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Bark beetle-associated fungi in Fennoscandia with special emphasis on species of *Ophiostoma* and *Grosmannia*

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Academic dissertation

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ABSTRACT

Global trade in untreated timber and wood products raises the risk of accidentally introducing forest pests and pathogens into new environments. Bark beetles (Coleoptera: Scolytinae) include several species that are regarded as forest pests. These insects are known to live in close association with fungi, especially species of ophiostomatoid fungi (Ascomycota). Several of these fungi are agents of blue stain in timber, and some are serious plant pathogens. However, only little is known regarding the fungal associates of bark beetles, or the interactions between the fungus, the bark beetle and the host tree in the boreal forests of Fennoscandia.

The aim of this study was to increase the knowledge regarding bark beetle-associated fungi in Fennoscandia, with special emphasis on the genera *Ophiostoma* Syd. & P. Syd. and *Grosmannia* Goid. Fungi associated with 13 different bark beetle species, infesting Norway spruce (*Picea abies* (L.) Karst.), Scots pine (*Pinus sylvestris* L.) and birches (*Betula* L. spp.) in the eastern parts of Finland and neighboring Russia, as well as southern Norway, were isolated and identified. The fungal identifications were based on morphological characteristics and DNA sequence comparisons.

The survey revealed the occurrence of at least 29 species of *Ophiostoma* and *Grosmannia*. All the bark beetle species considered in this study were frequently associated with a complex of ophiostomatoid fungi. Several species were recorded for the first time in the countries in the study. A surprisingly high number of previously undescribed fungal species were discovered. During the survey, eight of these species were described as new taxa. In addition, the study revealed new insect-fungus relationships. The number of taxa encountered, covering a relatively small geographical area, indicates that there are many more ophiostomatoid fungi occurring in the boreal forests of Fennoscandia than has previously been recognized. The study emphasizes the importance of developing a clear understanding of the possible threats of moving timber and wood products without knowledge of the micro-organisms that might also be moved.

Keywords: blue stain, insect-fungus interactions, molecular systematics, ophiostomatoid fungi, *Ophiostomatales*, symbioses

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Joensuu, April 2011

Riikka

LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which are referred to in the text by their Roman numerals **I-IV**:

I Linnakoski, R., de Beer, Z.W., Rousi, M., Niemelä, P., Pappinen, A., Wingfield, M.J. 2008. Fungi, including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. Mycological Research 112: 1475–1488. doi:10.1016/j.mycres.2008.06.007

II Linnakoski, R., de Beer, Z.W., Rousi, M., Solheim, H., Wingfield, M.J. 2009. *Ophiostoma denticiliatum* sp. nov. and other *Ophiostoma* species associated with the birch bark beetle in southern Norway. Persoonia 23: 9–15. doi:10.3767/003158509X468038

III Linnakoski, R., de Beer, Z.W., Ahtiainen, J., Sidorov, E., Niemelä, P., Pappinen, A., Wingfield, M.J. 2010. *Ophiostoma* spp., including five new species, associated with pine- and spruce-infesting bark beetles in Finland and Russia. Persoonia 25: 72–93. doi:10.3767/003158510X550845

IV Linnakoski, R., de Beer, Z.W., Tuan, D.A., Niemelä, P., Pappinen, A., Wingfield, M.J. *Grosmannia* and *Leptographium* spp., associated with pine- and spruce-infesting bark beetles in Finland and Russia. Manuscript.

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CONTRIBUTIONS OF THE AUTHORS

The table shows the major contributions of authors to the original articles and the manuscript. Other contributors are acknowledged in the relevant articles or the manuscript.

	I	II	III	IV
Original idea	MW	PN	MW	MW
Study design	PN, WB	HS, MR, RL	PN, WB	PN, WB
Bark beetle collections	MR, PN, WB	HS, MR, RL	ES, HR, PN, WB	HR, PN, WB
Fungal isolations	RL, WB	RL	ES, JA, RL, WB	JA, RL, WB
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ABBREVIATIONS

BI	Bayesian inference
BLAST	basic local alignment search tool
bp	base pair
CBS	Centraalbureau voor Schimmelcultures culture collection
CP	classic paradigm
DNA	deoxyribonucleic acid
EF1-α	elongation factor 1-alpha
GCPSR	genealogical concordance phylogenetic species recognition
ITS	internal transcribed spacer region of rDNA
LSU	ribosomal large subunit DNA
MAFFT	multiple sequence alignment program
MEA	malt extract agar
MEGA	molecular evolutionary genetics analyses program
ML	maximum likelihood
MP	maximum parsimony
MPB	the mountain pine beetle
NCBI	National Center for Biotechnology
NJ	neighbor-joining
OA	oatmeal agar
PCR	polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
sp.	species (singular)
sp. nov	new species
spp.	species (plural)
SSU	ribosomal small subunit DNA
TNT	tree analysis using a new technology program

1 INTRODUCTION

Bark beetles (Coleoptera: Scolytinae) include many primary pests, which can cause significant economic losses to forests and forestry. The majority of these species are harmless to healthy living trees, infesting mainly dead or dying trees in their native environment (Paine et al. 1997, Martikainen et al. 1999, Knížek and Beaver 2004). An interesting characteristic of bark beetles is their widespread association with fungi; the most notable are the associations with ophiostomatoid fungi (Ascomycota) responsible for discoloration of wood and serious tree diseases (Wingfield et al. 1993, Kirisits 2004). Bark beetles are known to greatly facilitate the spread of these fungi.

Both bark beetles and the fungi associated with them are easily transported through the movement of untreated wood products. Increased global trade in untreated timber and wood products raises the risk of accidentally introducing these forest pests and pathogens into a new environment (Tkacz 2002). Several examples of invasive bark beetle species and their associated fungi have shown that even species considered less harmful in their native environment can become potential threats to forests and their socio-economical importance to humans if accidentally introduced into a new environment (Ozolin and Kryokova 1980, Brasier 1983, Yin 2000, Li et al. 2001, Taylor et al. 2006, Lu et al. 2010). Considering the potential risks of introducing pests and pathogens in timber imported from Russia to Finland, a previous study identified a number of bark beetle species in the timber, including also potential pest species not native to Finland (Siitonen 1990). A changing environment can also increase the threats posed by these pests and pathogens (Williams and Liebhold 2002, Carroll et al. 2003, Berg et al. 2006).

Although a number of studies have been devoted to resolving the nature of bark beetle-fungus interactions since they were first recognized in the 19th century (Schmidberger 1836, Hartig 1844, Hartig 1878), these interactions remain poorly understood. The studies regarding bark beetle-associated fungi are mainly focused on the economically most important bark beetle species. This might have biased the observations of true fungal biodiversity in the studied regions, and also our understanding of these symbioses (Six and Wingfield 2011). Not all bark beetle-fungus interactions should be viewed as one type of symbiosis having similar function. Apparently bark beetles and fungi form complex and dynamic associations, which are shaped during long periods of co-evolution and which are strongly influenced by the environment. The research concerning these fascinating symbioses is at the point where we are just learning to understand the diverse roles of fungi and their importance in the lives of bark beetles.

2 REVIEW OF LITERATURE

2.1 Taxonomy and morphology of ophiostomatoid fungi

Ophiostomatoid fungi represent an artificial group of fungi that consist of c.a. 200 species distributed in the Ascomycete genera *Ceratocystis* Ellis & Halst. (Microascales), *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Grosmannia* Goid. and *Ophiostoma* Syd. & P. Syd. (Ophiostomatales). Adaptation to insect dispersal is typical for the majority of these fungi, and many of the species have a close association with their insect vectors (Wingfield et al. 1993). Ophiostomatoid fungi can be found on a wide variety of substrates in both the Northern and Southern Hemispheres.

Due to the relatively simple morphology and overlapping features between different species, it has been difficult to identify these species, and their classification has been complicated and regularly revised. These confusing taxonomic debates have surrounded the ophiostomatoid fungi since the descriptions of the two major genera *Ceratocystis* and *Ophiostoma.* Phylogenetic studies based on DNA sequence data have clearly shown that despite the morphological and ecological similarities, these two keystone genera are phylogenetically unrelated and represent different orders of fungi (Hausner et al. 1992, 1993a,b, Spatafora and Blackwell 1994). *Ceratocystis* belongs to the Microascales together with related but economically unimportant genera, such as *Gondwanamyces* G.J. Marais and M.J. Wingf., *Graphium* Corda and *Microascus* Zukal. With the confusion between *Ceratocystis* and *Ophiostoma* resolved by modern taxonomic techniques, recent studies have focused on the Ophiostomatales. Recent DNA sequence analyses have defined three distinct phylogenetic lineages supported by morphological features in the Ophiostomatales: *Ceratocystiopsis*, *Grosmannia* and *Ophiostoma* (Zipfel et al. 2006). As the recent studies have demonstrated, DNA sequence-based identification has become essential for the reliable identification and recognition of cryptic taxa amongst these morphologically similar ophiostomatoid fungi (Gorton et al. 2004, Grobbelaar et al. 2009). Analyses of DNA sequence data have thus redefined the status of several genera and species and have led to the discovery of several previously unrecognized taxa. This is a trend that is likely to continue as more sequence data become available.

Ophiostomatoid fungi have many morphological characters in common. These features are typically related to their adaptation for insect dispersal. The spore-bearing structures in both the teleomorph and anamorph states are in most cases long stalks, carrying the spores in slimy droplets. When possible, morphological identification has been based on the characteristics of both the anamorph and teleomorph structures. In many cases, the characterization is based on anamorph morphology only. Many species, particularly *Leptographium* Lagerb. & Melin spp., are not typically associated with a teleomorph, or the teleomorph is rarely observed. The typical teleomorphs of these fungi are characterized by globose ascomatal bases with elongated necks, evanescent asci and hyaline, one-celled ascospores having a wide variety of shapes (Hunt 1956, Upadhyay 1981, de Hoog and Scheffer 1984, Wingfield et al. 1993, Jacobs and Wingfield 2001, Zipfel et al. 2006). These fungi have a variety of different anamorphs, of which most also produce their conidia in slimy droplets. The sticky spore droplets can attach to the bodies of passing insects and thus facilitate the dispersal of the fungi. The morphological similarity of ophiostomatoid fungi is probably a result of convergent evolution, as adaptations to insect dispersal (Spatafora and Blackwell 1994).

Species of *Ceratocystis* are characterized by *Thielaviopsis* Went anamorphs and endogenous conidium development (Halsted 1890, Minter et al. 1983). In contrast, conidium development of species in the Ophiostomatales is exogenic (Minter et al. 1982). *Ceratocystiopsis* is characterized by *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. and *Sporothrix* Hektoen & C.F. Perkins anamorphs, and small perithecia with long, falcate ascospores (Upadhyay and Kendrick 1975, Zipfel et al. 2006). At present, eleven species of *Ceratocystiopsis* are known. The species of *Grosmannia* are characterized by *Leptographium* anamorphs, and the presence of intron 4 and the absence of intron 5 in the β-tubulin gene (Goidánich 1936, Zipfel et al. 2006). At present, 28 teleomorph species are recognized in *Grosmannia*, with many more *Leptographium* spp. for which no teleomorphs are known. The remaining genus in the Ophiostomatales, *Ophiostoma*, is the largest, including more than 120 species and a variety of ascospore shapes and anamorphs in *Sporothrix*, *Pesotum* J.L. Crane & Schokn. and *Hyalorhinocladiella,* or combinations of these.

The phylogenetic study of Zipfel et al. (2006) showed that the definition of *Ophiostoma* remains unsatisfactory. The study revealed that the genus is polyphyletic, forming lineages linked to morphological characters. *Ophiostoma* species with cylindrical or allantoid ascospores with pillow-shaped sheaths and a continuum of anamorphs, ranging from primarily *Hyalorhinocladiella*-type structures to more rare *Pesotum*-like synnematous structures, group with *Ophiostoma ips* (Rumbold) Nannf. and form the so-called *Ophiostoma ips*-complex (sensu stricto) (Zipfel et al. 2006). The species with relatively long allantoid ascospores and exceptionally long perithecial necks and *Sporothrix* anamorphs group within the *Ophiostoma pluriannulatum*-complex. The most challenging group to define is the *Ophiostoma piceae*-complex, which includes species with allantoid to cylindrical ascospores and a variety of anamorphs. This complex does not form a well-supported phylogenetic lineage. This is problematic, since the type species for *Ophiostoma*, *Ophiostoma piliferum* (Fr.) Syd. & P. Syd. falls in this group.

There are no clear characters that can be used to define *Ophiostoma* as a distinct genus. Several phylogenetic studies have shown that the hardwood-infesting isolates group together (Harrington et al. 2001, de Beer et al. 2003a, Grobbelaar et al. 2009, 2010). The *Sporothrix schenckii*-*Ophiostoma stenoceras*-complex of species, characterized by reniform ascospores without a sheath, a *Sporothrix* anamorph (de Beer et al. 2003b), and the absence of intron 4 and presence of intron 5 in the β-tubulin gene (Zipfel et al. 2006), also represent a discrete group. The habitat of species belonging to this group is in contrast to other Ophiostomatalean species, which are associated with bark beetles or other tree-infesting insects. The majority of the species in *S. schenckii*-*O. stenoceras*-complex are found in soil. A recent study has shown that the species in this complex should be recognized as a distinct genus (de Beer et al. 2010). Also, the monophyly supported by the morphological and possibly ecological characters of the other emerging groups within *Ophiostoma* remain unresolved. This is likely to remain the case until sequences of more species and more genes clarify the genetic status of these complexes.

Ophiostomatoid fungi also differ in the chemical composition of their cell walls (de Hoog and Scheffer 1984). The cell walls of *Ophiostoma* contain cellulose and rhamnose, which is unusual for the Ascomycetes. In contrast, the cell walls of *Ceratocystis* consist mainly of chitin. In addition, *Ceratocystis* spp. are very sensitive to the antibiotic cycloheximide, which inhibits the protein synthesis in most eukaryotic organisms (Harrington 1981, de Hoog and Scheffer 1984, Zipfel et al. 2006). Species of *Ophiostoma* are able to tolerate high concentrations of cycloheximide and this feature is commonly applied when these fungi are isolated from soil or insects.

2.2 Ecology of ophiostomatoid fungi

2.2.1 Sapstain

Ophiostomatoid fungi are also known as "blue-stain fungi" or "sapstain fungi", referring to the bluish, grey, brown or black discoloration of sapwood caused by them (Münch 1907, Seifert 1993). Other groups of fungi causing sapstain are black yeasts and dark molds (Seifert 1993). Sapstain-causing fungi are especially important in conifer trees in the Northern Hemisphere (Seifert 1993, Butin 1996). The discoloration lowers the value of timber, but unlike the structural damage caused by soft-rot or decay fungi, the damage is mainly cosmetic. Staining is caused by fungal hyphae usually growing in the ray parenchyma cells and resin ducts (Münch 1907, Gibbs 1993, Seifert 1993). At later stages of infection, the tracheids are also colonized (Liese and Schmid 1961, Ballard et al. 1982). Discoloration is due to melanin, a pigment existing inside the walls of the fungal hyphae, and not due to staining of the wood tissues (Zink and Fengel 1989, 1990).

2.2.2 Plant pathogens

Several species of ophiostomatoid fungi are serious forest pathogens. The pathogenicity of these fungi has been demonstrated to vary greatly from weak pathogens to species capable of killing healthy trees (Horntvedt et al. 1983, Solheim 1988, Kile 1993). The best-known examples of the latter group are the Dutch elm disease pathogens, *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier, species responsible for the disastrous pandemics killing millions of elm (*Ulmus* L.) trees in both Europe and North America during the past century (Gibbs 1978, Brasier 1991, Hubbes 1999, Brasier and Kirk 2001). Other severe pathogens include the host-specific varieties of *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf. causing black stain root disease in conifers in North America (Cobb 1988, Harrington 1993), *Leptographium calophylli* J.F. Webber, K. Jacobs & M.J. Wingf. causing Takamaka wilt disease (Ivory et al. 1996, Webber et al. 1999), and *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. that has been associated with a disease known as white pine root decline, but most likely only contributes to the disease (Kendrick 1962, Alexander et al. 1988, Wingfield et al. 1988). Species of *Ceratocystis* are also causal agents of tree diseases, such as *Ceratocystis fagacearum* (Bretz) J. Hunt. causing oak wilt (Hepting 1955, Kile 1993) and members of the *Ceratocystis fimbriata*-complex causing canker stain and vascular wilt diseases in a wide range of host trees (Kile 1993). Several species of *Ceratocystis* are also economically important pathogens of food and crop plants. A recent review has summarized the current knowledge regarding diseases caused by *Ceratocystis* spp. (Roux and Wingfield 2009).

2.3 Interactions

Fungi are heterotrophs that acquire their food from other organisms. They have developed various life strategies. To date, plant-fungi interactions are known to be older than interactions between fungi and insects (Taylor and Osborn 1996, Engel and Grimaldi 2004, Heckman et al. 2001). The terrestialization of the Earth by land plants might not have been possible without mutualistic plant-fungal interactions (Jeffrey 1962, Pirozynski and Malloch 1975). It has been hypothesized that the initial fungal associates of plants were saprobes with an

invasive mycelium, having the ability to penetrate dying and dead cells (Taylor and Osborn 1996). As these plant-fungal interactions developed, fungi might have overcome the defensive mechanisms of plants, so that parasitic and eventually biotrophic interactions evolved. The earliest fungi were present in the Precambrian period (Heckman et al. 2001), and first examples of plant defense responses to fungal parasites come from the Devonian period (Taylor et al. 1992). While fungi and plants were forming symbiotic relationships at a very early stage in terrestrial evolution, insects had just originated in the Silurian period (Engel and Grimaldi 2004). None of the early insect fossils are known to have fungal associates (Taylor and Osborn 1996). Therefore, it can be assumed that fungi were first adapted to plants and that interactions with insects developed much later. Examples show that since these interactions started to develop, they have often led to complex and rather sophisticated associations (Hughes et al. 2010).

The association between bark beetles and fungi was first recognized in the 19th Century (Schmidberger 1836, Hartig 1844, 1878). Due to the often destructive nature of these interactions, a number of studies have been devoted to resolving the nature of the associations. At present it is known that bark beetles, fungi and host trees form complex interactions, of which many are still only poorly understood.

