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Ophiostomatoid fungi associated with *Ips sexdentatus* on *Pinus sylvestris* in Poland

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Abstract: The six-toothed bark beetle (*Ips sexdentatus*) is known to be associated with ophiostomatoid fungi. However, very little is known about these fungi in Poland. The aim of this study was: 1) to identify fungi of *Ophiostoma s. lato* carried by this bark beetle and 2) to test the pathogenicity of several commonly occurring fungi. Isolations were carried out from the beetles and their galleries at three sites in Poland. Samples yielded a total 3162 cultures, which included 10 species of *Ophiostoma s. lato*. The most frequently encountered fungal associates of *I. sexdentatus* were *L.* cf. *truncatum*, *O. brunneo-ciliatum* and *O. ips*. *Ophiostoma* cf. *rectangulosporium*, *O.* cf. *abietinum*, *O. quercus* and *O. floccosum* appeared to be also specifically associated with *I. sexdentatus*. *Leptographium* cf. *truncatum* and *O. minus* were most virulent and can be considered as serious pine pathogens.

Additional key words: bark beetles; insect-fungal associations; *Leptographium*; *Ophiostoma*

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Introduction

Many species of bark beetles (Coleoptera: Scolytinae), also known as scolytids, infest *Pinus sylvestris* L. in Poland. Among them, the six-toothed bark beetle, *Ips sexdentatus* (Börn.) has caused little economic damage of pine forests in Poland because as a secondary pest only attacks dead or weakened trees. Occasionally, however under high population densities this beetle is able to attack healthy trees often in association with other pests, e.g. *Ips acuminatus* (Gyll.) or *Tomicus piniperda* (L.) (Michalski and Mazur 1999).

Many various microbes, especially ophiostomatoid fungi and species from *Geosmithia* genus (Ascomycota, Hypocreales) are associated with phloeophagous bark beetles (Kirisits 2004; Kolařík 2006). These groups include several serious pathogens of plants (e.g. *Ophiostoma novo-ulmi* Brasier or *Geosmithia morbida* Kolařík, Freeland, Utley & Tisserat) and many species that are agents of sapstain in trees and freshly cut logs (Kirisits 2004; Kolařík et al. 2011). The association between bark beetles and fungi suggests mutual benefit to both partners. Fungi associated with bark beetles play very important roles in the degrading hardly decomposing plant structures and provide main or additional nutrient source for insects development and/or reproduction. They may protect bark beetle larvae against detrimental fungi, balance moisture in bark beetle galleries, and due to their phytopathogenicity they may kill or accelerate the decline of stressed host trees (Beaver 1989; Six and Klepzig 2004; Six and Wingfield 2011). Six and Wingfield (2011) suggested that virulence of the ophiostomatoid fungi did not have an important role in ecology of bark beetles but rather supported their survival in fresh tissues of living trees and helped them overcome the host active defence. In turn, beetles act as vectors carrying fungal propagules with them to the nutrient-rich inner bark. Majority of phloem-feeding bark beetles disperse propagules of ophiostomatoid species on the surface of

their body or through the digestive system (Whitney 1982; Paine et al. 1997). Some of them (e.g. *Hylastes cunicularis* Er. or *Ips acuminatus* (Gyll.), similar as ambrosia beetles, have also highly specialized structures for fungal transmission – mycangia. *Ips sexdentatus* also belongs to this group and possesses mycangia as puncture pits on the mandibles, the pronotum and the elytra (Lévieux et al. 1989).

Ophiostomatoid fungi represent a group of morphologically similar genera, including *Ophiostoma, Grosmannia, Ceratocystiopsis, Ceratocystis, Gondwanamyces* and *Cornuvesica*. The most common conifer-associated species occurring in Europe belong to the genera *Ophiostoma* with *Hyalorhinocladiella*, *Pesotum* and *Sporothrix* anamorphs, and *Grosmannia* with *Leptographium* anamorph (Kirisits 2004; Zipfel et al. 2006; Linnakoski et al. 2012).

A high diversity of fungi associated with *I. sexdentatus* has been found in Europe (Francke-Grosmann 1952; Mathiesen-Käärik 1953; Levieux et al. 1989; Lieutier et al. 1989, 1991; Kirisits et al. 2000; Kirschner 2001; Romón et al. 2007; Bueno et al. 2010; Linnakoski et al. 2010). These studies showed that *Graphium pseudormiticum* (M. Morelet) K. Jacobs, Kirisits & M.J. Wingf., *Ophiostoma ainoae* H. Solheim, *O. brunneo-ciliatum* Math.-Käärik, *O. ips* (Rumbold) Nannf. and *O. minus* (Hedgc.) Syd. & P. Syd. were consistently and regularly found associated with *I. sexdentatus*. In Poland, there is only one report on fungi associated with *I. sexdentatus*. Siemaszko (1939) found that *Ambrosiella ips* (J.G. Leach, L.W. Orr & C.M. Chr.) L.R. Batra, *O. ips* and *O. minus* were associated with *I. sexdentatus* in pine stands in Anin.

