Biodiversity in the dark: root-associated fungi in the Arctic

Synnøve Smebye Botnen

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Department of Biosciences
Faculty of Mathematics and Natural Sciences
University of Oslo

Department of Arctic Biology
The University Centre in Svalbard
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Paper I

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Summary

Almost all higher plants form some sort of root symbiosis with fungi. For plant survival and growth, this symbiosis might be especially important for plants nutrient uptake under stressful environmental conditions, such as in the Arctic. Climate change are especially pronounced in the Arctic and may have detrimental consequences for the organisms living there, including the belowground fungal diversity, which in general is poorly explored. As fungi likely are key players in Arctic ecosystems, it is of pivotal importance to obtain more basic knowledge on fungal diversity and biogeography in these ecosystems and how climate variability may affect them. One important new tool used to survey belowground fungal diversity is DNA-metabarcoding, which is based on high throughput DNA sequencing (HTS) techniques. This approach enables in-depth analyses of complex microbial communities at an unprecedented scale, but are still limited by some methodological challenges. The overarching aims of this thesis were to investigate (1) methodological challenges related to the DNA metabarcoding technique, including sequence clustering and choice of primers; (2) the effect of climatic and other abiotic, as well as biotic factors on fungal community composition; and (3) patterns in fungal richness, taxonomic diversity, and life history strategies along environmental gradients. We conclude that sequence clustering threshold has little effect on the recovered community structure of root-associated fungi, as well as bacteria (Paper I). Hence, the DNA-metabarcoding approach is very robust for looking into compositional structure and changes (i.e. beta-diversity). This is because the community structure is mostly driven by a few dominant taxa that are not sensitive towards sequence clustering level. On the other hand, we observed that the recovered fungal community is to a large extent affected by the choice of DNA-marker; in paper IV we observed major discrepancies between the commonly used rDNA ITS marker and the 18S marker. The recovered fungal community compositions and patterns in richness and diversity were related to both climatic and soil edaphic factors (papers II-IV). Hence, changes in these factors due to ongoing climate change will have large effects on arctic fungal communities. No sign of host preference was observed for the fungi associated with ectomycorrhizal (ECM) plant hosts in the Arctic (paper III), whereas host identity was an important structuring factor for the root associated fungal communities of non-mycorrhizal plants, or plants with unclear mycorrhizal status (paper IV). Thus, while Arctic ECM fungi easily can switch between plant hosts, other types of root associated fungi are more dependent on the presence of specific plants. The phyla Basidiomycota and Ascomycota, including many known ectomycorrhizal and endophytic species, dominated across the study sites and host plant
species (paper II-IV). In general, many of the detected fungi had poor matches towards known reference sequences, indicating that far more efforts are needed to map this poorly explored diversity. Taken together, the results from this thesis show there is a huge and largely unexplored diversity of fungi associated with Arctic plants that will be affected by the coming climate change.
Introduction

**Root-associated fungi**

Fungi are incredible organisms, which can be found in almost all ecosystems, from sea ice in the Arctic (Gunde-Cimerman et al., 2003), and soil in the Mojave Desert (Titus et al., 2002), to deep-sea hydrothermal vents (Burgaud et al., 2009). Fungi fill all sorts of niches, and widely different nutritional modes have evolved. They are the major decomposers and nutrient cyclers in many ecosystems (Baldrion and Valášková, 2008), they are devastating parasites/pathogens (Fisher et al., 2012; Warnecke et al., 2012), and they form essential symbiotic relationships with other organisms (Smith and Read, 2008). Almost all plants host fungi in their root systems. Mycorrhiza, a root symbiosis between plant and fungi probably facilitated plant’s establishment on land (Field et al., 2015; Strullu-Derrien et al., 2018). This association between plant and fungi is one of the most ancient symbioses among multicellular organisms on land. In some cases, the plants depend on their mycorrhizal partner to grow in areas they would not be able to without their fungal partners, widening their realised niche (Smith and Read, 2008). The extramatrical mycelia of the fungi extend the root system of the plant, increasing the surface by several magnitudes (Agerer, 2001). The mycorrhizal fungi provide increased uptake of water and facilitates nutrient acquisition, such as nitrogen (N) and phosphorous (P), while in return, the plant provides photosynthetic products to the fungi (Smith and Read, 2008).

The most common mycorrhizal type is arbuscular mycorrhiza (AM), which is found in a wide variety of host plants, but mainly formed by one fungal phylum: Glomeromycota (Smith and Read, 2008). Typical structures of AM include: fungal arbuscules or hyphal coils between the plant cell wall and cell membrane, where the nutrient transfer occurs; and vesicles, which are storage structures (Fig. 1). This symbiosis is thought to be especially important when P is a limiting factor for the plant (Latef et al., 2016). Fungi forming AM do not produce above-ground structures (sporocarps) and we therefore rely on microscopy and DNA to identify them. Ectomycorrhiza (ECM) is the most widespread type of mycorrhiza among woody perennials in temperate and boreal sites (Smith and Read, 2008). Although it is only found on around 2% of the world plant species, ECM is estimated to be formed by 60% of all tree stems on earth (Steidinger et al., 2019). ECM is characterized by the fungus forming an intercellular net of hypha (called a Hartig-net) between the outer cells in plant roots, and a mantel around the root-tip of the plant (Fig. 1). This mycorrhizal type is thought to be especially important when N is the limiting factor for the plant (Smith and Read, 2008; Steidinger et al., 2019). It has been estimated that the ECM symbiosis has evolved independently in at least 30 monophyletic fungal
lineages, including 335 genera and around 8500 species (Tedersoo and Brundrett, 2017).

