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Ophiostomatoid fungi isolated from fallen shoots of Scots pine pruned by *Tomicus* species in Poland

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Ophiostomatoid fungi are known to be associated with *Tomicus* spp. on *Pinus sylvestris*. However, very little is known about the fungi present in the pine shoots damaged by these insects. The aim of this study was to survey species of *Ophiostoma* s.l. associated with fallen shoots of Scots pine pruned by *Tomicus* spp. in Poland. The study was conducted in four pure Scots pine stands in southern and south-western part of the country. Fungi were identified based on morphology and DNA sequence comparison for two gene (the ITS rDNA region and ß–tubulin). In total, 64 isolates obtained, represent seven species of ophiostomatoid fungi, including their asexual states of the genera. Two of them, *Ophiostoma* sp. 1 and *Sporothrix* sp. 1 probably represent new taxa. All species were found at very low frequencies. Among them, *Ophiostoma minus*, *Ophiostoma* sp. 1 and *Sporothrix* sp. 1 were the most frequently isolated, with a frequency of 2.0%. Occasionally, isolated species were: *Leptographium piriforme*, *Ophiostoma canum*, *O. floccosum* and *Grosmannia cucullata*-like. Association of species of *Ophiostoma* s.l. with *Tomicus* spp. and the taxonomic status of two new species are discussed.

Key words: *Ophiostoma*, *Sporotrix, Pinus sylvestris*, new taxa, bark beetles

INTRODUCTION

Many species of bark beetles (Coleoptera: Scolytinae) have been reported from pine stands of Poland. These insects are generally considered secondary colonizers, which attack stressed, dying or dead trees. Among them, *Tomicus piniperda* (L.) and *T. minor* (Hart.) are able to attack healthy standing Scots pine trees under favourable conditions. The damage by bark beetles of the genus *Tomicus* in pine trees is caused by two feeding strategies. As in many species of bark beetles

one strategy is penetration of the protective bark and nutritive phloem of tree stems, and transmission of blue-stain fungi in the sapwood. Another strategy occurring only in *Tomicus* beetles is penetration and maturation feeding of adults in living shoots of healthy trees. The shoots become hollow and easily break off. During this period of maturation-feeding, each new adult feeds on the current-year or last year's shoots, mostly in the upper half of the crown. Adults bore into the bark of shoots and hollow out the pitch to a length of 2-3 cm. At strong winter, most of the injured shoots break off and fall to the ground. When shoot feeding is severe, tree height and diameter growth are reduced (Borkowski 2001).

Species described in the past in the genus *Ophiostoma* s.l. represent a group of morphologically similar genera characterized by ascomata with elongated perithecial necks. In fact, according to the recent taxonomic studies most of them belong to the genus *Ophiostoma* Syd.& P. Syd. s. str. with *Pesotum* J.L. Crane & Schokn. and *Sporothrix* Hektoen & C.F.Perkins anamorphs, *Grosmannia* Goid. with *Leptographium* Lagerb. & Melin anamorphs and *Ceratocystiopsis* H. P. Upadhyay & W. B. Kendr. with *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. anamorphs (Upadhyay 1981; Zipfel et al. 2006). These genera and the members of the genus *Ceratocystis* Ellis & Halst. s. str. have been referred to as the ophiostomatoid fungi (Wingfield et al. 1993). Ophiostomatoid fungi are the predominant associates of *Tomicus* Latreille in Europe. Specific relationships between fungi and *Tomicus* spp. are the most clear for *T. minor*. The ambrosia fungus, *Ambrosiella tingens* (Lagerb. & Melin) L.R. Batra and *Ophiostoma canum* are common and consistent associates of *T. minor* in Europe (Mathiesen 1950, 1951; Francke-Grosmann 1952; Mathiesen-Käärik 1953; Kotýnková-Sychrová 1966; Kirisits et al. 2000; Jankowiak 2008). In the recent studies, the association of *T. minor* also with *Ophiostoma minus*, *O. piceae* (Münch) Syd. & P. Syd., *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., *L. lundbergii* Lagerb. & Melin (Jankowiak 2008) and *Ophiostoma canum*-like species (Linnakoski et al. 2010) has been reported. The association between *T. piniperda* and ophiostomatoid fungi is looser because this insect carries numerous fungal species, but with low and inconsistent frequency. Among them, *O. minus* and *Leptographium wingfieldii* M. Morelet were dominant fungal species in many populations in Europe (Lieutier et al. 1989; Gibbs, Inman 1991; Solheim, Långström 1991; Wingfield, Gibbs 1991; Jankowiak 2006; Jankowiak, Kurek 2006; Jankowiak, Bilański 2007).