2.3.1 Fungal-bark beetle interactions

Bark beetles are among the first insects that attack a dead or a weakened tree. They include species that reproduce in the inner bark (phloephagous species), and ambrosia beetles (xylomycetophagous species), which bore tunnels into the wood and cultivate and feed on symbiotic ambrosia fungi (Knížek and Beaver 2004). Bark beetle species are geographically widely distributed (Knížek and Beaver 2004), and occur in a wide range of host trees (Kirkendall 1983). In Nordic countries and Russian Karelia, entomological research has a long and intensive tradition, and the biology of forest pest fauna and their host range is well known (Lekander et al. 1977, Heliövaara et al. 1998, Mandelshtam and Popovichev 2000, Voolma et al. 2004). Probably due to the host choice behavior of the beetles, phloephagous species are normally specific to one tree genus, and only some species attack trees from closely related genera (Sauvard 2004, Bertheau et al. 2009). However, bark beetles are well suited for movement across national boundaries, and have adaptation capabilities that allow them to switch to novel host tree species if introduced to a new environment (Marchant and Borden 1976, Tribe 1992, Sauvard 2004, Yan et al. 2005). These potential new interactions are a matter of concern, as they can result in extensive insect outbreaks and damage in forest ecosystems.

Most of the bark beetle species are harmless to healthy living trees, but some are regarded as important forest pests, causing significant economic losses (Knížek and Beaver 2004). Conifer bark beetle species are the most important forest pests in the temperate zones (Grégoire and Evans 2004). Bark beetle species that infest hardwood trees are considered less harmful, with the exception of the species vectoring the fungi responsible for the Dutch elm disease pandemics.

In their native environment and during non-outbreak conditions, several bark beetle species are regarded as secondary, infesting dead or dying trees (e.g. *Ips pini* (Say), *Scolytus ventralis* LeConte, Paine et al. 1997, Martikainen et al. 1999, Knížek and Beaver 2004). They are organisms that have an important role in forest ecosystems accelerating the natural recycling of nutrients (Martikainen et al. 1999). Several bark beetles are keystone species driving forest succession, e.g. *Ips typographus* L. in Eurasia. A number of other organisms,

such as arthropods and fungi, are associated with *I. typographus* (Weslien 1992, Viiri 1997). Bark beetle species can become economically important when they transfer pathogenic fungi to living trees, when their populations build to outbreak levels, or when they are introduced into new environments (Wingfield and Swart 1994, Knížek and Beaver 2004). A relatively small number are considered primary bark beetles (e.g. *Dendroctonus ponderosae* Hopkins in North and Central America and *I. typographus* in Europe) that attack living, healthy trees, seedlings or seeds of commercial crops (Coulson 1979, Wood 1982, Paine et al. 1997, Knížek and Beaver 2004). The majority of bark beetle species have only minimal contact with living trees. These species are saprophagous, which colonize only dead trees (Raffa et al. 1993, Paine et al. 1997).

Ophiostomatoid fungi are common and relatively well-known associates of bark beetles (Münch 1907, Harrington and Cobb 1988, Wingfield et al. 1993, Paine et al. 1997, Jacobs and Wingfield 2001, Kirisits 2004). Ophiostomatoid fungi are commonly found in galleries constructed by bark beetles and their larvae in the phloem and wood of mainly coniferous trees (Kirisits 2004). Fungi sporulating in the galleries can be carried in mycangia, special organs of bark beetles (Francke-Grosmann 1967, Beaver 1989), attached to the surface of their exoskeletons (Beaver 1989), in the digestive tracts of the beetles (Furniss et al. 1990), or on mites phoretic on bark beetles (Moser et al. 2010). Usually bark beetles are associated with more than one fungus. Each bark beetle can transfer several fungal species, and thousands of conidia and ascospores, but great variation occurs between individuals (Solheim 1993a). The association of ophiostomatoid fungi with particular bark beetle species can be either specific or more casual. Bark beetle species with more casual associations can vector numerous fungi, but none of these fungal species is found consistently in high frequencies in bark beetle populations (Mathiesen-Käärik 1953, Solheim and Långström 1991, Gibbs and Inman 1991). For example, *T. piniperda* is a vector of numerous ophiostomatoid fungi, of which many are reported only occasionally and in low numbers (Kirisits 2004). In specific associations between fungi and bark beetles, a large number of individual bark beetles regularly carry spores of certain ophiostomatoid fungi. The diversity of ophiostomatoid fungi associated with hardwood-infesting bark beetles is still poorly understood, especially in the Northern Hemisphere. Most studies have focused on the *Scolytus* Geofroy spp. vectoring the Dutch elm disease fungi (Gibbs 1978, Brasier 1991, Hubbes 1999, Brasier and Kirk 2001). In this unusual fungus-vector system, the hardwood-infesting bark beetles have rather fixed associations with non-native fungi.

Studies of beetle-associated flora are generally focused on reporting the fungal associates of different bark beetle species. Lieutier et al. (2009) suggested that the host tree has a more important role than the beetle in the speciation of ophiostomatoid fungi. In the evolutionary sense, plant-fungi interactions are known to be older than interactions between fungi and insects (Taylor and Osborn 1996, Engel and Grimaldi 2004, Heckman et al. 2001). Studies regarding the origin of associations between ophiostomatoid fungi, the host tree and the vector insect are lacking. In the light of knowledge from plant-fungal interactions in general, it is possible to conclude that the adaptation of ophiostomatoid fungi to trees is also older than their adaptations to bark beetles (Harrington and Wingfield 1998, Lieutier et al. 2009).

Interactions between bark beetles and their fungal associates are diverse, ranging from antagonism and commensalism to mutualism (Klepzig et al. 2001, Klepzig and Six 2004). In many cases, the symbiosis is thought to be mutualistically benefitting for both the beetles and the fungi (Francke-Grosmann 1967, Beaver 1989, Berryman 1989, Ayres and Lombadero 2000). The dispersal of the ophiostomatoid fungi almost completely depends on the insect vectors, and therefore the fungi benefit from the association with the beetle vectors by transport

to new host trees (Dowding 1969, Paine et al. 1997, Klepzig and Six 2004). Ophiostomatoid fungi have evolved adaptations to facilitate this transfer between trees. The fruiting structures of ophiostomatoid fungi are usually long stalks bearing spores in slimy droplets and concave shapes to allow multiple contact points, which can easily attach to the surface of the insect vector (Malloch and Blackwell 1993). Sticky ascospores ensure that they adhere tightly to the body of the insect and disperse in the resin of the new host, not in water (Whitney and Blauel 1972). Besides rapid transport to a suitable habitat, insect dispersal provides protection from desiccation and UV light (Klepzig and Six 2004). Furthermore, for some mutualistic fungi, sexual recombination has become apparently no longer necessary, and they lack or rarely possess sexual reproduction (Wulff 1985). These morphological features are considered as adaptations to insect dispersal and to the bark beetle habitat (Francke-Grosmann 1967, Whitney 1982, Beaver 1989, Malloch and Blackwell 1993).

The evolution of mycangia, the special spore-carrying structures of bark beetles, indicates that some beetles also benefit from the association with fungi (Paine et al. 1997, Harrington 2005). In their nutrition-poor substrates of wood tissues, some bark beetles are dependent upon their fungal associates as a source of nutrients, or benefit from feeding on the fungi (Ayres and Lombardero 2000, Six and Paine 1998). Female ambrosia beetles carry the primary fungus in the mycangium, often together with an assemblage of other fungi, yeasts and bacteria (Batra 1966, Haanstad and Norris 1985). In the new host tree, bark beetles plant and tend the primary fungus in their galleries (Norris 1979). The ways bark beetle species benefit from the association with fungi include feeding on the fungi, modifying the substrate to be more suitable for the larval diet providing compounds such as nitrogen, sterols and proteins, and by limiting the growth of harmful fungal species (Beaver 1989, Paine et al. 1997, Ayres and Lombardero 2000, Klepzig and Six 2004).

Besides the apparently positive benefits to bark beetles, some ophiostomatoid fungi are antagonists of bark beetles. The most widely studied example is *Ophiostoma minus* (Hedgc.) Syd. & P. Syd., which presence is known to greatly reduce the reproductive success of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Barras 1970, Franklin 1970, Lombardero et al. 2003, Hofstetter et al. 2005). The southern pine beetle is typically associated with three fungi. Two species are mycangial fungi, *Ceratocystiopsis ranaculosa* T.J. Perry & J.R. Bridges and *Entomocorticium* sp., which are nutritional mutualists (Barras 1970, Hofstetter et al. 2005). The third species, *Ophiostoma minus* (Hedgc.) Syd. & P. Syd., is transported on the beetle's exoskeleton, or actively transported by mites phoretic on beetles (Lombardero et al. 2000, 2003). *Ophiostoma minus* is a strong nutritional mutualist of mites, and therefore more intimately associated with the mites than the southern pine beetle. When transported to phloem tissue, *O. minus* competes the same resources with the beetle-mutualistic fungi (Klepzig and Wilkens 1997, Klepzig et al. 2004). The recent studies have shown that bark beetles, mites and associated fungi form complex chains of interactions (Lombardero et al. 2000, Hofstetter et al. 2005), which could be altered by temperature (Hofstetter et al. 2007).

The possible benefits of fungal associates to bark beetles in the process of successful colonization of living trees have been the subject of continuing debate. Several bark beetle-associated fungi have been considered to facilitate the bark beetle colonization by helping to overcome host resistance and killing the tree (Nebeker et al. 1993, Paine et al. 1997). This classic paradigm (CP) suggests that many bark beetle-fungus associations are mutualistic, based on the phytopathogenicity of the fungal associates (Six and Wingfield 2011). The results of several studies focused on these host tree-bark beetle-fungi interactions have been controversial and without conclusive evidence to support the CP.

2.3.2 Fungal-host tree-bark beetle interactions

Our current understanding of the interactions between bark beetles, fungi and host trees is insufficient and thus beset with controversy. Here I will discuss only a few aspects of the presented arguments. The varying aspects have been discussed in more detail in several articles (e.g. Whitney 1982, Harding 1989, Raffa and Klepzig 1992, Harrington 1993, Paine et al. 1997, Lieutier 2002, 2004, Kirisits 2004, Lieutier et al. 2009, Six and Wingfield 2011), and the debates will certainly continue in future.

In Fennoscandia, the dispersal and the host finding phase of the bark beetle life cycle is averagely in May-June (Saalas 1949, Heliövaara et al. 1998). Bark beetles overwinter in the forest litter or under the bark of trees and begin their dispersal flight to seek suitable host trees in which to reproduce (Byers 1996). Bark beetles locate the suitable host tree by random landing and testing the tree and its resistance capability (Moeck et al. 1981, Wood 1982). Bark beetles have a pheromone-based communication system that helps them to select and colonize suitable host trees (Moeck et al. 1981, Bakke 1983). After the selection of the host tree, they release pheromones that attract mates and additional colonists, leading to a rapid aggregation of a large number of beetles on the potential host tree (Raffa and Berryman 1983).

Mutualistic relationships between phytopathogenic fungi have been proposed to be essential for bark beetles to successfully colonize living trees (Francke-Grosmann 1967, Graham 1967, Raffa and Berryman 1983). The tree killing hypothesis suggests that virulent fungi are responsible for tree death by blocking water conduction in the colonized tree (Långström et al. 1993, Paine et al. 1997). According to another hypothesis, fungi cause tree death indirectly by stimulating induced defense mechanisms of the host tree (Lieutier et al. 2009). Since the early propositions, the assumption was for many years that fungi are responsible for killing the trees attacked by bark beetles before the bark beetles can successfully continue the colonization (Berryman 1982, Coulson 1979, Wood 1982). The importance of ophiostomatoid fungi in host tree infestation by bark beetles has been tried to study developing fungal-free progenies of bark beetles, but with no success (Harding 1989). It has been demonstrated that the presence of ophiostomatoid fungi is not a prerequisite for successful reproduction of some bark beetle species (Grosmann 1931, Harding 1989, Colineau and Lieutier 1994). Additionally, tree-killing bark beetles are able to kill trees without virulent fungal associates (Hetrick 1949, Bridges et al. 1985). Even when virulent fungal associates do occur, they are usually inconsistent associates, such as *Ceratocystis polonica* (Siemaszko) C. Moreau associated with *I. typographus* during the outbreaks (Harding 1989, Jankowiak and Hilszczański 2005).

The role of fungi associated with bark beetles has been aimed to be shown in a number of studies attempting to mimic bark beetle attacks by artificially inoculating living host trees with symbiotic fungi (e.g. Christiansen 1985, Solheim et al. 1993, Yamaoka et al. 1995, Krokene and Solheim 1998, Kirisits 1998). The lesion length caused by the fungal infestation has been used as a measure to study the virulence of a fungus (Matsuya et al. 2003, Rice et al. 2007). Under the tree killing hypothesis, the most virulent fungal associates are believed to be the most effective in killing the tree, and therefore the most useful for bark beetles (Yamaoka et al. 1990, Solheim and Safranyik 1997). The defense exhaustion hypothesis suggests that the most virulent fungal associates are the most effective in exhausting tree defense mechanisms. Studying these hypotheses included in the CP has several difficulties, and the results from the studies have been controversial. However, the CP has strongly influenced the research on bark beetle-fungus symbiosis during the last decades. Recently, the CP has been proposed

to be fundamentally flawed (Six and Wingfield 2011). Six and Wingfield (2011) suggest that fungal phytopathogenicity has a more important role for the fungi, rather than supporting the bark beetles in tree killing.

Fungal pathogenicity may be a factor helping the fungi to survive in a living tree (Six and Wingfield 2011). Pioneer fungal species need to be able to colonize tissues that are still living, or be able to tolerate the defensive reactions of trees formed in response to the beetle attack. Highly virulent fungi might need to be able to survive in a living tree, because they live in association with bark beetles completing their entire life cycle in living trees (Six and Wingfield 2011). Fungi that do not display high levels of virulence might be those invading tree tissues later and more slowly, following pathogenic fungal associates. For example, species such as *C. polonica,* shown to be highly virulent in artificial inoculation studies, are the first species that invade sapwood (Solheim 1992a, 1992b, 1993a). Typical of these species is the fact that they have rapid growth rates and tolerance to low oxygen levels.

2.3.3 Fungal-fungal interactions

One relatively well-known example of fungal-fungal interactions is that between mycangial species and other fungi. Fungi carried in the mycangia of ambrosia beetles compete with other fungi carried by the beetles, and can positively affect the fitness of bark beetles by limiting growth of co-occurring fungi (Norris 1979, Mueller et al. 2005). Ambrosia beetles carry one primary fungus intended for cultivation, and the other fungi are possible weeds that soon contaminate and overgrow the cultivated fungal gardens, if they remain untended. Mycangial fungi are considered low-virulent species (Paine et al. 1997).

Trees attacked by bark beetles are subjected to colonization by several fungal species competing for the same resources. Ophiostomatoid fungi are known to be more tolerant to terpenes in conifer resin than other co-occurring early colonizing fungi, and thus some species may actually benefit from these defense reactions in the competition with other fungi (Cobb et al. 1968, De Groot 1972, Harrington 1993, Klepzig and Six 2004, Lieutier et al. 2009). Competition between pioneer fungi, including interspecific competition between ophiostomatoid species, might play an important role in the successful colonization and pathogenic properties of fungal species (Owen et al. 1987, Parmeter et al. 1989, Harrington 1993).

Bark beetles typically have multiple fungal associates. If competition between fungal symbionts is the only mechanism shaping the bark beetle-fungus interactions, there would be a strong evolutionary selection pressure driving the selection of the most competitive fungal associate (Six and Wingfield 2011). One hypothesis for the occurrence of multiple fungal associates at the same time is that although the fungi seem to occupy the same niche, separation into niches actually exists. This separation into niches reduces competition and thus allows the coexistence between several fungi. The niche separation might be a result of different temperature tolerance; resource use, such as the use of carbon and nitrogen sources; and a different degree of virulence between fungi (Six and Paine 1997, Solheim and Krokene 1998, Bleiker and Six 2007, Six and Wingfield 2011).

2.4 Impact of globalization and environmental change

The majority of the bark beetle species are considered rather harmless species in their native ranges, colonizing mainly weakened or dead trees. However, these species pose potential risks in changing or new environments. Therefore, they should not be ignored when evaluating risks and threats to ecosystems and the services they provide to humans or when determining quarantine measures for pests and pathogens. Forest pest insects and their associated micro-organisms are capable of movement through national boundaries. International trade and travel between and within continents has increased the rates of these forest pest introductions to new environments. For example, a recent study has listed 109 exotic phytophagous insect species originated from North America and Asia that successfully invaded and established themselves on Europe's woody plants (Vanhanen 2008). The risk of successful establishment in a new environment is highest when the main host species for the introduced pest species occurs naturally or is also introduced and widely cultivated. Changes in the climate might also induce invasions of both native and exotic insect pests from southern locations to northern locations, and increase the frequency and intensity of forest insect outbreaks (Ayres and Lombardero 2000). For example, a temperature increase can significantly affect the reproduction and population dynamics of *I. typographus* in Northern Europe (Jönsson et al. 2007).

A classic example of the impact of invasive species is found in the Dutch elm disease fungi. It has been hypothesized that these fungi were originally native to the Asia (Brasier 1983), from where the pathogen was accidentally introduced into America and Europe. Elm species in America and Europe do not display resistance to the pathogen (Ozolin and Kryokova 1980, Heybroek 1981), which resulted in two destructive pandemics wiping out millions of the elm trees.

