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Abstract

Fungal pathogens associated with bark beetles represent one of the most significant problems to forest health. Among these fungi, ophiostomatoid group are known to be most commonly associated with bark beetles. Some of these pathogens, such as Dutch elm disease-causing fungi, have a very long history of extensive research, while fungi causing other diseases are poorly or only partially understood and substantial knowledge gaps can exist in their ecology and spread.

The aims of the studies were to provide new knowledge on indigenous and newly massively tree-devastating bark beetles, vectored communities of fungi, and their pathogenicity to host trees. The principal methods included fungal culturing and rDNA sequencing, which allowed the investigation of composition of fungal communities associated with bark beetles. In particular, both ophiostomatoid and other phytopathogenic fungi were the most commonly detected groups. The use of pathogenicity tests revealed the ability of different ophiostomatoid fungi to infect *Pinus sylvestris* saplings, but with a varying degree of success for different fungal species. Long-term trials carried out in Sweden showed that *Scolytus multistriatus* is a vector for Dutch elm disease caused by invasive *Ophiostoma ulmi* complex that recently invaded the island of Gotland causing the disease to *Ulmus minor*.

It can be concluded that i) bark beetles are vectors for different fungi and that ophiostomatoid fungi are the most closely associated symbionts; ii) bark beetles can vector different functional groups of fungi including aggressive pathogens; iii) highly virulent fungi can help bark beetles to overcome the tree defence. The work presented in this thesis demonstrates that some fungi associated with bark beetles may significantly contribute to tree mortality. Understanding the nature of fungal-insect interactions may help more effectively to manage potential threats.

Keywords: ophiostomatoid fungi, bark beetle, pathogenicity, Dutch elm disease

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Förekomst av, och virulens hos, trädpatogena svampar som sprids av barkborrar

Abstrakt

Svampskadegörare associerade med barkborrar är ett av de mest allvarliga problemen för skogens hälsa. Ophiostomatoida svampskadegörare är antagligen de mest kända barkborre-associerade svampskadegörarna. För några av dessa skadesvampar, som svampen som almsjuka, finns det omfattande forskning kring, medan svampar som orsakar andra sjukdomar mindre välstuderade och därför finns det stora luckor i vår kunskap om deras ekologi och spridningsvägar. Syftet med arbetet i denna avhandling var att samla ny kunskap om existerande och invasiva och barkborrar som orsakar omfattande skador på träd, deras associerade svamp och svamparnas patogenicitet i värdträd. De viktigaste metoderna i arbetet var svampisolering och odling samt rDNA-sekvensering, vilket möjliggjorde analyser av vilka grupper av svampar som associerade med de undersökta barkborrearterna. Ophiostomatoid- och andra patogena svampar var de vanligast förekommande grupperna. Användningen av patogenicitetstester visade förmågan hos olika ophiostomatoida svampar att infektera *Pinus sylvestris* plantor, som skiljde sig åt mellan svamparterna. Långtidsstudier som genomförts i Sverige visade att *Scolytus multistriatus* är en vektor för almsjukan som orsakas av det invasiva *Ophiostoma ulmi*-komplexet som nyligen invaderade Gotland och orsakar sjukdom på *Ulmus minor*. Från arbetet följer slutsatserna att i) barkborrar är vektorer för olika svampar och att ophiostomatoid svampar är de vanligaste associerade symbionerna; ii) barkborrar kan sprida olika funktionella grupper av svampar inklusive aggressiva patogener; iii) vissa svampar, särskilt med hög virulens, kan hjälpa barkborrar att övervinna trädförsvaret. Arbetet som presenteras i denna avhandling visar att vissa svampar som är associerade med barkborrar kan bidra till utbredd förlust av träd. Att bättre förstå svamp-insektsinteraktioner kan bidra till effektiva metoder att hantera potentiella hot.

Nyckelord: barkbagge, holländsk almsjukdom, patogenicitet

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Dedication

To my mother Ludmyla, for all the love and support that you have given to me.

Contents

List of publications

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

I. **Davydenko K.**, Vasaitis R., Meshkova V., and Menkis A. (2014). Fungi associated with the red-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Curculionidae) in the forest-steppe zone in eastern Ukraine. European Journal of Entomology, 111(4), pp. 561-565. doi: 10.14411/eje.2014.070

II. **Davydenko K.**, Vasaitis R., and Menkis A. (2017). Fungi associated with *Ips acuminatus* (Coleoptera: Curculionidae) in Ukraine with a special emphasis on pathogenicity of ophiostomatoid species. European Journal of Entomology, 114, pp. 77-85. doi: 10.14411/eje.2017.011

III. Menkis A., Östbrant I.-L., **Davydenko K.**, Bakys R., Balalaikins M., and Vasaitis R. (2016). *Scolytus multistriatus* associated with Dutch elm disease on the island of Gotland: phenology and communities of vectored fungi. Mycological Progress, 15:55. doi:10.1007/s11557-016-1199-3

IV. **Davydenko K.**, Vasaitis R., Elfstrand M., Meshkova V., Menkis A. Fungi associated with bark beetle *Ips sexdentatus* attacking declining *Pinus sylvestris* in Ukraine: focus on occurrence and pathogenicity of ophiostomatoid spp. (manuscript)

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The contribution of Kateryna Davydenko to the papers included in this thesis was as follows:

I. Major participation in sampling, laboratory work, data analysis. Writing manuscript with supervision, responsible for correspondence with journal.

II. Major participation in planning, sampling, laboratory work data analysis and interpretation results. Writing manuscript with supervision and responsible for correspondence with journal.

III. Participation in sampling, laboratory work, data analysis.

IV. Major participation in planning, sampling, laboratory work data analysis and interpretation results. Writing manuscript with supervision.

In additional to the listed papers included in this thesis, Kateryna Davydenko has contributed to the following papers within the time frame of the thesis work:

I. Marčiulynienė, D., **Davydenko, K.**, Stenlid, J., & Cleary, M. (2017). Can pruning help maintain vitality of ash trees affected by ash dieback in urban landscapes? *Urban Forestry & Urban Greening*, 27, pp. 69-75.

II. Marčiulynienė, D., **Davydenko, K.**, Stenlid, J., Shabunin, D., & Cleary, M. (2017). *Fraxinus excelsior* seed is not a probable introduction pathway for *Hymenoscyphus fraxineus*. *Forest Pathology*, 48, e12392.

III. Stenlid, J., Elfstrand, M., Cleary, M., Ihrmark, K., Karlsson, M., **Davydenko, K.**, & Brandström Durling, M. (2017). Genomes of *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus* encode surprisingly large cell wall degrading potential, balancing saprotrophic and necrotrophic signatures. *Baltic Forestry*, 23(1), pp. 41-51.

IV. **Davydenko, K.**, Nowakowska, J., Kaluski, T., Gawlak, M., Sadowska, K., García, J., Diez, J, Okorski A. & Oszako, T. (2018). A Comparative study of the pathogenicity of *Fusarium circinatum* and other *Fusarium* species in Polish provenances of *P. sylvestris* L. *Forests*. 9(9), 560.

V. **Davydenko, K.**, Borysova, V., Shcherbak, O., Kryshtop, Y. and Meshkova V. (2019). Situation and perspectives of European ash (*Fraxinus excelsior*) in Ukraine: focus on eastern border. *Baltic Forestry* 25(2), pp. 193-202.

Abbreviations

BLAST	Basic local alignment search tool
CA	Correspondence analysis
CCA	Canonical correspondence analysis
CP	Classic paradigm
DED	Dutch elm disease
ITS	Internal transcribed spacer
MEA	Malt extract agar
NMDS	Non-metric multidimensional scaling
PCR	Polymerase chain reaction

1. Introduction

An increasing frequency and extent of forest disturbances, caused by different agents, such as storms, drought, fires, or insect outbreaks have recently been recorded in Europe. Moreover, most of the European forests are now increasingly threatened by climate change, which greatly intensifies forest disturbances such as bark beetle outbreaks (Sommerfeld et al. 2021, Schafstall et al. 2020). Bark beetles (Coleoptera: Curculionidae, Scolytinae) are well-known insects worldwide that are among the most economically and ecologically important primary pests of the forests, especially for stressed and weakened trees. Colonization by most of bark beetles are limited to weak or dying trees, but a few species can attack and kill healthy trees (Stursova et al. 2014, Teich et al. 2019, Six and Wingfield 2011a).

One of the most distinguished characteristics of bark beetles is the ubiquitous association with different fungi that were recognized more than a hundred years ago (Six and Wingfield 2011a). Therefore, bark beetles could be considered as key species in complicated symbiotic assemblages that encompass filamentous fungi and host plants. These sustained and complex associations between bark beetles and fungi still remain intriguing for almost a century. These fungal communities vectored by bark beetles could be both evolutionarily and incidentally associated with bark beetles. Among the first group, phytopathogenic blue-stain fungi, or so-called ophiostomatoid fungi, are intimately associated with bark beetles, which act as the most important vectors.

Bark beetles are well-known to contribute greatly to the spread of these fungi into the living trees as well as to untreated timber and wood products (Linnakoski 2011, Kirisits 2004). Increasing the global trade of timber can significantly increase the risks of incidental invasion of new bark beetle species and/or fungal pathogens into new geographical areas. There are few dramatic examples of the invasion of bark beetle species and vectored fungi into new areas where they became aggressive and caused threats to forests after they have been accidentally introduced (Linnakoski 2011, Thompson, Bodart and Gruner 2019, Hulcr et al. 2020, Bezos et al. 2015). Therefore, a few bark- and wood-boring insects have been identified as potentially invasive species in northern Europe by the means of import of deciduous wood chips from North America (Flo et al. 2014).

Although a great deal of work, covering multiple generations of scientists, has been carried out focusing on fungi and bark beetle interactions (Biedermann et al. 2019, Linnakoski 2011, Six and Elser 2019), these interactions still remain poorly understood (Linnakoski 2011, Six and Elser 2019, Six 2020), especially in the context of climate change that can also significantly increase the threats to forest ecosystems caused by bark beetles and their fungal symbionts.

1.1 Bark beetles in forest ecosystems

Bark beetles comprise a highly diverse group of insects with a worldwide distribution. Bark beetles mate and oviposit within the phloem where they construct galleries under the bark in the phloem and sapwood of trees, and can successfully breed after the death of these tissues. The overwhelming majority of bark beetle species are capable to attack weakened or dying trees when their populations are still low in abundance, but a mass-attack of large numbers of healthy trees can take place once their populations are high in abundance (Hlásny et al. 2019). To categorize the life-history and strategies, bark beetles were often divided into contingent clusters: primary, secondary, and saprophytic, or aggressive and non-aggressive beetles (Grove 2002, Huang et al. 2020, Paine et al. 1997, Six and Wingfield 2011a). However, both climate change and anthropogenic pressure resulted in the shift in ecological roles as e.g. non-aggressive bark beetles became aggressive (Six and Wingfield 2011a, Six 2020). Consequently, previously known as non-aggressive beetles, can now cause massive outbreaks and the dieback of a large number of healthy trees (Colombari et al. 2012, Havasova et al. 2017, Krokene and Solheim 1998b). In case of unfavourable conditions, most of bark beetles including aggressive ones, can exist at low population densities, surviving in dying trees, windfalls, or in the harvesting debris (Christiansen et al. 1987).

1.1.1 Bark beetles in conifers

Among aggressive bark beetles, most of them have developed adaptations to colonize trees from the family *Pinaceae*. Notwithstanding strong mechanism of tree defence, these insects are economically and ecologically important as during outbreaks, they can devastate both managed and natural forests (Raffa et al., 2015). The abundance of windthrows or conifer trees weakened by drought, as e.g. Norway spruce (*Picea abies* L) and Scots pine (*Pinus sylvestris)*, can also trigger the rapid growth of the bark beetle population. In case of the lack of fallen or stressed trees with low level of defence responses, this makes bark beetles to attack healthy trees (Havasova et al. 2017, Temperli et al. 2013, Siitonen 2014). For example, the European spruce bark beetle (*Ips typographus* L.) (Coleoptera: Curculionidae, Scolytinae), is capable of killing trees in large numbers, what makes it one of the most important and destructive bark beetles in both economic and ecological terms in the coniferous forests of the Palearctic region (Wermelinger 2004, Temperli et al. 2013). Various abiotic and biotic factors such as drought, windstorm, fire, infectious diseases and extensive forest management can make trees more susceptible to attacks by bark beetles and among these factors, drought is in particular considered to be a trigger for many outbreaks.