Although *Tomicus* spp. mycobiota is relatively well recognized in Europe (Kirisits 2004), we have no information about fungal taxa associated with pine shoots damaged by these insects. It is unknown whether shoots damaged by adult individuals feeding in Scots pine crowns are colonized by fungal species carried by *Tomicus* beetles. We often observed that numerous arthropods penetrated fallen pine shoots and therefore we presume that these organisms can act as vectors for fungal species not closely associated with *Tomicus* spp.

The studies were aimed to survey the ophiostomatoid fungi associated with pine shoots damaged by *Tomicus* spp. collected from Poland. We determined isolates to the species level using their morphological characteristics, as well as DNA sequencing data.

MATERIALS AND METHODS

Isolation of fungi from pine shoots. The investigations were carried out in 2007-2008 in four pure Scots pine stands in Poland (Fig. 1). In October, the fallen Scots pine shoots (100 per site) damaged by beetles were gathered from the forest floor and placed in separate clean paper bags. Later, shoots were stored for maximum 48 hours in a cool room at 4° C until they were used for the fungi isolation.

Shoot samples were cut into 5 cm long sections and surface disinfected by immersing in 95% ethyl alcohol. After drying, small pieces of shoots (about 5x5 mm) were removed from each shoot section (six pieces per one shoot) and placed in Petri dishes containing a selective medium for *Ophiostoma* spp. (20 g malt extract, 20 g agar, and 1L distilled water, amended with 0.05% cycloheximide). Resulting isolates were purified by transferring mycelium from the edges of single colonies to fresh 2% MEA (Tab. 1). The representative strains were deposited in the Culture Collection of Fungi, Department of Forest Pathology, Agricultural University of Cracow.

Culture morphology and DNA sequencing. Isolates were initially identified and grouped based on the culture morphology. Representatives of each of the groups obtained were selected for DNA sequencing (Tab.1).

Fungi were identified on the basis of morphological characteristics by comparison with published data and reference cultures deposited at Department of Forest Pathology, Agricultural University of Cracow.

Identification based on morphology was confirmed by DNA sequencing of the representative isolates (Tab.1). ITS rDNA region (ITS1-5.8 S-ITS2) were amplified using the primers ITS1 and NL4 as described by Kolařík et al. (2006). A partial ß-tubulin sequence was determined for a subset of isolates, including an unknown *Ophiostoma* sp. 1 and *Sporothrix* sp. 1 isolates using the primers T1 or bt2a and bt2b (Glass, Donaldson 1995; O'Donnell, Cigelnik 1997). The amplicons were purified and both strands were sequenced using the same primers by Macrogen Inc. (Seoul, Korea). The sequences were compared with data from GenBank using a Blast similarity search. Sequences generated in this study and sequences of other, related species from GenBank were aligned using Clustal W (Thompson et al. 1994). Phylogenetic trees were constructed with MEGA 5.04 using the Neighbour-joining (NJ) method and Kimura two-parameter with a transition to transversion ratio (Ti/Tv=2) (Tamura et al. 2011). Bootstrap values were calculated for 1000 replications.

Fig. 1. Map of sampling sites: Chrośnica (15°59′36″N, 52°17′13″E); Babimost (15°50′33″N, 52°09′11″E), Jaroszowiec (19° 36′45″N, 50°20′18″E), Mielec (21° 29′36″N, 50°18′53″E).

