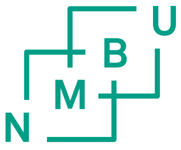
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Preface

Finally, two years of study for the master degree at the Norwegian University of Life Science is completed. This thesis is the end of my education in forestry science. It has been a long and sometimes challenging journey, but this is it. It started as a "worst case scenario", when I had to change my thesis assignment. Two months of fieldwork resulted in nothing due to unlucky circumstances. I was saved by the hero Dr. Halvor Solheim who is my main supervisor. He came up with the idea of study fungi associated with some bark beetle species. This thesis has given me the opportunity to practice and learn about new fields, that I thought I never would bother to try to understand. I've been working with tree samples, beetle species and DNA! Normally I was the one who usually prayed, that DNA wouldn't be a subject in the examinations.

In additions to Dr. Halvor Solheim, many people have inspired, helped and motivated me through the whole process. They have answered all my questions and have been there for me when I needed help. Dr. Paal Krokene for advisements and borrowing out literature. Senior Engineers Helge Meissner and Anne Eskild Nilsen for instructions and advising in the biochemistry lab. Senior Research Scientist Ari M. Hietala for advising me about the laboratory methods and the writing process. Lead Engineer Inger M. Heldal for advisements and mixing of different solutions, such as primers and enzymes. Lead Engineer Gro Wollebæk for the advisements and instructions for the laboratory work. Scientific Adviser Torstein Kvamme for assisting with the fieldwork, beetle identification and comments on the manuscript. Research Scientist Jørn Henrik Sønstebø for instructions in use of BLAST. Scientist Adam Vivian-Smith for helping with the fieldwork. My friend, Ruben A. Lindseth for the cooperation with fieldwork and laboratory work. My friend, Pål Hanssen for helping me with maps. Dr. Wilhelm de Beer and Dr. Miroslav Kolařík for details on the undescribed species (*Graphilbum* sp.1 and *Geosmithia* sp.1). Forestry manager in SB-Skog, Jens A. Randem and Forestry manager in Glommen Skog SA, Kjell I. Iversen, for help to find locations. I am truly grateful to all of you.

I am also very grateful that Norwegian Forest and Landscape institute made available an office desk, laboratories, and all kind of equipment and stuff I needed.

Max Emil Waalberg

15.05.2015

Abstract

Pityogenes bidentatus, *P. quadridens* and *Ips acuminatus* are known to be associated with fungi. However, little is known about the associations between the three beetle species and fungi in Norwegian Scots pine forest. This study reports species associated with the three beetle species in Norway. In total, 60 specimens *P. bidentatus,* 66 of *P. quadridens,* and 30 of *I. acuminatus* were collected and dissected. A total number of 560 fungal isolates including 46 species were obtained. In this study, ten ophiostomatoid species were found in association with the three investigated bark beetle species. *Pityogenes bidentatus* was found to be associated with 24 species of fungi. Five of these species were ophiostomatoid fungi. These were *Graphilbum* sp.1, *Grosmannia europhioides*, *Ophiostoma ips*, *O. minus* and *O.* sp. 1. *Graphilbum* sp.1 was the most abundant species, found in 31.7% of the samples. Two other fungi species with high frequency of occurrences were *Fuscoporia* sp.1 (15%) and *Geosmithia* sp.1 (20%). *Pityogenes quadridens* was found to be associated with 30 species of fungi. Six of these species were ophiostomatoid fungi. These were *Leptographium chlamydatum, Graphilbum* sp.1, *Grosmannia olivacea, O. bicolor, O. minus* and *O. saponiodorum*. However, these were isolated in low frequency. The species with highest frequency of occurrences were *Allantophomopsis* sp.1 (31.8%), *Geosmithia* sp.1 (31.8%) and *Phacidiopynis* sp.1 (30.3%). *Ips acuminatus* was found to be associated with eleven species of fungi. Four of these were ophiostomatoid fungi. These were *Graphilbum* sp. 1, *Graphium* sp. *O. minus* and *O. macrosporum*. The fungi with highest frequency of occurrences were *Graphilbum* sp. 1 (87%) and *Phoma* spp. (60%).

Some of the ophiostomatoid species found in this study have not been registered in Norway before. *Ophiostoma* sp. 1 might be a new species and not yet described. *Graphilbum* sp.1 and *Geosmithia* sp.1 are undescribed species that are found in other studies.

Sammendrag

Pityogenes bidentatus, *P. quadridens* og *I. acuminatus* er kjent for å være vektorer for ulike sopparter. Det har aldri blitt gjort noen studier på disse billene og deres assosierte sopper i norske furuskoger. Dette studiet tar for seg hvilke sopparter som er assosiert med de tre nevnte barkbillene. Totalt ble 60 *P. bidentatus*, 66 *P. quadridens* og 30 *I. acuminatus* kontrollert for sopper. Totalt ble det tatt 560 isolater fra billene.

Dette resulterte i 46 forskjellige sopparter, og av disse var ti ophiostomatoide. Totalt var det 24 ulike sopper som var assosiert med *P. bidentatus,* der fem av disse var ophiostomatoide. De fem ophiostomatoide soppene var *Graphilbum* sp.1, *Grosmannia europhioides*, *Ophiostoma ips*, *O. minus* og *O.* sp. 1. *Graphilbum* sp.1 var den arten som var assosiert med flest biller (31,7). *Fuscoporia* sp.1 (15%) og *Geosmithia* sp.1 (20%) var også isolert med høy frekvens. Totalt ble 30 sopper isolert fra *P. quadridens,* seks av disse var ophiostomatoide. De ophiostomatoide artene var *Leptographium chlamydatum, Graphilbum* sp.1, *Grosmannia olivacea, O. bicolor, O. minus* og *O. saponiodorum*. Disse artene var assosiert med et fåtall av billene. De artene som var assosiert med høyest frekvens var *Allantophomopsis* sp.1 (31.8%), *Geosmithia* sp.1 (31.8%) og *Phacidiopynis* sp.1 (30.3). Totalt ble elleve ulike sopparter isolert fra *I. acuminatus*, fire av disse var ophiostomatoide. De ophiostomatoide artene var *Graphilbum* sp.1, *Graphium* sp. *O. minus* og *O. macrosporum*. De artene som var assosiert med flest av billene var *Graphilbum* sp.1 (87%) og *Phoma* spp. (60%).

Flere av de ophiostomatoide artene som ble funnet i denne oppgaven har ikke blitt registrert i Norge. *Ophiostoma* sp.1 kan være en ny art som sannsynligvis ikke er beskrevet enda. *Graphilbum* sp.1 og *Geosmithia* sp.1 er to arter som ikke er beskrevet enda, men de er tidligere funnet i andre studier.

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Introduction

1.1 Bark beetle generally

Bark beetles (*Coleoptera: Curculionidae: Scolytinae*) are a highly diverse subfamily of weevils that spend most of their life history within plants (Raffa et al. 2015). *Scolytinae* taxonomy seems difficult since new species are described and since the taxonomy above species level is changed due to new taxonomic knowledge and studies of DNA (Hulcr et al. 2015). However, Hulcr et al. (2015) have gathered old information from earlier studies to describe the taxonomy. There are approximately 6.000 *Scolytinae* species in this hyper-diverse weevil subfamily, divided in 26 recognized tribes (Hulcr et al. 2015; Kirkendall et al. 2015). In Norway there are 71, documented, established outdoor-living bark beetle species, but more species are expected to occur or expected to arrive (Kvamme & Lindelöw 2014). Different species of bark beetles prefer different host species or different parts of the host tree. Some bark beetle species are able to colonize and breed in more than one host tree species (Kirisits 2014; Pfeffer 1995, Lekander et al. 1977). However, all bark beetles do not necessarily depend on wood. Many species can utilize and breed in grass, fruits, seeds and flowers (Kirkendall et al. 2015). The ecology of feeding amongst the bark beetles differs greatly and the groups are classified as; phloeophagy (breeding in inner bark), xylomycetophagy (feeding on wood-inhabiting ectosymbiotic fungi), xylophagy (breeding in wood), herbiphagy (feeding on the soft tissues of herbaceous plant), myelophagy (pith of twigs), spermatophagy (seed breeders), mycophagy (fungus feeders), beetles breeding in monocots (Kirkendall et al 2015). Although most species live in dead or weekened host material, some species live and breed in live hosts (Kirkendall et al. 2015).

Bark beetles are often divided in aggressive and nonaggressive beetles (Krokene & Solheim 1998; Raffa et al. 1993). They have also been divided in “primary” or “secondary” pests after time of arriving, on the host (Raffa et al. 1993). This generally, but not entirely, corresponds to the aggressive versus nonaggressive gradient (Raffa et al. 1993). Nonaggressive beetles, or secondary insects in general, prefer dead, dying, or weakened trees as breeding material (Alamouti et al. 2007; Raffa et al. 1993). However, under certain conditions, populations of these less aggressive beetles can increase, causing sporadic outbreaks, killing healthy trees (Alamouti et al. 2007; Raffa et al. 1993; Rudinsky 1962). Aggressive beetles are

known to attack, damage and even kill healthy trees (Bridges et al. 1985; Raffa et al. 1993). However, the majority of bark beetle is restricted to dying or very weak trees (Krokene & Solheim 1998; Weed et al. 2015). Less than 1% of the ca. 6000 described *Scolytinae* species regularly kill healthy standing tress. From the literature it seems unlikely that more than 5-10% occasionally do so (Kirkendall et al. 2015). However, the few existing aggressive beetles are known to have an important role to the ecology and create great economic losses (Krokene & Solheim 1998). Phloem- feeding bark beetles are considered to be amongst the most economically important forest pest, especially when it comes to conifer trees (Wood & Bright 1992). Bark beetles are important contributors to ecology and several of the species are described as "landscape engineers" (Raffa et al. 2015). The species contribute to nutrition cycling, canopy thinning, hydrology, disturbance regimes and successional pathways (Raffa et al. 2015). Bakke & Kvamme (1993) described the sequences of arrival of beetles associated with trees under attack by *Ips typographus* (Linnaeus, 1758). Their results indicate that the beetle may act as key stone species. *Ips typographus* kill and make trees suitable for other insect species. As the decomposition starts, the tree may serve as microhabitat for decades as the decomposition enables different decomposition species to inhabit the dead wood.

