Bark Beetles Facilitate the Establishment of Wood Decay Fungi

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Cover: Norway spruce high stumps and Norway spruce stem sections outside and inside a cage (photo: Y. Strid)

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Abstract

Forests in the northern hemisphere are largely dominated by conifers and provide a key habitat for a multitude of species. Wood decay fungi, i.e. basidiomycetes, are important for nutrient cycling. Saproxylic insects may facilitate the fungal colonization by opening up bark layer, provide a court for introduction, or they can specifically or loosely vector fungal propagules to the substrate.

The aim of the thesis was to clarify the role of insects for dispersal of fungal spores and propagules to Norway spruce stem sections, determine whether mycelial establishment was aided by holes in the bark created by the bark beetle, examine the early succession of fungal diversity in dead wood, and determine whether the hibernation environments for *Ips typographus* have an impact on the fungal community dispersed by the bark beetle.

Further analysis was conducted on wood material from high stumps and stem sections, mycelia from high stumps, and bark beetles from high stumps, stem sections, standing trees and forest litter. Pure culture isolation, T-RFLP, cloning and 454-sequencing were methods used to explain the fungal community composition.

It was concluded that (i) bark beetles contribute to the establishment of wood decay fungi and act as random vectors; (ii) the fungal community vectored by the bark beetle is depending on the hibernation environment; (iii) beetle entrance and emergence holes on their own have little or no effect on the substrate availability for air-dispersed fungal species; (iv) the fungal community changes drastically in newly created dead wood and the ecological interaction between fungi and dead wood is complex; and (v) the complexity of the fungal community detected is influenced by the method used to analyze it.

Overall, bark beetles have a major impact on fungal dispersal and colonization success of wood-decay fungi.

Keywords: Fungal succession, Wood-decay fungi, Bark beetle, *Picea abies*, T-RFLP, 454-sequencing

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Dedication

To myself, for finally being ready… Family and Friends

Be strong enough to stand alone, be yourself enough to stand apart, but be wise enough to stand together when the times comes

Mark Amend

Contents

List of Publications

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

I Ylva Persson, Katarina Ihrmark, Jan Stenlid (2011). Do bark beetles facilitate the establishment of rot fungi in Norway spruce? *Fungal ecology ournal* 4, 262-269.

II Ylva Persson, Rimvydas Vasaitis, Bo Långström, Petter Öhrn, Katarina Ihrmark, Jan Stenlid (2009). Fungi vectored by the bark beetle *Ips typographus* following hibernation under the bark of standing trees and in the forest litter. *Fungal Microbiology* 58, 651-659.

III Ylva Strid, Martin Schroeder, Björn Lindahl, Katarina Ihrmark, Jan Stenlid. Bark beetles have a decisive impact on fungal community in Norway spruce stem sections. (Manuscript).

IV Ylva Strid, Katarina Ihrmark, Jan Stenlid. The primer fITS9 avoids chimera formation when amplifying ITS in a bark beetle DNA background (Manuscript).

The contribution of Ylva Strid to the papers included in this thesis was as follows:

I Collecting samples and molecular work in the laboratory with the advice from supervisors. Analysis of data and writing of manuscript together with supervisors.

II Participating in pure culturing and analyzing of these samples together with Rimvydas Vasaitis. All molecular work in the laboratory. Analyzing of data and writing of manuscript assisted by co-authors.

III Participating in the experimental design assisted by supervisors. Implemented of field study assisted by supervisors. All laboratory work. Analyzing of data and writing the manuscript assisted by co-authors.

IV All molecular work. Analysis of data and writing the manuscript together with the supervisors.

1 The forest in Sweden

Forests in the northern hemisphere are largely dominated by conifers and provide a key habitat for a multitude of species. In the Fennoscandian forest region, about 30% of the 25 000–30 000 multicellular forest species depend on dead or dying wood to be able to complete their life cycle (de Jong *et al.* 2004). The most species-rich groups of saproxylic organisms in this region are fungi and insects, which are represented by more than 2500 and 3000 taxa, respectively (Petersen 2003; de Jong *et al.* 2004). Microorganisms such as bacteria and fungi carry out more than 95% of the litter decomposition and are the most important decomposers of lignified woody compounds (Persson *et al.* 1980).

The forestry industry in Sweden is based on the production of timber, pulp and paper. However, at the same time the forest should also fulfill a multitude of other roles, including CO_2 sequestration, production of non-forestry goods such as berries and mushrooms, harboring biodiversity, and provide recreation such as hiking and hunting. The public is allowed to use the production forest as a place for recreation owing to “The right of public access”. This is a unique institution that gives the public the right to roam the forests as long as they show consideration for the landowners' rights and for the other people in the countryside in accordance with the Swedish Environmental Protection Agency. This means that the public are allowed to use all private and public forest as long as they do not damage the trees and bushes or disturb the wildlife.

A range of different landscapes exist in Sweden (Table 1); however, the majority of the land is suitable for forest production owing to the prevailing climatic conditions of the northern hemisphere (Loman 2011).

Table 1. *Land use in Sweden* .

Swedish land use in Millions of Ha	
22.5	Productive forest
4.4	Bog and marsh
0.9	Rock surface
3.5	Mountains and Alpine coniferous forest
3.5	Cropland and grazing
4.2	Protected areas*

*Protected areas within National parks and Nature reserves

In total, the standing volume is estimated to 2.9 billion cubic meters of productive forest land, which is dominated by Norway spruce (*Picea abies* L. Karst) (42%) and Scots pine (*Pinus sylvestris*) (39%). Birch (*Betula* spp.) is the most common deciduous tree species (12%) (Loman 2011).

As a country, Sweden has a long history of using resources taken from a naturally regenerated forest environment and the forest has been affected indirectly by human activity for centuries. Various products for domestic use, such as wood fuel, bark for food and fodder, and wood for fencing, were taken out of the forest nearby the villages and during the summers the farmers used the forests as pastures for their cattle (Linder & Östlund 1998). In the late 18th century, the farmers started to use the forest beyond the nearby grazing area for the local production of potash and charcoal to support the iron works (Linder & Östlund 1998). During the industrial revolution the potash produced from deciduous trees was highly in demand to produce glass and soap and was exported mainly to Europe (Östlund *et al.* 1998). Logging on a larger scale was introduced in the late 19th century when the Swedish Forest Service began to set up generous contracts with private sawmills to be able to sustain the needs of industries for raw materials (Östlund *et al.* 1997). At the beginning of the 20th century, new forestry practices were developed with small clear cuts; fire was often used as soil scarification followed by sowing, which was the main regeneration strategy. By the 1950s, the availability of seedlings was increasing and planting seedlings became the dominant regeneration method (Östlund *et al.* 1997). During the 1950s and 1970s, clear cuts increased in size owing to the development of mechanical soil scarification, and herbicides were used against deciduous trees such as birch. The majority of dead or dying trees were removed from the forest because they were seen as trash and only as a habitat for pathogenic fungi and pest insects that could spread and infest healthy trees in the area (Linder & Östlund 1998; Fridman & Walheim 2000). The chemical treatment against deciduous trees was used until the late 1970s

when protests from the general public led to a halt in the use of herbicides (Östlund *et al.* 1997). During this time, clear-cut areas were mostly regenerated by planting seedlings and it was not until the 1990s that the forestry industry was encouraged to use a more 'natural approach' with smaller clear cuts, more deciduous trees and increasing the area of naturally regenerated forest (Linder & Östlund 1998).