Table 1
List of strains of fungi species isolated from pine shoots infested by *Tomicus* spp. used for DNA sequencing and their GenBank accession numbers

Taxon	Isolate	Site[a]	Primer used for amplification	Accession no.	Closest match in BLAST	Accession of match	Identity %
Grosmannia cucullata-like (H. Solheim) Zipfel, Z.W. de Beer & M.J Wingf.	183BRJ	C	ITS1F/NL4	JQ292836	*G. cucullata*	GU067758.1	98.9
Leptographium piriforme Greif, Gibas & Currah	215RJ	J	ITS1F/NL4	FM992031	*L. piriforme*	DQ885243	100
Ophiostoma canum (Münch) Syd. & P. Syd.	219RJ	J	ITS1F/NL4	JQ292826	*O. canum*	AJ538342.1	100
Ophiostoma floccosum Math.-Käärik	145RJ	C	ITS1F/NL4	JQ292830	*O. floccosum*	AJ538343.1	100
	148RJ	C	ITS1F/NL4	JQ292829	*O. floccosum*	AJ538343.1	100
Ophiostoma minus (Hedgc.) Syd. & P. Syd.	168RJ	C	ITS1F/NL4	JQ292827	*O. minus*	JF440585.1	100
	190bRJ	C	ITS1F/NL4	JQ292835	*O. minus*	AM943886.1	100
Ophiostoma sp. 1	105RJ	C	ITS1F/NL4	JQ292833	*O. quercus* (Georgev.) Nannf.	HM051398.1	96.6
			Bt2a/Bt2b		*O. psedotsugae* (Rumbold) Arx	AY542510.1	81.8
	116RJ	C	ITS1F/NL4	JQ292834	*O. quercus*	HM051398.1	96.6
	159RJ	C	ITS1F/NL4	JQ292822	*O. quercus*	HM051398.1	96.6
			Bt2a/Bt2b		*O. breviusculum* W.Hsin Chung, Yamaoka, Uznovic & J.J. Kim	AB200428.1	78.8
	183ARJ	C	ITS1F/NL4	JQ292823	*O. quercus*	HM051398.1	96.9
			Bt2a/Bt2b		*O. psedotsugae*	AY542510.1	77.8
	188RJ	C	ITS1F/NL4	JQ292824	*O. quercus*	GU062272.1	96.9
			Bt2a/Bt2b		*O. psedotsugae*	AY542510.1	79.6
	198RJ	C	ITS1F/NL4	JQ292825	*O. quercus*	HM051398.1	96.9
	235RJ	J	ITS1F/NL4	JQ292831	*O. quercus*	GU797205.1	96.7
Sporothrix sp. 1	55RJ	M	ITS1/NL4	JQ292821	*S. inflata* de Hoog	JN618188.1	93.3
			Bt2a/Bt2b		*S. inflata*	AY495437.1	88.1
	85RJ	M	ITS1/NL4	JQ292832	*S. inflata*	JN618188.1	93.2
			Bt2a/Bt2b		*S. inflata*	AM498345.1	86.2
	152RJ	C	ITS1/NL4	JQ292828	*S. inflata*	JN618188.1	96.5
	190aRJ	C	ITS1/NL4	JQ292820	*S. inflata*	JN618188.1	93.6

[a] J – Jaroszowiec, C – Chrośnica, M – Mielec

RESULTS

Identification of ophiostomatoid species. Morphological investigation showed that seven ophiostomatoid species were collected, namely *Leptographium piriforme*, *Ophiostoma canum*, *O. floccosum, O. minus*, *Grosmannia cucullata*-like and two unknown fungi, *Ophiostoma* sp. 1 and *Sporothrix* sp. 1.