1.2 Bark beetles vector fungi species

Bark beetles are associated with various organisms such as mites, nematodes, viruses, bacteria and fungi (Jankowiak & Kot 2011). Normally bark beetles are associated with several fungi species (Kirisits 2004). Some fungi species are constant associates with the beetle, while others probably are causal and play little or no role to the bark beetles biology (Kroken & Solheim 1996). There are also fungi species that occurs in low frequency during endemic periods and increases during epidemic (Solheim 1993). Bark beetles have a widespread association with fungi, especially with ophiostomatoid fungi (*Ascomycota*) (Linnakoski et al. 2012). Ophiostomatoid fungi are in the group of "blue-stain fungi", referring to the discoloration of the sapwood, were the wood seems bluish, grey, brown or black (Seifert 1993). Other groups of fungi causing discoloration are black yeast and dark molds (Seifert 1993). Some fungi species are considered more important than others. Some species may contribute to kill healthy trees, resulting in potential economic loss (Jankowiak et al. 2014). The pathogenic species are considered to be the worst, since they are proven to cause death on healthy trees (Solheim 1993). Some of the ophiostomatoid species are economically important as tree pathogens and agents of wood discoloration (Alamouti

et al. 2007). Many species associated with bark beetles have shown to be pathogenic to conifers when artificially inoculated into seedlings or (Christiansen 1985; Harrington 1993b; Solheim & Långström 1991; Solheim & Safranyik 1997). Association with aggressive blue-stain fungi seems to be necessary for those bark beetles (aggressive bark beetle) that attack living trees in order to overwhelm the defense system of the host tree (Christiansen et al. 1987; Raffa & Berryman 1987). Some ophiostomatoid fungi are suspected to be pathogenic species, which are suitable to overwhelm the defense system of the host tree (Harrington 1993a; 1993b).

The relationship between fungi and bark beetles has been recognized for more than one century, still there are many fundamental aspects poorly known around the subject (Kirisits 2004). The relationship appears to vary between different species of both fungi and beetles (Six 2003). However, in some cases the relationship between bark beetles and fungi appears to be mutualistic, each part benefits from each other, when colonizing the host (Raffa & Klepzig 1992). The pathogenic fungi can play an important part in exhausting the host defense and increase the aggressiveness of each beetle (Krokene & Solheim 1998). At the same time the beetles transports the fungi (long-distance movement) and play an important part in inoculating the fungi into suitable host-trees (Malloch & Blackwell 1993; Six 2003). Fungi are also reported to be an important nutrition for some bark-beetle species (Francke-Grossmann 1952; Six 2003). There are also fungi species that occur as antagonists, as they make the phloem unsuitable for larval nutrition or preventing the adult beetles to breed (Kirisits 2004).

1.3 Investigated beetles

Two-toothed pine beetle *P. bidentatus* (Herbst, 1783) are found in most of Europe (Knížek 2011; Lekander et al. 1977; Pfeffer 1995). Normally the flight period starts in June and the beetle usually hibernates under the bark or in the litter (Lekander et al. 1977). The species is known to breed in young trees and in branches of old, Scots pine (*P. sylvestris*) trees (Ehnstöm & Axelsson 2002; Jankowiak & Rossa 2008) and is also reported to attack *Pinus* species (Lekander et al 1977). Very little is known about the fungi associated with *P. bidentatus* (Jankowiak & Rossa 2008). Association between fungi and *P. bidentatus* has been reported, in young, managed Scots pine stands, in Poland, by Jankowiak & Rossa 2008. Their results indicate that *Penicillium* sp. 1 and *Geosmithia* sp.1 are the most common fungi associated with the *P. bidentatus*. *Geosmithia* sp.1 and *Hormonema dematioides* Lagerb. & Merlin was the most abundant fungus species associated with galleries. Three *Ophiostoma* species

(*Ophiostoma minus* (Hedgc) Syd. & P. Syd, *O. piceae* (Münch) Syd. and *Graphium* sp. "W") occurred occasionally from the isolations from both beetles and galleries.

Pityogenes quadridens (Hartig, 1834) is common all over Europe, also common all over Norway, but less common in northern Norway (Lekander et al. 1977, Pfeffer 1995, Knížek 2011). The Flight of the beetle begins in the latter half of May-beginning of June (Lekander et al. 1977). Usually the imagines hibernate in the litter. Also hibernation under the bark close to the galleries may take place (Lekander et al. 1977). *P. quadridens* is reported to attack only thin-barked parts of dead trees (Lekander et al 1977). Normally the beetle attack Scots pine, but are also reported to attack other pine species (Ehnstöm & Axelsson 2002). Associations between the beetle and fungi are poorly reported. However, Mathiesen (1950) and Mathiesen-Käärik (1953) have studied this topic. Mathiesen (1950) described associations between fungi and 11 specimens of *P. quadridens*. Her findings indicate Grosmannia olivacea (Mathiesen-Käärik) Zipfel, de Beer & Wingfield, *Leptographium lundbergii* Lagerb. & Merlin, *O. tingens* (Lagerb. & Merlin) de Beer & Wingfield, *Ophiostoma canum* (Münch) Syd. & P. Syd., *O. minus,* [*O. penicillatum* f. *pini*] and yeast to be associated with the beetle.

Ips acuminatus (Gyllenhal, 1827) is distributed in Asia, Japan, Thailand and most of Europe (Lekander et al 1997; Pfeffer 1995, Knížek 2011). The species is also common in Norway (Lekander et al. 1997). *Ips acuminatus* mainly attack Scots pine (*P. sylvestris*) and occasionally Norway spruce (*Picea abies*) (Ehnstöm & Axelsson 2002; Lekander et al. 1997). The beetle usually attacks the thin-barked parts of the bole/stem or branches of Scots pine (Kirisits 2004). *I. acuminatus* is also recorded to attack living trees (Lekander et al. 1977). Attacked parts usually become strongly discolored by blue-stain fungi (Lekander et al. 1997). The beetle usually flies in May-June when the air temperature reaches about 18°C (Lekander et al. 1997). The hibernation takes place either under the bark or in the litter (Lekander et al. 1997). Females and males of *I. acuminatus* are known to have paired membranous pouches at base of mandible (Kirisits 2004 and reference therein). The mycangium is an organ for fungus transport (Hulcr et al. 2015). The association between fungi and *I. acuminatus* has been investigated multiple times and the beetle is known to be associated with several species (Kirisits 2004). Eight *Ophiostoma* species are earlier found to be associated with the beetle species; *G. piceae* (Francke-Grosmann 1952; Mathiesen 1950; Mathiesen-Käärik 1953) *O. brunneo-ciliatum* Math-K (Lieutier et al. 1991), *O. canum* (Mathiesen 1950; Mathiesen-Käärik 1953), *O. clavatum* Math-K

(Francke-Grosmann 1952, 1963; Mathiesen 1950,1951; Mathiesen-Käärik 1953; Rennerfelt 1950), *O. ips* (Rumbold) Nannfeldt (Lieutier et al 1991; Mathiesen-Käärik 1953), *O. minus* (Lieutier et al. 1991; Mathiesen 1950; Mathiesen-Käärik 1953; Rennerfelt 1950), and *O. piliferum* (Fr.) Syd. & P. Syd. (Francke-Grosmann 1952; Mathiesen 1950; Mathiesen-Käärik 1953). Two other fungi species are also found to be associated with the beetle; *Leptographium* lundbergii (Lieutier et al. 1991; Mathiesen 1950; Mathiesen-Käärik 1953) and *O. macrosporum* (Francke-Grosmann.) de Beer & Wingfield (Cassar & Blackwell 1996; Francke-Grossmann 1952; Mathiesen 1951). *Ophiostoma macrosporum* is known to be important as nutrition for *I. acuminatus* imagines and the larvae is also reported to feed on *O. clavatum* (Francke-Grossmann 1952).

1.4 Aim of the study

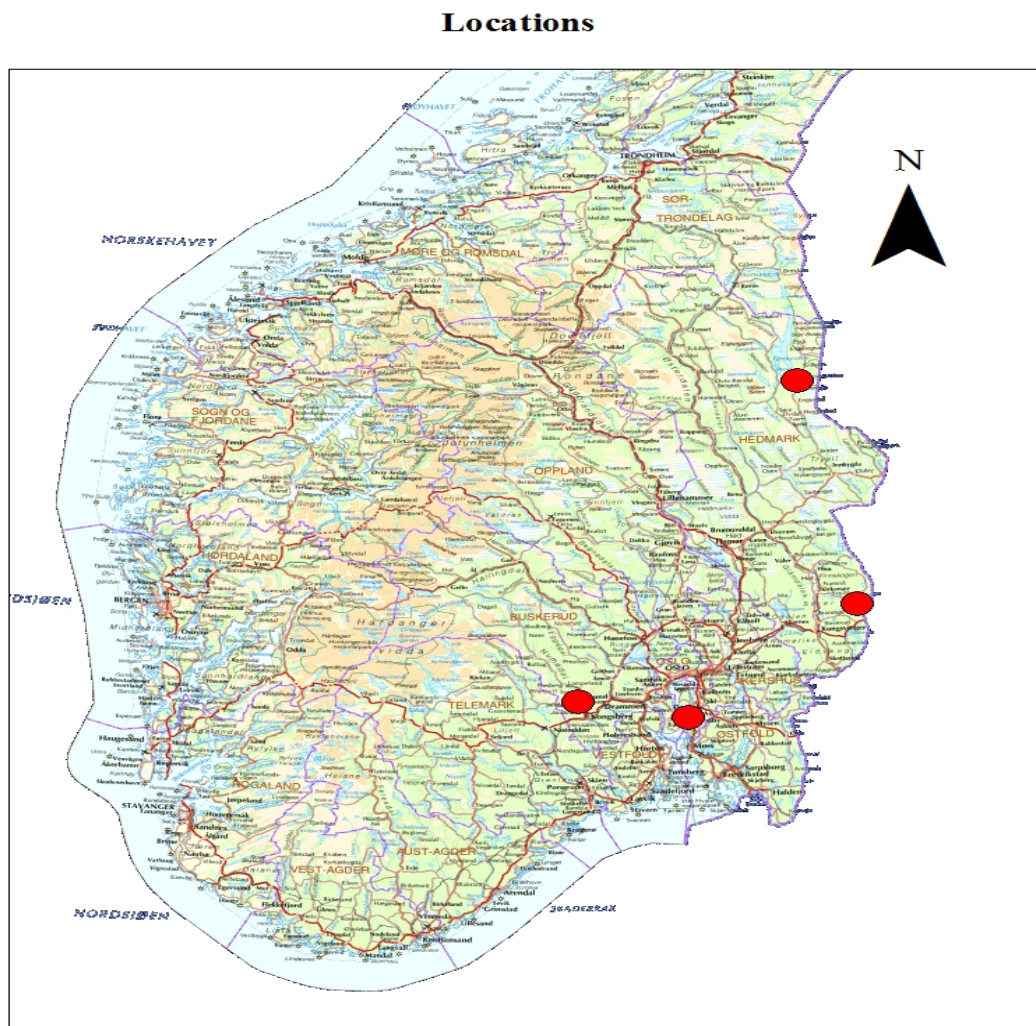
Few studies have studied associations between nonaggressive bark beetles and fungi compared to the aggressive beetles (Kroken & Solheim 1996). Associations between fungi and *Pityogenes bidentatus, Pityogenes quadridens* and *Ips acuminatus* have been studied in other countries but never in Norway. This study will be the first to investigate the topic.

The aim of this study was to examine occurrences of fungi associated with *P. bidentatus*, *P. quadridens* and *I. acuminatus,* attacking branches and thin barked parts, of Scots pine in Norway. The main goal was to study and discuss the occurrence of ophiostomatoid fungi. The most abundant species of other fungi will also be discussed.

