

Back to the roots:

Diversity and community composition of root-associated fungi explored by high throughput sequencing

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Summary

Interactions between plant roots and fungi are well known from most terrestrial ecosystems. Mycorrhizal association is the most prominent plant-fungi interaction, where the fungal partners increase the water and nutrient uptake of their host plants. This symbiosis might be especially important in marginal habitats like arctic and alpine environments. The structure, diversity and spatial patterns of the root-associated fungal communities are to a large extent unknown due to previous methodological limitations. The main objective in this thesis was to implement high throughput DNA sequencing to assess the community structure, richness and spatial distribution of root-associated fungal communities in arctic and alpine environments. We focused on one host plant species, namely the ectomycorrhizal herb *Bistorta vivipara*. Its small and condensed root system enabled us to analyze the entire fungal assemblages associated with individual root systems, using 454 pyrosequencing of ITS1 and/or ITS2 amplicons. All the five studies included in this thesis revealed that the most prominent fungal groups were well-known ectomycorrhizal fungi such as Agaricales, Sebaciales and Thelephorales. Furthermore, ascomycete fungi of the order Helotiales were also recovered frequently across all root systems. Although a high patchiness in fungal community composition generally was observed, some systematic compositional changes along gradients were observed. In a 2x2 m² local scale study, a spatial autocorrelation was observed at small scales (<0.34 m). Furthermore, a significant compositional difference was observed between the root-associated fungal communities and adjacent soil fungal communities. Along two primary succession gradients in arctic and alpine areas, a systematic compositional shift was observed. The fungal richness increased along the chronosequences towards the climax vegetation. In a biogeographic survey, where the root-associated fungi were analyzed across Svalbard, a compositional shift was observed that was associated with the latitudinal gradient. Moreover, the fungal richness increased westwards in the more climatic favorable habitats. Overall, the different studies indicate that stochastic processes, possibly related to aerial spore dispersal, are important during fungal community establishment. The conducted studies exemplify that high throughput sequencing is a powerful approach for analyzing complex microbial communities.

List of Papers

The thesis is based on the following five papers, which will be referred to in the text by their Roman numerals:

I. Blaalid R, Kumar S, Nilsson RH, Abarenkov K, Xiaoli Z, Kirk P, Kauserud H. 2012. ITS1 versus ITS2 as DNA metabarcodes for fungi. *Accepted in Molecular Ecology Resources*.

II. Blaalid R, Heegaard E, Halvorsen R, Kauserud H. 2012. Community structure of plant root associated fungi at small spatial scales in an alpine ecosystem. *Submitted to MicrobiologyOpen*

III. Blaalid R, Carlsen T, Kumar S, Halvorsen R, Ugland KI, Fontana G, Kauserud H. 2012. Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing. *Molecular Ecology*, **21**, 1897-1908.

IV. Eidesen PB, Blaalid R, Carlsen T, Kauserud H. 2012. Does latitude make a difference? Comparison of primary succession of root associated fungi along chronosequences in arctic versus alpine glacier forelands. (*Manuscript*)

V. Blaalid R, Carlsen T, Eidesen PB, Halvorsen R, Høiland K, Kauserud H. 2012. Biogeography of root associated fungal communities in the Arctic Archipelago Svalbard, Norway. (*Manuscript*).

Introduction

There is little consensus about how many fungal species that exist on earth, but the well-known estimate of 1.5 mill species (Hawksworth, 1991) seems to be a dramatic underestimate (Blackwell, 2011; Bass & Richards, 2011). Fungi are present in all biomes and most ecosystems, here playing important functional roles as saprotrophs, parasites and symbionts. Large proportions of all fungi are associated with plants (Hawksworth, 1991) and have plants as their main carbon source. Plants and fungi have a long history together and have co-evolved for some 450 million years since plants established on land (Smith & Read, 2008). Fungi interact with plants in various ways, having both negative and positive effects on their host plants. However, while there are many assumptions about plant-fungi interactions we have still very little knowledge about how most fungi interact with plants. Mycorrhiza is probably the most prominent interaction between plants and fungi and is a symbiosis of uttermost importance in most terrestrial ecosystems. It is assumed that both partners benefit from this relationship; the fungus by gaining photosynthesis products from their host and the plants by having an increased uptake of nutrients and water (Smith & Read, 2008). However, in an ideal 'plant world' with no nutrient and water limitation, competition pressure, or plant predation, plants would probably not enter into this symbiosis. The fungus on the other hand needs the plant regardless as carbon source. Mycorrhizal symbiosis is divided into different types, based on which plant and fungal partner being involved. Arbuscular mycorrhiza, formed by the monophyletic group Glomeromycota (AM), is found in nearly all plant families (Wang & Qiu, 2006; Krüger *et al.*, 2012). Hyphal fungi, forming vesicles and arbuscles very similar to today's Glomeromycetes, have been found in 400 million years old plant fossils (Remy *et al.*, 1994). Hence, this group may have played an important role during plants establishment on land (Nicolson, 1975; Smith & Read, 2008). Ericoid mycorrhiza (ErM), found among Ericaceous plants, is formed by the ascomycetous group Helotiales. Ectomycorrhiza (ECM) can be formed by diverse phylogenetic groups, including zygomycetes, ascomycetes and, most prominently, basidiomycetes. ECM includes a variety of plant partners across Plantae, usually trees and shrubs. The ECM symbiosis is thought to have arisen simultaneously as the gymnosperm family Pinaceae close to 130 mya (Axelrod, 1986; Trappe, 1987; Smith & Read, 2008). Today the ECM diversity includes over 6000