There are also several current examples of the major devastation that bark beetles and their fungi caused as a result of environmental changes or where they have been introduced into new environments. One example is the mountain pine beetle (MPB) outbreak in Canada. The mountain pine beetle (*Dendroctonus ponderosa* Hopk.) is native to pine forests in western parts of North America (Safranyik and Carrol 2006). It primarily infests lodgepole pine (*Pinus contorta* Dougl. Ex. Loud.), but can colonize most pine species occurring in the region. Lodgepole pine is widely distributed in Canada, and therefore the occurrence of the beetle species in western Canada is not restricted by the availability of a suitable host tree. Climate is the major factor limiting the MPB to expand to northern and eastern parts of Canada (Safranyik 1978). Normally the MPB infests weakened and dying trees. However, periodical large-scale outbreaks on healthy trees are also part of the normal behavior of the MPB (Safranyik and Carrol 2006). Current outbreaks in British Columbia, Canada are more severe and larger in area than any of the previous outbreaks recorded (Taylor et al. 2006). The outbreak is occurring in areas previously considered climatically unsuitable for the MPB (Safranyik et al. 1975). This shift to formerly climatically unsuitable areas during the last two decades has been explained by climate change. The sufficient changes in the climatic conditions, such as increased temperatures and reduced summer precipitation have allowed the mountain pine beetle to establish and form continuous populations in new areas (Williams and Liebhold 2002, Carrol et al. 2003). Another example of a bark beetle outbreak-induced by climate change which has led to significant damage in North America, Alaska, is the spruce beetle (*Dendroctonus rufipennis* Kirby) (Berg et al. 2006). As a result of increased temperatures, the reproduction time of the spruce beetle has halved and led to extensive and unprecedented damage to spruce forests.

An example of a beetle and its associated fungi recently introduced into a new environment is the red turpentine beetle (*Dendroctonus valens* LeConte). In its native range the bark beetle attacks living conifers, mainly *Pinus ponderosa* Dougl. ex Laws. in North America, without killing the trees (Smith 1961). It was introduced from the pine forests of North and Central America to China around 1980 (Pajares and Lanier 1990). In China, it spread rapidly since the first outbreak in 1999, causing significant damage in over half a million hectares of pine stands (Yin 2000, Li et al. 2001, Miao et al. 2001). In China, the main host tree species for *D. valens* is *Pinus tabuliformis* Carr. (Li et al. 2001). The red turpentine beetle vectors an ophiostomatoid fungus, *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., which is non-pathogenic in the USA, but has become a serious pathogen of pine in China (Lu et al. 2010). The invasive strains of the fungi tolerate higher concentrations of monoterpenes and are thus better adapted to the host's defense response. There is also evidence that the fungus may increase beetle fitness by increasing the weight of the larvae that feed on the fungus.

Numerous contemporary examples illustrate that bark beetles previously considered minor pests can become substantial threats in changing or new environments. Thus, all bark beetle species and the fungi they carry should be considered as potentially threatening. This is at least within the context that they may not necessarily behave similarly in their native and introduced ranges.

2.5 Occurrence of *Ophiostoma* spp. and *Grosmannia* spp. in Fennoscandia

Previous studies have recorded 15 species of *Ophiostoma* and 12 species of *Grosmannia* and *Leptographium* occurring in association with pine-, spruce- and birch-infesting bark beetles in Fennoscandia (Table 1). The investigations thus far have included 15 bark beetle species, of which 14 infest conifers and one infests hardwood species. The most extensively studied bark beetle species is *I. typographus*. The investigations conducted in entire Europe have recently been reviewed by Kirisits (2004).

The diversity of ophiostomatoid fungi that bark beetles vector in Fennoscandia shows some differences compared to southern parts of Europe. The species diversity appears to be lower in northern parts of Europe. Several ophiostomatoid fungi have been regarded as more common associates in northern parts of Europe, including species such as *C. polonica*, *Grosmannia penicillata* (Grosman) Goid., *Ophiostoma piceae* (Münch) Syd. & P. Syd., *Grosmannia piceiperda* (Rumbold) C. Moreau, *O. minus*, *Ophiostoma ainoae* H. Solheim and *Ophiostoma bicolor* R.W. Davidson & D.E. Wells. However, studies on ophiostomatoid fungi in Finland and neighboring Russia are limited. Reports of *Ophiostoma* and *Grosmannia* species from Russia are more numerous, but to our knowledge, none of the studies have been conducted in the Fennoscandian parts of Russia. The majority of the studies in Russia have focused on middle Siberia and southeastern parts of the vast country. Bark beetles and host trees that are common in the boreal forest of Siberia are not widely distributed in the European parts of Russia. The distribution of bark beetle that is considered quarantine pests in Europe, *Ips subelongatus* Motschulsky, follows the distribution of larch (*Larix* Mill. sp.) (Stark 1952). However, several ophiostomatoid species reported from conifer bark beetles in Siberia are also typical to Fennoscandia. These include *O. ainoae*, *O. bicolor*, *O. minus* and *O. piliferum* (Pashenova et al. 1995, 2004). On the contrary, although elms (*Ulmus* spp.) occur in southern Finland and parts of Russian Karelia, none of the elm-infesting *Scolytus* spp. have been found in this region (Jakovlev and Siitonen 2005). There are also no current reports of the occurrence of species responsible for Dutch elm disease from Finland. *Ophiostoma ulmi*

was once present in Finland, but successfully eradicated (Hintikka 1974).

Studies regarding bark beetle-associated fungi in Fennoscandia are rather limited and their main focus on the fungal associates of aggressive bark beetles might have biased the true fungal biodiversity in the region. Based on previous studies, boreal forests in Fennoscandia and Russia appear to have a rather similar bark beetle-associated fungal flora mycobiota, which show some differences compared to fungal assemblages occurring in southern parts of Europe. The variations at different locations in Europe migh reflect different sampling strategies and other subjective factors such as fungal isolation methods (Kirisits 2004). In general, the migration patterns of taxa in Northern Europe have been strongly affected by periods of glaciations (Hewitt 1996). For example, recent molecular analyses and fossil records have revealed that the Norway spruce populations in Northern and Central Europe form two distinct lineages, which have been isolated from each other for a long time (Tollefsrud et al. 2008). The populations in Northern Europe have originated from Russia, and spread from there to Scandinavia. Similar studies to fungal populations are limited. A recent study has shown that the European population of *C. polonica* could be treated as a single unit, and therefore no congruence with the genetic structure of its host tree *Picea abies* have been detected in Europe (Marin et al. 2009).

Table 1. Previous reports of *Ophiostoma* and *Grosmannia* spp. in association with different bark beetles infesting *P. abies*, *P. sylvestris* and *Betula pendula* in Fennoscandia. Identifications in all these studies were based on morphology, and only those marked with * included DNA sequence comparisons at least in one study.

Fungus	Beetle	Host tree	Reference
Grosmannia (?) *aureum*	*Hylastes ater*	*Pinus sylvestris*	Mathiesen-Käärik 1953
G. cucullata	*Ips typographus*	*Picea abies*	Solheim 1986, Ahtiainen 2008
G. galeiformis	*Hylastes cunicularius*	*P. abies*	Mathiesen-Käärik 1953
G. olivacea	*H. cunicularius*	*P. abies*	Mathiesen-Käärik 1953
*G. penicillata**	*H. ater*	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	Hylurgops palliatus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	H. cunicularius	*P. abies*	Mathiesen-Käärik 1953
	Ips duplicatus	*P. abies*	Valkama 1995, Krokene & Solheim 1996
	I. typographus	*P. abies*	Mathiesen 1950, Rennerfelt 1950, Mathiesen-Käärik 1953, Solheim 1986, 1992a, 1992b, 1993b, Furniss et al. 1990, Viiri & Weissenberg 1995, Krokene & Solheim 1996, Viiri 1997, Persson et al. 2009
	Polygraphus poligraphus	*P. abies*	Krokene & Solheim 1996
	Trypodendron lineatum	*P. abies*	Mathiesen-Käärik 1953

Table 1. Continued.

Fungus	Beetle	Host tree	Reference
G. piceiperda (=G. europhioides)	*H. palliatus*	*P. abies*	Krokene & Solheim 1996
	I. duplicatus	*P. abies*	Krokene & Solheim 1996
	I. typographus	*P. abies*	Solheim 1986, 1992b, 1993b, Viiri & Weissenberg 1995, Viiri 1997, Ahtiainen 2008
	P. poligraphus	*P. abies*	Krokene & Solheim 1996
	Tomicus piniperda	*P. sylvestris*	Solheim & Långström 1991
	I. typographus	*P. abies*	Persson et al. 2009
*L. chlamydatum**	*Dryocoetes autographus*	*P. abies*	Jacobs et al. 2010
	H. cunicularius	*P. abies*	Jacobs et al. 2010
*L. curvisporum**	*D. autographus*	*P. abies*	Jacobs et al. 2010
	H. cunicularius	*P. abies*	Jacobs et al. 2010
L. guttulatum	*H. palliatus*	*P. sylvestris*	Mathiesen 1950
L. lundbergii	*not reported*	*P. abies*	Hallaksela 1977
	Ips acuminatus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	Orthotomicus proximus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	Pityogenes quadridens	*P. sylvestris*	Mathiesen-Käärik 1953
	Tomicus minor	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	T. piniperda	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
L. procerum		*Picea abies*	Hallaksela 1977
L. wingfieldii	*T. piniperda*	*P. sylvestris*	Solheim & Långström 1991
O. ainoae	*I. typographus*	*P. abies*	Solheim 1986, 1992a, 1992b, 1993b, Viiri & Weissenberg 1995, Viiri 1997
		P. abies	Hallaksela 1977
*O. bicolor**	*Ips amitinus*	*P. abies, P. sylvestris*	Savonmäki 1990
	I. duplicatus	*P. abies*	Valkama 1995, Krokene & Solheim 1996
	I. typographus	*P. abies*	Solheim 1986, 1992a, 1992b, 1993b, Furniss 1990, Savonmäki 1990, Krokene & Solheim 1996, Viiri 1997, Ahtiainen 2008, Persson et al. 2009
	P. chalcographus	*P. abies*	Savonmäki 1990, Krokene & Solheim 1996
	P. poligraphus	*P. abies*	Krokene & Solheim 1996
*O. borealis**		*Betula pendula*	Kamgan et al. 2010
O. brunneo-ciliatum	*Ips sexdentatus*	*P. sylvestris*	Mathiesen-Käärik 1953

Table 1. Continued.

Fungus	Beetle	Host tree	Reference
O. canum	*I. acuminatus*	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	P. quadridens	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	T. piniperda	*P. sylvestris*	Mathiesen 1950, Rennerfelt 1950, Mathiesen-Käärik 1953
	T. minor	*P. sylvestris*	Mathiesen 1950, Mathiesen 1951, Rennerfelt 1950, Mathiesen-Käärik 1953
O. clavatum	*I. acuminatus*	*P. sylvestris*	Mathiesen 1950, Mathiesen 1951, Rennerfelt 1950, Mathiesen-Käärik 1953
	I. sexdentatus	*P. sylvestris*	Mathiesen-Käärik 1953
	O. proximus	*P. sylvestris*	Mathiesen-Käärik 1953
	T. piniperda	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
O. flexuosum	*I. typographus*	*P. abies*	Solheim 1986
O. floccosum	*I. typographus*	*P. sylvestris*	Mathiesen 1950, Mathiesen 1951, Mathiesen-Käärik 1953
	P. chalcographus	*P. abies*	Mathiesen 1950, Mathiesen 1951, Mathiesen-Käärik 1953
	T. minor	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
O. ips	*H. ater*	*P. sylvestris*	Mathiesen-Käärik 1953
	I. acuminatus	*P. sylvestris*	Mathiesen-Käärik 1953
	O. proximus	*P. sylvestris*	Mathiesen-Käärik 1953
	T. piniperda	*P. sylvestris*	Mathiesen-Käärik 1953
O. minus	*H. ater*	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	I. acuminatus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	I. typographus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	O. proximus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	P. quadridens	*P. sylvestris*	Mathiesen-Käärik 1953
	T. minor	*P. sylvestris*	Mathiesen 1950, Rennerfelt 1950, Mathiesen-Käärik 1953
	T. piniperda	*P. sylvestris*	Mathiesen 1950, Rennerfelt 1950, Mathiesen-Käärik 1953, Solheim & Långström 1991

Table 1. Continued.

Fungus	Beetle	Host tree	Reference
*O. piceae**	*H. ater*	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	H. cunicularius	*P. abies*	Mathiesen-Käärik 1953
	H. palliatus	*P. abies*	Savonmäki 1990, Krokene & Solheim 1996
		P. sylvestris	Mathiesen 1950, Mathiesen-Käärik 1953, Savonmäki 1990
	I. acuminatus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	I. amitinus	*P. abies, P. sylvestris*	Savonmäki 1990
	I. duplicatus	P. abies	Valkama 1995, Krokene & Solheim 1996
	I. typographus	*P. abies*	Mathiesen 1950, Rennerfelt 1950, Mathiesen-Käärik 1953, Solheim 1986, 1992b, 1993b, Savonmäki 1990, Viiri & Weissenberg 1995, Krokene & Solheim 1996, Viiri 1997, Persson et al. 2009
	not reported	not reported	Wegelius 1938
	O. proximus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	P. chalcographus	*P. abies*	Mathiesen 1950, Mathiesen-Käärik 1953, Savonmäki 1990, Krokene & Solheim 1996
	T. piniperda	P. sylvestris	Savonmäki 1990
	T. lineatum	*P. abies, P. sylvestris*	Savonmäki 1990
	P. quadridens	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	T. minor	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	T. piniperda	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953, Solheim & Långtröm 1991
	T. lineatum	P. abies	Mathiesen-Käärik 1953
O. piliferum	*H. ater*	*P. sylvestris*	Mathiesen-Käärik 1953
	I. acuminatus	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
	I. typographus	*P. abies*	Savonmäki 1990
O. pluriannulatum	*I. typographus*	*P. abies*	Mathiesen-Käärik 1953
	T. minor	*P. sylvestris*	Mathiesen 1950, Mathiesen-Käärik 1953
O. stenoceras	*I. typographus*	*P. abies*	Mathiesen 1950, Mathiesen-Käärik 1953

Table 1. Continued.

Fungus	Beetle	Host tree	Reference
O. tetropii	*I. typographus*	*P. abies*	Solheim 1986, 1992b, Savonmäki 1990, Viiri & Weissenberg 1995, Viiri 1997
	P. chalcographus	*P. abies*	Savonmäki 1990

2.6 Fungal species concepts

The concept of species is ambiguous in mycology. Species is commonly used as the basic rank in taxonomy, but what is considered to be a species can vary widely (Guarro et al. 1999). Asexual reproduction and hyphal anastomosis are common characters in fungi, and therefore an individual is not always easy to distinguish from a population in mycology (Carlile et al. 2001). Attempts to create a universal definition of species have failed, and are most likely bound to remain unresolved (Hey 2001). Thus, several different approaches for delineating species have been used. The most widely accepted approaches are the morphological species concept, the biological species concept, and the phylogenetic species concepts (e.g. Guarro et al. 1999, Taylor et al. 2000).

The classic and most widely used concept by mycologists has been the morphological species concept. The approximately 100,000 identified fungi have mainly been defined and described based on morphological characters (Kirk et al. 2008). The weakness of the application of this concept is that morphologically-defined species often comprise more than one taxon, and it cannot be counted on to diagnose evolutionary meaningful species of fungi (Taylor et al. 2000). The morphological species concept cannot be used as the only approach to define and delineate ophiostomatoid species, because of their relatively simple morphology and overlapping features between different species. Since the monograph by Upadhyay (1981), the taxonomic understanding of ophiostomatoid fungi has improved, and it is now clear that several morphology-based species descriptions are much too broad. In many cases, the biological species concept is less ambiguous in mycology and has held a prominent place in species recognition (Taylor et al. 2000). Ernst Mayr (1970) defined that "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." In ophiostomatoid fungi, the biological species concept was first applied to show the species delimitations in the *Ophiostoma ulmi*-complex (Brasier 1986), and the morphologically similar *O. piceae*-complex (Brasier and Kirk 1993, Halmschlager et al. 1994, Pipe et al. 1995). A serious problem with fungi, including ophiostomatoid fungi, is that mating tests are not possible to apply to fungi if they lack meiospores, are homothallic, cannot reproduce in cultivation or cannot be cultivated (Reynolds 1993). According to Taylor et al. (2000), an even deeper problem with the biological species concept is that fungi can be genetically isolated in nature, but retain the ancestral character of interbreeding.

The phylogenetic species concept has been used increasingly. Phylogenetic approaches and analyses of DNA sequence data have helped in resolving confusing taxonomic debates and they have greatly increased the taxonomic understanding of fungi (e.g. Zipfel et al. 2006, Grobbelaar et al. 2010). However, the definition of the phylogenetic species concept is not without complications. Ranking individuals in order to determine whether they can be considered different species by using phylogenetic analysis of a single gene without

including additional information, such as mating tests, is uncertain due to the possibility of polymorphism (Taylor et al. 2000). The subjectivity can be avoided by using comparisons of more than one gene genealogy a phylogenetic approach referred as genealogical concordance phylogenetic species recognition (GCPSR). Recent studies of ophiostomatoid fungi have widely adopted multi-gene phylogenies in combination with an evaluation of morphological and biological characteristics (Zipfel et al. 2006, Grobbelaar et al. 2010).

2.7 Tools for molecular identification

For phylogenetic species recognition, the genes encoding nuclear and mitochondrial ribosomal RNA genes and associated spacer regions are widely used. The universal and conserved nature of these genes makes them useful in studying fungi, as well as plants and animals (White et al. 1990). The nuclear ribosomal genes are relatively easy to study, because they are arranged in long tandem repeats, which means the gene is already amplified in the genome and only a moderate amount of initial template DNA is needed. The nuclear large subunit (LSU; 28S or 25S) and the mitochondrial small and large subunit genes (SSU, LSU) are used at intermediate taxonomic levels, e.g. to show the position within the genus and the order (Geiser 2004). Analyses of SSU and LSU have been used to identify major monophyletic groups and to suggest their branching orders (Sogin et al. 1996). Nuclear small ribosomal RNA subunit genes, including the 18S gene and internal transcribed spacer regions (ITS) have widely been used for species level studies in many fungi. The three subloci of the ITS regions have different rates of evolution: a highly variable ITS1, a very conserved 5.8S gene and a variable to a semi-conserved ITS2 (Hillis and Dixon 1991, Hershkovitz and Lewis 1996). The two spacer regions (ITS1 and ITS2) are transcribed but do not encode a gene product, and thus evolve faster than the ribosomal subunit genes (Geiser 2004).