In the 21st century, intensive management of forest stands typically involves clear-cutting to remove all the trees and snags and replanting with a monoculture in evenly distributed rows to produce high-quality timber. This gives a relatively uniform forest with regard to tree species, size, and spacing, often excluding dead wood (Hansen *et al.* 1991). However, the removal of dead wood and the efficient suppression of forest fires, which normally increases the amount of dead coarse wood (Fridman & Walheim 2000), have consequences for biodiversity because dead wood is the main habitat of many saproxylic organisms (Siitonen 2001; Grove 2002). During the historical forest clean-up in the mid-20th century, when all dead wood was removed and herbicides were commonly used to kill deciduous trees, the biodiversity of fungal and saproxylic species suffered a drastic decline (Jonsell & Weslien 2003). The *Swedish Forestry Act* strongly recommended that forest management should be striving to equate environmental goals, including conservation of native species, with production goals to prevent further losses of biodiversity (Anon. 2010). This includes leaving standing and dying trees, corridors of trees along creeks and lakes, creating high stumps and snags, and burning clear-cut areas (Larsson & Danell 2001; Anon. 2010). To sustain biodiversity, the Forest Stewardship Council (FSC)-Sweden and the Swedish Programme for the Endorsement of Forest Certification (PEFC) recommends the creation of at least 5 m^3 of dead wood and approximately three high stumps or girdle trees per hectare when clear cutting forests.

Norway spruce is a shadow-tolerant tree species, native to the northern hemisphere, and suitable for monoculture forest management. It is one of the most widely planted conifer trees, both within and outside its native range, and is one of the most economically important species in Europe because of its wide range of uses (i.e. pulp, paper and timber production) and as an indoor ornament at Christmas. During a lifetime that spans many decades, the Norway spruce tree encounters a variety of stresses of both abiotic and biotic origin.

Abiotic stress factors include drought, storms, high water levels in the soil and nutrient shortages; biotic stresses are caused by, for example, fungi or herbivores, mostly mammals or insects (Jactel *et al.* 2009). The most extreme stress that occurs in monoculture production forests occurs following final felling when a clear cut is created. The clear cut generates an automatic edge

effect where trees are highly exposed to the sun, wind, and high water levels owing to the harvesting of a large area of mature trees. High water levels in the soil lower the oxygen content and make the forest ground more unstable, which in turn predisposes trees to windthrow. Water stress together with windthrown trees creates a suitable habitat for many saproxylic organisms such as insect and fungal species. Even though modern forestry tries to maintain a sustainable management that reduces the damage of abiotic stress, it only favors one aspect of the biodiversity, those species that are opportunistic on conifers or specialists in Norway spruce. The succession of biodiversity on dead wood is a reflection of the species composition in the surrounding area. The intensity, abundance and the prior effect of the first arriving species are likely to affect the succession rate of the later arriving species, which can explain the species variation found in similar environmental conditions (Niemela *et al.* 1995; Chase 2010). For example, early decay agents have been shown to have an impact on the succeeding fungal decay community in the degradation of dead wood (Niemela *et al.* 1995).

1.1 Wood-decay fungi

All fungal species involved in wood decay can change the structural components of the host tree, (i.e. cellulose, hemicelluloses and lignin). Wood-decay fungi digest wood causing it to rot and then recycle the nutrients and minerals back to the forest ecosystem. The rots are divided into brown rot, white rot and soft rot depending on their ability to degrade the major cell wall components (Cooke & Rayner 1984). Brown-rot and white-rot fungi are typically basidiomycetes with some exceptions (e.g. *Xylariaceae*); the microbes responsible for soft-rots are typically ascomycetes but also include bacteria. These three basic classes of wood-decay fungal species are taxonomically closely related (Boddy *et al.* 2008). Most of the wood-decay fungal species are dependent on dead wood materials but under certain circumstances, for example, when trees are under severe stress, white-rot, brown-rot and soft-rot fungi are able to colonize living trees (Butin 1995; Boddy *et al.* 2008).

Brown-rot fungi are able to metabolize sugars from the hemicelluloses and cellulose chain molecules, leaving the lignin slightly chemically modified, which causes the cell structure to crumble and crack into brown-colored squares (Cooke & Rayner 1984; Butin 1995; Deacon 1997; Boddy *et al.* 2008). The brown-rot fungi degrade the hydrocarbon polymers in wood but leave most of the lignin with only minor structural changes. Brown-rot fungi are able to grow on cellulose but normally do not degrade the substrate, instead they

utilize hydrogen peroxide (H_2O_2) to perform this process, which is produced when hemicelluloses are degraded. The small molecules of H_2O_2 diffusing through the cell walls in the wood cause a generalized decay, which is an efficient way of accessing the limited nitrogen resources in wood (Cooke & Rayner 1984; Deacon 1997). By contrast, the white rots are able to utilize all the structural components of wood (cellulose, hemicelluloses and lignin) almost simultaneously, leaving the wood a moist, soft, spongy consistency with a bleached coloration (Cooke & Rayner 1984; Butin 1995; Deacon 1997; Boddy *et al.* 2008). The degradation of lignin is dependent on oxygen availability because of the fungal enzymes, such as glucose oxidase, manganese peroxidase and ligninase, that oxidize structural components of the wood (Deacon 1997). Although brown-rot fungal species dominate the coniferous wood-decay process they are also found in broadleaved forest ecosystems, whereas the white rots are important wood-decay species in both coniferous and broadleaved forests (Boddy *et al.* 2008) but tend to be more common in broadleaved trees (Cooke & Rayner 1984).