In the above-mentioned papers, identification of the fungal species was based on the morphological characteristics only. According to Grobbelaar et al. (2009) using DNA sequence comparisons to identification of morphologically similar species of ophiostomatoid fungi is essential tool to reliable determination of these species, especially cryptic species. A recent studies using DNA sequencing reported the association of *I. sexdentatus* with *Ophiostoma canum*-like (Münch) Syd. & P. Syd., *Ophiostoma floccosum* Math.-Käärik and *O. minus* in Russia and Finland (Linnakoski et al. 2010), and with *Grosmannia olivacea* (Math.-Käärik) Zipfel, Z.W. de Beer & M.J. Wingf., *Leptographium guttulatum* M.J. Wingf. & K. Jacobs, *Leptographium truncatum*-like (M.J. Wingf. & Marasa) M.J. Wingf., *Ophiostoma ips*, *O. minus*, *O. pluriannulatum* (Hedgc.) Syd. & P. Syd., *O. rectangulosporium*-like Ohtaka, Masuya & Yamaoka and *O. stenoceras* (Robak) Nanf. in Spain (Romón et al. 2007) .

The results of the inoculation studies with fungal associates of bark beetles indicate that *O. brunneo-ciliatum*, *O. minus* and *O. ips* are pathogenic to *P. sylvestris* (Lieutier et al. 1989; Solheim and Långström 1991; Solheim et al. 1993; Guérard et al. 2000; Solheim et al. 2001; Fernández et al. 2004). However, *O. minus* seemed to be more virulent than two other fungal species. There is no information about the pathogenicity to *P. sylvestris* other associates of *I. sexdentatus*.

The studies were aimed to survey the ophiostomatoid fungi associated with *I. sexdentatus* in Poland. We determined isolates to the species level using their morphological characteristics, as well as DNA sequences data. In addition, the pathogenicity of several of the ophiostomatoid isolates was investigated by inoculating Scots pine seedlings.

Materials and methods

Study areas

During 2009–2011, beetles and galleries of *I. sexdentatus* were collected from infested logs in three 60 to 80-year-old stands of *P. sylvestris* in Poland: Babimost (49°27'07" N, 20°57'40" E), Pateraki (49°38'40" N, 20°48'49" E) and Wierzchosławice (49°27'07" N, 20°57'40" E). Four weeks before the flight period of *I. sexdentatus*, six to ten trap logs (2 m long and 0.25 m in diameter) of *P. sylvestris* were laid on the forest floor in each stand.

Fungal isolation from beetles

The first flight of *I. sexdentatus* in each year occurred between April 26th and May 7rd. Overwintered adults were collected from the pine logs surfaces or from the phloem at the time of gallery construction. The beetles were collected with sterilised tweezers and then stored individually in sterile microtubes (1.5 ml) for later isolations. For isolations, each beetle was removed from storage microtubes with sterilized tweezers, and without surface-sterilisation were squashed onto the surface of a selective medium for *Ophiostoma* spp. (CMEA: 20 g malt extract, Biocorp Sp. z.o.o., Warszawa, Poland; 20 g agar, Biocorp Sp. z.o.o., Warszawa, Polska; 0.2 g tetracycline, Polfa Tarchomin SA, Poland and 0.2 g cycloheximide, Sigma-Aldrich, St. Louis, USA; all per litre of distilled water).

Fungal isolation from gallery systems

The samples from the logs were taken 6 to 8 weeks after the main beetle attack, at which time the beetle broods were in the larval stages. Four sections of logs (20 cm long) containing beetle galleries were cut from the each beetle-infested log and transported to the laboratory. In the laboratory, the bark was separated from the wood under sterile conditions and each section was split into separate galleries. The galleries were disinfected using cotton wool saturated with 96% ethyl alcohol (15 sec.) and then were dried on filter paper. Isolation of fungi was made from phloem fragments (six one's per gallery) taken from

and around galleries of *I. sexdentatus*. Fragments (4 mm × 4 mm) were removed with a sterile scalpel, and placed in Petri dishes containing a culture medium (CMEA). Altogether, 1,644 samples of phloem were collected in this study. Fragments were taken from a total of 274 gallery systems of *I. sexdentatus* (64–110 galleries/site).

Cultural procedures, fungal identification and sequences analyses

All isolations were made on 2% CMEA medium. 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 (22–24°C) in the dark. Purified cultures were grouped according to culture morphology using microscope Nikon Eclipse 50*i* (Nicon® Corporation, Tokyo, Japan) and Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) with software Coolview 1.6.0 (Precoptic®, Warsaw, Poland). Fungal structures (conidiophores, conidia, perithecia, ascospores), and colony characteristics were compared with the species descriptions given in the literature (Batra 1967; Upadhyay 1981; Jacobs and Wingfield 2001). From each morphological group, isolates were selected for DNA sequencing (Table 1). They were deposited in the Culture Collection of Fungi of the Laboratory of Department of Forest Pathology, Hugo Kołłątaj University of Agriculture, Cracow, Poland.