plant species and probably closer to 20,000 fungal partners. In addition to mycorrhiza, most plants harbor an enormous diversity of fungal endophytes both in above- and belowground parts. Dark septated root endophytes (DSE fungi) have been reported for >600 plant species within all habitats, emphasizing their status as important fungal members in the plant rhizosphere (Jumpponen & Trappe, 1998). Whether DSE fungi enhances host fitness has been debated, and there is seemingly differences between the host responses to these fungi (Jumpponen, 2001). Today, DSE fungi are largely regarded as mycorrhiza-like fungi (Jumpponen, 2001; Newsham *et al.*, 2009; Newsham, 2011) increasing the biomass of their hosts with 52-138% (Newsham, 2011).

Fungi in Alpine and Arctic environments

Alpine and Arctic environments are cold ecosystems with low nutrient availability, unstable conditions and short growth seasons. In most areas, less than ten plant species comprise more than 90% biomass (Chapin & Körner, 1995), and most of them form mutualistic relationships with fungi (Smith & Read, 2008). Many potential ECM or ErM host plants are co-occurring within both arctic and alpine systems (Gardes & Dahlberg, 1996). Still, nearly no host specificity has been observed for either ECM fungi (Gardes & Dahlberg, 1996) or ErM fungi (Walker *et al.*, 2005). A high degree of heterogeneity and richness of ECM fungi within arctic and alpine areas has been observed (Gardes & Dahlberg, 1996; Kjølner *et al.*, 2010; Bjorbækmo *et al.*, 2012) emphasizing their importance for plant survival in alpine and arctic ecosystems (Gardes & Dahlberg, 1996; Haselwandter & Read, 1987; Nara, 2006). AM is rare in high altitudes (Haselwandter & Read, 1982; Haselwandter *et al.*, 1983) and arctic environments (Väre *et al.*, 1992), while ECM and ErM are widespread. Typical ECM forming fungal genera in alpine/arctic environments are *Cortinarius*, *Inocybe*, *Laccaria*, *Tomentella* and *Hebeloma* (Väre *et al.*, 1992; Jumpponen *et al.*, 2002; Bjorbækmo *et al.*, 2010; Geml *et al.*, 2012). Although no fungal genera are shown to be exclusive to alpine and arctic environments, adaptations towards living within these harsh environments have been observed (Tibbett *et al.* 1998,1999).

DSE fungi (defined as anamorphic Helotiales; Smith & Read, 2008) have shown to be highly abundant in both arctic (Väre *et al.*, 1992, Bledsoe *et al.* 1990; Newsham *et al.*, 2009; Bjorbækmo *et al.*, 2010) and alpine (Haselwandter & Read, 1980, 1982; Currah & Van Dyk, 1986; Jumpponen & Trappe, 1998; Schmidt *et al.* 2008) environments.

Although, DSE fungi are common in all biomes, they may fill a specific niche here, due to the general absence of glomeromycetes. Their functional status has been proposed to be pathogenic (Wilcox & Wang, 1987; Stoyke & Currah, 1993) and mutualistic (Haselwandter & Read, 1982; Jumpponen *et al.*, 1998), dependent on host-fungus combinations (Wilcox & Wang, 1987). Based on these observations, DSE fungi might be considered partly mycorrhizal, as host responses to mycorrhizal fungi are considered to be a continuum ranging from parasitism to mutualism (Jumpponen, 2001; Mandyam & Jumpponen, 2005).

Recent studies of fungal communities within arctic and alpine areas have mainly focused on fungal community responses to gradients (Jumpponen *et al.*, 2002; Jumpponen, 2004; Cazares *et al.*, 2005; Nara & Hogetsu, 2004; Nara, 2006; Bjorbækmo *et al.*, 2010) and colonization of fungi in remote areas (Jumpponen *et al.*, 1999; Geml *et al.*, 2012). Given the rising concern of climate change, particularly in arctic and alpine environments, enumerating the fungal diversity present and determining responses of these communities to abiotic change is of urgent importance.

