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OPHIOSTOMATOID FUNGI ASSOCIATED WITH BARK BEETLES (COLEOPTERA: SCOLYTIDAE) COLONIZING BRANCHES OF *PINUS SYLVESTRIS* IN SOUTHERN POLAND

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Abstract. Bark beetles (Coleoptera: Scolytidae) infesting branches of Norway spruce (*Picea abies*) are known to be associated with fungi, especially species of *Ophiostoma* s.l. and *Ceratocystis*. Very little is known about these fungi colonizing branches of Scots pine. In this study we examined the ophiostomatoid species associated with three species of bark beetles infesting Scots pine (*Pinus sylvestris*): *Pityogenes bidenatus*, *P. chalcographus* and *Pityophthorus pityographus*. Fungi were isolated from the beetles and their galleries at six sites in southern Poland. A total of 264 fungal isolates belonging to 12 ophiostomatoid species were identified, including 4 associations between fungi and *P. bidenatus* not previously recorded. Except for *P. chalcographus*, all bark beetles species were rarely associated with ophiostomatoid fungi. The most commonly encountered fungal associate of *P. chalcographus* was *Ophiostoma ainoae*. Nine other ophiostomatoid species were isolated at low frequencies. *Pityogenes bidentatus* vectored six and *P. pityographus* vectored two ophiostomatoid species. These species were occasionally isolated from beetles and their galleries, suggesting a non-specific relationship.

Key words: ophiostomatoid fungi, insect-fungus interactions, Scots pine

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INTRODUCTION

Beetles of the family Scolytidae are associated with various organisms such as mites, nematodes, viruses, bacteria and fungi. Phloem-feeding bark beetles are frequently associated with specific fungi which are transmitted in specialized structures or on the insect's body. The most common mycelial fungi associated with bark beetles are ophiostomatoid fungi, including the morphologically similar genera *Ceratocystis* Ellis & Halst., *Ceratocystiopsis* H. P. Upadhyay & W. B. Kendr., *Cornuvesica* C. D. Viljoen, M. J. Wingf. & K. Jacobs, *Gondwanamyces* G. J. Marais & M. J. Wingf., *Grosmannia* Goid. and *Ophiostoma* Syd. & P. Syd. These genera are intimately associated with many bark beetle species infesting *Pinus* L., *Picea* A. Dietr. and *Larix* Mill. species in Europe (Kirisits 2004). These fungi are transferred to the tree during beetle attacks and due to their phytopathogenicity may compete with other organisms for food and survive in fresh tissues of trees (Six & Wingfield 2011). Apart from ophiostomatoid species, anamorphic fungi of the genus *Geosmithia* Pitt (Ascomycota: Hypocreales) recently were found associated with beetle species on conifers (Kirschner 2001; Kolařík 2006; Jankowiak & Rossa 2008; Jankowiak & Kolařík 2010).

Insects from the genera *Pityogenes* and *Pityophthorus* are frequent pests of thin branches of Scots pine (*Pinus sylvestris* L.) in Poland. Among them, the two-toothed pine beetle *Pityogenes bidenatus* (Herbst), the six-toothed spruce bark beetle *Pityogenes chalcographus* (L.) and the fir bark beetle *Pityophthorus pityographus* (Ratz.) have been commonly reported on branches of mature Scots pine (Korczyński & Kuźmiński 2007). Although they are generally considered secondary pests, *P. chalcographus* can kill healthy trees in some Norway spruce stands of the Polish mountains (Grodzki 2004).

Little is known about ophiostomatoid fungi associated bark beetle species colonizing Scots pine branches. In Europe there were only four

reports on fungal isolation from *Pityogenes* species on *Pinus sylvestris*. Two studies reported loose associations of *Pityogenes quadridens* (Hartig) with *Leptographium lundbergii* Lagerb. & Melin, *Ophiostoma canum* (Münch) Syd. & P. Syd. and *O. piceae* (Münch) Syd. & P. Syd. (Mathiesen 1950; Mathiesen-Käärik 1953). Similar loose associations with ophiostomatoid fungi were found in *P. bidentatus* on young Scots pine trees (Jankowiak & Rossa 2008). Recently, Linnakoski *et al.* (2010) found *O. brunneo-ciliatum* Math.-Käärik, *O. canum*, *O. canum*-like, *O. floccosum* Math.-Käärik and *O. minus* (Hedgc.) Syd & P. Syd., and described the new species *O. fuscum* Z. W. de Beer & M. J. Wingf., *O. saponiodorum* Z. W. de Beer & M. J. Wingf., and *O. tapionis* Linnakoski, Z. W. de Beer & M. J. Wingf. associated with *P. chalcographus* in Finland and Russia. They collected the samples from *P. sylvestris* as well as Norway spruce [*Picea abies* (L.) H. Karst.].