2.0 Materials and methods

2.1 Sampling areas

Dead wood of Scots pine, maximum one year old, was collected in five different locations. The locations were located in four different Norwegian municipalities (Map 1 & 2).

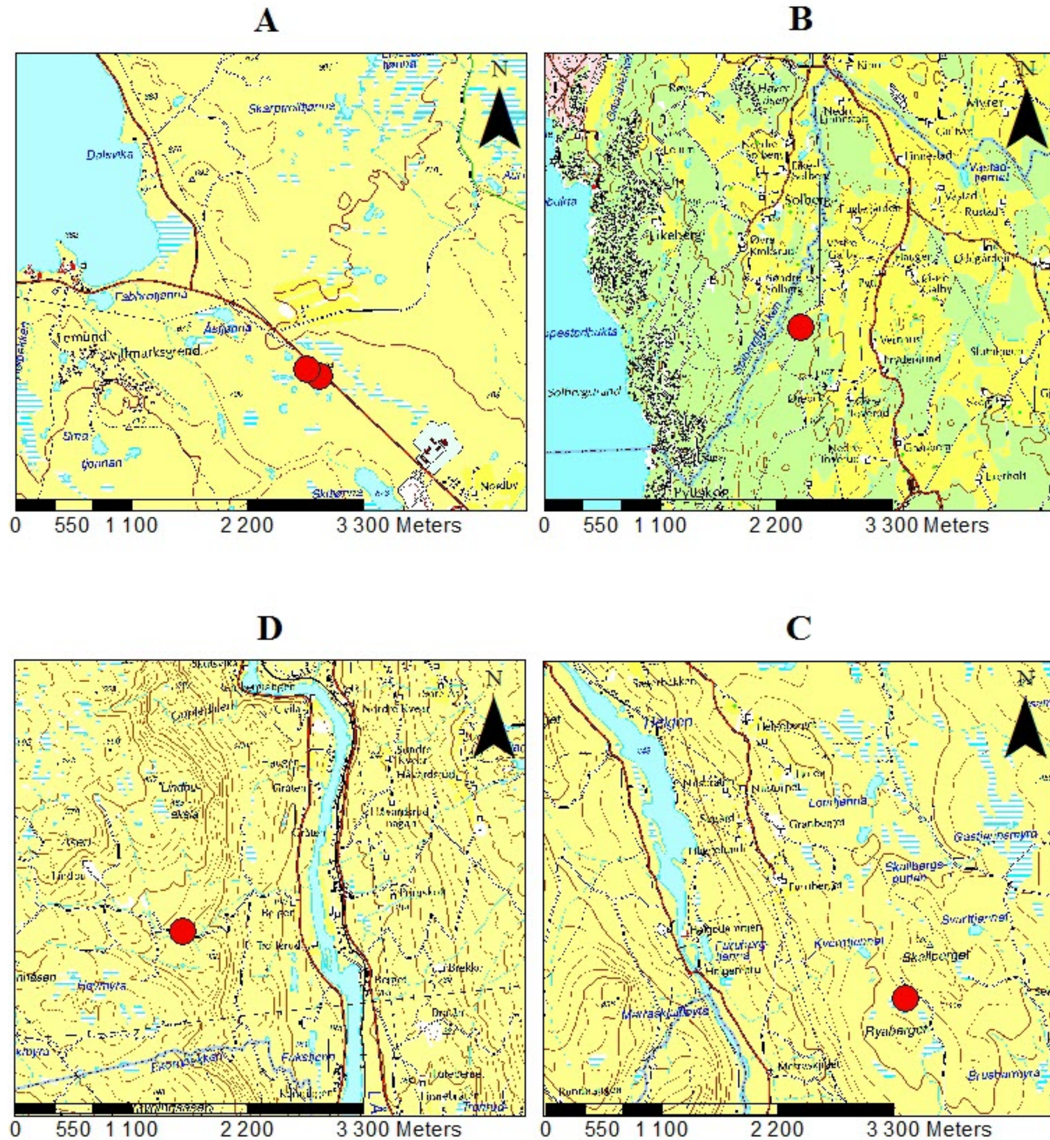


Map 1. Illustrates southern Norway and the location of the sampling areas. Red spots indicate the different locations.

2.1.1 Engerdal, Hedmark County

The sampling area (61°54’36.2”N 11°58’55.0”E) at Engerdal (*Map 2)* is located 695 m.a.s.l. (meters above sea level). The sampling area had probably been harvested for several of years ago. Although, the area was kind of open, because lack on the regrowth. Collected material from Engerdal was between 3 and 4 m high Scots pine,

dead or dying, and attacked by various bark beetles. The ground vegetation was covered with lichen (*Cladonia spp.)* and heather (*Calluna vulgaris* ((Linné) Hull, 1808)). The mean temperature for October, in this area, was 4.0 °C (yr.no 2014a). Total rainfall was 108.1 mm and normal rainfall for this period is 47.0 mm (yr.no 2014a).



Map 2. The map illustrates a closer overview of the sampling areas. Red spots indicates sampling areas; (A) Engerdal, (B) Frogn, (C) Flesberg and (D) Grue.

2.1.2 Frogn, Akershus County

The sampling area (59°37'33.1"N 10°40'40.6"E) at Frogn (*Map 2*) is located 91 m.a.s.l. In Frogn the material was collected from leftovers, from a harvesting area. Thin barked branches were collected and 10-15 diameter stumps. The ground vegetation on this area was mainly covered with mosses. The mean temperature for October, in this area, was 8.9 °C (yr.no 2014b). Total rainfall was 252.0 mm and normal rainfall for this period is 100.0 mm (yr.no 2014b).

2.1.3 Flesberg, Buskerud County

The sampling area (59°44’37.2”N 9°33’52.0”E) at Flesberg (*Map 2*) is located 354 m.a.s.l. Material from Flesberg was mainly collected from leftovers after clear cutting. The diameter on the collected trees was 10-15 cm. In this area, the ground vegetation was dominated with mosses (*Hylocomim splendens* ((Hedwig) Schimper, 1852)), blueberry plants (*Vaccinium myrtillus* (Linné)) occurred occasionally. The mean temperature for October, in this area, was 6.4 °C (yr.no 2014c). Total rainfall was 149.1 mm and normal rainfall for this period is 88.0 mm (yr.no 2014c).

2.1.4 Grue, Hedmark County

The sampling area (60°22’24.4”N 12°27’41.2”E) at Grue (*Map 2*) is located 421 m.a.s.l. Branches were collected from a windfelled tree. Most of the needles were still attached to the crown, although partly discolored. This indicates that the tree was felled by wind less than a year ago. The area was harvested four years ago, and trees were left to serve as seeding trees. Seeding trees are commonly thrown by wind due to a combination of wind exposure and weak roots. The ground vegetation on this location consisted of mosses, blueberry plants and some plants of lingberry (*Vaccinium vitis-idea* (Linné)). The mean temperature for October, in this area, was 7.7 °C (yr.no 2014d). Total rainfall was 123.8 mm and normal rainfall for this period is 75.0 mm (yr.no 2014d).

2.2 Fieldwork

Fieldwork carried out in autumn, 2014.

2.3 Beetle sampling method and species identification

In order to ensure that the collected material contained bark beetles, approximately 4 x 4 cm squares of bark were removed in the field. Only wood, which contained beetles and galleries, were brought back to the institute for further studies and sampling. At the Norwegian Forest and Landscape Institute the wood materials were stored outside under natural climatic conditions. During the following 14 days the wood was brought to the laboratory where the bark was removed by use of a knife and beetles were picked out by use of tweezers. All bark beetle specimens were kept separate in 1.5 ml Eppendorf tubes (Eppendorf, Hamburg, Germany) and stored at -18°C for species identification.

Bark beetles were identified to species. The nomenclature follows Kvamme & Lindelöw (2014) and Knížek (2011). The identifications were done in agreement

with the identification keys in Spessivtseff (1922) and Pfeffer (1995). A good quality stereoscopic microscope (Leica Micro-systems, Brannockburn, IL) was used. The zoom had a magnification ranging from 20 to 80 times. Thereafter the beetle were kept separate in 1.5 ml *Eppendorf tubes* and stored at -18°C for extraction of fungi. The beetles were kept frozen from September to January.

2.4 Laboratory work

Laboratory work carried out in January- April of 2015.

2.5 MEA, Beetle dissection and fungal isolation

Malt extract agar (MEA) was made by dissolving 6,25 g Bactomalt™ malt extract (Beckton, Dickinson, USA), 10 g Bactomalt™ agar (BD) (1.25 % malt, 2 % agar) in 0.5 l neutralized water. The mixture was stirred in a 2.0 l bottle, and autoclaved for 20 minute at 121°C (TOMY SS-325, Tomy Seiko Co. Ltd, Tokyo, Japan).

Before dissection the beetles were studied under a microscope and cleared for mites using a heat-burned needle. Bark beetles were individually dissected under a microscope, using sterile tweezers, scalpels and a bent needle-tool. Cleansed bark beetles were dissected in three parts; head/middle part, cover wings and gut/back part. The different parts were placed in individual 9 cm diameter Petri dishes (Heger AS, Rjukan, Norway), containing 2 % MEA. The cover wings were individually streaked on the surface of the media. All samples were stored in room temperature for later purifying.

After two days the Petri dishes where controlled for growth of mycelium and spores, and purified if necessary. The Petri dishes were regularly controlled and new subcultures were taken as mycelium started to grow (The first dishes were controlled at least four times) or in case of contamination (caused by mites or airborne spores). Yeast was excluded under the purification procedure. Cultures where purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh MEA. Afterwards the samples were stored in room temperature for later use. Purified cultures were regularly controlled by visual inspection. Size, colour, colony texture and conidiophore and conida morphology were used to group the isolates. Five samples from each group were further subcultured and placed in Petri dishes containing MEA coated with a cellophane membranefilament. For fast growing isolates; a single inoculum was placed in the center of the Petri dish, while for slow growing isolates three inocula were used per Petri dish. The cultures were incubated

until the mycelium was collected with a sterilized scalpel and placed in 1.5 ml *Eppendorf* tubes. The tubes were stored at -18°C for later DNA extraction.

2.6 DNA extraction and Polymerase Chain Reaction (PCR)

2.6.1 DNA extraction

DNA was extracted in batches, 20-24 samples per batch. DNA was extracted using Protocol 8#- Isolation of DNA from Mouse Tails (Easy-DNA^{TM} Kit; Invitrogen, San Diego, CA), with a slight modification. The lysis-buffer mastermix contained per sample 320 µl *TE*, 20 µl Solution A, 10 µl Solution B and 5 µl Protein Degrader mixed together in a 12 ml *Falkon* tube and stirred (vortexed) on a *Vortex-GENIE* 2 (Scientific industries, Inc., Bohemia, NY). About 0.03 g *Fluka* sand (Seesand, purum, Fluka; Switzerland) and 100 µl lysis-buffer mastermix were added to a set of labelled 2.0 ml *Eppendorf* tubes (marked 1-24). Fungal samples were then placed individually to the tubes with aid of a tweezers. The hyphal cultures were manually ground into small pieces with aid of a sterile pestle. After grinding, each pestle was washed in the Eppendorf tube, to reduce loss of valuable material, with 255 µl lysis-buffer mastermix (Eppendorf pipette 200-1000 µl). The pestle was then removed and sterilized for later use (next batch). The tubes were then vortexed for 1 minute. Thereafter the tubes were placed in a rack. The rack was placed for 12-20 hours in an incubator shaker (INFORS HT, Bottmingen, Switzerland), in 60°C, at 200 RPM (revolutions per minute).