Both brown- and white-rot fungi have representatives in all the major taxonomic classes of the Basidiomycotina (Cooke & Rayner 1984; Floudas *et al.* 2012). Typical brown-rot species found in the coniferous forests of the northern hemisphere are *Fomitopsis pinicola* and *Antrodia serialis*; white-rot species include *Armillaria* sp., *Bjerkandera adusta*, *Heterobasidion annosum* and *Trichaptum abietinum.*

The third class of fungal decay species is the soft-rot fungi, which is mainly represented by ascomycete species. Soft-rot fungi, like the brown-rot fungi, are only able to degrade cellulose and hemicelluloses, leaving the lignin slightly altered (Butin 1995; Deacon 1997; Boddy *et al.* 2008). Soft-rot fungi are the least specialized wood-rot fungi but are common in litter degradation (Deacon 1997). The major difference between brown-rot and soft-rot decay is that the soft-rot decay progresses much more slowly than brown rot (Cooke & Rayner 1984) and occurs at or near the surface of the wood where it causes well-defined softening (Butin 1995) that is spongy under wet conditions and brownish and crumbly under dry conditions (Cooke & Rayner 1984; Boddy *et al.* 2008). Soft-rot species are able to colonize wood with sufficient water content and a high nitrogen content where the brown- and white-rot species are more or less excluded (Cooke & Rayner 1984), such as fence posts enclosing farm animals (Deacon 1997). Soft-rot fungi are found more commonly in broadleaved forests but are able to utilize coniferous wood to a minor extent (Boddy *et al.* 2008). Typical soft-rot species found in spruce forests are *Fusarium* spp. and *Phialophora* spp.

1.2 Blue-stain fungi

Most of the blue-stain fungi, also known as the 'ophiostomatoid fungi' (Wingfield *et al.* 1993), are filamentous ascomycetes species belonging to the genera *Ceratocystis*, *Ceratocystiopsis*, *Ophiostoma* and *Grosmannia* (Wingfield *et al.* 1993; Butin 1995; Ayres *et al.* 2000; Kirsits 2004). The ophiostomatoid fungi evolved approximately 200 million years ago (Berbee & Taylor 2001) and the pathogenic groups of *Ophiostoma* and *Ceratocystis* split from the main division 30 million years later (Farrell *et al.* 2001). The fungal complex degrades non-lignified cellulose structures and unlike the decay fungi, is not able to utilize structural components of the wood (Cooke & Rayner 1984; Seifert 1993; Butin 1995; Kirsits 2004). However, it is likely that blue-stain fungi facilitate the establishment of secondary decay species because trees colonized by blue-stain fungi are under greater stress (Cooke & Rayner 1984). The growth of hyphae in the sapwood causes a dark blue-grayish discoloration of the wood, hence the common name of these fungi, which is considered a serious problem in timber production (Seifert 1993; Butin 1995).

Blue-stain fungi are often associated with bark beetles. Some species of bark beetle transport fungal spores in special structures on their body (Carlile & Watkinson 1994; Paine *et al.* 1997); however, in general, spores are transported either attached to the exoskeleton of the bark beetle or by passing through the gut undigested (Furniss *et al.* 1990; Paine *et al.* 1997). The selection of sticky ascospores and conidia, long perithecial neck and well-developed shield in the blue-stain fungi are seen as adaptations towards a relationship with a bark beetle partner (Upadhyay 1981; Harrington 1987; Jacobs & Wingfield 2001).

1.3 Bark beetles

Bark beetles (Curculionidae, Scolytinae) lay their eggs and the larvae develop in the phloem layer of dead or dying trees directly under the outer bark. Bark beetles in Sweden emerge from their winter hibernation, either from under the bark of trees or from the forest litter, and start to 'swarm' in April–June. After finding suitable breeding material some species release aggregation pheromones, which are strongly attractive to other individuals of the same species. The female bores a gallery where the eggs are evenly distributed and fungi are inoculated. The gallery systems are species specific and, hence, can be used to identify the species of bark beetle.

Normally in Sweden only one generation of bark beetles are produced per year; however, during favorable weather conditions two generations a year can be produced by some species (Hedgren & Schroeder 2004; Jonsson *et al.*

2007). More than 80 different bark beetle species have been recorded in Sweden, the majority of which only reproduce in dying or newly dead trees. Only a few bark beetle species are regarded as pest insects owing to their ability during outbreaks to mass colonize and kill healthy trees. In Europe, *Ips typographus* is regarded as the most devastating bark beetle on Norway spruce.

During mass outbreaks the economic consequences can be devastating for forestry (Christiansen & Bakke 1988) because the bark beetle mother galleries and larval galleries go deep into the phloem, disturbing the nutrient flow of the tree, and are associated with the primary aggressive pathogenic fungal species *Ceratocystis polonica* and *Grosmannia europhioides.* A number of different trials have shown that these fungi can kill trees even in the absence of bark beetles (Solheim 1988; Hardings 1989; Solheim 1991; Hardings 1995; Krokene & Solheim 1996; Solheim & Krokene 1998; Salle *et al.* 2005).

Pathogenic blue-stain fungi also reduce the defense response of the tree during bark beetle attacks, which is a key factor in breeding success when outbreaks of *I. typographus* occur (Hardings 1989; Solheim 1991, 1993). As well as the pathogenic fungal species, *I. typographus* has also been shown to vector more than 30 other fungal species to Norway spruce stems (Kirsits 2004).

1.4 Bark beetle associations

The associations between various kinds of ascomycetous fungal species and bark beetles are well recognized (Francke-Grosmann 1967; Krokene & Solheim 1996; Paine *et al.* 1997; Six 2003). Associations between insects and specific fungal species are believed to reach far back in evolutionary history. The fungi are thought to have developed an interaction with different arthropods and weevil ancestors before the Scolytinae beetle evolved and firm associations formed (Farrell *et al.* 2001). The cooperation between two different organisms such as a fungus and a beetle allows an extension of an already known habitat or an opportunity to utilize a new niche and avoid competition (Bruno *et al*. 2003; Mueller *et al*. 2005).

Some bark beetles are not able obtain all their essential nutrients by consuming wood and, therefore, rely on nutrient contributions from another food source such as a symbiotic fungus to complete the life cycle (Clayton 1964). Insects in general are composed of between 6% and 10% nitrogen and, therefore, are dependent on a high nitrogen intake to be able to reproduce. It has been shown that bark beetle growth and reproductive success are connected with dietary intake during the larval stage (Mattson 1980; Ayres *et al.* 2000), with a high concentration of nitrogen giving the highest ratio of larvae to pupae

survival and the largest adults (Ayres *et al.* 2000). During the larval stage the nitrogen content in the dietary intake can be boosted by a mutualistic inoculated fungus, which can give the larva an advantage in life during favorable conditions. However, it has also been shown that the wrong fungus can have the opposite effect on larval survival: for example, the antagonist *Ophiostoma minus* has a detrimental effect on the bark beetle *Dendroctonus frontalis* (Ayres *et al.* 2000). A successful *D. frontalis* larva feeding on a mutualistic fungus creates a small feeding chamber; by contrast, a *D. frontalis* larva feeding on phloem infected with *O. minus* results in a meandering feeding pattern that often ends with the larva dying (Ayres *et al.* 2000).