The use of ITS sequences for species level studies is sometimes problematic, because in some groups of fungi, ITS sequences have been observed to be either too variable to determine a major group (den Bakker et al. 2004), or too conserved to distinguish between species (Du et al. 2005). Therefore, the ITS region is commonly used as the first step in molecular identification of fungi, and in several cases, another gene or genes are needed for a precise identification. Depending on the fungi, the use of additional genes needs to be determined. In studies of ophiostomatoid fungi, the ITS region was successfully used to resolve the confusing taxonomy of *Ceratocystis* and *Ophiostoma* (Hausner et al. 1993a, b, Spatafora and Blackwell 1994). Between more closely related species, the ITS region is sometimes too conserved and fails to separate very closely related phylogenetic species, such as *O. piceae* and *O. canum* (Harrington et al. 2001).

In recent studies, the use of ITS sequence data together with protein-coding genetic data, such as β-tubulin and translation elongation factor 1-α (EF1-α), have become the norm (Lim et al. 2004, de Meyer et al. 2008, Roets et al. 2008, Grobbelaar et al. 2010). These intron-rich, highly conserved genes provide more resolution at the species level identification than the ITS region (Geiser 2004). The introns of these protein-coding genes evolve at a higher rate than the introns of the ITS region.

3 AIMS OF THE PRESENT STUDY

Despite the economic and ecological importance of forests in Finland, there is very little information on the occurrence of ophiostomatoid fungi on the commercially important tree species. Basically, previous studies have listed the fungal associates of the spruce bark beetle, *I. typographus* (Savonmäki 1990, Viiri 1997, Ahtiainen 2008). Apart from these studies, almost nothing is known regarding fungal associates of other, less-aggressive bark beetle species native to the region. For approaches to understand bark beetle-fungus interactions better there is a need to study interactions that include also other than economically important bark beetle species (Six and Wingfield 2011). Most bark beetle species are known to carry spores of ophiostomatoid fungi. Depending on the habits of their bark beetle vectors, these fungi can cause damage either on trees, logs or lumber. The fungi can weaken or kill trees and/or decrease the value of the wood due to sapstain. Also, the presence of these insects and fungi in the imported lumber raises concerns in countries importing wood products, especially if they do not naturally occur in the importing country. Finnish forests cannot supply the demand of the industry and the country relies heavily on Russia as source of raw timber. The risks of introducing pests and pathogens are difficult to assess because there are only limited studies concerning the possible pest insects and pathogenic fungi on timber imported from Russia to Finland. For example, a large number of bark beetle species, including potential pest species not native to Finland, were identified by Siitonen (1990). However, studies on fungal associates of these beetles in Finland and Russia are limited and the species identifications were made at a time when DNA sequence comparisons were not commonly applied. Furthermore, we are not aware of any study where the bark beetles and their fungal mycobiota in the two countries have been compared.

The general objective of this study was to provide more information on associations between fungi and bark beetles, including both aggressive and non-tree-killing bark beetles. The first aim was to isolate ophiostomatoid fungi associated with the most common bark beetle species infesting the dominant and commercially most important tree species in the boreal forests, Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*) and birch (*Betula pendula* Roth, *Betula pubescens* Ehrh.). The second objective of this study was to identify all ophiostomatoid fungi collected during this survey using morphological characteristics and DNA sequence comparisons for the rDNA gene regions and part of the β-tubulin and EF-1α genes. During the study, we found that the number of ophiostomatoid species is far more than previously thought. Also, a need for redefining some taxonomic groups emerged in this study. Therefore, some of the groups originally intended to be included in this study were left for future investigations. These taxonomic issues are discussed in the summary. The focus of this study is to report species of *Ophiostoma* and *Grosmannia*, which are the economically most important genera reported in several previous studies. Several new species of *Ophiostoma* and *Grosmannia* were found among the obtained isolates, which raised the third objective of describing these newly discovered species. The final objective was to compare the species diversity in Finland and Russia, and assess possible risks involved in the import of timber. The fungi included species from different groups within the ophiostomatoid fungi, which caused problems in DNA sequence alignment. Therefore, we decided to publish the results in smaller and more meaningful parts. Studies **I-II** present the bark beetle and fungal associates of hardwood trees (*Betula* spp.). In study **I**, we reported the fungal associates of the birch bark beetle (*Scolytus ratzeburgi*) and described one new, apparently consistent fungal associate of this scolytinea. In study **II**, the ecology and distribution of this newly described species was

further investigated. The study also revealed a new species, which was formally described, and several other ophiostomatoid taxa. Studies **III-IV** present the fungal associates of conifers (*Pinus sylvestris* and *Picea abies)*. Study **III** presents all species of *Ophiostoma* associated with pine and spruce-infesting bark beetles, and includes a description of five new *Ophiostoma* spp. In study **IV,** we report all species of *Grosmannia* associated with pine and spruce-infesting bark beetles, and describe one new species.

4 MATERIALS AND METHODS

4.1 Bark beetle occurrence and identification

The bark beetle collections for this study were mainly obtained from eastern parts of Finland and Russian Karelia in June-July 2005 (Table 2). Additional collections were obtained from Russian Karelia in June 2004 and July 2007, and southern Norway in July 2007. The landscape of the main study region is covered by mainly coniferous boreal (taiga) forests fragmented by open mires and lakes, cultivated land and sparse settlements. The climatic conditions in the region are characterized by warm summers and cold winters. Biogeographically, all the studied regions in this study (Finland, Russian Karelia and Norway) belong to the same area, Fennoscandia. During the last glaciations, Fennoscandia was covered by an ice sheet that started to retreat about 10,000 years ago. The tree species and their associated pests spread along the same postglacial routes from east-southeast (Hewitt 1996) Therefore, the tree-species composition in the area is rather uniform, with Scots pine, Norway spruce and birches (*B. pendula* and *B. pubescens*) being dominant.

The largest collections of bark beetles were obtained in eastern Finland and Russian Karelia in July 2005 (Table 2–3). Despite the geographical closeness, the forestry practices differ substantially on the different sides of the border, between Finland and Russia. Forests in Finland have been intensively harvested over the last decades, while on the Russian side of the border forests have not been subject to similar intensive forestry management, and relatively high proportions of patches close to their natural state can still be found. Also, higher amounts of dead wood occur in the forests of Russian Karelia. The proportion of dead wood can have an effect on the bark beetle and fungal populations (Martikainen et al. 1996). These features make the region ideal for studying species diversity in both areas of intensive forestry practices and areas where human influence has been slight. Before the flight period (averagely May-June) of bark beetles in the region (Heliövaara et al. 1998), freshly cut trapping logs were laid on the forest floor the previous autumn to allow for their natural colonization. Different bark beetles and their galleries were collected from birch, spruce and pine trapping logs and/or naturally infested trees at four different sites in Finland and six in Russia (Table 2). The sampling strategy in this study was opportunistic (no fixed number of samples per site to be collected). Therefore, direct comparisons between different locations should only be done with caution.

Approximately 600 hectares of spruce-dominated forest was felled by a storm in Lake Vodla national park in Russian Karelia during summer 2000 (Roininen et al. 2005). The majority of these storm-felled trees remained on the forest floor. These storm-felled trees were soon mass-attacked by *I. typographus*, and in autumn 2003, large numbers of healthy standing trees attacked and killed by *I. typographus* were also recorded. Large areas of killed standing trees were also observed outside the storm-affected region. Samples of *I. typographus* collected by H. Roininen from this extensive spruce bark beetle damage area in the Ohtama and Pilmazero regions of Russia were included in this study (Table 2).

Also, collections of *Tomicus piniperda* L. from Volosovo region in Russian Karelia in July 2007 obtained by E. Sidorov were investigated (Table 2). Bark beetles were collected from felled pines in a pine stand that was 80-100 years old. An additional collection of *Scolytus ratzeburgi* Jans. infesting birch in southern Norway was obtained. The sampling was conducted in July 2007 in Akerhus and Østfold counties, Norway (Table 2).

Table 2. Locations and years of sampling of bark beetle this study.

Location	Region	Country	Host trees	Year
Ohtama	Russian Karelia	Russia	*Picea abies*	2004
Ilomantsi, Parissavaara	North Karelia	Finland	*P. abies, Pinus sylvestris*	2005
Jouhteninen, Varparanta	North Karelia	Finland	*P. abies*	2005
Pyhäselkä, Kumpu	North Karelia	Finland	*P. abies*	2005
Laukansaari, Punkaharju	Southern Savonia	Finland	*Betula pendula, P. abies, P. sylvestris*	2005
Kivennapa, Lintula	Russian Karelia	Russia	*P. abies, P. sylvestris*	2005
Lisino-Corpus	Russian Karelia	Russia	*P. abies, P. sylvestris*	2005
Nurmoila	Russian Karelia	Russia	*P. abies, P. sylvestris*	2005
Manga	Russian Karelia	Russia	*B. pendula, P. abies, P. sylvestris*	2005
Roikonkoski	Russian Karelia	Russia	*P. abies*	2005
Uuksujärvi	Russian Karelia	Russia	*B. pendula, P. abies, P. sylvestris*	2005
Volosovo	Russian Karelia	Russia	*P. sylvestris*	2007
Hobøl	Øsfold county	Norway	*Betula* spp.	2007
Spydeberg	Øsfold county	Norway	*Betula* spp.	2007
Vestby	Akerhus county	Norway	*Betula* spp.	2007

4.2 Fungal isolation and identification

Ophiostomatoid fungi were isolated directly from bark beetles, as well as from their galleries on *Betula* spp., *Picea abies* and *Pinus sylvestris*. To prevent the colonization by secondary fungi, samples should be processed soon after they are collected. After bark beetles and galleries were collected, they were stored at 4 °C and the fungal isolations were done within two weeks. The bark beetle galleries were placed in moist chambers and incubated in room temperature for 4 to 6 weeks to allow fungi to sporulate. Each moist chamber consisted of a plastic Petri dish (9 cm) containing moist filter paper. During the incubation period, mycelium and/or fungal spore masses that formed in the galleries were subsequently detected under a dissecting microscope (32 × magnification). A sterile needle or fine sterile forceps were used to isolate fungi from aerial mycelium, from masses of spores on perithecia as well as from mononematous and synnematous conidiophores. The samples were transferred to 2 % malt extract agar (MEA) containing 1.5 % agar and 0.05 % cycloheximide or streptomycin to obtain a selective medium for *Ophiostoma* and *Grosmannia* species. Adult male and female beetles were squashed and streaked on the surface of the same media. Malt extract agar with cycloheximide is selective for *Ophiostoma* species, and it often results in a good sporulation.

Species of *Ceratocystis* are sensitive to cycloheximide (Seifert et al. 1993). Therefore, MEA containing streptomycin was also used in this study, but to a lesser extent.

Majority of the fungal isolates were obtained as described above. Fungal isolations from *Ips typographus* obtained from an extensive outbreak area in Russia were done following the isolation method described by Furniss et al. (1990). Samples of stained wood were transferred to 2 % MEA containing 1.5 % agar and 0.04 % streptomycin.

Different fungal structures from mixed cultures obtained from the beetles and galleries were transferred to new MEA. It should be noted, that the isolation method sometimes results more than one cultures of each species per beetle and/or gallery. These are further considered as separate isolates in the fungal frequency computations in this study (Tables 4–5). Once the resulting fungal isolates were purified, they were grouped based on the morphology. The isolates having similar aerial mycelium, mononematous of synnematous conidiophores, growth rates, growth patterns, colony margins and colors were grouped together, representing potentially the same fungal species. All isolates were transferred also to oatmeal agar (OA) and to MEA, to which sterilized birch, pine or spruce (depending on the origin of the strains) twigs were placed. Both media are suitable for some fungi to sporulate well (Seifert et al. 1993).

4.2.1 Morphological characteristics

The cultural characteristics of species described in the study are based on the colony description of the representative isolates grown at 20°C in the dark. The colony colors were defined according to Rayner's (1970) color charts. The microscopic characteristics were examined using a phase contrast microscope (Nikon Corporation, Tokyo, Japan). For the species descriptions, anamorph and teleomorph (where present) fruiting structures were mounted in 85 % lactic acid on glass slides and examined with a 10×, 25×, 40× objective or a 100× oil-immersion objective. Measurements were made of 50 of each of the relevant morphological structures so that the ranges and averages could be computed. The 0.5 μm scale was used in studies **I-IV** (the theoretical resolution for a light microscope is 0.2 μm). The photographic images were captured with A Nikon DS-F11 camera system (Nikon Corporation, Tokyo, Japan).

4.2.2 DNA sequence data

For molecular identification, one isolate from each morphological group was chosen for DNA extraction and sequencing. The widely sequenced DNA region in fungi, the internal transcribed spacer (ITS) region of the rDNA, was chosen as the starting point for molecular identification. NCBI BLAST searches were conducted for the preliminary identifications. The GenBank database provides an increasing number of fungal ribosomal DNA sequences, particularly for the ITS region (Geiser 2004). A fungus can be identified reasonably well at least to genus level by submitting its ITS sequence and performing a BLAST search in the GenBank.

Based on the BLAST results and the preliminary phylogenetic analyses using ITS sequences, the fungi were further grouped and the need for further sequencing was determined. In several cases, the ITS region did not resolve phylogenetic species very well. Therefore, protein coding genes, β-tubulin (partial gene) and in some cases EF1-α (partial gene), were sequenced to provide more resolution and to confirm the results obtained from the analyses of the ITS region. Based on the BLAST results and the preliminary phylogenetic analysis, some

sequences were novel, representing previously undescribed taxa. Some of those possible new species were also subjected to sequencing the LSU gene to show their placement at higher taxonomic levels. Also, more isolates representing different species were chosen for sequencing of the ITS region. When possible, isolates were selected for DNA sequencing to represent as wide ecological and morphological variation as possible including the following characteristics: different locations, host trees, bark beetle vectors and morphological groups.

4.2.3 Phylogenetic analyses

BLAST searches were conducted for the preliminary identifications, after which datasets were assembled that included reference sequences from GenBank. All datasets were compiled and the preliminary phylogenetic analyses were done using Molecular Evolutionary Genetic Analyses (MEGA) v3.1 (Kumar et al. 2004). Phylogenetic trees used in studies **I-IV** were also edited using MEGA. Prior to the phylogenetic analyses, the datasets must be aligned. The compared sequences usually have different lengths, which means the locations of insertions and deletions must be inferred by introducing gaps in the DNA sequence alignment (Nei and Kumar 2000, Salemi and Vandamme 2003). In the multiple sequence alignment, the idea is to identify homologous regions within several related sequences. Divergent sections in sequences are sometimes problematic in multiple sequence alignments. The error rate in the alignment increases as divergence increases, and can cause the related part of the sequences to show lower similarity than they actually have. This is a problem also within ophiostomatoid fungi, which comprises fungi distributed to different genera. Based on preliminary phylogenetic analyses, isolates could be designated to different genera and complexes of species amongst them. Therefore, separate analyses of sequences for isolates representing taxa in different genera and also different complexes of species were necessary because of their differences in the presence and absence of introns. All datasets in studies **I-IV** were aligned using the online version of MAFFT v6 (Kumar et al. 2002). MAFFT is a fast and accurate multiple sequence alignment program, which has achieved the best results in alignment accuracy in a comparison of several multiple alignment programs (Nuin et al. 2006). After alignment, the datasets were manually edited in MEGA.

Phylogenetic analyses of DNA (or protein) sequences are important tools for studying the evolutionary history of different organisms (Nei and Kumar 2000, Salemi and Vandamme 2003). The true phylogenetic tree is almost always unknown. None of the tree-building methods is perfect or superior to others, and different data sets seem to favor different algorithms. Therefore, it is advisable to employ more than one method for each data set, a practice we applied in this thesis. In studies **I-IV**, a combination of distance methods (neighbor-joining analysis), parsimony methods (maximum parsimony), likelihood methods (maximum likelihood) and Bayesian inference were used. In studies **I-II**, three different approaches for phylogenetic analyses were employed. A neighbor-joining analysis (NJ) with the Kimura 2-parameter (K80) substitution model switched on and a maximum parsimony (MP) analysis were performed using MEGA and Bayesian inference (BI) with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). The reliability of each interior branch of the tree was examined using a bootstrap test (Felsenstein 1985). Phylograms presented in studies **I-II** were obtained from the NJ analyses. In studies **III-IV**, a maximum likelihood analysis (ML) was performed using RAxML 7.0.4 (Stamakis et al. 2008), run on the CIPRES Portal at the San Diego Supercomputing Center (Miller et al. 2009), and a MP analysis using TNT v1.1 (Goloboff et al. 2008) was run on the computer clusters of the CSC, ITS Center for Science, Espoo, Finland, and BI with MrBayes v3.1.2. Phylograms presented in studies **III-**

IV were obtained from the ML analyses. The reliability of each interior branch of the tree was examined using a bootstrap test (Felsenstein 1985). In the TNT parsimony analysis, gaps were coded as a fifth state (using gaps as information). In parsimony analyses, a fifth state coding has been reported to recover a more accurate tree reconstruction compared to treating gaps as missing data (Odgen and Rosenberg 2007). However, in many cases no difference has been reported to occur in the topological accuracy of the different methods of gap coding.

5 RESULTS AND DISCUSSION

5.1 Bark beetle occurrence and identification

Investigations in eastern Finland, Russian Karelia and southern Norway did not reveal any bark beetle species that have not previously recorded in the studied regions. All the species detected during the study represented commonly occurring bark beetle species in the region (Heliövaara et al. 1998, Voolma et al. 2004). The most numerous and most widely occurring bark beetle species were *I. typographus*, *Hylurgops palliatus* gyll., and *Pityogenes chalcographus* (Table 3). Bark beetles colonize trap trees during the dispersal flight period in Fennoscandia, approximately between May-June, a typical time for the dispersal flight for the majority of species (Heliövaara et al. 1998). At the time of collection, galleries of bark beetles were distinguishable in the phloem and cambium tissues of the infested materials (Figure 1–2). Most of the trees sampled contained beetles at a similar stage of development. At the time of the collection (Table 2), both the female gallery stage and the larval galleries of conifer-infesting bark beetles were visible. Only the female galleries could be observed for *S. ratzeburgi*. Altogether 13 bark beetle species and their galleries were collected from Finland, Russia and Norway during the course of this study (Table 3). The majority of the conifer-associated bark beetle species were collected from both pine and spruce. *Ips typographus* and *Ips* sp. (samples for identification purposes were lost; these samples probably present *Ips duplicatus* Sahlb.) were collected only from spruce, and *Ips sexdentatus* Boerner, *Orthotomicus suturalis* Gyll., *T. minor* and *T. piniperda* only from pine. Species that were collected only in Finland included *Hylastes brunneus* Er. and *O. suturalis*, while *I. sexdentatus* and an unidenfied *Ips* sp. and an unidentified *Pityogenes* sp. (samples for identification purposes were lost) were found only at Russian collection sites. The birch bark beetle, *S. ratzeburgi*, was collected from birch (*Betula* spp.) in Finland, Russia and Norway.