Other types of mycorrhiza include: ericoid (ErM), orchid (OM), arbutoid and monotropid mycorrhiza, all characterized by fungal hyphae growing into the roots and the plant cells, and forming specialized structures where the nutrient exchange happens (Smith and Read, 2008). In addition, several fungi may reside within plant roots without forming typical mycorrhizal structures. These fungi are called root endophytes, and less is known about their function and roles in ecosystems. Different definitions of endophytes exist; they are most commonly described as harmless fungi living in plant tissue without causing any structural changes in the root, or alternatively producing specific organs for nutrient transfers (Schulz and Boyle, 2005; Rodriguez et al., 2009). Endophytes may often be commensalists, i.e. there are no obvious beneficial effects on the host plant. However, so-called dark septate endophytes (DSE), named after their melanized hyphae, can be important non-mycorrhizal partners, as they can potentially enhance plant growth and nitrogen acquisition for the plant (Jumpponen et al., 1998; Newsham, 2011; Hill et al., 2019).

Symbiotic relationships between fungi and plants can be especially important in stressful environments (Smith and Read, 2008), such as arctic and alpine areas. In arctic environments, ECM fungi supply up to 61-86 % of their host plants’ N need (Hobbie and Hobbie, 2006), and can survive even lower temperatures than their host (Lehto et al., 2008; Korhonen et al., 2013). The most important mycorrhizal types present in the Arctic include: ECM, associated with shrubs and a few herbs; ERM found with ericaceous plants; and AM, mainly associated with herbaceous plants (Väre et al., 1992; Gardes and Dahlberg, 1996;
Newsham et al., 2017). A large fraction of the arctic flora is apparently non-mycorrhizal (Väre et al., 1992; Gardes and Dahlberg, 1996), but several of plants host DSE with tentative beneficial effects (Newsham, 2011; Hill et al., 2019). Still, the root associated fungal (RAF) communities of these apparent non-mycorrhizal plants are less studied than in their mycorrhizal counterparts, especially by molecular methods.

**Drivers of fungal diversity**

As RAF are important players in ecosystems, understanding drivers of their diversity patterns also lead to a better understanding of the whole ecosystem. Numerous biotic and abiotic factors affect RAF composition, structure and diversity, often in highly complex interplays. Climatic parameters, such as precipitation and temperature, are shown to be strong predictors of RAF richness and community compositions at large scales (Tedersoo et al., 2014; Timling et al., 2014; Steidinger et al., 2019). In addition, soil chemistry parameters, such as pH, P, C and N content, often impact or are impacted by the fungal community composition and diversity (Tedersoo et al., 2014; Mundra, Halvorsen, et al., 2015; Mundra, Bahram, et al., 2016; Soudzilovskaia et al., 2019). The degree of impact of these factors on diversity patterns vary across habitats (Mundra, Bahram, et al., 2016), scales (Tedersoo et al., 2014; Mundra, Halvorsen, et al., 2015) and fungal nutritional modes (Tedersoo et al., 2014). Tedersoo et al. (2014) found, for example, that while richness of EcM was highest in slightly acidic to neutral soils, saprotrophs were more diverse in moderately to strongly acidic soils.

Abiotic factors are not independent of biotic ones. A recent study found that climate related decomposition processes (and N availability) drives large-scale distribution of mycorrhizal types (Steidinger et al., 2019). Host identity can also play an important role for RAF diversity, but is seems to vary depending on which type of fungal root association the plant forms (Walker et al., 2011; Becklin et al., 2012; Fujimura and Egger, 2012; Botnen et al., 2014), and across different ecosystems (Ishida et al., 2007; Botnen et al., 2014). Mycorrhizal host plants’ evolutionary history has, for example, been shown to affect their associated fungal communities (Hoeksema et al., 2018). However, the relative importance of host species seems to vary in different ecosystems: for example, host specificity of ECM fungi seems fairly common in boreal and temperate areas (Molina and Trappe, 1982; Ishida et al., 2007; Linde et al., 2018), whereas little host preference of ECM fungi has been observed in the Arctic (Ryberg et al., 2009, 2011; Timling et al., 2012; Botnen et al., 2014). This may again be related to differences in growth conditions due to both nutrient and moisture availability, and length of
growing season related to temperatures. Fungal dispersal also plays a prominent role in community assembly and for the richness of fungi observed in a system (Gleason, 1926; Peay et al., 2010), including their biogeographic structure. In relatively isolated arctic islands, with fluctuating climate, it may be particularly important, and observations by Geml et al. 2012 indicate that arctic fungi consist of long-distance dispersers.

Plant roots do not only host fungi – they also host other organisms, such as non-fungal microeukaryotes. These may interact with the fungi, e.g. the presence of mycophagous amoeba has been shown to reduce ectomycorrhizal colonization in seedlings (Chakraborty et al., 1985). In addition, certain amoeba and microfloral grazers (such as nematodes) have been shown to have positive effects on plants (Elliott et al., 1979; Gould et al., 1979; Ingham et al., 1985; Bonkowski and Brandt, 2002). These effects include increased nitrogen uptake (amoeba and nematodes: Elliott et al., 1979; Ingham et al., 1985), increased phosphatase activity (amoeba: Gould et al., 1979) and changes in root growth and structure (amoeba: Bonkowski and Brandt, 2002). It probably exists numerous complex interactions between plant host, RAF and other microeukaryotes, which may affect the fungal community composition. However, beyond a few examples little is known about such biotic interactions.