Form 2010s onwards, two species of bark beetles were shown to be the ones that became the most destructive pests in *P. sylvestris* forests, i.e. becoming aggressive, attacking and killing healthy trees in Finland, Italy, Poland, Spain, Germany, Slovakia (Bueno et al. 2010, Dobor et al. 2020, Jankowiak et al. 2017, Colombari et al. 2012, Siitonen 2014) and also in Ukraine. In Ukraine, massive *P. sylvestris* dieback and considerable economic damage were caused by the pine engrave beetle (*Ips acuminatus*) (Gyllenhal, 1827) (Coleoptera: Scolytidae) and six-toothed bark beetle (*Ips sexdentatus*) (Börner, 1767) (Coleoptera: Scolytidae) (Meshkova 2020). For many years, both of these bark beetles were considered to be of minor significance, however, recently they were reported as commonly causing extensive damage to young plantations and stands of *P. sylvestris* (Levieux et al. 1989, Siitonen 2014). Therefore, *I. sexdentatus* and *I. acuminatus* can kill a considerable number of healthy trees given that their population density is high. The drought-induced weakening of *P. sylvestris* trees and their further infestation by bark beetles resulted in a rapid tree dieback in Ukraine, resulting in about 70 thousand hectares of lost plantation forests (Figure 1). *Ips acuminatus* affects the thin bark in the upper part of *P. sylvestris* throughout Europe (Colombari et al. 2012), while *I. sexdentatus* colonize lower part of *P. sylvestris* trees (Levieux et al. 1989).

Figure 1. *Pinus sylvestris* dieback in 2018 caused by *Ips acuminatus* in Kyiv region, Ukraine. Photo by Kateryna Davydenko.

Moreover, bark beetles from the genera *Hylurgus* (*Hylurgus ligniperda*) and *Hylastes* (*H. angustatus* and *H. ater*) are the serious pest causing extensive damages to young plantations, stands and timber of *P. sylvestris*. *Hylurgus ligniperda* is among the most common bark beetles in Ukraine. *Hylurgus ligniperda* breeds in logging residues including stumps, roots and logs of *P. sylvestris* trees (Reay & Walsh 2001, 2002). Besides, larvae of *H. ligniperda* may also feed on roots and butts of healthy-looking and diseased seedlings and saplings.

1.1.2 Bark beetles in hardwood trees with a special emphasis on *Scolytus multistriatus*

Many bark beetles have evolved to breed in angiosperms, but most of those are saprophages (Raffa et al. 2015). Since 19th century, there are numerous studies on conifer bark beetles and their economic and ecological importance to conifer forests (Linnakoski 2011, Villari et al. 2012, Kirisits 2004). Although there are a multitude of species of bark beetles that attack hardwood trees and there is no species capable of ecological or economical losses in hardwood forest at the same extent as compared to conifer bark beetles (Ohmart 1989) with exception to the large and small elm bark beetles *Scolytus scolytus* (F.) and *S. multistriatus* (Marsham), respectively, that are the most common and important species spreading the Dutch elm disease worldwide (Santini and Faccoli 2015, Menkis et al. 2016).

Scolytus multistriatus (Scolytinae: Scolytini) is native to Europe, the Middle East, and northern Africa (Menkis et al. 2016), but was introduced with elm (*Ulmus* spp.) wood to other continents including North America, New Zealand, and Australia and generally occurs within the distribution area of *Ulmu*s spp. (Menkis et al. 2016). *Scolytus multistriatus* attacks dying, stressed or trees weakened by different abiotic or biotic factors while transmitting destructive Dutch elm disease (DED), caused by *Ophiostoma ulmi* s.l., which during the last 100 years have destroyed billions of *Ulmus* spp. trees worldwide. *Scolytus multistriatus* usually oviposit in the phloem of weakened trees and hatching larvae immediately construct larval galleries, which extend from the maternal gallery. Larvae feed in the phloem for about 30 days, and then metamorphose into pupae in the pupal chamber in the external part of the sapwood and after about two weeks turn into adults (Menkis et al. 2016, Santini and Faccoli 2015). After the emergence, adults fly and look for healthy trees for maturation feeding and mate. Elm bark beetles may be mono- or even bivoltine in case of favourable climatic conditions (Santini and Faccoli 2015). Active spreading of DED and infestation of *Ulmus* spp. occurs as a result of a synchrony between life cycles of *Ulmus* trees, fungal pathogen and elm bark beetles that are the main vectors for these fungi (Santini and Faccoli 2015).

1.2 Ophiostomatoid (blue-stain) fungi

Most bark beetles are well-known to be associated with specific fungi, so-called ophiostomatoid or blue-stain fungi, which represent genera that are morphologically similar, although not phylogenetically closely related (genera *Ophiostoma*, *Ceratocystiopsis*, *Graphilbum*, *Raffaelea* and *Leptographium* in the Ophiostomatales, and *Ceratocystis* and *Graphium* in the Microascales) (De Beer et al. 2013). Moreover, fungi from the genus

Geosmithia, which belong to *Hypocreales* (*Ascomycota*) and produce dry-spores, are also known as associates of conifer-infesting bark beetles (Dori-Bachash et al. 2015, Jankowiak and Rossa 2008). The ophiostomatoid fungi evolved approximately 200 million years ago and passed a very long evolution path together with their vector beetles (Persson et al. 2011). Now most of the ophiostomatoid fungi are also well-known as sapstain or blue-stain fungi, due to the blue, brownish, greyish, or black discoloration of colonized phloem and sapwood (Linnakoski 2011).

Fungal diseases associated with bark beetles are some of the most significant problems appearing in the forest ecosystems. Some of these diseases are destructive, such as DED, having a history of extensive research on them for nearly a century (Kirisits et al. 2001, Menkis et al. 2016). The ophiostomatoid fungi include well-known conifer tree pathogens, which are responsible for discoloration of wood, serious tree diseases and high rates of tree mortality (Kirisits 2004, Linnakoski et al. 2012). For example, *I. typographus* is known to be associated with the pathogenic and primary invader fungus *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. (previously known as *Ceratocystis polonica*), which can be an aggressive pathogen and kill trees (Viiri and Lieutier 2004). Other severe pathogens are *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf. causing black stain root disease in conifers in North America and *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. that were often found as the cause of white pine root decline (Linnakoski et al. 2012, Wingfield et al. 2016, Wingfield et al. 1993). Among hardwood tree species, *Ceratocystis fagacearum* (Bretz) J. Hunt. causing oak wilt, and *Ceratocystis fimbriata*-species-complex causing vascular wilt diseases in a wide range of angiosperms (Wingfield et al. 2017). By contrast, other diseases caused by ophiostomatoid fungi are still poorly or only partially understood and new species belonging to blue-stain fungi were found, while others are under investigation (Ploetz et al. 2013, Linnakoski and Forbes 2019, Jankowiak et al. 2019, Aas et al. 2018, Jankowiak et al. 2018). Forest diseases associated with bark beetles are still the subject of extensive research due to the significant knowledge gaps existing in their ecology, epidemiology, and management, as well as due to the insufficient knowledge on different types of interactions between fungi and beetles that attack living trees (Ploetz et al. 2013, Six and Wingfield 2011a, Six and Elser 2019).

1.2.1 Ophiostomatoid fungi associated with *Pinus sylvestris* bark beetles

One of such bark beetles, that is still poorly investigated, is *I. acuminatus*, that was shown to be associated with several *Ophiostoma* species (Figure 2), which can cause reduced increment growth, crown thinning, needle chlorosis or even death of the trees (Colombari et al. 2012, Villari et al. 2012). Among the most common fungal species associated with *I. acuminatus* are the two morphologically similar species including *Ophiostoma clavatum* Math. and *O. brunneo-ciliatum* Math.-Käärik (Mathiesen-Käärik 1953, Francke-Grosmann 1963, Villari et al. 2012).

Figure 2. Adults of *Ips acuminatus* and associated blue-stain fungi colonizing the wood on *Pinus sylvestris*. Photo by Kateryna Davydenko.

Ips sexdentatus is one of the most abundant scolytid species of the central and southern European countries, affecting *Pinus* spp., but can also attack *Picea* spp. (Jankowiak 2012, Bueno et al. 2010). A high diversity of fungal species associated with *I. sexdentatus* was studied in Poland (Jankowiak 2012), Sweden (Mathiesen-Käärik 1953), France (Kirisits 2004) and Spain (Moraza et al. 2013), but there were no data about the fungi vectored by *I. sexdentatus* in Ukraine. A few previous studies demonstrated that some ophiostomatoid fungi were associated with *I. sexdentatus*, e.g. four species in Spain (Bueno et al. 2010) and ten species in Poland (Jankowiak 2012). Some of these saproxylic bark beetles carry different fungi on the specialized structure known as mycangia or on their body surface. *Ips sexdentatus* is also in this group and has fossae on the mandibles, the pronotum and the elytra (Bueno et al. 2010, Jankowiak 2012) to vector different fungi. *Hylurgus ligniperda* was shown to be associated with several *Ophiostoma* species, which can cause weakening or dieback of the tree (Kim et al. 2010, Kirisits 2004). It may also vector several *Leptographium* species, which were shown to cause root diseases (Kim et al. 2010, Kirisits 2004, Linnakoski 2011).

1.2.2 Ophiostomatoid fungi associated with *Scolytus multistriatus*

Scolytus multistriatus is one of the most effective vectors for DED (Menkis et al. 2016, Santini and Faccoli 2015) caused by fungi from the genus *Ophiostoma*. DED is a lethal vascular wilt disease comprised of three distinct fungal pathogens, less virulent *Ophiostoma ulmi (*Buisman) Nannf., and highly virulent *O. novo-ulmi* Brasier and *O. himal-ulmi* Brasier &Mehrotra, a species endemic to the western Himalayas (Brasier and Mehrotra 1995). Conidia, which are the infection source of DED pathogens, are transmitted on the body surface of the beetles into the tree, and a new generation of beetles is only infested if the DED fungus is present in the galleries. Conidia are produced in sticky masses that facilitate their attachment and transportation by beetles as they emerge from the trees (Ploetz et al. 2013, Menkis et al. 2016). After the tree infestation by bark beetles the fungus enters into the sapwood and spreads within the tree causing leaves and branches to wilt and die due to the blockage of the xylem vessels and the production of toxins, and eventually causing the death of a tree (Menkis et al. 2016).

The Swedish island of Gotland has a large and a highly valuable wild population of elms (mainly *Ulmus minor*), which until recently was not affected by DED. In 2005, arrival of DED fungi (*O. ulmi* and *O. novo-ulmi*) to Gotland started to cause a massive mortality of elm trees (Menkis et al. 2016). Despite the numerous data for DED causing *O. ulmi* s.l. species and worldwide research for the last 100 years, little is known about when *S. multistriatus* is most active in Gotland (Sweden) and especially what proportion of those beetles vector conidia of DED fungi as well as about other fungal species vectored by *S. multistriatus*. This information was needed to provide guidance for the disease management.

1.3 Bark beetle fungi interaction

Bark beetles and fungi form complex and dynamic associations, which have been shaped during long periods of co-evolution (Six 2020). The interaction between bark beetle and ophiostomatoid fungi were the first time described by von Schrenk (1903) and further developed and confirmed by Leach et al. (1940), who found that two bark beetles i.e. *Ips pini* and *I. grandicollis*, introduced blue-stain fungi into freshly cut logs of *P. abies*. Generalized life cycle of a bark beetle and associated fungi (Figure 3) consists of 1) dispersal; 2) attack of trees and construct galleries under the bark in the sapwood and inoculation of trees by associated fungi into phloem; 3) colonization phase where bark beetle mate, lay eggs and their brood feed and develop together with the growth of ophiostomatoid fungi (Six and Wingfield 2011a).

