Foliar Fungi of Scots Pine (*Pinus sylvestris*)

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Abstract

Scots pine (*Pinus sylvestris*) is an ecologically and economically important tree species in Fennoscandia. Scots pine needles host a variety of fungi, some with the potential to profoundly influence their host. These fungi can have beneficial or detrimental effects with important implications for both forest health and primary production. In this thesis, the foliar fungi of Scots pine needles were investigated with the aim of exploring spatial and temporal patterns, and development with needle age and health status.

Using 454 sequencing, diverse fungal communities were detected from Scots pine needles sampled along latitudinal and altitudinal gradients in Sweden and from forests along the Lithuanian coast. Latitude and altitude, as well as the forest edge, were found to influence the fungal community. Needle age and needle heath status affected the fungal community, while OTU (operational taxonomic unit) richness increased with needle age and in symptomatic needles. In addition, Dothistroma needle blight (DNB) was examined under Nordic conditions on seedlings of Scots pine and Lodgepole pine (*Pinus contorta*). The disease development of DNB was found to be comparable to what has been reported in studies from Europe and New Zealand.

Findings in this thesis show that the fungal community associated with Scots pine needles is species rich, but the majority of these fungi have unknown ecological roles. Our results contribute to the understanding of spatial variability and dynamics of these fungi, which is important for better understanding of their potential effect on ecosystem processes.

Keywords: Scots pine, needles, endophyte, pathogen, fungal community, next generation sequencing, altitude, latitude, edge effects, *Dothistroma*, *Lophodermium*.

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Dedication

Till mamma och pappa

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I Millberg H., Boberg J., Stenlid J. (2015). Changes in fungal community of Scots pine (*Pinus sylvestris*) needles along a latitudinal gradient in Sweden. *Fungal Ecology* 17, 126-139.

II Millberg H., Boberg J., Bakys R., Stenld J. Edge effects on fungi of Scots pine (*Pinus sylvestris*) needles at the Lithuanian coast. (*manuscript*)

III Millberg H., Boberg J., Gadjieva R., Stenlid J. Influence of altitude and needle age on foliar fungal communities associated with Scots pine (*Pinus sylvestris*). (*manuscript*)

IV Millberg H., Anna JM Hopkins AJM., Boberg J., Davydenko K., Stenlid J. Disease development of Dothistroma needle blight in seedlings of *Pinus sylvestris* and *Pinus contorta* under Nordic conditions. *Forest Pathology.* (*In press*)

The contribution of Hanna Millberg to the papers included in this thesis was as follows:

I Planned the study together with supervisors. Collected samples and performed the laboratory work. Analysed the data and wrote the manuscript with co-authors.

II Performed the laboratory work. Analysed the data and wrote the manuscript together with co-authors.

III Planned the study together with supervisors, responsible for collection of samples. Analysed the data and wrote the manuscript in collaboration with co-authors.

IV Took part in planning of the study, collection of samples and laboratory work. Analysed the data and wrote the manuscript in collaboration with co-authors.

Abbreviations

DCA	Detrended correspondence analysis
DNA	Deoxyribonucleic acid
DNB	Dothistroma needle blight
ITS	Internal transcribed spacer of rDNA
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction

1 Background

1.1 Scots pine

Pinus is an important conifer genus in the northern hemisphere that is found from the subalpine zone across the boreal, temperate and subtropical zones (Richardson, 1998). Scots pine (*Pinus sylvestris*) has the largest natural distribution of all pine species, covering northern and central Europe and reaching far into eastern Siberia. Tree species diversity in the Fennoscandian boreal forests is low and the dominating tree species, both economically and ecologically, are Scots pine and Norway spruce (*Picea abies*). Both species have broad habitat preferences, although Scots pine predominates on drier sites (Esseen *et al.*, 1997). In Sweden, almost 50 % (23 million hectares) of the land area is covered with forest and Scots pine constitutes about 39 % of the standing forest volume (Statistical Yearbook of Forestry, 2014). Forestry is one of the most important industries in Sweden and Scots pine trees are used for saw timber and pulpwood. In the 2013/2014 felling season Scots pine represented about 32 % of the annual harvest (total 85 million m^3) from Swedish forests (Nilsson *et al.*, 2015).

1.1.1 Scots pine needles

Unlike deciduous trees and some conifers such as *Larix* spp., pines are evergreen trees with needles that are retained for several years. Every summer a new needle cohort develops on the tree and in the autumn the older needles are shed. The number of needles and the length of the shoot produced each year is determined by the conditions of the previous year when the terminal buds were formed (Salminen & Jalkanen, 2005; Lanner, 1985). On average, one needle cohort is shed every year, causing the tree to have a more or less constant number of needle cohorts over time (Jalkanen, 1998). Senescence is the process that leads to needle death, and it is an internally regulated process

involving the disintegration of the structures of the cells, the transportation of mobile nutrients from the yellowing needle back to the tree (Noodén, 1988) thereafter an abscission layer is formed and the needles is shed. Needle senescence in Scots pine is characterized by the yellowing of the needles (Kivimaeenpaeae & Sutinen, 2007).

Depending on the pine species and environmental conditions, needles are typically retained for between two and eight years. However, some species retain needles for even longer (Richardson, 1998). Scots pine needles are normally retained for between two and five years and needle retention time is usually shorter at more productive sites. Needle longevity increases with increasing altitude and latitude, and is largely a phenotypic response. Individuals of the same species that were planted together, but which were of different latitudinal or altitudinal origin have been shown to exhibit similar needle retention time, although there were also individual variations (Reich *et al.*, 1996). Needle life-span is shorter at more nutrient-rich sites. There are also variations within the crown, where needle life-span is shorter in sunny positions (Reich *et al.*, 1995). At higher altitudes and latitudes, longer needle life-span has also been suggested to be a nutrient conservation mechanism, giving the trees competitive advantages in cold habitats (Oleksyn *et al.*, 2003). The photosynthetic capacity decreases with needle age. In Scots pine, net photosynthetic capacity is highest in one-year-old needles as well as in current year needles in late summer (Aagren *et al.*, 1980). Consequently, the younger needle cohorts are the main contributors to tree growth (Drenkhan *et al.*, 2006).

1.2 Foliar fungi

The foliage of forest trees hosts a diverse fungal community representing a variety of ecological functions and lifestyles. Foliar pathogens infect and cause disease that is limited to the needles or leaves, but can, at high infection levels, cause growth reductions as a consequence of reduced photosynthetic capacity (Hanso & Drenkhan, 2012; Manter *et al.*, 2003; Van der Pas, 1981). Only in severe cases will foliar pathogens cause tree mortality. By weakening the tree, foliar diseases can contribute to higher susceptibility to biotic and abiotic stresses (Bednářová *et al.*, 2013; Kowalski, 2013). Based on trophic interaction with the host, different types of pathogens can be distinguished; for example necrotrophic fungi live on dead cells whereas biotrophic fungi derive carbon and nutrients directly from living cells (Deacon, 1997).

