylogenetic diversity (II, III, IV, V)

Ascomycota ruled the fungal communities in the marine wooden substrates with 83% of the culturing OTUs and 74% of the pyrosequencing OTUs belonging to this phylum. This was expected, but the taxonomic profile detected at the class level was partly different from the profile of obligatory marine fungi (Figure 3). For example, Leotiomycetes taxa were much more frequent in North Norwegian driftwood based on both culturing and pyrosequencing. It seems that traditional survey methods based on morphological identification have overlooked Leotiomycetes taxa, which perhaps rarely sporulate on wood. Sordariomycetes is the most species-rich class based on fruiting body surveys, and it also proved a common taxon in the North Norwegian driftwood, although relatively less frequent than globally. The taxonomic profile of the culturing survey corresponds better to the taxonomic profile of obligate species than the culture independent survey. This was expected, since with the latter non-fruiting and inactive species can also be detected. With pyrosequencing unique classes of dikaryan fungi, for examples Neolectomycetes, were observed for the first time in the marine environment.

More differences in taxonomic profiles were observed at lower taxonomic levels. The most striking was the high frequency of Helotiales (Leotiomycetes) taxa detected both with culturing and culture independent approaches. One of the genera explaining the high frequency of Helotiales was *Cadophora*, which represents a new dominant group of wood-inhabiting marine fungi. Other novelties detected with the two main methods include rare OTUs belonging to novel environmental fungal clusters, and more explicitly to the Pezizomycotina clone group and the hydrothermal and/or anaerobic fungal group (Manohar and Raghukumar 2013). These sequences have been found in marine sponges and deep-sea habitats (Lai *et al.* 2007, Gao *et al.* 2008, Nagano *et al.* 2010). The same clusters have also been detected at hydrothermal vents along the mid-Atlantic ridge in the North Atlantic Ocean (López-García *et al.* 2003, López-García *et al.* 2007, Le Calvez *et al.* 2009), but not along the Norwegian coast.

It is obvious that high throughput sequencing enables deeper sampling of fungal communities on marine wooden substrates than culturing (**IV**). However, the taxonomic profiles are comparable at a higher level. It is desirable to sample the total fungal community, or at least as many of its members as possible, in order to draw sound conclusions about the community. If only one study method is selected, the high throughput sequencing approach should be used. On the other hand, reference sequence data is lacking for marine fungi and fungi in general, and *in vitro* experiments on cultures are needed. Combining high throughput sequencing with culturing (and also morphological studies) is of paramount importance, if we intend to develop better sequence databases and get better insights into, for example, ecophysiological responses of fungi to environmental factors.

