

Effects of Tree Species Diversity on Foliar Fungal Distribution

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Cover: Tree species mixture from monoculture of a tree species to mixture of four tree species

(Illustration: Diem Nguyen)

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Abstract

European forest ecosystems span many different ecological zones and are rich in tree species. The environment in which the trees grow similarly affects fungal communities that interact with these trees. Fungal pathogens can cause severe damage to trees and potentially impair forest stability. In particular, pathogens that damage the foliage will affect the tree's photosynthetic ability, partly or completely. At the same time, pathogens can create niches for different plants by removing dominant species. The foliage community also comprises fungal species whose ecological functions are not entirely known and may either positively or negatively impact the tree's health status.

The aim of this thesis was to understand the effect of tree species diversity in mitigating fungal pathogen damage and in affecting the fungal community distribution. To achieve this, visual assessment of leaves for pathogen damages was carried out on 16 different tree species from six European forests. The fungal communities of Norway spruce needles from four European forests were studied by using next generation sequencing technology, and fungal communities of birch leaves by sequencing and morphological assessment. In this thesis, foliar fungal pathogen damages were positively correlated with tree species richness – latitude interaction, suggesting that tree species diversity may regulate pathogens but was dependent on the forest. Additionally, foliar fungal community composition was found to differ significantly in different forests, which may be attributable to local environmental effects or reflect the evolutionary history of the host tree, and thereby this study contributes to the understanding of biogeographic patterns of microorganisms. Finally, methods used to study fungal communities revealed that the sequencing-based method provided a richer picture of the fungal community than morphological assessment of fungal structures and symptoms, though neither method informed the distribution patterns as it relates to tree species diversity. Overall, impact of tree species diversity on foliar fungal distribution may not be strong, but it invites us to consider other factors that interact with fungal communities and how fungi may in turn shape their environment.

Keywords: Tree species diversity gradient, Foliar fungal pathogens, Fungal community, Next generation sequencing, *Picea abies*, *Betula pendula*, Insurance hypothesis, Generalists, Specialists, Latitude gradient

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Dedication

To Adabelle and Penelope Thi Buonocore

Today me will live in the moment, unless it's unpleasant, in which case me will eat a cookie.

Cookie Monster

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Baeten, Lander; Verheyen, Kris; Wirth, Christian; Bruelheide, Helge; Bussotti, Filippo; Finer, Leena; Jaroszewicz, Bogdan; Selvi, Federico; Valladaresh, Fernando; Allan, Eric; Ampoorter, Evy; Auge, Harald; Avacariei, Daniel; Barbaro, Luc; Barnoaiea, Ionu; Bastias, Cristina C.; Bauhus, Jurgen; Beinhoff, Carsten; Benavides, Raquel; Benneter, Adam; Berger, Sigrid; Berthold, Felix; Boberg, Johanna; Bonal, Damien; Braggernann, Wolfgang; Carnol, Monique; Castagneyrol, Bastien; Charbonnier, Yohan; Checko, Ewa; Coomess, David; Coppi, Andrea; Dalmaris, Eleftheria; Danila, Gabriel; Dawud, Seid M.; de Vries, Wim; De Wandeler, Hans; Deconchat, Marc; Domisch, Timo; Duduman, Gabriel; Fischer, Markus; Fotelli, Mariangela; Gessler, Arthur; Gimeno, Teresa E.; Granier, Andre; Grossiord, Charlotte; Guyot, Virginie; Hantsch, Lydia; Haettenschwiler, Stephan; Hector, Andy; Hermy, Martin; Holland, Vera; Jactel, Herve; Joly, Francois-Xavier; Jucker, Tommaso; Kolb, Simon; Koricheva, Julia; Lexer, Manfred J.; Liebergesell, Mario; Milligan, Harriet; Mueller, Sandra; Muys, Bart; **Nguyen, Diem**; Nichiforel, Liviu; Pollastrini, Martina; Proulx, Raphael; Rabasa, Sonia; Radoglou, Kalliopi; Ratcliffe, Sophia; Raulund-Rasmussen, Karsten; Seiferling, Ian; Stenlid, Jan; Vesterdal, Lars; von Wilpert, Klaus; Zavala, Miguel A.; Zielinski, Dawid; Scherer-Lorenzen, Michael (2013). A novel comparative research platform designed to determine the functional significance of tree species diversity in European forests. *Perspectives In Plant Ecology Evolution And Systematics* 15(5), 281-291.

- II **Nguyen, D.**, Castagneyrol, B., Bruelheide, H., Bussotti, F., Guyot, V., Jactel, H., Jaroszewicz, B., Valladares, F., Stenlid, J., Boberg, J. Tree diversity effects on fungal pathogens change across latitude in European forests (submitted).
- III **Nguyen, D.**, Boberg, J., Ihrmark, K., Stenström, E., Stenlid, J. Scale-dependent distribution of fungal communities in Norway spruce needles in mature European forests (submitted).
- IV **Nguyen, D.**, Boberg, J., Cleary, M., Hönig, L., Bruelheide, H., Stenlid, J. Foliar fungi of Birch in mixed tree species stands: comparing high-throughput sequencing and morphological assessment (manuscript).

Paper I is reproduced with the permission of the publisher.

The contribution of Diem Nguyen to the papers included in this thesis was as follows:

- I Involved in the planning of the design of the study.
- II Planned the study together with supervisors. Collected and analysed the data with input from co-authors. Wrote the manuscript with supervisors and input from co-authors. Responsible for correspondence with the journal.
- III Planned the study together with supervisors. Collected samples and performed a large portion of laboratory analyses. Analysed the molecular data and performed statistical analyses. Wrote the manuscript with input from supervisors. Responsible for correspondence with the journal.
- IV Planned the study together with supervisors. Collected samples and performed all laboratory analyses. Analysed the molecular data and performed statistical analyses. Wrote the manuscript in collaboration supervisors.

Abbreviations

ANOSIM	Analysis of similarity
GLMM	Generalized linear mixed effects model
ITS	Internal transcribed spacer region of the rRNA gene
LMM	Linear mixed effect model
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PERMANOVA	Permutational multivariate analysis of variance
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR

1 European Forests

Forests in Europe are important ecosystems that cover more than 1 billion hectares, the largest forest area of any continent in the world (FAO, 2015). The Food and Agriculture Organization of the United Nations (FAO) defines a forest as any land spanning more than 0.5 hectares with trees higher than 5 meters and a canopy cover of more than 10% (FAO, 2010). These forests provide a number of important ecosystem functions, including production of wood, fibre, bio-energy and/or non-wood forest products, protection of soil and water, conservation of biodiversity, provision of social services, or multiple uses, i.e. forests managed for any combination of previously mentioned functions (FAO, 2010). Each country varies in the amount of forested area and in the primary use of their forests (Table 1). For example, Finland is predominantly covered by forest and much of its forests are managed for production purposes. On the other hand, Germany may have one third of its land area covered by forests, but concentrates nearly 75% of its activities on diversifying the usage of forests, which include production, protection of soil and water, social services, while 25% of its activities are directed towards conservation of biodiversity. Additionally, European forest types vary across a number of ecological zones, from the subarctic boreal forests to the subtropical dry forests. As a consequence of the soil and climatic gradients, varying species assemblages are created that are sometimes unique to that specific forest type.

The hallmarks of natural mature forests are long time scales, diversity in species compositions and heterogeneity in structure. Forest trees are similarly long-lived, immobile organisms, often with long generation times, and interact with other organisms in a varied environment. These characteristics of trees make them vulnerable to many abiotic and biotic factors in the changing landscape of forests.

Table 1. Description of forest areas for selected countries in Europe used in this thesis. The primary designated functions, according to FAO, Global Forest Resources Assessment Country reports in 2010, include Production, Protection of soil and water, Conservation of biological diversity, Social services and Multiple use (FAO, 2010). Included here are three of the functions as percent of forest area designated for each category.

Country	Forest area (1000 ha)	Forest area (% total land area)	Production ¹ (% of forest area)	Conservation of biodiversity ¹ (% of forest area)	Multiple use ¹ (% of forest area)
Finland	22157	73	87	9	4
Poland	9337	30	40	5	1
Germany	11076	32	0 ²	26	74
Romania	6573	29	48	5	0 ²
Italy	9149	31	45	36	0 ²
Spain	18173	36	20	12	46

1. These primary designated functions are individually assessed for each country by the respective management units. Other primary functions not included in this table include protection of soil and water, social services, and other uses of the forest.

2. The “0” notation in this table does not suggest that there are no forest areas designated for “Production” as is the case of Germany or “Multiple use” as is the case for Romania and Italy. For instance in Germany, within the “multiple use” category, the forest area is designated *primarily* for more than one purpose and where none of these alone is considered as the predominant designated function. Forest areas in Germany are also used for production activities. Likewise, for Romania and Italy, the primary designated functions are explicitly specified, instead of categorized as “multiple use.”

Fungal pathogens as a group are one such biotic factor that have been identified as an emerging threat to various ecosystems, including forests and their products and services (Fisher *et al.*, 2012). Furthermore, climate change is thought to increase the damaging effects of forest pathogens (Sturrock *et al.*, 2011; La Porta *et al.*, 2008). In the last century, European forests have been invaded by forest pathogens at an exponential rate that have threatened and endangered trees (Santini *et al.*, 2013). Some few examples include *Ophiostoma ulmi* and *O. novo-ulmi* causing Dutch elm disease, *Fusarium circinatum* (pine pitch canker) and *Hymenoscyphus fraxineus*, the causal agent of ash dieback. Fungal pathogens can affect all parts of trees, from the roots to the canopy. Canopy foliage (i.e. leaves of broadleaved trees and needles of conifer trees) is the site of transpiration and photosynthesis and is susceptible to damage by pathogens and other biotic and abiotic agents. However, other members of the fungal community that coexist with the pathogens in the foliage may play important roles in protecting trees from pathogens (Ganley *et al.*, 2008; Arnold *et al.*, 2003).

Various methods can be used to detect and study the foliar fungal pathogens and the associated fungal community. These include traditional isolation and

culturing methods from leaves and needles and visually characterizing the fungal structures and damage symptoms. Modern day advances in technology has allowed the use of “next generation” high-throughput DNA sequencing methods to simultaneously identify multiple fungal species from complex environmental samples. This has permitted detection of less-abundant, cryptic, non-culturable and/or slower growing members of the community.