The study system

Bistorta vivipara (Figure 1.) is a perennial herb in the family Polygonaceae common in alpine and arctic environments. This species has an important ecosystem function within these environments, as a common part of the diet of reindeer and ptarmigan (Moss & Parkinson, 1975; Pardoe, 1995). ECM associations on the roots of *B. vivipara* were first described by Hesselmann (1900), and have later been confirmed by numerous studies (Read & Haselwandter, 1981; Lesica & Antibus, 1986; Väre *et al.*, 1992; Eriksen *et al.*, 2002; Massicotte *et al.*, 1998; Mühlmann *et al.*, 2008). The distributional range of *B. vivipara* is circumpolar, and in alpine and arctic environments it is a very common plant community member, here often growing together with plants such as *Salix spp.*, *Dryas octopetala* or *Betula nana*. In an evolutionary time span *B. vivipara* might be relatively young ECM plant compared to e.g. *Salix spp.*, *D. octopetala* and *B. nana*, as other members of Polygonaceae rarely form ECM. Hence, a host switch of the ECM fungi from co-existing ECM forming plants might have happened.

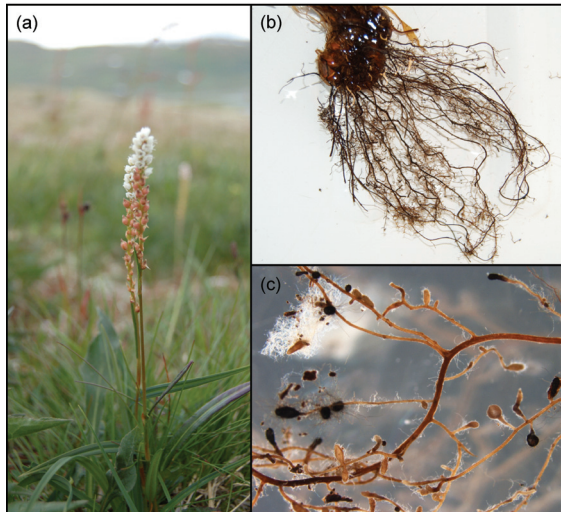


Figure 1. *Bistorta vivipara* and its roots, (a) the above ground part of the plant including the inflorescence (b) washed root system and (c) magnified picture of the roots indicating typical ECM structures.

Spatial distribution of fungi

According to Wolfe *et al.* (2009) and Pickles *et al.* (2009), the importance of spatial scales has largely been overlooked by fungal ecologists. Often spatial scales are defined to be local, landscape, or regional (Wolfe *et al.*, 2009). Different factors govern the richness and structure of fungal communities at various scales. At a local scale, intermediate disturbance through root disruption, nutrient availability and soil properties are important (Bruns, 1995). At landscape scale, substrate availability has been proposed as a driving factor (Burrows & Pflieger 2002; Lilleskov *et al.*, 2004; Wolfe *et al.*, 2009). At regional scales, various climatic drivers might set limits for species occurrences and co-existence (Wollan *et al.*, 2008).

Fungal community responses to gradients (Tedersoo *et al.*, 2003; Green *et al.*, 2006; Toljander *et al.*, 2006) indicate spatial dependency, hence fungal communities are spatially structured and a deterministic model opposed to a stochastic model of fungal distribution is likely. However, numerous studies have indicated that fungi in general are patchily distributed (Horton & Bruns, 2001; Jumpponen *et al.*, 2002; Lilleskov *et al.*, 2004; Stukenbrock & Rosendahl, 2005; Taylor *et al.*, 2010) at both local and landscape scale. Patchiness is usually associated with lack of spatial structure. According to Wolfe *et al.* (2009) spatial patterns are easier to observe at regional scale due to the high degree of heterogeneity and local diversity within local/landscape scales. This heterogeneity potentially blurs out spatial patterns, and fungal communities are therefore often

considered to lack structure at smaller spatial scales (e.g. landscape/local scales). Historically, community ecology studies have ignored spatial scales (Pickles *et al.*, 2009; Wolfe *et al.*, 2009) and as such, inferences of spatial dependency remain largely unresolved, presenting future challenges for fungal ecology.

High throughput sequencing of fungal communities

Molecular tools such as PCR and Sanger sequencing revolutionized fungal research from the early 90s (Horton & Bruns, 2001). During the last 20 years, these techniques have been used in fungal taxonomy as well as fungal ecology, for identification of new species and understanding complex ecological relationships. However, these methods are limiting fungal ecological studies because they are time consuming and expensive, typically capturing only the most abundant taxa, normally leaving the rare ones unaccounted for (Taylor, 2002). This proposes a dilemma, as most fungal communities seem to be composed of a few abundant- and many rare species (Taylor, 2002).