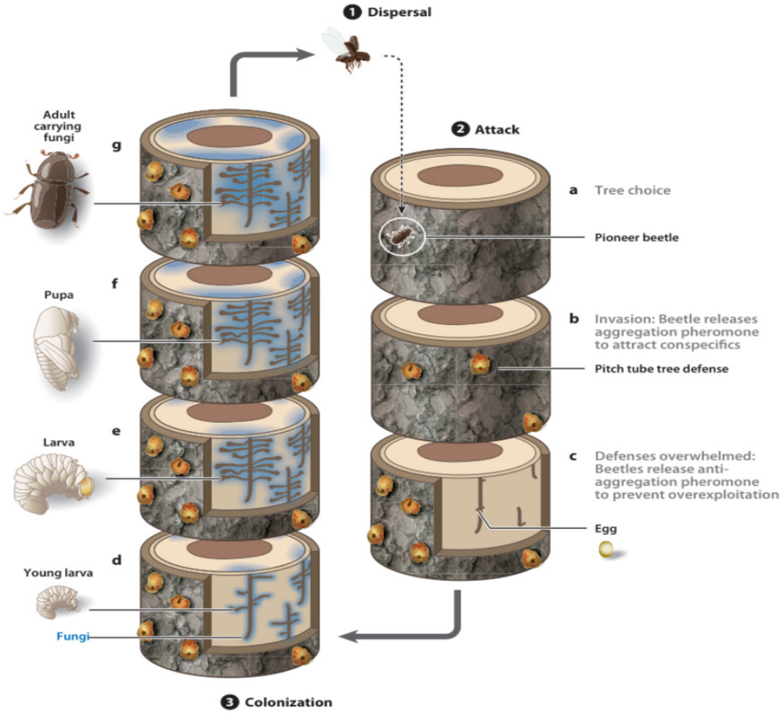

Figure 3. Generalized life cycle of a tree-killing bark beetle and associated blue-stain fungi (Six and Wingfield 2011b): a) tree choice by first arriving bark beetle; b) damage of a tree and the use aggregation and other additional pheromones; c) overcoming tree

defences (the point of no return); d) egg gallery construction, egg-laying, and inoculation of fungi into phloem; e) construct of larval galleries, allowing extensive colonization by ophiostomatoid fungi; f) pupal chambers and pupation when ophiostomatoid fungi form conidia in pupal chambers; g) conidial feeding by young emerged adults and getting of fungi in mycangia or on exoskeleton.

Based on over 100 years ago started observations on bark beetle and ophiostomatoid fungi, it was concluded that these fungi might play key role in the weakening and dieback process of attacked trees or even, in the nutrition of bark beetles (Kirisits 2004, Jacobs et al. 2003, Biedermann et al. 2019, Six and Wingfield 2011a).

Later, the main hypothesis explaining fungus-beetle interactions, has received most attention over the years as the classic paradigm (CP) that states that tree-killing aggressive bark beetles demand fungal pathogens to overcome the tree defence and associated fungi contribute to the tree mortality (Hulcr et al. 2020, Six and Wingfield 2011a). The most studies of that time focusing on bark beetle-associated fungi were mainly centred on the economically important bark beetle species that attack conifers, thereby affecting conclusions. Another and broadened hypothesis was suggested by Diana Six (Six and Wingfield 2011a, Six and Elser 2019), who suggested that pathogenicity is an important factor, which can determine both the success of competition among different fungi colonising living sapwood and the success in overcoming the tree defence.

Bark beetles were found to be associated with a complex of fungi, and the composition of these fungi could vary significantly in time and space, depending on species, region, host and season (Linnakoski et al. 2016, Bezos et al. 2018, Bleiker and Six 2009, Bracewell and Six 2015). Anyway, the association of fungi and bark beetle species can be either typical or occasional depending on the several factors including the dependence between the insect and the fungus (Bracewell and Six 2015, Six 2020). Diana Six hypothesized that functional interactions between bark beetles and associated fungal species vary greatly and can include antagonism, commensalism and mutualism, wherein they can change significantly and might be determined by biotic and abiotic conditions (Bleiker and Six 2009, Six and Wingfield 2011a). Many xylem-feeding bark beetles co-evolved symbiotic mutualistic relationship with fungi, which provide nutrition, which is essential for survival of larvae. as e.g. ambrosia beetles, which usually breed in the sapwood of trees and require fungal mycelium for nutrition. These fungi that directly provide nutrients for larvae are well-known as “ambrosia fungi”, hence ambrosia beetles store these fungi in specific organs, called mycangia, and transport and cultivate them into the wood in their galleries for further feeding. Sapwood is a poor substrate for larval nutrition due to the inability to digest lignin and cellulose (Kirisits 2004). To overcome this, bark beetles transport and inoculate spores from mycangia during construction of breeding galleries. In response, fungi produce abundantly conidia, that are consumed by larvae and sometimes by beetles (Kirisits 2004, Paine et al. 1997). In contrast to ambrosia beetles, phloeophagous bark beetles do not require fungal symbiotic species for feeding of larvae and/or young beetles, as they are able to extract and digest nutrients from the sapwood.

In general, there is still limited information about the fungi associated with different bark beetles, because the majority of studies were done for aggressive and economically important bark beetles, while a considerable number of bark beetle species occur seldom and exist only as unique samples (Klepzig and Six 2004, Six and Elser 2019). The lack of information about the real fungal biodiversity associated with bark beetles might have biased our understanding about bark beetle-fungi symbioses, resulting in the tendency to see all bark beetle–fungi relationships as a mutualistic and similar in function (Six 2012). However, the increasing number of studies focused on interactions among bark beetles and associated fungi, that are based on DNA sequencing, allows to better understand the actual diversity of these symbioses (Paine et al. 1997, Biedermann et al. 2019, Bleiker and Six 2009, Linnakoski 2011, Kirisits 2004). New and a more comprehensive paradigm extended CP statements, adding new knowledge about the diversity of interactions, suggesting that although the actual modalities are still not sufficiently understood, the symbiotic relationship between bark beetles and associated fungi varies significantly (Paine et al. 1997, Biedermann et al. 2019, Bleiker and Six 2009, Jacobs et al. 2003).

Therefore, a new comprehensive hypothesis suggests that associated fungi can benefit from bark beetles, e.g. for the transportation to nutritional sources, which otherwise could not be accessible. Another important benefit for fungi from the symbiosis with bark beetle is the possibility to be introduced into the living or slightly weakened trees. Early introduction enables fungi to avoid strong competition from different fungal species, which colonize the wood at a later stages (Six and Wingfield 2011a), e.g. saprotrophic or pathogenic fungi.

Moreover, various symbioses among bark beetles and fungal species can also include antagonistic relationships. For example, *Ophiostoma minus* can compete with the other fungi after their inoculation into phloem by bark beetles and it also appears to be a highly antagonistic to bark beetles themselves (Klepzig and Six 2004) causing strong negative effects on the survival and development of larvae (Six and Wingfield 2011a, Bracewell and Six 2015).

In summary, the recent information on bark beetle-fungi symbioses demonstrate that associations may vary from beneficial or mutualistic to neutral and even negative, depending on many different factors (Six and Wingfield 2011a, Six 2020).

Presumably, bark beetles and associated fungi usually build up a complex and multifunctional associations, which co-evolved during the long period of time and which are strongly affected by environmental factors. The research presented in this thesis focused on bark beetles and associated fungal communities that were poorly studied before owing to understand the diverse roles of associated fungi and their importance for bark beetles. Generally, the information from Eastern Europe about fungi associated with *I. acuminatus*, *I. sexdentatus* and *H. ligniperda* and their symbioses with fungi was scarce. As the damage caused by the abovementioned *P. sylvestris* bark beetles is increasingly important (Siitonen 2014, Colombari et al. 2012, Jankowiak 2012), the investigation of fungal communities associated with these bark beetles and assessment of pathogenicity of ophiostomatoid fungi to host trees is of particular interest. The latter may demonstrate that fungi vectored by *I. acuminatus*, *I. sexdentatus* and *H. ligniperda* can be of particular importance to forest health, yet they have never been investigated in the regions of Ukraine, which represent the south-eastern timberline of *P. sylvestris* distribution in Europe.

1.4 Pathogenicity of ophiostomatoid fungi

The pathogenicity of ophiostomatoid fungi was found to vary significantly between non- or weakly pathogenic and highly pathogenic species capable of killing healthy trees, while the pathogenicity of different isolates within the same species can also differ substantially. For example, previous research has demonstrated that the pathogenicity for aggressive and highly virulent fungus *Endoconidiophora polonica* can be lost after a serial of vegetative transfers (Krokene and Solheim 2001).

Overall, there is a significant number of infectious tree pathogens among the ophiostomatoid fungi (Dori-Bachash et al. 2015, Fourie et al. 2019, Jankowiak and Kolarik 2010, Krokene and Solheim 1998b, Hulcr and Dunn 2011), that can colonize coniferous and deciduous hardwood trees. The most aggressive pathogens among the ophiostomatoid fungi were considered to be the species that can cause vascular wilt of trees (Hulcr and Dunn 2011). One of the first and probably best-known examples of lethal vascular wilt pathogens, involved in insect-fungus symbioses, are *O. ulmi* and *O. novo-ulmi* that are vectored by the elm bark beetles such as *S. multistriatus*. Infection of elm trees by *O. ulmi* s.l. complex during elm berk beetle attacks results in a rapid wilt due to the formation of tyloses in the xylem vessels and the production of toxins, eventually causing the death of a tree (Brasier, Kirk and Tegli 1995, Menkis et al. 2016). Epidemic and invasion of DED into non-native regions led to the dieback of numerous *Ulmus* spp. trees in North America and Europe. According to many investigations on ophiostomatoid fungi, these fungi can often cause sapstain in the sapwood of conifers and rarely in hardwoods. In addition to DED, such aggressive insect-fungi symbioses have led to the dieback of living trees as many of these fungi appears to be responsible for the epidemics of oak wilt (caused by *Ceratocystis fagacearum*), black stain root disease (caused by *Leptographium wageneri*) in North America, and wilt and canker diseases on a variety of woody plants, including Eucalyptus (caused by *Ceratocystis fimbriata*) in Africa (Kirisits 2004). However, as mentioned previously, most of fungal species are considered as moderately or weakly pathogenic or even non-pathogenic, and thus, resembling saprophytes. Such fungal species mostly cause damage to stored timber, fresh logs and lumber (Krokene and Solheim 1998b, Kirisits 2004). The most pathogenic ophiostomatoid fungi are the primary invaders and usually can colonize fresh sapwood. Then, less pathogenic ophiostomatoid fungi can follow them as secondary invaders, which are also rapidly replaced by saprotrophic fungi that colonize the sapwood at later stages of the temporal succession. It was shown that following the death of a tree, in sapwood most of ophiostomatoid fungi are unable to adapt to the saprophytic life style, thus these are replaced by other fungal species (Krokene and Solheim 1996, Krokene and Solheim 1997, Solheim, Krokene and Langstrom 2001). Since the degree pathogenicity of ophiostomatoid fungi can increase the aggressiveness of bark beetles, this can have an impact on forest health. Thus, the assessment of pathogenicity in these fungi is an important part of understanding bark beetle and fungal symbioses (Kirisits 2004). Artificial inoculation of ophiostomatoid fungi into their host trees and observation of characteristic symptoms are used to assess the pathogenicity of these isolates (Krokene and Solheim 1998b, Solheim and Krokene 1998). Krokene and Solheim have developed methods for inoculation of trees (Krokene and Solheim 1997, Krokene and Solheim 1998b, Solheim and Krokene 1998), which are similar to those seen after natural bark beetles attacks and infections by ophiostomatoid fungi (Krokene and Solheim 1998b). The latter indicates that inoculation of tree saplings can be used as a reliable, low-cost and convenient method for the assessment of virulence of bark beetle-associated ophiostomatoid fungi (Krokene and Solheim 1998a). Therefore, for almost all studies on pathogenicity of ophiostomatoid fungi, *P. sylvestris* and *P. abies* seedling were commonly used for inoculations (Jankowiak 2012, Liu et al. 2017, Repe et al. 2015, Jankowiak and Kolarik 2010). The evaluation was done by measuring the necrotic lesions and recording typical symptoms after inoculation (Figure 4), as well as by evaluating the mortality of the saplings.