In conifers, where the needles are retained for several years, the severity of a needle disease is determined by which needle cohort is affected. In Scots pine, where the youngest needle cohorts are the main contributors to tree

growth, disease in the younger needles (last year's needles in spring and summer and the current year's needles in late summer and autumn) will have a larger impact on the tree than disease in the older needle cohorts (Drenkhan *et al.*, 2006; Aagren *et al.*, 1980). At higher latitudes where the shorter vegetation period will force the trees to flush later in the season, the loss of last year's needles could potentially have a larger impact on tree growth. At more southern latitudes where the needles flush earlier, and there is a longer vegetation period, a larger proportion of the year's sunlight will be absorbed by the current year's needles and loss of last year's needles might be less severe.

Apparently healthy foliage also hosts a diverse fungal community, consisting of species that are not necessarily pathogenic fungi. Fungi that colonize the internal tissue of plants without causing any visible symptoms are referred to as endophytes. Used in a broader sense, as defined by De Bary in the 1880s, the term endophyte refers to an organism colonizing internal plant tissue (Petrini, 1991). However, here an endophyte is considered to be an organism that asymptomatically colonizes the internal tissue of a plant, at least for a part of its life cycle (Petrini, 1991). Thus, the term endophyte rather describes the momentary nature of interaction with the host (Schulz & Boyle, 2005).

Foliar fungal endophytes comprise fungi with varying ecological roles, representing a continuum of host interactions that range from mutualistic to pathogenic (Rodriguez *et al.*, 2009; Arnold, 2007; Sieber, 2007; Schulz & Boyle, 2005). Some endophytes are saprotrophs that are present in healthy needles as small colonies awaiting tissue ageing or senescence (Deckert *et al.*, 2001; Stone, 1987). In needles of Norway spruce, Rajala *et al.* (2014) found that one third of the endophytes detected in samples from healthy needles were also detected in samples from decomposing needles. Pathogenic fungi can have an endophytic (latent) phase and other endophytes may be weak pathogens. For some fungi, host conditions and environmental factors can cause the interaction with the host to shift from neutral to pathogenic (Sieber, 2007). However, the ecological role of many endophytes is still unknown or poorly defined.

Fungi present on the surface of needles and leaves are referred to as epiphytes. Although occupying habitats in close proximity, epiphytes and endophytes have been observed to represent predominantly different assemblages of fungi (Zambell & White, 2014; Santamaria & Bayman, 2005; Legault *et al.*, 1989a). Legault *et al.* (1989a) noted that whereas epiphytes typically are generalist saprotrophs that can be recovered from the surface of many different host species, endophytes are typically host specific (Legault *et al.*, 1989b).

1.2.1 Some examples of fungi in Scots pine needles

Lophodermium is a well-known fungal genus from pine needles and at least three members of this genus are found in needles of Scots pine in Fennoscandia: *L. seditiosum*, *L. pinastri* and *L. conigenum* (there are possibly more species present, see Paper I). Of these three, only *L. seditiosum* is pathogenic, causing Lophodermium needle cast, a serious needle disease in southern and central Sweden (Stenström & Ihrmark, 2005; Minter & Millar, 1980; Minter *et al.*, 1978). The fungus infects the current year's needles in the summer and autumn. The following spring and early summer, these needles turn red-brown and are shed prematurely (Fig 1) (Diwani & Millar, 1990). The disease is limited to the needles. During the summer, new shoots and needles will emerge; however, the loss of last year's needles may reduce the tree's photosynthetic capacity. The disease is a serious problem for seedlings and young plants. However, the vulnerability to the disease decreases as the trees age (Hanso & Drenkhan, 2012; Stenström & Ihrmark, 2005). This could be a result of an increased resistance as the trees grow older, or an increased distance from the canopy to the forest floor, where the spores are produced on needle litter. The conditions at the forest floor are also more humid, which would favour fungal infections on seedlings and young plants.

Figure 1. Lophodermium needle cast on a small seedling in early summer, where last year's needles are dead. The shoot and current year needles are not affected by the disease and have started to elongate. (photo: H. Millberg)

Unlike the pathogenic *L. seditiosum*, both *L. pinastri* and *L. conigenum* are non-pathogenic fungi that live endophytically within pine needles, but with somewhat different ecological strategies. Fruit bodies of *L. pinastri* are found on needles that have died after the process of senescence, whereas fruit bodies of *L. conigenum* are predominantly found on healthy needles that have died prematurely: for example as a consequence of snow break or brashing (Minter & Millar, 1980). Although our knowledge of the biology of *L. conigenum* still limited, it has been shown that *L. pinastri* has the ability to decompose both cellulose and lignin (Boberg *et al.*, 2011; Osono & Hirose, 2011). Recent investigations have found at least three cryptic species within *L. pinastri* in Scotland, possibly with distinct ecological behaviour (Reignoux *et al.*, 2014). Knowledge about the distribution and habitat preference of these cryptic species outside Scotland is limited.

Dothistroma septosporum is a serious needle disease, causing Dothistroma needle blight (DNB) on a wide range of pine species (Watt *et al.*, 2009). In the southern hemisphere it is a major pathogen in exotic pine plantations. Since the 1990s, the severity and prevalence of the disease has increased in the northern hemisphere, causing severe outbreaks in both North America and Europe (Fabre *et al.*, 2012; Brown & Webber, 2008; Woods *et al.*, 2005). Recently, the presence of *D. septosporum* on Scots pine has been reported in the Nordic countries (Solheim & Vuorinen, 2011; Drenkhan & Hanso, 2009; Müller *et al.*, 2009). In Sweden, *D. septosporum* was discovered for the first time in 2007 on needles of Scots pine in Fagersta, in central Sweden (Stenlid, unpublished). Since then, the disease has been observed at a few sites in the same area. In this geographic area, symptoms are predominantly visible in the spring and early summer on two-year-old needles (Fig 2). To date, little is known about the disease cycle under Nordic conditions.

Differences in weather conditions have been linked to variations in the infection rate and severity of the disease between years. It is possible that the cold climate in the Nordic countries could be limiting for DNB. Modelling the possible geographic range of DNB based on its climatic requirements suggests that the climate in the Nordic countries, except for the northernmost part, is suitable, but not optimal, for the disease (Watt *et al.*, 2009). Predictions about climate change suggests that mean temperatures and precipitation could increase in the Nordic countries and, hence, the environment would become more favourable for the disease and the area suitable for the disease would expand northwards (Watt *et al.*, 2011). It is thus important to learn more about the disease under Nordic conditions.