5.2 Fungal isolation and identification

A surprisingly high number of bark beetle associated ophiostomatoid species were found in the study areas, including several previously undescribed species (Table 4). As expected based on previous investigations conducted in various parts of Europe, a wide collection of fungi were found in association with different bark beetle species infesting spruce, pine and birch in Fennoscandia. Ophiostomatoid fungi represented the majority of the fungi collected. In total, at least 29 species of *Ophiostoma* and *Grosmannia* were detected (Table 4). Several species were recorded for the first time in the studied countries. A total number of eight new species were described during the survey, including *S. ratzeburgi*-and birch-associated *Ophiostoma karelicum* Linnakoski, Z.W. de Beer & M.J. Wingf. and *Ophiostoma denticiliatum* Linnakoski, Z.W. de Beer & M.J. Wingf., and conifer-infesting bark beetle-associated fungi *Grosmannia taigensis* Linnakoski, Z.W. de Beer & M.J. Wingf., *Ophiostoma fuscum* Linnakoski, Z.W. de Beer & M.J. Wingf., *Ophiostoma pallidulum* Linnakoski, Z.W. de Beer & M.J. Wingf., *Ophiostoma rachisporum* Linnakoski, Z.W. de Beer & M.J. Wingf., *Ophiostoma saponiodorum* Linnakoski, Z.W. de Beer & M.J. Wingf. and *Ophiostoma tapionis* Linnakoski, Z.W. de Beer & M.J. Wingf. The identity of six more species and two species complexes remain unresolved. Only a few fungal species were more constant associates of certain bark beetle species, and if found together with other beetle vectors, they were detected only occasionally. The majority of the fungi occurred in association with a wide variety

Figure 1. Exposed galleries of *Ips typographus* on *Picea abies*. Photo by Wilhelm de Beer.

Figure 2. Fungal sporulation surrounding *Ips typographus* in pupal chambers of the beetle gallery. Photo by Wilhelm de Beer.

Table 3. Bark beetles infesting *Picea abies*, *Pinus sylvestris* and *Betula* spp. collected in this study.

Beetle* species →	1		2		3		4		5		6		7		8		9		10		11		12		13		
Location ↓	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	**Total no.**
FINLAND																											
Ilomantsi					16	12	23	15	20	12					13	16			7								134
Jouhteninen	8	5									5	5									29		9		15	4	13
Pyhäselkä	1										9				9												11
Punkaharju	23				18		17		19																		152
RUSSIA																											
Kivennapa	5	5			10	10			5	7	10	8			3	1					6	8	4	7			89
Lisino-Corpus	24	28			12				12	12	9	8									13	11	3	3			135
Nurmoila	5	5	2	1							12	10	5	3													43
Manga	4	6	5	7					10	4	4	3			1		10	9							4		67
Ohtama	40																										40
Roikonkoski											10	7															17
Uuksujärvi	13	13							1	1			5	7							9	8	9	9	4		79
Volosovo																							15				15
NORWAY																											
Hobøl																									11	10	21
Spydeberg																									13	13	26
Vestby																									10	10	20
Total no.	123	62	7	8	56	22	40	15	67	36	59	41	10	10	26	17	10	9	7	0	57	27	40	19	57	37	**862**

Numbers in table refer to number of bark beetles or their galleries.

Host trees of bark beetles: Blue = spruce, Green = pine & spruce, Yellow = pine, Purple = birch

* Bark beetle species: 1 = *Ips typographus*; 2 = *Ips* sp.; 3 = *Dryocoetes autographus*; 4 = *Hylastes brunneus*; 5 = *Hylurgops palliatus*; 6 = *Pityogenes chalcographus*; 7 = *Pityogenes* sp.; 8 = *Trypodendron lineatum*; 9 = *Ips sexdentatus*; 10 = *Orthotomicus suturalis*; 11 = *Tomicus minor*; 12 = *Tomicus piniperda*; 13 = *Scolytus ratzeburgi*.

b = beetle; g = galleries.

Table 4. *Ophiostoma* and *Grosmannia* spp. isolated from different bark beetles infesting *Picea abies*, *Pinus sylvestris* and *Betula* spp. in this study.

Beetle* species →	1		2	3		4	5		6		7	8		9	10	11		12		13			
Fungus species ↓	F	R	R	F	R	F	F	R	F	R	R	F	R	R	F	F	R	F	R	F	R	N	
F = Finland; R = Russia; N = Norway																							**Total no. isolates**
Grosmannia cucullata-complex [a,b]	4	10		44	5	17	14				1	15			4	1	1		2				118
G. galeiformis [a]						2	1					5											8
G. olivacea [a,b]	26	2	10		1									1									40
G. piceiperda-complex [b]		4						3	1	2													10
G. taigensis [a,b,c]		3			2			2		6	1												14
Leptographium chlamydatum [a,b]	8	2		2	1	1	5		18	11									1				49
L. lundbergii				4	1	6	5	3	1	2													22
L. procerum-like [b]								1															1
L. truncatum				1																			1
Ophiostoma abietinum-like																	1						1
O. ainoae		51			2			7									3						63
O. bicolor		25																					25
O. borealis [a]																				2			2
O. brunneo-ciliatum [b]		55								11							2		3				71
O. canum [a,b]		7				1	1	14		4		1				10	35		5				78
O. canum-like	17	16	1	15	48	2	15	41	9	33	4	7		13		27	10	8	54			2	322
O. denticiliatum [c]																						3	3
O. flexuosum-like																						1	1
O. floccosum [a,b]		3	5					13		4	2			5					1				33
O. fuscum [a,b,c]		2							1	2													5
O. karelicum [a,b,c]	2															1	3			15	6	87	114
O. minus [a]		4			5		1	3		3	1		1	2			16	1	16				53
O. multiannulatum-like																						1	1
O. pallidulum [a,c]				1		5	1					1				1							9
O. quercus [a,c]																				1		5	6
O. rachisporum [a,c]							1					10	1										12
O. saponiodorum [a,c]	3								10	1													14
O. tapionis [a,b,c]	7					1	10	5	1							2							26
Ophiostoma sp. I				1																			1
Total isolates	67	184	16	68	65	35	54	92	41	79	9	39	2	21	4	42	71	9	82	18	6	99	**1103**

Numbers in table refer to number of fungal isolates.
Bold font = novel taxa found and described during this survey. Host trees of bark beetles: Blue = spruce, Green = pine & spruce, Yellow = pine, Purple = birch
A new report from: [a] Finland, [b] Russia, [c] Fennoscandia
* Bark beetle species: 1 = *Ips typographus*; 2 = *Ips* sp.; 3 = *Dryocoetes autographus*; 4 = *Hylastes brunneus*; 5 = *Hylurgops palliatus*; 6 = *Pityogenes chalcographus*; 7 = *Pityogenes* sp.; 8 = *Trypodendron lineatum*; 9 = *Ips sexdentatus*; 10 = *Orthotomicus suturalis*; 11 = *Tomicus minor*; 12 = *Tomicus piniperda*; 13 = *Scolytus ratzeburgi*.

of bark beetle species. In general, bigger differences in the fungal diversity were observed between different host trees than between different bark beetle species.

5.2.1 Ophiostomatoid fungi

Amongst the ophiostomatoid fungi, species belonging to the genera *Ophiostoma* were most numerous. In total, 20 *Ophiostoma* spp. were found (Table 4). This result is consistent with previous investigations conducted in Europe, which have listed *Ophiostoma* spp. as the most numerous fungal associates of bark beetles (Kirisits 2004). Species that have been reported in previous studies from Fennoscandia included *O. ainoae*, *O. bicolor*, *Ophiostoma borealis*, *Ophiostoma brunneo-ciliatum* Math.-Käärik, *O. canum* (Münch) Syd. & P. Syd., *Ophiostoma floccosum* Math.-Käärik and *O. minus* (Table 1). The only known species not previously reported from Fennoscandia was *Ophiostoma quercus* (Georgev.) Nannf. The new *Ophiostoma* species discovered in this study included seven species, described as *O. karelicum* (study **I**), *O. denticiliatum* (study **II**), *O. fuscum*, *O. pallidulum*, *O. rachisporum*, *O. saponiodorum* and *O. tapionis* (study **III**). In total, seven *Ophiostoma* spp. were found together with bark beetles on hardwoods (*Betula* spp.), while 15 were associated with conifers (*Picea abies*, *Pinus sylvestris*).

Species of *Grosmannia* were also relatively common associates of pine- and spruce-infesting bark beetles (Table 4). None of the *Grosmannia* spp. was found on birch. Species of *Grosmannia* in Europe are mainly known from conifer-infesting bark beetles; only one species is known to infest hardwoods (Davidson 1971, Jacobs and Wingfield 2001, Kirisits 2004). At least nine species of *Grosmannia* or *Leptographium* were detected in this study. These included species in the *G. cucullata*-complex, *Grosmannia galeiformis* (B.K. Bakshi) Math.-Käärik, species in the *G. piceiperda*-complex, *Leptographium chlamydatum* K. Jacobs, M.J. Wingf. & H. Solheim, *Leptographium lundbergii* Lagerb. & Melin, *Grosmannia olivacea* (Math.-Käärik) Zipfel, Z.W. de Beer & M.J. Wingf., a species closely related to *L. procerum* and *Leptographium truncatum* (M.J. Wingf. & Marasas) M.J. Wingf. All of the known species have previously been reported to occur in Fennoscandia in at least one country considered in this study (Table 1). One new *Grosmannia* sp. was discovered in this survey, and was described as *G. taigensis* (Study **IV**). There may be more novel species in the *G. cucullata* and *G. piceiperda* complexes, but their phylogenetic position could not be fully resolved in this thesis.

Another occasionally found ophiostomatoid fungus was *C. polonica*, which was detected in low numbers in association with *I. typographus* infesting spruce in Russia (Table 5). A further species occasionally reported from Europe is *Pesotum fragrans* (Math.-Käärik) G. Okada & Seifert (Mathiesen-Käärik 1953, Romón et al. 2007). The species was first described by Mathiesen-Käärik (1953) from *Pinus sylvestris* infested by *I. sexdentatus* and *Orthotomicus proximus* Eichh. in Sweden. During this study, 125 isolates similar to *P. fragrans* were found in association with several bark beetle species infesting pine and spruce (data not shown). Recent studies have indicated that *P. fragrans* is only distantly related to other Ophiostomatales (de Beer et al. 2010, Paciura et al. 2010). It is also morphologically different compared to other species forming *Pesotum*-anamorphs. Based on our preliminary analyses, the so-called *P. fragrans* isolates collected from Finland and Russia together with closely related species, such as *Ophiostoma rectangulosporium* Ohtaka, Masuya & Yamaoka, form a complex of species. Our early proposition is that the complex should be recognized as a distinct genus. Further studies including an inspection of the type material and comparisons of sequences for additional gene regions will be needed to clarify the taxonomy of this group. Therefore, these isolates are not further discussed in this thesis.

Table 5. Fungal associates of *Ips typographus* in outbreak and non-outbreak areas.

Fungus	Outbreak areas	Non-outbreak areas	
	R	F	R
Ceratocystis polonica	4		3
Grosmannia cucullata-complex	1	4	9
G. piceiperda-complex	4		
Leptographium chlamydatum		8	2
G. olivacea		26	2
G. taigensis			3
O. ainoae	14		37
O. bicolor	25		
O. brunneo-ciliatum	50		5
O. canum			7
O. canum-like		17	16
O. floccosum			3
O. fuscum			2
O. karelicum		2	
O. minus			4
O. saponiodorum		3	
O. tapionis		7	

The numbers in the table refer to number of fungal isolates
Bold font = novel taxa found and described during this survey
F = Finland; R = Russia

5.2.2 Sibling species within the ophiostomatoid fungi

This study clearly demonstrates the importance of DNA based methods in the identification of ophiostomatoid fungi. The methods used in this study revealed several previously unrecognized species. The results of this study also raised a need for clarifying the status of both previously known species such as *Grosmannia cucullata* (H. Solheim) Zipfel, Z.W. de Beer & M.J. Wingf., *G. piceiperda*, *O. minus* and *O. piceae*, and several apparently novel species.

In general, the evolution of species is considered to lead to the formation of diverse morphological features, such as colors or shapes, which distinguish different species from each other (Givnish and Sytsma 2000). Sometimes species do not look distinct and are difficult or impossible to identify based on morphology. Such examples of ophiostomatoid species, which share a relatively simple morphology with frequent overlapping characters and size ranges. Some species have so subtle differences that they are remarkably difficult to identify morphologically. Therefore, confusing taxonomic debates have surrounded the ophiostomatoid fungi even at the order and genus level of classification, ever since the description of *Ceratocystis* (1890) and *Ophiostoma* (1919). Up to date, ca. 200 ophiostomatoid species have been identified worldwide. DNA sequence based identification has become an essential tool for the reliable identification and recognition of cryptic taxa amongst these fungi (Gorton et al. 2004, Grobbelaar et al. 2009). Molecular techniques have revealed several

previously unrecognized species and helped in resolving species boundaries for complexes of cryptic taxa. Examples of these cryptic species with a confusing taxonomic history are *O. piceae* and *O. quercus*. The species were described as two different taxa, but later *O. quercus* was treated as a synonym to *O. piceae* for over 30 years (Münch 1907, Georgevitch 1926, 1927, Hunt 1956). During this period, several other morphologically similar species were also listed as synonyms of *O. piceae*. Recent DNA sequence analysis confirmed that mainly conifer-infesting *O. piceae* and hardwood-associated *O. quercus* are distinct taxa (Harrington et al. 2001), and more recent multi-gene phylogenies were able to delimit *O. quercus* sensu stricto and clarify the status of its synonymous species (Grobbelaar et al. 2009).

In this study, most cases of rDNA based phylogenetic and of morphological species recognition were consistent. In some cases, morphologically similar species formed phylogenetically distinct lineages, revealing cryptic species. Also the opposite situation, when morphologically delimited species had identical or almost identical ITS sequences, was encountered. Moreover, DNA based identification has certain challenges. In many cases, the ITS sequence data alone were not variable enough for species recognition. The ITS sequences of the morphologically and biologically distinct taxa *O. piceae* and *O. canum* are identical, and cannot be used for distinguishing these two species (Harrington et al. 2001). Morphologically delimited isolates representing *O. canum* and a species that we have referred to as *O. canum*-like had ITS sequences identical to each other and identical to an authentic isolate of *O. canum* (CBS 133.51) (Study **III**). The morphological characteristics of isolates representing *O. canum* corresponded well to those of *O. canum*, which could easily be distinguished by its globose conidia produced by the *Pesotum* anamorph (Mathiesen 1950, 1951, Harrington et al. 2001). The isolates representing the *O. canum*-like fungus were different from *O. canum*, producing obovoid conidia that were more similar to *O. piceae* (Harrington et al. 2001). Sequencing the protein coding gene β-tubulin provided sufficient resolution in most other cases but could not fully distinguish between *O. canum* and the *O. canum*-like species. Based on phylogenetic analyses of the β-tubulin gene, the sequences of isolates representing *O. canum* were identical to the sequence of the authentic *O. canum* strain. The sequences of the *O. canum*-like isolates were almost identical, differing only in a single base pair at the 30th position of exon 5. Despite several attempts, we did not manage to amplify the EF 1-α gene region for the *O. canum*-like species. We consider it as a possible new taxon, but additional gene regions should be explored further to clearly distinguish it from *O. canum.*

Interestingly, the unidentified *O. canum*-like species was the most frequently isolated fungus in this study. It is also surprising that despite the relatively large number of different bark beetles species and locations sampled in this study, not even a single isolate of *O. piceae* could be found, even though several earlier studies have reported its common occurrence in Russia and Scandinavian countries (Table 1). The suggestion of this survey is that the *O. canum*-like taxon detected in this study might be the same as isolates from Russia and Scandinavia previously identified as *O. piceae* based on morphology only, and the putative identification of the *O. piceae* isolates in previous studies in Russia and Scandinavia should be confirmed by DNA sequence comparisons of the β-tubulin gene. Our suggestion is supported by the fact that β-tubulin sequences of *O. canum*-like isolates differ substantially from those of authentic *O. piceae* isolates in other studies (Jacobs and Kirisits 2003, Kim et al. 2005, Bommer et al. 2009, Grobbelaar et al. 2009).

Another example of a sibling species within the ophiostomatoid fungi is *O. minus*. Recent phylogenetic analyses have shown that *O. minus* isolates collected in Europe and China are distinct from isolates collected from North America that include also the type of *O. minus* (Hedgcock 1906, Gorton and Webber 2000, Gorton et al. 2004, Lu et al. 2009). This distinction was observed also in our study (study **III**). The isolates collected during

this survey in Russia and Finland grouped with the European clade of *O. minus*. European isolates probably represent *Ophiostoma pini* (Münch) Syd. & P. Syd. *Ophiostoma pini* was described from pine by Münch (1907), but since 1956 has been considered a synonym of *O. minus* (Hunt 1956, Olchowecki and Reid 1974, Upadhyay 1981). Further studies including an inspection of the type material, examination of additional isolates of both species, and comparisons of sequences for gene regions additional to those previously considered, will be needed to clarify the taxonomy of this group.

A similar situation to *O. minus* was also observed within species of *Grosmannia*. Comparisons of the β-tubulin and EF 1-α sequences for isolates representing *G. piceiperda* revealed distinct phylogenetic lineages (study **IV**). Isolates from Europe and America formed two distinct lineages. The isolates obtained in this study grouped either within the European clade of species or within a distinct clade, the status of which remains unresolved. In addition, species of *Grosmannia* also included *G. cucullata* sensu lato and *L. procerum* sensu lato, which appear to represent complexes of species that could not be resolved with certainty in this study.