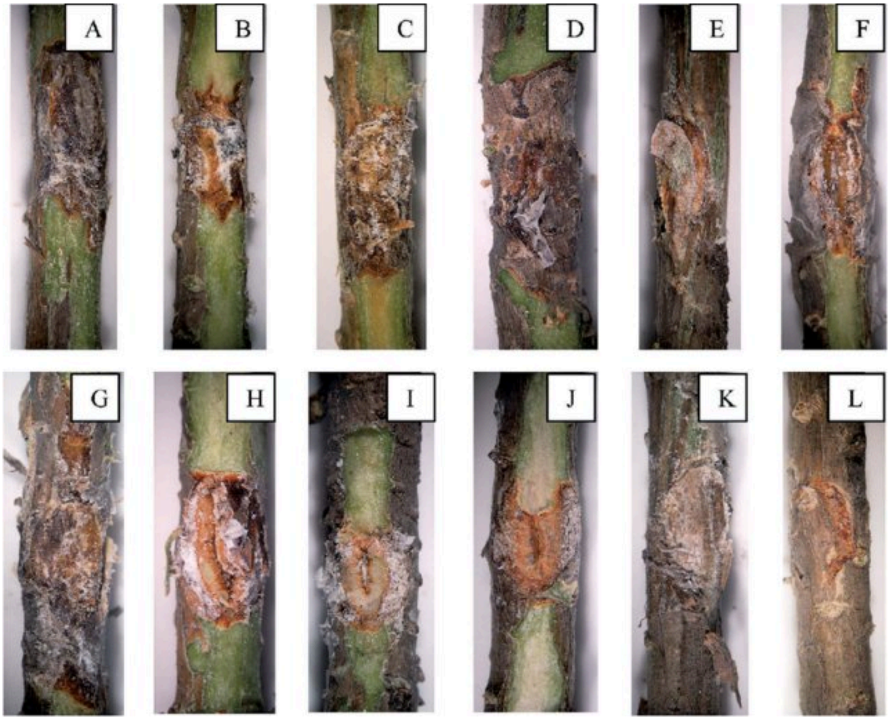

Figure 4. Lesions on the surface on stems of *Pinus sylvestris* saplings caused by (A) *Grosmannia radiaticola*, (B) *Leptographium lundbergii*, (C) *Leptographium procerum*, (D) *Leptographium truncatum*, (E) *Ophiostoma cf. abietinum*, (F) *Ophiostoma fl occosum*, (G) *Ophiostoma minus*, (H) *Ophiostoma piceae*, (I) *Ophiostoma piliferum*, (J) *Ophiostoma quercus*, (K) *Sporothrix inflata*, and (L) control (Jankowiak, 2013).

The risk to forest health by both bark beetles and associated fungi are commonly determined after the development of the beetle outbreaks and the infection of trees by the associated fungal species. The pathogenicity tests of fungal species include the isolation of fungi and the completion of Koch's postulates (Ploetz et al. 2013). Due to the common occurrence of other, non-aggressive bark beetle species, their roles as a vectors may be less clear, requiring both Koch's and Leach`s postulates to be used (Ploetz et al. 2013, Bezos et al. 2015). To establish the type of interaction between bark beetles and associated fungal species, and to elucidate the possible role of each bark beetle species in transmission of tree diseases, Leach` rules can be used (Bezos et al. 2015, Ploetz et al. 2013). Leach (1940) has developed four postulates to confirm that an insect is a vector of a pathogen that can cause the disease to plants as follow: 1) a close, although not constant, association between the insect and the disease hosts; (2) the presence of regular visits by bark beetles to healthy trees; 3) the presence of the pathogen on the insect in nature; and (4) whether insect can successfully transmit the pathogen to the host under the laboratory conditions.

2. Objectives

I. Investigate the composition of fungal communities associated with the pine bark beetles (*Ips acuminatus* and *Ips sexdentatus*) in Ukraine, and to investigate the effects of fungal colonisations in the phloem and sapwood of infested trees.

II. Understand pathological process and virulence of fungi vectored by *Ips sexdentatus* and *Ips acuminatus* to *Pinus sylvestris*.

III. Investigate composition of the fungal community associated with the red-haired bark beetle (*Hylurgus ligniperda*) in Ukraine, which represent the south-eastern timberline of *Pinus sylvestris* distribution in Europe.

IV. Identify seasonal flying patterns of the smaller elm bark beetle (*Scolytus multistriatus*) and to assess communities of vectored fungi, particularly focusing on Dutch elm disease pathogens in Gotland.

3. Material and methods

3.1 Project description

Insects and microorganisms are among the most evolutionary successful and diverse organisms, with a great variety of relationships and unique adaptation capabilities, but despite huge fundamental and practical significance, our understanding of the insect – microorganisms' interactions is still relatively limited.

The overall purpose of the project was to provide new fundamental and practical knowledge on indigenous and newly occurring (invasive) and massively tree-devastating bark beetles, vectored communities of fungi, and their pathogenicity to host trees.

The present work aimed to focus on economically and ecologically important bark beetles in the Baltic Sea area including Ukraine. The main research activities included: **Project 1** - Diversity and impact of mycobiota associated with the red-haired bark beetles (*Hylurgus ligniperda*) in stands of Scots pine (*Pinus sylvestris*), **Project 2** - Fungi associated with *Ips acuminatus* and *Ips sexdentatus*, and their succession in the phloem and sapwood of infested trees; **Project 3** - Phenology and communities of vectored fungi by the smaller elm bark beetle (*Scolytus multistriatus*), and **Project 4** - Pathogenicity of fungi associated with bark beetles *Ips sexdentatus* and *Ips acuminatus*.

3.1.1 Sampling of *Pinus sylvestris* bark beetles in Ukraine (Papers I, II and IV)

The study sites for papers **I**, **II** and **IV** were *P. sylvestris* forest stands located in the different regions of the forest-steppe zone of Ukraine. The sampling sites for papers **I** and **II** were situated at Kharkiv and Luhansk region, while for the paper **IV** at Sumy, Kharkiv, Donetsk and Kyiv regions (Table 1).

Table 1. General information on bark beetle sampling from *Pinus sylvestris* in Ukraine

Sampling site	**Latitude, Longitude**	**Beetle species**	**No. of beetles sampled**
Eastern Ukraine			
Luhansk	N 48°43', E 039°05	*Hylurgus ligniperda* *Ips acuminatus*	48 192
Kharkv	N 49°10', E 037°14'	*Hylurgus ligniperda* *Ips acuminatus*	48 192
Kharkv	N 49°48'40, E 36°21'24	*Ips sexdentatus*	96
Donetsk	N 48°57'12, E 37°54'58	*Ips sexdentatus*	96
Northern Ukraine			
Sumy 1	N 51°57'18, E 33°34'27	*Ips sexdentatus*	96
Sumy 2	N 51°23'02, E 33°23'33	*Ips sexdentatus*	96
Sumy 3	N 50°50'40, E 33°55'41	*Ips sexdentatus*	96
Central Ukraine			
Kyiv	N 50°55'40, E 30°02'27	*Ips sexdentatus*	96

All study sites in Ukraine were situated within a radius of ca. 300 km. Stands at all sites were ca. 50-70 year-old monoculture plantations of *P. sylvestris*.

Sampling of bark beetles was carried out from randomly selected, standing trees of *P. sylvestris*. Trees were living, but slightly or severely weakened by bark beetles and drought. The time of sampling coincided with the period when the dispersal flying period of young generation bark beetles was completed and their galleries have already been built under the bark. Samples of the bark with bark beetle galleries, phloem and sapwood were taken from the relevant parts of standing trees using sterilized forceps, individually placed in sterile Ependorff tubes and transported to the laboratory. Half of the beetles from each site were stored at 4°C for fungal culturing and the other half at -20°C for direct DNA sequencing of fungi.

3.1.2 Sampling of *Scolytus multistriatus* in Gotland (Paper III)

The study sites for *S. multistriatus* were located at Vallstena (N57°36′, E18°41′) and Hogrän (N57°31′, E18°18′) on the Baltic Sea island of Gotland (Sweden). The distance between the sites was ca. 26 km. The site at Vallstena was a mixed forest composed of *P. sylvestris*, *P. abies*, *Betula pendula*, *Ulmus* spp. and *Alnus* spp. Both sites were characteristic to Gotland in terms of landscape and trees species composition and were in the areas characterised by a high incidence of DED. Sampling of bark beetles was carried out using pheromone traps. At each site, 50 transparent delta traps with a sticky insert (Pherobank, Wijk bij Duurstede, The Netherlands) on the bottom and a P188 pheromone lure (Synergy Semiochemcials Corp., Burnaby, Canada) were placed every 50 m along a transect, which was 2.5 km long. Lures consisted of two semi-permeable plastic pouches containing a mixture of cubeb oil, 1-hexanol, multistriatin and 4-methyl-3- heptanol. The lure used attracts *Scolytus* spp. beetles. Sampling was carried out from the beginning of June until the end of August in the years 2012, 2013, and 2014. During the sampling period, traps were visited once a week and sticky inserts with trapped insects were collected and replaced with new inserts. Collected beetles of *S. multistriatus* were individually placed into 2-mL screw-cap centrifugation tubes and stored at −20 °C until further DNA processing.

3.2 Studies of fungal communities (Paper I, II, III and IV)

Our understanding of fungal communities associated with bark beetles is based on numerous observations of ophiostomatoid fungi and their sexual fruiting bodies (ascocarps). Many studies have characterized the ecological requirements and pathogenicity of ophiostomatoid fungi (Dori-Bachash et al. 2015, Kirisits 2004, Paine et al. 1997, Wingfield et al. 2017). Therefore, habitat preferences and associations with particular insects are relatively well known for many species, mostly associated with conifers. Thus, the role of vectored fungi vary widely and some of them are more sensitive to habitat than generalist fungal species that is known to be associated with different types of insects and can utilize both conifers and hardwood trees (Raffa et al. 2020, Six 2012). While the study of the fungal community should provide high-quality data on the species richness, many of the fungi that are associated with bark beetles remain undetected. Therefore, both classical methods of fungal isolation and direct sequencing were used in presented studies.

3.2.1 Isolation and morphological grouping of fungi from *Pinus sylvestris* bark beetles and sapwood (Paper I, II and IV)

The half of bark beetles of each species and from each site were used for fungal isolation into pure culture on agar media. Isolation from bark beetles and wood samples with beetle galleries were used to isolate primarily ophiostomatoid fungi for further pathogenicity tests (Persson et al. 2009). Fungal cultures were divided into groups based on their morphology, and for species identification representative cultures from each group were subjected to sequencing of the internal transcribed spacer regions of the fungal ribosomal RNA (ITS rRNA). Isolation of DNA, amplification and sequencing followed methods described previously (Menkis et al. 2006). Amplification by PCR was done using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al., 1990). In addition, β-tubulin gene and the partial elongation factor 1-alpha (EF1-a) gene were amplified and sequenced for fungi from genera *Ophiostoma*, *Graphilbum* and *Grosmannia*. The β-tubulin gene was amplified using the primers Bt2a and Bt2b (Glass and Donaldson 1995) and EF1-a gene was amplified using the primers EF1F and EF2R following the published protocols (Jacobs et al. 2004). Sequences were quality-filtered and assembled into different contigs (at ≥98% similarity representing different fungal species), which were used for analysis. Databases at GenBank were used to determine the identity of ITS rRNA sequences. The criteria used for identification were: sequence coverage > 80%; similarity to species level 98-100%, similarity to genus level 94-97%.