Several of the studies on RAF communities in the Arctic and alpine areas report a large fraction of unexplained variation (Mundra, Bahram, et al., 2015, 2016; Mundra, Halvorsen, et al., 2015, 2016), which could be linked to unknown biotic interactions. However, there are usually also many abiotic factors not accounted for, including e.g. freeze-thaw processes and microscale differences in soil properties. On the other hand, much of the variation unexplained could also be related to stochastic processes, e.g. random dispersal events and assembly processes. Much remains to be explored.

Climate change

Ongoing climate change is expected to be a major threat to biodiversity and ecosystem functioning. On a global scale, average temperature increased with 0.85 °C from 1880 to 2012, and precipitation patterns have also been altered (Pachauri et al., 2015). This has led to a shift in geographical range, seasonal activities, migration patterns, abundances, and species interaction of several terrestrial, freshwater and marine species (Sturm et al., 2001; Parmesan and Yohe, 2003; Perry et al., 2005; Post et al., 2009; Poloczanska et al., 2016; Cristofari et al., 2018). In the Artic, both a greening and browning have been observed, likely induced by climate change (Xu et al., 2013; Myers-Smith et al., 2015; Phoenix and Bjerke, 2016; Epstein et al.,
2017). The tight connection between climate and distribution of mycorrhizal types observed by Steidinger et al. 2019 indicates that changes in climate will affect their future large-scale distributions, where e.g. the AM symbiosis may expand northwards on the cost of ECM. Changes in temperature and precipitation patterns are especially pronounced in several arctic areas (ACIA, 2005; Bilt et al., 2019), and the effect on the fungal diversity here is thus, potentially large. Altered precipitation patterns may lead to an increase in snow cover, which may provide more insulation and protection from winds for the biota, in addition to more moisture available at the start of the growing season. However, consequences might be detrimental: a recent study reported the highly disturbing news of ecosystem-wide reproductive failure of plants and animals due to increased snow cover and late melting snow (Schmidt et al., 2019). This may obviously affect their associated fungal communities. Experimentally increased snow depth has been shown to induce shifts in fungal richness (Morgado et al., 2016; Mundra, Halvorsen, et al., 2016), while conflicting results have been reported when it comes to the effect on ECM fungal community composition (Morgado et al., 2016; Mundra, Halvorsen, et al., 2016). Also, stronger effects of increased snow depth have been observed in dry compared to moist sites (Morgado et al., 2016). Furthermore, numerous warming experiments have shown changes in fungal community composition across temperature gradients (Clemmensen et al., 2006; Deslippe and Simard, 2011; Deslippe et al., 2011; Geml et al., 2015), suggesting that climate change will affect fungal communities. Climate change is also leading to an accelerated pace of glacier retreat in the arctic (Martin-Moreno et al., 2017; Bourriquen et al., 2018), and the habitat of glacier forelands may change and eventually disappear. Many of the early colonizing plants moving in after glacial retreat form mycorrhiza. As such, the newly exposed land may represent an opportunity for both the plants and their mycorrhizal partners. However, previous studies have found that RAF community composition is habitat dependent (Ryberg et al., 2011; Mundra, Bahram, et al., 2016), and with the accelerated pace of glacier retreat, this habitat may change, and eventually disappear. This potentially represents a threat for the early fungal colonizers.

Shifts in fungal community composition will likely influence nutrient cycling, C-N dynamics and ecosystem respiration, and thus C emissions and C fixation, including dramatic effects on the stored permafrost carbon (Vonk et al., 2012). In addition, potential changes in community composition will also likely affect interactions between species: Species have different niches, and range tolerances, and it is unlikely that these changes will affect their distribution in a similar way. To understand these changes, it is of pivotal importance to obtain
baseline knowledge on fungal diversity and biogeography in vulnerable arctic ecosystem; it is only then possible to understand how climate variability affects them.

**How to assess fungal communities?**

The ecological roles of microorganisms can only be fully understood when they are studied in their natural environment. Microbial ecology has over the last decade(s) taken great steps forward due to the introduction of high throughput DNA sequencing (HTS) techniques that enable in-depth analysis of complex microbial communities. Fungi are increasingly detected, identified, and quantified through their DNA, and our understanding of the ecology of fungal communities has in this way been greatly enhanced. Early studies in fungal ecology relied on sporocarp identification (e.g. Molina *et al.*, 1992), morphotyping of root tips (e.g. Hesselman, 1900; Read and Haselwandter, 1981; Väre *et al.*, 1992), and later, on traditional Sanger sequencing (e.g. Ishida *et al.*, 2007; Ryberg *et al.*, 2009). Sanger sequencing has been successfully used to identify parts of the fungal diversity, particularly when it comes to new species, and is of immense importance for creating reliable reference databases (Horton and Bruns, 2001; Vandenkoomhuyse *et al.*, 2002; O’Brien *et al.*, 2005; Lindahl *et al.*, 2007). A challenge with this approach, however, is that it is extremely laborious to obtain a high sequence number, which is needed to cover the immense belowground diversity. This makes Sanger sequencing less suitable for environmental samples (but see: Taylor *et al.*, 2014; Linde *et al.*, 2018). High-throughput sequencing techniques, the basis of the DNA metabarcoding approach, allows for a high number of DNA molecules to be simultaneously sequenced. In DNA metabarcoding analyses, we use group-specific primers that amplify a specific DNA region from all target organisms in the environmental sample. Since thousand and millions of sequences can be generated from the PCR amplicons, we can obtain a deep characterization of organisms present. Hence, this makes it possible to identify dominant as well as rare species in a sample. DNA metabarcoding has been successfully implemented in numerous fungal ecology studies over the last decade (Walker and Del Moral, 2003; Junpponen *et al.*, 2010; Wallander *et al.*, 2010; Walker *et al.*, 2011; Davey *et al.*, 2013; Lindahl *et al.*, 2013), and is used in this thesis to reveal fungal and microeukaryote communities associated with plant roots.