DNA was extracted using PrepMan Ultra Sample preparation reagent (Applied Biosystems, Foster City, CA, USA) using the manufacturer's protocol. The ITS rDNA region (ITS1-5.8 S-ITS2) was amplified using the primers ITS 1F (Gardes and Bruns 1993) and ITS 4 (White et al. 1990). The ITS gene region were amplified in 25 μL reaction mixture containing 0.25 μL of Phusion High-Fidelity DNA poly-

Table 1. List of the isolates associated with *Ips sexdentatus* used for DNA sequencing and their GenBank accession numbers

Taxon	Isolate	Site*	Accession no.	Closest match in BLAST	Accession of match	Identity** %
Leptographium procerum	1257RJ	W	JQ289014	*L. procerum*	EU879143.1	100
Leptographium cf. *truncatum*	1069RJ	B	JQ288998	*L.* cf. *truncatum*	DQ539513.1	99
	1086RJ	B	JQ288999	*L.* cf. *truncatum*	DQ539512.1	99
Ophiostoma brunneo-ciliatum	1133RJ	P	JQ289009	*O. brunneo-ciliatum*	HM031499.1	99
	1134RJ	B	JQ289010	*O. brunneo-ciliatum*	HM031499.1	99
	1063RJ	B	JQ289011	*O. brunneo-ciliatum*	HM031499.1	99
	1114RJ	B	JQ289012	*O. brunneo-ciliatum*	HM031501.1	99
	1117RJ	B	JQ289013	*O. brunneo-ciliatum*	HM031499.1	99
Ophiostoma floccosum	1120RJ	B	JQ289004	*O. floccosum*	AJ538343.1	100
	1135RJ	B	JQ289005	*O. floccosum*	EF506933.1	100
	1111RJ	B	JQ289019	*O. floccosum*	EF506933.1	100
	1466RJ	W	JQ289024	*O. floccosum*	EF506933.1	100
Ophiostoma ips	1121RJ	B	JQ289015	*O. ips*	AY194933.1	100
	1112RJ	B	JQ289016	*O. ips*	AY194940.1	100
	1130RJ	B	JQ289017	*O. ips*	AY194933.1	100
Ophiostoma minus	1071RJ	B	JQ289000	*O. minus*	GU134172.1	100
	1072RJ	B	JQ289001	*O. minus*	AM943890.1	100
Ophiostoma piceae	1075RJ	B	JQ289003	*O. piceae*	AF493249.1	100
	870RJ	P	JQ289025	*O. piceae*	AF493247.1	100
Ophiostoma quercus	1137RJ	B	JQ289002	*O. quercus*	AY466622.1	100
	1454RJ	W	JQ289022	*O. quercus*	EF429090.1	100
	1456RJ	W	JQ289023	*O. quercus*	EF506936.1	100
	1121RJ	B	JQ289018	*O. quercus*	AY466622.1	100
	1136RJ	B	JQ289020	*O. quercus*	EF429090.1	100
Ophiostoma cf. *abietinum*	1129RJ	B	JQ289006	*O.* cf. *abietinum*	HM031511.1	100
	1066RJ	B	JQ289007	*O.* cf. *abietinum*	HM031511.1	100
Ophiostoma cf. *rectangulosporium*	1132RJ	B	JQ289008	*O.* cf. *rectangulosporium*	DQ539538.1	99
	1138RJ	B	JQ289021	*O.* cf. *rectangulosporium*	DQ539537.1	99