The xylem-feeding ambrosia beetles and their associated fungi have a well-defined co-evolution and a successful partnership. The ectosymbiosis that is established between the beetle and a highly nutritious fungal species enables the beetles to feed their larvae on a specific fungus that was inoculated into the wood substrate when they laid their eggs (Francke-Grosmann 1967; Carlile & Watkinson 1994; Six 2003). The fungal propagules are transported in highly specialized pit structures on their exoskeleton called mycangia (Francke-Grosmann 1967; Carlile & Watkinson 1994; Paine *et al.* 1997; Six 2003). The beetle offspring are obligately dependent on the nutrient supply of amino acids, vitamins and sterols that are obtained from the fungus, which is cultivated by the female on the gallery walls (Clayton 1964; Kok *et al.* 1970).

Phloeophagous bark beetles can extract most of the nutrients they need from the phloem and are not obligated to have a fungal symbiont; however, larvae without a fungal symbiont must consume more phloem tissue to maintain their nutritional requirement (Clayton 1964; Ayres *et al.* 2000).

The basidiomycetes associated with bark beetles are less well-known than the ascomycetes even though it has been suggested that bark beetles feed more commonly on basidiomycete than on ascomycete species (Hsiau & Harrington 2003). Based on phylogenetic analyses and morphological comparisons, basidiomycetes associated with phloem-feeding bark beetles are suggested to have evolved at least twice in evolutionary history (Hsiau & Harrington 2003). Harrington *et al*. (1981a) reported that decay caused by *Fomitopsis pinicola* was accelerated when associated with bark beetle galleries of *Dendroctonus pseudotsugae.* Other studies have detected *F. pinicola* on several North American bark beetles, such as *Dendroctonus brevicomis*, *Dendroctonus ponderosae*, *Dendroctonus valens* and *Ips pini* (Pettey & Shaw 1986; Lim *et al.* 2005), as well as on *D. pseudotsugae* (Castello *et al.* 1976; Harrington *et al.* 1981b). *F. pinicola* is not dependent on being vectored by bark beetles, but is probably spread opportunistically by the beetles. The white-rot fungus *Phlebiopsis gigantea*, which is used as a biocontrol agent against

Heterobasidion spp. (Holdenrieder & Greig 1998), is an example of a basidiomycete species that is assumed to have evolved an insect-dispersal mechanism involving the bark beetle *Dendroctonus frontalis* (Hsiau & Harrington 2003). As well as normal wind-spread spores, *P. gigantea* is also able to form sticky asexual arthroconidia that appear to be suitable for grazing insects and attach to the exoskeleton fairly well (Hsiau & Harrington 2003).

2 Objectives

The overall goal of this thesis was to study the relationship between bark beetles and basidiomycetous fungi inhabiting wood, in particular high stumps and stem sections of Norway spruce. Despite the lack of a clear symbiotic relationship between basidiomycetes and beetles, the later can still significantly facilitate fungal establishment. We also sought to clarify the role of passive wind dispersal of fungal spores and propagules to Norway spruce stem sections and whether mycelial establishment was aided by holes in the bark created by the bark beetle.

We were also interested in examining the early succession of fungal diversity in dead wood

The specific objectives to this thesis were:

I – To investigate the fungal biodiversity and the species associated with bark beetles in high stumps of Norway spruce and to follow the succession across one, two and three years after the clear-cut felling and the creation of the high stumps (paper I).

II – To compare the fungal flora vectored by the spruce bark beetle *Ips typographus* from two different hibernation environments, under the bark of a standing tree and in the fungal flora of the forest litter, determined using two different methods, pure culture isolation and terminal-restriction fragment-length polymorphism (T-RFLP) (paper II).

III – To compare the fungal flora dispersed by wind or insects to Norway spruce stem sections that were protected from or exposed to bark beetles, and to investigate whether fungal colonization and succession was affected by mechanically created insect entrance or emergence holes or by the baiting the

stem sections with pheromone produced by the bark beetle *Pityogenes chalcographus* on (paper III).

IV – To determine whether the newly defined PCR primer fITS9 avoids chimera formation when amplifying the fungal internal transcribed spacer (ITS) region in a bark beetle DNA background to enable a less biased amplification of the fungal ITS to be performed from mixed samples compared with a nested PCR approach (paper IV).

3 Materials and Methods

3.1 Materials

All the materials collected had a close connection to Norway spruce. The bark beetles were collected directly from bark samples of randomly selected stems (paper II), stem sections (paper IV) or high stumps (paper I) of Norway spruce. In paper I, the samples were collected from high stumps of Norway spruce created between 2004 and 2006 from clear cuts of at least 5 ha in northern Uppland. Some of the bark beetles collected in the study reported in paper I were randomly selected for the analysis in paper IV to investigate the ability of fITS9 to bind to insect DNA. *Ips typographus* beetles in paper II were collected in southern Sweden from an area hit by the storm Gudrun in January 2005. The samples were collected from the storm-felled trees in March 2007. The logs studied in paper III were created at a site in Northern Uppland from Norway spruce trees with a diameter of at least 30 cm at breast height.

3.2 Methods

Paper I relies on molecular terminal-restriction fragment length polymorphism (T-RFLP) to analyze the fungal flora in the samples. T-RFLP allows different communities to be compared based on restriction endonuclease digestion of PCR products, generated with fluorescently labeled primers. Following the restriction reaction, the digest products were separated according to size by capillary gel electrophoresis using a genetic analyzer. T-RFLP gives individual 'fingerprints' of species and can be identified by comparison with a clonal library of known 'fingerprints'. This method gives a broader knowledge about the community of, in this case, fungal species compared with pure culture isolation.

T-RFLP was also used to analyze the fungal species present with bark beetles in paper II. The results obtained using T-RFLP were compared with those obtained by isolating fungal species from the bark beetles and subsequent sub-culture into pure culture.