2 Tree Species Diversity Effects

The Millennium Ecosystem Assessment defines ecosystem services as supporting, provisioning and regulating services, and cultural activities that ecosystems provide for the benefit of humans (Millennium Ecosystem Assessment, 2005). With the decline in biodiversity, the provision of these services in the future has been of great concern (Chapin III *et al.*, 2000). Biodiversity research has shown that multiple ecosystem functions and services are influenced by the diversity of species within the system (Gamfeldt *et al.*, 2013; Isbell *et al.*, 2011; Mouillot *et al.*, 2011; Bengtsson *et al.*, 2000). Ecosystem functions are activities, processes or properties of ecosystems influenced by the biota (Scherer-Lorenzen, 2005). These include primary production, nutrient cycling, decomposition and resistance to disease (Hooper, 2002). Different levels of diversity can be considered in the tree layer, including a number of different species (i.e. taxonomic richness) (Naeem *et al.*, 1995) that may give rise to functional diversity (Diaz & Cabido, 2001), genetic variability within a species (Mundt, 2005), or phylogenetic diversity among species (Gilbert & Webb, 2007). According to the **insurance hypothesis**, organism diversities in ecosystems can buffer against disturbances, such as damaging effects of pathogens (Yachi & Loreau, 1999), making an ecosystem more resilient to return to its equilibrium state following perturbations (Elmqvist *et al.*, 2003). The effects of species diversity can thus result in more stable ecosystems (Bengtsson *et al.*, 2000; Bengtsson, 1998). The more species present in an ecosystem, the higher the probability for overlapping effects across species (i.e. functional redundancy) (Diaz & Cabido, 2001). For instance, a lost nitrogen-fixing species can be substituted with another species, sustaining the ecosystem function. While presence of a particular species may change over time, ecosystem function can be maintained, given the inherent species diversity that insures greater variation in response to environmental fluctuations (Diaz & Cabido, 2001).

Recently, research platforms have been established to address the role of species diversity on forest ecosystem functions (Verheyen *et al.*, 2015; Scherer-Lorenzen *et al.*, 2007; Scherer-Lorenzen *et al.*, 2005). In these experiments, complexity was increased beyond two-species assemblages and parameters other than the traditionally studied plant productivity and growth (Pretzsch, 2005). These large-scale experimental forest plantations have been pivotal to show that tree species diversity can influence carbon pools and fluxes (Potvin *et al.*, 2011), regulate herbivores (Haase *et al.*, 2015; Castagneyrol *et al.*, 2014; Vehviläinen & Koricheva, 2006), are important in competition and facilitation processes in the early stages of establishment (Pollastrini *et al.*, 2013), affect the composition of understory vegetation (Amptooter *et al.*, 2014), and strongly influence the diversity of the soil biota (Tedersoo *et al.*, 2015). However, tree species diversity did not play a role in water use efficiency among species (Grossiord *et al.*, 2013). From these early efforts, tree diversity had positive, negative or neutral effects, depending on the ecosystem function addressed. One possible reason may be the study system, i.e. the experimental forest plantation.

Plantation forests for functional biodiversity experiments may relay a different story than mature forests in natural landscapes, the “real world”. These tree plantation experiments are in the early phase of stand development, and would reflect a forest ecosystem that is re-establishing post disturbance. According to Leuschner *et al.* (2009), they are far from maturity, have limited plot history and short time horizon (some ecosystem processes require more than a few decades to be realized (Jenkinson *et al.*, 1990)), lack the complexity in stand and age structure, and have evenly spaced plants in small sized plots. In contrast, mature forests are in later stages of stand development, i.e. late to mid stem exclusion stage, the understory reinitiation stage or old-growth stage. These forests have an older, uneven age and size distribution and have endured environmental fluctuations posed by biotic and abiotic factors for decades. Trees that have been tested and survived go on to reproduce, while those that failed no longer contribute to the gene pool. Consequently, the physical structure of the forest and the age of the trees are varied.

In these mature forests, tree species mixtures were found to be more productive (Jucker *et al.*, 2014) and more resilient to environmental stress (Grossiord *et al.*, 2014a; Grossiord *et al.*, 2014b) than monocultures. Possible reasons for these observations are more efficient resource partitioning or complementarity effects, and interspecific competition with a few dominant species driving the performance of the forest stand (Loreau & Hector, 2001), or alternatively presence of more species altering the local micro-environment

(Kelty, 2006). Additionally, tree diversity was shown to limit the impact by invasive organisms (Guyot *et al.*, 2015).

Examples of a number of different ecosystem services and functions have so far been enumerated above. For provisioning and supporting services, functions included biomass production, nutrient cycling and water fluxes, and for regulating services functions included carbon fluxes resistance to mammal and insect herbivores. Lacking still is the incorporation of fungal pathogens and the regulation plant diseases (Millennium Ecosystem Assessment, 2005). To better understand the effects tree species diversity on disease mitigation, studies in both plantation and natural systems are required.

Tree species diversity may result in creating a **dilution effect** (Keesing *et al.*, 2006), a pattern generally observed for different parasites (Civitello *et al.*, 2015). Parasite is used generally here to include virus, bacteria, insects, fungal pathogens. The dilution effect could be used inclusively to describe the net effect of species diversity reducing disease risk by any of a variety of mechanisms (Keesing *et al.*, 2006). The presence of a diversity of tree species may serve to reduce the encounter between a parasite and its host, interfere with transmission or dispersal of a parasite, and regulate the abundance of susceptible hosts via interspecific competition, and thus decreasing disease transmission (Keesing *et al.*, 2006). The targeted host tree of a particular parasite may be in lower proportion in relation to other tree species present in an area, thereby regulating the abundance of an important host species (Burdon & Chilvers, 1982). Monocultures of forest stands with a higher proportion of host species can lead to a situation predicted by the **resource concentration hypothesis** (Root, 1973) that states that specialist parasites should be more abundant in large patches of host plants. The increased density of host tree thus allows easier access to susceptible hosts, and subsequently increases disease risk.

Diversity of tree species may also lead to situations of **associational resistance or susceptibility** (Barbosa *et al.*, 2009), whereby neighbouring species somehow alter the micro-environment to make pest or pathogen attack less likely to occur. For fungal species that rely on insect vectors that actively find hosts, interference with chemical or visual cues by an admixture of host trees, is a situation comparable to associational resistance (Tahvanainen & Root, 1972). On the other hand, other host trees that enhance the discovery of a host for active insects or that serve as reservoirs for pathogen could lead to associational susceptibility. Thus, for associational susceptibility, neighbouring species could increase the probability that the focal species is damaged depending on the identity of the neighbours (species identity effect).

3 Fungi and Fungal Pathogens

Fungal species, in contrast to insects, do not actively seek out their hosts. Spore dispersal or mycelial spread allows the dissemination of inoculum to new hosts. Spores disperse within trees (e.g. conidia of *Ceratocystis fagacearum* spreading via root graphs (Kuntz & Riker, 1955)), between trees and across landscapes via air, water or soil (Tainter & Baker, 1996). Spore dispersal can also be mediated by insect vectors, such as *Hylurgopinus rufipes* and *Scolytus* species vectoring the Dutch elm disease pathogen *Ophiostoma ulmi* and *O. novo-ulmi* (McLeod *et al.*, 2005), nitidulid beetles that vector *C. fagacearum* which causes oak wilt in *Quercus* species (Jewell, 1956) and *Xyleborus glabratus*, the redbay ambrosia beetle introducing *Raffaelea* sp into redbay (*Persea borbonia*) and avocado (*P. americana*) (Mayfield *et al.*, 2008a). Fungal mycelial growth of *Heterobasidion annosum* from untreated stumps via roots leads to infection of new trees (Stenlid & Redfern, 1998).

Many spores that form do not disperse beyond the immediate area; the highest density of spores of *H. annosum* can be found within 5 meters from an infection center (Möykkynen *et al.*, 1997). Conidia that are sticky and rely on moisture to disperse (e.g. *Gremmeniella abietina*, (Laflamme & Rioux, 2015; Bergdahl, 1984)) also do not disperse to great distances. However, airborne spores, or those that spread by insects (White *et al.*, 2000), can disperse long distances, and those causing agricultural diseases can spread over hundreds or thousands of kilometers (Brown & Hovmøller, 2002), including the dispersal of coffee rust across the Atlantic Ocean (Bowden *et al.*, 1971). Some fungal species such as rust species produce spores on different hosts, so-called, alternate hosts. The management of forest trees against such organisms may be difficult under some situations. For example, the alternate hosts may be unknown, but later discovered, as exemplified by *Melampyrum sylvaticum* as the alternate host of pine stem rust *Cronartium flaccidum* (Kaitera & Hantula, 1998). The alternate host may be abundant in the environment (*Ribes* sp as a

host for *Cronartium ribicola*) or exist in the landscape outside of the immediate managed area, whereby rust spores can disperse as far as 500 km to new niches, and has the potential to cause infection. Spore dispersal, however, is one of the first of many steps of the disease cycle that needs to happen before a fungus establishes (Oliva *et al.*, 2013). Once arriving on a host substrate, the spore needs to be able to germinate and form a germ tube to be able to interact with the host, colonize and eventually disseminate. The interspecific interaction among host in the environment can influence each stage of the disease cycle.

Depending on their resource specialization (Jorge *et al.*, 2014), fungal species are classified as specialists, with a narrow host substrate range, or generalists, with a broad host substrate range. However, the behaviour of fungi is more of a continuum than a fixed categorization, and specialists may be considered as generalists in certain circumstances. The complication is that host range may be defined by phylogenetic association, whereby more phylogenetically similar trees are more susceptible than less similarly related species (Parker *et al.*, 2015). The range can be single species, or a genus or a family of species. Management of specialist pathogens, if their ecology were known, would be possible by decreasing the density of susceptible tree species. But for some fungal pathogens, this certainly is not the only way to manage these diseases. Generalist pathogens may induce “pathogen spill-over” (Daszak *et al.*, 2000) from non-susceptible hosts, such that some serve as inoculum reservoirs for susceptible hosts. In this case, tree species diversity may be ineffective to control these types of pathogens. Management strategies need to be pathosystem specific.

Specialized pathogens may also exert negative feedback on plant communities, thus influencing the diversity of such communities (Mangan *et al.*, 2010). For example, conspecific seedlings around an adult plant were selected against, while heterospecific seedlings were preferentially recruited due to the activities of accumulated soil pathogens (Bever, 2003; Bever *et al.*, 1997). The incursion of pest and pathogens into a system could make the targeted plant species vulnerable to their damaging effects, for which there has been no selection pressure against the new invaders. For other plant species not impacted by the pathogen, a niche is now open for colonization. For example, the devastating impact of *Hymenoscyphus fraxineus* on European ash (*Fraxinus excelsior*) has allowed the recruitment of other tree species into what was predominantly ash stands (Lygis *et al.*, 2014) or the effects of the laminated root rot fungus *Phellinus sulphurascens* in shifting plant community composition by killing off Douglas fir (*Pseudotsuga menziesii*) trees (Holah *et al.*, 1997).