*B Babimost, W Wierzchosławice, P Pateraki

**derived from the pairwise alignment of each isolate sequence with the closest BLAST match in GenBank

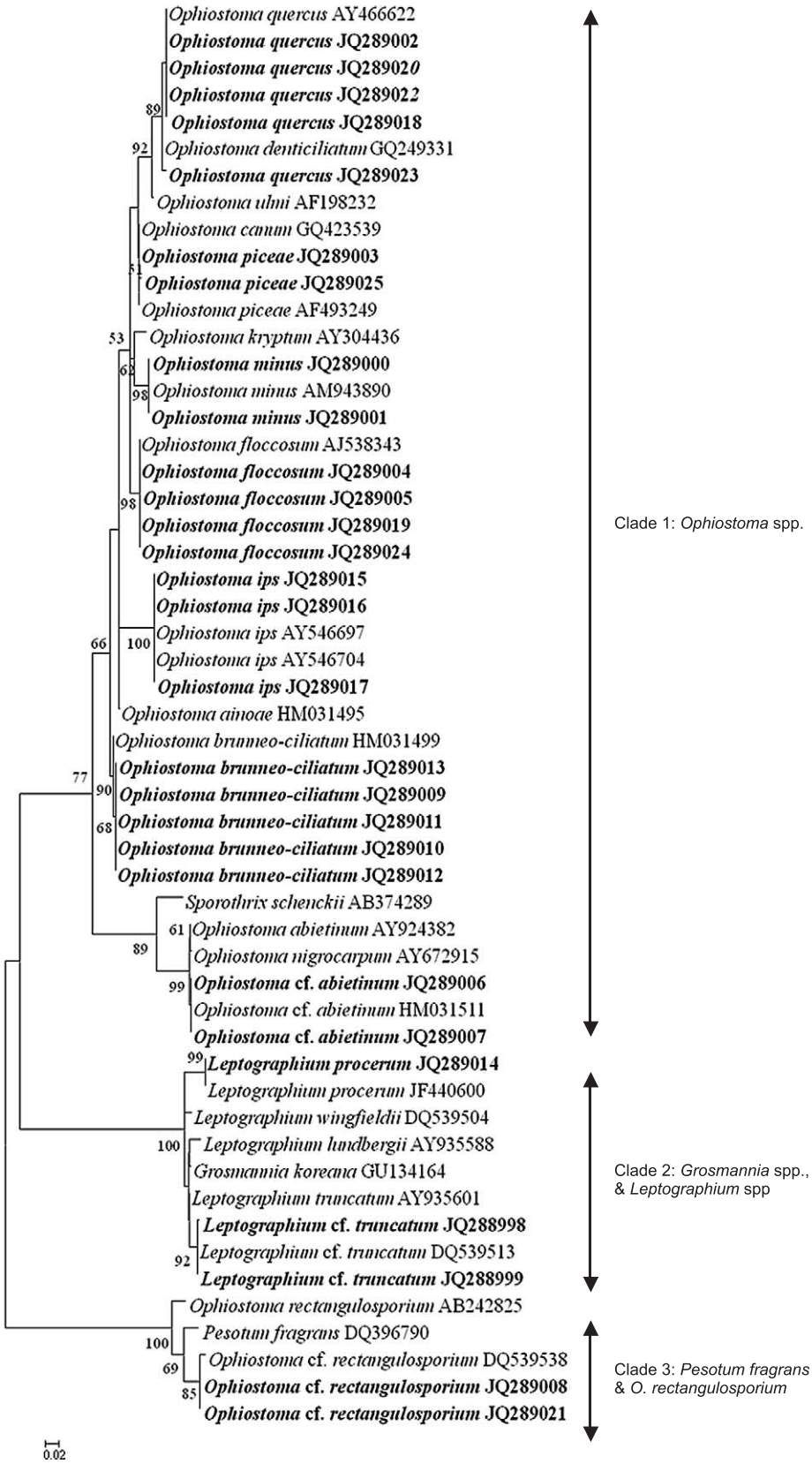

Fig. 1. Polygram obtained from ML analyses of the ITS region. ML bootstrap support values (1000 replicates) above 50% are indicated at the nodes. Scale bar=total nucleotide difference between taxa. Sequences obtained during this study with accession no. for NCBI database are bold printed

merase (Finnzymes, Espoo, Finland), 5 μL Phusion HF buffer (5×), 0.5 μL of dNTPs (10 mM), 0.75 μL DMSO (100%) and 0.5 μL of each primer (25 nM). Amplification reactions were performed using Biometra T-Personal 48 Thermocycler (Biometra GmbH, Goettingen, Germany). The PCR conditions were following: an initial denaturation step at 98°C for 30 sec., followed by 35 cycles of 5 sec. at 98°C, 10 sec. at 57°C and 30 sec. at 72°C, and a final chain elongation at 72°C for 8 min. The PCR products were visualized under UV light on 2% agarose gel stained ethidium bromide. Amplified products were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), using the same primers used for the PCR. Sequences (Table 1) were compared with data from GenBank using a Blast similarity search. All sequences were aligned using ClustalW (Thompson et al. 1994). Datasets were analysed using maximum likelihood (ML). ML analyses were performed using MEGA 5.04. assuming the K2 + G substitution model (Tamura et al. 2011). The best-fit model of nucleotide substitution was calculated in MEGA 5.04. under default parameters using the Bayesian information criterion (BIC, Schwarz 1978). Support for the nodes was estimated from 1000 replications. Sequences were deposited in GenBank (Table1). Accession numbers for sequences from reference isolates obtained from GenBank are presented in the phylogenetic tree (Fig. 1) and in the Table 1.

Pathogenicity test

To investigate the impact of the fungal associates of *I. sexdentatus* on Scots pine, infection experiments were conducted on 2-year-old *P. sylvestris* seedlings obtained from a nursery. Seedlings were growing in containers with a mixture of peat : perlite (8.5 : 1.5). The plants were maintained outdoor under ambient conditions and watered as required. Stem diameters at the inoculation site ranged from 3.08 to 5.80 mm (the mean diameter was 4.17 mm).

Four species of ophiostomatoid fungi commonly associated with *I. sexdentatus*: *L.* cf. *truncatum, O. brunneo-ciliatum, O. ips* and *O.* cf. *rectangulosporium* were used for the inoculation (Table 2). Additionally, *O. minus* a known pathogen of *P. sylvestris* was used as a positive control to estimate fungal virulence. Two random isolates of each fungus and one isolate of *O. minus* were used. On the 15th of August 2011, 135 seedlings were inoculated with selected isolates (15 seedlings for each isolate). Fifteen plants were inoculated with sterile MEA as negative controls. Inoculations were made by cutting out a bark flap (4 x 8 mm) with a sterile scalpel, placing inoculum on the exposed sapwood surface and covering it up with the bark flap and a Parafilm® M strip, as described by Krokene and Solheim (1998). The wounds were made on the first-year shoot of plants, four cm above the root collar. The inoculum consisted of a 3 mm disc of fungus growing on 2% MEA or sterile 2% MEA (negative control). The inoculum was taken from the margin of 12-day-old cultures grown at 22°C in dark.

Observations of plant mortality were performed at weekly intervals for 8 weeks. A seedling was considered dead when the stem and needles above the inoculation site were brown discoloured. After 8 weeks, all plants were harvested, and the bark was removed around the inoculation site. The length of the necrotic lesion on the sapwood surface was measured. The data were analysed using analysis of variance (ANOVA). Significant treatment differences were further evaluated by Fisher's (LSD) test (STATISTICA® 8.0 (StatSoft, Inc., USA).