Strain classified as *Grosmannia cucullata*-like produced in culture dark brown perithecia with straight or curved neck ended ostiolar hyphae. Ascospores were hyaline, one-celled, lunate, with a thick hyaline sheath. This fungus formed *Pesotum* anamorph, stipe branched at the apex , with cylindrical conidia (Figs 2-5). *Sporothrix* sp. 1 (Fig. 6) was characterized by producing micronematous conidiogenous cells arising orthotropically from undifferentiated hyphae. Conidia were produced directly on denticles, clavate; no ascomata were observed. Strains referred to as *Ophiostoma* sp. 1 presented both *Pesotum* and *Sporothrix* synanamorphs in culture (Figs 7-10) while no ascomata were observed. Conidiophores were synnematous, branched at the apex, single but often also in loosely arranged groups. Conidiogenous cells of *Sporothrix* state were micronematous, mononematous, hyaline, arising ortho- or slightly plagiotropically from undifferentiated aerial hyphae. Conidia were produced directly on denticles, clavate, slightly curved.

Comparison of ITS sequences obtained for isolates in this study, with sequences from GenBank, indicate that our isolates could also be assigned to seven taxa. DNA sequences of the majority of species identified here were identical with reference sequences, confirming their identity (Tab. 1). ITS rDNA sequences of *G. cucullata*-like isolate showed 99% similarity with *G. cucullata* strains from GenBank. In the ITS tree, *Ophiostoma* sp. 1 grouped most closely to *Ophiostoma quercus* in *O. piceae* complex (Fig. 11). Similarity search using a ß-tubulin showed highest relatedness with *Ophiostoma pseudotsugae* (<81.8%) and *Ophiostoma breviusculum* (78.8%) (Tab. 1). Based on sequence data of the ITS regions and ß-tubulin, the unknown *Sporothrix* sp. collected by us was most closely related to *S. inflata* and *S. schenckii* in *S. schenckii-O. stenoceras* complex (Tab. 1, Fig. 11).

Isolation frequency. All the species were found at very low frequency (Tab. 2). In culture, 43 % (28 isolates) of a total of 64 fungal isolates obtained from pine shoots

Table 2
Number of isolates and frequency (in parentheses)* of fungi species associated with pine shoots infested by *Tomicus* spp.

Fungal species	Sites				Total
	Mielec	Chrośnica	Jaroszowiec	Babimost	
Grosmannia cucullata-like		1(1)			1(0.3)
Leptographium piriforme	1(1)		2(2)		3(0.8)
Ophiostoma canum		1(1)	3(1)		4(1.0)
Ophiostoma floccosum		2(2)			2(0.5)
Ophiostoma minus		2(2)	10(5)	3(1)	15(2.0)
Ophiostoma sp. 1		17(6)		11(2)	28(2.0)
Sporothrix sp. 1	1(1)	6(3)		4(4)	11(2.0)
Total no. isolates	2	29	15	18	64
Total no. shoots and shoot fragments (in parentheses)	100 (600)	100 (600)	100(600)	100(600)	400 (2400)

*Frequency = (No. of shoots from which a particular fungus was isolated/Total number of shoots) x 100