3.1 Fungal Pathogens of Trees

There are different scenarios when tree species mixtures can be beneficial, in terms of resisting the effect of pathogens, and when non-mixtures (i.e. monocultures) are better (Pautasso *et al.*, 2005), and it usually depends on the pathosystem. In the latter case, where monocultures seem to have escaped pathogen damage, it is likely that the monoculture has not yet encountered a pathogen or the level of infectious agents has not reached critical levels. Maybe it is really a “lucky monoculture”. Though given time, they may not be so lucky. One reason may be the build up of enough inoculum potential, as likely the case for the invasive pathogen *H. fraxineus* (Bengtsson *et al.*, 2012) or the native pathogen *Dothistroma septosporum* of NW British Columbia (Woods *et al.*, 2005; Woods, 2003). At the other extreme, high susceptibility to damage has been observed despite high diversity. Some rust fungi provide special examples of this scenario. For heteroecious rust species, which require both hosts to complete their lifecycle, like pine twisting rust *Melampsora pinitorqua* that alternates between Scots pine (*Pinus sylvestris*) and aspen (*Populus tremula*), mixtures where both hosts are present highly increases the risk for pines to be infected (Mattila, 2005). Additionally, the presence of willow (*Salix* sp), which is not a host for *M. pinitorqua*, in the stands increased the probability of rust damage (Mattila *et al.*, 2001), though increased distance between aspens and pines decreased the probability of damage (Mattila, 2005). In another example, tree diversity did not protect pine species from the white pine blister rust pathogen *C. ribicola*. Rather it was the presence of an alternate host in the forest stand that influenced the disease outcome (Zeglen, 2002). Some rust fungi like *Chrysomyxa abietis* is autoecious and can complete its life cycle on one host, Norway spruce, and this has implications for whether mixtures are effective or not.

Intermediate between these two extremes is the situation where diversity insures reduced levels of susceptibility to disease (Bengtsson *et al.*, 2000). Diversity can be considered not only from the perspective of different hosts, but also from intraspecific variation within a species. *Melampsora epitea* is a rust species that causes epidemics on willow species (*Salix* sp). Plantations that incorporate mixed host genotypes in *Salix* sp have been able to escape the yield loss due to rust infections (Pei & McCracken, 2005).

3.1.1 Evidence from Roots

There have been a number of studies that considered tree diversity effects on fungal pathogens. The pathogen *C. fagacearum*, the causative agent of oak wilt, can be spread via root contacts. Modelling the disturbance caused by this pathogen indicated that the stand composition affected the mortality of

Quercus species, such that the increase in the proportion of the susceptible host above 50% was marked by an increase in total mortality (Menges & Loucks, 1984). The mechanism for the increased mortality was increase in root graph transmissions. Root and butt rot pathogens such as *Heterobasidion* sp and *Armillaria* sp cause considerable damage to ecologically and economically important tree species. In North America, a study found that the rate of spread of *P. sulphurascens* was slower in mixed western hemlock (*Tsuga heterophylla*) and other coniferous species forests than pure western hemlock forests (McCauley & Cook, 1980). Similarly, resistant hosts such as western red cedar (*Thuja plicata*) and paper birch (*Betula papyrifera*) may serve as barriers to disease spread between roots of susceptible conifers in *A. ostoyae* infested areas and reduce mortality (Cleary *et al.*, 2008; Simard *et al.*, 2005; DeLong *et al.*, 2002). Additionally, it has been observed that increased seedling mortality due to *Armillaria* infection correlated with the increased proportion of conifers, relative to broadleaved species that are less susceptible to *Armillaria* (Gerlach *et al.*, 1997).

Furthermore, studies in Europe also showed positive effects of tree diversity. The probability of root rot damage was slightly lower in mixed stands than in pure spruce stands (Thor *et al.*, 2005) and this was correlated with reduced proportion of spruce in the stands (Lindén & Vollbrecht, 2002; Huse & Venn, 1994; Piri *et al.*, 1990). Thus, proportion of spruce trees with *Heterobasidion* root rot was higher in pure stands than in mixed species stands. However, other studies, as summarized by Pautasso *et al.* (2005), have shown no effect of diversity. Diversity did not increase or decrease susceptibility to butt rot (Siepmann, 1984) and infection was equally high in high diversity stands (Korotkov, 1978). One study by Kató (1967) even found negative diversity effects; butt rot was present higher in mixed stands than in pure.

3.1.2 Evidence from Foliage

Few studies have thus far considered the effect of tree species diversity on fungal pathogens of foliage. The reason for this is likely a result of the difficulty to obtain living leaves from the canopy of trees. Hantsch *et al.* (2013) studied tree species diversity in relation to reducing pathogen load (i.e. the amount of pathogen damage) of powdery mildew species in young experimental plantations, but no found tree diversity effects were found. However, the identity of tree species in the mixture was observed to affect the pathogen community and load; the presence of *Quercus* sp positively correlated with higher fungal species richness and pathogen load (Hantsch *et al.*, 2013). Similarly, pathogen richness increased with the presence of *Acer platanoides*, while pathogen load increased with disease-prone tree species *A.*

platanoides, *P. tremula* and *Tilia cordata* and decreased with the presence of disease-resistant species (Hantsch *et al.*, 2014b). Furthermore, the local diversity of the tree species (i.e. neighbourhood effect) can also decrease the fungal species richness and level of pathogen infestation, evidenced by *T. cordata*, but the effects were not consistent for *Q. petraea* (Hantsch *et al.*, 2014a). For the generalist forest oomycete pathogen *Phytophthora ramorum*, the causative agent of sudden oak death, lower survival of reservoir inoculum in bay laurels (*Umbellularia californica*) leaves were found in a mixed evergreen forest compared with a redwood (*Sequoia sempervirens*) forest (Davidson *et al.*, 2011). A dilution effect was demonstrated whereby *P. ramorum* disease risk was reduced in forest stands with increased tree species, and the mechanism by which this happens may result from lower competency of alternative hosts to further transmit the disease (i.e. encounter reduction) (Haas *et al.*, 2011). Furthermore, a recent and more expanded study to identify key parameters that explained infection risk of susceptible oak species also found pathogen dilution effects (Haas *et al.*, 2015). Also shown to be important drivers of sudden oak death included large size of oak species, variation in inoculum production, and warmer and wetter rainy-season conditions in consecutive years (Haas *et al.*, 2015).

4 Foliar Fungal Community

4.1 Fungal Endophytes and Epiphytes

A fungal community is defined as an assemblage of fungal species, regardless of their ecological role, that share a habitat such as tree foliage. The community can include endophytes, epiphytes and pathogens. Fungal species can play important roles as bioindicators of tree species health conditions (Luchi *et al.*, 2015), or as mycoparasites or antagonists protecting leaves from pathogens (Topalidou & Shaw, 2015). Fungal endophytes are generally defined as species that infect and inhabit tissues without causing apparent disease in the host (Petrini, 1992; Carroll, 1986). They are ubiquitous in nature, can be found on a diverse array of plant hosts, and occur within living plant tissues (Saikkonen, 2007; Sieber, 2007). While fungal endophytes are typically thought to be beneficial to the host (Ganley *et al.*, 2008; Minter, 1981) by protecting against pests and pathogens (Gange *et al.*, 2012; Rodriguez *et al.*, 2009; Arnold *et al.*, 2003), they can also be detrimental to the host (Busby *et al.*, 2013; Arnold & Engelbrecht, 2007; Schulz *et al.*, 1998). Living in close proximity to endophytes are epiphytes, organisms found on the surface of leaves and needles. Epiphytes have been shown to occupy different niches from those of endophytes (Santamaría & Bayman, 2005; Legault *et al.*, 1989).

The way in which endophytes and epiphytes disperse to new hosts is by horizontal transmission of spores (Arnold & Herre, 2003). These fungi produce spores that infect foliage elsewhere, rather than by vertical transmission from parent to offspring via seeds typified by grass endophytes (Rodriguez *et al.*, 2009). Newly flushed needles have been shown to be endophyte-free (Hata *et al.*, 1998), though over time infection by endophytes increase with needle age (Rodrigues, 1994; Bernstein & Carroll, 1977).

The leaf is a niche for many fungi. It is exposed to the harsh environment surrounding it and is subjected to desiccation, radiation, and overcrowding

(Juniper, 1991). The conditions that affect leaves also affect the fungal community of the leaf. Different factors can influence the fungal community structure at different scales, beginning with the community members on the leaf surface. They compete with one another for the limited resources on the surface (Larkin *et al.*, 2012). Members of the fungal community are also influenced by their own population dynamics (Saikkonen *et al.*, 1998). At the plant host level, individual host tissues (i.e. the leaf) (Cordier *et al.*, 2012; Barengo *et al.*, 2000; Deckert & Peterson, 2000; Lodge *et al.*, 1996), leaf surface traits (Valkama *et al.*, 2005), tissue age (i.e. needle) (Espinosa-Garcia & Langenheim, 1990), tissue type, host genotype (Bálint *et al.*, 2013), and host defence response (Bailey *et al.*, 2005) vary and affect the community either by increasing or decreasing the number of species in the community or selecting for specific organisms. At the stand scale, environmental factors such as location of hosts in the landscape (Haas *et al.*, 2015; Haas *et al.*, 2011; Jumpponen *et al.*, 2010), site quality and nutrient availability (Martín-García *et al.*, 2011), light availability (Matson & Waring, 1984), fertilization (Desprez-Loustau & Wagner, 1997), drought stress (Jactel *et al.*, 2012) and species composition (Jules *et al.*, 2014; Hoffman & Arnold, 2008; Lodge & Cantrell, 1995) that include vegetation structure in the stand (Longo *et al.*, 1976), can influence interspecific interactions among the host trees. The plants in a stand can influence the canopy cover that may in turn affect the microclimate of the site, dispersal of fungal spores and moisture levels (Collado *et al.*, 1999). Likewise, forest management strategies such as thinning practices may create less suitable environments for some species such as *Dothistroma septosporum* (Bulman *et al.*, 2013), while promoting other species such as decay fungi (Vasaitis *et al.*, 2012). At larger spatial scales, the influence of pollution can increase or decrease different members of the fungal community, perhaps by interfering with growth or sporulation (Magan & McLeod, 1991).

Weather factors such as the occurrence of rain, which can influence tree growth or spore germination, and temperature, which can affect the longevity of spores, are important to the distribution of fungi (Desprez-Loustau *et al.*, 1998; Weissenberg & Kurkela, 1980; Kurkela, 1973). Furthermore, climate factors such as temperature and precipitation can influence the host-fungus interaction, either by changing the plant community composition, increasing stress on hosts or expanding the geographic range of fungal pathogens (Oakes *et al.*, 2014; Desprez-Loustau *et al.*, 2007; Pearson & Dawson, 2003).