Thereafter 300 µl Solution A and 120 µl Solution B was added to the tubes, and shaken until the solution was uniformly viscous. In order to purify the DNA, 750 µl chloroform was added (laminar was used when working with chloroform). Following the purifying process the samples were centrifuged (Eppendorf Centrifuge 5415R: Eppendorf, Hamburg, Deutschland) at 16100 RCF (relative centrifugal force) for 10 min at +4°C (separate chloroform and DNA-material). The upper phase (600 µl) was then replaced into 1.5 ml *Eppendorf* tubes. A volume of 1.0 ml of 96% ethanol was mixed into the solution and thereafter incubated on ice for 30 min. Following centrifugation the 96% ethanol was then removed with a pipette. A volume of 500 µl 80% ethanol was added, the tubes were inverted (3-5 times), followed by centrifugation (16100 RCF) for 3-5 min, at +4°C. The ethanol was then removed, followed by centrifugation (16100 RCF) for 1-3 min at +4°C. All residual of ethanol was thereafter removed with aid of a pipette. The tubes were then air dried

(approximately 15 min) in a sterilized laminar to dry the DNA pellet. Afterwards, 50 µl of the buffer TE were added to dissolve the DNA.

2.6.2 DNA quality and quantity

NanoDrop 2000 (Thermo Scientific, Wilmington, DE) was used to examine quality and quantity of DNA-samples. Samples with good DNA quality and quantity were stored at -20°C, to be later amplified (PCR).

2.6.3 PCR

For ribosomal DNA operon, the internal transcribed spacers (ITS) 1 and 2, including 5.8S gene, were amplified using primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). The β-tubulin gene was amplified for blue-stain fungi using primers Bt2a and Bt2b (Glass & Donalds 1995). A portion of the elongation factor 1-α (EF1-α) gene region was amplified using primers EF1F and EF2R (Jacobs et al. 2004) to further verify the ITS-based phylogeny. To optimize the PCR reaction and to obtain release of compounds inhibitory to PCR, a slight modification was made to the mastermix described in HotStarTaq 2006 (Qiagen, Hilden, Germany). Ingredients per sample; 5 µl 10x PCR buffer, 2 µl 25mM $MgCl_2$, 1 µl dNTP (10 µM of each), 5 µl BSA (0.4%), 5 µl TMACL (0.1 mM), 1 (10 µM) of each primer, 0.4 µl Hot Star Taq+ and 27.6 µl RNase-free water (5 Prime Gmbh, Hilden, Germany) were stirred on *Vortex-GENIE* 2. A volume of 48 µl mastermix and 2 µl template (DNA) was added in PCR strips (each tube holds 200 µl). The PCR reactions were performed on GeneAmp PCR System 9700, the program GeneSnap were used. The PCR conditions for ITS were; an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 30 sec 95°C, 30 sec 53°C, 1 min 72°C, final elongation at 72°C for 10 min and PCR product storage at 4 °C. The PCR conditions for β-tubulin and EF1-α genes were; an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 30 sec 95°C, 30 sec 56°C, 1 min 72°C, final elongation at 72°C for 10 min and PCR product storage at 4 °C.

After the process the PCR-samples were stored in machine or in a refrigerator.

2.6.4 PCR separation

Five microliter PCR product volumes were visualized under UV light (GENE GENIUS-SYNGENE) after separation on 1 % agarose gel supplemented with 0.01% ethidium bromide and run in 1xTAE buffer in Bio-rad Subcell GT horizontal electrophoresis gel apparatus (BIO-Rad, Laboratoties Inc., Hercules, CA) for 30 min

at 80 volt, using 7.5 µl *1Kbt* laddermix as a size reference. The remaining 45 µl volume of the PCR reactions were stored in 4°C.

2.6.5 Purifying PCR and adding of primers

Following methods were used to purify the PCR;

Purification method 1:

Modification of Bench Protocol: QIAquick PCR Purification Microcentrifuge and Vacuum Protocol (QIAquick 2006, Qiagen). Briefly, a volume of 45 µl of PCR reaction and 250 µl PB buffer were mixed in 2.0 ml Eppendorf tubes. The mixture was then replaced into a QIAquick column-tube (to bind the DNA). The tubes were then centrifuged (16100 RCF) at 23°C for 1 min. The columns were replaced into new 2 ml Eppendorf tubes (lid cut off). A volume of 750 µl *PE* buffer was added to the columns to wash the solution. All tubes were then centrifuged (16100 RCF) at 23°C for 1 min. The columns were then replaced into new 2 ml Eppendorf tubes (lid cut off). Tubes were then centrifuged (16100 RCF) at 23°C for 1 min. Thereafter each column was replaced into 1.5 ml Eppendorf. The tubes were then placed in a rack and air-dried (sterilized laminar) for 20 min. Afterwards, 30 µl EB buffer was added to the center of the filter column. The columns were then centrifuged (16100 RCF) in 23°C for 1 min and the flow-through containing the Purified PCR product was retained. Finally volume of 5 µl of each purified PCR product (5 µl for each primer) was placed in two Eppendorf tubes (1.5 ml): First tube was mixed with 5 µl primer *ITS1F* and the next with 5 µl ITS4.

Purification method 2:

Modification of MinElute Purification Kit Protocol (MinElute Handbook©, Qiagen). This method uses Min Elute columns and only 15 µl EB buffer to elute the DNA. The rest of the procedure is identical with method 1.

Purification method 3:

Rapid PCR Cleanup Enzyme Set (New England Biolabs and Boehringer, Mannheim) were used for β-tubulin and EF1-α genes. A volume of 2 µl of each enzyme (following the kit) was mixed with 5 µl of the PCR product in a 2 ml Eppendorf tube. The tubes were then incubated at 37°C for 5 min. Afterwards the tube were incubated at 80°C for 10 min. Then 5 µl of forward or reverse primer was added to each tube as described above

Afterwards each tube was ID-labelled and stored at -80°C prior to shipping to sequencing at GATC-Biotech in Germany.

2.7 Sequence analysis

Sequencing was performed in *CLC Main Workbench 7.6-Evalution* (CLC Bio©, Denmark). Sequences of isolates from this study were aligned with sequences in GenBank obtained using BLAST searches (http://blast.ncbi.nlm.nih.gov). The species that gave the highest identity match are presented in the results. None of the alignments were saved in the GenBank. In some causes the primers was unreadable together, because of disturbance on the nucleotide. In this cases the nucleotide were individually visualized and controlled. Only one of the primers was blasted in cases where one was unreadable/readable. Some samples were also unusable because of the disturbance. In cases of, suspected ophiostomatoid species, the related Petri dish was purified and a new process was started (earlier described under chapter 2.5-2.7). ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were used to compare alignments with data in GenBank. The web-tool was also used to detect differences between alignments that gave the same identity but had different accession number.

2.8 Maps

Coordinates were downloaded from GPS-Astro 320 (Garmin. Kansas, USA) into ArcGis 10.2.2. N50 Rastor maps were used. All map layers were downloaded from Statsenskartverk free website (2015). UTM zone 32 was used as coordinate system.

2.9 Tables and frequency

Microsoft® Excel® for Mac 2011 (14.4.8) was used to make the table and to calculate the frequency.

3.0 Result

A total number of 60 specimens of *P. bidentatus,* 66 of *P. quadridens,* and 30 of *I. acuminatus* were dissected. This resulted in 468 main dishes. All of the investigated beetles were associated with one, or more, species of fungi. The highest amount of fungi species associated with a beetle was six. In total, 560 isolates were taken from the 468 main dishes. Approximately 190 isolates were purified and placed on Cellophane. Nine potential groups, based on morphological characters and several of occasionally species were tested with ITS (Table 1 & 2). Twenty-four samples of ophistomatoid fungi, based on ITS, were tested with β-tubulin and 12 samples with

EF1-α. The results were tested against earlier result in the GenBank. A total of 26 samples containing ITS-data returned negative. The results from β-tubulin was mixed, five samples were unreadable and several of the samples were only readable one way. None of the EF1-α gens were readable.

Table 1 List of representative strains of ophiostomatoid species and results of their BLAST searches.

Taxon	Strain 2015	Closest match in BLAST	GenBank accession no. ITS rDNA	Identity (%)
Leptographium chlamydatum[a]	006/3/1	*Ophiostoma* sp. 131	DQ318206.1	99
Graphium sp.	248/1/2	*G. pseudormiticum*	FJ824623.1	99
Graphilbum sp. 1[b]	265/2/1	*O. rectangulosporium*	EU785449.1	99
Grosmannia europhioides	118/3/1	*G. europhioides*	EU879141.1	99
G. olivacea	049/1/2	*O. olivaceum*	AJ538337.1	99
Ophiostoma bicolor	227/2/1	*O. bicolor*	HE866712.1	99
O. ips	087/3/2	*O. ips*	AY546697.1	100
O. macrosporum	271/1/2	*Ambrosiella* sp. *2YT2P-A2*	DQ268585.1	
O. minus	067/3/2	*O. minus*	JF440585.1	99
O. saponiodorum	009/2/3	*O. saponiodorum*	HM031507.1	100
O. sp.1	077/1/1	*Ambrosiella* sp. 3YT7P- A1	DQ268583.1	95

[a] β-tubulin sequence gave 100% match with *Leptographium chlamydatum*

[b] Wilhem de Beer has seen the sequences, this is an undescribed *Graphilbum* species.

[c] β-tubulin sequence gave 100% match with *O. macrosporum*

Table 2 List of representative strains of other fungal taxa and results of their BLAST searches.