During 2007 when these two studies were conducted, T-RFLP was at the forefront of the methods available. T-RFLP, together with a constructed clonal sequenced library, gave a broader species flora with hits for more species than obtained previously using traditional methods. The complexity of nature became more visible and more questions were raised than answered about the role of the different species. However, not all the species isolated were also found using T-RFLP as might have been expected. The PCR bias before the actual transformation from the 'fingerprint' into different species probably selected species that were favorable to the molecular treatment and *vice versa* when using the isolation identification method. Some of the bias originated from the nested PCR approach but, to a large extent, was probably also due to the long PCR products obtained with primers ITS1f and ITS4 (400–1500 bp). We switched to the mass sequencing technique, 454 sequencing, when this technique had developed enough to be more cost efficient and less labor intensive. T-RFLP is now regarded as obsolete for dealing with community studies in the forest ecosystem.

Paper III uses 454 sequencing (FLX and Titanium), which generates much more data, gives the data directly as sequences instead of the more indirect method of T-RFLP, and gives a greater output of the species complex within a sample compared with earlier methods. These systems rely on the ability to mark each sample with specific tags, ligating adapters to the DNA fragments and attaching them to a specific bead. The DNA on the beads is then amplified by PCR and the DNA sequencing read by the pyrosequencing technology. The more sequences obtained of the same species, the more common the species is in the community. The earlier, time-consuming method of building up a clonal library of restriction patterns is now no longer needed because sequences can be compared with available sequence databases such as the NCBI GenBank.

The problem of PCR bias persisted using FLX-sequencing because the same nested approach and primer pair were used; however, Titanium mass sequencing involved the use of other primers (fITS9 and ITS4) that amplified much shorter products (270–350 bp). Shorter PCR products and a reduced size span is known to give less bias (Ihrmark *et al.* 2012). It would have been possible to have repeated the T-RFLP analysis using the new primer pair to reduce the PCR bias; however, given the technological advances that had occurred by that time, this option did not seem worthwhile. The shorter PCR products amplified by the new primers might also have been problematic using

T-RFLP because the restriction products in many cases would have been too short to reliably detect with capillary gel electrophoresis.

In contrast to papers I–III, which rely on T-RFLP and mass sequencing, paper IV is based on cloning and direct sequencing of the cloned DNA. In this paper we investigated the specificity of a new primer, and whether this new primer amplifies both the fungal and bark beetle ITS region or if it discriminates against the bark beetle ITS region. Only a few samples were needed for this study and, therefore, we chose a method that is more suitable for small-scale studies. To use this method for a community analysis involving a larger number of samples would be both time consuming and very expensive to exhaust the variation in the community compared with next-generation sequencing (NGS).

The reason that the analysis method changed twice during the course of this studies is because technical developments in the area of DNA sequencing were rapid during these years.

454 sequencing offers a relatively easy way of detecting the community of fungal species in a sample; however, a huge number of sequences are obtained using this method. For example, the total number of sequences obtained during the work reported in paper III exceeded 1.5 million, including all singletons. All the singletons were counted as reading faults and were removed to make the dataset more manageable.

Despite its many advantages, NGS technology does have some limitations such as relatively short sequence reads. The development of sequencing has continued to advance and today sequences of about 400–600 bp can be obtained using the Titanium platform compared with earlier sequences of about 200–300 bp, and the price per sequence has decreased.

This high-tech method of analyzing samples does indeed have advantages over the older strategies, but what is gained? Of course, a more complete picture of the species community is obtained; however, to draw any conclusions it is necessary to have another community to compare it with. Careful handling of the samples is required so as to avoid major contamination issues. Although this applies to all laboratory work, the chances of contaminating samples that are pooled together at the end, resulting in the loss of a significant amount of work, are much greater for samples analyzed using NGS methods than for samples that are handled individually, where the loss of a few contaminated samples would be immediately apparent.

4 Result and Discussion

4.1 Paper I

Mechanically created high stumps, often of Norway spruce, are created during final felling to promote biodiversity. They increase the amount of dead wood, which in theory sustains the diversity of saproxylic species. This study follows the succession of the fungal community in mechanically created high stumps of Norway spruce for the first 3 years after final felling. Bark beetles, mycelia and wood were sampled from the vicinity of insect galleries in the bark of *Picea abies* high stumps of four different age classes in southeastern Sweden.

Using the T-RFLP method, a total of 21 fungal taxa were detected from 203 samples, including 12 ascomycetes and 9 basidiomycetes. Of the filamentous fungal species, 50% were found both in bark and bark beetles, and 37% were found in bark, wood and bark beetles. Yeasts dominated in stumps that were 1-yr old and in control samples without insect activity. In 2- and 3-yr-old stumps, filamentous ascomycetes were present and common wood-decay basidiomycetes such as *Stereum sanguinolentum*, *Phlebiopsis gigantea*, *Trichaptum abietinum* and *Fomitopsis pinicola* were found as mycelia associated with insect galleries and on bark beetles. The results indicate that insects facilitating the establishment of wood-decay fungi cannot be neglected.

Fungal species were found in all three types of sample, i.e. wood, mycelia and from bark beetles, indicating that these have at least a random connection with insects given that the fungal communities were similar.

Initially yeasts of *Cryptococcus* spp. were the dominant bark-inhabiting fungi collected from year zero, which also corresponded with the fungal community detected in the control sample collected from undisturbed bark in each of the high stumps each year. This result indicates that provided the beetles carried spores with them, insect activity such as changing the

microclimate under the bark stimulates the development of wood-decay fungi in the high stumps during the 3 years following cutting. The inner bark of trees from 3-year-old high stumps was to a large extent disintegrated because of the primary saproxylic insects inhabiting the stump and was not collected in this study. Studies of beetle diversity on dead spruce wood in the region reported that the beetle assemblage was disproportionately dominated by the genus *Crypturgus*, and that *P. chalcographus* was the third most common species (Djupstrom *et al.* 2008).

Most of the fungal species found with the bark beetles were also detected in the other samples (i.e. the wood and mycelial samples). Eighteen species of fungi were detected in the *Crypturgus* and *Pityogenes* samples: 14 species were detected from both species of bark beetles and four were only associated with *Crypturgus* sp. This indicates that the insect-associated fungal community can be transported by emerging beetles, either from a dead wood substrate or from forest litter, during the spring migration.

There was low similarity between species detected in the control samples and the insect samples, probably because the control samples were collected where there was no evidence of insect activity. The species detected in the control samples, which were taken from two types of woody tissue, fresh high stumps that had not been colonized by insects and colonized high stumps that were sampled in a region of the stump without insect activity, were unsurprisingly the communities that most resembled each other given that they were collected from the same type of tissue.

The fungal species recorded for the mycelia and insect samples were similar to each other but dissimilar to the control samples, probably because the sampling methods used for the mycelia and the insect samples were different to that used for the wood and the control samples.