Latitudinal patterns can have an effect on biotic interactions (Kozlov *et al.*, 2015; Qian & Ricklefs, 2007). Biogeographic patterns exist among microbial communities (Martiny *et al.*, 2006), including fungi (Meiser *et al.*, 2014). The diversity of endophytes have been shown to decrease along a latitudinal

gradient from the tropics to northern boreal forests, and that conifers contained a higher incidence of cultivable endophytes than expected (Arnold & Lutzoni, 2007). However, Millberg *et al.* (2015) showed that across Sweden, there was an increase in diversity at higher latitudes. In conifer needles, species belonging to Ascomycota are more abundant than those of Basidiomycota (Terhonen *et al.*, 2011). Dothideomycetes are especially prevalent in boreal forests, and the Sordariomycetes are highly represented in temperate forests (Hoffman & Arnold, 2008; Arnold & Lutzoni, 2007).

4.2 Fungal Communities of Norway Spruce and Birch

Norway spruce (*Picea abies*) and birch (*Betula* sp) are important tree species in European forest that are hosts to diverse fungal communities in different tissues. In their needles and leaves, pathogens share a habitat with endophytes and epiphytes. The fungal community can be quite diverse on a single Norway spruce needle and can reveal polymorphisms within one fungal taxon, previously demonstrated for *Lophodermium piceae* (Müller *et al.*, 2001). Close to 100 species have been cultured from spruce needles from different forest stands (Sieber, 1988). Fungal species commonly found include *L. piceae* (Butin, 1986), *Tiarospora parca* (Müller & Hallaksela, 2000) and *Rhizosphaera kalkhoffii* (Livsey & Barklund, 1992). *L. piceae*, co-occurred with the rust pathogen *Chrysomyxa abietis* (Lehtijarvi *et al.*, 2001) and *R. kalkhoffii* as well, though at low frequencies (Livsey & Barklund, 1992). Furthermore, the needle associated fungal communities have been found to be affected by the host genotype, and tends to be more diverse in slower growing Norway spruce than faster growing trees (Rajala *et al.*, 2013; Korkama-Rajala *et al.*, 2008). Biotic factors that interact with spruce, such as insect pests can change the composition of the fungal community (Menkis *et al.*, 2015). Site conditions may also influence needle infection frequency, whereby infections occurred abundantly in pure spruce stands and dense virgin stands (Müller & Hallaksela, 1998).

The fungal community of birch leaves has been studied in number of ways. Isolation and culture methods frequently detected *Gnomonia setacea* and *Fusicladium betulae*, *Venturia ditricha* and *Melanconium betulinum* (Helander *et al.*, 2007; Saikkonen *et al.*, 2003). Additionally, the frequency of the endophytes *Fusicladium* sp and *Melanconium* sp were found to vary with birch families (Elamo *et al.*, 1999), while the genetic diversity of *V. ditricha* was affected by the genotype of birch; susceptible hosts were typically infected with genetically similar *V. ditricha* and resistant hosts with more genetically dissimilar genotypes (Ahlholm *et al.*, 2002). Furthermore, macro- and

microscopic analyses revealed that *Discula betulina*, *V. ditricha* and *Atopospora betulina* were present on the leaves and that their abundance was affected by the genetics of birch clones that have differential susceptibility to pathogens (Hantsch, 2013). The visual assessment of the leaf coverage of birch rust caused by *Melampsorium betulinum* was negatively affected by leaf surface traits such as trichome density and epicuticular flavonoid aglycones (Valkama *et al.*, 2005). Pathogens of birch leaves include *M. betulinum* that alternate with *Larix decidua*, *D. betulina*, and *Phyllactinia guttata* (Butin, 1995).

4.3 Tools for Describing the Fungal Community

An environmental sample is a complex sample that can include, for example, a gram of soil, pieces of shaved wood dust or a few spruce needles. The diversity of fungal species in environmental samples can be studied in a variety of ways to identify fungal taxa and understand the distribution patterns and their ecological role. Traditional methods to study fungal community have been accomplished by collecting fruiting structures and conducting macro- and microscopic analyses. Moreover, many fungal species have been isolated in culture and subsequently identified based on morphological characteristics. Some fungal species are amenable to being cultivated under laboratory conditions, predominately relying on identifying the optimal growth conditions (Sun & Guo, 2012). However, many more species cannot be isolated, perhaps given their lifestyle (obligate biotrophs require living hosts) or lack of knowledge of their optimal growth conditions. The inability to cultivate all organisms is a limiting factor in characterizing the entirety of the fungal community from their natural habitats, which is estimated to be large number, approximately 1.5 million species (Hawksworth, 2001). To be able to capture the abundance and diversity of such complex samples that may contain tens to hundreds of species or taxa, more advanced methods are required.

Researchers have relied on technology available at the time to overcome culturing biases. PCR amplification with fungal specific primers (White *et al.*, 1990) and cloning, followed by traditional Sanger sequencing (i.e. in which individual base pairs can be determined by selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during DNA replication of cloned amplicons) was one method used to study diversity that did not rely on growing fungi on agar plates (Sun & Guo, 2012). However, it was not always possible to pick enough clones to obtain a representative sampling of the community. With the advancement in technology and the economic accessibility of next generation sequencing, it is now possible to study the

diversity of microbial species, including fungi, with high-throughput sequencing to detect hundreds and thousands of species directly from complex environmental samples (e.g. soil, wood and foliage) (Millberg *et al.*, 2015; Ottosson *et al.*, 2015; Monard *et al.*, 2013).

High-throughput sequencing can generate millions of sequence reads. These reads can be clustered to group identical or similar sequences. Using either complete linkage or single linkage clustering methods, these millions of reads can become more manageable (Lindhahl *et al.*, 2013). In this thesis, the generated sequence clusters are called operational taxonomic units (OTUs) or taxa, without implying any phylogenetic relationship among the taxa. Thresholds for defining a cluster are specified by the clustering distance. Taxonomic resolution for most fungal groups can typically be delineated at 98% similarity (Koljalg *et al.*, 2013).

At the time of writing this thesis, Roche 454 pyrosequencing, one of the next generation sequencing technologies, could sequence approximately 500 base pairs. The evolution of these technologies has been rather rapid, and already there is the so-called “third” generation sequencing technology. Single molecule real time (SMRT) sequencing from Pacific Biosciences can yield average read lengths of > 10,000 base pairs. These two technologies are the least prone to sequence length biases. LifeTech’s IonTorrent and Illumina suffer from sequence length bias such that there is preferential sequencing of shorter amplicons.

Targeted sequencing of genes or gene regions may inform the diversity of a complex environmental sample. Fungal species, predominantly Ascomycota and Basidiomycota taxa, can be identified by the ribosomal RNA genes. The molecular barcode for fungi is the internal transcribed spacer (ITS) region of the ribosomal RNA genes (Schoch *et al.*, 2012; Bellemain *et al.*, 2010; Nilsson *et al.*, 2009; Bruns & Gardes, 1993; Gardes & Bruns, 1993; White *et al.*, 1990), which consists of the ITS1 and ITS2 regions, separated by the conserved 5.8S gene. The ITS region is considerably variable to allow molecular species identification (Nilsson *et al.*, 2008), and has been used in analyses of mixed fungal communities (Blaalid *et al.*, 2013; Mello *et al.*, 2011). Sequencing of the rDNA is typically done to determine the total fungal community of a sample, though may include resting propagules and dead organisms (Demanèche *et al.*, 2001; Stenlid & Gustafsson, 2001; England *et al.*, 1997). Furthermore, targeting of rRNA would reveal the metabolically active members of the community (Baldrian *et al.*, 2012; Rajala *et al.*, 2011; Pennanen *et al.*, 2004). The detection of precursor rRNA, which contains the ITS regions, is affected by RNA turnover rates; some species of RNA are more prone to degradation at a higher rate than others (LaRiviere *et al.*, 2006).

The lifespan of precursor rRNA containing ITS is a few minutes (Koš & Tollervey, 2010), as a result of post-transcriptional processing to remove ITS1 and ITS2 to have mature rRNA genes. It has since been shown that fungal ITS can be detected in RNA pools of fungal precursor rRNA molecules (Rajala *et al.*, 2011; Anderson & Parkin, 2007).

The primers selected for sequencing can have a profound effect on the organisms detected. Fungal-specific primers are often not fungal specific enough. For example, the fungal specific fITS7 (Ihrmark *et al.*, 2012) – ITS4 (White *et al.*, 1990) primer pair amplifies the ITS2 region of fungi and plants, though much less plant material than using gITS7 (Ihrmark *et al.*, 2012) – ITS4 pair. To use a general enough primer set to target as many taxa as possible has the trade off of not being able to detect other taxa of interest. For example, fITS7 – ITS4 cannot be used detect *Cantharellus* species and excludes many *Penicillium* species. Furthermore, primers that target the Basidiomycota and Ascomycota are not necessarily appropriate for studies of Glomeromycota (Krüger *et al.*, 2009).

The ITS2 region, in contrast to the ITS1 region, is fairly equal in length (250-400 bp). The ITS1 region of some species is subjected to insertions, making the PCR fragment rather long (Johansson *et al.*, 2010; Martin & Rygielwicz, 2005). The length variation of the entire ITS region may lead to PCR amplification biases against long amplicon fragments in mixed communities (Lindahl *et al.*, 2013; Ihrmark *et al.*, 2012) or may exclude some fungal taxa due to sequence length limitation (e.g. Illumina sequencing has been limited to sequencing ~250 bp). Additionally, one of the problems with targeting the entire ITS region is the potential for generation of chimeric sequences due to recombination at the conserved 5.8S gene region (Nilsson *et al.*, 2008). To overcome these and other biases, some studies have sequenced only the ITS1 region (Unterseher *et al.*, 2011; Jumpponen & Jones, 2009) or the ITS2 region (Menkis *et al.*, 2015; Ihrmark *et al.*, 2012).

However, not all species may be taxonomically resolved to species level by sequencing such a short region. Other gene regions (Krüger *et al.*, 2009), or even multiple genes are required for resolution of phylogenetic relationships among members of specific taxonomic groups (Schoch *et al.*, 2006; Spatafora *et al.*, 2006; Wang *et al.*, 2006). For example, *Fusarium* species have low resolution for the ITS2 region (O'Donnell & Cigelnik, 1997), and thus other genetic regions have been needed to resolve different *Fusarium* species (Wang *et al.*, 2011). However, using multiple target sequences for environmental samples may not help to increase the taxonomic resolution in the analysis of the fungal community since different sequences are drawn randomly from the pool of extracted template DNA.

