

MYCOBIOTA ASSOCIATED WITH *HYLURGOPS PALLIATUS* (GYLL.) ON *PINUS SYLVESTRIS* L. IN POLAND

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ABSTRACT

The assemblage and abundance of fungal species associated with *Hylurgops palliatus* on *Pinus sylvestris*, were studied in Poland. Fungi were isolated from beetles of *H. palliatus* and their galleries collected from three populations. In total, 1832 fungal isolates, including 38 species, were obtained. The most important group of fungi were the ophiostomatoid fungi. Among the seven species of ophiostomatoid fungi isolated, *Leptographium lundbergii* and an unknown species of *Graphium* sp. code-named 'W' were the dominant species. *Ophiostoma minus* and *O. piceae* were also relatively common. All ophiostomatoid species, except *L. lundbergii* occurred more frequently on the overwintered adults than in gallery systems. *Ophiostoma minus* and *O. piliferum* are documented here as new associates of *H. palliatus*.

KEY WORDS: *Hylurgops palliatus*, *Leptographium lundbergii*, *Ophiostomatoid* fungi, *Pinus sylvestris*.

INTRODUCTION

Many species of fungi are known to be associated with bark beetles (Kirisits 2004; Upadhyay 1981). Among them, blue-stain fungi, especially ophiostomatoid species, are most frequently associated with bark beetles infesting coniferous trees (Wingfield et. al. 1993). These fungi are an important fungal group because they cause blue-staining in freshly cut logs and some of them are capable of killing mature trees (Harrington 1993).

Many species of bark beetles, for example *Ips typographus* (L.), are considered the major forest pests. *Hylurgops palliatus* (Gyll.) is not considered to be an economically important pest in its natural range because it infests logs, stumps and dead or dying trees. However, when the population level of *H. palliatus* is high it may also attack live trees. In Europe, *H. palliatus* breeds in conifers of the family Pinaceae including species of *Abies* spp., *Larix* spp., *Picea* spp. and *Pinus* spp. *Hylurgops palliatus* may have one or two generations per year in Poland. In April-May and July-August adults establish galleries in the phloem (Michalski and Mazur 1999). During gallery construction, the beetles disseminate the spores of the blue-stain fungi. Propagules of blue-stain fungi are carried on the external surface of the exoskeletons of *H. palliatus* and in very primitive mycangia. Among the ophiostomatoid fungi transmitted by *H. palliatus*, there are pathogenic fungi which

may play a role in overcoming the resistance of trees attacked by the beetles (Kirisits 2004).

Several reports on the fungi associated with *H. palliatus* have been published in Europe (Harding 1989; Jacobs and Wingfield 2001; Jacobs et. al. 2001; Kirisits et. al. 2000; Kirisits 2001; Kirschner 1998, 2001; Kotýnková-Sychrová 1966; Krokene and Solheim 1996; Mathiesen 1950; Mathiesen-Käärik 1953; Wingfield and Gibbs 1991). These studies were focussed on the species of blue-stain fungi associated with *H. palliatus* on Norway spruce. *Ambrosiella* sp., *Leptographium lundbergii* Lagerb. & Melin, *Ophiostoma ainoae* H. Solheim, *O. cucullatum* H. Solheim, *O. neglectum* Kirschner et Oberwinkler, *O. penicillatum* (Grosn.) Siem., *O. piceae* (Münch) Syd. & P. Syd. and *O. piceaperdum* (Rumb.) von Arx were a dominant fungal species.

There was no report about mycobiota associated with *H. palliatus* in Poland. Some of the blue-stain fungi were isolated from other pine bark beetles by Siemaszko (1939). He reported, that in the Białowieża Forest, *Ophiostoma ips* (Rumbold) Nannf., *O. minus* (Hedgc.) Syd. & P. Syd. and *O. minus* were associated with *I. sexdentatus* (Börn.) and *Tomicus piniperda* (L.), respectively.

In this study, composition and frequency of fungi associated with *H. palliatus* on Scots pine were investigated. This was achieved by isolating fungi from the beetle bodies and their galleries.

MATERIALS AND METHODS

Study site

The investigations were conducted in 2005 in three study plots located in *Pinus sylvestris* stands in the Niepołomice Forest District (49°59'53" N, 20°19'56" E), Oleszyce Forest District (50°07'45" N, 22°57'17" E) and Olkusz Forest District (50°14'28" N, 19°31'25" E). All plots were located in 40-50 years old pine stands, where *P. sylvestris* was the dominant species. Fungal isolations were made from the beetles colonizing trees and tissues of trees colonized by the first generation of *H. palliatus*.