Taxon	Strain	Closest match in BLAST	GenBank accession no. ITS rDNA	Identity (%)
Allantophomopsis sp.1	060/3/2	*A. lycopodina*	AB041243.1	99
Beauveria bassiana	262/3/3	*B. bassiana*	KC768069.1	99
Botryosphaeriaceae sp.	045/1/1	*B.* sp. F16	JF439465.1	99
Cadophora sp.	006/2/1	*C. malorum*	DQ404350.1	99
Cladosporium cladosporioides	070/3/2	*C. cladosporioides*	JX981454.1	100
C. pini-ponderosae	022/3/3	*C. pini-ponderosae*	NR_119730.1	100
C. sp.	070/3/1	*C.* sp. TMS-2011	HQ631003.1	99
Entomocorticium sp.	021/3/3	*E. sp. ZK25/08*	FR837930.1	99
Epicoccum nigrum Link	242/3/1	*E. nigrum*	GU566259.1	99
Fuscoporia sp.1	007/3/1	*F. gilva*	DQ103884.1	99
Geosmithia sp.1	174/1/1	*G.* sp. 24 NL-2014	KF808311.1	100
G. sp.2	104/3/1	*G.* sp. 31 NL-2014	KF808318.1	100
G. sp.3	038/3/1	*G.* sp. MK1837	HE604165.1	100
Hypocrea parapilulifera	263/3/1	*H. parapilulifera*	AY241587.1	100
H. sp.	263/3/2	*H.* sp. IMI 206039	EU294196.1	100
Lecythophora sp.	088/1/5	*L.* sp. UBCtra1453C	AY219880.1	98
Leotiomycetes sp.	099/1/2	*L.* sp. ASR_H18_12A	JX421713.1	99
Microsphaeropsis olivacea	276/1/1	*M. olivacea*	JX681101.1	100
M. sp.	040/3/3	*M. proteae*	JN712495.1	99
Mortierella sp.	095/2/1	*M.* sp. 04NY05	JX270372.1	99
Mucor sp.	026/2/1	*M. hiemalis*	AJ876490.1	99
Neonectria sp.	008/1/2/1	*N. fuckeliana*	HQ840386.1	98
Penicillium raistrickii	043/1/1	*P. raistrickii*	KP152490.1	99
P. spathulatum	045/3/2	*P. spathulatum*	KC427190.1	100
P. spinulosum	007/1/1	*P. spinulosum*	JQ272372.1	100
P. sp. 1	021/1/3	*P. rugulosum*	GU566230.1	99
P. sp. 2	023/2/1	*P. brevicompactum*	AB479306.1	100
Pestalotiopsis funereal	008/1/2	*P. funerea*	EF055197.1	99
Phacidiopycnis sp.1	019/2/1	*P. washingtonensis*	JF732919.1	99
Phellinus sp.	070/1/1	*P.* sp. SA03	EF694973.1	99
	118/1/1	*P. herbarum*	FN868459.1	99
Phoma spp.	247/1/1	*P. macrostoma*	LN714588.1	99
	008/2/1	*P.* sp. E16	FJ903335.1	100

Rhizosphaera kalkhoffii Bubák	066/3/1	*R. kalkhoffii*	JX981459.1	100
Sphaeropsis sp.	7/3/1	*S. sapinea*	KF766159.1	100
Trichoderma sp.	268/1/1	*Trichoderma* sp. 16 BRO-2012	KF367557.1	100

3.1 *Pityogenes bidentatus* (Herbst, 1783)

Five different species of ophiostomatoid fungi were associated with the investigated beetle (T*able 3*). Three species were associated with beetles caught in Grue, but with a low frequency. The beetles from Engerdal were associated with *Graphilbum* sp.1 (25%) and *O. minus* (40%). *Graphilbum* sp.1 Grosmannia europhioides (Wright & Cain) Zipfel, de Beer & Wingfield were associated with the beetles from Flesberg. However, the frequency of *Graphilbum* sp.1 for this site (65%) was rather high in contrast to the other detected species.

Table 3 Number and frequency of ophiostomatoid fungi isolated from P. bidentatus *collected in Grue, Engerdal and Flesberg.*

	Number of beetles (% frequency) collected in:			
Ophiostoma species:	Grue	Engerdal	Flesberg	Total
Graphilbum sp.1	1 (5)	5 (25)	13 (65)	19 (31.7)
Grosmannia europhioides			2 (5)	2 (3.3)
Ophiostoma ips	5 (25)			5 (8.3)
O. minus	2 (10)	8 (40)		9 (15)
O. sp.1				
Number of investigated beetles	20	20	20	60

Pityogenes bidentatus was also associated with nineteen other fungal taxa (*Table 4*). Most of them were only isolated between 1-4 times (90%). The beetles caught in Grue were associated with 50% of the species and *Geosmithia* sp.1 (50%) and *Fuscoporia* sp.1 (30%) were the most abundant species. These species were also, in total, presented with the highest frequency, *Geosmithia* sp.1 (20%) and *Fuscoporia* sp.1 (15%). Beetles collected in Engerdal were associated with five (25%) species and the frequency was low (rated from 5-10 %). Flesberg were associated with the highest amount of species. Although, with a low frequency (rated from 5-20%). In total *Geosmithia* sp.1 (20%) and *Fuscoporia* sp.1 (15%) were the most abundant species.

Table 4 Number and frequency of other fungal taxa isolated from P. bidentatus *collected in Grue, Engerdal and Flesberg.*

Species	Number of beetles (% frequency) collected in:			
	Grue	Engerdal	Flesberg	Total
Allantophomopsis sp.1	2 (10)		1 (5)	3 (5)
Claudiosporium cladosporioides	1 (5)		1 (5)	2 (3.3)
Cylindrocarpon sp.		1 (5)		1 (1.6)
Fuscoporia sp.1	6 (30)		3 (15)	9 (15)
Epicoccum nigrum			1 (5)	
Geosmithia sp.1	11 (50)	1 (5)	1 (5)	12 (20)
Geosmithia sp.2	1 (5)			1 (1.6)
Geosmithia sp.3		1 (5)		1 (1.6)
Lecythophora sp.	2 (10)			2 (2)
Leotiomycetes sp.	6 (30)			6 (10)
Microsphaeropsis olivacea			1 (5)	1 (2)
Mortierella sp.		1 (5)		1 (2)
Penicillium raistrickii			2 (10)	2 (3)
P. spathulatum	4 (20)			4 (6.7)
P. sp.1	1 (5)			1 (1.6)
Phacidiopynis sp.1			2 (10)	2 (3)
Phoma spp.			4 (20)	4 (6.7)
Rhizosphaera kalkhoffii			1 (5)	1 (1.6)
Trichoderma sp.	1 (5)			1 (1.6)
Number of investigated beetles	20	20	20	60

3.2 *Pityogenes quadridens* (Hartig, 1834)

Six different ophiostomatoid species were found to be associated with the investigated beetle (*Table 5*). The total frequency for each species was low (1.5-12.1%). In total, *O. minus* was the most frequent ophiostomatoid species to be associated with *P. quadridens*. Five species of fungi were associated with the beetles from Grue. *Ophiostoma minus* was the most frequent to be associated with the beetles collected at Grue (23%). *Leptographium chlamydatum* Jacobs, Wingfield & Solheim, *Graphilum* sp., and *O. saponiodorum* Linnakoski, de Beer & Wingfield were less frequent (3.3-6.7%) species that occurred in Grue. *Grosmannia olivacea* and O. minus were isolated in low frequent from beetles collected in Frogn.

Table 5 Number and frequency of ophiostomatoid fungi isolated from P. quadridens *collected in Grue and Frogn.*

	Number of beetles (% frequency) collected in:		
Ophiostoma species	Grue	Frogn	Total
Leptographium chlamydatum	1 (3.3)		1 (1.5)
Graphilbum sp.1	2 (6.7)		2 (3)
Grosmannia olivacea		2 (5.6)	2 (3)
Ophiostoma bicolor	3 (10)		3 (4.5)
O. minus	7 (23.3)	1 (2.7)	8 (12.1)
O. saponiodorum	1 (3.3)		1 (1.5)
Number of investigated beetles	30	36	66

Pityogenes quadridens was also associated with twenty-four other species of fungi (*Table 6*). A total of 25 different taxa were found. Overall, *Allantophomopsis* sp.1 (31.8%), *Geosmithia* sp.1 (31%) and *Phacidiopynis* sp.1 (30.3%) were the most abundant species. The beetles caught in Grue were associated with 16 different species of fungi (64%). The three most abundant species in Grue were *Allantophomopsis* sp.1 (53.3%), *Geosmithia* sp.1 (33.3%) and *Phacidiopynis* sp.1 (30). The other species were less frequent (3.3-6.7 %). The three most abundant species in Frogn were *Geosmithia* sp.1 (30.5%), *Phacidiopynis* sp.1 (30.5%) and *Penicillium raistrickii* (G. Sm) (27.8%). Other species were less frequent, eight species in the range between 2.8-5.6% and five species in the range between 11.1-19.4%. In total, *Allantophomopsis* sp.1 (31.8%), *Geosmithia* sp.1 (31.8%) and *Phacidiopynis* sp.1 (30.3%) were the most abundant species.

Table 6 Number and frequency of other fungal taxa isolated from P. quadridens *collected in Grue and Frogn.*

	Number of beetles (% frequency) collected in:		
Species	Grue	Frogn	Total
Allantophomopsis sp.1	16 (53.3)	5 (13.9)	21 (31.8)
Botryosphaeriaceae sp.		4 (11.1)	4 (6.1)
Cadophora sp.	1 (3.3)		1 (1.5)
Cladosporium cladosporioides	1 (3.3)		1 (1.5)
C. pini-ponderosae	1 (3.3)		1 (1.5)
C. sp.		1 (2.8)	1 (1.5)
Entomocorticium sp.	2 (6.7)	7 (19.4)	9 (13.7)

Fuscoporia sp.1	1 (3.3)	7 (19.4)	8 (12.1)
Geosmithia sp.1	10 (33.3)	11 (30.5)	21 (31.8)
Microsphaeropsis olivacea		1 (2.8)	1 (1.5)
Microsphaeropsis sp.		1 (2.8)	1 (1.5)
Mucor sp.		1 (2.8)	1 (1.5)
Neonectria sp.	2 (6.7)		2 (3)
Penicillium raistrickii	2 (6.7)	10 (27.8)	12 (18,1)
P. spathulatum	2 (6.7)	2 (5.6)	4 (6.1)
P. spinulosum	1 (3.3)		1 (1.5)
P. sp. 1		1 (2.8)	1 (1.5)
P. sp. *2*		7 (19.4)	7 (10.6)
Pestalotiopsis sp.	1 (3.3)		1 (1.5)
Phacidiopynis sp.1	9 (30)	11 (30.5)	20 (30.3)
Phellinus sp.	1 (3.3)		1 (1.5)
Phoma spp.	1 (3.3)		1 (1.5)
Sphaeropsis sp.	1 (3.3)		1 (1.5)
Trichoderma sp.		1 (2.8)	1 (1.5)
Number of investigated beetles	30	36	66

3.3 *Ips acuminatus* (Gyllenhal, 1827)

Four ophiostomatoid species were associated with *I. acuminatus* (*Table 7*). *Graphium* sp. was the most commonly species from the isolates (87%). The other species were less common (3.3-10%).

Table 7 Number and frequency of ophiostomatoid fungi isolated from I. acuminatus *collected in Flesberg.*

Species	Number of beetles (% frequency) collected in:
	Flesberg
Graphilbum sp.1	26 (87)
Graphium sp.	1 (3.3)
Ophiostoma minus	3 (10)
O. macrosporum	2 (6.7)
Number of investigated beetles	30

Seven other species were associated with *I. acuminatus* (*Table 8*). The most common species was *Phoma* spp. with 60% frequency. The six other fungi species were less common (3.3-6.7%).