One of the limitations with available databases, such as NCBI Genebank (Benson *et al.*, 2013) and UNITE (Abarenkov *et al.*, 2010), to search sequences, is the lack of reliable and informative reference sequences or reference sequences in general, to compare query sequence data against. Sequencing environmental samples tend to generate a large pool of sequences for taxa for which no information exists. There are vast numbers of microorganisms that have not been cultured from these samples, or have been cultured but are undescribed. These taxa are thus under represented in the databases. While the UNITE database is a well-curated database, there are limited sequences available for Ascomycota species.

To generate reference genomes, it is plausible to pick single colonies from the foliage surface and sequence them. In this way, lesions or fungal fruiting structures that can be identified morphologically can be linked to a species nucleotide sequence. Of course, it would be less precise than culturing methods because there could be contamination by other organisms. But the benefit is phenotypic metadata (e.g. characteristics of lesions, and their incidence and prevalence) that can be affiliated with a sequence, which is especially useful if the fungus has not yet been described. Moreover multiple target sequences can be obtained from the same unit, thereby increasing the potential for taxonomic resolution. However, there may be some limitations, e.g. not all lesions or fruiting bodies have been attributed to a species taxonomically. It is therefore possible that morphologically identified taxa can only be resolved to a taxonomic rank, such as “Ascomycota sp” or “uncultured fungus clone”.

5 Objectives

The aim of this thesis was to investigate the effects of tree species diversity on foliar fungal species in European forests.

Specifically, the objectives of were to:

- I. Develop a method to study the effect of tree species diversity on fungal pathogen disease in large-scale research plots across Europe (Paper I).
- II. Determine the potential drivers of observed patterns of foliar fungal pathogen damages in mature forests across Europe (Paper II).
- III. Analyse fungal communities of Norway spruce needles in relation to tree species diversity in four mature forests (Paper III).
- IV. Compare methods for investigating the fungal community of birch and the effect of tree species diversity (Paper IV).

We hypothesized that 1) the prevalence of fungal pathogens is reduced with tree species diversity, perhaps as a result of dilution effects or associational resistance (Papers I, II); 2) the fungal community is affected by tree species diversity, such that diverse fungal communities will be found in the mixtures, rather than monoculture, perhaps resulting from horizontal transfer of fungal species among the tree species (Papers III, IV); 3) the fungal pathogen activity and fungal community diversity will decrease with latitude (Papers II, III); 4) the active fungal community will be sensitive to the micro-environment created by tree species richness and thus their diversity and composition will change along the gradient (Paper III); and 5) the fungal community of birch leaves will be more accurately assessed through molecular approaches, particularly fungal species that do not form visible structures (Paper IV).

6 Material and Method

A more detailed description of the methods used can be found in the different papers enclosed in the thesis and in the references cited therein.

6.1 Study Areas

The study areas chosen for this thesis were mature European forests and one tree plantation experiment (Figure 1). The mature forests defined in this thesis are those that are in the late to mid stem exclusion stage, understory reinitiation stage or old-growth stage of stand development (Oliver & Larson, 1990). These forests are also considered ancient forests, meaning they have been continuously forested at least since the oldest available land-use maps (Baeten *et al.*, 2013). To determine the effects of tree species diversity on foliar fungal pathogens (Paper II) and communities (Paper III) in mature forests, research sites were established as described in Paper I. Six forests span major European forest types along the gradient from Mediterranean forest to the boreal forest, and differed in their tree species composition and richness (Figure 1, Table 2). The tree species pool overall comprised 16 tree species that were regionally common and/or economically important (Table 2). Specific selection criteria were met to select plots for this study. Standardized plots of 30×30 m were delimited within each forest, within which a tree species richness gradient ranging from monoculture to five-species mixtures was created. Each richness level contained varying tree species assemblages. For example in Finland, a two-species mixture level can contain a combination of Scots pine-Norway spruce, Scots pine-birch and Norway spruce-birch. Focal trees of the largest diameter at breast height were randomly selected within each plot: six trees in monoculture plots and three trees per species in mixtures. Sampling was conducted over a two-week period for each forest site during the growing season, in 2012 and 2013. In total, 209 plots were sampled.

Table 2. Description of study sites used in this thesis.

Forest Type	Country, Region	Coordinates Latitude, Longitude (°)	Topography, Altitude ¹	MAT, MAP ²	Study area size (km x km)	Stand developmental stage ³	Species richness levels	Sampling period	Number of plots	Number of trees sampled	Tree species pool
Plantation	Finland, Satakunta	61.4, 21.6	Flat, 20-50 m	5.4 °C, 550 mm	1.5 ha	Stand initiation	5	August 2011	25	55	<i>Pinus sylvestris</i> , <i>Picea abies</i> , <i>Betula pendula</i> , <i>Alnus glutinosa</i> , <i>Larix sibirica</i>
Mature, Boreal	Finland, North Karelia	62.6, 29.9	Flat, 80-200 m	2.1 °C, 700 mm	150 x 150	Mid/late stem exclusion, Understory reinitiation	3	August 2012	28	180	<i>Pinus sylvestris</i> , <i>Picea abies</i> , <i>Betula pendula</i>
Mature, Hemiboreal	Poland, Białowieża	52.7, 23.9	Flat, 135-185 m	6.9 °C, 627 mm	30 x 40	Mid/late stem exclusion, Understory reinitiation	5	July-August 2013	43	378	<i>Pinus sylvestris</i> , <i>Picea abies</i> , <i>Betula pendula</i> , <i>Carpinus betulus</i> , <i>Quercus robur</i>
Mature, Beech	Germany, Hainich	51.1, 10.5	Mainly flat, 500-600 m	6.8 °C, 775 mm	15 x 10	Understory reinitiation, Old growth	4	July 2012	38	296	<i>Picea abies</i> , <i>Acer pseudoplatanus</i> , <i>Fagus sylvatica</i> , <i>Fraxinus excelsior</i> , <i>Quercus petraea</i> / <i>Quercus robur</i>
Mature, Mountainous beech	Romania, Râșca	47.3, 26.0	Medium-steep slopes, 600-1000 m	6.8 °C, 800 mm	5 x 5	Mid/late stem exclusion, Understory reinitiation	4	July 2013	28	207	<i>Abies alba</i> , <i>Picea abies</i> , <i>Acer pseudoplatanus</i> , <i>Fagus sylvatica</i>

Forest Type	Country, Region	Coordinates Latitude, Longitude (°)	Topography, Altitude ¹	MAT, MAP ²	Study area size (km x km)	Stand developmental stage ³	Species richness levels	Sampling period	Number of plots	Number of trees sampled	Tree species pool
Mature, Thermophilous deciduous	Italy, Colline Metallifere	42.2, 11.2	Medium-steep slopes, 260-525 m	13 °C, 850 mm	50 x 50	Mid/late stem exclusion	5	June 2012	36	292	<i>Castanea sativa</i> , <i>Ostrya carpinifolia</i> , <i>Quercus cerris</i> , <i>Quercus ilex</i> , <i>Quercus petraea</i>
Mature, Mediterranean mixed	Spain, Alto Tajo	40.7, -1.9	Flat-medium slopes, 960-1400	10.2 °C, 499 mm	50 x 50	Mid/late stem exclusion, Understorey reinitiation	4	June 2013	36	252	<i>Pinus nigra</i> , <i>Pinus sylvestris</i> , <i>Quercus faginea</i> , <i>Quercus ilex</i>

1. Altitude in meters above sea level.

2. MAT: mean annual temperature, MAP: mean annual precipitation.

3. Stand developmental stages according to Oliver and Larson (1990).



Figure 1. Map of six European forests and one experimental forest plantation. Filled circles represent mature forests in Papers I, II and III. Open circle represents the experimental forest plantation in Paper IV.

One plantation from the global network of tree diversity experimental research sites was established in 1999, and was incorporated into this thesis in Paper IV (Scherer-Lorenzen *et al.*, 2007; Scherer-Lorenzen *et al.*, 2005). The Satakunta Area 1 plantation is situated in the boreal zone in southwest Finland (Figure 1). At the time of sampling, the trees were about 15-years-old. The tree species pool consisted of five tree species that are economically important for Finland, functions as a nitrogen-fixing species and represents an exotic species (Table 2). Species mixtures were composed in such a way that they represent a gradient from completely coniferous forest (pine, spruce and larch) through mixed conifer/deciduous stands to deciduous ones (birch and alder), for a total of 19 treatments. The tree species gradient consisted of monocultures of each species, two-, three- and five-species mixtures, where tree species were mixed in equal proportions. Plots were 20 m x 20 m and contained 13 rows with 13 seedlings in each row. In August 2011, five trees of each species were randomly selected in each plot.

6.2 Studies of Fungal Pathogens and Communities

6.2.1 Foliar Pathogen Damage of Mature Forests (Papers I, II)

One of the many ecosystem functions targeted by designing a large-scale tree diversity experiment is the regulation of pathogen damage. To determine whether tree species diversity can mitigate the effects of fungal pathogens, foliage samples were assessed for damages from 16 tree species in the six mature forests. For each tree, two branches were cut from the southern exposition: one from the sun exposed part of the canopy, and one closer to the lower third. Leaves and conifer shoots were collected. In a total of 50 – 60 leaves or 20 shoots per tree were sampled.

Five damage types were assessed: oak powdery mildew, leaf spots, and unknown fungal damage type for the broadleaved tree species, and rust and needle cast for the conifer species. Visual inspection for these pathogen damage symptoms (or suspected damage caused by fungi) was conducted on fresh foliage within one day of sampling by one person. The proportion of sampled leaves or shoots with the respective damage types was recorded for each tree. There were instances where leaves had two types of damage, i.e. both leaf spots and powdery mildew, either on the same leaf or two different leaves. Therefore, to avoid over-counting the number of damaged leaves, the total number of leaves with damages, regardless of the type, was noted as well. Total pathogen damage was defined as the total number of leaves or shoots with any type of damage for each tree, regardless of the damage type. The final data set included 1605 trees.

6.2.2 Foliar Fungal Communities of Norway Spruce (Paper III)

Tree species diversity effects across a latitudinal gradient on the fungal communities in Norway spruce needles were tested. Current-year needles were collected from Norway spruce in Finland, Poland, Germany and Romania. The same trees and branches and shoots sampled for Paper II were used in this study. A total of ten shoots per tree were collected and dried, after which the needles detached from the shoots. Twenty needles were randomly selected per tree and washed in 0.1 % Tween-20 to remove surface debris. Needle samples were milled and subjected to DNA extraction with 3% CTAB buffer. The fungal ITS2 region was amplified using the primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990) that was uniquely barcoded for each sample. PCR amplicons were purified and mixed into equal mass proportion to generate two pools of samples (one pool from Finland and Germany, and another pool from Romania and Poland). These samples were 454 pyrosequenced.