3.2.2 Direct sequencing from bark beetles (Paper I, II, III and IV)

While the conditions vary significantly compared phloem and nutrient media, the cultural method is selective for fast-growing fungi and is biased towards those fungal species that are slow growers on artificial media. The use of molecular methods circumvents this problem and fills a gap by extracting DNA directly from bark beetles and then amplifying it using nested PCR reactions (Persson et al. 2009). The fungal specific primers NLC2 (GAGCTGATTCCCAAACAACTC) and NSA3 (AAACTCTGTCGTGCTGGGGATA) were applied first (Persson et al.

2009), then, in a second (nested) PCR, ribosomal internal transcribed spacer (ITS) region has been targeted for the identification of a fungal community (White et al. 1990). After Sanger sequencing, taxonomic identification of derived sequences was carried out by comparing with known sequences in databases such as GenBank.

Paper III focused on fungal community vectored by *S. multistriatus* utilizing high-throughput Ion Torrent sequencing, which generated far greater sequence data as compared with earlier methods. The system of high-throughput sequencing is built on the ability to label each sample with specific sequence tags, ligating adapters to the DNA fragments and attaching them to a specific bead (Persson et al. 2011). The marked DNA amplified by PCR and the DNA sequencing read by the Ion Torrent sequencing technology. Construction of the sequencing library and sequencing using a 316 chip was carried out by NGI SciLifeLab (Uppsala, Sweden). The sequences generated were subjected to quality control and clustering in the SCATA NGS sequencing pipeline (http://scata.mykopat.slu.se). Quality filtering of the sequences included the removal of short sequences (<200 bp), sequences with low read quality, primer dimers, and homopolymers. Sequences that were missing a tag or primer were excluded. The primer and sample tags were then removed from the sequence, but information on the sequence association with the sample was stored as metadata. The sequences were then clustered into different taxa using single-linkage clustering based on 98.5 % similarity. The most common genotype for clusters was used to represent each taxon. For clusters containing two sequences, a consensus sequence was produced. The fungal taxa were taxonomically identified using GenBank (NCBI) database and the Blastn algorithm.

3.3 Pathogenicity and vector tests (Paper II and IV)

3.3.1 Pathogenicity tests of ophiostomatoid fungi

As mentioned above, ophiostomatoid species can be pathogenic to their host trees. Therefore, to confirm pathogenicity and define its extent, the reappearance of the disease symptoms after artificial inoculation by tested species is defined. This reappearance of symptoms, used to define the infection processes similar to those in the nature, is the criterion for the determination of virulence of the pathogen (Wingfield et al. 1993, Solheim et al. 1993, Krokene and Solheim 1998a).

For this, ophiostomatoid fungi were inoculated into phloem by making wounds similar to bark beetle damage. This fungal inoculation may result in the formation of necrotic lesions in the phloem and colonization of sapwood, and in some cases, in the tree mortality (Jankowiak 2013). Thus, the virulence of the fungi was evaluated by measuring the size of lesions and by monitoring the mortality of host trees (Jankowiak 2013, Krokene and Solheim 1998a, Repe et al. 2015). The pathogenicity of ophiostomatoid fungi is commonly assessed by mass fungal inoculation in host saplings or even mature trees by making a wound and by placing mycelium or spore suspension into the wound and by covering it with a Parafilm to avoid contamination and desiccation (Krokene and Solheim 1998a, Krokene and Solheim 1998b). The inoculated fungus enters into the phloem and the sapwood forming necrosis that confirms the capability of the fungus to colonize and cause the disease to host trees.

In Paper **II** and **IV**, the degree of pathogenicity was estimated by measuring the length of necrotic lesion 2-6 months after inoculation, as well as by measuring the blue-stained surface area and desiccated sapwood. In addition, there was evaluated the development of visual decline symptoms (e.g. wilt, branch dieback, blue-grey discolouration of sapwood, resin flow for conifers) and the death of the host trees. Necrotic lesions were commonly darker and slightly depressed, or there was an uplifted necrotic phloem tissue surrounding the point of inoculation. Sapwood became blue- or black stained that was caused by deeply penetrating ophiostomatoid fungi (Seifert 1993). The reaction of host trees to blue-stain inoculation was manifested in the desiccation of sapwood followed by the release of terpenes (Jankowiak 2012, Paine et al. 1997).

To study the impact of the fungi associated with bark beetles on *P. sylvestris* trees, inoculation tests were conducted on 3-4-year-old saplings. Eleven species of ophiostomatoid fungi associated with *I. sexdentatus* and *I. acuminatus* were used for the inoculation, namely *Ceratocystis ips, Graphium sp., Grosmannia olivacea, G. penicillata, Leptographium* sp.*, Ophiostoma bicolor* and *O. canum, O.minus, O. pallidulum O. rectangulosporium* and *Pesotum piceae.* Inoculation was carried out with selected isolates by removing out a bark flap (10 × 15 mm, approximately) and placing inoculums (plugs with fungal mycelia growing on MEA) on the sapwood surface and covering it up with the bark and Parafilm® M, as previously described by Krokene and Solheim (1998b). In addition, control plants were inoculated with sterile MEA as negative controls. The decline or mortality of saplings was observed at weekly intervals for 4-6 months and afterwards all plants were harvested, and the size of the necrotic lesion on the sapwood was measured.

3.3.2 Vector test for ophiostomatoid fungi (Paper IV)

Leach's postulates (Ploetz et al. 2013, Bezos et al. 2015) were used to test if *I. sexdentatus* is a vector for fungal species infecting *P. sylvestris* trees. The association between *I. sexdentatus* and healthy and/or declining trees (postulate #1 and 2) were confirmed by surveying *P. sylvestris* forests to find *I. sexdentatus* on healthy trees and trees infested by pathogens. The presence of the pathogen on the insect was reaffirmed during identification of fungi from bark beetles (postulate #3). Adults of *I. sexdentatus* were collected individually from *P. sylvestris* at all sites to check for the presence of associated fungal pathogens. For the laboratory vector experiments (postulate #4), living bark beetles (adults) were collected in the forest and placed in plastic tubes individually. Two cultures (*O. minus* and *Graphium* sp.) were selected for testing. To confirm that the bark beetle is a vector for the pathogen, fresh and visually healthy logs under controlled conditions were subjected to attacks by *I. sexdentatus*. For this, twenty adults of *I. sexdentatus* were inoculated with a conidial suspension of *O. minus* and *Graphium* sp. Each beetle was inoculated with ca. 50 µl of the suspension obtained from a pure culture of *O. minus* or *Graphium* sp. growing on MEA. Thirty *P. sylvestris* logs 18-22 cm in diameter (ten per each isolate and ten for controls) were treated with paraffin at the ends to avoid the desiccation. *Pinus sylvestris* logs with inoculated specimens of *I. sexdentatus* were placed individually into plastic containers for 30 days to allow the colonization. Controls were established in the same way only using non-inoculated specimens of *I. sexdentatus*. At the end of the experiment, all logs were visually checked for the presence of blue-stain symptoms. Moreover, three pieces of wood tissue ca. 1 cm in length from each logs with visible entry holes and maturation feeding of *I. sexdentatus* were removed and plated on MEA containing antibiotics in order to re-isolate *O. minus* and *Graphium* sp. using classical phytopathological methods (Bezos et al. 2015).

3.4 Statistical analyses

Principal statistical analyses were carried out using the statistical software package PAST: Paleontological Statistics Software Package for Education and Data Analysis (Hammer et al. 2001). All obtained data were tested for adherence to normal distribution using the Kolmogorov–Smirnov test. Shannon and Simpson diversity indices as well as pairwise beta diversity index were used to characterise the diversity and composition of fungal communities. The Simpson diversity index was used to estimate the dominance in fungal diversity while taking into account both richness and evenness. Shannon diversity index and quantitative Sørensen similarity indices were used to characterise fungal communities present at different study sites (Brown and Williams 2016, Daly et al. 2018). The data from the inoculation and vector tests were analysed using analysis of variance (ANOVA). Significant differences between treatments were further evaluated by Fisher's exact test followed by Tukey`s HSD post hoc test. The significance was evaluated at the $p=0.05$ level.

4. Result and discussion

4.1 Fungal community associated with *Pinus sylvestris* bark beetles in Ukraine (Paper I, II and IV)

Ophiostomatoid and other fungi associated with bark beetles, that are infesting *Pinus sylvestris* in Ukraine, were investigated in **I**, **II** and **IV**. Among the countries neighboring Ukraine, the most comprehensive and detailed investigations on fungi associated with bark beetles, that are infesting hardwoods and conifers, including living trees, cut or fallen trees and logs, was carried out in Poland (Jankowiak and Hilszczanski 2005, Jankowiak 2006, Jankowiak and Rossa 2008, Jankowiak and Kolarik 2010, Jankowiak 2012, Jankowiak and Bilanski 2013, Jankowiak et al. 2017, Jankowiak et al. 2019). These studies indicated numerous ophiostomatoid fungi associated with bark beetles in Poland. Among other countries neighboring Ukraine, only one recent study was published by Slovakian researchers, focusing on ophiostomatoid fungi and *P. sylvestris* bark beetles (Pastircakova et al. 2018).

In the papers presented in this thesis, the use of both fungal culturing and sequencing methods allowed the discovery of a species-rich fungal communities associated with *P. sylvestris* bark beetles in Ukraine. These fungi encompassed different groups of the fungal kingdom and represented several different ecological roles. The overall fungal community (**I, II** and **IV** together) was composed of 76.3% Ascomycota, 10% Basidiomycota and 5% Mucoromycotina and 8.7% species remained unidentified. The most commonly detected groups of fungi were pathogens, ophiostomatoid fungi and saprotrophic fungi, while the frequency of fungal groups with other ecological roles varied between 0.63 and 7.02 % (Figure 5).

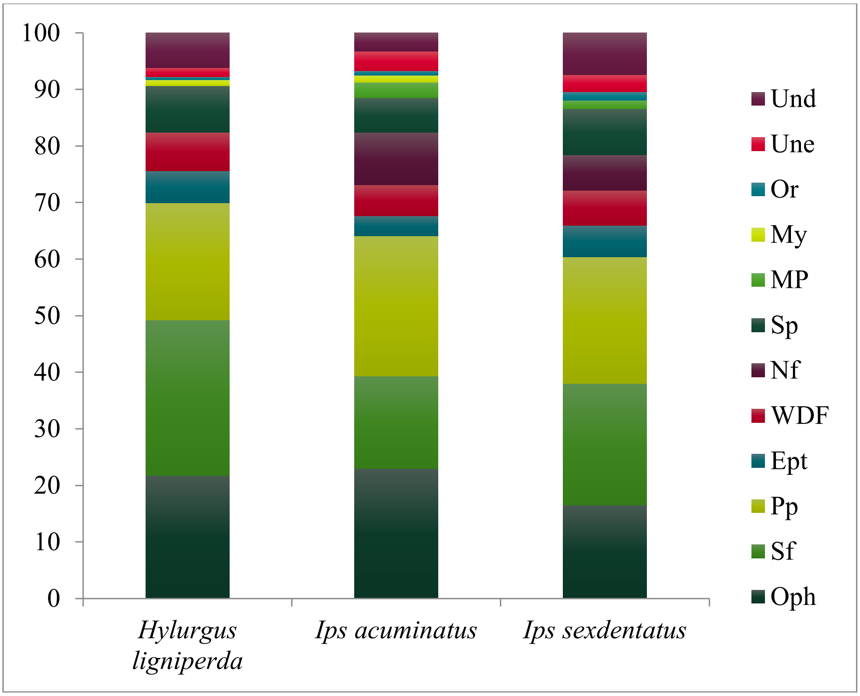

Figure 5. Composition of fungal groups with different ecological roles associated with different *Pinus sylvestris* bark beetles, Oph = ophiostomatoid fungi, Ept = entomopathogenic fungi; Wd = wood decay fungi; Sf = saprophytic fungi; Pp = Plant pathogens, Sp = fungi combined saprophytic and pathogenic strategy; Mp = mycoparasites; My = mycorrhizal fungi; Or = other ecological roles; Une = no data about ecology available, Und = unidentified fungi.