The fungal associates of *P. chalcographus* have been studied mainly on Norway spruce (Mathiesen 1950; Mathiesen-Käärik 1953; Kotýnková-Sychrová 1966; Krokene & Solheim 1996; Kirisits *et al.* 2000; Kirschner 2001; Kirisits & Kondrad 2006; Jankowiak *et al.* 2009). The known fungal associates of *P. chalcographus* on Norway spruce are ascomycetes in the genera *Grosmannia*, *Ophiostoma*, *Ceratocystis* and *Ceratocystiopsis*, and the anamorphic genera such as *Graphium* and *Leptographium* (Krokene & Solheim 1996; Kirisits *et al.* 2000; Kirschner 2001; Jankowiak *et al.* 2009).

The information about ophiostomatoid fungi associated with *P. pityographus* on Pinaceae in Europe is even more limited than for *Pityogenes* spp. There was only one report about fungal isolations from *P. pityographus*. Jankowiak *et al.* (2009) found that seven species of *Ophiostoma* s.l., especially *Ophiostoma ainoae* H. Solheim were associated with *P. pityographus* on Norway spruce in Poland. No association between *P. pityographus* and fungal species on Scots pine has yet been reported.

In this study, the species composition and frequency of occurrence of ophiostomatoid species associated with three species of bark beetles developing in branches of Scots pine were investigated.

Materials and methods

Collecting bark beetles and their galleries

During 2007, adult bark beetles *Pityogenes bidentatus*, *P. chalcographus*, *P. pityographus*, (Coleoptera: Scolytidae) and their galleries were collected from windblown Scots pine trees with a mean age of 50 years, in six stands in southern Poland. These stands were sampled to characterize the dominant bark beetles and their fungal associates. Three sites (Stanisławice – 50°00′50″N, 20°21′16″E; Hysne – 50°02′01″N, 20°16′41″E; Mielec – 50°19′08″N, 21°30′41″E) had stands of more than 90% Scots pine with a small share of Norway spruce, white birch (*Betula pendula* Roth.) and European larch (*Larix decidua* Mill.). One site (Czajowice – 50°12′02″N, 19°48′59″E) was located in old mixed stand dominated by European beech (*Fagus sylvatica* L.). For the last two sites (Rączna – 50°00′39″N, 19°45′41″E; Goszcza – 50°11′07″N, 20°04′20″E) the share of Scots pine over 60–80% was typical, with admixture of English oak (*Quercus robur* L.) and Norway spruce.

In May 2007, at each site a total of 18 sections of branches (40 cm long, 1.1–11.6 cm in diameter) were taken from three neighboring windblown Scots pine trees (six branches per tree crown). The same branches were used for collecting both adult bark beetles and their galleries.

Then the branches were transferred from the forest to the laboratory. Their outer bark was stripped within 48 hours of collecting. The adult bark beetles were removed from their galleries with sterilized tweezers. They were placed individually in sterile microtubes (1.5 ml) and identified, using a Nikon 1500SZ microscope, by external characters according to Nunberg (1981). A total of 393 individuals and 139 galleries of bark beetles were collected.

Isolation and identification of fungi

Within 48 hours of collection, the beetles without surface-sterilisation were crushed with a sterile scalpel on a microscopic slide, and the macerate was spread evenly over the surface of the medium placed in Petri dishes.

The galleries were disinfected using cotton wool saturated with 96% ethanol. The disinfection lasted *ca* 15 seconds. Then the galleries were dried on filter paper. The surface layer of phloem was removed with a sterile scalpel. Fragments of sapwood (4 × 4 mm in size) were cut out with a sterile chisel and placed on culture medium in Petri dishes. Samples were collected from the discoloured sapwood underneath the galleries up to the depth of 10 mm into the sapwood. Altogether, 834 samples of sapwood were collected.