During the last five years, new generations of DNA sequencing techniques, so called high throughput sequencing (HTS), have been developed and are now widely used in fungal ecology research (Buée *et al.*, 2008, Öpik *et al.*, 2009; Jumpponen & Jones, 2009; Tedersoo *et al.*, 2010; Kausserud *et al.*, 2012). The most promising new method is the 454-pyrosequencing techniques, where thousands of DNA sequences are generated in parallel, each from different single DNA molecule templates (Margulies *et al.*, 2005). Sequencing single DNA molecules within a mixture of molecules, allows for the identification of both the dominant taxa as well as rare taxa in environmental samples. The new sequencing techniques now make us able to analyze fungal communities both to greater detail and more extensively. Hundreds of environmental samples can now be processed in parallel and deep sequence coverage can be obtained from each sample. However, the new sequencing techniques have led to new challenges; until recently, the main focus of most researchers was to generate sequence data, now the main challenge is to handle, process and store huge sequence datasets.

Objectives

The overarching aim of this PhD-thesis was to assess the root-associated fungal richness and community structure at different spatial scales using high throughput sequencing (HTS).

More specific aims were to:

- Explore the usability of high throughput sequencing to analyse fungal communities (I-V)
- Evaluate the usability of ITS1 versus ITS2 as DNA metabarcodes for fungi (I)
- Reveal the diversity and spatial structure of root associated fungal communities at different spatial scales in alpine and arctic environments (II-V)
- Investigate the structural differences of fungal communities found within plant roots versus adjacent soil (II)
- Assess the fungal structure and diversity along primary successional gradients in alpine and arctic environments (III, IV)

Results

Paper I. ITS1 versus ITS2 as DNA metabarcodes for fungi

Within paper I, we evaluate the usability of ITS1 versus ITS2 as a DNA metabarcoding marker for fungi. ClustEx revealed that a 97% similarity cut-off represent a reasonable threshold for estimating the number of known species in the datasets for both ITS1 and ITS2, although no single threshold value worked well for all fungi at the same time. We found that the Operational Taxonomic Unit (OTU) concept is not easily translated into the level of species because many species are distributed on several clusters. 454 pyrosequencing data revealed a high similarity between the two datasets when it comes to taxonomic coverage, indicating that ITS1 and ITS2 to a large extent yield similar results when used as DNA metabarcodes for fungi.

Paper II. Fungal community structure at small spatial scales

In paper II we assessed the community structure and spatial distribution of fungi in a 2×2 m plot in an alpine area in Norway, using the ITS2 marker. Parallel analyses were made of fungi associated with *B. vivipara* roots and adjacent soil. Fungal diversity was higher in soil compared to roots, and the fungal community composition differed considerably. Fungi with taxonomic affinity to Sebaciniales and Helotiales were more frequent in plant roots while fungi belonging to Tremellales and Mortierellales were relatively more frequent in soil. Only at a fine scale (< 34 cm) we observed a spatial autocorrelation in the fungal community composition in plant roots. The high degree of patchiness and limited spatial autocorrelation indicates that random colonization events from aerial spores or soil erosion are likely the most important factors structuring the studied alpine fungal communities.

Paper III. Primary succession in alpine areas

In paper III our main objective was to assess the degree of variation in fungal richness and community composition along a primary succession gradient using the ITS1 as marker. Already in the first stage of succession a high fungal diversity was present in the *B. vivipara* root systems and the total fungal richness increased significantly along the gradient towards climax vegetation. The high degree of patchiness in distribution of fungal

OTUs across root systems indicated that stochastic processes to a large extent structure the fungal communities. However, time since deglaciation had impact on the fungal community structure, as a systematic shift in the community composition was observed along the chronosequence.

Paper IV. Primary succession in arctic versus alpine glacier forelands

In paper IV we compared a primary succession gradient in the Arctic (Svalbard) to the one studied in paper III. Both gradients showed high fungal diversity in early successional stages, and high patchiness in distribution of fungal OTUs both within root systems and among deglaciations stages. Time since deglaciation affected the fungal community along both gradients, but the systematic shift in species composition (β -diversity) was stronger at the arctic site. For example, Thelephorales, which was abundant in all stages in the alpine site, was missing from the youngest arctic stage. As in the alpine site, the α -diversity clearly peaked in the old, stabilized vegetation in the arctic site. With time, the composition of root-associated fungal communities in the more marginal arctic habitat seems to resemble the more climatic favorable alpine areas.