There are, however, several pitfalls and challenges associated with the DNA metabarcoding approach, summarized in e.g. Lindahl *et al.*, 2013; Calderón-Sanou *et al.*, 2019; and Nilsson *et al.*, 2019. One major challenge is to delineate appropriately between the different species, which is the basic taxonomic level in community ecology. Different markers are being
used for different groups, which differ in their taxonomic resolution and inter- and intraspecific variability. The internal transcribed spacer (ITS) of the rDNA is generally used as a fungal barcode (Nilsson et al., 2008; Schoch et al., 2012), the rDNA 18S region as a general eukaryotic barcode (Hadziavdic et al., 2014), and the rDNA 16S region for bacteria (Stackebrandt and Goebel, 1994; Tanabe and Toju, 2013). The 18S region is relatively conserved with low inter- as well as intraspecific variability, making it less useful for species delimitation. However, broad taxonomic groups can be recognized, typically at a class level and above, and 18S is therefore suitable to provide a comprehensive picture of all eukaryotes present in environmental samples (e.g. Mahé et al., 2017). In contrast to the 18S marker, the ITS region has relatively high inter- as well as intraspecific variation (Nilsson et al., 2008), and the degree of variation differs between species. As such, there is no general cut-off that can be used to delimit fungal species based on the ITS region. While some species and sister species share the same ITS sequence, others possess up to 10% intraspecific sequence divergence (Nilsson et al., 2008). A common approach to account for the intraspecific sequence variation in ITS, is to cluster sequences into groups (OTUs) that putatively represent different species. However, the threshold used to define OTUs differs between studies: typically, between 95-99 % sequence similarity have been used (Geml et al., 2009; Nemergut et al., 2011; Blaalid et al., 2013; Bonito et al., 2014; Lorberau et al., 2017; U’Ren et al., 2019). It is obvious that choice of threshold will affect the observed alpha diversity in the samples, but to what extent it affects beta diversity in fungal community studies is an open question.
Objectives

In this thesis, I investigate the diversity of fungi associated with plants in the Arctic. Arctic is expected to undergo dramatic changes in the future due to climate change (Bilt et al. 2019) and it is therefore important to establish baseline information about the current biodiversity, to be able to monitor how they may change. By studying hitherto unexplored locations and host species, I wanted to establish new knowledge about arctic fungal biodiversity and which factors influence them. The main study area, Svalbard, is a part of the High-Arctic where climate change is especially pronounced (Bilt et al. 2019). Compared to other organisms groups, like plants and vertebrates, we still now little about the Arctic fungal diversity, especially in remote locations. The more specific objectives in the thesis were to investigate (1) methodological challenges related to the DNA metabarcoding approach, including sequence clustering (paper I) and choice of primers (paper IV); (2) which climatic and other abiotic (paper II/III) and biotic factors (paper III/IV) influence the root associated fungal community composition; and (3) patterns in fungal richness (paper II/III), taxonomic diversity (paper II/III/IV), and life history strategies (paper IV) of fungi associated with arctic plant roots.
Materials and methods

Figure 2: The glacier Friederichbreen located in Bockfjorden at Svalbard. Plant roots were collected both from the glacier foreland (in paper III), and from the low-statured tundra seen in the forefront of this picture (paper IV). Photo by Peter Convey

Svalbard as a study system

High arctic habitats are characterised by high environmental resistance. Soils are nutrient poor (e.g. low levels of N, C and P), there is little precipitation, low temperatures, high soil movement due to frequent freezing and thawing events and a short growing season. In addition, wind exposure and periglacial processes (at distances < 1 m; Washburn, 1980) also influence the vegetation and soil conditions. This results in challenging growth conditions and limits vegetation development. For example, the vegetation in Svalbard is scarce and generally below 15 cm (Fig. 2). Only 184 plant species are native to the archipelago (Alsos et al., 2019). However, the richness and diversity of fungal communities are relatively high (Gardes and Dahlberg, 1996; Geml et al., 2012; Botnen et al., 2014; Davey et al., 2015; Mundra, Bahram, et al., 2015), and fungi play important roles as symbionts and decomposers in the ecosystem functioning. In Svalbard, a warming of up to six times higher than the global temperature increase has been observed (3-5 °C from 1971 to 2017; Bilt et al., 2019). By the end of this century the average temperature is estimate to increase with 7-10 °C if we continue with medium to high greenhouse gases emissions (Fig. 3a). Furthermore, a 35-65 % increase in
precipitation is estimated (Fig. 3b). This further highlights the need to understand and describe the present fungal diversity, and compare it across current climatic gradients (such as at a North-Atlantic scale), to better understand how fungal communities in this vulnerable ecosystem might change in response to climate change. Due to challenging sampling conditions, and remote locations, the fungi in large parts of the landmasses in Svalbard are undescribed. In addition, previous studies of RAF in the archipelago using high throughput sequencing (HTS) found a large degree of unidentified fungal sequences (e.g. Botnen et al., 2014; Mundra, Bahram, et al., 2015; Zhang and Yao, 2015; Dong et al., 2016). Svalbard represents the main study area in this thesis, and data from this archipelago are included in all papers.