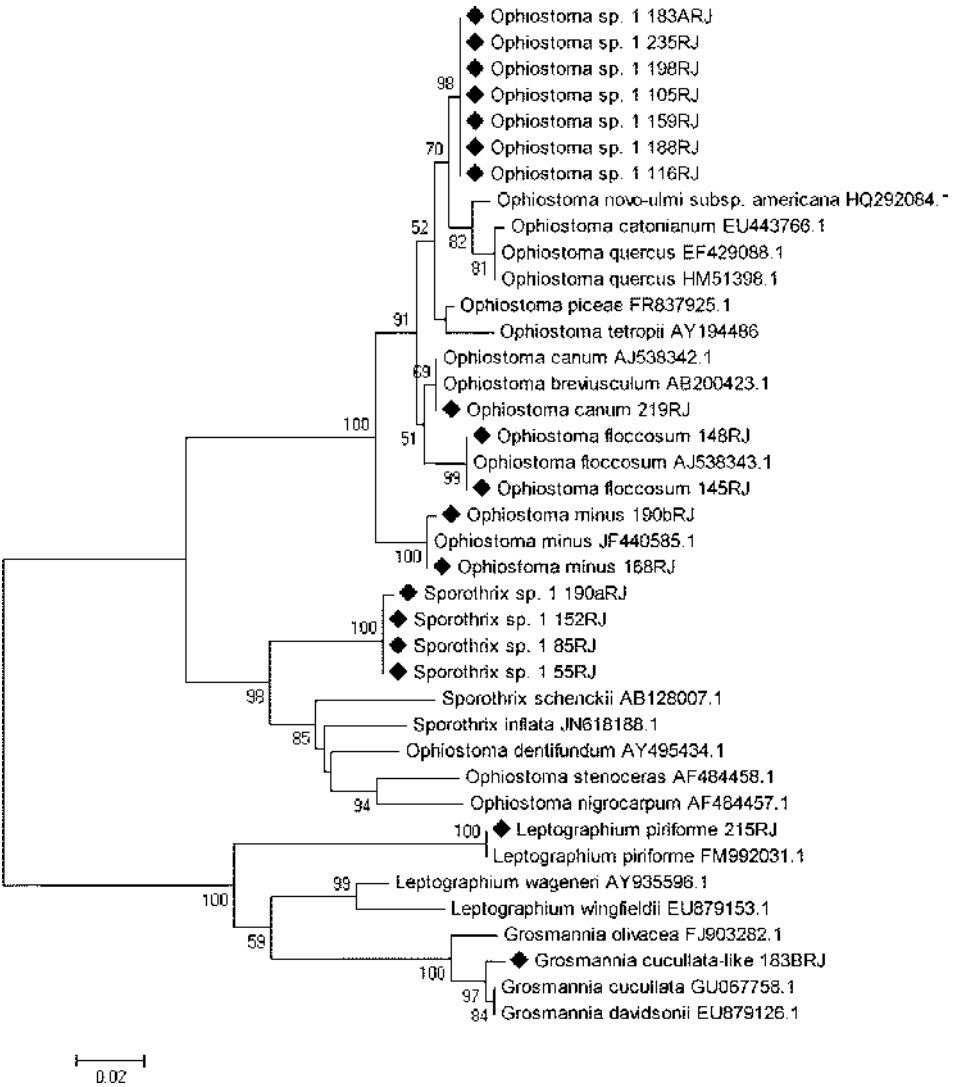
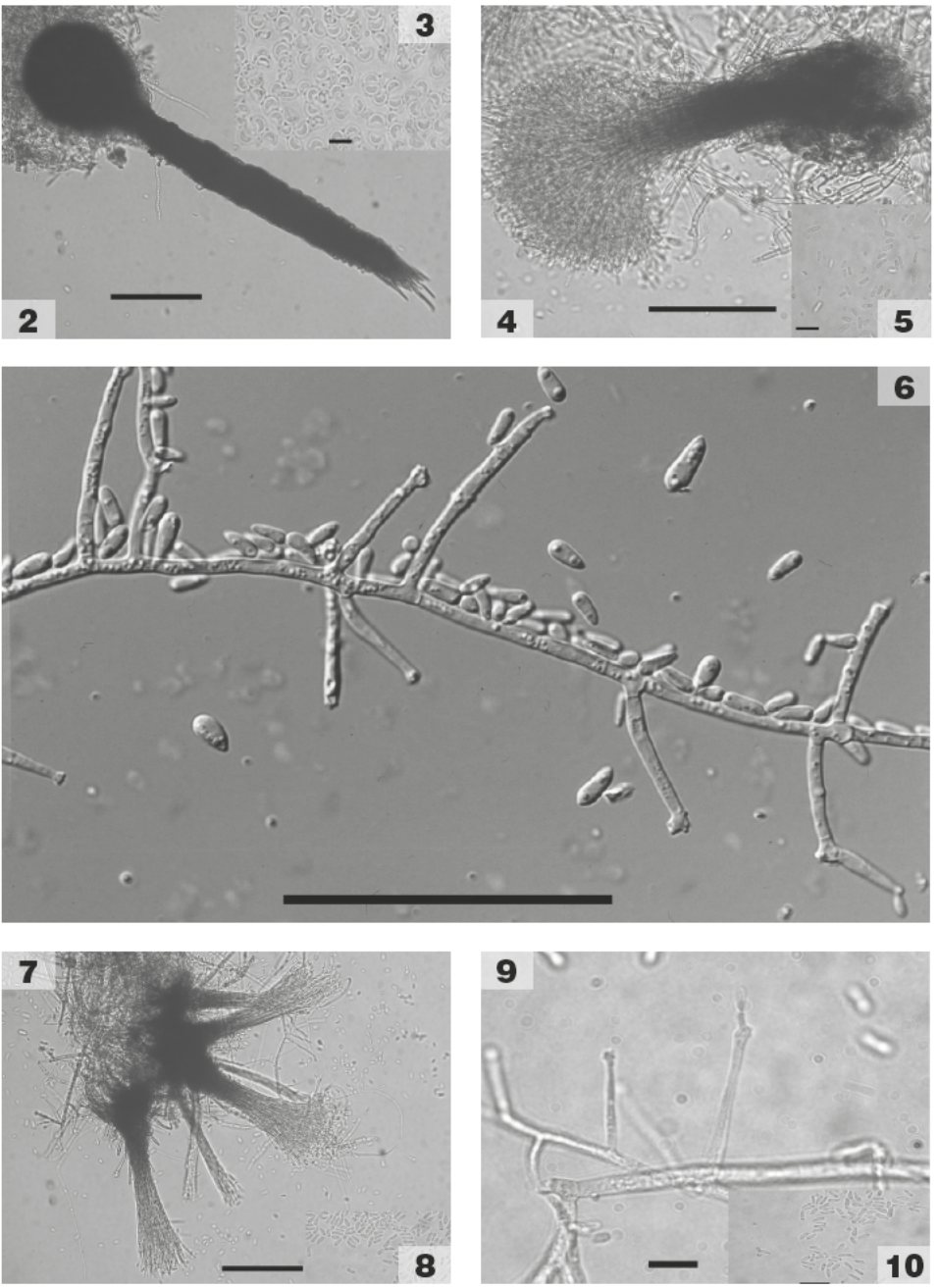