All isolations were made on 2% malt extract agar (MEA; 20 g malt extract, Difco, Detroit, Michigan; 20 g agar Fluka Chemie GmbH, Madrid, Spain; and 0.2 g tetracycline, Polfa Tarchomin SA., Poland; all per litre distilled water). When necessary, cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh 2% MEA. Cultures were incubated at room temperature in the dark. Fungi were identified on the basis of morphological characteristics of the ascomata, ascospores, conidiophores and conidia. Ascomata and conidiophores were mounted in lactophenol on glass slides and characterized using light microscopy. Fungal structures were compared with the species descriptions given in the literature (Upadhyay 1981; Solheim 1986; Jacobs & Wingfield 2001). Comparisons of fungal isolates with known cultures from the collection of the Laboratory of Department of Forest Pathology (Agricultural University of Cracow, Poland) were also made.

STATISTICAL ANALYSES

As in previous studies (Lee *et al.* 2006), the Simpson's index of diversity (Simpson 1949) was used to characterize fungal diversity:

$$D = 1 - \sum_{i=1}^{i=s} P_i^2$$

where: P_i is the probability of sampling species 'i', counted in the case of bark beetle adults, as the ratio between the number of individuals of a given bark beetle species carrying the respective fungus and the total number of individuals of a given bark beetle species; in the case of fragments from bark beetle galleries expressed as the ratio between the number of fragments from which a given fungal species was isolated and the total number of fragments examined. S is the number of species per sample. Fungal dominance was determined by Camargo's index (1/*S*) (Camargo 1993), where S represents the number of species (species richness). Particular species of fungi were considered as dominant in cases where $Pi > 1/S$.

RESULTS

A total of 393 individuals of three bark beetle species yielded a total of 120 fungal isolates (Table 1). A total of 139 galleries of bark beetles yielded a total of 144 isolates of fungi (Table 2). These 264 isolates included a total of 12 species of *Ceratocystiopsis*, *Ophiostoma* s.l. and/or their asexual stages (Tables 1 & 2).

From the *Pityogenes bidentatus* adults 10 fungal isolates comprising three species were obtained (Table 1). *Ophiostoma ainoae*, *O. minus* and *Leptographium* sp. had very low isolation frequency (< 3%). Ninety-nine species of fungal isolates, representing seven species, were found in association with the *P. chalcographus* bodies. The most dominant species was *O. ainoae*, isolated from 38% of the beetles. *Ophiostoma piceae* was isolated from 11% of the beetles, while *Graphium fimbriisporum* (M. Morelet) K. Jacobs, Kirisits & M. J. Wingf., *Grosmannia piceiperda* (Rumbold) Goid., *O. bicolor* R. W. Davidson & D. E. Wells, *Leptographium* sp. and *Ophiostoma* cf. *rectangulosporium* Ohtaka, Masuya & Yamaoka were found at low frequencies. Only a single ophiostomatoid species, *O. ainoae*, was found on the *P. pityographus* beetles. This species was present on 19% of the beetles (Table 1).

A total of 19 isolates, representing six species, were collected from galleries of *P. bidentatus*. All of the fungal species present on adults were also found in the galleries, however, three species [*O. canum* and *O. piliferum* (Fr.) Syd. & P. Syd. and *O.* cf. *rectangulosporium*] were occasionally found in galleries but were not isolated from insects. Other species were also occasionally isolated from galleries of *P. bidentatus* (Table 2). Nine species

Table 1. Isolation numbers and frequencies (in parentheses)[1] of ophiostomatoid fungi[2] associated with beetles of bark beetles occurring on *Pinus sylvestris.*

Fungi	*Pityogenes bidentatus*	*Pityogenes chalcographus*	*Pityophthorus pityographus*
Graphium fimbriisporum		9(6.8)	
Grosmannia piceiperda		9(6.8)	
Ophiostoma ainoae	2(1.0)	**50(37.9)**	11(19.0)
Ophiostoma bicolor		6(4.5)	
Ophiostoma minus	2(1.0)		
Ophiostoma piceae		14(10.6)	
Ophiostoma cf. *rectangulosporium*		1(0.8)	
Leptographium sp.	6(3.0)	10(7.6)	
Total no. of fungal isolates	10	99	11
Number of species (S)	3	7	1
Simpson's index of diversity (D)	0.62	0.70	0
Number of investigated beetles	203	132	58