Figure 2. Dothistroma needle blight on Scots pine in spring, before flushing. Symptoms appear on two-year-old needles. (photo: H. Millberg)

In Sweden, there are two commercially grown pine species that are susceptible to DNB, Scots pine and Lodgepole pine (*Pinus contorta*). Lodgepole pine is the most extensively planted exotic tree species in Sweden, and it is primarily grown in the northern parts of the country. At the beginning of the 21st century the area of Lodgepole pine plantations was about 600 000 ha (Elfving *et al.*, 2001). Lodgepole pine has been reported to be highly susceptible to DNB, whereas Scots pine has been reported to be both highly susceptible and slightly susceptible (Watt *et al.*, 2009). However, the impact of the disease on these species under Nordic conditions has not been evaluated.

1.3 Factors affecting abundance and distribution of needle fungi

Within a singe host species, environmental factors, host factors and fungal interactions can influence the abundance and distribution of fungi associated with needles. Weather factors, such as temperature and precipitation, have been shown to affect important steps in the lifecycle, such as spore germination, infection, fruit body formation and spore release, of most, if not all, fungi (Boateng & Lewis, 2015; Stone *et al.*, 2008; Osorio & Stephan, 1991; Gadgil, 1974). Climatic factors vary over large spatial scales, such as across countries and continents, and can consequently influence the geographic distribution of different fungal species. However, there are also other factors with potential influence on fungi that changes over larger areas, such as airborne pollution deposition rates, nutrient availability, length of growing season and land use history (Larkin *et al.*, 2012; Helander, 1995; Sieber-Canavesi & Sieber, 1987). At smaller spatial scales, between and within site variation can be attributed to

variations in microclimate, forest structure, host density, canopy cover, and surrounding vegetation (Saikkonen, 2007; Helander *et al.*, 2006; Helander *et al.*, 1994; Legault *et al.*, 1989b). Forestry practices, for example forest regeneration method after final harvest have been shown to influence foliar endophyte community composition where the transmission of endophytic fungi from the surrounding trees may be limited by clear cutting and replantation (Johnston, 1998). Within a single tree, variations in microclimate and light availability in the crown can affect the distribution of different fungal species (Saikkonen, 2007).

Foliar fungi are typically transmitted horizontally (Arnold & Herre, 2003; Diwani & Millar, 1990), that is, they infect needles or leaves with spores dispersed from other individuals and are not transmitted systemically and passed through somatically (vertically) to new plant individuals (Yan *et al.*, 2015). Hata *et al.* (1998) found that in needles of *Pinus thunbergii* and *Pinus densoflora*, sampled at the time when they started to flush, contained practically no endophytes.

Needle age may influence the abundance and distribution of needle associated fungi. This could be attributed to both longer time of exposure to fungal infections and colonization (Arnold & Herre, 2003) and to changes in the physiology of the needle with age (Hata *et al.*, 1998). Species richness of both endophytic and epiphytic fungi has been observed to increase with tissue age (Espinosa-Garcia & Langenheim, 1990; Legault *et al.*, 1989a) however, when comparing endophyte richness in leaves of *Sequoia sempervirens*, Espinosa-Garcia and Langenheim (1990) found that in the oldest leaves, endophyte richness decreased. Host defence against fungal infections, whether being pathogenic or endophytic, may also influence fungal communities. In the foliage, this host defence becomes weaker with age (Bell, 1980; Stavely & Slana, 1971). Physical protection i.e. wax layer on the needle surface are also degraded as needles age, these waxes starts to form as the needles elongate (Jalkanen *et al.*, 1981) and very young needles are also likely to be more susceptible to fungal infections before these are fully developed. During the maturity of leaves of *Populus* sp., Coleman (1986) identified the time of transition from sink to source (when leaves approach full expansion) to a stage of high susceptibility to abiotic and biotic stress, related to rapid anatomical, physiological and biochemical changes in the leaves. Leaves at this stage had the highest susceptibility to biotrophic leaf pathogens, but there was no clear relationship with necrotrophic pathogens. Arnold and Herre (2003) noted that the time of exposure influenced the endophyte colonization of leaves of *Theobroma cacao*, however, leaves of different age were equally susceptible to

endophyte infection and there appears to be no effect of prior endophyte infection for the probability of subsequent infections.

Stressful conditions to the tree may also influence the fungal community of needles and the effect of host stress may act differently on different groups of fungi. For example, drought stress can limit the resources available for tree defence, favouring necrotrophic pathogens by facilitating their access to carbon and nutrients. Biotrophic pathogens, on the other hand, are likely to be negatively affected by stressful conditions to the host since stress limits the resources available to the biotrophic interaction (Oliva *et al.*, 2014). Stress can also accelerate the onset of senescence (Reich *et al.*, 1995), something that could favour saprotrophic fungi.

Needle disease or damage to the needles may also influence the fungal communities of needles. It is plausible that diseased needles would provide additional habitats for fungal species compared with healthy ones (Marcais *et al.*, 2011). At the same time as parts of the needle remain healthy, diseased needles would include areas of dead tissue or areas where host defence is weakened, which could be colonized by fungi that are unable to colonize healthy tissue (Ragazzi *et al.*, 2003).

2 Aim

Fungi associated with Scots pine needles are an overlooked and sometimes even hidden component of biodiversity and represent various lifestyles and functions. Some of these fungi can profoundly influence their host, having beneficial or detrimental effects with important implications for both forest health and primary production. Although fungi associated with Scots pine needles have been the subject of several investigations, little is known about the spatial patterns and dynamics of this group of fungi, and the factors involved in shaping these communities. Recent methodological development provides an opportunity for a more comprehensive description of these fungal communities. In this thesis, fungi associated with Scots pine needles are investigated with the aim of exploring spatial and temporal patterns, and development with needle age and health status.

The specific objectives were:

I To investigate the identity and diversity of fungi associated with needles of Scots pine (Papers I, II and III)

II To examine patterns in community composition and the diversity of fungi associated with Scots pine needles at different spatial scales, and their possible relation to environmental factors (Papers I, II and III)

III To determine how the fungal communities of Scots pine needles develop with needle age (Papers I and III) and needle health (Papers I, II and III)

IV To study the disease development of Dothistroma needle blight on two pine species under Nordic conditions (Paper IV)

3 Project descriptions

3.1 Paper I: Latitudinal gradient through Sweden

In Paper I, the aim was to explore the fungal community of Scots pine needles along a latitudinal gradient through Sweden, from the middle subzone of the boreal zone in the north to the temperate zone in the south. We also aimed to investigate variations in the fungal community with needle health status and needle age, and the possible effects of the method used to regenerate the forest.