Paper V. Biogeography of fungal communities in the Arctic

In paper V we aimed at answering how the ECM fungal community structure and richness of *B. vivipara* vary at a larger biogeographic scale (Svalbard archipelago) using ITS1 as DNA metabarcoding marker. Roots of *B. vivipara* were sampled across Spitsbergen and Edgeøya, totaling 185 samples from 37 localities. Basidiomycetous groups including Agaricales, Thelephorales and Sebaciales were the most dominating groups. The diversity of the Svalbard mycoflora indicates weak patterns of biogeographical distribution along a north/south gradient. Fungal species richness was higher within western parts of Svalbard although species accumulation curves indicated insufficient sampling. We found most of the fungal diversity to be patchily distributed, indicating importance of random colonization events from aerial spores or soil erosion.

Discussion

The included studies within this thesis (I-V) indicate the large potential of HTS techniques for analyses of structure and diversity of fungal communities. However, there are numerous problems associated with the new techniques. These are discussed below followed by a discussion of the biological findings.

Methodological considerations

Sampling

In Fig. 2, an overview is provided of the localities where *B. vivipara* root systems and soil samples were collected. Three of the studies were conducted in alpine environments at Finse, central Norway (I, II and III), one study at the Arctic Archipelago Svalbard (V), and one study is comparing data from Finse and one locality (Ny-Ålesund) at Svalbard (IV). In all studies entire root systems of *B. vivipara* were collected. The small, condensed root system (Fig. 2) allows us to study the entire fungal community associated with each plant (Kausrud *et al.*, 2012) opposed to only parts of the roots systems (Tedersoo *et al.*, 2003; Tedersoo *et al.*, 2006; Tedersoo *et al.*, 2009). In this way, sampling bias may to a higher extent be avoided. Sampling strategies do affect how we perceive root associated fungal communities (Taylor, 2002), and in root tip based studies there is seemingly a close relationship between numbers of sampled root tips and observed species (Taylor, 2002).

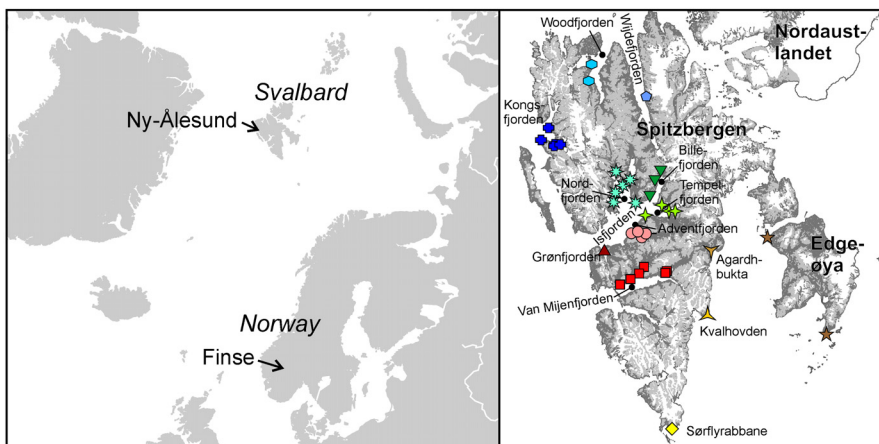


Figure 2. Locality map, indicating the sampling areas for studies I-III, IV and V. In total 410 plant roots and 64 soil samples were included within this thesis.

Replication is a fundamental issue in ecological studies but largely overlooked by most microbiologists (Prosser, 2010). In the studies included in this thesis a high number of samples and replicates are generally included, enabling us to perform rigorously statistical tests for compositional changes and diversity trends.

Extracting DNA

In all studies, DNA from plant roots was isolated using CTAB extraction (Murray & Thompson, 1980; Gardes & Bruns, 1993), while DNA from soil samples was extracted using a MO BIO soil isolation kit (**I-II**). Extraction method is known to affect the detected fungal community profile (Tedersoo *et al.*, 2010). Studies of bacterial communities have conflicting conclusions about the suitability of the CTAB method (Leuko *et al.*, 2008; Mitchell & Takacs-Vesbach, 2008). Because of the content of silt and clay within the soil samples, a specific soil isolation kit was used for DNA extraction of the soil samples (**I-II**). Based on current knowledge I would argue that the CTAB method is appropriate to use for extracting fungal DNA from plant root systems.