5.2.3 Other fungal associates

A number of other fungi were detected in association with bark beetles and their galleries. As the focus of this research was on ophiostomatoid fungi, no further efforts were made for their identification. In some cases, the ITS region was sequenced and the preliminary identification was based on the BLAST result. An exception is study **I**, which included all fungi associated with *S. ratzeburgi* on birch. All different fungi were isolated and the purified cultures are stored for future investigations at the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Previously synnematous *Pesotum* anamorphs of *Ophiostoma* were classified in *Graphium* (Upadhyay 1981, Seifert and Okada 1993). Species of *Ophiostoma* and *Graphium* are difficult to identify from each other, because both have similar micro-morphological and colony characters and are associated with bark beetles. This has led to the confusing taxonomic status of *Graphium*. Recent molecular studies have shown that *Graphium* and *Pesotum* are phylogenetically clearly distinct and unrelated taxa (Okada et al. 1998, 2000, Jacobs et al. 2003). In this survey, two species of *Graphium* were occasionally isolated. *Graphium fimbriisporum* (M. Morelet) K. Jacobs, Kirisits & M.J. Wingf. was found in association with *I. typographus* and *Pityogenes* sp. on spruce in Russia. The species was first described by Morelet (1995) from galleries of *I. typographus* on spruce. Its detection in this study is consistent with previous investigations in which *Gr. fimbriisporum* has been reported to occur mainly in association with *I. typographus* on spruce in Europe (Morelet 1995, Kirisits 1996, Kirisits et al. 2000), but is also also associated with several other spruce-infesting bark beetle species (Kirisits 1996, Kirisits et al. 2000, Jacobs et al. 2003, Kirisits 2004). The other species, *Graphium pseudormiticum* M. Mouton & M.J. Wingf., was described by Mouton et al. (1994) from galleries of *Orthotomicus erosus* (Wollaston) infesting pine in South Africa. Previous reports of *Gr. pseudormiticum* from Europe are limited (Kirschner 1998, Persson et al. 2009). *Graphium pseudormiticum* is assumed to be of European origin (Morelet 1995), and Jacobs et al. (2003) suggested that due to confusing taxonomy, the species might have been overlooked in previous surveys, and that it actually occurs in association with a variety of pine-infesting bark beetles in Europe. In this survey, *Gr. pseudormiticum* was occasionally detected in association with *I. typographus* on spruce, *T. piniperda* on pine and *Pityogenes* sp. on spruce.

Yeasts were commonly found both bark in association with beetles and their galleries at all locations. Several previous studies have also reported yeasts to occur as common associates of bark beetles (Grosmann 1931, Callaham and Shifrine 1960, Francke-Grosmann 1967,

Bridges et al. 1984, Furniss et al. 1990, Six 2003, Persson et al. 2009, Rivera et al. 2009). The taxonomy, distribution and role of bark beetle-associated yeasts are still poorly known. Yeasts may have an important role in bark beetles' digestive and detoxification processes and pheromone production (Borden 1982, Paine et al. 1997). Yeasts have rather unspecific associations with bark beetles (Callaham and Shifrine 1960, Six 2003, Riviera et al. 2009), and they are not known to be pathogenic to their host trees (Callaham and Shifrine 1960). All reported bark-beetle associated yeasts belong to the ascomycetes, to the genera *Candida* Berkhout, *Cryptococcus* Vuill., *Hansenula* Syd. & P. Syd., *Kuraishia* Y. Yamada, K. Maeda & Mikata, *Pichia* E.C. Hansen and *Saccharomyces* Meyen ex E.C. Hansen (Callaham and Shifrine 1960, Whitney 1982, Six 2003, Rivera et al. 2009). The yeast found in this survey included species of *Candida* and *Pichia*.

Several other species belonging to the Ascomycota were found. These included species of *Alternaria* Nees, *Biscogniauxia* Kuntze, *Beauveria* Vuill., *Cadophora* Lagerb. & Melin, *Cordyceps* Fr., *Epicoccum* Link, *Fusicoccum* Corda, *Geotrichum* Link, *Nectria* (Fr.) Fr., *Neosartorya* Malloch & Cain, *Paecilomyces* Bainier, *Phialophora* Medlar, *Phoma* Fr., *Rhinocladiella* Nannf., *Thysanophora* W.B. Kendr. and *Trichoderma* Pers. The majority of these species are saprotrophic or plant pathogenic fungi. *Beauveria* and *Paecilomyces* are insect pathogens. Species of *Trichoderma* are nonpathogenic, opportunistic plant symbionts. The occurrence of any these fungi in association with bark beetles is not a surprise. Bark beetles come into contact with a variety of other plant-associated fungi, which they occasionally vector. Insect pathogens such as *Beauveria* can attack bark beetles, and these fungi are also investigated as potential biological control agents against bark beetles (Glare et al. 2008).

One basidiomycetes species, a polypore fungus in the genus *Trametes* Fr. sp., was encountered. The association of basidiomycetes with bark beetles is rather poorly studied, and their diversity might be underestimated (Kirschner 1998, 2001). In some cases, bark beetles seem to have rather intimate associations with Basidiomycetes, such as *Dendroctonus* spp. and *Ips avulsus* (Eichhoff) (Six 2003). Bark beetles have been found to be casually associated with *Heterobasidion annosum* (Fr.) Bref., the causal agent of the Annosum root rot, which is considered to be the economically most important forest pathogen in the Northern Hemisphere (Bakshi 1950, Harding 1989, Kirschner 1998, Kirisits 2004).

Zygomycetes have previously been reported as casual associates of bark beetles (Whitney 1982, Harding 1989, Kirschner 1998, Persson et al. 2009). In this study, one zygomycetes fungus was found in association with bark beetles.

5.3 Fungal associates of spruce-infesting bark beetles

5.3.1 Ips typographus

Ips typographus was found in association with a high number of ophiostomatoid fungi, of which the majority appears to represent rather casual fungal associates (Table 4). The majority of research on the bark beetle associated fungi in Northern Europe has dealt with this economically most important bark beetle species on Norway spruce (e.g. Harding 1989, Furniss et al. 1990, Solheim 1993b, Viiri 1997, Kirisits 2004). The genus *Ips* (the engraver beetles) accommodates species that are distributed throughout coniferous forests of the northern hemisphere (Heliövaara et al. 1998). The species of *Ips* usually attack freshly harvested logs and felled trees, or stressed living trees. *Ips typographus* is by far the most aggressive insect pest in European forests, causing high economic losses by killing millions of Norway spruce trees. Occasionally it can be found also on pines (Saalas 1949, Heliövaara et

al. 1998). In this survey, *I. typographus* was found only on spruce, and was the most frequent beetle species collected. Extensive *I. typographus* outbreaks usually follow windfalls and drought (e.g. Saalas 1949, Christiansen and Bakke 1988, Heliövaara et al. 1998, Økland and Christiansen 2001, Schelhaas et al. 2003). Windfallen and damaged trees with low resistance are suitable as breeding material, and can promote a significant increase of the bark beetle population. At the same time, the risk of bark beetle attacks on healthy spruce trees increases.

Ips typographus is an example of a bark beetle species that has been consistently and regularly found in association with a range of different ophiostomatoid species (Table 3).. Individual bark beetles carry spores of at least one fungus, but many of these fungal species are reported only occasionally (Kirisits 2004). Based on a number of studies, a few ophiostomatoid species can be regarded as relatively constant associates. These include *C. polonica*, *O. ainoae*, *O. bicolor*, *Ophiostoma penicillatum* (Grosmann) Siemaszko and *G. piceiperda*. The present study is in agreement with the previous observations. *Ips typographus* was found in association with a high number of ophiostomatoid species (Table 4). In total, 17 different ophiostomatoid fungi were found in association with *I. typographus* in Finland and Russia. The majority of the fungi was found occasionally and were present only in low numbers. A few fungal species were found more consistently, most notably *O. brunneo-ciliatum*, *O. ainoae*, *O. canum*-like, *O. bicolor* and *G. olivacea*. The total absence of *G. penicillata* is noteworthy and surprising. Based on previous studies this species is a relatively specific and a common fungal associate of *I. typographus* throughout Europe (Rennerfelt 1950, Mathiesen 1950, Mathiesen-Käärik 1953, Solheim 1986, Harding 1989, Viiri 1997, Kirisits 2004). The isolation method migh have selected agains *G. penicillata* and made it difficult to detect the species in this study.

This study made it possible to compare two countries and both non-outbreak and outbreak areas (Table 5). The differences in associated fungi between Finland and Russia and between non-outbreak and outbreak areas were notable. The most frequent species found in Finland was *G. olivacea*, which was present only in low numbers in *I. typographus* collected from Russia. The most abundant species found only in Russia included *O. brunneo-ciliatum*, *O. bicolor* and *O. ainoae*. The total number of ophiostomatoid species in Russia was higher than in Finland. The most commonly encountered fungal associates in non-outbreak areas in Russia were *O. ainoae* and *O. canum*-like, while *O. brunneo-ciliatum* and *O. bicolor* were common in outbreak areas. Some ophiostomatoid species including *G. piceiperda* and *O. bicolor* were found only in outbreak areas. It is noteworthy that the ophiostomatoid taxa that were not found in association with any other conifer-infesting beetle species considered in this study included only two species, *C. polonica* and *O. bicolor*.

Several previous studies have focused on searching for a causal fungal agent responsible for killing Norway spruce trees in the course of *I. typographus* attacks. Based on studies conducted in Norway, *C. polonica* was found frequently and the fungus was suggested to be essential in the initiation of *I. typographus* outbreaks (Christiansen et al. 1987, Christiansen and Solheim 1990, Krokene 1996, Krokene and Solheim 1998). In this survey, only a few isolates of *C. polonica* were obtained, and no differences in the occurrence of this species in non-outbreak and outbreak areas were observed (Table 5). Previously, the species has been detected in low numbers in Finland (Viiri 1997). Similarly, a generally low and usually also variable frequency of *C. polonica* has been reported in several other studies conducted in Europe. In some localities, the species is considered as the most common fungal associate of the insect species (Kirisits 2010). Reasons for the variability of this fungus in different locations are not fully understood. One possible explanation is that the isolation methods employed by different researches could significantly influence the detection of this species (Kirisits 2004). The isolation methods used in this study are not the best to detect *C. polonica*,

and its frequency may therefore have been underestimated. The fungus is also known to be difficult to isolate directly from the beetles (Furniss et al. 1990).

Ceratocystis polonica is no longer considered an obligatory associate enabling *I. typographus* to successfully colonize Norway spruce trees (Harding 1989, Viiri 1997, Kirisits 2004, Six and Wingfield 2011). Numerous inoculation experiments have shown that *C. polonica* is the most virulent species compared to other fungi commonly associated with *I. typographus*, such as *O. bicolor* and *O. ainoae* (Horntvedt et al. 1983, Harding 1989, Kirisits 1998). *Ophiostoma bicolor* is a frequently found associate, especially during the early phase of an *I. typographus* attack on living trees, suggesting it might be an important associate of the insect (Solheim 1986, Harding 1989). Based on the results of this study, great variation of fungal associates of *I. typographus* between different localities could be observed (data not shown).

5.4 Fungal associates of pine-infesting bark beetles

5.4.1 Tomicus spp.

In this study, two *Tomicus* spp. infesting pine were found: *T. piniperda* and *T. minor*. Both bark beetle species vectored numerous fungi, of which the majority appear to be rather casual associates of the insects (Table 4). A few fungal associates were more commonly encountered. Species in the genus *Tomicus* (the pine shoot beetles) are economically important pests causing significant damage to pine forests in Eurasia (Saalas 1949, Postner 1974, Långström 1983, Ye and Lieutier 1997). *Tomicus* spp. reproduce in the phloem of the trunks and larger branches of dead or weakened trees, followed by a maturation feeding. During their long maturation-feeding period, adults tunnel in the branch tips of healthy host trees. A large number of individuals can feed on the same tree, causing loss of needles and depressed growth (Postner 1974). *Tomicus* spp. usually over-winter under thick bark at the bases of old pines, but occasionally cause damage by over-wintering at the bases of young standing trees (Saalas 1949). Both species are widespread in Europe, breeding normally in *Pinus* spp. (Heliövaara et al. 1998). *Tomicus piniperda* was recently introduced to North America, where it spread rapidly and is recognized as a potentially damaging exotic species (Haack 2006). *Tomicus piniperda* prefers thicker-barked trees than *T. minor* (Saalas 1949). Both species are considered as secondary bark beetles, although *T. minor* can occasionally occur as a primary species in standing trees.

Among the four strictly pine-infesting bark beetle species considered in this survey, *T. minor* was most frequently collected and therefore also most intensively studied. Eleven ophiostomatoid species were associated with *T. minor* in Finland and Russia. The most frequent fungal associates were *O. canum*-like, *O. canum* and *O. minus*. The other fungi were found only in low numbers. The results are in agreement with previous studies conducted in Europe, which reported *O. canum* as a rather consistent associate of *T. minor* (Rennerfelt 1950, Mathiesen 1950, Mathiesen-Käärik 1953, Kirisits 2001, Kirisits 2004). Also *O. minus* has been reported, but the fungus is more rarely found than *O. canum* (Rennerfelt 1950, Mathiesen 1950, Mathiesen-Käärik 1953). Some previous studies also concluded that *O. piceae* is a relatively common associate of *T. minor* (Mathiesen 1950, Mathiesen-Käärik 1953). The *O. canum*-like species detected in this study might be the same as the isolates from Scandinavia previously identified as *O. piceae* based on morphology only (see 5.2.2).

Seven ophiostomatoid species were isolated from *T. piniperda*. The most commonly encountered fungal associates were the *O. canum*-like fungus and *O. minus*, while the

other species were obtained in low numbers. A large number of ophiostomatoid species has previously been found in association with *T. piniperda* (Kirisits 2004). *Ophiostoma minus* seems to be a relatively consistent associate of *T. piniperda* in Europe, although the frequency of the fungus is very variable (Lieutier et al. 1989, Jankowiak 2006). In this survey, the *O. canum*-like species was the most numerous fungal associate of *T. piniperda*. Several studies conducted in Europe have recorded *O. piceae* in low numbers (Gibbs and Inman 1991, Solheim and Långström 1991, Kirisits 2001, 2004). The frequency of this species varies. In a recent study, *O. piceae* was recorded in relatively high frequencies in Poland (Jankowiak 2006). As discussed for *T. minor*, the previous reports of the association of *O. piceae* with *T. piniperda* in Scandinavia (Mathiesen 1950, Mathiesen-Käärik 1953, Solheim and Långström 1991, Gibbs and Inman 1991) may in fact represent the *O. canum*-like species, but this requires confirmation. A total absence of *Leptographium wingfieldii* M. Morelet in this study is surprising, since the species is regarded as one the most common associates of *T. piniperda* in previous studies (Solheim and Långström 1991, Gibbs and Inman 1991, Kirisits 2004).

5.4.2 Orthotomicus suturalis

The fungal associates of this bark beetle species have never been studied before in Europe. In this study, *O. suturalis* infesting pine was found at only one collection site in Finland (Table 3). *Orthotomicus* species are common conifer-infesting bark beetles. *Orthotomicus suturalis* is a relatively primary species, infesting mainly dead or dying standing thin-barked conifers, especially trees damaged by fire (Saalas 1949). It can also infest timber, but is not considered as an economically important species (Heliövaara et al. 1998). Very little is known about the fungal associates of this scolytine species. A previous study in Japan listed seven ophiostomatoid species, of which *G. olivacea* was the most frequently isolated fungus (Matsuya et al. 2009). Within the small number of *O. suturalis* found and examined in this study, the bark beetle was found in association with species in the *G. cucullata*-complex (Table 4). More comprehensive studies are needed to assess the ophiostomatoid fungal associates of this bark beetle species more thorougly.

5.4.3 Ips sexdentatus

Ips sexdentatus was found at one collection site in Russia, infesting pine (Table 3). The records of the species in Finland, Sweden and the Karelia region have been scarce during recent years, and in Sweden, *I. sexdentatus* is included in the national Red List (Gärdenfors 2000, Voolma et al. 2004). *Ips sexdentatus* is considered a secondary pest, usually attacking stressed or weakened trees (Saalas 1949). It prefers to attack large trees with thick bark. It has been reported to be capable of causing mass outbreaks and significant damage to pine forests (Saalas 1949, Browne 1972, Schönherr et al. 1983). Although it mainly infests pines, it can occasionally occur on spruce (Saalas 1949, Heliövaara et al. 1998).

Four ophiostomatoid fungi were associated with *I. sexdentatus* in Russia (Table 4). These species included *G. olivacea*, *O. canum*-like, *O. floccosum* and *O. minus*. A variety of ophiostomatoid species have been reported to be associated with *I. sexdentatus* in Europe (Mathiesen-Käärik 1953, Kirisits 2004). *Ophiostoma minus* is the only species recorded in this study which has previously been detected as a rather common associate of this insect. The most commonly encountered fungus in this survey was the *O. canum*-like species. *Ips sexdentatus* was found at only one collection site in Russia and the samples included only a small number of beetles. Therefore, definitive conclusions about the associated fungi of this scolytine species in Fennoscandia cannot be drawn.

5.5 Fungal associates of spruce- and pine-infesting bark beetles

5.5.1 Pityogenes chalcographus

Pityogenes chalcographus was a frequently collected beetle species (Table 3). It was found in association with various fungi (Table 4). *Pityogenes chalcographus* is the most common bark beetle species in southern Finland (Saalas 1949, Heliövaara et al. 1998). It mainly infests spruce, but can also occur on other conifers. It is a relatively primary species capable of killing healthy, living trees. Usually it prefers thin, weakened standing trees or felled trees. It commonly occurs together with *I. typographus*, causing significant damage (Saalas 1949). In this study, *P. chalcographus* was found both on pine and spruce. This scolytine species was an important vector of numerous ophiostomatoid species. This finding is in agreement with previous studies, which have reported *P. chalcographus* to vector numerous fungi (Kirisits 2001, Kirisits 2004). Some of these species, such as *O. ainoae*, *O. bicolor* and *G. piceiperda* are rather consistent associates of *P. chalcographus*. Twelve ophiostomatoid species were found to be associated with the beetle species in Finland and Russia in this study. The most numerous fungi were *L. chlamydatum*, *O. canum*-like, *O. brunneo-ciliatum* and *O. saponiodorum*. *Pityogenes chalcographus* was the most important bark beetle species considered in this study vectoring *O. saponiodorum,* a novel species described in this survey.