Needle samples from Scots pine were collected in the summer of 2008 from ten sites across Sweden, ranging from Skåne in the south (55.9°N) to Överkalix in the north (66.5°N) (Fig 3). At each site, two forest stands were chosen for sampling. The sampled forests ranged in age between 10 and 20 years. There were eight naturally regenerated forests and 12 planted forests. One-year- and two-year-old needles (i.e. needles that flushed in the summer of 2007 and 2006) were collected as separate categories, as were visibly healthy needles and needles with symptom of disease, resulting in four sampling categories. In each forest stand, the needles from each sampling category were collected from each of ten trees. From each forest, all the needles of the same sampling category, for example one-year old healthy needles, were pooled together to form one sample, resulting in four samples per forest. Each needle sample was freeze-dried and homogenized, and DNA was extracted from a small subsample of the homogenized material. The fungal community was described using 454 sequencing (further described in section 3.5).

3.2 Paper II: Edge effects

In Paper II, the fungal community of Scots pine needles collected in Palanga on the Lithuanian coast were studied. The fungal community of needles from the forest edge, adjacent to the Baltic Sea, was compared with that of needles

in the forest interior, with the aim of investigating possible edge effects on the fungal community.

Samples were collected from Scots pine forests situated adjacent to the Baltic Sea. Needles were collected from trees at the forest edge facing the shore, and from trees growing 80 m and 150 m trees further into the forest. Needle samples were collected in the autumn and only current year needles were collected. Ten healthy and ten symptomatic needles were collected from each tree and pooled to form two samples per tree. The fungal community was described using 454 sequencing (described in section 3.5).

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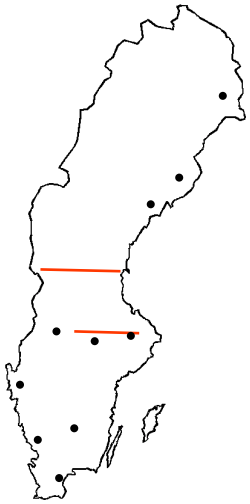

Figure 3. Map of Sweden with sampling sites in Paper I represented by black circles, and the two altitudinal gradients in Paper III are represented by red lines.

3.3 Paper III: Altitudinal gradients

In Paper III, variations in the fungal community of Scots pine needles were investigated along two altitudinal gradients. Along long gradients, such as the latitude gradient in Paper I, there are several factors potentially influencing the fungal community, such as temperature, precipitation, nitrogen deposition and day length, that change along the same gradient. The benefit of altitudinal gradients is that temperature, and related factors such as length of vegetation season, change along the gradient whereas the number of other, confounding, factors may be reduced

Needles were collected from two altitudinal gradients ranging from an elevation close to sea level up to 650 m above sea level. Sweden is fairly flat, and, therefore, to obtain this elevation range, the two gradients were long, 200 and 250 km respectively, and were positioned in east-west directions across Sweden (Fig 3). Thus, some factors that change from north to south but not from east to west, such as nitrogen deposition, will be constant along these altitudinal gradients. Samples were collected from four or five different elevations along the gradients and at each elevation from two or three forests. All needle cohorts were sampled and each needle cohort was sampled as a separate category. Samples were also separated based on needle health into two categories. In each forest, samples were collected from five trees and from each forest, all needles of the same sampling category, for example healthy current year needles, were pooled to form one sample. The fungal community of the needles was described using 454 sequencing (described in section 3.5). However, we also wanted to distinguish the fungi that were only present within the needles from the fungi on the needle surface. Therefore all needle pairs were split at the needle base and one of the needles from each needle pair was surface sterilized and the second was untreated before DNA extraction (further described in section 3.5).

3.4 Paper IV: Disease development of DNB

The aim of Paper IV was to examine the disease development of Dothistroma needle blight on seedlings of Scots pine and Lodgepole pine under Nordic conditions. Seedlings of Scots pine and Lodgepole pine were planted at two sites in Fagersta in central Sweden. Every second to third month, needle samples from each needle cohort present on the seedlings were collected. Seedlings were also examined for the distinctive symptoms of DNB: red bands and small black conidiomata. Although these symptoms are characteristic, symptoms at the earlier stages may be difficult to distinguish from other diseases and it would not be possible to identify any latent phase based solely on only visual symptoms. Furthermore, *D. septosporum* is difficult to isolate and grows slowly in culture. Additionally, DNB is caused by two closely related species, *D. septosporum* and *D. pini*, which cannot be separated based on morphological characteristics (Barnes *et al.*, 2004). Therefore we used species-specific primers and conventional PCR to detect infections of *D. septosporum* from the needle samples (Ioos *et al.*, 2010). Needles were surface sterilized (further described in section 4.2) prior to DNA extraction to remove spores from the needle surface so as to detect only true infections of *D. septosporum*.

3.5 Methods for studying needle fungi

Due to the cryptic lifestyle of many foliar fungi it is not possible to rely on fruit bodies or specific symptoms when studying the foliar fungal flora. Not all fungal inhabitants of needles are pathogens that cause specific symptoms. There are also pathogens that only cause symptoms under conditions conducive for disease development. Some foliar fungi are not known to produce fruiting structures, whereas others may only produce fruit bodies when the needles are shed or during a specific season. Many studies of the fungal flora of Scots pine needles have relied on culture-based methods (Romeralo *et al.*, 2012; Terhonen *et al.*, 2011; Peršoh *et al.*, 2010; Kowalski, 1993). These methods have several advantages, such as the possibility to identify individuals and determine colonization frequencies, as well as being a way of acquiring isolates that can be used in experimental studies. Nevertheless, culture-based methods suffer from biases by selecting for fast-growing fungal species, and are unable to capture all species present; fungi that grow poorly, or not at all, in culture are likely to be overlooked.

Culture-independent methods can provide a more comprehensive description of the fungal community and rely on DNA extraction directly from the needle material. Typically, in fungal community studies, the ribosomal internal transcribed spacer (ITS) region is amplified and sequenced. Sequences are then assigned a taxonomic identity by comparison with known sequences in databases such as the NCBI database (http://blast.ncbi.nlm.nih.gov). The development of various high-throughput sequencing methods that can generate thousands, and even millions, of sequences, enables detailed descriptions of fungal communities and permits large, sample-rich studies to be performed (Lindahl *et al.*, 2013). However, these methods also have biases. For example PCR is dependent on primers that can have different affinity to DNA from different species thereby causing primer biases. Different lengths of the PCR products from different species, and variations in the number of ribosomal gene copies present in different species can influence the relative proportions of the products.