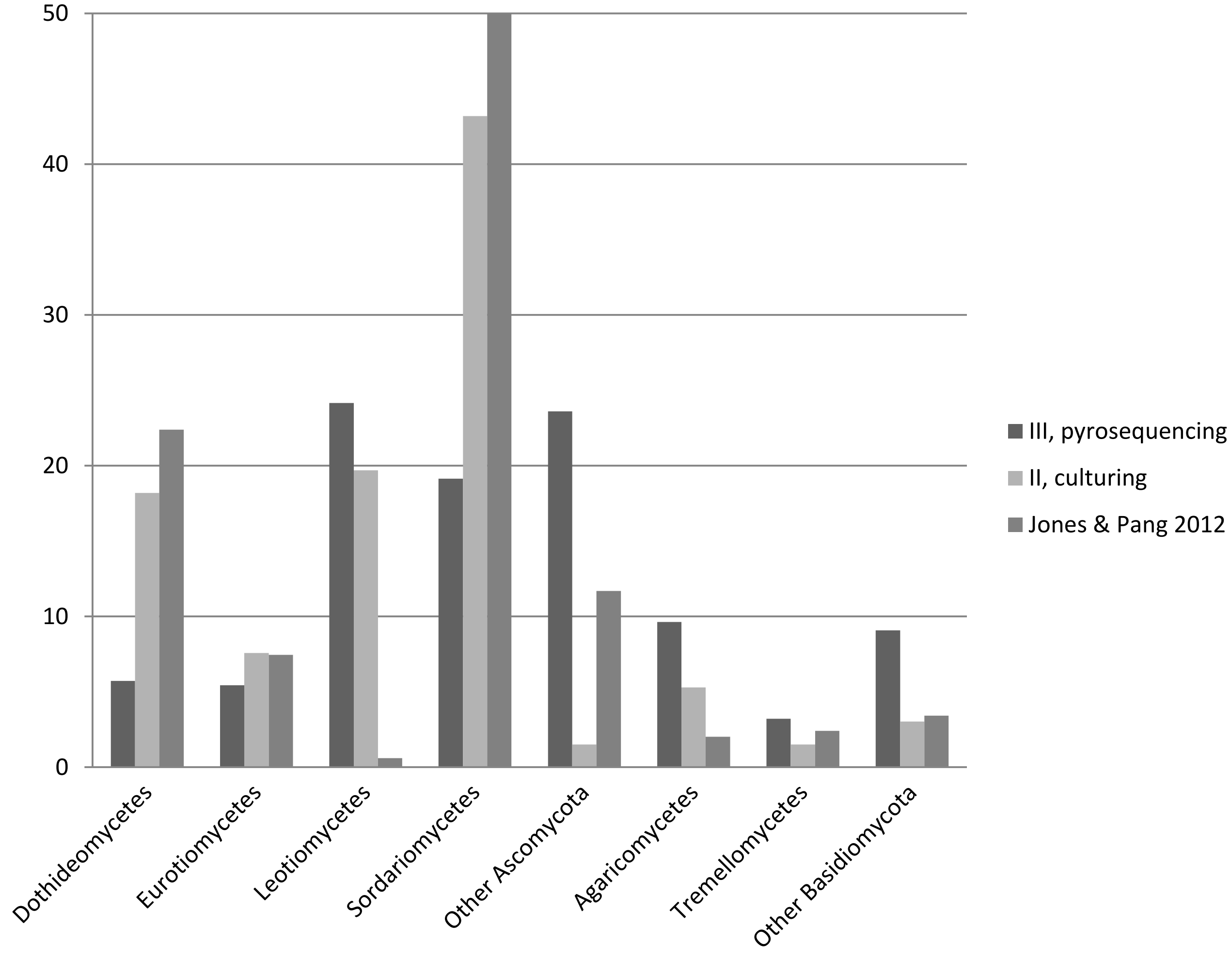

Figure 3. Classes of filamentous fungi in Ascomycota and Basidiomycota and their relative frequencies (%) within different studies. Results gained with the two main methods are compared with the current taxonomic profile of obligate marine fungi (Jones and Pang 2012).

A relatively modest effort was put into the morphological work in this study. Despite this, a previously unknown Lulworthiales taxon was detected in the cold waters of Svalbard (**V**). Other Lulworthiales taxa and especially the *Lulworthia* spp. complex was well represented among the studied specimens and in the culturing dataset, and clearly contains several species based on ITS phylogenies (**II,** Rämä *et al.* unpublished). Additionally, many determinations of other obligate marine fungi remained slightly uncertain (cf.), since often some species characteristics observed did not match with the reference literature. It might be that some of these species actually represent undescribed taxa. This applies especially for specimens collected in Svalbard, where new and undescribed taxa have recently been reported (Pang *et al.* 2008, Pang *et al.* 2009, Pang *et al.* 2011).

Ecology and adaptations of fungi to the marine environment (II, III, IV)

Approximately one half of the OTUs detected using culturing and amplicon pyrosequencing represented non-marine taxa. This is a very common result in marine mycological studies from early on (Elliott 1930, Siepmann and Johnson 1960, Johnson 1967, Shearer 1972). Physiological definitions for marine fungi were proposed, but failed to define this group (Johnson and Sparrow 1961, Tubaki 1969, Kohlmeyer 1974, Hughes 1975). The most commonly used practical division to obligate and facultative marine fungi (Kohlmeyer 1974) is still difficult to apply. Many taxa isolated from or molecularly detected in marine mycological studies cannot be put under the two categories due to i) lack of information on whether a taxon grew and sporulated in the sea or not, and ii) lack of reference sequences enabling accurate species identification. The ecological role of putatively non-marine (terrestrial or freshwater) taxa detected from the sea has been debated in the past. The more frequent than by chance isolation of non-marine taxa in the marine realm has been seen as proof that non-marine fungi are active and do have a role in the sea (Raghukumar and Raghukumar 1999). On the other hand, in order to prove morphologically that a fungus fruits, sporulates or actively grows in the sea, corresponding structures (fruit bodies, mycelium) should be directly observed in fresh material (Kohlmeyer and Kohlmeyer 1979, Shearer *et al.* 2007). The development and use of molecular techniques has improved the discussion about the role of putatively non-marine taxa in the sea, although the question has not been thoroughly addressed. It has been shown that the ecological adaptation to the marine environment has happened multiple times and that marine taxa are spread across the fungal tree of life

(Richards *et al.* 2012). RNA sequencing of deep-sea subsurface sediments suggests that some putatively terrestrial species are active in this marine habitat (Edgcomb *et al.* 2011). Moreover, *Aspergillus flavus* has been demonstrated to possess no phylogeographic structure between terrestrial and marine strains (Ramírez-Camejo *et al.* 2012). This fungus is obviously active in the sea, since it is the most common one isolated from sea fan (*Gorgonia ventalina*) tissues (Zuluaga-Montero *et al.* 2010) and can grow in 6% salt concentrations (Bayman *et al.* 2002). To conclude, it has been confirmed with molecular methods that a single fungus can be ecologically active both in a terrestrial and marine habitat and many other fungi may show similar environmental plasticity.