Table 8 Number and frequency of other fungal taxa isolated from I. acuminatus *collected in Flesberg.*

Species	Number of beetles (% frequency) collected in:
	Flesberg
Beauveria bassiana	1 (3.3)
Hypocrea parapilulifera	1 (3.3)
Hypocrea sp.	1 (3.3)
Microsphaeropsis olivacea	1 (3.3)
Penicillium raistrickii	2 (6.7)
Phacidiopynis sp.1	1 (3.3)
Phoma spp.	18 (60)
Number of investigated beetles	30

4.0 Discussion

4.1 Ophiostomatoid species

In this study, ten ophiostomatoid species were found in association with the three investigated beetles. These were *Leptographium chlamydatum, Graphilbum* sp.1*, Grosmannia olivacea, Grosmannia europhioides, O. bicolor* Davidson & Wells*, O. ips, O. macrosporum, O. minus, O. saponiodorum* and *O.* sp.1.

Leptographium chlamydatum was isolated from one *P. quadridens* (1.5%). The beetle was collected in Grue. ITS-data gave 99% identity match on *Ophiostoma* sp. 131 WIN(M) (DQ318206.1) and 99% on *Leptographium* sp. 2YT2P-L2 (DQ268598.1). β-tubulin-data gave 100% identity on *Leptographium chlamydatum* (JF280029.1). However, *L. chlamydatum* has not earlier been sequenced with the internal transcribed spacers (ITS) 1 and 2. Other studies have used other regions such as ITS2 and 28S, together with β-tubulin and EF1-α (Linnakoski et al. 2012a: Halvor Solheim per. comm.). ITS2 and 28S should therefore have been used in addition to β-tubulin. The ITS-data from this study and older ITS 2 alignments in the GenBank, do not show any similarity. The fungi might therefore be a new species, but more tests are needed to determine this hypothesis. However, in this study the fungus are presented as

Leptographium chlamydatum, because of the 100% match with β-tubulin-data in the GenBank. The result is supported by other isolates (Ruben A. Lindseth; unpublished master thesis 2015). He has also sequenced this fungus. *Leptographium chlamydatum* appears to occur in Finland, Norway and Russia (Jacobs et al. 2010; Linnakoski 2011). *Dryocetes autographus* (Ratzeburg, 1837) and *Hylastes cunicularius* (Erichson, 1836) are known to vector *L. chlamydatum* (Jacobs et al. 2010; Linnakoski 2011). The fungus is only reported from beetles that were collected from Norway spruce (Jacobs et al. 2010; Linnakoski 2011). The result from this study might indicate that *L. chlamydatum* is also associated with *P. quadridens* attacking Scots pine.

Graphium sp. was only isolated from one specimen of *I. acuminatus* (3.3) collected in Flesberg. *Graphium pseudormiticum* (Mouton & Wingfield, 1994) was the closest match (97%) according to the ITS-data. *Graphium pseudormiticum* might earlier have been overlooked or confused, in other studies, with the related species *Graphium fimbriasporum* (Morelet) Jacobs, Kirisits & Wingfield and *Graphium laricis* Jacobs, Kirisits & Wingfield (Jacobs et al. 2003). *Graphium fimbriasporum* (96%) and *Graphium laricis* (99%) score also high on identity (although with lower query cover). However, the three different Graphium species seem to have different ecological niches (Jacobs et al. 2003). *Graphium pseudormiticum* is associated with pine, *Graphium fimbriasporum* with spruce and *Graphium laricis* with European larch (*Larix decidua* Miller) (Jacobs et al. 2003 and references therein). Although, new studies indicate *Graphium pseudormiticum* also to infest Norway spruce (Jankowiak & Kolařík 2010b; Persson et al. 2009) *I. acuminatus* has not earlier been reported to vector *Graphium pseudormiticum*. However, *Graphium pseudormiticum* is suspected to be associated with a wide variety of pine bark beetles in Europe (Jacobs et al. 2003; Linnakoski et al. 2012b). In this study the fungus is presented as *Graphium* sp. because of a low identity score, with other sequenced species, in the GenBank. The results indicate that *Graphium pseudormiticum* are found in Norwegian Scots pine forest.

Graphilbum sp.1 was isolated from one (5%) *P. bidentatus* collected in Grue, five specimens from Engerdal (25%) and thirteen specimens from Flesberg (65%). *Graphilbum* sp.1 was also isolated from two specimens of *P. quadridens* (6.7%) collected in Grue. Most of the *I. acuminatus* (87%), collected in Flesberg, were associated *Graphilbum* sp.1 The results indicates *Graphilbum* sp.1 to be a very common fungus in Flesberg. The results indicate also that *P. bidentatus, P.*

quadridens (occasionally) and *I. acuminatus* are suitable vectors for the fungus. *Graphilbum* sp.1 is described as unidentified and is also known to be present in China and Poland (Wilhelm de Beer, per. comm.). The closest alignment match with in the GenBank, according to the ITS and β-tubulin-data, was *O.* cf. *rectangulosporium* strain CMW26258 (identity 99-100%). This fungus is described to be associated with *Dendroctonus valens* (LeConte, 1860), infesting *Picea abies tabuliformis* (Carriére), in China (Lu et al. 2009). Other studies have earlier reported *O. rectangulosporium* spp. to be associated with weevils and other types of bark beetles (Jankowiak & Bilański 2013; Jankowiak & Kolařík 2010b; Ohtaka et al. 2006). However, this might not be the same species. The results from this study are the first to confirm the occurrences of the presented *Graphilbum* species in Norway.

Grosmannia europhioides was associated with two specimens of *P. bidentatus* (6.7%). Both beetles were caught at Flesberg. ITS-data gave an identity match with two different isolates of *Grosmannia piceaperda* (Rumbold) Goid. 1936. The taxonomy of these species have been unclear (Linnakoski et al. 2012b), and *Grosmannia piceaperda* and *G. europhioides* were earlier treated as two separate related species (de Beer et al. 2013). However, the two different lineages are now presented by the name *G. europhioides* and are a part of the *G. piceaperda* complex (de Beer et al. 2013 and references therein). Several studies have reported the occurrences of either *G. piceaperda* or *G. europhioides* (Linnakoski et al. 2012b and references therein). The occurrences of these two lineages will be determined as one (*G. europhioides*) since this study follows the nomenclature of de Beer et al. (2013). *Grosmannia europhioides* is found in Finland, Norway, Sweden, Russia and North America (Linnakoski et el. 2012a & Linnakoski et al. 2012b; Lu et al. 2009). The fungus has earlier been described to be associated with *I. typographus, I. duplicatus* (Sahlberg, 1836)*, P. poligraphus* and *Hylurgops palliates* (Gyllenhal, 1813) on spruce (Krokene & Solheim 1996; Linnakoski et al. 2012b and references therein). *Grosmannia europhioides* has not been reported to be associated with *P. bidentatus.* This study shows that *G. europhioides* occurs also in Norwegian Scots pine forest and that *P. bidentatus* is a suitable vector.

Grosmannia olivacea was isolated from two *P. quadridens* (3%). Both beetles were caught in Frogn. ITS-data gave 99% identity match on *O. olivaceum* (AJ538337.1). *Ophiostoma olivaceum* was first described by Mathiesen (1951). Following the new nomenclature, the species is renamed to *G. olivacea.* Reports of *G. olivacea* are limited (Linnakoski 2011). However, the fungus is known to be common associate of

pine- and spruce infesting bark beetles, such as *Dryocoetes autographus* (Ratzeburg, 1837)*, Hylastes cunicularius* (Erichson, 1836)*, I. sexdentatus* (Börner, 1767) and *I. typographus* (Linnakoski 2011; Mathiesen 1951). *Grosmannia olivacea* has not been reported to be associated with *P. quadridens*, until now. This is also the first time *G. olivacea* has been isolated from a beetle in Norway. *Grosmannia olivacea* is a species complex which is studied more thoroughly with four different gene regions. Those are not yet published in GenBank (Halvor Solheim, pers. comm.)

Ophiostoma bicolor was associated with three *P. quadridens* (4.5%). All three beetles were collected in Grue. *Ophiostoma bicolor* has not earlier been isolated from *P. quadridens*. However, *Ophiostoma bicolor* is one of the most common species to be associated with spruce infesting *I. typographus* (Krokene & Solheim 1996; Linnakoski et al. 2010: Persson et al. 2009; Solheim 1992 & 1993). *Ophiostoma bicolor* is common to find in Finland, Norway, Sweden and Russian Karelia (Linnakoski et al. 2012b). In Norway, the fungus is reported to be associated with four beetle species (Krokene & Solheim 1996). The fungus is regarded as a specific associate of the aggressive *I. typographus* (Krokene & Solheim 1996; Solheim 1988). *Ips duplicatus*, *P. chalcographus* (Linnaeus, 1761) and *Polygraphus poligraphus* (Linnaeus, 1758) is also species that vector this fungus in Norway (Krokene & Solheim 1996). The level of aggressiveness for this fungus is reported to be low (Solheim 1988). However, the result from this study indicates *P. quadridens* to be a suitable vector species for *Ophiostoma bicolor*.

Ophiostoma ips was associated with five specimens of *P. bidentatus* (8.3%). This fungus was only isolated from beetles collected in Grue. The fungus is not earlier found to be associated with *P. bidentatus*. However, *Ophiostoma ips* is earlier reported to be associated with pine-infesting bark beetles in northern parts of Sweden (Mathiesen-Käärik 1953). Mathiesen-Käärik (1953) detected this fungus to be associated, at low frequency, with *I. acuminatus* and *Orthotomicus proximus* (Eichhhoff, 1868). The fungus is only recorded from Sweden, in Fennoscandia (Linnakoski et al. 2012b). *Ophiostoma ips* has shown to have low levels of aggressiveness to *Pinus* spp. in South Africa (Zhou et al. 2002). However, it has been reported to be pathogenic to pine spp. in other countries (Zhou et al. 2002 and references therein). The aggressiveness of *Ophiostoma ips*, in Norway, is not studied. This study is the first to confirm that *Ophiostoma ips* occurs in Norway. The study is also the first to isolate *Ophiostoma ips* from *P. bidentatus*.

Ophiostoma macrosporum was isolated from two *I. acuminatus* beetles (6.7%). The ITS-data (only ITS-4) gave 96% identity match on *Ambrosiella* sp. (2YT2P-A2). β-tubulin-data gave 100% identity on *Ambrosiella macrospora* strain CBS367.53 (EU977465.1). However, *Ambrosiella macrospora* has not earlier been sequenced with ITS primers. Therefore it was important to test the isolated fungus with β-tubulin-data. *Ambrosiella macrospora* has been reclassified to *Ophiostoma*, because of a closer relationship to *Ophiostoma* genera (de Beer et al. 2013). Therefore the species is presented as *O. macrosporum*. *Ophiostoma macrosporum* is earlier reported to be a common associate of *I. acuminatus* (Cassar & Blackwell 1996; Francke-Grossmann 1952; Mathiesen 1951). The fungus is known to grow on the mycangium of the beetle and to be an important nutrition for the beetle (Francke-Grossmann 1952). The fungus is actually only known to be associated with *I. acuminatus,* and is only reported from Sweden (Linnakoski et al. 2012b). However, the result from this study confirms that *Ophiostoma macrosporum* is also associated with *I. acuminatus* in Norway.