Irrespective of whether a culture-based or a culture-independent method is used, sample treatment before DNA extraction or isolation will affect which fungi are retrieved from the needles. Fungi are present both on the needle surface and inside the needle and surface sterilization by immersing needles in ethanol and sodium hypochlorite is believed to kill fungi on the needle surface. This method has traditionally been used in culture-based studies to kill fungi on the needle surface before isolation of fungi present inside the needle. The same method can also be applied before attempting to extract fungal DNA

from the interior of the needles because sodium hypochlorite not only kills fungi on the surface, it also destroys the DNA (Prince & Andrus, 1992). If the needles are green and symptom free, fungi recovered from needles after surface sterilization can be considered to be endophytes.

In this thesis, high-throughput sequencing by 454 pyrosequencing of the ITS 2 region in the ITS was used to detect the needle fungal community in Papers I, II and III. In Papers I and II, the fungal-specific primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990) were used to amplify the ITS region. Sequencing started from the ITS4 primer and because the generated sequences were short (mean read length 244 bp), only the ITS 2 region was covered. In Paper III, the primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 were used to amplify the ITS 2 region.

The derived sequences were analysed and clustered in the computer package SCATA (Sequence Clustering and Analysis of Tagged Amplicons, https://scata.mykopat.slu.se/). Sequences not passing the quality check, and sequences with missing primers and tags were discarded. The remaining sequences were clustered into operational taxonomic units (OTUs) using single-linkage clustering at 98 % similarity, which gives a reasonable taxonomic resolution for most fungal groups (Kõljalg *et al.*, 2013). The OTUs were assigned a taxonomic identity by performing a nucleotide BLAST search in the NCBI database (http://blast.ncbi.nlm.nih.gov).

4 Results and discussion

4.1 Spatial patterns

4.1.1 Large scale patterns (Papers I and III)

The spatial patterns in the fungal community of Scots pine needles were investigated at large spatial scales across Sweden along a latitudinal gradient (Paper I) and altitudinal gradients (Paper III). In Paper I, the latitudinal gradient through Sweden covered three different vegetation zones. We expected variations in the needle fungal community along the gradient given that several other factors, potentially influencing the abundance and distribution of fungi, change along the gradient. Such factors included climatic factors, e.g. temperature and precipitation, length of the vegetation period, amount of sunlight, needle nutrient content and nitrogen deposition. A detrended correspondence analysis (DCA) ordination revealed a gradual change in community composition along the north-south gradient and community composition was significantly influenced by both latitude and vegetation zone (Permanova, $P < 0.05$). In other studies, variations in community composition of foliar fungi have been attributed to variation in temperature and precipitation (Cordier *et al.*, 2012b; Zimmerman & Vitousek, 2012). However, in this study several factors varied along the latitude gradient and, therefore, it was not possible to identify specific environmental factors shaping the fungal community.

In Paper III, the fungal community of Scots pine needles was investigated along two altitudinal gradients through Sweden, attempting to reduce the number of confounding factors that may vary along the latitude gradient in Paper I. Temperature, which decreases with increasing altitude, has been shown to affect important steps in the lifecycle of foliar fungi, and therefore, we expected variations in the needle fungal community along the gradients. Changes in foliar fungal community composition along altitudinal gradients

have been observed in previous studies (Davey *et al.*, 2013; Cordier *et al.*, 2012b) and, likewise, we found that altitude significantly affected the community composition detected along both altitude gradients, in both surface-sterilized and untreated needles (Permanova, $P < 0.05$). Studying the foliar endophyte community of *Metrosideros polymorpha* in Hawaii, Zimmerman and Vitousek (2012) found variations along an elevation gradient that could be correlated with variations in temperature and rainfall. The here observed changes in community composition along the altitudinal gradient could possibly be related to temperature since temperature changes along the gradient. Still, as was the case for the latitude gradient (Paper I), several confounding factors change along the same gradient, such as the length of vegetation period, land use history and landscape composition. However, other factors such as nitrogen deposition and day length should be (more or less) constant along the altitude gradient, suggesting that these are not main factors involved in shaping fungal communities at larger spatial scales.

Generally, species richness increases with decreasing latitude (Hillebrand, 2004). This pattern has been observed, for example, among fungi associated with Scots pine needles in Finland (Terhonen *et al.*, 2011) and for foliar endophytes a continental scale (Arnold & Lutzoni, 2007). By contrast, we found that estimates of OTU richness and Shannon's diversity index increased with latitude in healthy needles from naturally regenerated stands (Paper I). Exceptions to the latitudinal diversity gradient, similar to the pattern observed here, have been found in other studies. Tedersoo *et al.* (2014) found that several classes of saprotrophic soil fungi increased in diversity towards the poles. The increase in fungal diversity in naturally regenerated stands observed in Paper I could have several explanations. For example, the longer needle retention often observed in northern Sweden could enable fungi to colonize a more diverse habitat over a longer period of time, allowing the needle to host a greater diversity of fungi. The observed pattern could also be an effect of the variation in the forest landscape across Sweden, where the distribution of pine is more discontinuous in southern Sweden, which may also have a negative effect on leaf-inhabiting fungal communities (Helander *et al.*, 2007). This pattern was only observed among healthy needles in naturally regenerated forest stands. Forest regeneration method has been shown to influence foliar endophyte communities where replanting after clear cutting can limit the transmission of endophytic fungi from the surrounding trees (Johnston, 1998; Sieber-Canavesi & Sieber, 1987). Plants could also acquire a priming effect from the forest nursery that influences the fungal community for an extended period of time. Such an effect has been observed in the fungal community of

conifer roots (Menkis *et al.*, 2006). Among symptomatic needles there was no relationship between OTU richness and latitude (Paper I) or altitude (Paper III).

4.1.2 Edge effects (Paper II)

In Paper II, spatial patterns were examined at local scale, where we studied edge effects on the fungal community of Scots pine needles in forest stands along the Lithuanian coast facing the Baltic Sea. We hypothesized that OTU richness would be higher in the forest interior, as a direct effect of the less favourable environmental conditions for fungi at the forest edge. A DCA ordination showed that there were clear differences in the fungal community composition at the forest edge compared with that of the forest interior, and distance from the forest edge was found to significantly influence the fungal community composition (Permanova, $P < 0.05$). As expected, OTU richness was lower at the forest edge and several environmental factors could contribute to this, such as lower humidity at the forest edge as well as larger temperature fluctuations, higher levels of solar radiation and higher levels of salt deposition from the sea (Scattolin & Montecchio, 2009; Newsham *et al.*, 1997; Chen *et al.*, 1995; Osorio & Stephan, 1991). However, it was not possible to determine the relative importance of these factors. There was no clear separation between the fungal communities detected in samples collected at 80 and 150 m into the forest interior, indicating that the influence of the edge reached less than 80 m into the forest. This is comparable to the results by Siitonen *et al.* (2005) who found that edge effects on the frequency of white rot fungi did not reach further than 50 m into the forest.