6.2.3 Active Fungal Community of Norway Spruce (Paper III)

To study the active fungal community in Norway spruce needles, current-year needles were collected from the same 60 Norway spruce trees sampled in Finland in Paper II. Twenty needles were collected from the same shoots as previously mentioned but were immediately stored in RNALater to preserve RNA integrity. Needle samples were subjected to co-extraction of rRNA and rDNA. The fungal ITS2 region was amplified using the primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990) that was uniquely barcoded for each sample and prepared for 454 sequencing as above.

6.2.4 Foliar Fungal Community of Birch (Paper IV)

The fungal community of birch leaves were determined by two different methods. A molecular high-throughput sequencing approach of describing the community was compared to morphological assessments to determine tree species diversity effects. Furthermore, tree species diversity effects on specific fungal taxa were also tested.

Birch leaves, five from each of the four branches at two different levels of the canopy, 20 leaves in total, were collected from the plantation in Satakunta, Finland and dried at 60 °C for three days. Ten leaves from each tree were subjected to high-throughput community sequencing, and the remaining ten leaves were subjected to macroscopic assessment. The leaves were milled and DNA was extracted, fungal ITS2 region was amplified using the primers fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990) that was uniquely barcoded for each sample and prepared for 454 sequencing as above. The primer fITS7 was used instead of gITS7 because gITS7 preferentially amplified birch DNA.

As Hantsch (2013) previously described, fungal species on the leaf surface were identified to the species level by macroscopic analyses (Braun *et al.*, 2012; Brandenburger, 1985; Ellis & Ellis, 1985). Furthermore, using a stereomicroscope, fungal pathogen infestation was surveyed on the upper and lower leaf surface. The total damaged area caused by each fungus species was estimated (Hantsch *et al.*, 2013; Schuldt *et al.*, 2010).

6.2.5 Data Analysis

A variety of analysis methods were used in this thesis, to understand the effects of tree species diversity on foliar fungal species distribution. In Paper II, statistical models were used to disentangle to possible explanatory variables for pathogen damage from a complex hierarchical study design. The design was such that there were the six mature forests with the same richness levels in the different forests, replication of plots for each richness level within one forest,

replication of species composition within one forest and across the forests, and several trees (the sampling unit) within a plot. To further complicate the statistical model, tree species response was different for a tree species that was in monoculture or mixture. Thus to account for non-independence generalized linear mixed-effect models (GLMMs) were used that allowed for nested and crossed random-effect terms (Schielzeth & Nakagawa, 2013; Zuur *et al.*, 2009).

The fungal communities associated with Norway spruce needles, both from the four countries and the active community from Finland (Paper III) and the fungal community in birch (Paper IV) were analysed in similar ways. Sequence reads (“reads”) from the sequencing facilities were parsed using the bioinformatics pipeline SCATA (<https://scata.mykopat.slu.se/>). Reads were subjected to quality filtering to remove short sequences, low quality reads and those missing primer sequences. The remaining reads were clustered by single linkage clustering into operational taxonomic units (OTUs) at 98.5% similarity. Taxonomic identification of OTUs relied on nucleotide BLAST searches in the NCBI database (Altschul *et al.*, 1997) where the ITS homology for defining taxa were set to 98-100% for species level, 94-97% for genus level and 80-93% for order level.

In Paper III, fungal species diversity 1) across countries, 2) in each country for each tree species richness level, and 3) for fungal community type (i.e. active and total) was analysed using Hill numbers (Bálint *et al.*, 2015). Hill numbers are a way to measure and incorporate richness (Hill 0), exponent of Shannon Index (Hill 1) and inverse Simpson (Hill 2) (Legendre & Legendre, 1998). Fungal species diversity in Paper IV was estimated with Fisher’s alpha, a parameter of the log series model that is robust to sample size variation (Magurran, 2004; Fisher *et al.*, 1943). Analysis of multivariate abundance data by regression models is currently not possible for complex sampling designs, though statistical packages are available to perform analyses for simpler designs such as *mvabund* in R (R Core Team, 2013; Wang *et al.*, 2012). Thus, to analyse the community composition, the ordination method non-metric multidimensional scaling (NMDS) was used to visualize the fungal compositional variation among countries and among tree species richness levels with countries and community type. Analysis of similarities (ANOSIM (Clarke, 1993)) aided in determining differences in fungal community composition among tree species richness levels. Additionally, permutational multivariate analysis of variance (PERMANOVA (Anderson, 2001)) allowed partitioning of the variance contributed by the explanatory variables, and thus tested significance of the difference among countries, levels of tree species richness and community type. Though multivariate analysis cannot be easily

done, GLMMs can be applied to individual fungal taxa to analyse tree species diversity effects. Fungal communities can be influenced, not only by plot scale processes, but also by their local environment. To study any possible neighbourhood effects on specific fungal taxa in Paper IV, the proportion of birch in the immediate vicinity of the focal tree, i.e. the eight trees surrounding the focal tree, was determined and tested using GLMMs and linear mixed effect models.

7 Results and Discussion

7.1 Tree Species Diversity Effects on Pathogen Damage (Papers I, II)

The planning of large-scale research plots to study the forest ecosystem's services, including regulation of foliar fungal pathogens, led to the establishment in 2011 of a series of plots in six mature forests with various levels of tree species mixtures (Paper I). Besides a tree diversity gradient within each forest site (“country”), a latitudinal gradient between the 40 °N and 63 °N, corresponding to the different forest types was also established (Figure 1). The installed plots from Paper I permitted study of fungal pathogen damage (Paper II) and fungal communities (Papers III, IV) in the context of tree diversity and latitudinal gradients.

While developing a method to study fungal pathogens in these plots, measurements for other ecosystem services and functions were performed in parallel and sometimes on the exact same trees. The approach to “make all measurements in all plots” allowed the opportunity to correlate possible explanatory factors to patterns observed for foliar fungal pathogen damage on mature forest trees. These explanatory factors included leaf nitrogen content, carbon to nitrogen (C/N) ratio, specific leaf area, leaf thickness, leaf area index (Guan & Nutter Jr, 2002) and chlorophyll fluorescence that would indicate photosynthetic performance (Luque *et al.*, 1999), tree height, tree crown architecture, understory vegetation and leaf litter.

Decreased leaf nitrogen content and increased C/N ratio has earlier been found to correlate with decreased leaf spot disease severity on *Acer rubrum* (McElrone *et al.*, 2005). Tree height and canopy architecture creates vertical stratification that influences the light availability and microclimate within the canopy (Saikkonen, 2007). For example, Swiss needle cast, a foliage disease of Douglas fir (*Pseudotsuga menziesii*) caused by *Phaeocryptopus gaeumannii*

decreased needle retention in the upper crown, but not so much the lower crown (Shaw *et al.*, 2014). Mixed species stands naturally create these micro-environmental gradients. The understory vegetation may include alternate hosts for rust fungi such as *Chrysomyxa ledi* that can cause severe defoliation and even seed loss (Kaitera *et al.*, 2010; Crane *et al.*, 1998). Regardless of the trees in a mixture, the presence of such alternate hosts would lead to disease in Norway spruce. Lastly, the leaf litter composition may be a source of pathogen inoculum as observed for European ash (*Fraxinus excelsior*), in which infected fallen leaves serve as the primary habitat of the ash dieback pathogen, *Hymenoscyphus fraxineus* (Kowalski, 2006). Decreased litter input by ash trees in mixed stands would be a way to decrease inoculum load and maybe minimize the potentiation of the disease.

In Paper II, prevalence of foliar pathogen damage along a tree diversity and latitudinal gradient was determined. Foliage from 16 tree species (Table 1) were visually assessed for four types of fungal pathogen damage: leaf spots, powdery mildew, rusts and needle cast. Foliar fungal pathogens were detected on both conifers and broadleaved tree species in all six countries. The highest amount of pathogen-induced damage on foliage, regardless of the damage type, was detected on trees in Finland, while the lowest occurred in Spain and Romania.

The four damage types were present in the plots, though needle cast was the least prevalent. Needle cast would not be detected in the canopy, but would be found on the forest floor. It is likely that needles shed prematurely before the sampling of conifer trees in this study, which are typical symptoms of needle cast diseases (Hansen & Lewis, 1997), or during the sampling when the cut branches fall to the forest floor and needles detach, and thus the impact of needle cast pathogens would be underestimated. Different types of needle cast diseases can occur in these sampled forests. *Dothistroma* needle blight can infect Scots pine needles and *Gremmeniella abietina*, while primarily infecting and killing buds and shoots, can grow into needles and cause needle shed. *Rhizosphaera kalkhoffii* was found to infect Norway spruce needles and cause premature needle loss (Livsey & Barklund, 1992; Livsey & Barklund, 1985). Rust infections can also cause premature needle shedding. *C. ledi* (Crane, 2001) and *C. abietis* infect newly flushed Norway spruce needles (Murray, 1953). To increase the detection of needle cast the installation of litter traps and periodic collection of needles, preferably earlier in the vegetation season would be advised, as Livsey and Barklund (1992) had done to detect both *R. kalkhoffii* and *Lophodermium piceae*. Later in the vegetation season, it would be difficult to distinguish between senesced needles and damaged needles.

In Paper II, *C. ledi* was found on Norway spruce in Finland in all plots, and signs of *G. abietina* were observed in Spain and Finland on Scots pine. Major pathogens detected on of broadleaved species detected included *Erysiphe* sp [oak powdery mildew] on sessile oak (*Quercus petraea*) and pedunculate oak (*Quercus robur*) in Germany and Poland, respectively. The effect of each of these fungal species were tested to determine whether the damage they caused responded to tree species richness, but none were significantly influenced by tree diversity. One possible reason for this could be a result of site history. For example, powdery mildew was first detected in 1907 and caused severe outbreaks (Marçais & Desprez-Loustau, 2012). Over the last 100 years, oak trees have experienced the selective pressure of these pathogens, and those that are still alive are to some degree tolerant to the disease. The trees in the landscape today are either survivors from before the outbreaks or offspring of those that have survived.