Fig. 11. Phylogram obtained from Neighbour-joining analyses of DNA sequences of nuclear ITS region. Bootstrap support values (1000 replicates) above 50% are indicated at the nodes. Isolates with markers represent those collected and sequenced in this study.

infested by *Tomicus* spp. in Poland, represented *Ophiostoma* sp. 1. This species was isolated from shoots with an average isolation frequency of 2% (from 0% at Jaroszowiec to 6% at Chrośnica). The second most frequent species (15 isolates) was *O. minus*, with frequencies ranging from 0% at Mielec to 5% at Jaroszowiec. The third taxon, *Sporothrix* sp. 1 (11 isolates) occurred at an average frequency of 2% (from 0% Jaroszowiec to 4% Babimost). *Leptographium piriforme*, *O. canum, G. cucullata*-like strain and *O. floccosum* were isolated from shoots infested by *Tomicus* spp. with very low frequencies (Tab. 2).



Figs 2–5. *Grosmannia cucullata*-like, (photo by R. Jankowiak): 2. Ascocarp (scale bar = 100 μm), 3. Ascospores (scale bar = 10 μm), 4. *Pesotum* anamorph (scale bar = 100 μm), 5. Conidia (scale bar = 10 μm). Fig.6. *Sporothrix* sp.1 (photo byT. Kowalski): conidiogenous cell with conidia (scale bar = 100 μm). Figs 7–10. *Ophiostoma* sp.1 (photo by R. Jankowiak): 7. *Pesotum* anamorph (scale bar = 100 μm), 8. Conidia (scale bar = 10 μm), 9. *Sporothrix* synanamorph (scale bar = 100 μm), 10. Conidia (scale bar = 10 μm).