![Figure 3: Predicted changes in A) mean temperature (°C) and B) mean precipitation (%) from 1971-2000 to 2071-2100 in Svalbard assuming continued medium to high emissions of greenhouse gasses. Figure modified from: Climate in Svalbard 2100 (Bilt et al. 2019)](image)

**Study systems and sites**

In papers II and III we investigated RAF in the widespread ectomycorrhizal forming, perennial herb *Bisorta vivipara* (L.) Delarbre (Polygonaceae; syn.: *Polygonum viviparum* L. and *Persicaria vivipara* (L.) Ronse Decr.). Because of its small and condensed root system, the plant has been used as a model-system in several studies of arctic and alpine RAF (Blaalid et al., 2014; Botnen et al., 2014; Davey et al., 2015; Mundra, Bahram, et al., 2015, 2016; Mundra, Halvorsen, et al., 2015, 2016). DNA can be extracted from the entire root system, and it is thus ideal for investigating the whole RAF community associated with one ectomycorrhizal forming plant. In paper III, *B. vivipara* was analysed together with *Salix polaris* Wahlenb. They are both pioneer-species and important for ecosystem functioning in Svalbard, and listed as two of the
key species there by the Climate-Ecological Observatory for Arctic Tundra (Ims et al., 2013). *Bistorta vivipara* is e.g. an important food-source for Rock Ptarmigan - *Lagopus mutus* (Moss and Parkinson, 1975), barnacle goose - *Branta leucopsis* (Kuijper et al., 2006), and reindeer - *Rangifer tarandus platyrhynchus* (Lindwall et al., 2013). In paper II, we also took advantage of several previous studies on *B. vivipara* in Svalbard (Blaalid et al., 2014; Botnen et al., 2014), and mainland Norway (Blaalid et al., 2012; Yao et al., 2013), and used data from these studies together with new samples collected from Jan Mayen, Iceland, Scotland and Austrian Alps to investigate broad-scale biogeography RAF of a single ECM forming plant at a North-Atlantic scale.

Our sampling trips in 2013 and 2014 allowed us to access several remote areas across Svalbard. In papers III and IV, we investigate fungal diversity across in total 15 geographically widespread locations in the Archipelago (for example Bockfjorden, fig 2). The patchy and scarce vegetation in Svalbard, made it relatively easy to dig up and clean whole, intact roots systems, which allowed us to survey whole root RAF communities of 31 different non-ECM plant species in paper IV.

**Workflow**

DNA metabarcoding was used to study microbial communities in this thesis (papers I-IV). The general workflow of DNA metabarcoding include: collecting and preparing environmental samples; library preparation; high throughput sequencing; bioinformatics and statistical analyses, followed by ecological interpretations (Fig 4; Fig 5).

![Figure 4](image.png)

*Figure 4:* Picture of ongoing root washing for paper IV.
Figure 5: Overview of workflow using high throughput sequencing and DNA metabarcoding. First, DNA is extracted from an environmental sample, then a DNA marker of interest are amplified using PCR. Primers used include molecular identifiers (MIDs), unique for each sample included in the same sequencing library, to be able to tell the samples apart. The sequence libraries are then sequenced on an HTS platform of choice. Obtained sequence reads are being processed bioinformatically, before ecological analyses and biological interpretations are conducted. Drawing of computer is based on https://www.drawingforall.net/how-to-draw-a-macbook/
Results and discussion

In the following part, I will summarize and discuss the main findings of this thesis. Firstly, I will start with a discussion regarding clustering (paper I) and primer choice (paper IV) in DNA metabarcoding, with a main emphasis on fungal communities. I will then provide a general overview of the recovered taxonomy and functional lifestyles in this thesis (papers II-IV), before I focus on drivers of the recovered fungal diversity patterns (II-IV).

HTS methods for microbial ecology: Considerations in the lab and on the computer

As mentioned in the introduction, to correctly identify species from a genetic DNA barcoding marker is challenging. Both intra- and interspecific variation can be different across targeted taxonomic groups. Hence, using a general sequence similarity threshold during clustering therefore is problematic. Alternatively, the use of amplicon sequence variants (ASVs), representing an approximation for the underlying genotype/haplotype, has been suggested an input for ecological analyses (Callahan et al., 2016). The use of ASVs might be a suitable approach for relatively conserved markers, like 18S and 16S. However, this approach becomes problematic when the used marker possesses high intraspecific variation, such as the fungal ITS region, where a high number of ITS alleles often exist within species. To approach the species level, which typically is the goal in community ecology analyses, it will be important to group the sequence reads or ASVs further into OTUs. Clustering based on a certain similarity threshold can be a practical way of grouping sequences, but where to put the threshold to use is debated (Gevers et al., 2005; Hanage et al., 2006; Koeppel et al., 2008; Fraser et al., 2009; Koeppel and Wu, 2013; Patin et al., 2013; Powell and Sikes, 2014; Yamamoto and Bibby, 2014; Ryberg, 2015; Tikhonov et al., 2015). However, whether this problem is relevant for ecological interpretation is poorly known. We therefore set out to investigate whether clustering threshold affected the ecological interpretation when it comes to compositional structure (paper I). Our analyses revealed that, when it comes to estimation of beta diversity (community structure), the results are very robust across different clustering thresholds (paper I). This is in agreement with some previous and newer studies focusing on the effect of different clustering thresholds or species delineation methods (Koeppel and Wu, 2013; Lekberg et al., 2014; Glassman and Martiny, 2018; Calderón-Sanou et al., 2019). Nilsson et al. (2019) and Blaalid et al. (2013) observed that a clustering threshold of 97-98% in general balances intraspecific variance and PCR errors for the ITS region in fungi. Taken together, I suggest that the 97 % clustering level used on the ITS marker in this thesis (papers II-IV) represent a good basis for
answering our research questions related to community composition. Also, for more conserved markers, such as 18S and 16S, correct species delineation will likely not affect the recovery of microbial community structure to a large degree, especially when the ecological gradients are strong. However, it is important to note that we only investigate the effect on compositional gradient structure in paper I. Other aspects of diversity may show different responses to variation in clustering thresholds (Calderón-Sanou et al., 2019).