Ophiostoma minus was found occasionally with the tree investigated beetle species. The result was expected since earlier studies also indicates *O. minus* to be associated with the three different beetles (Jankowiak & Rossa 2008; Lieutier et al. 1991; Mathiesen 1950; Mathiesen-Käärik 1953; Rennerfelt 1950). The fungus is common in whole of Fennoscandia (Linnakoski et al. 2012b). *Ophiostoma minus* is known to be associated with a numerous of bark beetle species attacking spruce and pine (Jankowiak & Kolařík 2010b; Linnakoski et al. 2012b). This fungus is known to cause great damage to Scots pine and is even reported to be able to kill the host tree (Solheim & Långström 1991; Solheim et al. 1993). However, the taxonomy of *Ophiostoma minus* is more defuse because the North American and European isolate reside in distinct phylogenetic lineages (Lu et al. 2009; Linnakoski et al. 2010). The *O. minus* found in this study is probably belonging to the European clade of *O. minus*.

Ophiostoma saponiodorum was isolated from two Petri dishes, both dishes were belonging to one *P. quadridens* specimen. The beetle was caught in Grue. The ITS-data gave 100% match on *O. saponiodorum* (CMW29497). *Pityogenes quadridens* has not been reported to be associated with this fungus. *Ophiostoma saponiodorum* has only been registered in Finland, Russia and Poland (Jankowiak, R. & Bilański. 2013; Linnakoski et al. 2010; Linnakoski et al. 2012b). Little is known about the occurrences and of which beetle species who are suitable to vector *O. saponiodorum* (Linnakoski et al. 2012b). The fungus is earlier isolated from *I. typographus* on Scots

pine (Linnakoski et al. 2012b and references therein). *Ophiostoma saponiodorum* is also known to be one of the most common species associated with *P. chalcographus* on Norway spruce (Linnakoski et al. 2012b). Jankowiak, R. & Bilański (2013) isolated *O. saponiodorum* from *Pissodes piniphilus* (Herbst, 1779) that was infesting pine habitats. Jankowiak, R. & Bilański (2013) and Linnakoski et al. (2012b) results indicate *O. saponiodorum* to potentially have wide host range in Europe. The result from this study might be a closer step to confirming this hypothesis. This study can confirm that *O. saponiodorum* occurs in Norway, with *P. quadridens* that are infesting Scots pine.

Ophiostoma sp.1 was isolated from two *P. bidentatus* (3.3%). Both beetles were collected in Grue. ITS and β-tubulin-data gave 95% identity match on *Ambrosiella* sp. 3Y7TP-A1 (DQ268583.1). The identity match was low considering the identity determination of this species. *Ophiostoma* sp.1 might be a new species, but more genes must be sequenced (Halvor Solheim per. comm.). In this study the fungus was presented as *Ophiostoma* sp.1, because of the replacement of *Ambrosiella* in the taxonomy (de Beer et al. 2013). *Ophiostoma* sp.1 has also been isolated from *P. chalcographus* collected in Ullensaker (Ruben Lindseth; unpublished master thesis 2015). This indicates that this species might be common in Norway, since the fungus is isolated from two different beetle species and at two different locations.

4.2 Other fungal associations

A total of thirty-six fungal (This under chapter will not include *Ophiostoma* species in the summarizing) species other than ophiostomatoid fungi were found to be associated with the three investigated beetles (*Table 4, 6 & 8*). *Pityogenes bidentatus* was associated with 19 different fungi species and *P. quadridens* was associated with 24 different species. *Ips acuminatus* was associated with seven different species of fungi. All of the species found, in this study, will not be discussed. Some of the isolated species are common while other species are very difficult, with limited litterateur (Halvor Solheim, pers. comm.). The most abundant fungi to be associated with the beetles will be discussed.

Some of the species were more abundant then other species *Allantophomopsis* sp.1, *Geosmithia* sp.1, *Penicillium* species, *Phacidiopynis* sp.1 and *Phoma* spp. were the most abundant species. *Allantophomopsis* sp.1 was associated with the two investigated *Pityogenes* species. The fungi occurred occasionally with *P. bidentatus*. However, it was one of the most abundant species associated with *P. quadridens*. The

fungus was associated with 53.3% of the *P. quadridens* caught at Grue and with 13.9% of the beetles from Frogn. In total, the fungus was isolated with the frequency of 31.8%. The nearest match, with search in GenBank, with this fungi species was *Allantophomopsis lycopodina* (Höhnel) Carris. The search gave a 99% identity match. *Allantophomopsis lycopodina* is described as a black rot fungus (Carris 1990). The fungus is earlier found to infest *Larix occidentalis* (Nuttall), *Lycopodium complanatum* (Linné), *Pinus monticola* (Douglas ex. D. Don), *Pinus nigra* sp., *Pseudotsuga menziesii* (Mirbel), *Vaccinium macrocarpon* (Aiton) (Carris 1990). Some newer studies describe *Allantophomopsis lycopodina* also to be associated with litter of Scots pine (Koukol et al. 2009; Koukol & Baldrian 2012). However, the species is not earlier found to be a common associate of the two investigated *Pityogenes* species. The observed fungi from this study fits with the morphological description of the *Allantophomopsis lycopodina* (Carris 1990). However, the identity is not 100% with earlier ITS alignments from the GenBank. In this study the specie is concluded to be close relative of *Allantophomopsis lycopodina*. The decision is taken because of finding from earlier studies and since the identity was 99%. More studies are needed to determine the identity on the presented species.

Three different *Geosmithia* species were associated with *P. bidentatus,* and these were *Geosmithia* sp. 1*, Geosmithia* sp. 2 and *Geosmithia* sp. 3. However, *Geosmithia* sp. 2 and *Geosmithia* sp. 3 were only associated with one beetle. *Geosmithia* sp.1 had 99% (ITS-data) identity match with Geosmithia sp. 24 NL-2014 strain (KF808311.1). *Geosmithia* sp. 2 had 100% (ITS-data) identity match with Geosmithia sp. 31 NL-2014 strain RJ21K (KF808318.1). *Geosmithia* sp. 3 had 100% (ITS-data; ITS1-F) identity match with *Geosmithia* sp. MK1837 (HE604165.1). *Geosmithia* sp. 1 was commonly found in Grue, with a 50% frequency. The species were less frequent in Engerdal and Flesberg, with only 5% frequency at each site. *Pityogenes quadridens* was only associated with one *Geosmithia* species, and this was *Geosmithia* sp. 1. The frequency of *Geosmithia* sp. 1 associated with *P. quadridens* was 33.3% for Grue and 30.5 % for Frogn. The result indicates *Geosmithia* sp.1 to be common associates of the two investigated *Pityogenes* species, although, *Geosmithia* sp.1 did occur as less frequent in Engerdal and Flesberg. The frequency difference may have been caused by differences amongst the collected material (branches vs. logs) or the exposer of the material. *Geosmithia* species are commonly found in open areas (branches) that are exposed to rapid desiccation (Jankowiak 2014; Jankowiak & Rosa 2008). This area description is well suited with Grue, Frogn and Engerdal but the description does not

match with Flesberg (see chapter 2.1). This theory can therefore only explain the lack of *Geosmithia* species in Flesberg and not for Engerdal. More studies are probably needed to explain this topic.

Geosmithia species are earlier found to be associated with *P. bidentatus* (Jankowiak 2014; Jankowiak & Rosa 2008). *Pityogenes quadridens* are not earlier found to vector *Geosmithia* species. However, beetles caught in thin barked parts have earlier been described to vector *Geosmithia* species (Jankowiak & Kolařík 2010a; Jankowiak et al. 2014). *Pityogenes bidentatus, P. chalcographus* and *Polygraphus poligraphus* are species which breed under thin and thick barked parts, these beetle are also known to vector *Geosmithia* (Jankowiak et al. 2014). Jankowiak et al. (2014) suspected that the total number of *Geosmithia* species varied significantly along the gradients towards the thickness of wood substratum preferred by pine-and spruce-infesting beetles. Information about bark beetle-associated *Geosmithia* species on trees of the *Pinaceae* family is very limited (Jankowiak et al. 2014). This is the main cause of the difficulty of determining the *Geosmithia* species. However, a group of scientists are currently working with describing *Geosmithia* species, also including the species found in this study (Miroslav Kolařík, pers.com.)

Penicillium species were isolated from *P. bidentatus, P. quadridens* and *I. acuminatus*. Two species of *Penicillium* were associated with *P. bidentatus* and five different species of *Penicillium* were associated with *P. quadridens*. Only two specimens of *I. acuminatus* were associated with *P. raistrickii. Penicillium raistrickii* was associated with all three of the beetle species and was the fourth most abundant species to occur with *P. quadridens*. However, most of the *Penicillium* species occurred with low frequency. The result indicates that *P. bidentatus, P. quadridens* and *I. acuminatus* are suitable vector species for *Penicillium*. This is also confirmed by other studies (Mathiesen-Käärik 1953: Jankowiak & Rossa 2008). *Penicillium* is widely distributed in nature and is also known to be associated with other insects (Jankowiak & Rossa 2008).

Phacidiopynis sp.1 was associated with two specimens of *P. bidentatus*, twenty *P. quadridens* and with one specimen of *I. acuminatus*. The fungi were found on all locations except on Engerdal. The nearest match with this fungi species was *Phacidiopycnis washingtonensis*, with a 99% identity match. *Phacidiopycnis washingtonensis* was first found in Washington State and described as new *Phacidiopycnis* species by Xiao et al. (2005). Normally this fungus is associated with pome fruits. The species is not earlier described to be associated with conifers trees

(Weber 2011; Xiao et al. 2005). The *Phacidiopycnis* sp.1 found in this study is therefore most likely not *Phacidiopycnis washingtonensis*, but a close relative to the fungi.

Phoma spp. were associated with four specimens of *P. bidentatus*, one specimen of *P. quadridens* and with eighteen specimens of *I. acuminatus.* The species was difficult to align in BLAST searches. PCR-products from this species were sent to GATC-Biotech although the result was negative. It was too much disturbance on both of the nucleotides. This resulted in new purification of older samples, and a two-week setback, since the whole procedure had to be done again. The problem with the identification may have been caused by pollution, content of other species or use of wrong primers. The two first theories are more likely to cause the problem. In most cases the nucleotides were double and showed a high grade of disturbance. This is normal for samples containing more than one type of DNA.

Basidiomycetes fungi were associated with two of the investigated beetle species. *Fuscoporia* sp.1 was associated with 15% of the investigated *P. bidentatus* beetles. *Entomocorticium* sp.1 and *Fuscoporia* sp.1 *Phellinus* sp. were associated with *P. quadridens*. *Phellinus* sp. was only associated with one specimen and will therefore not be discussed. However, the results from this study confirm that *Basidiomycetes* species are suitable to be associated with bark beetles. *Basidiomycetes* have earlier been reported to be associated with bark beetles, although with few beetle species (Harrington 1993a; 1993b). Some of the *Basidiomycetes* are considered to be important sources of nutrients for larva or young adults (Harrington 1993a; 1993b).