5.5.2 Hylastes brunneus

Previous studies have shown that several root-infesting *Hylastes* spp. are vectors of ophiostomatoid fungi, especially *Leptographium* spp. (Harrington and Cobb 1988, Wingfield and Gibbs 1991, Reay et al. 2002, Kirisits 2004, Zhou et al. 2004), but to the best of our knowledge, the fungal associates of *H. brunneus* have not been studied prior to the present investigations. *Hylastes* species are considered to be insects displaying low agressivenes to live trees, breeding mainly in roots, stumps and logs of decaying conifers (Saalas 1949, Heliövaara et al. 1998). The adults can also damage young seedlings and in some cases cause serious local losses. *Hylastes brunneus* is a common species in Northern Europe, infesting mainly pines. In this survey, *H. brunneus* was found only in Finland, infesting both pine and spruce (Table 3). *Hylastes brunneus* was found to be an important vector of several ophiostomatoid fungi, having rather casual fungal associates (Table 4). A total of twelve ophiostomatoid fungi were found in association with this bark beetle. The most commonly encountered fungi were species in the *G. cucullata*-complex. All the other species were found in lower numbers. *Hylastes brunneus* was the most important bark beetle species considered in this study vectoring *O. pallidulum,* which was one of the new species described in this survey.

5.5.3 Trypodendron lineatum

The majority of *T. lineatum* were collected in Finland (Table 3). In total, seven ophiostomatoid species were found in association with this wood-infesting bark beetle species (Table 4). The most common fungi included species in the *G. cucullata*-complex and a novel species described in this study, *O. rachisporum*. *Trypodendron lineatum* was also the most important bark beetle species vectoring *O. rachisporum*. The other ophiostomatoid fungi were present in lower numbers. Based on previous studies, *O. piceae*, *O. piceaperdum* and the ambrosia fungus *Ambrosiella ferruginea* (Math.-Käärik) L.R. Batra are common associates of the beetle species (Mathiesen-Käärik 1953, Kirschner 1998, 2001).

Trypodendron species are ambrosia beetles that live in a nutritional symbiosis with fungi (Saalas 1949, Heliövaara et al. 1998). The majority of ambrosia beetles colonize the xylem of dead or dying coniferous wood, particularly one or two years after tree death. The beetles bore tunnels into the wood, in which they establish and tend fungal gardens of their ambrosia fungus, *A. ferruginea*, on which the adult insects and developing larvae feed on. Therefore, the total absence of the ambrosia fungus in this study was a surprise. *Trypodendron lineatum* is a common species, which infests both pine and spruce. In this survey, *T. lineatum* was mainly found in Finland, on both conifer species (Table 3). It is a secondary species which infests dead or dying standing trees, typically the next summer following *I. typographus* attacks or during the same summer after *T. piniperda* infestations. It also colonizes felled trees, stumps and logs. *Trypodendron lineatum* can cause economically significant damage by boring tunnels and vectoring sapstain fungi, thereby lowering the value of logs and timber.

5.5.4 Hylurgops palliatus

Hylurgops palliatus was found to be an important vector of ophiostomatoid fungi, associated with a high number of species (Table 4). In total, fifteen different ophiostomatoid species were recorded. The most commonly encountered species included species in the *G. cucullata*-complex, *O. canum*, *O. canum*-like, *O. floccosum* and a novel species, *O. tapionis*. Isolates of *O. tapionis* were found only occasionally in association with other bark beetle species, indicating that *H. palliatus* might be an important vector of this newly described fungus. Based on the previous studies, the ophiostomatoid mycobiota of this bark beetle species includes numerous fungi. Some of these are rather consistent associates, including species such as *G. cucullata*, *G. piceiperda, L. lundbergii, O. ainoae* and *O. piceae* (Kirschner 1998, Mathiesen-Käärik 1953, Harding 1989, Krokene and Solheim 1996.

The species of *Hylurgops* are common bark beetle species, which infest several coniferous trees (Saalas 1949, Heliövaara et al. 1998). They are secondary species, infesting dead trees and stumps. In this survey, *Hylurgops palliatus* was a common species found on both pine and spruce. As *T. lineatum*, *H. palliatus* appears often a year after *I. typographus* has infested a spruce tree, or during the same summer if a pine tree is attacked by *T. piniperda*. *Hylurgops palliatus* is not known to cause any direct economic losses. On the contrary, *H. palliatus* can be considered a beneficial species competing with more harmful bark beetle species (Heliövaara et al. 1998).

5.5.5 Dryocoetes autographus

A total of eleven different ophiostomatoid species were found in association with *D. autographus* in Finland and Russia (Table 4). The dominant fungal associates were species in the *G. cucullata*-complex and the *O. canum*-like taxon. All the other species were found only in low numbers. This is consistent with previous studies conducted in Europe, in which *G. cucullata* and *O. piceae* were amongst the species mentioned as common and rather constant associates of *D. autographus* (Kirschner 1998, 2001, Kirisits 2001, 2004). The genus *Dryocoetes* includes secondary bark beetle species that infest dying trees, mainly conifers (Saalas 1949, Heliövaara et al. 1998). *Dryocoetes autographus* is a common species in Scandinavia, infesting the bases and roots of dying or damaged standing trees, as well as felled or windfallen trees. It mainly infests spruce, but is known to colonize also other conifers. In this survey, *D. autographus* was frequently collected and mainly found on spruce, but also commonly detected on pine. It was also found in galleries of *T. piniperda*. This insect is a secondary or even tertiary bark beetle species colonizing trees long after other scolytines. It is not known to cause any direct economic losses.

5.6 Fungal associates of the birch-infesting bark beetle

5.6.1 Scolytus ratzeburgi

The only fungus that could be regarded as a highly consistent and rather specific fungal associate of a particular bark beetle species considered in this study is *O. karelicum*, a novel species described during this study. The fungus was found in association with each individual *S. ratzeburgi* beetle or its galleries at all studied locations and countries, including Finland, Russia and Norway (Table 4). The type of the association is similar to those observed between ophiostomatoid fungi and other *Scolytus* species. The taxonomy and biology of the hardwood-infesting *Scolytus* spp. on elm species and the ophiostomatoid fungi associated with them have been the focus of extensive studies (Webber and Brasier 1984, Webber 1990, Brasier and Mehrotra 1995). The Dutch elm disease fungi responsible for disastrous disease pandemics, *O. ulmi* and *O. novo-ulmi* are primarily vectored by three *Scolytus* spp. (Webber and Brasier 1984, Webber 1990). Unlike most conifer-infesting bark beetles, hardwood-infesting *Scolytus* spp. are known to have rather consistant associations with their particular fungal associates. Elm and other hardwood-infesting *Scolytus* spp. often also have a more casual association with several other ophiostomatoid species. Several ophiostomatoid fungi have been detected from birch, but previous studies have not reported their possible insect vectors. The birch bark beetle, *Scolytus ratzeburgi*, is the only *Scolytus* species known to infest birch. It is considered a secondary species that primarily infests weakened or dying standing trees, felled trees and logs in various parts of Europe, Siberia and Japan (Saalas 1949, Heliövaara et al. 1998). It commonly occurs also as a primary species, killing living or weakened trees and causing local damage to forests and ornamental trees. In this study, *S. ratzeburgi* was found infesting *B. pendula* and *B. pubescens* in Finland, Russia and Norway (Table 3). Fungal associates of this insect have not been previously investigated in detail. However, there are remarks in literature that *S. ratzeburgi* can cause sapstain in timber (Löyttyniemi 1983, Verkasalo 1993).

Other ophiostomatoid species found in association with *S. ratzeburgi* in this study, *O. quercus* and *O. borealis*, were present in low numbers (Table 4). As the number of samples was relatively low, we decided to conduct a more extensive survey in other geographical areas where the bark beetle occurs (study **II**). Finland and Russia border Norway in the north, but are in south separated by the Baltic Sea and its offshoots. The climate also varies. The sampling of *S. ratzeburgi* in southern Norway gave very similar results as the investigations in Finland and Russia (study **I**). *Ophiostoma karelicum* was consistently isolated from every beetle and gallery sampled. Other ophiostomatoid species that were infrequently found included *O. quercus* and four previously unknown taxa, of which one species (*O. denticiliatum*) was described during the survey. Of the remaining taxa the numbers of isolates were too low for formal species descriptions, and therefore they remained undescribed for the moment.

The results of the surveys in Fennoscandia suggest that *O. karelicum* is rather specifically associated with *S. ratzeburgi* and that the fungus may occur across the geographic range of the beetle species. It should me noted, that *O. karelicum* occurs occasionally also on conifers (Table 4). These findings are similar is similar to previous investigations of hardwood-infesting *Scolytus* spp., which have an intimate association with one of their fungal associates and a more casual association with a variety of other ophiostomatoid species. In addition, being morphologically and phylogenetically closely related to the highly aggressive Dutch elm disease fungi, *O. karelicum* also seems to occupy the same ecological niche on *Betula* spp. as the Dutch elm disease fungi vectored by *S. scolytus* on *Ulmus* (Webber 1990). The

difference is that in the *S. ratzeburgi*-*O. karelicum* association both the insect and the fungus are native. It should be also noted that the most aggressive ophiostomatoid fungi are associates of hardwood trees. Aside from the Dutch elm disease fungi, other notable hardwood-infesting ophiostomatoid species include fungi that cause vascular wilt and canker diseases, such as *Ceratocystis fagacearum*. Hunt causing oak wilt and *Ceratocystis fimbriata* Ellis & Halst. causing wilt and canker diseases on woody plants such as *Eucalyptus*. The relatedness to the Dutch elm disease fungi further suggest that *O. karelicum* has the potential to cause a vascular disease, and its virulence especially to exotic *Betula* spp. should be considered. The possibility of *S. ratzeburgi* and its associated fungi being introduced from European timber to North America is relatively high. Several native *Betula* spp. occur in North America, but thus far *S. ratzeburgi* has not been detected there. The introduction of *S. ratzeburgi* and *O. karelicum* to North America could lead to a situation analogous to the introduction of the Dutch elm disease pathogens, which presumably originated in the southern Asia and were accidentially moved to Europe and North America (Brasier 1983). Since the introduction of *S. ratzeburgi* and its associated fungi to non-native areas is likely to cause economic or environmental harm, regulatory tactics should be designed to prevent the introduction of this bark beetle species.

5.7 Overview on the intimacy of observed fungal-bark beetle associations

All the bark beetle species investigated were associated with an assemblage of several ophiostomatoid fungi (Table 4). The intimacy of the associations of bark beetles with ophiostomatoid fungi can be broadly divided into constant and more casual associations (Kirisits 2004). Only one constant association, in which the majority of individual bark beetles carry spores of ophiostomatoid fungi and favor one fungus over the whole assemblage of fungal associates, was observed during this study. Several species of *Ophiostoma* were found in association with the birch bark beetle (*S. ratzeburgi*), but only one fungus, *O. karelicum,* was detected from every individual bark beetle investigated. This kind of association is rather rare; one particular fungus is not always present in such high frequencies even when considered as a constant associate, indicating that *S. ratzeburgi* is an effective vector of the fungus. It is not a surprise that the other known examples represent bark beetles from the same genera that also vector closely related fungal species; the elm-infesting *Scolytus* spp. vectoring the Dutch elm disease pathogens.

In general, relatively similar assemblages of fungal associates between the different conifer-infesting bark beetle species were observed. Pine-and spruce-infesting bark beetle species were more loosely associated with ophiostomatoid fungi. In many previous studies rather consistent associations between ophiostomatoid fungi and conifer bark beetles have been observed, e.g. for *I. typographus* and *T. minor* (Rennerfelt 1950, Mathiensen 1950, Mathiesen-Käärik 1953, Solheim 1986, Harding 1989, Furniss et al. 1990, Solheim 1986, 1992a, 1992b, 1993, Viiri 1997). Only in one example a relatively specific association was observed, with *O. bicolor* associated with *I. typographus* on spruce. The fungus was not detected together with any other bark beetles. Compared to the intimacy of association between *S. ratzeburgi* and *O. karelicum*, the occurrence of *O. bicolor* was very variable between different study sites. The species was detected only at one collection site, where an outbreak of *I. typographus* occurred (Tables 4–5). In several cases, a few fungal associates were more common over the range of ophiostomatoid species associated with a particular bark beetle species. These more consistent and more specialized fungal associates included

species such as *O. canum* and *O. minus* associated with *T. minor*, species in the *G. cucullata*-complex associated with *D. autographus*, *L. chlamydatum* associated with *P. chalcographus*, as well as *G. olivacea*, *O. ainoae*, *O. bicolor* and *O. brunneo-ciliatum* associated with *I. typographus*. One ophiostomatoid species, *O. canum*-like, was clearly a generalist, occurring at relatively high frequencies with a wide range of different bark beetles and on both conifer host trees at various locations.

5.8 Comparison of fungal associates of tree-killing and non-tree-killing bark beetles

The results of this study indicate that both aggressive and non-tree-killing conifer-associated bark beetles vector relatively similar assemblages of fungi (Table 4). Also, the less-aggressive bark beetle species were associated with several ophiostomatoid species. This is agreement with several previous studies conducted in Europe (Mathiesen 1950, Mathiesen 1953, Harding 1989, Krokene and Solheim 1986, Krokene 1996, Kirschner 1998, Kirisits et al. 2000). The most aggressive bark beetle species considered in this study, *I. typographus*, was found in association with 17 ophiostomatoid species, while a non-tree-killing scolytine *D. autographus* was found in association with twelve species and another less-aggressive insect, *H. palliatus,* with 16 species. Of the ophiostomatoid fungi species associated with *I. typographus*, a number of species were the same as those detected together with less-aggressive bark beetles: six species overlapped with *D. autographus* and ten species overlapped with *H. palliatus*. Differences could also be observed. Common species associated with *I. typographus*, *G. olivacea*, *O. ainoae*, *O. bicolor* and *O. brunneo-ciliatum* were found only in low numbers, if present at all, in association with *D. autographus* and *H. palliatus*. On the contrary, both *D. autographus* and *H. palliatus* were found in association with fungi not occurring with *I. typographus*. These include several species of *Grosmannia* (*G. galeiformis*, *L. lundbergii*, *L. procerum* and *L. truncatum*) and also novel *Ophiostoma* spp. described in this study (*O. pallidulum* and *O. rachisporum*).

Previous studies have mainly focused on economically important bark beetle species, and less is known about the symbiosis between fungi and non-tree killing species in Northern Europe. Based on the classic paradigm, tree-killing bark beetles are associated with fungi that are responsible for overwhelming the host tree defenses, and therefore important for successful colonization of trees by bark beetles (Six and Wingfield 2011). This hypothesis has influenced the research and our views of the bark beetle-fungus symbiosis for the last decades. One prediction under the classic paradigm is that tree-killing bark beetles would have virulent pathogenic fungal associates, and beetles that do not kill trees are lacking fungal associates or they carry nonpathogenic or low virulent fungal species. The most widely studied example for the classic paradigm in Northern and entire Europe is the search for a fungus contributing to the successful colonization of Norway spruce trees by *I. typographus*. The virulent fungus *C. polonica* was suggested to be essential for the initiation of *I. typographus* outbreaks (Christiansen et al. 1987, Christiansen and Solheim 1990, Krokene 1996, Krokene and Solheim 1998). This fungus typically occurs in low, but variable frequencies, indicating that its presence is not obligatorily necessary for outbreaks of *I. typographus*. A low frequency of *C. polonica* in association with *I. typographus* was also observed in this study, in both non-outbreak and outbreak areas (Table 4). It should ne noted, that there are a few areas in Europe, where *C. polonica* is common and the dominant fungal associate of *I. typographus* (Solheim 1986, Furniss et al. 1990, Kirisits 2010). The inconsistent results might be due to different fungal isolation methods employed by different researches (Kirisits 2004).

In this study, *I. typographus* was more commonly associated with nonpathogenic or weakly virulent species such as *O. bicolor*, *O. ainoae* and *O. brunneo-ciliatum* (Horntvedt et al. 1983, Harding 1989, Kirisits 1998, Guérard et al. 2000). This finding is consistent with previous studies, which reported *O. bicolor* as an abundant fungal associate, especially during early phases of *I. typographus* attacks on living trees (Solheim 1986, 1992a, 1992b, 1993a, Harding 1989). Contrary to the classic paradigm, it is more commonly observed that aggressive bark beetle species are most consistently associated with low virulent fungi, and nonaggressive bark beetles sometimes with virulent fungi (Six and Wingfield 2011). Based on this hypothesis, the associates of the nonaggressive bark beetles *D. autographus* and *H. palliatus* should include virulent species. The most constant fungal associates of *D. autographus* were species in the *G. cucullata*-complex and the *O. canum*-like fungus. Knowledge on the pathogenicity of *G. cucullata* is minimal, but it has been reported to be nonpathogenic (Jankowiak and Kolařík 2010). Similarly, *O. piceae* (*O. canum*-like found in this study most likely resembles the species previously identified as *O. piceae* in Scandinavia and Russia) was nonpathogenic in previous inoculation experiments (Krokene and Solheim 1998). Based on this study, *O. canum*-like, *O. canum*, species in the *G. cucullata*-complex, *O. floccosum* and *O. tapionis* can be considered as the most constant fungal associates of *H. palliatus*. For *O. floccosum* and the newly described *O. tapionis*, information on their phytopathogenicity is not available. Previous studies have shown that *O. canum* is a nonpathogenic fungus (Solheim et al. 2001). Also, the less frequently found fungi that were not detected in association with *I. typographus*, *L. lundbergii*, *L. procerum* and *L. truncatum* are not considered to be virulent species (Kaneko and Harrington 1990, Zhou et al. 2002, Wingfield 1986. *Ophiostoma minus* was the only species associated with *D. autographus* and *H. palliatus* that is known to be pathogenic (Solheim and Lånström 1991). The fungus was found only inconsistently and occasionally also in association with *I. typographus*. Species in the *G. piceiperda*-complex associated with *D. autographus* were not recorded in this study, and only rarely with *H. palliatus*. In previous studies they have been found as rather consistent fungal associates of these two insect species and also of other bark beetle species, including *I. typographus*. *Grosmannia piceiperda* has displayed relatively high levels of virulence in some inoculation experiments (Harding 1989, Kirisits 1998, Sallé et al. 2005).