PCR primers and barcode region

The ITS marker is an obvious choice for the analyses of fungal diversity and communities, as it has functioned as the ‘unofficial DNA barcode region’ for fungi for almost 20 years (Seifert, 2009; Bellemain *et al.*, 2010) and is now accepted as the official barcode of fungi (Schoch *et al.*, 2012). Because of its popularity as a marker in phylogenetics at low taxonomic level, a high number of reference sequences are available in the ISDN databases, as well as in more focused DNA barcoding databases such as UNITE (Abarenkov *et al.*, 2010). The ITS region consists of three subparts; ITS1, the conserved 5.8S and ITS2, and in this thesis, both ITS1 (**I+III-V**) and ITS2 (**I-II**) have been used as DNA metabarcoding markers. Because most of the current HTS methods can only generate short (<500 bp) reads, i.e. not covering the entire ITS region, decisions whether ITS1 or ITS2 is more suitable as DNA metabarcoding marker is important. A recent study by Mello *et al.* (2012) found taxonomical differences in the fungal community when analyzing ITS1 and ITS2 in parallel. These observed discrepancies may be attributed to difference in variability within the two sub regions, ITS1 being in general more variable

compared to ITS2 (Nilsson *et al.*, 2008). There are also more ITS2 sequences available for comparison in the ISDN databases (Nilsson *et al.*, 2008), which may make taxonomical inference easier for the ITS2 region compared to the ITS1 region. Also, the ITS1 primers have shown to be biased towards basidiomycetes whereas ITS2 primers were biased toward ascomycetes (Bellemain *et al.*, 2010).

We demonstrate that choice of region between ITS1 and ITS2 doesn't necessarily affect conclusions in community level ecology as the observed differences between the markers were relatively small, and similar structural patterns were obtained across markers (I).

PCR errors and conditions

PCR errors are known to occur due to non-perfect polymerase activity and switching of templates during polymerase activity within mixed templates, the latter known as chimeric sequences (Wintzingerode *et al.*, 1997). These chimeras are largely associated with conserved regions, which we have avoided by either working with ITS1 or ITS2, not including the conserved 5.8S region across all studies (I-V). Gonzales *et al.* (2012) found that low abundant taxa are under-represented due to PCR biases. In fact, compositional changes within mixed templates during PCR have been proved in several studies (Amend *et al.*, 2010; Avis *et al.*, 2010; Engelbrekton *et al.*, 2010). We have refrained from using read abundance data throughout all studies (I-V) to account for potential PCR introduced biases.

Sequencing errors

DNA fragments may switch tags during laboratory setup (van Orsouw *et al.*, 2007; Carlsen *et al.*, 2012), most likely prior to the emulsion PCR. If not controlled for, this will cause numerous false positives in downstream analyses. We have tagged the amplicons in both ends before pyrosequencing, to control for potential tag switching across all studies (I-V). These tag switches presents themselves as low frequency OTUs within other samples. To account for these switches, low frequency OTUs (< 5 reads) has been omitted from downstream analyses per individual sample in some studies (I, II, V).

Although the 454-methods have improved regarding error rates (Huse *et al.*, 2007), low quality reads are still present, some of them probably due to sequenced PCR errors (Balzer *et al.*, 2011). However, miscounted homopolymers (Kunin *et al.*, 2010) during the

454-sequencing step have been shown to overestimate diversity. We have accounted for this by collapsing all homopolymers >6 across our studies (I-V). To avoid including potential PCR errors and sequencing errors as true diversity, low quality reads have been removed from further downstream analyses (I-V) as recommended by Huse *et al.* (2007), Quince *et al.* (2009) and Tedersoo *et al.* (2010).

Clustering and the OTU concept

Clustering of sequences into Operational taxonomic units (OTUs) as crude approximation of species proposes many challenges. The terms OTU has been widely adopted and is a preferred entity measure when working with molecular ecology of fungi because an OTU is merely a sequence similarity based surrogate for a species (Sharpton *et al.*, 2011). But how well do the OTUs represent true species? Low sequence coverage will yield more OTUs, and a threshold of 50% sequence coverage is observed as a minimum requirement to avoid this effect (Kumar *et al.*, 2011). Required sequence similarity is much more debated. The “3% golden rule” of sequence dissimilarity to demarcate distinct species has been used in many studies (O’Brien *et al.* 2005; Morris *et al.* 2008; Ryberg *et al.* 2008; Walker *et al.* 2008; Bjorbækmo *et al.* 2010; Tedersoo *et al.* 2010) although plenty of data show that many lineages of the fungal kingdom differ in terms of distance among species (Nilsson *et al.* 2008; Gazis *et al.* 2011). Choice of clustering algorithm and overly stringent parameter choice may influence the number of OTUs (Huse *et al.*, 2010; White *et al.*, 2010). In study I, we observed that species (i.e. Latin binomials in the reference sequence datasets analyzed) often were split into several clusters and that many clusters included multiple species. New clustering algorithms, not primarily based on sequence similarity, may offer a solution to avoid multispecies clusters (Pommier *et al.* 2009; Zinger *et al.* 2009; Powell *et al.* 2011).