Fungal isolation from the beetles

The main attack of *H. palliatus* started on 2 April. On 27 and 28 April, four trees attacked by *H. palliatus* at each location were felled and adults were collected from its galleries. The bark of felled trees was peeled with a sterilized hatchet. The galleries of *H. palliatus* were not well developed and overwintered adult beetles, eggs and young larvae were found. The beetles were collected with sterilized tweezers and then stored individually in sterile microtubes (1.5 ml) for isolations. In total, 192 adults were picked up from galleries.

Before isolation of fungi, each beetle was bathed in sterile water for 30 seconds. After drying on a sterile blotting paper, the beetles were crushed on a microscopic slide and evenly spread on the surface of medium, with a sterile scalpel.

Fungal isolation from the gallery systems

Samples were taken from gallery systems found on the trees felled on 27 and 28 April. From parts of the stem infested by *H. palliatus* four sections (30 cm long) were cut from each tree and transported to the laboratory. The sections were cut from the tree stems between 2 and 4 m above the ground. In the laboratory, the bark was separated from the wood under sterile conditions. Fragments of the gallery from which isolations were intended were disinfected using cotton wool with 96% ethyl alcohol. The disinfection lasted approximately 15 sec. and then gallery fragments were dried on filter paper. Isolation of fungi was made from phloem fragments taken from egg and larval galleries of *H. palliatus* and around them. Isolations of fungi were also made from the stained or discoloured sapwood underneath insect galleries up to a depth of 20 mm into the sapwood. After drying of samples, the surface layer of phloem tissues was removed with a sterile scalpel. Subsequently, small fragments of phloem or sapwood (about 4×4 mm) were cut with a sterile scalpel or a chisel, and placed on a culture medium in Petri dishes. In total, 828 plant pieces were used in this study.

Cultural procedures

All of the isolations were made on 2% malt extract agar (2% MEA; 20 g malt extract, 20 g agar, 1000 ml distilled water) supplemented with the antibiotic tetracycline (200 mg per 1 litre of culture medium) to inhibit bacterial growth. Where necessary, cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh 2% MEA. The primary isolation plates were incubated at room temperature in the darkness. Emerging fungi were identified on the morphological cha-

acters such as perithecia, ascospores, conidiophores and conidia.

Frequency of occurrence

Frequencies were computed using the following formula: $F = (NF/NT) \times 100$, where F represents the frequency of occurrence (%) of each fungal species, NF represents number of beetles or plant fragments, from which a particular fungus was isolated and NT represents total number of beetles or plant fragments from which fungal isolation was attempted. Fungal frequencies were computed together for phloem and sapwood fragments.

RESULTS

In this study, 1832 fungal isolates were obtained from adult beetles and gallery systems. In total, 31 fungal species were isolated from the beetles and 20 from the gallery systems. These fungi represented mainly ascomycetes and anamorphic fungi, but a few zygomycetes were also isolated. The most important group of fungi were ophiostomatoid fungi, represented by seven species: *Ophiostoma minus*, *O. piceae*, *O. piliferum* (Fr.) Syd. & P. Syd., *Ceratocystiopsis alba* (DeVay, R.W. Davidson & W.J. Moller) H.P. Upadhyay, *Leptographium lundbergii*, *Graphium* sp. 'W' and *Graphium* sp. 'D' (Tables 1, 2).

Fungal isolations from beetles

Approximately 96% of the 192 beetles carried fungi (Table 1). Among the species isolated from the beetles, five ophiostomatoid fungi were identified: *O. minus*, *O. piceae*, *L. lundbergii*, *Graphium* sp. 'W' and *Graphium* sp. 'D'. All ophiostomatoid species, except *Graphium* sp. 'D' were isolated from beetles in all populations. *Leptographium lundbergii* was the most commonly isolated fungus (isolated from 34.4 to 73.4% of beetles). *Graphium* sp. 'W' was the second most abundant fungus (31.3-35.9%). Two other ophiostomatoid fungi, *O. minus* and *O. piceae* were isolated from 12.5 to 29.7% and 15.6-23.4% of beetles, respectively (Table 1). Among other species, *Trichoderma* spp., *Penicillium* spp., *Mucor* sp. and *Mortierella ramanniana* var. *ramanniana* (A. Möller) Linnem. were most abundant (Table 1).