Different fungal species have different abilities to colonize high stumps. A species arriving first at a site has advantages over other species until the substrate becomes less suitable (i.e. microclimate change and nutrient limitation). *P. gigantea* and *S. sanguinolentum* occur more frequently over the years and are more common in 3-year-old high stumps than in one- and two-year old high stumps. It could perhaps be argued that the use of the white rot fungus *P. gigantea* in production forests as a biocontrol agent against *Heterobasidion annosum* would increase the number of *P. gigantea* spores in the air, which could explain the increased occurrence of *P. gigantea* detected in the samples over time. However, Samils *et al.* (2009) did not find that the introduction of *P. gigantea* to forests had a strong influence on the local population structure of the fungus away from the local forest stands where it had been used.

The occurrence of the white rot and pathogenic fungus *S. sanguinolentum* normally increases over time in wounded trees and stumps (Vasiliauskas & Stenlid 1998), as seen in this study. Theoretically, the fungus is able to enter the high stump through bark damaged by exit holes bored by the bark beetle or vectored by the bark beetle during flight.

The brown rot *Fomitopsis pinicola* is typically found on dead, standing conifer wood or on fallen logs, which are rapidly decayed by the fungus (Hogberg *et al.* 1999; Jonsell *et al.* 2005). The fungus occurred in association with insects and was found more frequently in 2-yr-old stumps than in 1-yr-old stumps; however, it was not present in 3-yr-old stumps. Even though the bark beetles that were found at the test sites were not connected with *F. pinicola* fruiting bodies, the bark beetles are likely to transport mycelia on their body when moving from an infested tree. *F. pinicola* has been detected on beetles in flight (Pettey & Shaw 1986) and commonly fruits on high stumps of Norway spruce (Jonsell *et al.* 2005; Abrahamsson *et al.* 2008). *F. pinicola* and the white rot fungus *Trichaptum abietinum* were detected in about 2% of the insect galleries examined. Despite this low frequency, the vectoring of decay species by insects should not be ignored. Given the hundreds or thousands of insect galleries that can be found in the bark of a dead spruce tree, the likelihood is relatively high for insect facilitation of decay fungi in dead trees or logs. In theory, insect facilitation could account for the inoculation of up to 19 different genotypes of these species found in a single standing or fallen tree (Norden 1997; Kauserud & Schumacher 2003). In the present study it was apparent that bark beetles acted as facilitators of wood decay fungi. Of the 21 fungal species detected in the high stumps, 18 were associated with insects. Either these fungi could have been directly introduced by bark beetles or they could have gained entry via the bark beetle entry hole in the bark when dispersed by air or rain.
The bark beetles role as a vector of root-rot fungi should not be neglected.

4.2 Paper II

Ips typographus is known to spread a variety of fungal species and has different hibernation environments, either under the bark of standing trees or in the forest litter, which is likely to affect the beetle-associated fungal flora. We isolated fungi from beetles collected from standing *I. typographus*-attacked trees and from forest litter below the attacked trees. Fungal identification was performed using cultural and molecular methods. The fungal species that were detected using the two different methods were compared. Fungal communities associated with *I. typographus* differed considerably depending on the

hibernation environment. In addition to seven taxa of known ophiostomatoid *I. typographus*-associated fungi, 18 ascomycetes and anamorphic fungi, five wood-decaying basidiomycetes, 11 yeasts, and four zygomycetes were detected. Of those, 14 fungal taxa were detected exclusively from beetles that hibernated under bark, and six taxa were detected exclusively from beetles hibernating in forest litter.

Several European studies have also reported associations between populations of *I. typographus* and the ophiostomatoid species detected in this study (Mathiesen-Käärik 1953; Solheim 1991; Krokene & Solheim 1996; Kirschner 2001; Kirsits 2004; Jankowiak 2005; Salle *et al.* 2005). In our study, only low levels of the tree pathogens *Ceratocystis polonica* and *Grosmannia europhioides* (0.9% and 11.0%, respectively) were vectored by *I. typographus*. This finding is rather surprising given that the beetle was collected from spruce stands attacked by *I. typographus* and that numerous attacks on living trees had been observed in the stand during the previous growing season of 2006. This finding indicates that the beetle can successfully attack and kill trees without the help of ophiostomatoid fungal species.

The more frequent detection of *Grosmannia europhioides* in samples of *I. typographus* that were hibernating under the bark of standing trees compared with *I. typographus* hibernating in the litter was statistically highly significant, and the possibility that the observed shift in the overwintering niche affected which fungal community was vectored to a new substrate cannot be overlooked. In general, this study demonstrated that the fungal communities associated with *I. typographus* could show considerably variation depending on the beetle hibernation environment.

The detection method also had a notable impact on the recorded fungal community structure. Pure-culture isolations seemed more suited to isolating fast-growing fungi than the direct molecular T-RFLP method, which detected yeast species exclusively. The pure-culture media was not a suitable growth environment for several of the beetle-associated yeasts.

The yeasts remained unidentified despite analyzing them using the molecular T-RFLP method because there was no close matches with identified ITS sequences in the available databases. However, three of the yeasts detected in *I. typographus* samples were identical to yeasts (BAF 22, BAF5 and BAF6) detected on other bark beetles species in Canada (Lim *et al.* 2005).

The role of yeasts in the lifecycle of the beetle is unknown but it has been suggested that certain yeasts are likely to influence the distribution of *D. ponderosae* mycangial fungi in the host tree, which may affect the fitness of the beetle (Adams *et al.* 2008).

This study is the first to report that wood decay fungal species are dispersed, albeit randomly, by *I. typographus*. The species detected in the bark beetle samples were the polypores *Bjerkandera adusta*, *Fomitopsis pinicola*, and *Trichaptum abietinum* and the corticoids *Phlebiopsis gigantea* and *Stereum sanguinolentum*. These are active wood decomposers and produce abundant sporocarps in managed forests on various types of woody debris (stumps, logs, snags, slash), and sometimes on wounded stems of living trees and are not strictly dependent on insects.

Basidiomycete decay species have occasionally been mentioned as possible associates of *I. typographus* in other studies; however, in most cases, the basidiomycetes were not identified and although they were reportedly found within or beneath the beetle galleries, there is no direct evidence that these fungi were carried by insects (Kirsits 2004; Jankowiak 2005; Adams *et al.* 2008). Of the basidiomycetes found on *I. typographus* during the present work, *F. pinicola* has previously been detected on several North American bark beetles, such as *Dendroctonus pseudotsugae* (Castello *et al.* 1976; Harrington *et al.* 1981a), *Dendroctonus brevicomis*, *D. ponderosae*, *Dendroctonus valens*, and *Ips pini* (Pettey & Shaw 1986; Lim *et al.* 2005). *P. gigantea* has been identified as a mycangial fungus of *Dendroctonus approximatus* and has also been found in the galleries of *D. ponderosae* (Hsiau & Harrington 2003; Harrington 2005). *T. abietinum* has been isolated from wood beyond the galleries of *D. pseudotsugae* (Kim *et al.* 2005). This study is the first to report an association between bark beetles and the wood-decay fungi *B. adusta* and *S. sanguinolentum*.