Re-isolations of the inoculated fungi from each seedlings were attempted by removing two small sapwood pieces (1x1 mm) above and below the points of inoculation at distances of 0.5–1 cm and incubating them on 2% MEA. In total, 270 plant pieces were used to re-isolations of fungi. Plates were stored for 4 weeks at 22 °C in dark and then checked for the presence of the inoculated fungi.

Results

Identification of *Ophiostoma s. lato* species

After collecting and morphological identifying 567 adults and their galleries, isolations yielded a total of 3162 cultures, which included 10 species of *Ophiostoma s. lato* or their asexual states: *Leptographium* cf. *truncatum, Leptographium procerum* (W.B. Kendr.) M.J. Wingf., *Ophiostoma brunneo-ciliatum, Ophiostoma floccosum, Ophiostoma ips, Ophiostoma minus, Ophiostoma piceae* (Münch) Syd. & P. Syd., *Ophiostoma quercus* (Georgev.) Nannf., *Ophiostoma* cf. *abietinum* Marm. & Butin and *Ophiostoma* cf. *rectangulosporium* Ohtaka, Masuya & Yamaoka.

PCR of the ITS regions delivered products ranging from about 500 to 600 bp in size. Comparison of the ITS sequences with GenBank sequences confirmed the identities of ten species of *Ophiostoma s. lato*. ITS rDNA sequences of *L. procerum, O. floccosum, O. ips, O. minus, O. piceae* and *O. quercus* were identical to reference sequences while sequences of *O. brunneo-ciliatum, L.* cf. *truncatum* and *O.* cf. *rectangulosporium* strains showed 99% similarity with published sequences (Table 1). The phylogenetic tree using ML analysis placed the fungal taxa into tree major clades (Fig. 1). Within clade 1 all selected isolates represented *Ophiostoma* spp. with *Hyalorhinocladiella, Sporothrix* and/or *Pesotum* anamorphs (*O. brunneo-ciliatum, O. floccosum, O. ips, O. minus, O. piceae, O. quercus* and *O.* cf. *abietinum*) and were clustered together with the others members

Table 2. List of isolates used in the pathogenicity test

Species	Isolate	Substrate[a]
Leptographium cf. *truncatum*	1069RJ	*Ips sexdentatus*, adult
	1086RJ	*Ips sexdentatus*, adult
Ophiostoma brunneo-ciliatum	1014RJ	*Ips sexdentatus*, adult
	1017RJ	*Ips sexdentatus*, adult
Ophiostoma ips	1112RJ	*Ips sexdentatus*, galleries
	1121RJ	*Ips sexdentatus*, galleries
Ophiostoma cf. *rectangulosporium*	1132RJ	*Ips sexdentatus*, galleries
	1138RJ	*Ips sexdentatus*, galleries
Ophiostoma minus	1071RJ	*Ips sexdentatus*, adult

[a]All isolates were collected in 2010 at Babimost site

of *Ophiostoma* spp. The last species, *Ophiostoma* cf. *abietinum* had a *Sporothrix* anamorph and was most closely placed to *O. abietinum* in the phylogenetical analysis (Fig. 1). Within clade 2, isolates produced *Leptographium* anamorphs (*L.* cf. *truncatum* and *L. procerum*) and corresponded to other known *Grosmannia* and *Leptographium* species. The *O.* cf. *rectangulosporium* isolates with *Pesotum* and *Leptographium*-like anamorphs grouped in a clade 3 containing published sequences of *Pesotum fragrans* (Math.-Käärik) G. Okada & Seifert and *O. rectangulosporium* (Fig. 1).

Fungal isolation from *I. sexdentatus* beetles

A total of 518 fungal isolates were obtained from *I. sexdentatus* adults. Nine *Ophiostoma s. lato* species were identified among the isolates, including *L.* cf. *truncatum*, *L. procerum*, *O. brunneo-ciliatum*, *O. ips*, *O. minus*, *O. quercus*, *O. floccosum*, *O.* cf. *abietinum* and *O.* cf. *rectangulosporium* (Table 3). *Leptographium* cf. *truncatum* and *O. brunneo-ciliatum* were most frequently isolated from *I. sexdentatus* adults (64 and 47%, respectively) (Table 3). *Leptographium* cf. *truncatum* was isolated at frequency from 46% at Babimost to 94% at Wierzchosławice, while *O. brunneo-ciliatum* occurred at frequency from 38% at Babimost to 59% at Pateraki (Table 3). *Ophiostoma ips* was also common species, which was isolated from 35% of the beetles (from 28% at Babimost to 53% at Wierzchosławice). *Ophiostoma* cf. *abietinum* and *O.* cf. *rectangulosporium* were isolated from 8 and 11% of the beetles, respectively. These species were found most frequently at Wierzchosławice (Table 3).