Fungal isolations from gallery systems

Overall, 97.6% of the 828 plant fragments taken from colonized trees contained fungi (Table 2). Seven species of ophiostomatoid fungi, *C. alba*, *O. minus*, *O. piceae*, *O. piliferum*, *L. lundbergii*, *Graphium* sp. 'W' and *Graphium* sp. 'D' were isolated from gallery system. All species, except *C. alba*, *O. piliferum* and *Graphium* sp. 'D' were isolated from *H. palliatus* gallery systems in all populations. *Leptographium lundbergii* was isolated most frequently and was found in 81.8% of all samples. *Graphium* sp. 'W' was isolated from 33.7% of plants fragments. The remaining ophiostomatoid species were isolated less frequently. *Ophiostoma minus* and *O. piceae* were isolated from 15.2 and 6.6% of all samples, respectively. Species not belonging to the ophiostomatoid fungi were relatively rare. Among these fungi, one ascomycetes *Dipodascus aggregatus* Francke-Grosz. as well as anamorphic fungi (*Trichoderma* spp., *Penicillium* spp.) were most abundant (Table 2).

TABLE 1. Frequency of occurrence (%) of fungi isolated from beetles of *Hylurgops palliatus* collected at three locations.

Fungi	Niepołomice	Oleszyce	Olkusz	Total
Ophiostomatoid fungi				
<i>Leptographium lundbergii</i> Lagerb. & Melin	46.9	34.4	73.4	51.6
<i>Ophiostoma minus</i> (Hedgc.) Syd. & P. Syd.	23.4	15.6	17.2	18.8
<i>Ophiostoma piceae</i> (Münch) Syd. & P. Syd.	23.4	12.5	29.7	21.9
<i>Graphium</i> sp. 'W'	31.3	35.9	34.4	33.9
<i>Graphium</i> sp. 'D'			6.3	2.1
Other				
<i>Acremonium</i> sp.			1.6	0.5
<i>Acremonium striatum</i> W. Gams	1.6	1.6		1.0
<i>Alternaria alternata</i> (Fr.: Fr.) Keissler			3.1	1.0
<i>Arthrinium</i> state of <i>Apiospora montagnei</i> Sacc.		1.6		0.5
<i>Aspergillus niger</i> van Tieghem	1.6			0.5
<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries	6.3	1.6	1.6	3.1
<i>Cladosporium sphaerospermum</i> Penz.	3.2		1.6	1.6
<i>Dipodascus aggregatus</i> Francke-Grosmann	6.3	12.5	1.6	6.8
<i>Epicoccum nigrum</i> Link	1.6			0.5
<i>Fusarium</i> sp.	1.6			0.5
<i>Harposporium</i> sp.			1.6	0.5
<i>Homonema dematioides</i> Lagerb. & Melin	1.6	1.6		1.0
<i>Lecythophora hoffmannii</i> (van Beyma) W. Gams & McGinnis		1.6		0.5
<i>Mortierella ramanniana</i> var. <i>ramanniana</i> (A. Möller) Linnem.		12.5	14.1	8.8
<i>Mucor</i> sp.	4.7	1.6	18.8	8.3
<i>Myceliophthora</i> sp.	1.6			0.5
<i>Oidiodendron truncatum</i> G.L. Barron			1.6	0.5
<i>Penicillium</i> sp.	28.1	35.9	17.2	27.1
<i>Phialocephala</i> cf. <i>dimorphospora</i> Kendrick	1.6			0.5
<i>Sepedonium chrysospermum</i> (Bull.) Link	3.2			1.0
<i>Trichoderma</i> spp.	17.2	14.1	4.7	12.0
<i>Verticillium chlamydosporum</i> Goddard	1.6		1.6	1.0
Yeasts	7.8	1.6	3.1	4.2
Unidentified				
Other (3 species)	6.3	12.5	1.6	6.8
Percentage of "sterile" beetles	12.5	9.4	0	3.6
Number of investigated beetles	64	64	64	192