The results of this study do not support the classic paradigm regarding the argument that tree-killing coniferous bark beetles are closely associated with pathogenic fungi. For several ophiostomatoid species found in this study, nothing or very little is known regarding the pathogenicity to their host trees. As Six and Wingfield recently (2011) stated, due to strong evolutionary selection pressure, bark beetles should have a highly consistent association with virulent fungal associates, if virulence of the fungi is required in the tree-killing process. This kind of a highly consistent association between any of the conifer-infesting bark beetles and their fungi could not be confirmed. Also, non-tree-killing bark beetle species were associated with numerous fungi, which is also contradictory to the classic paradigm. We could, however, not confirm one of the arguments against the classic paradigm that nonaggressive bark beetles have a more consistent association with virulent fungal species. The rather consistent association of some nonaggressive bark beetle species with a relatively virulent fungus *G. piceiperda* observed in previous studies may be seen as an argument against the classic paradigm (Harding 1989, Kirisits 1998, Sallé et al. 2005). Six and Wingfield (2011) suggested that virulence might play a more important role for the fungus itself than for the bark beetle in tree killing. Virulence might be an advantage in the competition between tree-infesting fungi and increase the fitness of the fungus in a living tree. Considering also the evolutionary history of these symbioses, this hypothesis could explain a great deal of the inconsistencies

observed in several studies, including this study. The complex of the fungi associated with bark beetles appear to share a similar niche. However, differences in virulence could be one property affecting niche separation, and thus allowing coexistence of several fungi showing different ecological strategies (Six and Wingfield 2011).

5.9 Importance of the host tree for the ophiostomatoid mycobiota of bark beetles

The results of this survey suggest that the host tree has more importance for the qualitative and quantitative composition of the ophiostomatoid mycobiota of bark beetles than the individual bark beetle species themselves. From an evolutionary perspective, plant-fungal interactions in general are older than fungal-insect symbioses (Taylor and Osborn 1996, Engel and Grimaldi 2004, Heckman et al. 2001). It can therefore be assumed that also fungal virulence is rather an adaption to the host tree than to the insect vector (Lieutier et al. 2009). The importance of the host tree for the mycobiota of bark beetles becomes most clear, when comparing conifers and hardwoods. Hardwood-infesting ophiostomatoid species, such as *O. karelicum* infesting mainly birch, are only occasionally detected in association with conifer-infesting bark beetles, and found sporulating in their galleries on conifers (Table 4). This is also, at least partly, a result of the host specifity of bark beetle species. Most phloephagous bark beetles are normally specific to one tree genus (Sauvard 2004). Even polyphagous beetle species generally attack either different conifers or different broadleaved trees. Although most bark beetle species have one main host tree, there is still more chance for the fungi to be transferred between different conifers in Northern Europe.

In general, two conifer species belonging to the same genus are likely to have similar assemblages of bark beetle-associated ophiostomatoid fungi, even if the beetle species represent different genera (Kirisits 2004). Also, differences can be observed between different host tree genera infested by closely related bark beetle species. For example*, I. typographus* has been reported to share more fungal associates with another spruce-infesting beetle, *P. chalcographus*, than with the more closely related, pine-infesting *I. sexdentatus* (Kirisits 2004, Lieutier et al. 2009). Based on the results from this survey, a clear pattern between pine and spruce-infesting bark beetles regarding their associated fungi could not be detected. Bark beetles only occurring on pine (*I. sexdentatus*, *T. minor* and *T. piniperda*) vectored 13 ophiostomatoid fungi, of which ten overlapped with bark beetle species solely found on spruce (*I. typographus*, *Ips* sp.). A comparison of the mycobiota of all pine- and spruce-infesting bark beetle species examined in this study, excluding the species represented by single isolates, shows that five species can be regarded as spruce-specific and only one species as pine-specific. The species strictly associated with pine was *O. saponiodorum*, a new species described in this survey. The fungal species that were strictly associated with spruce included *C. polonica*, *Gr. fimbriisporum*, *G. piceiperda*, *O. bicolor*, as well as the newly described *O. fuscum* and *O. saponiodorum*. In previous studies, *C. polonica*, *G. piceiperda* and *O. bicolor* have been found in association with bark beetles occurring commonly on spruce, but also on other host tree species (Mathiesen 1950, Mathiesen-Käärik 1953, Krokene and Solheim 1996, Viiri 1997, Kirisits 2004, Jankowiak and Hilszczański 2005).

When looking at the overall host tree preference of the fungi, a clearer pattern emerges. A number of fungi were found in association with those bark beetle species infesting both pine and spruce, but were more commonly encountered either in association with pine or spruce (the majority of fungal isolates were obtained from one host tree species) (data not shown). The fungi that were more commonly obtained from spruce-infesting beetles included five

species: *G. olivacea*, *G. taigensis*, *O. ainoae*, *O. brunneo-ciliatum* and *O. tapionis*. Reports of *G. olivacea* in Europe are limited. This species has been previously found only in association with spruce-infesting bark beetles (Mathiesen-Käärik 1953, Kirisits 2004). *Ophiostoma ainoae* and *O. brunneo-ciliatum* have been reported from both spruce and pine-infesting bark beetles (Kirisits 2004, Jankowiak and Hilszczański 2005). The fungi that were most typically associated with pine included four species: *O. canum, O. floccosum*, *O. minus* and *O. rachisporum*. Also, previous reports of *O. canum* and *O. minus* mainly come from pine-infesting bark beetles; *O. floccosum* has been reported from both pine and spruce-infesting scolytine species (Kirisits 2004).

Several previous studies have mainly focused on reporting fungal associates of one or a few bark beetles infesting typically one host tree species. Similar, more comprehensive surveys covering several bark beetles species and different host trees in the Northern Europe have not been conducted before. Previous studies have also strongly been focused on the bark beetle benefits of the association with fungi; benefits to fungi have until recently received less attention (Lieutier et al. 2009, Six and Wingfield 2011). In addition, the virulence of fungi might be just one fungal characteristic, which probably does not play a significant role in the ecology of bark beetles. Artificial inoculation trials have mainly focused on testing the virulence of fungi against the same tree species, from which the bark beetles or fungi have been originally collected. Much less attention has been paid to what happens if fungal associates are inoculated onto other tree species, or if it may be moved from its native habitat. Previous studies have shown that different tree species differ in their responses to fungal inoculation (Christiansen and Solheim 1990, Raffa and Smalley 1995, Zhou et al. 2002). Own preliminary results suggest that if some hardwood-infecting fungi are inoculated onto a coniferous tree, some fungi are not able to tolerate the tree responses and do not cause symptoms (Selochnik et al. 2010). In contrast, species such as *O. quercus*, which is non-virulent to its original hardwood host trees, can display a high level of virulence towards conifer trees in inoculation experiments (Selochnik et al. 2010).

Apparently ophiostomatoid fungi have diverse roles in ecology of bark beetles (Six and Wingfield 2011). The results of this study show that the importance of the host tree for the occurrence of fungi might have been underestimated. Previous research has been mainly focused on the possible benefits of fungal associates for bark beetles. Considering effects to bark beetles, in several cases ophiostomatoid fungi might be just "weeds" that take advantage to be dispersed by their insect vectors. As a result of long time co-evolution with bark beetles, the fungi have developed structures that facilitate their spread to new host trees. If these fungi accidentally come in contact with new host tree species and are introduced to new areas, the consequences might be unpredictable, as previous examples have shown (Lu et al. 2009, Selochnik et al. 2010).

5.10 Assessment of the pest and pathogen risks connected to timber imports from the Karelia region

The study was mainly conducted in eastern Fennoscandia, on both sides of the Finnish-Russian border (Table 2). Although the forestry practices differ significantly on these two sites of the border, the region is geographically rather uniform and no natural barriers prevent the movement of species spreading from east to west. Based on previous studies, timber imports from the Russian part of Karelia and adjacent areas do not pose an evident risk of pest introductions into Finland (Siitonen 1990, Jakovlev and Siitonen 2005). Economically

important pests not found in Finland are not known to occur in the European parts of Russia. The forest pest fauna in the region is generally well known, but the abundance and western distribution limits of species occurring in other parts of Russia are not well-studied. Several new pest species to Finland have been detected on timber transported from Siberia (Siitonen 1990). During this survey, we did not observe bark beetle species non-native to the region.

Between Finland and Russia, considerable differences could be observed in the assemblages of ophiostomatoid fungi vectored by the various bark beetle species. The total number of *Ophiostoma* and *Grosmannia* species was similar in both countries. In total, 20 species were found in Russia, and 19 in Finland. Seven of the species were only found in Russia, and five species were found only in Finland. Interestingly, the number of *Grosmannia* isolates was higher in Finland than in Russia, while *Ophiostoma* isolates were more commonly found in Russia. This result likely reflects differences in the spectrum and number of bark beetle species collected in the two countries (Table 3). The study revealed a surprisingly high number of previously unknown taxa. Also, new bark beetle vectors were recognized for several fungi. In comparison to previous studies in Europe, similar assemblages of ophiostomatoid fungi associated with individual bark beetles could be observed. For well-investigated bark beetles species, especially *I. typographus*, considerable variation in the assemblages of fungal associates between different localities in Europe have been documented. It appears that variation in the assemblages of ophiostomatoid fungi associated with certain bark beetle species such as *I. typographus* is a common phenomenon. Although a number of hypotheses have been proposed the factors influencing this variation and how it affects the ecology of bark beetles remain poorly known.

The recorded variation in the diversity and assemblages of ophiostomatoid species between Finland and Russia might be a result of different forestry practices leading to different forest structures and different amounts of dead wood. However, it should be noted that the sampling strategy and the study design does not allow doing comparisons between Finland and Russia using inferential statistics. Likely the most important factor explaining most of the differences between Finland and Russia are differences in the assemblages of bark beetles obtained in the two countries. The diversity of ophiostomatoid species detected in this study was higher than previously thought, but apparently represents typical fungi that are native to the region. The pathogenicity of many of these fungi to their endemic host trees is unknown. Therefore, the risks involved in timber imports from Russian Karelia to Finland are difficult to predict based on the current knowledge. As recent examples of invasive bark beetle species and their associated fungi have shown (Lu et al. 2010), even species that are considered less harmful in their native environment pose potential risks in changing or new environments. Therefore, all the bark beetle species and their fungal associates investigated in this study should be considered potential threats to the health and ecology of forests and their socio-economic importance for humans, especially if accidentally introduced to new environments.

6 CONCLUSIONS

Fungi associated with bark beetles infesting the dominant tree species in Fennoscandia, Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), and birch trees (*B. pendula*, *B. pubescens*), were studied in order to broaden the knowledge of the bark beetle associated fungal diversity in the region. The present study is one of the most comprehensive investigation of its kind conducted in the Northern Europe thus far, including both aggressive and less-aggressive bark beetles typical to the region. A focus was to investigate the diversity of ophiostomatoid fungi, with special emphasis on the genera *Ophiostoma* and *Grosmannia*. A further aim was to compare the ophiostomatoid species diversity between Finland and Russia, where forestry practices differ substantially. The major findings of this study are summarized as follows:

All bark beetle species investigated in this study, including both aggressive and non-aggressive species, were associated with a complex of ophiostomatoid fungi. Pine- and spruce-infesting bark beetles were typically associated with numerous fungi, of which the majority was present in low numbers. Moreover, they were not consistently found at different locations and are regarded as casual fungal associates. A few species occurred at higher frequencies with particular bark beetle species and more consistently at different locations. They are considered constant associates. Some fungi were generalists and were found at several locations in association with a wide range of bark beetles, and infecting different host trees. Other species were specialists occurring in association with one or a few bark beetle species. Compared to conifer-infesting bark beetles, the assemblage of fungi vectored by the birch-infesting bark beetle, *S. ratzeburgi*, was different. The total number of associated fungi was lower for this scolytine. One fungus, *O. karelicum*, was, however, found in association with each individual beetle or its galleries at all studied locations, and this species is thus regarded as rather specific and consistant fungal associate of the insect.

The results of this study indicate that the host tree has more importance for the ophiostomatoid fungal diversity than the bark beetle vectors themselves. Considering the benefits to the bark beetles, many fungi might be only incidental passengers, which have developed structures to facilitate the transfer to new host trees. The classic paradigm suggests that fungi associated with aggressive bark beetle species are critical for a successful colonization and the tree-killing process of the host tree (Six and Wingfield 2011). Several findings in this study argue against the classic paradigm. First, there was a lack of consistency in the occurrence of virulent fungal associates with aggressive bark beetles. It should be noted that in many cases, the virulence of the fungi identified in this study is unknown, but in general such consistency predicted by the classic paradigm could not be observed. If a certain fungal associate is of critical importance to a bark beetle or even obligatorily required for successful colonization of the host tree, the absence of that fungus would soon lead to the extinction of the bark beetle population (Six and Wingfield 2011). The fungus that could be considered the most virulent species found in this study, *C. polonica*, was only a rare associate of the aggressive bark beetle species *I. typographus*. Second, also the non-aggressive bark beetles were commonly associated with ophiostomatoid fungi. The classic paradigm predicts that non-aggressive bark beetles have either no, non-pathogenic or low virulent fungal associates (Six and Wingfield 2011). As much as is known about the virulence of the fungi found in this study, non-aggressive bark beetles such as *D. autographus* and *H. palliatus* were casually associated with a virulent fungus, *O. minus*. Although differences between the fungal assemblages of various bark beetle species were observed, the general

spectrum of fungi was very similar for aggressive and non-aggressive bark beetle species. This study thus supports the view recently presented by Six and Wingfield (2011), which suggests that the majority of ophiostomatoid fungi associated with bark beetles are not of cruitial importance to their insect vectors.

The diversity of ophiostomatoid fungi in Finland and Russia was found to be much higher than previously thought. Several species were recorded for the first time in the studied countries, but only one previously described species, *O. quercus*, was recorded for the first time from Fennoscandia. In addition, a surprisingly high number of new taxa from a relatively small geographic area were encountered during this survey. In total, at least 29 species of *Ophiostoma* and *Grosmannia* were found in association with 13 different bark beetles on birch (*B. pendula*, *B. pubescens*), spruce (*Picea abies*) and pine (*Pinus sylvestris*). Of these, eight species were described as novel taxa during the study, and several putatively new species remain unidentified. Species belonging to the genus *Ophiostoma* were most numerous. Also, species belonging to the genus *Grosmannia* were commonly found. So far, only a small portion of bark beetle species occurring in boreal forests have been studied and the sampling does not cover the whole diversity of the habitats. The outcome of our survey work likely indicates that even many of the relatively common ophiostomatoid species have yet to be discovered and described.

Comparing the results of this study with previous reports, the ophiostomatoid species diversity in the boreal forests of Fennoscandia show only some differences compared to forests in more southern parts of Europe. The differences migh reflect different sampling strategies and subjective factors in the various studies. Although fungi associated with bark beetles have most likely followed the post-glacial distribution routes of their host species and insect vectors, little differentiation between populations of the fungi have been found and no congruence with the geographically isolated conifer host, *Picea abies* have been detected in Europe (Tollefsfud et al. 2008, Marin et al. 2009). The total number of ophiostomatoid species was similar between Finland and Russia. Interestingly, *Ophiostoma* spp. were more common in Russia, while *Grosmannia* spp. were more frequently isolated from Finland. The observed differences in bark beetle and fungal species diversity might be a result of differences in the assemblages of bark beetle species found and collected in the two countries. Different forestry practices in the two countries can also influence the spectrum and populations of bark beetles (Martikainen et al. 1996) and probably also the assemblages and frequency of fungal associates.

Most ophiostomatoid fungi that were isolated during the course of this study were designated either to known species or to previously unknown taxa. In most cases, the phylogenetic species recognition was essential for a reliable identification of previously unrecognized taxa. This emphasizes the importance of the DNA sequence based recognition of ophiostomatoid fungi. However, one should be cautious to underemphasize the importance of morphological characters and to misleadingly use the molecular methods, especially ITS data. In some cases, the ITS region was not sufficiently variable for the identification of morphologically similar or virtually identical species. The opposite situation also occurred, in which morphologically well-delimited species had identical ITS sequences. Although the β-tubulin gene provided more resolution and usually helped to resolve species identities, in some cases it was also not sufficiently variable to distinguish two morphologically delimited species. A number of the previously unknown species were formally described during the course of this study. However, some species clearly represented new taxa, but were found in far too small numbers to justify formal species descriptions. Additional isolates need to be collected for the description of these fungi. The identities of some other fungal isolates also

remained unresolved. In some cases, this was due to a lack of phylogenetic resolution using both ITS and β-tubulin data. Considering their morphology and ecology, it seems possible that these fungi represent either new haplotypes of known species or hitherto undescribed taxa. Additional isolates and sequences for additional gene regions will be necessary to fully clarify the identity of these isolates. Based on the phylogenetic analysis, some known species formed clearly distinct lineages, and the status of these species or species complexes needs to be clarified. Further studies including an inspection of type material, additional isolates from various regions, and DNA sequence comparisons for gene regions additional to those previously considered, will be needed to clarify the taxonomy of isolates representing species complexes.

Overall, the results of this study clearly indicate that there are many more fungi associated with bark beetles in the boreal forests of Fennoscandinavia than was previously recognized. We believe that these species represent a fungal diversity typical to the region, but it is difficult to predict the risks involved in timber imports from Russia to Finland. For many species, little or nothing is known regarding their pathogenicity to endemic host trees. In addition, nothing is known about their pathogenicity to potential new host trees, if they are accidentally moved to new areas and environments. Bark beetles and the fungi associated with them are well-adapted to be moved across national boundaries in unbarked and untreated timber. Although the roles of these fungi for the biology and ecology of their insect vectors are poorly known, it is well-documented that several of the fungi vectored by bark beetles are capable of killing trees. Therefore, all the bark beetle species and their fungal associates investigated in this study should be considered as potential threats to forests and socio-economic well-being, especially if accidentally introduced to areas, where they are not native.

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