The observation that the wood-decay species *P. gigantea* and *T. abietinum* occurred on *I. typographus* hibernating in the forest litter agrees with our previous study that demonstrated that several wood-decay basidiomycetes, including *P. gigantea*, can colonize fine tree roots in the soil *in vivo* and can form mycorrhizal-like associations *in vitro* (Vasiliauskas *et al.* 2007). The results of the current study have provided additional evidence that wood-decay basidiomycetes thrive in the soil environment, contributing new knowledge about the ecology of this group of fungi.

4.3 Paper III

Few studies have focused on the role played by insects in the colonization success of air-dispersed basidiomycete fungal species or on the association between the occurrence of fruiting structures of particular basidiomycete decay fungi and certain early-succession insect species (Abrahamsson *et al.* 2008; Weslien *et al.* 2011). It has been suggested that several bark beetle species that

fly during basidiomycete sporulation periods may randomly pick up basidiomycete spores and, thus, act as vectors (Pettey & Shaw 1986). It has also been suggested that rot fungi have an effect on the beetle diversity in dead wood (Jonsell *et al.* 2005). In contrast to the insect–basidiomycete connection, a variety of studies have investigated the association between pathogenic ophiostomatoid ascomycete species and insects such as the bark beetle *Ips typographus*, which is a destructive pest and known to facilitate the establishment of a variety of *Ophiostoma* spp. and non-pathogenic fungal species in Norway spruce (*Picea abies*) stems (Solheim 1991, 1993; Persson *et al.* 2009).

When a tree dies, the wood will be colonized by fungi that either were present in the wood prior to tree death or established after the tree died. This process can be extended over time and gives rise to succession patterns. Insects may facilitate fungal colonization by opening up the bark layer, providing an infection court, or they can specifically or loosely vector fungal propagules to arrive at the substrate. In our investigation, we distinguished between stem sections that had been exposed to insects or protected from insects by being placed inside a netted cage. One treatment involved baiting stem sections with the pheromone of the bark beetle *Pityogenes chalcographus* to induce attacks by this species. The results clearly showed that excluding insects from fresh stem sections had a significant effect on the fungal community. One of the experimental treatments involved making small wounds in the bark to mimic insect entrance or emergence holes. These holes only had a minor measurable effect on the fungal community that developed in stem sections protected by the cages and no effect on the exposed sections. This suggests that insects do contribute to the development of early fungal succession on dead wood but that creating small disturbances in the bark such as entry or exit holes only has a minor effect.

In our study, among the 200 species detected most frequently in the samples, 60 species were basidiomycetes and 139 species were ascomycetes, which were clearly dominant in terms of species number. The stem sections were colonized by basidiomycete species with different ecological strategies: 21 white-rot fungi, 2 brown rot, 5 saprotrophs, 5 mycorrhizal, 22 basidiomycetous yeasts and 5 had unknown ecological strategies. The ascomycetes were represented by 28 saprotrophs, 10 Ophiostomatoid, 18 endophytes, 23 true yeasts, 8 soft-rot fungi and 46 had unknown ecological strategies.

The permutation test clearly showed that protecting stem sections with cages had a major effect on the fungal community. In a Detrended Corresponding Analysis (DCA), samples taken from stem sections inside the

cages formed a separate cluster from those taken from outside the cages and the latter were similar to stem sections that had been baited with pheromone. A high proportion of the detected fungal species showed a higher incidence in stem sections that were exposed to insect activity.

Several species of wood-decay fungi were less frequent in stem sections protected by cages and some of these fungi were also detected in the bark beetle samples. The early wood-decay species included some of the most common fungi (*T. abietinum* and *F. pinicola*), some fungi that are known to colonize trees prior to death (*Heterobasidion parviporum*, *S. sanguinolentum* and *Amylostereum chailletii*) and some that are usually found on angiosperm trees (*B. adusta and Stereum purpureum*).

Müller *et al.* (2002) studied the colonization of bolts that were either protected by cages or exposed to insects for 28–30 months following an initial colonization phase of 3 weeks without net cages. The netting procedure used by Müller *et al*. did not exclude insects as successful as our system. The fungal flora detected by Müller *et al.* using isolation and molecular identification methods had some similarities with our observations. Their principal basidiomycete flora included *Hypholoma capnoides* and *Antrodia serialis* in addition to our most common species. In our study, the treatment with greatest effect on fungal diversity was the pheromone baited stem sections, which were separated from the other treatments in the DCA analysis.

The fungal community of the bark beetles that were collected from the sections showed most similarity with the fungal flora in the outer samples of the pheromone-baited stem sections, although not all species were present in both sets of samples. This finding indicates that bark beetles carry a fungal community on the surface of their body or in their gut depending on their life history and the fungal community of their ancestors. These fungi may be inoculated into the bark and wood when the insects colonize new trees. In theory this would affect the ability of other fungal species, such as rot fungi, to colonize and inhabit the wood.

In our study, ascomycetes were very common and outnumbered the basidiomycetes in terms of species diversity. This is in contrast to the findings of most studies on early succession where fruiting bodies have been studied. Ascomycete fungi are frequently detected in the fungal flora of studies that involve isolation work and are even more pronounced in studies using direct molecular detection (Allmer *et al.* 2006; Lindner *et al.* 2011; Kubartová *et al.* 2012; Rajala *et al.* 2012). Although most of the ascomycetes are not major degraders of wood structures, they still play a decisive role in determining the environment for the following succession of wood-decay fungi.

In our study, white-rot fungi were the dominant ecological group among the basidiomycetes identified. This supports the findings reported by other studies that in terms of species numbers, white-rot fungi are more common than brown-rot fungi in early succession in Norway spruce wood (Renvall 1995; Rajala *et al.* 2012). This might be explained partly by the tendency of white-rot species to associate with insects. Ascomycetous yeasts and particularly basidiomycetous yeasts also associate with insects. We also detected an interesting group of fungi that are known to be mycoparasites, for example, *Trichoderma pleuroticola* and *Tremella encephala*, which are known to be associated with *Pleurotus* and *Stereum sanguinolentum*, respectively (Pippola & Kytoviita 2009; Sobieralski *et al.* 2012). Some mycorrhiza-forming fungi were also identified in our dataset. There were no plants growing on the stem sections that were able to form mycorrhiza in this early decompositional stage, so it is not clear what role these fungi played in this fungal community.