The marine environment is a stressful habitat for fungi. The multivariate analyses showed that the number of fungi is decreasing with the duration of submersion in the marine realm. The comparison between marine and terrestrial communities confirmed that the marine environment favours more stress-tolerant Ascomycota over wood-inhabiting Basidiomycota dominant in terrestrial logs. The multivariate analyses also showed that tree type (coniferous or broadleaved) and attachment type in the intertidal zone (fixed or loose) structures the communities, as do geography and correlated geographical gradients. Also, other substrate and site level factors, partly different for culturing and culture independent approaches, proved to be important in the analyses. The geographical variables likely reflect different biogeographical background of the logs and/or changes in environmental factors (such as tree type), as coniferous logs with Siberian origin were much more common in the east, whereas local broadleaved wood was better represented in the west of the study area. Due to the explorative sampling design, general conclusions about the main drivers cannot be drawn. However, it seems that factors previously found to be important for fungi in terrestrial logs, such as log diameter (Nordén *et al.* 2013), are also important for marine wood-inhabiting mycota.

In general, relatively few ecological and physical adaptations of marine fungi have been demonstrated. The morphological adaptations of marine Ascomycota with appendaged spores and deliquescing asci, are classical examples, but many of the OTUs found in the marine logs have affinities to fungi not possessing these characteristics in fruiting structures. Additionally, some of the physical adaptations (discussed in the Introduction) are not unique for marine fungi. So, what are the main adaptations of fungi to the marine environment? Recently, Jones and Choeyklin (2008) suggested that the soft-rot strategy of many microscopic Ascomycota is advantageous over the rot strategies of terrestrial Basidiomycota (white and brown rot), which cause leaching of lignocellulolytic enzymes and their loss into the surroundings. The adaptations of marine Basidiomycota include highly reduced fruit bodies (Hibbett and Binder 2001), and likely also a yeast-like growth form, which is possessed by a high number of marine species (Fell 2012). These adaptations seem to fit for the majority of taxa found in this study, but likely there are many more to be discovered and scrutinized, especially at the cell level. For example, genomic analyses have shown that a marine ascomycete has much more diversity in particular transmembrane proteins than terrestrial ascomycetes (Derek Johnson unpubl.). RNA sequencing is the next step to be taken to better understand the ecophysiological adaptations of fungi to the marine milieu. Genome-enabled mycology will be of great help here (Hibbett *et al.* 2013), since it can be used to reveal mechanisms and previously unrecognized adaptations coded in genomes of fungi thriving on land and in the sea. If molecular results are translated for traditional marine mycologists to understand, we will soon have a more accurate definition for marine fungi.

Conclusions and future perspectives

This thesis focuses on exploring the largely unknown diversity of marine wood-inhabiting fungi in northern waters. A rich and diverse mycota was found, and the thesis greatly contributed to the global and national knowledge of marine fungi. Previously unrecognized, frequent and abundant taxa were detected both at the first nodes of Dikarya and at the terminal branches. However, many OTUs remained unidentified to the species level due to poor resolution in molecular species identification. Consequently, it is important that more effort be made in morphological identification and culturing coupled with sequencing of marker genes, as this is fundamental in building up a representative reference library for marine fungi in the northern waters. For the marine order Lulworthiales, this kind of work was already launched during the doctoral studies: in an ongoing collaborative project the systematics of this order is revised world-wide using a multi-locus dataset and new taxa will be described for science. A good reference library for all marine fungi would really enable better insights into the taxonomic diversity of fungi found in marine habitats, especially when combined with high throughput sequencing of environmental samples.

It was demonstrated that an integrated approach combining different study methods is needed in holistic diversity assessment of fungi, as the two main methods that were used detected different parts of the fungal community. However, culturing-based or culture independent methods can be separately applied for rough taxonomic profiling of marine wood-inhabiting fungi. Similarly, both methods revealed consistent results for the frequency of putatively terrestrial OTUs. It seems obvious that many more terrestrial fungi are able to live in the marine environment than currently acknowledged in marine mycology. RNA sequencing of environmental samples should be applied to these communities in order to reveal the fungi that are active in driftwood. This would also give clues about currently unrecognized adaptations of fungi to the marine milieu and the evolutionary history of fungi.

The implementation of the integrated approach in community ecology analyses of marine wood-inhabiting fungi showed that the fungal communities are structured along geographical and environmental factors and gradients, which are partly different for the culturable and the total fungal community. Unfortunately, two factors that are shown to have a major influence on the distribution of marine fungi, sea water temperature and salinity, were incompletely scrutinized in this study. These variables are difficult to measure due to high seasonal and annual fluctuations, and a paucity of hydrographical stations along the studied coastline. Hence, only rough values for temperature and salinity, taken as close as possible to the sampling site and day, could be used. A better approach would have been to measure these variables in the field when sampling the logs. The next step is to study the community ecology of marine fungi in permanent stations. Temperature and salinity will be recorded at each sampling occurrence throughout more than one year. Moreover, these studies will be carefully designed and aimed at testing *a priori* defined hypothesis.

With the results from this thesis included, 61 species of obligatory marine fungi have been recorded in Norway since 1895. This low number of species reflects the small study efforts made to map them, not the true number of marine fungal species occurring in Norway. The long-term objective rising from this doctoral thesis work is to establish a research group focusing on marine fungi in polar areas and base it in Tromsø. Many areas in the biology of these fungi need to be further studied and many more marine mycologists are needed to assist in this, as well as in applied science research projects gaining momentum world-wide.

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Original Chapters

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