The ITS region has been used to study fungal diversity since the 1990’s (White et al., 1990; reviewed in Nilsson et al., 2014), and has been denominated as the main fungal barcode (Schoch et al., 2012). Hence, a huge amount of references sequences exits. The most recent version of fungal ITS sequences in the UNITE (Abarenkov et al., 2010) and INSDC (Karchesz-Mizrachi et al., 2018) databases available at unite.ut.ee (visited 18.11.2019) exits of 887 395 number of sequences (UNITE Community, 2019). Thus, it is the obvious choice for studying fungal communities and diversity. However, different biases exist related to the amplification of the ITS1/ITS2 maker (Bellemain et al., 2010; Tedersoo et al., 2015; Nilsson et al., 2019). Even if the primer sites are situated in more conserved parts of the rDNA, mismatches in the most commonly used primers exists in several taxonomic groups, such as Archeorhizomycetes (Rosling et al., 2011), Chytridiomycota and Glomeromycota (Tedersoo et al., 2015). This results in some groups being poorly amplified or not amplified at all. The length of the ITS region also vary across species (Bellemain et al., 2010; Tedersoo et al., 2015; Nilsson et al., 2019). This represent another bias, because short fragments are preferentially amplified in the PCR, resulting in poor amplification of taxa with the longest fragments (Nilsson et al., 2019). The more conserved V4 18S marker is likely more unbiased when it comes to amplification of all fungal groups. Therefore, we used this primer to get a more comprehensive overview of fungal groups present in plant roots, as well as other microeukaryotes (paper IV). As we used general eukaryote primers, we expected the plant DNA to dominate in the data. However, host plant DNA accounted for around 99 % of the sequence reads, resulting in a small non-host sequence dataset. Thus, compositional analyses were not conducted on this data (paper IV). Even if the sequencing depth was likely too shallow to detect the whole eukaryotic community, we still find it interesting that a relatively larger diversity of Chytridiomycota and Mucoromycota was recovered with the 18S primers compared to the ITS2 primers (paper IV). We also find value in reporting these results, so that future studies using similar methods might use blocking-primers for plant DNA, or other primers.

Methodological issues are not limited to species delineation and marker/primer biases – biases can emerge in other steps of DNA metabarcoding studies. However, comprehensive
literature discussing potential biases regarding DNA metabarcoding of fungi exists (Tedersoo et al., 2015; Tedersoo and Nilsson, 2016; Calderón-Sanou et al., 2019; Nilsson et al., 2019), and I will not discuss this further in this thesis.

**Taxonomy and lifestyles**

By targeting the ITS1 region (paper II), ITS2 region (papers III/IV) as well as the 18S region (paper IV) we surveyed fungal communities associated with plant roots in Svalbard (papers II-IV) and in the North-Atlantic (paper II, *B. vivipara*). The detected fungi were taxonomically as well as functionally annotated, and I will focus on the recovered ITS data in the following section.

![Figure 6: Taxonomic distribution of fungal ITS OTUs across papers II-IV at phylum (A and B) and order level (C and D). A and C are based on OTU occurrences in samples, while B and D are based on read abundance. This figure was constructed in R, and is based on rarefied data including all fungal groups detected.](image)

The majority of known ECM fungi belongs to Dikarya (Tedersoo et al., 2010). Numerous studies have reported a relatively high diversity of both these phyla in plant roots...
(e.g. Dickie et al., 2004; Bjorbækmo et al., 2010; Kjøller et al., 2010; Walker et al., 2011; Botnen et al., 2014; Mundra, Bahram, et al., 2015). This agrees with the recovered taxonomy in this thesis (papers II-IV, Fig. 6). Ectomycorrhizal basidiomycetes, closely followed by ECM ascomycetes, dominated in the ECM forming host plant studied (papers II/III). Contrasting these results, Ascomycota was clearly the dominating phylum in the RAF communities of host plant assumed not to form mycorrhiza, or with unclear mycorrhizal associations (paper IV). However, more endophytes and parasites are expected in these root systems, and Ascomycete dominance is typically observed in studies of endophytic fungi (Rodriguez et al., 2009; Zhang and Yao, 2015; Sun and Guo, 2012, and references therein), likely explaining the difference. Dark septate endophytes have, for example, been found to commonly occur in cold environment (Read and Haselwandter, 1981; Jumpponen and Trappe, 1998; Newsham, 2011; Pandey, 2019), where they may have a role in nutrient acquisition (Jumpponen et al., 1998; Jumpponen, 2001; Upson et al., 2009; Hill et al., 2019). In contrast to our expectations, only nine ITS OTUs were assigned as DSE in the plant roots of the non-mycorrhizal plants, or plants with unclear mycorrhizal associations (paper IV). Still, a large part of the OTUs could not be classified at an order or family level, and thus not be functionally annotated. We suspect that several of these “unknown” OTUs represent undescribed DSEs.