Fungal isolation from *I. sexdentatus* galleries

A total of 2644 isolates were collected from *I. sexdentatus* galleries. These isolates representing the

Table 3. Frequency of occurrence (%) of *Ophiostoma s. lato* species isolated from adults of *Ips sexdentatus* and their galleries

Taxon	Frequency of occurrence (%)*							
	Babimost		Wierzchosławice		Pateraki		Total	
	Adults	Galleries	Adults	Galleries	Adults	Galleries	Adults	Galleries
Leptographium procerum	2			2			1	1
Leptographium cf. *truncatum*	46	62	94	100	77	77	64	79
Ophiotoma brunneo-ciliatum	38	70	58	69	59	72	47	70
Ophiostoma floccosum	1	6	2	4	4	5	2	5
Ophiostoma ips	28	42	53	45	33	47	35	44
Ophiostoma piceae		1				2		1
Ophiostoma quercus	3	10	2	5	10	9	4	8
Ophiostoma minus	6	2			7		5	1
Ophiostoma cf. *abietinum*	4	16	19	10	9	5	8	11
Ophiostoma cf. *rectangulosporium*	7	19	22	24	12		11	16
Total isolates	213	1158	159	917	146	567	518	2644
Number of adults/galleries	160	110	64	100	69	64	293	274

*Frequency of occurrence of each fungal species isolated from beetle bodies and galleries was calculated using formula: F = (NS/NTs) × 100; where F represents the frequency of occurrence (%) of the fungus from each niche; NS represent the number of beetles or galleries from which a particular fungus was isolated and NTs represents the total number of beetles or galleries

Table 4. The results of the inoculation experiments with fungi associated with *Ips sexdentatus* on two-year-old seedlings. The lesion length with the same letter were not significantly different according to the Fisher's test (P=0.05) following ANOVA

Species	Isolate	Mean lesion length (mm)	% dead plants	% successful re-isolation
Leptographium cf. *truncatum*	1086RJ	33.0^{ef}	46.7	73.3
	1069RJ	–	93.3	100
Ophiostoma brunneo-ciliatum	1114RJ	15.9^{c}	0	86.7
	1117RJ	19.3^{cd}	6.7	100
Ophiostoma ips	1112RJ	24.0^{de}	33.3	80
	1121RJ	33.5^{f}	33.3	100
Ophiostoma minus	1071RJ	–	100	80
Ophiostoma cf. *rectangulosporium*	1132RJ	8.9^{b}	0	66.7
	1138RJ	17.8^{c}	13.3	73.3
control		0.4^{a}	0	

same species as were isolated from the beetles and *O. piceae* (Table 3). All fungal species, except *O. minus* were isolated from galleries at higher frequency than from the beetles. *Leptographium* cf. *truncatum* and *O. brunneo-ciliatum* were most frequently isolated from galleries (79% and 70%, respectively) (Table 3). *Leptographium* cf. *truncatum* was most frequently (100%) obtained from *I. sexdentatus* galleries in Wierzchosławice, while *O. brunneo-ciliatum* occurred at frequencies about 70% at all sites. The third most common species in *I. sexdentatus* galleries was *O. ips* with an average frequency of 44% (from 42% at Babimost to 47% at Pateraki). *Ophiostoma* cf. *abietinum* and *O.*cf. *rectangulosporium* were found in 11 and 16% of galleries, respectively (Table 3).

Pathogenicity test

Leptographium cf. *truncatum* 1069RJ and *O. minus* killed 93% and 100% of the 2-year-old plants within 8 weeks after inoculation. *Ophiostoma ips* (isolate 1112RJ and 1121RJ) and *L.* cf. *truncatum* 1086RJ killed 33-47% of the seedlings (Table 4). First symptoms of plants dying, wilting of the new shoots of the current's years growth and yellow-brownish needles, were observed 3–5 weeks after inoculation. *Ophiostoma* cf. *rectangulosporium* 1138RJ and *O. brunneo-ciliatum* 1117RJ caused the dead of 13 and 7% seedlings, respectively (Table 4). No control plants or plants inoculated with *O.* cf. *rectangulosporium* 1132RJ and *O. brunneo-ciliatum* 1114RJ died (Table 4).

All the inoculated fungi caused necrotic lesions in the two-year-old seedlings and all fungi induced longer lesions than sterile inoculated control plants. *Leptographium* cf. *truncatum* 1086RJ, and both isolates of *O. ips* induced significantly larger necrotic lesions than *O. brunneo-ciliatum* and *O.* cf. *rectangulosporium* (Table 4).

The inoculated fungi were successfully re-isolated from 67–100% of the plants (Table 4).

Discussion

Based on morphological characteristics and DNA sequences comparisons, ten species of *Ophiostoma s. lato*, including *Ophiostoma* and *Leptographium* spp., were found associated with the *I. sexdentatus* in Poland. The fungal taxa obtained in this study resided in the three clades representing *Ophiostoma* spp., *Grosmannia/Leptographium* spp. and *P. fragrans* & *O.* cf. *rectangulosporium* group. Majority of them were placed in *Ophiostoma* genus including *O. piceae-*, *O. minus-*, *O. ips* and *S. schenckii* – *O. stenoceras* complexes (Harrington et al. 2001; de Beer et al. 2003a; Zipfel et al. 2006).