Taxonomy

Only a small fraction of the fungal diversity is present in ISDN databases, meaning that only a small fraction of the detected OTUs within HTS studies can be matched to annotated sequences (Nilsson *et al.*, 2009; Hibbett *et al.*, 2009). The approximate number of newly described taxa per year (1223; Hibbett *et al.*, 2009) are in contrast to the millions of sequences generated by HTS methods, indicating that a solution to this problem does not lie within near future. Ecologists not taxonomists are therefore currently discovering

new fungal taxa, however, the term OTU is far from satisfactory to work with, as it is difficult to make ecological inferences. To add insult to injury to this taxonomical conundrum, many errors are present in sequence reference databases, especially in the uncurated INSD databases (e.g. GenBank; Nilsson *et al.*, 2006).

The OTU concept is based on sequence similarity within a cluster of sequences. But are clusters true representative of species? Clustering sequences with known taxonomic affiliation have indicated that the sequence rich clusters often contains several biological species (**I**). In addition to this, studies have indicated that within many fungal lineages, the used marker (ITS) is often not able to separate between closely related taxa and closely related species can have identical ITS sequences (Nilsson *et al* 2008). This advocates for multi-locus approaches when doing HTS studies (Vrålstad, 2011).

Fungal communities associated with *Bistorta vivipara*

Taxonomy of the root-associated fungal communities

Within all studies (**I-V**) we detected many typical ECM forming taxa such as Agaricales, Thelephorales and Sebaciales. Our consistent findings of Sebaciales and Thelephorales as frequent members of the community (**I-V**) are in contrast with sporocarp studies (Väre *et al.*, 1992; Gardes & Dahlberg 1996; Gulden & Torkelsen, 1996). However, members within these orders have small and inconspicuous fruit bodies, which would explain their previous absence. However, Sebaciales and Thelephorales are frequently detected in studies using molecular tools for taxon identification, especially in arctic and alpine environments (Bjorbækmo *et al.*, 2010, Geml *et al.*, 2012).

Typical dark septated endophytes (DSE) fungi like *Phialocephala* and *Rhizoscyphus* were observed within all of our studies (**I-V**). Many of these OTUs are usually assigned to a low-level taxonomy (order level), which makes it difficult to separate true DSE fungi from other Helotialen groups within our studies (**I-V**). It is known that DSE fungi are important within arctic and alpine environments (Jumpponen & Trappe, 1998; Newsham *et al.*, 2009), and they are found widely associated with plants within these areas (Read and Haselwandter, 1981; Väre *et al.*, 1992; Schmidt *et al.*, 2008; Bjorbækmo *et al.*, 2010). Many of the OTUs within our studies (**I-V**) show affinity to Helotiales, although these OTUs are in many cases only assigned approximately at an order level. Several of these Helotialen fungi may very well represent undescribed DSE fungi, as well as ECM fungi, however more studies are needed to confirm this.

The composition of the root associated fungal communities was similar at larger spatial scales (Fig. 3; **III-V**) indicating that the 454-pyrosequencing method is able to capture the representative taxonomic composition across sites. The large fraction of unidentified ascomycetes across all studies emphasizes the importance of further taxonomic work of these groups, including addition of more reference sequences. Unfortunately, the field of taxonomy has over the last three decades been marginalized. In the new HTS era we are totally dependent of good reference sequence data that only trained taxonomist ultimately can provide for most fungal groups.

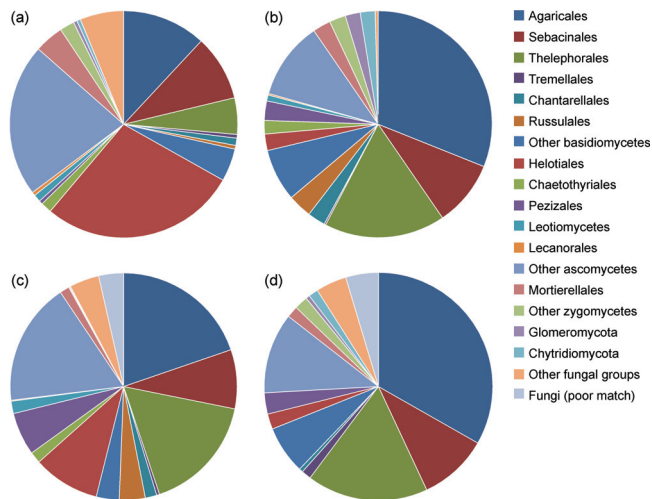


Figure 3. Taxonomic composition within *Bistorta vivipara* roots from (a) the local community study (**II**), (b) alpine primary succession (**III, IV**), (c) arctic biogeography study (**V**) (d) arctic primary succession (**IV**).