The environment at the forest edge would presumably also be less favourable for the host trees, which was indicated by a higher proportion of symptomatic needles at the forest edge and a reduced number of retained needle cohorts. A stressful environment for the tree would weaken its defences and this could indirectly influence the needle fungal community. We hypothesized that edge effects acting indirectly on the fungal community through a weakened host defence would enhance the ability of necrotrophic pathogens to colonize the needles by facilitating their access to carbon resources in the tree (Oliva *et al.*, 2014). By contrast, biotrophic pathogens, which derive carbon and nutrients from living cells, were expected to be negatively affected by host stress because it would lead to a decrease in the carbon available in the cells (Oliva *et al.*, 2014). We found that among the most abundant OTUs there were three OTUs with higher relative abundance at the forest edge: two of these OTUs also had a higher relative abundance in symptomatic needles. These OTUs could possibly represent necrotrophic

pathogens. However, it was not possible to assign these OTUs with a species identity and, hence, it was not possible to make a conclusion about their ecological role. Another possibility is that these fungi represent saprotrophic fungi, which would be likely to be favoured by host stress given that stress may accelerate the onset of needle senescence (Reich *et al.*, 1995).

Edge effects are complex and can influence the fungal community both directly and indirectly and further studies are needed to determine the specific factors influencing the fungal community and the effects on specific species and their associated function. The clear edge effect on foliar fungi observed in Paper II suggests that edge effects may play an important role in the distribution of foliar fungi at a landscape level. This calls for further studies where also edge effects on foliar fungi are investigated in forest edges created by anthropogenic activity, such as edges to roads and clear cuts.

4.2 Needle age

Effects on needle age on the fungal community were studied both in needles from the latitudinal gradient through Sweden (Paper I) and in needles from the altitudinal gradients (Paper III). Needle age was also considered in Paper IV and is further discussed in section 4.4.1. Time of exposure to fungal infections increase with needle age and the physiology of the needles and needle defence also change over time. Therefore we expected variations in the fungal community composition with needle age. We hypothesised that OTU richness should increase with needle age, something that has been reported from other host species (Espinosa-Garcia & Langenheim, 1990; Legault *et al.*, 1989b).

Both along the latitudinal gradient (Paper I) and the altitudinal gradients (Paper III) effects of needle age was found to have a significant effect on community composition (Permanova, $P < 0.05$). In needles from the altitudinal gradient the pattern was more pronounced in untreated needles than in surface sterilized needles (Paper III). The effect was also more pronounced in untreated needles from the altitude gradients (Paper III) than in needles from the latitude gradient, that were also untreated prior to DNA extraction (Paper I). A possible explanation for this could be the investigated age of the needles. From the latitudinal gradient, one-year-old and two-year-old needles were compared, while in current-year needles and one-year-old needles were compared along the altitudinal gradients. It is possible that the fungal community associated with the current year needles is more distinct than other cohorts. These needles were collected in the early autumn and at that time the current year needles had not been exposed to potential fungal infections across different seasons.

Comparing all needle cohorts present on the trees along the altitudinal gradients (Paper III), as expected, both OTU richness increased with needle age, however only in needles that were untreated prior to DNA extraction. It is possible that the surface sterilization procedure is too powerful on older needles, exposing also the needle interior to sodium hypochlorite, which would reduce the number of detected OTUs. There could also be an ecological explanation to this pattern. The oldest needles in this study were dead, but still attached to the tree, or dying. It is likely that, at least in senescent needles where host defence is weak, some fungi with high competitive abilities, colonize the needle excluding and replacing other fungi in the needle interior. This has been observed among wood decay fungi, in an experiment investigating competition between different species *Resinium bicolor* was found to successfully replace other fungi (Holmer & Stenlid, 1996). Several studies have shown that endophyte colonization frequency increase with needle age. In needles of *Sequoia sempervirens*, Espinosa-Garcia and Langenheim (1990) noted that endophyte richness increased with needle age but in the oldest needle cohorts endophyte richness decreased at the same time as the frequency of two fungal species increased. It has also been suggested that saprotrophic epiphytes colonize the needles and leaves as death occur replacing some of the endophytes (Cabral, 1985). Still, a more thoroughly analysis of the data is needed.

4.3 Needle health status

The effect of needle health status on the fungal community of Sots pine needles was investigated in Papers I, II and III. Disease could introduce additional habitats for fungi in the needles and symptoms of disease was expected to increase species richness of the needles since new species, together with some of the fungi already present in the needle, may colonize parts of the needle at the same time as species can remain in healthy tissue (Ragazzi *et al.*, 2003; Cabral, 1985).

Both along the latitudinal gradient (Paper I) and at the Lithuanian coast (Paper II) needle health status was found to have significant effects on community composition (Permanova, $P < 0.05$), and OTU richness was higher in symptomatic needles. The same pattern was observed in untreated needles (needles that were not surface sterilized prior to DNA extraction) from the altitudinal gradients (Paper III). Disturbance is an important process in the dynamics of communities (Johnson & Miyanishi, 2007) and pathogenic infection could be considered a small-scale disturbance leading to a change of the fungal community. Such changes have been observed in the community

dynamic of wood decay fungi where the activity of primary colonizers of the functional sapwood opens up the habitat for secondary colonizers (Boddy, 2001).

However, needle heath status had no effect on community composition or OTU richness in surface sterilized needles (Paper III). As discussed in the previous section, at needle senescence or when a needle dies, fungi either already present in the needle or new species arriving from the exterior of the needle could colonize the needle. This could explain why there is no change in OTU richness between healthy and symptomatic needles. However, it does not explain why there is no effect on community composition and these results need to be further explored.

4.4 Specific species (Papers I and IV)

4.4.1 Dothistroma needle blight under Nordic conditions (Paper IV)

In Paper IV, the disease development of DNB, caused by *D. septosporum* was followed on seedlings of Scots pine and Lodgepole pine. It is possible that the disease is limited by the cold climate in the Nordic countries, and symptoms of DNB on the surrounding older trees were predominantly visible on two-year-old needles. Therefore, we hypothesized that the disease would primarily affect older needle cohorts, either as a consequence of a longer disease development time or because the needles become infected at an older age. Unexpectedly, *D. septosporum* began to infect needles of both pine species as they started to elongate during their first summer. The characteristic symptoms of DNB became visible the following spring. This pattern is comparable to the observations reported in studies in both Europe and New Zealand (Karadžič, 1989; Butin, 1985; Gilmour, 1981). Thus our results show that DNB has the potential to survive and proliferate under Nordic conditions. On Scots pine the disease development was found to be even shorter if the needles were infected at an older age. This is possibly reflecting a weakened defence with needle age, something that has been found in needles and leaves among other species (Bell, 1980; Stavely & Slana, 1971) and which could probably accelerate the disease development. At one of the sites, at some time points, infections of *D. septosporum* were detected in more than 50 % of the seedlings, but the characteristic symptoms of DNB were only observed on a small number of seedlings throughout the study period. These results highlight that apparently healthy seedlings can carry latent infections of this potentially serious disease. This might have implications for the movement of seedling over large distances. However, in our study, seedlings from the forest nursery were all free of infections of *D. septosporum*.