Entomocorticium species are earlier reported to be associated with *Dendroctonus ponderosae* (Hopkins, 1902), *D. brevicomis* (LeConte, 1876), *D. jeffreyi* (Hopkins, 1909) (Hsiau & Harrington 2003). Some of the *Basidiomycetes* are also considered to infest pine species (Hsiau & Harrington 2003). The result of this study provides that *Entomocorticium* species are also found to be associated with *P. quadridens*. However, *Entomocorticium* might be difficult to identify. *Entomocorticium* species are described as a new group of fungi that have evolved from a *Peniophora* ancestor (Hsiau & Harrington 2003). Hsiau & Harrington (2003) have described the new species to be more adapted to beetle dispersal, then the earlier related specie. The species found here in Norway might be closely related to these species, but more studies are needed to confirm this theory.

Fuscoporia sp.1 was associated with *P. bidentatus* (15%) and eight *P. quadridens* (12.1%). The fungi species occurred in four different sampling areas. This result indicates *Fuscoporia* sp.1 to be a common associate of the two beetle species. Although, the closest match with this fungus species was *Fuscoporia gilva* (99% identity match). This species is not known to exist in Europa (Ryvarden & Gilbertson 1994). The fungus found in this study is therefor presented as *Fuscoporia* sp.1. However, more investigations on the presented species are needed to identify the species.

4.3 *Pityogenes bidentatus* and *P. quadridens*

Pityogenes bidentatus was associated with several of fungi. These results indicate that the *Pityogenes* species are more generalized in their association with fungi. Phloem-feeding bark beetles are commonly associated with various fungi (Kirisits 2004). Results from Jankowiak & Rossa (2008) supports this result, as they found 34 fungus species to be associated with *P. bidentatus*. The high amount of associated fungus taxa might be explained by the time of the beetle collecting. The beetles from this study and the beetles from Jankowiak & Rossa (2008) were collected late in the year. Beetles collected late in the year might be associated with more fungus species, especially with secondary fungus species, than beetles caught earlier in the year. However, the fungi that are associated with the beetle in late autumn might also be associated with beetles in the spring/early summer. The time of the collecting might be important, but the hypothesis needs to be studied further. The ophiostomatoid fungi are known to have evolved adaptions to the symbiosis with their host, such as sticky ascospores and conidia (Malloch & Blackwell 1993; Six 2003). Fungi species without these adaptions might therefore easier be worn off when the beetles are flying or when developing their galleries. The theory might not be suitable with the result in this study, because the ophiostomatoid species were less frequent with the two *Pityogenes* species. However, earlier studies indicate ophiostomatoid species to occur occasionally with *P. bidentatus* (Jankowiak & Rossa 2008; Jankowiak et al. 2014). *Pityogenes quadridens* seems also to be loosely associated with ophiostomatoid fungi (Mathiesen-Käärik 1953). Jankowiak et al. (2014) describes ophiostomatoid species to be less associated with beetles caught in drier substrata, particularly *P. bidentatus*, which infest thin rapidly desiccating branches. Bark beetle species that breed in drier substrate are not able to maintain mutualism with *Ophiostoma* species, because these types of fungi are more sensitive to desiccation (Jankowiak et al. 2014). This theory is in agreement with the results of Mathiesen-Käärik (1960). Mathiesen-Käärik (1960)

observed that different ophiostomatoid fungi have very specific nutritional and moisture requirements, as well development times. Those two theories might as well apply for *P.* quadridens. However, this theory is based on the facts that P. *bidentatus* and *P. quadridens* prefer the same host and habitat (Ehnstöm & Axelsson 2002). In this study, the two theories suit better with *P. quadridens*. Because *P. bidentatus* was highly associated *Graphilbum* sp.1.

Associations between fungi and *P. quadridens* are poorly investigated (as earlier mentioned). The studies of Mathiesen (1950) and Mathiesen-Käärik (1953) and the determination of the fungi are based on morphological characters. This study relays on DNA. Therefor it is difficult to compare the results from this study with Mathiesen (1950) & Mathiesen-Käärik (1953). Associations between fungi and *P. quadridens* need to be more studied.

4.4 *Ips acuminatus*

Ips acuminatus was associated with fewer fungi then the other two investigated beetles. This was also seen at an early stage in the purification procedure. The result corresponds well with the study by Mathiesen-Käärik (1953). She investigated 86 specimens of *I. acuminatus* and found 11 species of fungi to be associated with the beetle species. However, *O. macrosporum* was expected to be one of the most frequent species to be associated with *I. acuminatus* (Francke-Grosmann 1952). The fungus is known to grow in the mycangia of *I. acuminatus*. The isolated fungus might have been associated with the mycangia of the two beetles. The beetles should have been studied more thoroughly under microscope to determine this phenomenon. In this study *O. macrosporum* was associated with two specimens of *I. acuminatus* so the frequency was low. There can be several explanations to why the frequent of isolates were low. The beetles in this studied were frozen in three months at 18°C, which might have had an impact on the cultures of *O. macrosporum* and other cultures. *Pityogenes bidentatus* and *P. quadridens* were associated with a high occurrence of different fungus. The three different beetle species were handled in the same why, and frozen under the same conditions, but *I. acuminatus* was the last beetle to be investigated. Therefor the beetle were frozen for a longer period then the other beetles. *Ophiostoma macrosporum* grows in mycangia while other species survive as spores. *Ophiostoma macrosporum* might be more sensitive to sudden temperature drop, then other fungi species. This theory has also been discussed in other studies (Kirisits 2004 and references therein). This may explain the low frequency of *O. macrosporum*. However, the fungus is also known to be capable to survive in

different winter conditions. The temperature differences might therefore not be so important. Slow growth of *O. macrosporum* and overgrown by other fungus may also be a possibility to the low occurrences of the fungus. Fast growing fungi will use lesser time to grow hyphae in the MEA. In some cases this types will dominate the Petri dish and other fungus species may not show their presence before a later stage. Some fast growing species needed only three days until the hyphae were possible to transfer to new Petri dishes. Therefor some species may still be presence in the MEA, but not been isolated. *Ophiostoma macrosporum* might not occur before several months later than other fungi species and therefore not been registered. It is also a possibility that *O. macrosporum* is less common in Norway. However, investigations of more *I. acuminatus* specimens and other samplings areas are needed to determine the occurrence of this species, in Norway.

I. acuminatus was highly associated with spp. and *Graphilbum* sp.1. The relationship with *I. acuminatus* and the two isolated fungus are unclear. More studies are needed to determine the relationship between *I. acuminatus* and the two isolated fungus.

4.5 General

Beetles are not easy working with, as they are small, very active and often occur in great numbers. Each part of a dead tree can host several galleries from different beetles. Some tunnels might go closely beside each other or even overlap. This causes a potential for the fungi spores to infect other vector species. Potential contact with other beetle galleries or contacted with infected areas. The question will therefore be if the first invader was caught or if it was a second invader. Some of the earlier invaders might have left the tree, for hibernation in the litter. While the second invaders still might be found in the wood. The potential for exchanging spores between beetle species is high and will be higher for beetles caught late in the year. Aggressive beetle vs. nonaggressive beetle may vector different species. Total number of beetle species hosted in the tree sample may therefore be important for the result.

Many mite species are associated with bark beetles (Hofstetter et al. 2015; Lieutier et al. 2015). Most mite species can attach themselves to the cuticle of the emerging beetles, in purpose of transport to a suitable host (Lieutier et al. 2015). *I. acuminatus* is known to vector mites (Lieutier et al. 2015). This was a problem with the investigated *I. acuminatus* beetles. Numerous of mites were attached to different body parts. The problem was not so common with the *Pityogenes* species. Although, several of the main Petri dishes, from all three species, were destructed because of

containing mites (or traces in the MEA). Mites are suitable to vector fungi (Hofstetter et al. 2015). Phoretic mites are known to play an important role in transmissions of ophiostomatoid fungi (Kirisits 2004 and references therein). Some mites have even developed specialized structures, such as sporotheca; organ for transmission of fungi (Kirisits 2004 and references therein). However, the impact of mites in this study will be low since the beetles were cleansed for mites (Chapter 2.5) and most of the main Petri dishes were purified 1-4 times before getting destructed.

Several of the samples were not sequenced because of unreadable alignments. Some species might therefore not be identified. The problem might be less important because of the high frequency of fungi. All of the samples that came back negative were less frequent species. However, the species might appear as different species, based on morphologic characters, because of contamination by other fungi. In this cases the species might already be identified, but have been thought to be different because of the morphologic characters. It is also a possibility that some fungi have not been purified and isolated. Some species might be slow growing and occur at a later stage. The theory was also confirmed by an examination of the main dishes. The observed fungi were not purified because of limited time.

ITS primers had some problems with identifying species within the ophiostomatoid fungi. In several cases, the ITS data did not distinguish clearly between closely related species. This phenomenon is also been reported from an earlier study (Linnakoski et al. 2010). Some species have not been sequenced with ITS-primers and therefore give various matches in the GenBank. Therefore it will be important to use other primers in addition to the ITS-primers. β-tubulin gave some good results, but most of the samples were only readable one way. However, β-tubulin were important in determine *O. macrosporum.* Sequencing the EF1-α results was not successful. This might have been caused by something wrong with the primers or with the method that were used in this study. The last hypothesis is most likely. The problem might be explained by the different temperatures used for the amplification process (see method; under chapter 2.6.2). Linnakoski et al. (2012) used a different amplification process for the EF1-α that gave good results.

Kirisits (2004) discusses how the isolation method might be important for the outcome of the result. Some studies use antibiotic solutions mixed in the MEA, such as tetracycline and cycloheximide (Jankowiak et al. 2014; Lu et al. 2009). Some fungi are reported to be sensitive to cycloheximide, such as *Ceratocystis* species (Linnakoski et al. 2012). These chemicals might therefore be selective to the

occurrences of fungi. Methodological factors should therefore be considered, when comparing results with other results (Kirisits 2004). However, in this study chemicals were not used. Therefore the result might have higher occurrences of fungi then other studies.

5.0 Conclusion

The investigated beetles were association with several of fungi species. Some of the fungi species might be new for science, although more investigations are needed to confirm this. However, the result from this study shows that bark beetles in Norway are associated with a variety of fungi species. Some of the species are not earlier been reported from Norway. More beetle specimens from the investigated beetles and other bark beetles species should therefore be investigated. There are certainly more fungi species in Norway, that yet not have been reported.

More time would have given more opportunities to control the main dishes and opportunities to learn about and test other gene regions. The choice of primers was based on earlier reports. However, there are other primers that should have been tried, such as TEF-1a. Other studies should therefore investigate different possibilities in use of primers. Studies have started to sequence several of different gene regions (Yin et al. 2014). However, comparing the sequences with earlier GenBank results might be a problem, because of available data.

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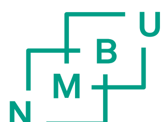

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