DISCUSSION

In this study, the ophiostomatoid fungi were the most common fungal associates of the *H. palliatus* and they were represented by seven species. These fungi were isolated from the beetles as well as from gallery systems. Among them, *L. lundbergii* and *Graphium* sp. 'W' had high frequency of occurrence, suggesting a close association with *H. palliatus*. Kotýnková-Sychrová (1966) described only *L. lundbergii* as fungal associate of *H. palliatus* on *Picea abies* and *P. sylvestris* trees in formerly Czechoslovakia. In contrast to Kotýnková-Sychrová's investigations, Wingfield and Gibbs (1991) reported a wide variety of *Leptographium* and *Graphium* species to be associated with *H. palliatus* in England. These authors found that *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., *L. truncatum* (M.J. Wingf. & Marasas) M.J. Wingf., *L. wingfieldii* M. Morelet, *L. guttulatatum* M.J. Wingf., K. Jacobs and the two undescribed species *Graphium* sp. were associated with new adults of *H. palliatus* trapped in *P. sylvestris* billets. *Hylurgops palliatus* is also associated with a number of blue-stain fungi on Norway spruce (Jacobs and Wingfield 2001; Jacobs et al. 2001; Harding 1989; Kirisits et al. 2000; Kirisits 2001; Kirschner 1998, 2001; Krokene and Solheim 1996; Mathiesen 1950; Mathiesen-Käärik 1953). Among

them, *L. lundbergii*, *O. ainoae*, *O. cucullatum*, *O. neglectum*, *O. penicillatum*, *O. piceae* and *O. piceaperdum* were dominant fungal species. These ophiostomatoid fungi are known to be commonly associated with bark beetle species infesting Norway spruce in Europe (Harding 1989; Jankowiak 2005; Kirisits 2001; Kirschner 1998; Sallè et al. 2005; Solheim 1986; Viiri 1997; Viiri and Lieutier 2004). Among them, only *L. lundbergii* and *O. piceae* were also found to be associated with *H. palliatus* on *P. sylvestris* in this study. It may suggest that *L. lundbergii* and *O. piceae* are the most consistent but unspecific associates of *H. palliatus*. However, the populations of insects occurring in *P. sylvestris* forests may carry additionally *O. minus*, *O. piliferum* and *Graphium* sp. 'W' but other *H. palliatus* populations breeding in *P. abies* trees transmitted also ophiostomatoid fungi associated with other bark beetle species on spruce.

In this study, *L. lundbergii* was the most commonly isolated species from overwintered adults constructing new galleries. It suggests that propagules of this fungus are especially frequently introduced to Scots pine by beetles of *H. palliatus* in Poland. Kotýnková-Sychrová (1966) considered that *L. lundbergii* may be frequently isolated shortly after the initiation of the brood systems. In this phase of brood development it caused yellow-brown discoloration

TABLE 2. Frequency of occurrence (%) of fungi isolated from gallery systems of *Hylurgops palliatus* collected at three locations.

Fungi	Niepołomice	Oleszyce	Olkusz	Total
Ophiostomatoid fungi				
<i>Ceratomyces alba</i> (DeVay, R.W. Davidson & W.J. Moller) H.P. Upadhyay			0.3	0.1
<i>Leptographium lundbergii</i> Lagerb. & Melin	62.5	81.3	94.7	81.8
<i>Ophiostoma minus</i> (Hedgc.) Syd. & P. Syd.	28.1	14	8.3	15.2
<i>Ophiostoma piceae</i> (Münch) Syd. & P. Syd.	6.3	1.2	13.0	6.6
<i>Ophiostoma piliferum</i> (Fr.) Syd. & P. Syd.			0.3	0.1
<i>Graphium</i> sp. 'W'	34.9	17.6	50.7	33.7
<i>Graphium</i> sp. 'D'			0.7	0.2
Other				
<i>Cladosporium cladosporoides</i> (Fresen.) G.A. de Vries			0.3	0.1
<i>Dipodascus aggregatus</i> Francke-Grosmann	2.1	3.0	12.7	6.3
<i>Doratomyces</i> sp.			0.3	0.1
<i>Epicoccum nigrum</i> Link			0.7	0.2
<i>Mortierella ramanniana</i> var. <i>ramanniana</i> (A. Möller) Linnem.	2.6	0.9	2.3	1.8
<i>Mucor</i> sp.	1.0	1.5	0.3	1.0
<i>Penicillium</i> spp.	5.2	10.7	16.3	11.5
<i>Pezicula eucrita</i> P. Karst.			0.3	0.1
<i>Phialocephala</i> cf. <i>dimorphospora</i> Kendrick		0.3	0.3	0.2
<i>Phialophora</i> sp.			0.3	0.1
<i>Trichoderma</i> spp.	17.2	13	5.7	11.4
<i>Tysanophora penicillioides</i> (Roum.) Kendrick			0.3	0.1
Unidentified				
Other (1 species)		0.3	1.3	0.6
Percentage of "sterile" plant fragments	0	5.9	0	2.4
Number of investigated plant fragments	192	336	300	828