In particular, Ascomycota fungi were commonly associated with all investigated beetle species and at all sampling sites. Among ophiostomatoid fungi, only *Ceratocystis ips* (syn. *Ophiostoma ips*) and *P. picea* (syn. *O. picea*) were detected in association with all beetle species and sites, while *G. penicillata* and *Leptographium* sp. were associated exclusively with *I. sexdentatus* and *O. pallidulum* with *I. acuminatus* (Figure 6). Moreover, *G. penicillata, G. olivacea, Graphium sp., O. canum, O. minus, O. pallidulum, Graphilbum rectangulosporium* and *Leptographium* sp. were found for the first time in Ukraine. Other ophiostomatoid fungi i.e. *C. ips* and *P. picea*, were mentioned to be associated with *P. sylvestris* logs in national database (Cybertruffle's Robigalia, Observations of Fungi and their Associated Organisms: www.cybertruffle.org.uk/robigalia/ukr).

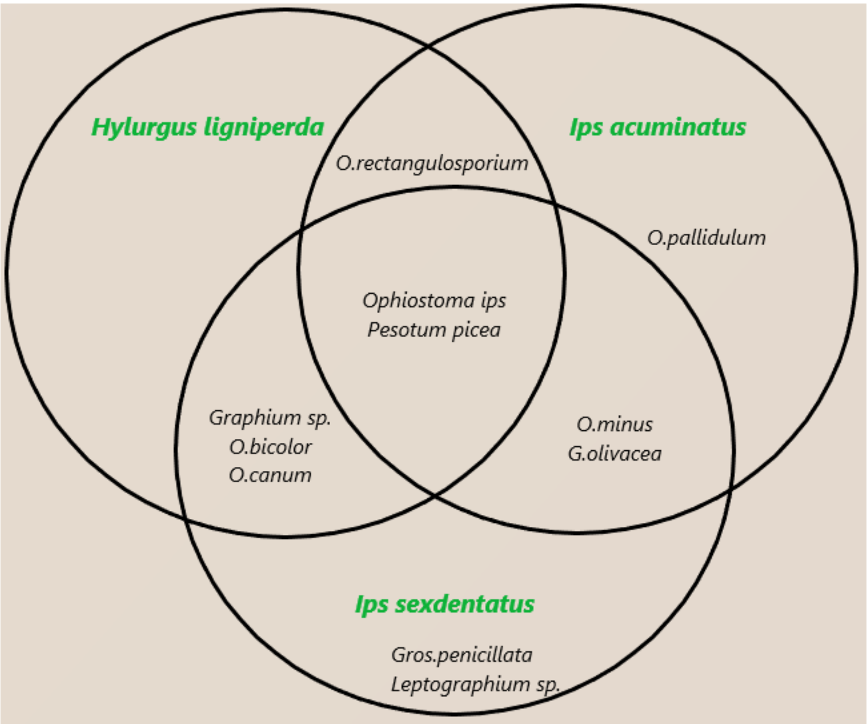

Figure 6. Venn diagram showing the association of ophiostomatoid fungi with three *Pinus sylvestris* bark beetles, i.e. *Hylurgus ligniperda, Ips acuminatus* and *Ips sexdentatus*.

Among the basidiomycetes, species belonged to *Polyporales*, i.e. species within *Tremellales, Corticiales, Agaricales* and *Russulales*, were also detected. Within Basidiomycota, wood-decay fungi (*Bjerkandera adusta, Fomitopsis pinicola, Heterobasidion annosum* and *Phlebiopsis gigantea*), mycorrhizal fungi (*Hebeloma* sp.), plant pathogenic species (*Cryptococcus* sp.) and a nutritional fungus *Entomocorticium* sp. were detected.

Heterobasidion annosum s.l., which can cause root and stem rot in conifers, has also previously been isolated from *H. ligniperda* (**I**), but not from *I. acuminatus* (**II**). This can be explained by the difference of localization of bark beetles (*H. ligniperda* and *I. acuminatus*) in the stem. *Hylurgus ligniperda* colonises the lower part of the stems and mycelia of root rot fungi can grow into this wood, and subsequently appear on the body surface or in the stomach of these bark beetles.

Entomocorticium sp. was the most common fungus associated with *I. acuminatus* (24.5%) **(II)** and *I. sexdentatus* (12.3%) (**IV**), while no nutritional fungi were found in association with *H. ligniperda* (**I**). In both cases, *Entomocorticium* sp., was detected by direct sequencing from *I. acuminatus* and *I. sexdentatus*. It is a mycangial fungus, which was previously reported in association with *Dendroctonus ponderosa* Hopkins, (Coleoptera: Curculionidae) and *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae) (Klepzig et al. 2001, 2004, Six and Klepzig 2004). *Entomocorticium* sp. may provide nutritional benefits to larvae of bark beetles. By contrast, *O. minus* results in a poorly developed larvae, which often fail to reach the adult stage (Klepzig et al. 2001, 2004, Six and Klepzig 2004). *Entomocorticium* sp. may provide some protection for larvae against the negative impact of *O. minus* (Kirisits 2004). *Ophiostoma minus* was found to be a highly virulent fungus that threatens normal development of larvae because it can stop the increase of nutrient concentrations of the phloem (Bracewell and Six 2015). By contrast, *Ambrosiella macrospora* (Franke-Grosm.) is primarily known as a food source for larvae and is thought to be non-pathogenic, and was found to be commonly associated with *I. acuminatus* in Italy (Villari et al. 2012), but was not detected in association with *I. acuminatus* from Ukraine. The latter examples demonstrate that *I. acuminatus* and *I. sexdentatus* may vector rather different fungal communities in different parts of Europe, including unique nutritional fungi that can serve as an additional source of nutrients for both adults and larvae (Jankowiak 2012, Bueno et al. 2010, Villari et al. 2012, Guérard et al. 2000).

Diplodia sapinea was found to be associated with *I. acuminatus* at a high frequency (24.0%). However, *D. sapinea* was rarely isolated from *I. sexdentatus* and never from *H. ligniperda*, which can be attributed to the absence of *H. ligniperda* and *I. sexdentatus* during maturation feeding in the crown. *Diplodia sapinea* is a widely distributed pathogen of conifers causing tip blight and stem canker disease (Luchi et al. 2014). Previously, associations between *D. sapinea* and *Tomicus piniperda* L. (Coleoptera: Curculionidae), *Hylastes attenuatus* Erichson, (Coleoptera: Curculionidae), *Hylurgops palliates* (Gyll.) (Coleoptera: Curculionidae), and *Xyleborus dispar* (F) (Coleoptera: Curculionidae) beetles have been reported from northern Spain (Goldazarena et al., 2012). A possible interaction between an exotic insect *Leptoglossus occidentalis* Heidemann (Hemiptera:

Coreidae) and *D. sapinea* was postulated as both of these species cause damage to pine cones in Italy (Luchi et al. 2012). Moreover, it was demonstrated that *D. sapinea* was effectively vectored by *Hylastes ater*, *Hylastes opacus* Erichson (Coleoptera: Curculionidae) and *H. ligniperda* in Poland (Jankowiak 2013). Study **II**, therefore, for the first time provided the evidence that *D. sapinea* is commonly associated with *I. acuminatus* in Ukraine.

Generally, a comparison among different bark beetle species showed that diversity indices were higher for *I. sexdentatus* and *I. acuminatus* than for *H. ligniperda.* A few fungal species that were not identified, occurred at low frequencies, therefore making them less important in terms of the spread by bark beetles. While fungal community composition differed among different bark beetles, these fungal communities were found to be similar in similar habitats colonized by bark beetles (Figure 7). However, a core community of fungal species, e.g. ophiostomatoid group, entomopathogenic fungi and Mucoromycotina species seemed to be present in associated with all bark beetle species (Figure 7). This core community includes the nine most abundant species from the three pine bark beetle species and included *Ceratocystis ips, Pesotum picea, Ophiostoma pinus, Fomitopsis pinicola, Sydowia polyspora, Umbelopsis ramanniana, Alternaria* sp. and *Beauveria bassiana.*

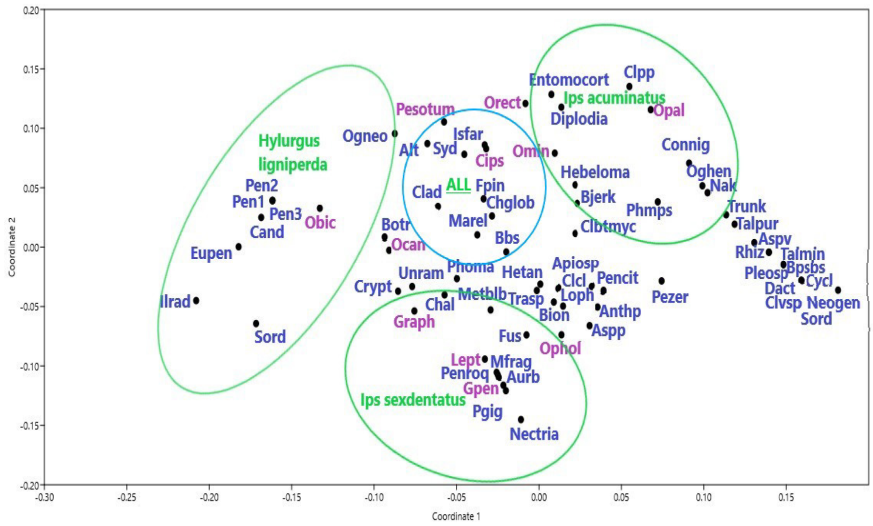

Figure 7. Results of non-metric multidimensional scaling (NMDS) of fungal community compositions for the three bark beetle species (points are labelled by the ranked distance (dissimilarity) in the original space., stress value = 0.2057, p=0.0129). Abbreviations: Alt - *Alternaria* sp., Anthp - *Anthostomella pinea*, Apiosp - *Apiospora montagnei*, Aspv - *Aspergillus versicolor*, Aspp - *Aspergillus pseudoglaucus*, Aurb - *Aureobasidium pullulans*, Bbs - *Beauveria bassiana*, Bpsbs - *Beauveria pseudobassiana*, Bjerk - *Bjerkandera adusta*, Bion – *Bionectria* sp., Botr - *Botryotinia fuckeliana*, Cand - *Candida* sp., Chglob - *Chaetomium globosum*, Chal – *Chalara* sp., Clbtd - *Cladobotryum dendroides*, Clbtmyc - *Cladobotryum mycophilum*, Clcl - *Cladosporium cladosporioides*, Clpp - *Cladosporium pini-ponderosae*, Clad - *Cladosporium* sp., Clvsp - *Clavispora lusitaniae*, Connig - *Coniella nigra*, Cycl - *Cyclaneusma niveum*, Crypt - *Filobasidium magnum,* Dact - *Dactylonectria macrodidyma*, Diplodia – *Diplodia sapinea*, Entomocort – *Entomocorticium* sp., Fpin – *Fomitopsis pinicola,* Fus - *Fusarium avenaceum*, Eupen - *Eupenicillium* sp., Graph - *Graphium* sp., Grpen - *Grosmannia penicillata*, Hebeloma – *Hebeloma* sp., Hetan – *Heterobasidion annosum* s.l., Ilrad - *Ilyonectria radicicola*, Isfar - *Isaria farinose*, Lept - *Leptographium* sp, Loph - *Lophodermium seditiosum*, Marel - *Mariannaea elegans*, Metblb - *Metapochonia bulbilosa*, Mfrag – *Mucor fragilis* Nak - *Nakazawaea holstii*, Nectria - *Nectria* sp, Neogerm - *Neocatenulostroma germanicum*, Oghen - *Ogataea henricii*, Ogneo - *Ogataea neopini*, Obic - *Ophiostoma bicolor,* Ocan - *Ophiostoma canum*, Cips - *Ceratocystis ips*, Omin - *Ophiostoma minus*, Ophol - *Grosmannia olivacea,* Opal - *Ophiostoma pallidulum,* Orect - *Ophiostoma*

rectangulosporium Pencit - *Penicillium citreonigrum*, Penroq - *Penicillium roqueforti*, Pen1 - *Penicillium* sp. HK36 7, Pen2 - *Penicillium* sp. HK80 14, Pen3 - *Penicillium* sp. HK83 22, Pesotum - *Pesotum piceae,* Pezer - *Pezicula eucrita*, Pgig - *Phlebiopsis gigantea*, Phoma - *Phoma macrostoma*/*Didymella macrostoma*, Phmps - *Phomopsis* sp., Pleosp - *Pleosporales* sp., Rhiz - *Rhizoctonia* sp., Sord - *Sordariomycetes* sp., Syd - *Sydowia polyspora*, Talmin - *Talaromyces minioluteus*, Talpur - *Talaromyces purpureogenus*, Trasp - *Trichoderma asperellum*, Trunc - *Truncatella* sp., Umram - *Umbelopsis ramanniana*.