Interestingly, in other recent fungal community studies, mycorrhizal species have also been detected from logs without any obvious living host plants for forming a symbiotic relationship (Kubartová *et al.* 2012; Rajala *et al.* 2012).

Several species of blue-stain fungi are known to be vectored to Norway spruce trees by bark beetles (Solheim 1988; Furniss *et al.* 1990; Solheim 1992; Krokene & Solheim 1996; Jankowiak 2005; Persson *et al.* 2009). However, most previous studies have focused on *Ips typographus* and reports about *Pityogenes* sp. are scarce. Only one of the Ophiostomatoid fungi detected in the molecular analysis occurred significantly more frequently in stem sections that were exposed to insect activity. It was also surprising that the blue-stain fungi, which appeared to be highly abundant, based on a visual examination of the stem sections, were not detected that frequently using molecular methods. It is likely that blue-stain fungi would have been identified more frequently if the identification had been performed on the basis of fungal cultures isolated from the wood samples because blue-stain fungi grow well on media and out compete other species when grown on media giving a falsely high impression of the frequency.

There are several reports on successional patterns in the fungal community on decaying wood. Most of these studies are based on observations of macroscopic fruiting bodies. The general pattern observed is that the number of species initially increases during colonization of the newly dead wood until intermediate decay and then decreases again when the logs start to become highly degraded. In a study followed over 15 years, Weslien *et al.* (2011) reported that insect species such as *Hylurgops palliatus* positively influence the probability of *F. pinicola* fruiting on high stumps whereas *Monochamus sutor* negatively influences the probability of *F. pinicola* fruiting on high stumps.

Müller *et al*. (2002) found that there was a negative correlation between the number of *H. palliatus* marks on logs and the diversity of the fungal flora after 28–30 months colonization.

Our findings indicate that despite the distance between them, the two field sites shared a similar fungal community. This indicates that the processes that drive colonization are general.

The structure of the fungal community changed over time. Species that were one of the top ten most frequently detected species in 2009 may not have been present at all in 2010 or *vice versa*. This suggests that the primary species in 2009 had a greater ability to colonize the stem sections at the beginning of the study, but as time went on and the microclimate changed, other secondary species were able to colonize and the primary species were suppressed because of their low combative ability.

The ability to combat species already present in woody material is crucial for secondary species. Primary invaders, which are often fast growers and specific to newly dead materials, initially have an advantage over secondary fungal species, which are typically slow growers or not favored by the fresh wood material. The secondary fungal species are often more long lived than the primary invaders (Rayner & Boddy 1988; Boddy 2000; Olsson *et al.* 2011).

4.4 Paper IV

To eliminate the formation of chimeras between fungal and insect DNA when using primer pair ITS1F and ITS4, a nested PCR was conducted with fungal primers (NLC2 and NSA3) that bind specifically to fungal DNA. However, this method increases the handling of DNA, which can increase the risk of contamination and underestimate the fungal community. To determine whether a nested PCR step could be avoided, we compared primer pair ITS1F and ITS4 without a nested PCR against primer pair fITS9 and ITS4. Our analysis showed that performing a nested PCR is unnecessary when using the recently developed fungal primer fITS9. The formation of fungal–insect chimeras is not a substantial problem when using the fITS9 primer compared with the ITS1F without nested PCR because the fITS9 primer binds to the 5.8S ribosomal gene of the ITS region, which reduces the risk of chimeras. The fITS9 primer does bind to algae and lichens to some extent; however, the frequency is low compared with the frequency of chimera formation when using the ITS1F and ITS4 primer pair and has only a small effect on the result.

5 Conclusion

I. Bark beetles not only facilitate the establishment of ascomycete species and ophiostomatoid species, but also contribute to the establishment of wood decay fungi and act as random vectors for the guild.

II. The fungal community vectored by the bark beetle is depending on the hibernation environment.

III. Beetle entrance and emergence holes on their own have little or no effect on the substrate availability for air-dispersed fungal species

IV. The fungal community changes drastically in newly created dead wood and the ecological interactions between fungi and dead wood are complex.

V. The complexity of the fungal community detected is influenced by the method used to analyze it.

Different fungi have different dispersal strategies. Most basidiomycete species use air as their dispersal method but, as shown in papers I–III, the random dispersal of some basidiomycetes by different bark beetle species does have an effect on the diversity of fungal species. There will always be a shortage of dead wood in productive forests. However, the mechanical creation of high stumps, as in paper I, is one way of increasing the amount of dead wood available to saproxylic organisms. The majority of high stumps are created from Norway spruce; however, in terms of nature conservation, this action will only support one part of the ecosystem, i.e. Norway spruce-specialized species. When developing management plans for the productive forest that include nature conservation, all aspects of the dispersal of insect and fungal species need to be considered.

In paper II, the variety of fungal species detected in standing dying trees was more diverse compared with that detected in high stumps in paper I. This may suggest that the diversity is greater in standing dying trees. Together with paper III, which investigated the impact of excluding insects, these studies show that forest fungal biodiversity is most likely to increase with a combination of high stumps, dying trees and logs. Increased forest fungal biodiversity is necessary to maintain the ecology of the saproxylic organism diversity.

As we have shown in papers I–III, bark beetles not only have an impact on the dispersal of fungal species but can also have an impact on whether the substrate is suitable for fungal decay species to infect. Insect damage (i.e. the hole in the bark) is not sufficient at the early stages of colonization to increase infection by air-dispersed fungal species; however, when the bark loosens because of insect activity under the bark, the microclimate of the substrate becomes more suitable for colonization. The standing dying trees in paper II were exposed to sunlight as well as to shadow from the surrounding trees and are probably the best way of sustaining the majority of species dependent on dead wood given that the trees will eventually fall to the ground, which will create new substrates that can sustain even more species.

When investigating species diversity, in this case fungal species diversity, the method used is highly significant with regard to obtaining an accurate view of the diversity. The method that has been used historically (i.e. pure culturing) does not reveal the whole diversity of fungal species, only those that are favored by the specific media used. As new techniques such as the NGS techniques continue to develop, the succession of species is being clarified even though the ecology of different species interactions is often still unknown. NGS sequencing has enabled us to clarify and study fungal community changes over time and, with the aid of specific primers such as the fungal-specific fITS9, the fungal community has been shown to be highly diverse and complex.

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