in the sapwood of Scots pine. This study confirms Kotýnková-Sychrová's observation because *L. lundbergii* was a dominant fungus in the phloem and sapwood samples taken from early galleries of *H. palliatus*. This may indicate that *L. lundbergii* is able to colonize rapidly the sapwood of Scots pine and is the most important invader of the phloem and sapwood of trees attacked by *H. palliatus*. This fungus was also found in gallery systems of *H. palliatus* with high frequency in Denmark (Harding 1989). *Leptographium lundbergii* was associated with stained sapwood on pine and spruce, and it was isolated from the gallery systems of many bark beetles (Jacobs and Wingfield 2001; Kirisits 2004). It was first described by Lagerberg and Melin (Lagerberg et al. 1927). Recently, Jacobs et al. (2005) have made a taxonomic re-evaluation of this fungus. On the basis of DNA sequence and morphology they gave a new and expanded description of the species. The morphological characteristics of *L. lundbergii* isolates from this study are very similar to the characteristics of the cultures examined by Jacobs et al. (2005).

The two unidentified species *Graphium* sp., code-named 'W' and 'D' are used here in the broad sense (Seifert and Okada 1993). The taxonomic position of the *Graphium* sp. 'W' and *Graphium* sp. 'D' is currently under investigation and will be discussed in a later report. The synnemata of *Graphium* sp. 'W' had lightly pigmented stipes with cylindrical conidia. This species was also sporadically isolated from *Tomicus piniperda* L. galleries in Scots pine forests in Poland (Jankowiak 2006). The two unknown *Graphium* spp. were also isolated from *H. palliatus* by Wingfield and Gibbs (1991) in England but the authors did not give any description of these species.

The results presented here suggest that *H. palliatus* may be effective vector for *O. minus*. It is interesting because this fungus had never been reported in association with *H. palliatus*. So far this species has been described as associated with other bark beetle species on *Pinus* spp. (Mathiesen 1950; Mathiesen-Käärik 1953; Lieutier et al. 1989; Rennerfelt 1950; Siemaszko 1939; Solheim and Långström 1991). In Poland this fungus was found in connection with *T. piniperda*, *Ips sexdentatus* (Jankowiak 2006, Jankowiak unpublished, Siemaszko 1939) and *T. minor* (Hrtg.) (Jankowiak, unpublished). *Ophiostoma minus* associated with *T. piniperda* on *P. sylvestris* is able to kill trees in experimental mass inoculations (Långström et al. 1993; Solheim et al. 1993). In contrast to *O. minus*, *L. lundbergii* is not highly virulent and may be a secondary pathogen (Harrington and Cobb 1988). In this study, inoculation tests with the ophiostomatoid fungi isolated from *H. palliatus* have not been conducted. Studies on pathogenicity of the fungi against Scots pine are conducted.

Ophiostoma piceae was relatively frequently isolated from galleries of *H. palliatus*. Thus, the relatively high frequency of occurrence of *O. piceae* was not a surprise because this fungus was frequently isolated from beetles and galleries of other bark beetle species attacking Norway spruce in Europe (Harding 1989; Kirschner 1998; Mathiesen-Käärik 1953). This study showed that *O. piceae* is also an important associate of *H. palliatus* in Poland.

Penicillium and *Trichoderma* species were also commonly isolated from galleries of *H. palliatus* in this study. Adult insects usually spend the winter in the soil and can easily introduce litter and soil fungi such as *Trichoderma*, *Penicillium* and *Mortierella ramanniana* var. *ramanniana*

to the pine trunks. This group of fungi is not associated with any specific species of bark beetles on forest trees (Kirschner 2001).

Some of the remaining species isolated from phloem and sapwood represent endophytes associated with *P. sylvestris*. Such species as *Epicoccum nigrum* Link, *Pezicula eucrita* P. Karst. or *Phialocephala* cf. *dimorphospora* W.B. Kendr. were frequently isolated from symptomless and uncolonized Scots pine trees (Kowalski and Kehr 1996). The other non-ophiostomatoid fungi were rarely associated with *H. palliatus* and represent entomochoric fungi (*Dipodascus aggregatus*), nematode-destroying fungi (*Harposporium* sp.), wood colonizing fungi (*Phialophora* sp.) and other ecological groups (Kirschner 2001).

In conclusion, the results of the study confirmed previously reported associations between many ophiostomatoid fungal species and *H. palliatus* in other parts of Europe. However, the pathogenic *O. minus* and *O. piliferum* have not been previously reported. The majority of ophiostomatoid fungi isolated from *H. palliatus* infesting Norway spruce were not found in this study.

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