Given that Lodgepole pine is reported to be highly susceptible to DNB (Watt *et al.*, 2009), we expected that Lodgepole pine seedlings would be more severely affected by the disease than the Scots pine seedlings. On the contrary, although there was no clear difference between the two species in terms of disease development and the proportion of seedlings with detected infections, the mortality of Scots pine was higher than Lodgepole pine. This was likely caused by other stresses and the presence of the pathogen *L. seditiosum* which only infected Scots pine.

DNB has only been observed in a few forest stands in Fagersta in central Sweden, however. In material that was not presented in the four papers in this thesis, we found that *D. septosporum* is present across most of the country. Using 454 sequencing *D. septosporum* was detected from Scots pine needles at several sites, ranging from Fredrika in northern Sweden to Vaggeryd in southern Sweden. This was not unexpected; the disease has been found at several locations across both Norway and Finland (Solheim & Vuorinen, 2011; Müller *et al.*, 2009), and the wide distribution of this pathogen challenges the assumption that the disease represents a newly introduced species. Although the specific symptoms of DNB on needles are characteristic, the disease is likely to be overlooked since it is not severely affecting Scots pine in Sweden.

4.4.2 *Lophodermium* spp. (Paper I)

Lophodermium spp. was found in all 454 sequencing studies and in Paper I, were needle samples were collected from a latitudinal gradient through Sweden, this genus was more thoroughly examined. Four of the more sequence rich OTUs could be assigned to *Lophodermium* spp.. Among these, two different OTUs was assigned to *L. pinastri*, L. pinastri ID 6 and L. pinastri ID 47, supporting the findings by Reignoux *et al.* (2014) who identified three cryptic species of *L. pinastri*, designated *L. pinastri* I, II and III. In a neighbour-joining tree, *L. pinastri* ID 6 was grouped with sequences of *L. pinastri* I (GenBank accession number HM060657, HM060655) and *L. pinastri* ID 47 was grouped with sequences form *L. pinastri* II (GenBank accession number HM122036, HM06066). These two *L. pinastri* also had different abundance patterns (Fig 4), where the relative abundance of *L. pinastri* ID 6 increased with latitude (tested in a generalized linear mixed model, $P < 0.05$) while *L. pinastri* ID 47 was only observed in the southern parts of the country, which could reflect different habitat preferences.

Lophodermium seditiosum is generally considered to have a more southern distribution in Sweden since severe attacks by this fungus has been reported mostly from southern and central Sweden (E. Stenström, personal communication). Our findings, where the relative abundance of *L. seditiosum*

decreased with increasing altitude support this (Fig 4). However, the pathogen was also found at low relative abundance as far north as the middle boreal zone. Probably *L. seditiosum* is present in northern Sweden but causes less problems. Possibly the mild winter are favouring the diseases in southern Sweden (Stenström & Arvidsson, 2001). It is possible that under anticipated climate change, which predicts increased mean temperatures and precipitation in the Nordic countries (Swedish Meteorological and Hydrological Institute, 2014), Lophodermium needle cast may become a problem to young pines also in northern Sweden

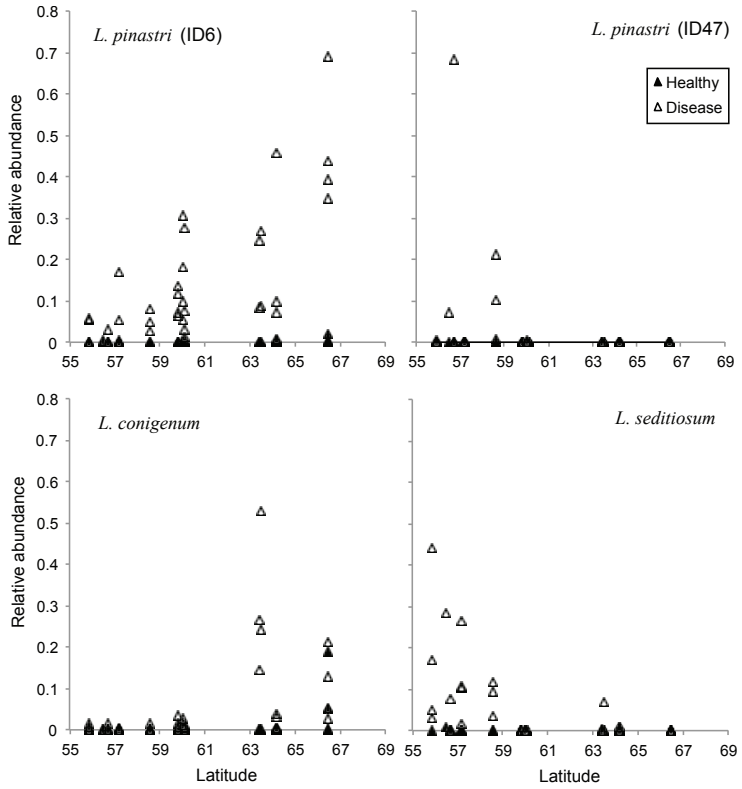

Figure 4. The relative abundance at different latitudes of the four OTUs designated to the genus *Lophodermium*. Filled symbols represent healthy needles and empty symbols represent diseased needles. triangles represent one-year old needles and circles represent two year old needles.

4.5 Fungal community of Scots pine needles (Papers I, II and III)

Both along the latitude gradient and altitude gradients in Sweden (Papers I and III), and in forests at the Lithuanian coast to the Baltic Sea (Paper II), 454

sequencing revealed species-rich fungal communities of Scots pine needles (clustered at 98 % similarity 602 OTUs in Paper I, 665 OTUs in Paper II and 1397 OTUs in Paper III, singletons excluded). None of the constructed rarefaction curves reached the asymptote, suggesting that the fungal communities were not exhaustively sampled. Individual rarefaction curves for each samples based on the number of sequence reads reached the asymptote for some samples but not for all, suggesting that the sequencing depth (number of sequence reads) were not always sufficient.