In the present study, were isolated more diverse fungal associates of the *I. sexdentatus* than previously reported. While *L.* cf. *truncatum, O. brunneo-ciliatum, O. floccosum, O. ips, O. minus* and *O. piceae* have previously been reported from *I. sexdentatus* (Siemaszko 1939; Mathiesen-Käärik 1953; Lieutier et al. 1989; Kirisits et al. 2000; Kirschner 2001; Romón et al. 2007; Bueno et al. 2010; Linnakoski et al. 2010), additionally were isolated *L. procerum, O. quercus*, and *O.* cf. *abietinum*. In contrast to previous investigations, this study did not find the presence of *O. canum*-like, *Ceratocystiopsis minuta* (Siemaszko) H.P Upadhyay & W.B. Kendr., *Ophiostoma ainoae, O. araucariae* (Butin) de Hoog & R.J. Sceff., *O. clavatum* Math.-Käärik, *O. japonicum* Yamaoka & M.J. Wingf., *O. obscurum* (R.W. Davidson) Hendr., *O. piceaperdum* (Rumbold) Arx in association with *I. sexdentatus* on *P. sylvestris* (Mathiesen-Käärik 1953; Kirschner 2001). Possibly that *O. piceae* emerging from this study might be the isolates of *O. canum*-like. According to Linnakoski et al. (2010) these two species could not be distinguished based on ITS sequences and morphology. ß-tubulin sequences must be used for identification of *O. piceae* and *O. canum*-like. In the comparative Finnish and Russian studies (Linnakoski et al. 2010), besides *O. canum*-like only *O. floccosum* and *O. minus*

have been found in association with *I. sexdentatus* on *P. sylvestris*. Different sampling, geographic locations of study sites and different host trees could possibly influence the number of ophiostomatoid fungi associated with *I. sexdentatus*.

Surprisingly, in the present study, the most commonly encountered species was *L.* cf. *truncatum*. In the ITS tree (Fig. 1), *L.* cf. *truncatum* grouped most closely to *L. truncatum*. However, *L.* cf. *truncatum* is distinguished from *L. truncatum* by the sizes and shape of conidia. All isolates examined displayed the characteristic obovoid conidia with a size of 14.7–27.1 × 6.2–12.0 μm. This is in contrast to *L. truncatum* which have broadly ellipsoid conidia with a size of 3-5 x 2-4 μm (Jacobs et al. 2005). In addition, conidiophores of *L.* cf. *truncatum* most often are arranged in close groups on the hyphae like sporodochia. Despite a very high isolation frequency of *L.* cf. *truncatum* in this study, no isolates of this fungus have not been previously reported in association with *I. sexdentatus* or *P. sylvestris*. *Leptographium* cf. *truncatum* had been only sporadically recorded from Spain in earlier study (Romón et al. 2007). Interesting that, *L.* cf. *truncatum* had not been also found in earlier polish studies (Siemaszko 1939). Possibly that *L.* cf. *truncatum* emerging from this study might be the same as *Ambrosiella ips* identified by Siemaszko on the basis of morphology only. The shape and size range of *L.* cf. *truncatum* conidia agrees closely with that given by Siemaszko (1939) for *A. ips* (15–25 × 5.4–12.5 μm). Unfortunately, we have no original fungal isolates determined as *A. ips* by Siemaszko (1939). *Ambrosiella ips* had yet been reported from *I.sexdentatus* in Scandinavian studies only (Mathiensen-Käärik 1953). In 2010 Harrington et al. on the basis of morphology and LSU rDNA sequences comparisons transferred *A. ips* to the anamorph genus *Hyalorhinocladiella* as *H. ips* (J.G. Leach, L.W. Orr & C.M. Chr.) T.C. Harr. The American isolates of *A. ips* used in this study, similarly as isolates of *L.* cf. *truncatum,* formed sporodochia in galleries but sequence analyses of *A. ips* placed these isolates among *Ophiostoma* species with *Hyalorhinocladiella* anamorphs, such as *O. ips* or *O. bicolor* R.W. Davidson & D.E. Wells.

The other *Leptographium* species, *L. procerum* was isolated at very low frequency from adult *I. sexdentatus* and their galleries. This suggests that this fungus is weakly associated with *I. sexdentatus* in Poland. *Leptographium procerum* is quite often found in Poland. So far this fungus was recorded in association with *Tomicus* spp. (Jankowiak 2006, 2008), cerambycid beetles (Jankowiak and Kolařík 2010; Jankowiak and Rossa 2007) and root-feeding beetles, such as *Hylastes* spp., *Hylurgus ligniperda* (Fabr.) and *Hylobius abietis* (L.) (Jankowiak and Bilański unpublished).

Ophiostoma brunneo-ciliatum and *O. ips* were found on the beetle's bodies as well as in their galleries with relatively high frequency and seem to be a close associate of *I. sexdentatus* in Poland. These associations were previously recorded by other authors (Siemaszko 1939; Francke-Grosmann 1952; Mathiesen-Käärik 1953; Lieutier et al. 1989,1991; Kirschner 2001; Bueno et al. 2010), but were not found in Finland, Russia (Linnakoski et al. 2010) and Spain (Romón et al. 2007). In Poland, *O. brunneo-ciliatum* had been also reported from *Larix decidua* (Mill.) as the main associate of *Ips cembrae* (Heer) (Jankowiak et al. 2007b) and from *P. sylvestris* infested by *Ips typographus* (L.) (Jankowiak and Hilszczański 2005).