Fungal communities associated with *I. acuminatus* and *I. sexdentatus* were also found to differ significantly between different sampling sites, while fungal communities associated with *H. ligniperda* were similar to each other at different sampling sites (data not shown) (**I**, **II**). The latter demonstrates that for certain bark beetle species, the habitat could have a profound effect on the composition and diversity of associated fungal species. For example, the diversity of fungal species associated with different bark beetles was found to be higher in wet habitats (northern Ukraine) as compared to lower humidity habitats (data not shown).

4.1.1 Specificity of fungi associated with *Hylurgus ligniperda*

The study on fungal communities associated with *H. ligniperda* was carried out in the forest-steppe zone in eastern Ukraine (Table 1) and resulted in 40 fungal species among which *Pesotum piceae* (10.3%), *Alternaria alternata* (9.7%), *Ogataea neopini* (8.0%), *Botryotinia fuckeliana* (5.1%) were the most common. Ophiostomatoid fungi were among the most abundant and included five different taxa i.e. *P. piceae, O. bicolor, C. ips, O. canum* and *Graphilbum rectangulosporium* (Figure 6), which all are known to be little- or non-pathogenic to the trees. Insect pathogens *Isaria farinose* and *Beauveria bassiana* were also detected. Therefore, *H. ligniperda* vectors diverse communities of fungi majority of which appear to be little or non-pathogenic to the *P. sylvestris* trees.

4.1.2 Specificity of fungi associated with *Ips acuminatus*

Identification of the fungal community associated with *I. acuminatus* revealed the presence of 60 fungal species. Among these, the most common were *Entomocorticium* sp. (24.5%), *D. sapinea* (24.0%), *C. ips* (16.7%), *S. polysporsa* (15.1%), *Graphilbum rectangulosporium* (15.1%), *O. minus* (13.8%). It may be concluded that *I. acuminatus* vectors a species-rich fungal community including needle and shoot pathogens such as *D. sapinea A. alternata, B. fuckeliana (Botrytis cinerea), Cyclaneusma niveum, Dactylonectria macrodidyma, Fusarium avenaceum, Lophodermium seditiosum, Nectria* sp., *Neocatenulostroma germanicum.* This can suggest that these fungi may negatively affect the health of forest trees following attacks and maturation feeding by *I. acuminatus*. The fungal community was found to be different from these in other regions of Europe.

4.1.3 Specificity of fungi associated with *Ips sexdentatus*

The study on fungi vectored by *I. sexdentatus* showed the presence of 56 fungal species among which the most common were *Entomocorticium* sp. (12.33%), *Cladosporium* sp. (11.1%) and *Ceratocystis ips*. Moreover, nine ophiostomatoid species were identified in association with these bark beetles and seven were isolated from their galleries. The similarity index between fungal communities at different sites was low indicating significant differences between sites, while Shannon-Weiner and Simpson diversity indices reflected a high uncertainty of individual fungal species and high diversity for all sites.

4.2 Fungal community associated with *Scolytus multistriatus* (Paper III)

The fungal community vectored by *S. multistriatus* was comprised by 67.6% *Ascomycota*, 31.0% *Basidiomycota*, 0.7% *Mortierellomycotina*, 0.4% *Chytridiomycota*, 0.2% *Glomeromycota*, and 0.1% *Mucoromycotina*. The most common taxa were *Cladosporium* sp. 2170_0 (37.9%), *O. novo-ulmi* (9.0%), *Aureobasidium pullulans* (7.5 %), *Dioszegia fristingensis* (4.9 %), and *Cryptococcus wieringae* (3.9 %). Both DED pathogens, less virulent *O. ulmi* and highly virulent *O. novo-ulmi*, were detected by ITS rDNA sequencing of *S. multistriatus* beetles. However, *O. ulmi* was detected in a single (0.6 %) beetle, while *O. novo-ulmi* was detected in 79 (44.6 %) beetles (difference significantly at $p<0.0001$). *O. novo-ulmi* was detected during the entire sampling period.

The occasional occurrence of *O. ulmi* suggests that, as elsewhere, it is being replaced by *O. novo-ulmi*. The data showed that association between *S. multistriatus* and *O. novo-ulmi* is very dynamic and may change over time. It appears that the number of the beetles infested by *O. novo-ulmi* is significantly dependent on the efficiency of the control measures implemented. The study also revealed the flying intensity and seasonality of *S. multistriatus* (Figure 8), which provided important practical information required for efficient control of DED. Furthermore, it was also demonstrated that the continuity of the control measures is of paramount importance as the population of *S. multistriatus* vectoring *O. novo-ulmi* can recover in a single flying season.

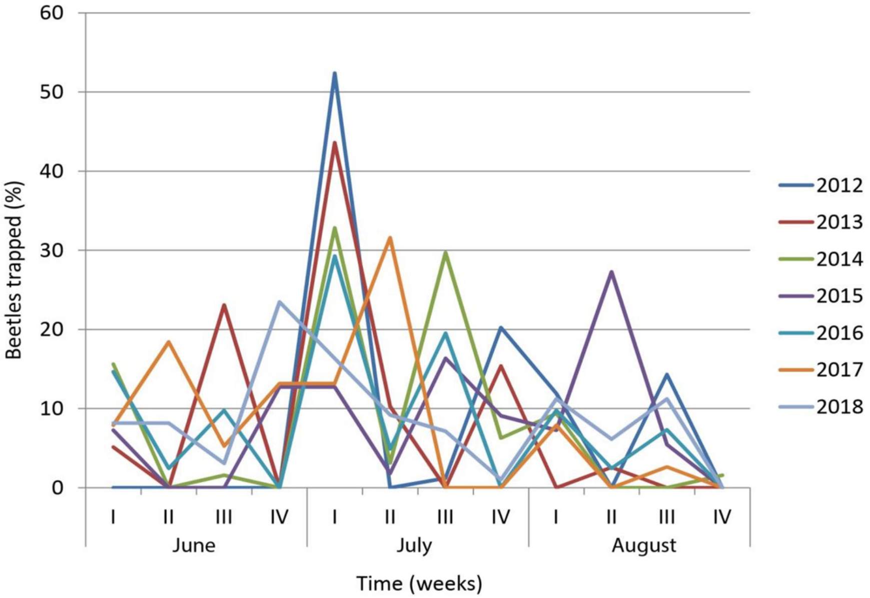

Figure 8. Flying patterns of *Scolytus multistriatus* shown as a relative abundance (data pooled) during June-August of 2012-2018 on the island of Gotland.

The data provided the evidence that *Ascomycota* fungi, especially ophiostomatoid fungi, are predominantly associated with *S. multistriatus*. In addition to ophiostomatoid fungi, *Geosmithia* spp. were also detected, which are well-known to establish stable symbioses with different bark beetle species. Among other fungi, yeasts from the genera *Dioszegia, Cryptococcus, Udeniomyces, Candida*, and *Sporobolomyces* were also common.

4.3 Pathogenicity and vector tests (Paper II and IV)

The focus in paper **II** and **IV** was on ophiostomatoid species that were found in close associations with *P. sylvestris* bark beetles. Firstly, it was found that fungi can be present on the body surface of bark beetles or in their mycangia or stomachs, and thus, could be vectored by bark beetles. Secondly, tree attacks by bark beetles resulted in the establishment of ophiostomatoid fungi in sapwood. The early established of ophiostomatoid fungi in phloem can be determined by their competitive abilities, which can be considered as an important mechanism shaping fungal communities in the wood. Understanding the degree of virulence of these fungi can provide insights into potential effects on forest health as well as a deeper knowledge on the nature of bark beetle-fungi symbioses.

Several methods can be used to assess the virulence of the ophiostomatoid fungi associated with bark beetles. The most common method is to measure the size of lesions forming after the inoculation of these fungi into the wood. The assessing the health status of the inoculated saplings is another useful measurement, which was carried out in **II** and **IV**. The separate evaluation of both of these two parameters is an essential element in the pathogenicity test (Paine et al. 1997). Inoculation of *P. sylvestris* saplings with the eleven ophiostomatoid fungi resulted in the formation of necrotic lesions, resin flow from the stems, and needle and branch dieback. Consequently, pathogenicity test revealed that different fungal species possessed different degree of virulence at the same time showing the colonization patterns of the plant tissue (Table 2). *Leptographium* sp., *Graphium* sp. and *O. minus* caused the highest mortality of the inoculated saplings (75.0%, 58.3% and 45.0%, respectively) and symptoms of decline on the remaining saplings (Table 2), including wilting of shoots and discoloration of needles. One of the most interesting findings of the present study is the fact that a few ophiostomatoid isolates that exhibited the highest pathogenicity scores towards *P. sylvestris* were unidentified ophiostomatoid species, namely *Leptographium* sp. and *Graphium* sp. (Table 2). Both of those fungi can lead to lethal outcome of saplings (75.0% and 58.3%, respectively) and to a significant extent to sapling decline. *Grosmannia penicillata, G. olivacea* and *O. bicolor* caused the dieback on 33.3%, 16.7% and 8.3% of saplings, respectively, and the symptoms of decline on the remaining saplings. Inoculation with *G. rectangulosporium* resulted in decline of 10% of saplings (Table 2). *Ceratocystis ips, O. canum O. pallidulum* and *P. piceae* did not cause any decline or dieback of *P. sylvestris* saplings. No dieback or decline of the saplings was observed in the control. All fungi tested resulted in lesions of different sizes (Table 2) and these lesions were significantly higher than in control saplings (p<0.0001).

Table 2. Effect of inoculation of ophiostomatoid fungi on 3-4 year-old *Pinus sylvestris* saplings (values for blue-stain depth and lesion length are shown as a mean ± one standard error). Values followed by the same letter are not significantly different at p=0.05.

Species	**Length of the lesion, mm**	**Dead plants, %**	**Declining plants, %**
***Ceratocystis ips* (*Ophiostoma ips*)**	3.9 ± 0.4^{a}	0	0
Graphium sp.	14.7 ± 1.2^{b}	58.3	42.7
Grosmannia olivacea	6.5 ± 0.2^{c}	16.7	16.7
Grosmannia penicillata	9.6 ± 1.9^{d}	33.3	16.7
Grosmannia rectangulosporium	$11.0+0.2^{d}$	0	10.0
***Leptographium* sp.**	25.9 ± 1.2^{c}	75.0	25.0
Ophiostoma bicolor	10.2 ± 0.3^{d}	8.3	25.0
Ophiostoma canum	3.4 ± 0.1^{a}	0	0
Ophiostoma minus	19.6 ± 0.9^{e}	45.0	25.0
Ophiostoma pallidulum	$2.9+0.2^{a}$	0	0
***Pesotum piceae* (*Ophiostoma picea*)**	$3.2+0.1^{a}$	0	0
Control	0^{f}	0	0

Leptographium sp. and *O. minus* caused significantly larger necrotic lesions than other ophiostomatoid fungi (Table 2). Therefore, all inoculated fungi showed the capability to infect *P. sylvestris* tissues and caused lesions (Table 2). The lesions were generally covered with resin and extended vertically in both directions from the point of inoculation.