Pezizales represented the most common ascomycete genera in the innermost glacier forelands (paper III), which contrast other studies finding Helotiales to be more common among RAF in the Arctic (papers II/IV; Walker et al., 2011; Mundra, Halvorsen, et al., 2015; Zhang and Yao, 2015). This may be explained by the relatively high abundance of the stress-tolerant genera Geopora (paper III), which have been commonly detected in other marginal environments (Fujimura et al., 2005; Hrynkiewicz et al., 2009; Gordon and Gehring, 2011). This genus likely possesses special adaptations to thrive under marginal conditions, such as in glacier forelands (paper III), post-fire succession (Fujimura et al., 2005), and coastal sand dunes (Botnen et al., 2015). We also found the abundance of Glomeromycota (AM fungi) to vary slightly across the study systems in this thesis (papers II-IV). This is not surprising, since paper IV included typical AM-forming host genera, such as Ranunculus, and Taraxacum. These genera have recently been confirmed to form AM at Svalbard (Newsham et al., 2017).

As mentioned above, due to primer/maker biases with the ITS, we used the 18S marker in paper IV, to get a more comprehensive picture of the fungal diversity in the plant roots.
**Drivers of fungal diversity – abiotic factors**

The relatively large geographical scales studied in papers II-IV allowed for investigation of relations between fungal diversity and gradients in the major climatic factors temperature and precipitation. Our results agree with several other large-scale studies, finding climatic variation to play an important role for the structure of RAF communities (Timling et al., 2012, 2012; Tedersoo et al., 2014; Steidinger et al., 2019; Větrovský et al., 2019). Temperature and moisture are basic regulators of fungal growth (Maynard et al., 2019), and thus, likely for community composition as well (Andrew et al., 2018). Climatic control of decomposition has recently been suggested to drive global biography of mycorrhizal symbiosis (Steidinger et al., 2019), highlighting the importance of climatic factors as large-scale drivers of fungal community composition. We also observed climatic factors to be predictive of the recovered RAF richness at a North-Atlantic scale (paper II); and diversity (measured as Shannon diversity index) across Svalbard (paper III). Taken together, these results highlight the sensitivity of fungal communities to climate change.

At Svalbard, both more precipitation and higher temperatures are expected in the future (Bilt et al., 2019), generally providing better conditions for fungal growth. The poleward expansion of shrubs in the Arctic (Sturm et al., 2001; Stow et al., 2004) due to climate change, implies an expansion of their ECM partners as well. Long-term experimental warming of shrub vegetation has been found to affect the diversity of ECM fungi (Clemmensen et al., 2006; Deslippe et al., 2011; Geml et al., 2015), including an increase of ECM fungi with proteolytic capacity (Deslippe and Simard, 2011). This may lead to a higher turnover of organic matter. It has been shown that certain ECM fungi have an enzymatic machinery able to decompose soil organic matter (SOM), leading to a quicker turnover of SOM (Bödeker et al., 2009, 2014, 2016; Lindahl and Tunlid, 2015). Together with melting of permafrost, which release latent SOM, substantial loss in the carbon storage of arctic soils is expected (Vonk et al., 2012). Thus, this reinforcing feedback mechanism of further increase in CO2 emissions (Grosse et al., 2011), will likely cause ecosystem-wide effects. Since AM fungi primarily are associated with warmer regions (Smith and Read, 2008; Steidinger et al., 2019) increasing temperatures might also lead to a larger portion of AM colonization in the Arctic, partly related to a potential increase in available host plants and a longer growing season (Newsham et al., 2009; Steidinger et al., 2019). Soils dominated by AM fungi have also been found to have lower amount of carbon sequestration compared to soils dominated by e.g. ECM fungi (Averill et al., 2014).

In contrast to observations of greening, browning (a die-back of vegetation), has also recently been reported in the Arctic (Phoenix and Bjerke, 2016; Epstein et al., 2017). Hence, a
die-back of plant hosts may lead to a decline in RAFs. On the other hand, browning and melting of permafrost leads to more available dead organic material (Vonk et al., 2012; Phoenix and Bjerke, 2016; Epstein et al., 2017), opening up opportunities for saprotrophic or partly saprotrophic fungi.

Climate change has also led to an accelerated pace of glacier retreat in the Arctic (Martín-Moreno et al., 2017; Bourriquen et al., 2018), and in the long term they might all together disappear. Thus, the dynamics of the early fungal pioneers of glacier forelands may change. Glacier forelands in Svalbard are generally characterized by high environmental stress, which probably results in limited competition (Davey et al., 2015). Previous studies of successional patterns in Svalbard, show that the RAF of glacial forelands follow a directional, non-replacement successional pattern (Hodkinson et al., 2003; Davey et al., 2015), which is likely a result of this limited competition. Climate change may result in less environmental stress in the Arctic, facilitating an increase of strong competitors. As such, we might see the more traditional directional, replacement successional pattern (Matthews, 1978) in the future, which may represent a threat for poor competitors adapted to extreme habitats, like the members of the stress-tolerant Geopora frequently observed in paper III.

In addition to regional-scale climate variation, we also assessed the effects of local scale variation in edaphic factors in papers III and IV. We found that both SOM (paper III), pH, C:N (papers III/IV), and N (paper IV) were related to our recovered RAF community composition. In agreement with our results, soil edaphic factors have previously been shown to be predictors of RAF diversity and distribution (e.g. Tedersoo et al., 2014; Timling et al., 2014; Mundra, Halvorsen, et al., 2015). Soil organic matter and C:N ratio are related to nutrient availability (Cleveland and Liptzin, 2007; Yoshitake et al., 2007). In systems with high C:N, nutrients are often bound up in organic material (Clemmensen et al., 2013). Since arctic soils are generally nutrient poor, it is reasonable that small changes in SOM and C:N affect the fungi living in the especially nutrient poor environments of arctic glacier forelands (paper III). Mundra, Bahram et al. (2016) found that soil edaphic factors were more important for the community structure of RAF in B. vivipara in edge-habitats (host plant less frequent) compared to core habitats (host plant frequent) in Svalbard (Mundra, Bahram, et al., 2016). They explained this by that the plant and associated fungi are more sensitive to change in edge habitats which represents extremes in nutrient availability, like glacier forelands certainly represents. Additionally, our analyses revealed that pH and C:N negatively correlated with OTU richness in glacier forelands (paper III). pH has earlier been shown to be of the most important factors for predicting differences among fungal community and richness (Tedersoo et al., 2014), and is known to
change, or covary, with both nutrient and enzyme activity in the soil (Frankenberger and Johanson, 1982; Rousk et al., 2009). Slightly acidic to neutral soils have been found to represent peaks of ECM richness (Tedersoo et al., 2014). Thus, the negative correlation between pH and OTU richness observed, is likely due to the relatively high pH observed in the glacier forelands (average: 7.33, in paper III).