Fungal richness

Across all studies the fungal species richness was high (**I-V**) (Fig. 4). Not surprisingly, the fungal richness at local scale (**II**) was clearly lower compared to the richness found at a biogeographical scale (**V**) regardless of number of samples, verifying the species-area hypothesis (Arrhenius, 1921). Habitat heterogeneity plays an important role when it comes to fungal species richness (Bruns, 1995). All the large-scale studies (**III-V**) have close to linear accumulation curves, indicating insufficient sampling. However, the high heterogeneity of the fungal community may be the reason for why a horizontal asymptote will never be reached. If the fungal species are distributed mainly due to stochastic events, that patchy structure is merely a reflection of the fungal community structure. Larger areas share, in general, less species compared to smaller areas. In spite of this, a low degree of spatial autocorrelation was detected even at small spatial scales (**II**).

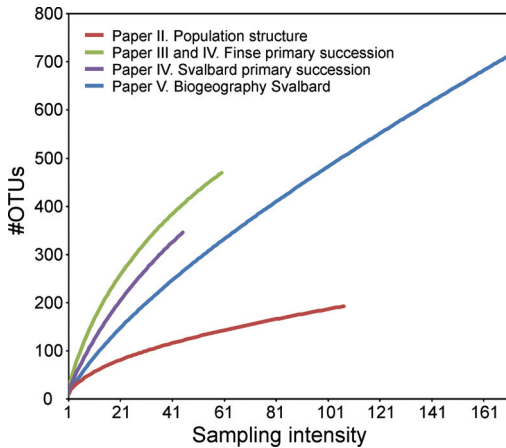


Figure 4. OTU accumulation curves from the local community (II), alpine primary succession (III, IV), arctic primary succession (IV) and arctic biogeography (V).

Spatial structure of the fungal communities

Species responses to gradients are a central point in all studies dealing with community ecology. Many factors are thought to govern these communities, such as dispersal ability, habitat remoteness, ability to colonize, competition, and predation. Differences between fungal communities found in plant roots and adjacent soil samples indicated that the fungal communities are not only randomly distributed, but habitat specific. We also found structural trends along gradients (III, IV, V) at landscape and regional scale, and along primary succession gradients, favoring a deterministic distribution model, at least for some members within the fungal community.

However, in all studies (II-V), most of the fungi was patchily distributed, indication lack of spatial responses. This means that a stochastic distribution model governs large fractions of the fungal community. We cannot exclude the possibility that we are operating on too coarse scales for detection the spatial dependencies and fungal responses to gradients.

The common denominator across all studies (II-V) is that even though structure was observed, the heterogeneity of the data is very large and a high degree of patchiness was observed even on small scales (II). Many fungal species must be considered rare, thus it is impossible to infer spatial patterns of OTUs occurring in single root systems (I-V). Explanations for these observations may be that most fungi rather co-exists rather that out-

compete each other due to the niche partition theory (Bruns, 1995). According to this theory, we will observe (1) high fungal richness, (observed across **II-V**) and (2) low degree of structure (observed across **II-V**).

The take home message from the studies in this thesis is that there are some widespread and common fungi that respond in a predictable way to known ecological gradients. But the majority of fungi are rare and patchily distributed. Perhaps by looking for structure in our datasets biologists are overlooking the main pattern governing the distribution of most fungi. Which is stochasticity.

Concluding Remarks

Although the 454-pyrosequencing technique has proved to be a very powerful approach, there is still considerable room for improvement of the methodology. In addition to methodological challenges, accurate taxonomic assignment is difficult. In many studies using HTS technology, many of the sequences generated do not have good blast matches to any species within reference databases, and therefore are annotated at a higher taxonomic level than what gives ecological information. Throughout all studies included in this thesis, the term OTU has been applied and annotating these with unambiguous species names has been impossible due to the low number of taxonomic reference sequences. How informative are the number of OTUs, and “What’s in a name”? I would argue a lot lies within species names! We cannot disregard the importance of naming an organism for further ecological inferences; hence more taxonomical studies across all fungal groups are crucial.

Future perspectives

The use of pyrosequencing techniques imposes two major challenges. The first is directly connected to the method itself, the comparison of fungal communities across studies. The inconsistency in data treatment, and different preferential molecular marker among mycological “schools”, makes large-scale inferences of fungal communities difficult. The other issue is the lack of taxonomical resolution of the fungal biota. This proposes challenges in accurately characterize the fungal communities found by the 454-method. The need for taxonomic becomes even more prevalent due to this new methods.

Within the near future even more cost efficient methods such as nanopore sequencing may further revolutionize mycological research. The new (third generation) sequencing technologies have the potential to dramatically improve molecular ecology. In the future, the introduction of these techniques will mean that sequencing will not be the limiting factor in studies. However, the main challenges of these new methods will probably be in downstream analyses, including bioinformatics and more importantly – biostatistics.

There is a bright future for fungal ecology!

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A handwritten signature in blue ink, reading "Rakel Blaaid", is centered on a light gray rectangular background.

Rakel Blaaid

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