The detected fungal communities were all dominated by ascomycetes, which represented 70-80 % of the OTUs (Papers I, II and III) and is consistent with what has been reported form other studies of foliar fungal communities (Bálint *et al.*, 2015; Cordier *et al.*, 2012a; Terhonen *et al.*, 2011). Particularly abundant were *Capnodiales* within the *Dothideomycetes* and *Rhytismatales* and *Helotiales* within the *Leotiomycetes*. Among the basidiomycetes, *Tremellales* within the *Tremellomycetes* was the most abundant order. A large proportion of the detected OTUs could not be taxonomically assigned further than to subdivision or class, something that has also been reported from other studies (Bálint *et al.*, 2015; Menkis *et al.*, 2015; Cordier *et al.*, 2012a). This highlights the need for further studies of foliar fungi, and the development of more complete curated databases,

The detected OTUs do not all necessarily reflect 'true' species, for example PCR artefacts and intraspecific variation within the ITS region for some species may contribute to an overestimation of species richness. When needles are not surface sterilized the method also captures fungi that may not be directly associated with pine needles, such as spores that can land on the needle surface by chance. However, the method used only gives a momentary picture of the fungal community, and low relative abundance does not always imply that a specific species have a weak association with the host. For example, endophytes may be present in a healthy needle only as very small colonies and may only colonize the needle extensively when the conditions are right, for example at needle senescence (Deckert *et al.*, 2001). Because of small biomass during phases of their lifecycle, these species may only be detected at low relative abundance, or in some samples not at all. This might have been the case for *L. pinastri* (ID 6 and 47) in Paper I. Although *L. pinastri* has frequently been recovered from healthy needles in culture-based studies it was only detected at low relative abundance, or not at all, in healthy needle samples (Fig 4).

Surface sterilization had a strong effect on the fungal community, which was expected since the procedure would remove fungi from the needles surface and fewer OTUs were also detected from surface sterilized needles compared to untreated needles (Fig 5).

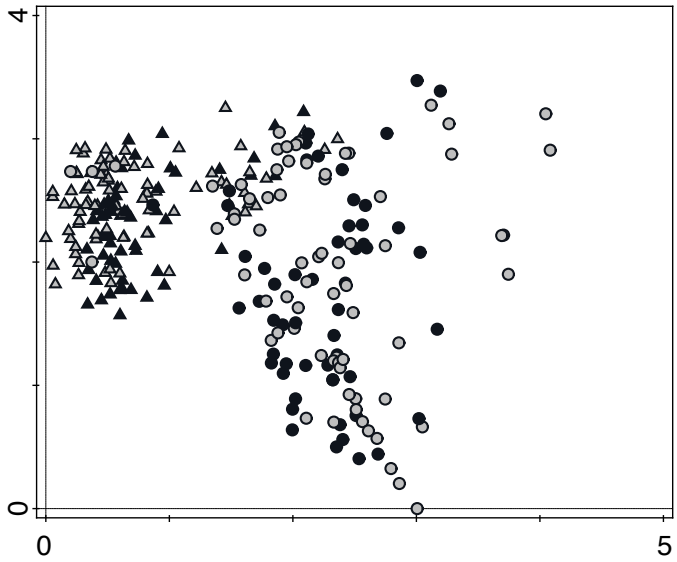

Figure 5. DCA ordination including all samples from the two altitude gradients in Paper III. Circles represents surface sterilized needles, where yellow represent samples from gradient 1 and orange represent samples from gradient 2. Triangles represent needle samples that were untreated prior to DNA extraction and blue represent gradient 1 and purple represent gradient 2.

In Paper I, one OTU (ID 0) was widespread and was found across the country, in all samples but one. There was no match to any identified species in GenBank. However, by sequencing the large subunit we were able to assign the OTU to the Arthoniomycetes, a class containing many lichenised fungi. Also in Paper III, this OTU was among the more abundant OTUs that were found and was present in about 90 % of untreated needle samples. However, it was only present in 28 % of the surface sterilized needle samples, suggesting that this OTU may represent a species predominantly found on the needle surface. This OTU was also found in samples from Lithuania (Paper II). The 100 % match to an uncultured fungus from needles of Scots pine in Finland (present in samples from all sites: Terhonen, personal communication) and from saproxylic beetles in Gotland in the Baltic sea, suggests that this fungus is

widespread in the region with a possible connection to forest trees. However, the ecological role of this OTU (ID 0) remains unknown.

5 Conclusions and future prospects

This thesis investigates foliar fungi associated with Scots pine needles and contributes to knowledge of spatial variability and dynamics of these fungi, which is important for understanding their potential effect on ecosystem processes (Martiny *et al.*, 2006). Using 454 sequencing, a species rich fungal community associated with Scots pine needles was revealed. The fungal community composition was found to change along a north-south gradient as well as along altitude gradients. Geographic patterns could also be distinguished for specific species where the relative abundance of *L. seditiosum* was found to decrease with latitude whereas relative abundance of *L. pinastri* (ID 6) increased with latitude. At a local spatial scale, we identified edge effects on the fungal community associated with needles of Scots pine in forests at the Lithuanian coast facing the Baltic Sea. Community composition at the forest edge was different from that in the forest interior and OTU richness was lower at the forest edge. These findings may have implications also for large-scale patterns of fungi, and needs to be further investigated. Additional studies are needed to identify specific factors influencing the abundance and distribution of foliar fungi at different spatial scales.

Comparing the fungal community of needles of different age and health status, we found patterns that suggests that the fungal community associated with Scots pine needles develops and changes over time, both as the needles age and finally become senescent, but also when needles are damaged by, for examples foliar pathogens. Interestingly, the same patterns were not observed for surface sterilized needles. This finding is intriguing and more thorough analysis is needed before further conclusions can be drawn.

Examining the disease development of DNB we found that the lifecycle of *D. septosporum* under the colder conditions in the Nordic countries was similar to that observed in other parts of the world. Thus, *D. septosporum* may have the potential to cause severe damage to pines also in the Nordic countries. Still,

symptoms on older trees predominantly appear on the older needle cohorts compared to last year needles on the seedling in our study. Whether this difference is the result of increased resistance with tree age, or if disease is more severe on young seedling because of the higher spore pressure and more favourable conditions for the disease by the forest floor remains unclear.

Our result revealed a species rich foliar flora associated with Scots pine needles. However, a large proportion of the detected OTUs were not possible to identify further than to class or order level. In order to understand possible implications of the observed patterns and dynamics of foliar fungal communities increased knowledge about specific species and their ecological function is needed. Future studies also need to focus on experimentally testing and identifying specific factors influencing the abundance and distribution of specific species, and also on their associated function and potential effects on the host. This is particularly important in the light of global change and when considering the important role of Scots pine in northern European forests.

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