[1] Frequency = (no. of individual beetles carrying the respective fungus / total number of beetles) × 100
[2] Bold type indicates dominant ophiostomatoid species

of ophiostomatoid fungi were identified in 124 isolates from gallery systems of *P. chalcographus*. *Ophiostoma ainoae* was the most numerous species, isolated from 27% of investigated fragments of galleries. In contrast to the results from beetles, *Ceratocystiopsis minuta* (Siemaszko) H. P. Upadhyay & W. B. Kendr. and *L. lundbergii* were also found in galleries of *P. chalcographus*. These and other species were isolated at very low frequencies. Only a single ophiostomatoid species, *Leptographium* sp., was found in the *P. pityographus* galleries (Table 2).

The fungal community associated with *P. chalcographus* beetles showed the highest number of species and the highest diversity. It was dominated by *O. ainoae*. In contrast to the results from the beetles, the highest fungal diversity in gallery systems was found in *P. bidentatus*. The lowest fungal diversity was typical of *P. pityographus* populations (Tables 1 & 2).

Discussion

The study demonstrates the low diversity of ophiostomatoid fungi associated with the three species of bark beetles in the branches of Scots pine in southern Poland. Twelve species of ophiostomatoid fungi, including *Ceratocystiopsis*, *Grosmannia*, *Ophiostoma*, *Leptographium* and *Graphium* spp., were isolated from bark beetles.

The community of the ophiostomatoid fungi associated with *P. chalcographus* on *P. sylvestris* was similar to the spectrum of fungi reported from *Picea abies* in other parts of Europe (Mathiensen 1950; Mathiesen-Käärik 1953; Kotýnková-Sychrová 1966; Krokene & Solheim 1996; Kirisits *et al.* 2000; Kirschner 2001; Kirisits & Konrad 2006; Jankowiak *et al.* 2009). Similar to the Polish study (Jankowiak *et al.* 2009), *O. ainoae* was the most commonly encountered fungal associate of bark beetles. However, we isolated other fungal associates of *P. chalcographus* than Linnakoski *et al.* (2010). In Finnish and Russian studies, *Ophiostoma brunneo-ciliatum*, *O. canum*, *O. canum*-like, *O. floccosum*, *O. fuscum*, *O. minus*, *O. saponiodorum* and *O. tapionis* have been recorded in association with *P. chalcographus*, possibly due to different fungal identification methods used. In our study, the identification of fungi based only on their morphology could limit the number of species, especially cryptic taxa. The climatic conditions and different host plants could also influence the fungal associates of *P. chalcographus* (Six & Bentz 2007) in the boreal forests of Karelia and in Poland.

Unexpectedly, the assemblage of fungi associated with *P. bidentatus* was different from that in an early Polish study (Jankowiak & Rossa 2008). While *O. minus, O. piceae* and *O.* cf. *rectangulosporium* (named as *Graphium* sp. 'W') were isolated from the two-toothed pine beetle in accordance with the previous study (Jankowiak & Rossa 2008), we also isolated *O. ainoae*, *O. canum*, *O. piliferum* and *Leptographium* sp. These species were not previously known as associates of *P. bidenatus*. The isolation substrates in each study might have affected the species found. In this work, the beetles and galleries were collected from Scots pine branches where *P. bidenatus* cohabit with the other species of bark beetle (e.g. *P. chalcographus*, *Tomicus* spp.). In the previous study the beetles were collected from 4- to 8-year-old pine trees infested only by *P. bidenatus*. Therefore, the presence of a higher number of ophiostomatoid species on the two-toothed pine beetle on Scots pine branches is likely due to cross-contamination with fungal associates of other bark beetles.