DISCUSSION

This is the first survey of ophiostomatoid species associated with pine shoots damaged by adult individuals of *Tomicus* spp. yielded in 64 strains isolated from four pine forests stands in Poland. Based on morphological characteristics and DNA sequence comparison, four species of the genus *Ophiostoma* s.str. and after one *Grosmannia*, *Leptographium* and *Sporothrix* were confirmed as associated with fallen pine shoots. Three species, namely *O. canum*, *O. floccosum* and *O. minus,* have previously been recorded in Europe in association with *Tomicus* spp. beetles (Kirisits 2004). However, *O. floccosum* is reported here for the first time from Poland. This fungus is widely distributed in Europe primarily on pine trees, but usually it is recorded at low frequency (Linnakoski et al. 2010).

Two species, *Ophiostoma* sp. 1 and *Sporothrix* sp. 1 probably represent new taxa. According to molecular data, isolates of *Ophiostoma* sp. 1 obtained in this study resided in the *O. piceae*-complex including species having allantoid ascospores and both *Pesotum* and *Sporothrix* synanamorphs. *Ophiostoma* sp. 1 seems to be closely related to *O. quercus*. The morphological characteristics of *Sporothrix* sp. 1 broadly resemble species of the *S. schenckii–O. stenoceras* complex. Sequence data and phylogenetic analyses also placed *Sporothrix* sp. 1 in the *S. schenckii–O. stenoceras* complex. Sequences of *Sporothrix* sp. 1 even formed a separate lineage within *S. schenckii–O. stenoceras* complex.

Only single isolate represented *Grosmannia cucullata*-like, thus its taxonomical status remains unclear and requires confirmation based on greater number of isolates. Based on ITS sequences this strain is closely related to isolates of *G. cucullata*. This species was also morphologically similar to *G. cucullata* but had shorter perithecial neck and larger ascospores. *Grosmannia cucullata* has been reported from *Picea abies* (L.) Karst., *P. sylvestris* and *Larix decidua* Mill. in association with various species of bark beetles (Kirisits 2004) and cerambycids (Jankowiak, Kolařik 2010a). The taxonomic position of *Ophiostoma* sp. 1, *G. cucullata*-like and *Sporothrix* sp. 1 will be further discussed after a more critical study has been concluded.

We presumed that new *T. piniperda* and *T. minor* individuals may efficiently introduce spores of ophiostomatoid fungi to young pine shoots during maturation-feeding. Unexpectedly, the frequencies of the ophiostomatoid species were very low. None species was consistently isolated from each of the studied pine stands. Unfavorable growth conditions in very fresh tissues of shoots could be the reason the lower than expected frequency of these fungi. Our study demonstrates that among fungal associates of *Tomicus* spp. in Poland only *O. minus* may infest fresh pine shoots during maturation-feeding. These results confirmed the findings of Jankowiak and Kurek (2006), who isolated *O. minus* from *T. piniperda* galleries at relatively high frequencies in 10 weeks after the main beetles attack. The ability of *O. minus* to colonize the shoots results from its ability to tolerate low concentration of oxygen (Solheim et al. 2001). This physiological feature agreed with the high level of virulence of this species to pine (Lieutier et al. 1989; Solheim, Långström 1991; Solheim et al. 1993; Solheim et al. 2001; Jankowiak et al. 2007). However, low and very variable frequency of *O. minus* indicates that its role in damaging of pine shoots during the shoot feeding phase is rather limited.

Our studies showed that shoots laying on the forest floor may be relatively often colonized by ophiostomatoid fungi that are not closely associated with *Tomicus* spp. beetles, primarily by *Ophiostoma* sp. 1 and *Sporothrix* sp. 1. Ecology of these fungi is however unknown. We presume that various antropods as spiders, ants and dipterans may vectored propagules of ophiostomatoid species. In nature, we often observed unidentified spiders in the pitch of shoots damaged by *Tomicus* spp. Similar observations were made by Greif et al. (2006), who isolated *L. piriforme* from various arthropods taxa. The presence of *L. piriforme* in pine shoots demonstrated in our study, could be the result of such vectoring as well. Interestingly, this fungus has been described by Greif et al. (2006) from various arthropods collected in an aspen-dominated forest in western Canada and until 2010 it has been known only from this country. Our study showes that *L. piriforme* occurs pine forest habitat in Europe and may suggest that the species can develop on a wider range of host plants. Its pathogenicity has not been fully recognized yet. According to Jankowiak and Kolařik (2010b), *L. piriforme* appears to be a weak pathogen of *Pinus sylvestris*.