A statistical modeling approach was used to study the correlation between tree species richness across all countries and prevalence of fungal pathogen damage on all tree species. Results revealed tree species richness – latitude interactions as significant factors (Figure 2), suggesting that the prevalence of foliar fungal pathogen damage may change when forests changed. The model parameter estimate was significantly positive, albeit small, indicating that there was a trend for increasing foliar damage with increasing latitude. Despite the overall positive trend, tree species richness played a weak role in mitigating the effects of fungal pathogen damages; it was confounded by site-specific factors. There is building evidence that other factors are important to take into consideration that are specific to the pathosystem in question, regardless of the diversity of tree species, which Vacher *et al.* (2008) found not to be a major determinant for pathogen richness. Instead, host abundance and composition of host species have direct effect on pathogen richness. Future studies should also consider the importance of host genetic diversity within a host species (Bálint *et al.*, 2013; Mundt, 2005; McCracken & Dawson, 2003; Garrett & Mundt, 1999), genetic diversity of the pathogen (Bengtsson *et al.*, 2012; Ahlholm *et al.*, 2002), understory diversity especially in relation to rust species, and the influence of climatic factors. Vacher *et al.* (2008) found that high winter and low summer mean temperatures positively correlated with disease. Additionally, Hantsch *et al.* (2014a) found inter-annual variation in weather conditions to affect foliar fungal species richness and fungal disease infections, though patterns were not consistent for the two tree species examined. Thus forest management to diversify forests to protect against a diversity of pathogens may be less effective than considering other variables and a limited array of pathogens.

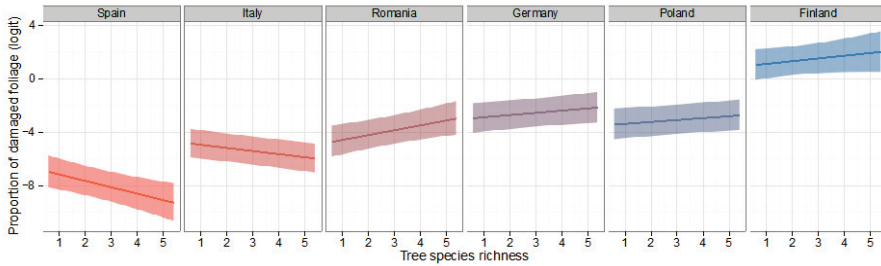


Figure 2. Predicted proportion of foliar fungal damage along a tree species richness gradient across mature European forests. The odds of having fungal damaged foliage in higher species richness levels relative to the odds of foliage damage in the monoculture (solid line) was computed for each country. The higher the logit values are, the greater the amount of pathogen damage. The effect of tree species richness was extrapolated beyond the observed range (always up to five, whereas it was three or four in some countries). The shaded area shows the corresponding confidence interval.

The experimental design of future studies should also consider the role of management has in promoting damages to trees. Forest management techniques can create infection courts whereby fungal pathogens overcome the natural constitutive barriers, physical and chemical, that plants have. During tree harvesting processes, stumps exposed, for example of Norway spruce, serve as an infection court for basidiospores of *Heterobasidion annosum* (Redfern & Stenlid, 1998), and the mycelium can subsequently spread to other trees through root contacts (Stenlid & Redfern, 1998). Wounds created by machines or damage to crop trees during pre- or commercial thinning operations, can be routes for decay fungi such as *Stereum sanguinolentum*, *Amylostereum areolatum*, and *Nectria fukeliana*, among others (Arhipova *et al.*, 2015; Vasaitis *et al.*, 2012; Zeglen, 1997). Residual crop trees, or trees left in the forest instead of removal to processing plants, can be breeding habitats for insects that vector pathogens. Examples of the devastating effects of leaving diseased elms in the forest have been observed in Europe. *Scolytus* beetles that carry the Dutch elm disease fungus *Ophiostoma ulmi* and *O. novo-ulmi* breed in the dying trees and fly to new trees infecting them as well, thus continuing the cycle (Webber & Brasier, 1984). Diseased trees may be removed, as in the case of dead/dying ash, but to leave behind the leaves and rachises where the pathogen *Hymenoscyphus fraxineus* is found is not advisable. Fruiting and spore dispersal can occur long after the leaves have detached from trees and the removal of trees from the forest (Kirisits, 2015).

7.2 Foliar Fungal Communities of Norway Spruce (Paper III)

Endophytic communities of different tree species spanning a latitudinal gradient were shown to exhibit latitudinal effects in fungal species composition and fungal species diversity (Arnold & Lutzoni, 2007). However, within one tree species, an opposite trend was observed (Millberg *et al.*, 2015) whereby fungal diversity of Scots pine needles increasing latitude. Thus, needle associated fungal communities of Norway spruce was expected to be differently diverse along a latitudinal gradient. In contrast to both of the studies listed above, the work in this thesis found no effect of a latitudinal gradient on fungal diversity.

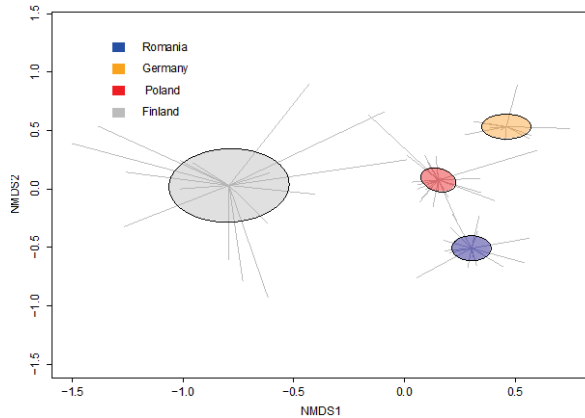


Figure 3. Non-metric multidimensional scaling (NMDS) ordination of the fungal community in the four countries in Paper III. Stress value was 0.17, indicating a reliable fit of the data. The 95% confidence intervals of the country's centroid is a coloured ellipse. Lines connect the pooled plot-level samples to the country's centroid. Romania is represented by blue, Germany by orange, Poland by red, and Finland by grey.

While the fungal diversity did not differ along a latitudinal gradient, fungal community composition differed drastically. The fungal community in Finland was the most separated from communities from the other three sites (Figure 3). The separation visually reflects the geographic location of each of these samples, which may result in differences in climate factors (U'Ren *et al.*, 2012). However, differences in fungal community composition may also reflect on the genetic origin of Norway spruce. Recolonization of Norway spruce since the last Ice Age occurred from several distinct refugia, which can be traced from the pollen records and fossil macro-remains (Huntley & Birks, 1983). Genetic studies have revealed that the entire northern range of Norway spruce was colonized from a single glacial refugium located in European Russia that established in Scandinavia following the glacial retreat,

corresponding to Finland in Paper III (Tollefsrud *et al.*, 2008). Other refugia contributed to the establishment of Norway spruce in central Europe. Several pockets of Norway spruce populations between the Alps and Carpathian Mountains may have been sources for colonization in Germany and Poland, and differently for Romania (Huntley & Birks, 1983). In Paper III, the fungal communities of Norway spruce in Germany, Poland and Romania were more similar to each other than the community in Finland. The consequence of these different origins of the host tree may contribute to variation in the fungal communities that established with these different Norway spruce populations, and the fungi may have subsequently undergone independent speciation events in their respective settlement areas, which has been previously proposed in other plant species (Higgins *et al.*, 2007).

Within single needles of Norway spruce, up to 25 fungal taxa were identified from symptomless needles (Müller *et al.*, 2001). One study of the fungal community in Norway spruce needles suggests that there could be approximately 200 fungal taxa from one tree (Müller & Hallaksela, 2000). When Sieber (1988) sampled Norway spruce needles from sites in Switzerland, he isolated close to 100 fungal taxa. Studies with using culture-independent approaches from decaying needles found 26 taxa belonging to Ascomycota and Basidiomycota (Korkama-Rajala *et al.*, 2008). In Paper III, 513 OTUs were detected. Different studies may yield variable numbers of taxa depending on the methods used. Culture-dependent methods can give a more restricted estimation of the fungal diversity, while the number of taxa detected using culture-independent methods can vary depending on the methods used to analyse the DNA markers, i.e. Sanger sequencing individual clones, performing restriction digestion, or high-throughput sequencing.

The more abundant fungal taxa in the community were examined for potential correlation with the tree species richness effects. Seven OTUs were either positively or negatively affected by tree diversity but these same patterns were not consistent among all four counties. Positive effects were seen for OTU_7 (*Aureobasidium pullulans*) in Germany, but nowhere else, despite its presence in the other countries as well. The rust species *C. ledi* (OTU_1) was prevalent in Finland, but was not correlated with any diversity effects, consistent with its long distance dispersal of spores, at least from beyond the 30 m x 30 m plot boundary. It is likely that the needles were infected by spores dispersed from the alternate host *Ledum palustre*, that is locally present in wet sites in the coniferous forest but that grew outside the sampling plots.

A majority of these OTUs could not be identified to species level by sequencing ITS2. This is one of the limitations of sequencing such a short region, though including the entire ITS region would have had its drawbacks in

terms of community biases due to sequence length variation in the ITS1 region and would thus hinder comparison of relative abundance of the fungal community (Lindahl *et al.*, 2013).

The importance of understanding the diversity and composition of the fungal community (both endophytes and epiphytes) and the patterns that shape their distribution cannot be understated. Some fungal species are known to associate with important fungal pathogens. For example, mycoparasites *Ampelomyces/Phoma* sp have been observed to be in close association with oak powdery mildew species (Topalidou & Shaw, 2015). Foliar endophytes of Norway spruce have been found to be useful to protect trees against insect pests, such as spruce budworm (*Choristoneura* sp) (Miller, 2011). However, the fungal community of Norway spruce twigs and needles can be drastically affected following infestation by the spruce bud scale (*Physokermes piceae*) (Menkis *et al.*, 2015). The potential use of species in foliar communities as biocontrol agents against forest pathogens has been considered an attractive option in integrated pest and disease management programs (Witzell *et al.*, 2014). Certain limitations, however, make their practicality less tractable such as their cryptic lifestyles, stability, robustness and impact on the environment.

7.3 Active Fungal Community of Norway Spruce (Paper III)

Studying metabolically active organisms by extracting their RNA offers the possibility to address which taxa are part of the active community and what activity they could be performing, and not only what is present; rRNA is constantly synthesized by active cells. The active members include those that respond the quickest and strongest to changes in the environment (Pennanen *et al.*, 2004). In this part of Paper III, the active fungal community was expected to be sensitive to the environment created by tree species richness, whereby their diversity and composition will change along the gradient. To address this, the fungal community was sequenced to reveal the functionally active fungal species in Norway spruce needles. The corresponding total fungal community was also sequenced to determine those taxa that were present in the same samples. We found that the number of OTUs shared by each community type was quite similar; both the active and total community shared 286 OTUs out of 313 OTUs. While fungal community composition in terms of OTU identity was similar, the relative abundance of the OTUs in the different communities may be the factor driving the small but significant variation in the fungal community defined by their activity and presence. The total community accounted for approximately 50,000 reads in 303 OTUs while the active community comprised about 17,500 reads in 293 OTUs. Despite the difference

in relative abundance, both communities were not differentially diverse and there was no observed correlation between tree species richness and either fungal community type. This is in contrast to what has previously been observed for soil microbial communities. Baldrian *et al.* (2012) found differentially diverse communities revealed by sequencing DNA and RNA, despite similar richness in taxa. They also observed that some active species were sometimes less abundant or were not even detected in the DNA community. They further observed that the composition and activity of the active soil fungal community varied depending on the soil type, which is consistent with Rajala *et al.* (2011) who found in decaying wood different composition patterns of metabolically active fungi. Thus there was an expectation in Paper III that the active community would be able to respond to changes in their local environment. The fact that no effects were seen may reflect the uneven distribution of taxa in the landscape perhaps due to dispersal limitations of some key species, considering that the plots of Norway spruce were between 150 m to 90 km apart in Finland. Perhaps the local environment of the trees influence the fungal communities as previously discussed.