Soil edaphic factors are not necessarily independent of each other, and their effects on fungal community composition are therefore difficult to tease apart. Results related to this must therefore be interpreted with some level of caution.

**Drivers of fungal diversity – biotic factors**

Not only abiotic factors affect fungal communities – they are also affected and structured by biotic factors. In papers III and IV we investigate host plants effect on RAF diversity. The importance of host plant identity on the RAF community structure depends on which type of fungal root association is present, as well as the habitat (Ishida et al., 2007; Walker et al., 2011; Becklin et al., 2012; Fujimura and Egger, 2012; Botnen et al., 2014; Hoeksema et al., 2018; Linde et al., 2018). In paper III, we confirmed previous findings, showing low degree of host preference of ECM fungi, and no difference in OTU richness between the ECM host plant species in the Arctic (Ryberg et al., 2009, 2011; Timling et al., 2012; Botnen et al., 2014). This may be related to a reduced probability of ECM colonization, due to short growing seasons in the Arctic and potential long distance between suitable hosts (Botnen et al., 2014). Both for the ECM fungi and plant it may then be more favorable to associate with multiple hosts to increase the probability of finding a suitable partner, which may lead to increased probability of survival. However, when investigating the RAF community of 31 apparent non-ECM (mostly non-mycorrhizal) forming plant species (paper IV), we found host species alone to explain 33 % of the compositional variation. Compared to the low levels of total variation explained in the other papers in this thesis (papers II/III, 11% and 14 %), as well as other studies of RAF in ECM plant hosts in Svalbard (Mundra, Bahram, et al., 2015, 2016; Mundra, Halvorsen, et al., 2015, 2016), this percentage is high. The roots of the plant host species investigated in paper IV are probably dominated by endophytes and parasites, which in general are more tightly associated with the plant hosts compared to the ECM fungi. Parasites are typically very host specific due to the “arms-race” and co-evolution between host and parasite (Mode, 1958; reviewed in Woolhouse et al., 2002), which may explain strong host effects (paper IV). Likewise, in a study of above-ground endophytes in Svalbard a large degree of host specificity was observed (Zhang
and Yao, 2015), in agreement with our results (paper IV). Related to this, we also observed that the phylogenetic distance between the plant hosts correlated with the RAF community composition distances (paper IV). These results suggest that the evolutionary history of the host plant is important for which root colonizing fungi are present. In this respect, our finding agrees with observations of mycorrhizal plants in non-arctic ecosystems (Hoeksema et al., 2018), suggesting that evolutionary history of both plant and fungi predict the strength of the mutualism.

Another biotic factor that may influence on the community assembly of RAF is the fungi’s dispersal abilities. Long-distance dispersal is thought to be especially important for plant and fungal establishment in polar areas (Alsos et al., 2007; Geml et al., 2012; Cox et al., 2016), due to the fluctuating climate and potential long geographical distances between suitable habitats. The biogeographical structuring of the fungal communities observed in papers II-IV may largely be driven by the abiotic factors discussed above. However, it may also be related to dispersal processes. The biogeographical patterns were especially distinct in paper II, which represents the largest scale studied in this thesis, with varying degrees of geographic isolation between the studied localities. For example, we observed that the fungal community composition in the small, isolated island of Jan Mayen represented a mixture of those present in Svalbard and Iceland (paper II). In relation to biogeography and dispersal patterns, we also observed that plants in the smallest and most isolated island (Jan Mayen) hosted the lowest OTU richness (paper II). This is generally in line with classic island biogeography theory (MacArthur and Wilson, 1967), that states that richness decreases with isolation and area.

**Future perspectives**

This thesis revealed a high diversity of fungi associated with Arctic plant roots. Most of this diversity is poorly explored, both when it comes to their taxonomy and phylogenetic belonging, and their functions. Presently, we do not know what we may lose with climate change. Hence, there is a need for more research mapping the diversity and exploring the functions of the arctic fungal diversity. We need continuous and reinforced efforts with traditional methods, including sporocarp based fungal systematics. In this way, the reference sequence databases can be better propagated with arctic fungi. However, there is also an enormous diversity of belowground arctic fungi that cannot be investigated with traditional means; many of these fungi never or rarely produce aboveground sporocarps. To study and communicate this diversity, a novel sequence-based classification and systematics must be
implemented. In future studies, environmental sequencing in the form of metabarcoding, metagenomics, and metatranscriptomics can be combined with advanced imaging analyses (e.g. FISH) to obtain improved knowledge about the diversity, niche preferences and functionalities. To gain more precise knowledge on their functions, experimental hypothesis-driven research is also needed. For example, experimental studies may give us more insight in the fungi’s nutritional modes, as well as how they affect the host plants in the Arctic. By this multitude of approaches, we can get a better understanding of the dark arctic fungal diversity and their ecosystem effects.

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References


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