Sporothrix species placed in the *O. stenoceras-S. schenckii* complex are reported to live mostly as saprotrophs on wood or as soil fungi (de Beer et al. 2003). Six species that had been isolated directly from soil but one of the recent studies (de Meyer et al. 2008) have revealed next three new species associated with wood and soil: *Sporothrix stylites* de Meyer, Z.W. de Beer & M.J. Wingf., *S. humicola* de Meyer, Z.W. de Beer & M.J. Wingf. and *S. lignivora* de Meyer, Z.W. de Beer & M.J. Wingf. We suspect that *Sporothrix* sp. 1 may have a similar ecological niche as above mentioned species because this fungus was isolated from pine shoots laying often directly on the sandy soil.

CONCLUSIONS

The aim of this study was to characterize ophiostomatoid species associated with pine shoots damaged by *Tomicus* spp. in Poland. The diversity of reported was low, with seven species found. In addition, all the species were recorded at low frequencies. Our research show that the pine shoots damaged by *Tomicus* spp. beetles are poorly colonized by fungal associates of these insects. Only *Ophiostoma minus*, the most important associate of *T. piniperda* in Poland, was relatively often isolated from fallen pine shoots. Two other frequent species, *Ophiostoma* sp. 1 and *Sporothrix* sp. 1 probably represent undescribed taxa. This is also the first report of *Ophiostoma floccosum* from Poland.

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Grzyby z rodzaju *Ophiostoma* s.l. wyizolowane z opadłej cetyny sosnowej w Polsce

Streszczenie

Grzyby należące do grupy gatunków w przeszłości zaliczanych do rodzaju *Ophiostoma* s.l. są znane ze współżycia z owadami z rodzaju *Tomicus* żerującymi na sośnie zwyczajnej *Pinus sylvestris* L. Jednakże bardzo mało wiemy o grzybach występujących w pędach sosny, uszkodzonych przez te owady. Celem badań było poznanie składu gatunkowego i frekwencji występowania grzybów z tej grupy związanych z pędami sosny zwyczajnej uszkodzonymi przez żer uzupełniający cetyńców. Badania przeprowadzono w latach 2007-2008 w czterech drzewostanach sosnowych zlokalizowanych w różnych częściach Polski. Grzyby były identyfikowane na podstawie cech morfologicznych, a wybrane, reprezentatywne szczepy grzybów poddano także amplifikacji i sekwencjonowaniu fragmentów ITS1, 5.8S, ITS2 oraz fragmentów genu ß-tubuliny. Ogółem, z pędów uszkodzonych przez cetyńce, otrzymano 64 izolaty grzybów reprezentujące siedem gatunków grzybów należących do *Ophiostoma* s.l. Dwa z nich, *Ophiostoma* sp. 1 i *Sporothrix* sp. 1 prawdopodobnie reprezentują taksony nowe dla nauki. Niepewny pozostaje również status taksonomiczny izolatu zaklasyfikowanego jako *Grosmannia cucullata*-like.

Wszystkie stwierdzone gatunki charakteryzowały się niską częstością występowania. Wśród nich, *Ophiostoma minus*, *Ophiostoma* sp. 1 i *Sporothrix* sp. 1 były izolowane z 2.0% pędów. Pozostałe gatunki grzybów (*Leptographium piriforme*, *Ophiostoma canum*, *O. floccosum* i *Grosmannia cucullata*-like) zasiedlały pędy sosny sporadycznie. Wśród zidentyfikowanych grzybów *O. floccosum*, *Ophiostoma* sp. 1 i *Sporothrix* sp. 1 zostały stwierdzone w Polsce po raz pierwszy.