In this work we isolated only two ophiostomatoid species (*O. ainoae* and *Leptographium* sp.) from *P. pityographus*. In the previous report they were also found on the beetles and galleries of *P. pityographus* on *P. abies* (Jankowiak *et al.* 2009). However, apart from these two species, Jankowiak *et at.* (2009) reported also *Graphium fimbriisporum*, *Grosmannia piceiperda*, *Ophiostoma bicolor* and *O. piceae* as fungal associates of *P. pityographus* on *P. abies*. In this study, major factors that might have contributed to the differences in *P. pityographus* mycobiota were the different species of host plant. The fir bark beetle breeds mainly in Norway spruce but has also been recorded on other conifers (Michalski & Mazur 1999). It seems that fungal associates of *P. pityographus* are more adapted to colonize spruce trees than pine trees. In the present study a similar phenomenon was also observed for *P. chalcographus*, because ophiostomatoid species occurred less frequently than in a complementary study on Norway spruce (Jankowiak *et al.* 2009). Similar relations have also been documented for

Table 2. Isolation numbers and frequencies (in parentheses) [1] of ophiostomatoid fungi[2] associated with galleries of bark beetles occurring on *Pinus sylvestris.*

Fungi	*Pityogenes bidentatus*	*Pityogenes chalcographus*	*Pityophthorus pityographus*
Ceratocystiopsis minuta		4(1.2)	
Graphium fimbriisporum		10(3.1)	
Grosmannia piceiperda		6(1.9)	
Leptographium lundbergii		1(0.3)	
Ophiostoma ainoae	4(1.1)	**86(26.5)**	
Ophiostoma bicolor		5(1.5)	
Ophiostoma canum	2(0.5)		
Ophiostoma minus	6(1.6)	2(0.6)	
Ophiostoma piceae		6(1.9)	
Ophiostoma piliferum	1(0.3)		
Ophiostoma cf. *rectangulosporium*	2(0.5)		
Leptographium sp.	4(1.1)	4(1.2)	1(0.7)
Total no. of fungal isolates	19	124	1
Number of species (S)	6	9	1
Simpson's index of diversity (D)	0.83	0.51	0
Number of investigated fragments	372	324	138
Numbers of galleries	62	54	23

[1] Frequency = (no. of fragments from which a given species was isolated / total number of fragments) × 100
[2] Bold type indicates dominant ophiostomatoid species

Ips typographus (L.) (Jankowiak & Hilszański 2005) and *Hylurgops palliatus* (Gyll.) (Jankowiak 2006; Jankowiak *et al.* 2009).

Apart from *P. chalcographus*, other bark beetles species were rarely associated with ophiostomatoid fungi. The relatively weak relationships with ophiostomatoid fungi were similar to those recorded for *P. bidentatus* (Jankowiak & Rossa 2008) and *P. quadridens* (Mathiesen-Käärik 1953) on *P. sylvestris*. In Polish studies (Jankowiak & Rossa 2008), *P. bidentatus* carried ophiostomatoid species very rarely and this bark beetle was frequently associated with fungi of the genus *Geosmithia*. In our study *Geosmithia* fungi were also isolated from *P. bidenatus* and *P. pityographus* at high frequencies. The community of the *Geosmithia* fungi associated with these bark beetles is currently under investigation and details will be given in a later report. Probably, bark beetles, such as *P. bidenatus* and *P. pityographus* which breed in rapidly desiccating branches of trees are not able to maintain the mutualism with ophiostomatoid fungi, which are more sensitive to desiccation than *Geosmithia* species (Jankowiak & Rossa 2008). Recently this hypothesis has been confirmed by studying *in vitro* the effects of temperature on growth and survival of colonies of two *Geosmithia* species isolated from *P. sylvestris* (Jankowiak 2011). This test showed that *Geosmithia* species grew well at temperatures ranging from 25 to 35°C. Additionally, a few colonies of *Geosmithia* spp. survived 4 days of exposure to 55°C (Jankowiak 2011). These results suggest that *Geosmithia* spp. as heat-tolerant species can dominate in the fungal communities associated with bark beetle species infesting branches subject to strong insolation. Similar competition between ophiostomatid fungi has also been observed in *Dendroctonus ponderosae* Hopkins (Six & Bentz 2007).

This study confirmed that *P. bidenatus* and *P. pityographus* infesting branches of Scots pine were rarely associated with ophiostomatoid fungi. These bark beetles species were more commonly associated with *Geosmithia* spp. In contrast to previous work, we have shown that the fungal community of *P. bidentatus* was more diverse in branches of old trees than in stems of young pine trees. This may indicate that the composition of fungal associates of *P. bidentatus* was strongly affected by the type of substrate infested by this insect. This study confirmed also that *O. ainoae* was a common associate of *P. chalcographus*.

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