In this study, the most abundant OTUs in a fairly robust dataset were tested for tree diversity effects. Interestingly one taxon, OTU_16 (*Heterobasidion parviporum*), had a negative relationship with tree species richness; it was less likely to detect *H. parviporum* in mixture plots than in monocultures. *H. parviporum* is an important root and butt rot pathogen in coniferous forests and causes severe damages in Norway spruce stands (Asiegbu *et al.*, 2005). The finding in this study was not a surprising one, however and confirms what has been observed from fruiting body inventories. The fruiting bodies of *H. parviporum* are affected by amount of spruce in a stand (Gonthier *et al.*, 2001). Furthermore, the spores of *H. parviporum* do not disperse very far from their inoculum source, typically within 5 m (Möykkynen *et al.*, 1997) and detection of this fungus in spruce needles would thus suggest that the spore source is likely within the plot, rather than outside. Thus the lack of susceptible hosts in plots where Norway spruce was in mixtures with other tree species, and hindrance to dispersal, is a clear demonstration of the dilution effect. The observed relationship between the fungus and tree species richness has also been previously observed (Thor *et al.*, 2005; Huse & Venn, 1994; Piri *et al.*, 1990). *H. parviporum* (OTU_16) was also detected in the total community of these samples, but was about two times as abundant in the active community than the total community. It was also found in the dataset analysed for Paper III section 7.2 of this thesis, though rarely, occurring in three of the 16 samples at a frequency of less than 0.12%.

Another important pathogen of Norway spruce needles was detected in the active community, OTU_1 (*Chrysomyxa ledi*). *C. ledi* did not exhibit any response to tree species richness. This heteroecious rust fungus requires the shrub *Ledum palustre* as the alternate host to finish its life cycle (Butin, 1995). *L. palustre* is a common evergreen shrub in the northern latitudes and in peatlands of Finland. As previously described, and in contrast to *H. parviporum* that has dispersal limitations, *C. ledi* may be infecting Norway spruce from an inoculum source outside of the studied plots in Finland. While *L. palustre* was not detected within the plots, infection points to a source elsewhere.

In this part of Paper III, the active fungal community was determined to be not affected by environmental effects created by tree species mixture. However, one important fungal pathogen was shown to be present and active in Norway spruce needles. At the scale of this study, namely examining plot-scale responses, it may not be appropriate to study the activity of the fungal community that may be more influenced by local effects. Perhaps studying the diversity of the surrounding trees and determining the proportion of those trees that are Norway spruce or other tree species may help to disentangle local diversity effects.

7.4 Foliar Fungal Community of Birch (Paper IV)

In Paper IV, the fungal community of birch leaves was evaluated to determine whether it can be more accurately assessed through molecular approaches compared with morphological assessments to reveal tree species diversity effects, either at the plot level or locally within the neighbourhood of focal trees. The most dominant taxon found by sequencing ITS2 was OTU_3 *Venturia ditricha* or *Fusicladium peltigericola*. Sequencing this short ITS2 region, and even including ITS1, would preclude further species delimitation. The genus *Fusicladium* is recognised as anamorph of *Venturia* (Crous *et al.*, 2010) and both species are considered sister taxa, with very high sequence homology. Sequencing other genes may further shed light on the identity of OTU_3. However in a mixed environmental sample, it may be difficult to target just *V. ditricha* or *F. peltigericola*. Culturing these fungi from other sources and comparing their reference sequences would be more informative to design primers to specifically sequence both of these species in the leaf samples. *Venturia ditricha* was the second most common species found by macroscopic assessment of leaves. Another species detected by visual methods was *Discula betulina*. However, *D. betulina* was not detected in the sequencing dataset. One reason for this could be that the primer ITS4 does not match the

primer-binding site and thus *D. betulina* DNA would not be amplified or sequenced. *Atopospora betulina* was detected on leaves visually, but was also absent from the sequencing data. There are no available reference sequences for this species in the reference database, which highlights one of the major limitations of sequencing from environmental samples; identification of taxa is only as good as the databases available (Weinstock, 2012).

The sequencing of the ITS2 region approach revealed fewer OTUs than sequencing fungi from Norway spruce needles in Paper III, 45 OTUs in the birch dataset of 55 samples compared with either the active or total community in Finland with the same number of samples (Paper III and section 7.3 of this thesis) that had 293 and 303 OTUs, respectively. It could be a reflection of the decreased diversity in birch, or broadleaves in general, though unlikely the case. Leaves of a single beech tree (*Fagus sylvatica*) host about 400 taxa (Cordier *et al.*, 2012), whereas over 2000 OTUs were detected in poplar (*Populus balsamifera*) (Bálint *et al.*, 2015). This may reflect more of a methodological constraint; the primers used in this study were the fITS7 – ITS4 pair. In particular, fITS7 was designed to be more specific for fungi (Ihrmark *et al.*, 2012), than the gITS7 used in Paper III, though detected an overwhelming amount of birch sequences. The most abundant non-fungal OTU with the highest amount of sequence reads was one birch that accounted for 75% of the sequence reads that passed quality filtering. Fungal OTUs may thus have been under sequenced, though the rarefaction curves in Paper IV would suggest that sampling of the community was sufficient.

Fungal community composition of the sequencing data was distinct among the tree species richness levels, though differences were weak. There were no observed differences in the diversity of the communities from each of the richness levels. Testing the dominant OTUs revealed only one (OTU_7, Dothideales sp) that was negatively affected by tree species richness. Interestingly, the local neighbourhood of birch positively affected this same taxon. The increased proportion of birch in the vicinity of the sample birch tree resulted in the increased prevalence of OTU_7. While it may be interesting to speculate widely about the ecological role of OTU_7 as a generalist or specialist fungus, so little is known about the identity of the taxon except that it is a Dothideales sp. Needless to say, tree diversity effects, at the plot scale for tree richness or at the local scale with neighbourhood analysis, was not a general pattern observed. Furthermore, while more fungal taxa were detected by sequencing than by visual assessments, neither informed the tree species richness effects.

8 Conclusion

The work in this thesis has focused on two important microbial components of foliage of forest trees: fungal pathogens and fungal communities. Management of forests for the future in a changing climate cannot consider tree species diversity alone. As highlighted in this thesis, tree species identity effects and environmental factors also influence fungal communities and pathogens. There is a need to consider host heterogeneity such as phenotypic and genetic diversity within and among trees species (Jules *et al.*, 2014; Bálint *et al.*, 2013; Ahlholm *et al.*, 2002), structural diversity of the forests (Saikkonen, 2007; Castello *et al.*, 1995), management factors that influence stand age, density and composition (Bulman *et al.*, 2013; Helander *et al.*, 2006) and landscape factors such as environmental fragmentation (Condeso & Meentemeyer, 2007; Helander *et al.*, 2007). Perhaps by studying fungal pathogens and fungal communities of clonal populations of tree species and their respective responses to infection (Hantsch, 2013; Danielsson *et al.*, 2011), one can better understand the roles of host genetic diversity and environment in mitigating the effects of fungal infections.

Forest management tends to make fungal pathogens the enemy. However, fungal pathogens have their place in the ecosystem as drivers of species composition shifts (Holah *et al.*, 1997) and as agents of plant species diversity (Bever *et al.*, 2014). From an ecological point of view, pathogens should be maintained in the landscape, though may not be an economically sound strategy from the forestry perspective. Invasive fungal pathogens, however, pose a special threat to forest ecosystems. They lack the co-evolutionary history with their host in the new environment that they have invaded and established. As a consequence, there could be widespread decimation of an entire species or family of species, regionally or globally, as exemplified by laurel wilt disease, ash dieback and Dutch elm disease, (Smith *et al.*, 2009;

Mayfield *et al.*, 2008b) with cascading effects ecological effects (Mitchell *et al.*, 2014; Snyder, 2014; Jönsson & Thor, 2012).

Studies of fungal species distribution should be considered across varying spatial scales. Fungal species disperse within small areas and across landscapes, and not just the artificial boundaries of forest plots. The observed limited effect of tree diversity in this thesis work is perhaps a result of three things: 1) insufficient replication of tree species composition to disentangle species identity effects, and the lack of consideration for 2) the spatial scale in which fungi disperse and 3) confounding effects of environmental conditions on fungal species. The cryptic lifestyle of many of the fungal species here precludes understanding the spatial scale necessary to manage for these organisms. A step back to study the spore dispersal patterns in the forest landscape (Edman *et al.*, 2004) and a more comprehensive sampling scheme within one forest site, taking into account various factors that can affect foliar fungal species, can provide a better understanding of the distribution of the fungal community. Fungi may be studied through the establishment spore traps or high-throughput sequencing of diverse environmental samples (not only tree leaves, but also forest soils) with the goal to measure and monitor forest diseases.

The future of applying next generation sequencing methods is to go beyond single genes for identifying fungi. Constant development of next generation technology is ongoing. What began with cloning and Sanger sequencing of environmental samples has developed to include a vast array of sequencing platforms (454, Illumina, PacBio and IonTorrent). Targeted sequencing of the rRNA gene has been a powerful tool to assess the microorganisms that are present in a given habitat. What is still lacking from this approach are functional and genetics aspects. Not all sequence reads match sequences in databases. A majority of organisms have not been well studied, particularly those that are not yet cultured or have fastidious growth conditions, and thus hindering the ability to characterize them or sequence them. Consequently no reference genomes are available. On the other hand, there are also lots of sequences in the databases for organisms for which we know nothing about. Inference about their ecological function is still limited but may be improved. Moving beyond single gene sequencing is necessary. Metagenomic analysis, and comparison of gene and predicted gene product information against databases such as KEGG (Kyoto Encyclopedia of genes and genomes) and CAZy (carbohydrate-active enzymes) of members of the community is the next step. In the future, the interaction among the species, and between species and hosts, can be more easily studied in a holistic and organic way.

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