In summary, *O. minus, Leptographium* sp. and *Graphium* sp. were most virulent pathogens, while *G. olivacea, G. penicillata* and *O. bicolor* were moderately virulent pathogens. These results are in agreement with other studies, which found that *Grosmannia* and *Leptographium* species could have high virulence to conifers in Europe including Scandinavia, where they are well-known to exist in symbiosis with *I. typographus* (Linnakoski 2011, Solheim et al. 1993, Wingfield et al. 1993). The capability of these fungi to degrade phenolic compounds of the host trees could be one of the factors determining their high virulence (Zhao et al. 2019). *Grosmannia olivacea* appears to be a significantly less virulent as compared to *G. penicillata*, while inoculation of *P. sylvestris* saplings with *C. ips, O. canum O. pallidulum* and *P. piceae* resulted in relatively small lesions, indicating that these species were weak pathogens though the size of lesions were longer and deeper compared to control saplings. Finally, these results corroborate previous studies, suggesting that pathogenicity is not a specific ability and that large differences in virulence exist between closely related, but different species of fungi (Jankowiak and Kolarik 2010, Jankowiak 2013, Zhao et al. 2019). Moreover, the interesting fact of our study is that two ophiostomatoid fungi, *Leptographium* sp. and *Graphium* sp. (Table 2), are to date unidentified, although ophiostomatoid group is one of the most extensively investigated on the worldwide scale (Yamaoka 2017). For instance, closest ITS matches for Leptographium sp. in GenBank were found *Leptographium celere*, *L. pistaceae* and *L. procerum*, while for *Graphium* sp. – *Graphium pseudomiticum* and *G. fimbriasporum*. It's very probable that we have two previously unknown fungi associated with *I. sexdentatus*, and that these species are very likely to be alien and have the potential to pose serious threat to health of *P. sylvestris* trees in Europe.

Vector test (**IV**) was confirmed using Leach`s postulates (Leach 1940). These postulates were fulfilled as there was a close association between the *I. sexdentatus* and infested (postulate #1) and healthy (postulate #2) trees; the presence of the fungus on the bark beetle in nature (postulate #3); and that *I. sexdentatus* can successfully vector the fungus to fresh logs under the laboratory conditions (postulate #4). Visual signs of attacks and maturation feeding by adults of *I. sexdentatus* were found on 19 out of 20 logs, and on 9 out of 10 control logs exposed to bark beetles. In most cases, isolation of the fungi in pure culture led to the recovery of the same fungi (*Ophiostoma minus* and *Graphium* sp.) that had been used for inoculation of bark beetles. The control samples (using non-inoculated beetles) showed the presence of ophiostomatoid fungi, namely *O. canum, O. picea* and *O. ips*, indicating that bark beetles were already infected with these ophiostomatoid species at the time of sampling in nature.

5. Conclusions and Future Directions

The importance of fungal-bark beetle symbioses has been increasingly recognized as it is illustrated by the rapid increase in the number of scientific papers on this topic during the last decades. The studies presented in this thesis have, for the first time, characterised fungal communities associated with *P. sylvestris* bark beetles in Ukraine, thereby contributing to new knowledge and broadening our understanding on the specificity of insect and fungal symbioses. Furthermore, the study on fungi associated with *S. multistriatus* provided new valuable information on symbioses and ecology of this bark beetle species on Gotland. The study also revealed the flying periods and flying intensity of *S. multistriatus*, what in practical terms is very important for planning of management measures and the conservation of *Ulmus* spp. population on Gotland.

Studies presented in this thesis showed that *P. sylvestris* bark beetles in Ukraine vector a species-rich fungal community, which was dominated by tree pathogens including ophiostomatoid fungi. The community composition of ophiostomatoid fungi appeared to be rather different for each species of bark beetles and several of these fungi were found for the first time in Ukraine. Moreover, ophiostomatoid fungi showed different degree of virulence to *P. sylvestris* saplings. *Leptographium* sp., *Graphium* sp. and *O. minus* caused the highest rate of mortality and decline of the inoculated saplings. These pathogenic fungi are likely to play an important role in overcoming the tree defence and facilitating the establishment of *I. sexdentatus* and *I. acuminatus* after the attack of *P. sylvestris* trees. Assessing the virulence for the rest of the tested fungi showed the presence of both moderately and weakly pathogenic species, which did not significantly increase the mortality of the saplings. The association between fungal pathogen *D. sapinea* and *I. acuminatus* can be of considerable practical importance for forest health and requires further attention.

Although different fungi associated with bark beetles may have various dispersal and life strategies, basidiomycetes appear to be randomly associated with different bark beetle. However, among the known associations of basidiomycetes and bark beetles, only the corticioid fungus *Entomocorticium* sp. seems to be beneficial as its mycelium or conidia may serve as a food source for larvae. However, further work is needed to clarify the relationship between *Entomocorticium* sp. and *I. sexdentatus* and *I. acuminatus* i.e. to identify how *Entomocorticium* sp. has adapted to the lifestyle of bark beetles.

The obtained data suggests that fungi associated with bark beetles encompass a high diversity of different functional groups, including mutualistic, antagonistic and neutral associations. The community of fungi associated with bark beetles from different regions was found to differ significantly, indicating that environmental conditions play an important role shaping these fungal communities and that bark beetles can acquire new microbes, many of which could be specific and/or new for each region. For the detected pathogenic fungi, which can provoke tree diseases, improved detection, prediction, and forest management plans are needed to maintain productive and sustainable forestry and nature conservation. These tasks should be among major goals of future research, where all aspects of bark beetle - fungal symbiosis should be considered. Although bark beetles can use their symbionts to facilitate the establishment in attacked trees, bark beetles may utilize various fungal compositions and/or in different ways, which require further attention. The pathogenicity tests provided important tools for recognition of the multitude and the nature of fungal roles. Therefore, further investigations and especially with new organisms, e.g. yeast symbionts, or mites associated with fungi and bark beetles, could be a new research direction in the subject area.

Finally, it is important to remain cautious interpreting research findings, as a lot of fungal species may be randomly associated with bark beetles. On the other hand, many ophiostomatoid fungi appears to have co-evolved with their bark beetles, forming a close mutualistic relationship and benefiting each other.

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Popular science summary

The studies presented in this thesis were carried out in response to the alarmingly increasing the number of outbreaks of bark beetles and associated fungi in European forests. With these studies it was aimed to generate new knowledge on tree-devastating bark beetles and understand the possible role of vectored fungi. European forests provide essential ecosystem services and a great economic value for centuries. Unfortunately, these forests are subjected to difference disturbances, the most important being windstorm, bark beetles, fire and drought. The obtained data can contribute to the increased knowledge and allow adequately respond to an expected intensification of bark beetle disturbances, as well as may allow to better understand tree diseases and how to manage them.

Over the past decades, the bark beetle spread was almost unchecked, leading to destroying forest ecosystems and high economic losses to the timber industry worldwide. Some bark beetle species can easily damage living trees, but another great problem arises from fungi that bark beetles carry on their surface. Molecular analysis revealed that a few such beetle's partners "in crime" may help the insect to overcome the tree defence, providing additional pressure on the tree resistance system.

These fungi, so-called blue stain fungi, are closely associated with bark beetles and use the insect to reach their habitats on the body of an insect, which is usually a bark beetle. Bark beetles usually prefer to attack stressed or dying trees to avoid a tree defence system. Although a vectored fungus and a bark beetle may be more successful in a weakened tree, some fungi may help bark beetles to overwhelm the tree defences, and in this way, to colonize even healthy trees. Besides, different fungal species associated with bark beetle can kill living trees. For example, *Ophiostoma novo-ulmi* can cause massive mortality of elm trees and vector by the elm bark beetle. Moreover, *Leptographium* sp, *Graphium* sp. and *O. minus* can cause mortality of pine trees and could be carried out by pine bark beetles.

Bark beetles can also transfer many other aggressive pathogens that can cause crown and root decline. Therefore, highly virulent and aggressive fungi can strengthen the negative impact of bark beetles on forest and contribute to the ability of these bark beetles to overcome the tree resistance. The work presented in this thesis demonstrated the importance of insect-fungi symbioses as important biotic factor that may have a strong negative effect on forest health and sustainability.

Populärvetenskaplig sammanfattning

Arbetet som presenterades i den här avhandlingen genomfördes på grund av det allt mer oroande antalet utbrott av barkborrar och barkborre-associerade skadesvampar i Europeiska skogar. Våra Europeiska skogar har levererat ekosystemtjänster och skapat ett stora ekonomiska värden i århundraden. Tyvärr utsätts våra skogar också för olika störningar, det viktigaste är vind/storm, barkborrar, eld och torka som påverkar både skogarnas ekosystemtjänster och ekonomiska värde negativt.

Syftet med arbetet var att skapa ny kunskap om barkborrar och deras associerade skadesvampar. Resultaten från min forskning bidrar till en ökad förståelse för olika barkborrar och deras associerade skadesvampar. De underlättar också, förhoppningsvis, en bättre hantering av framtida attacker och utbrott av sjukdomar på angripna träd. Angrepp som blir allt vanligare. Under de senaste decennierna har barkborrespridningen varit nästan okontrollerad, vilket lett till att skogsekosystem förstörts och stora ekonomiska förluster för träindustrin över hela världen. Vissa barkborrearter kan på egen hand orsaka omfattande skador levande träd, men omfattande problem orsakas av skadesvampar som barkborrarna bär på ytan av sin kropp. Molekylär analys visade att vissa sådana ”medbrottslingar” kan hjälpa insekten att övervinna trädens försvarssystem. Dessa svampar, så kallade blånadssvampar, är nära förknippade med barkbaggar och ”liftar” på insektens kropp till sina livsmiljöer inuti trädet. Barkborrar föredrar vanligtvis att attackera redan stressade eller döende träd för att sådana träds försvarssystem är svagare jämfört med dem i friska vitala träd. Även om medföljande svampar kan hjälpa barkborrar att vara mer framgångsrika även i ett redan försvagat träd, kan vissa svampar hjälpa barkborrarna att överväldiga trädförsvaret och på detta sätt ge dem möjlighet att kolonisera även friska träd. Olika svamparter som är associerade med barkborrar har kapaciteten döda levande träd i princip på egen hand. Exempelvis kan *Ophiostoma novo-ulmi* som sprids med den tandade almsplintborren orsaka omfattande dödlighet hos almträd. *Leptographium* sp, *Graphium* sp. och *O. minus* är exempel på svampskadegörare som kan döda tallar och som sprids av tallbarkborrar.

Barkborrar kan också överföra andra aggressiva svampskadegörare som kan orsaka kron- och/eller rotskador. Arbetet som presenterades i den här avhandlingen visar att insekt-svamp-symbioser kan ha en stark negativ effekt på träds och skogens hälsa och hållbarhet.

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The aim of the present thesis was to study indigenous and newly occurring and massively tree-devastating bark beetles, vectored communities of fungi, and their pathogenicity to host trees. Investigations showed that bark beetles can vector different functional groups of fungi including aggressive tree pathogens, which may help bark beetles to overcome the tree defence, and thus, may contribute significantly to tree mortality.

Kateryna Davydenko, the author of this thesis, received her graduate education at the Department of Forest Mycology and Plant Pathology, SLU, Uppsala. Her undergraduate degree is from V. N. Karazin Kharkiv National University.

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