Consistently with Romón et al. (2007) data, *I. sexdentatus* carried spores *O.* cf. *rectangulosporium*. In Europe up to the present study it was known only from *Pinus radiata* D. Don (Romón et al. 2007) and from *P. sylvestris* (Jankowiak and Kot 2011). In contrast to results of Romón et al. (2007), *O.* cf. *rectangulosporium* was frequently isolated from *I. sexdentatus* adults and their galleries in this study. This results suggest that *O.* cf. *rectangulosporium* is vectored by *I. sexdentatus*. This species is close to *Pesotum fragrans* and *O. rectangulosporium* in the phylogenetic analysis (Fig. 1). These fungal taxa form a distinct lineage in the Ophiostomatales and probably represent a new genus (Paciura et al. 2010). It is very probable that *O.* cf. *rectangulosporium* is widely distributed on Scots pine trees in Poland because this fungus was found in association with several bark beetle and weevils species infesting Scots pine (Jankowiak and Kot 2011; Jankowiak, unpublished data). Results of this study showed also that *I. sexdentatus* is associated with *O.* cf. *abietinum*, which is first reported from Poland. Up to the present study it was known only from Russia (Linnakoski et al. 2010).

Due to strong contamination of *I. sexdentatus* beetles by fast growing micro-organisms like *Mortierella* spp., *Trichoderma* spp. or *Mucor* spp., the selective medium (CHMA) for *Ophiostoma* spp. has been used in this study for isolation purposes. Therefore ophiostomatoid species sensitive to cycloheximide (e.g *Ceratocystis* nad *Graphium* spp.) could not be detected. Nevertheless in galleries of *I. sexdentatus* synnemata of *Graphium pseudormiticum* M. Mouton & M.J Wingf. have often been observed. This fungus was also a common associate of *I. sexdentatus* in Germany (Kirschner 2001) and Austria (Kirisits 2004).

The higher isolation frequency of *O. quercus* than *O. piceae* in this study was unexpected. According to Harrington et al. (2001), *O. piceae* is mostly isolated from a variety of Pinaceae tree hosts, while *O. quercus* mostly colonized tissues of hardwood trees. However, recent studies have shown that *O. quercus* is much more widely distributed on woody substrates than previously recognized, with reports of the fungus coming from pines plantations (de Beer et al. 2003b; Zhou et al. 2006). *Ophiostoma piceae* was

indentified from Poland in several earlier studies (Jankowiak 2006, 2008) based only on morphology. However, the DNA sequence comparisons used in this study suggest that in Poland *O. quercus* can be more widespread in *P. sylvestris* stands than *O. piceae*. Similar results have also been obtained for other species of bark beetles and weevils infesting *P. sylvestris* in Poland (Jankowiak and Bilański, unpublished). Detailed ecological studies are needed to define the host range for *O. quercus* and *O. piceae*.

Another species collected from *I. sexdentatus* adults and their galleries in this study was *O. floccosum*, which was found at relatively low frequency. *Ophiostoma floccosum* is known to be a common associate of phloephagous bark beetles but usually occurring at low frequencies (Kirisits 2004; Linnakoski et al. 2010). Up to the present study it was known in Poland only from fallen shoots of Scots pine pruned by *Tomicus* species (Jankowiak and Kolařík 2011). The fungus seems to be an infrequent associate of conifer-infesting bark beetles in Poland. The other blue-stain species, *O. minus* was isolated at very low frequencies from adult *I. sexdentatus* and their galleries. In Poland, this fungus has been isolated from pine attacked by *T. piniperda* with variable frequencies (for example, Jankowiak 2006).

The fungal species tested in this study differed in their virulence to Scots pine. *Ophiostoma minus* and *L.* cf. *truncatum* were much more virulent than other fungi evaluated in this study. The high virulence of *O. minus* is in accordance with results of other investigations (Lieutier et al. 1989; Solheim and Långström 1991; Solheim et al. 1993; Solheim et al. 2001; Jankowiak et al. 2007a). However, the high virulence of *L.* cf. *truncatum* to pine seedlings was a surprising. It seems that this fungus due to its high phytopathogenicity may survive in fresh tissues of trees after beetle attack and may effective compete with other organisms for food resources. To fully explain pathogenic capability of *L.* cf. *truncatum*, further pathogenicity studies should be conducted using mature Scots pine trees although Krokene and Solheim (1998) gave a clear evidence that inoculation of seedlings was a suitable method for determining the virulence of bark beetle-associated blue-stain fungi. However, results from seedling inoculations must be carefully interpreted because young trees do not have the well-developed defense system found in older trees (Sandnes and Solheim 2002). Therefore some authors suggested that fungi appear to be more pathogenic to seedlings than to larger trees (Basham 1970).

Consistent with previous results (Fernández et al. 2004), *O. ips* had also pathogenic capability, while isolates of other inoculated fungi appeared to have low virulence against Scots pine seedlings.

In conclusion, this study showed that *L.* cf. *truncatum*, *O. brunneo-ciliatum*, *O. ips* were a common associates of *I. sexdentatus*. Among the other species, *O.* cf. *rectangulosporium*, *O.* cf. *abietinum*, *O. quercus* and *O. floccosum* seemed to be also more closely associated with *I. sexdentatus*, while *L. procerum*, *O. piceae* and *O. minus* appear to be sporadic associates. *Leptographium procerum*, *O. quercus* and *O.* cf. *abietinum* are documented here as a new associate of *I. sexdentatus*, whereas *L.* cf. *truncatum* was isolated for the first time from *P. sylvestris* trees. Inoculation of Scots pine seedlings indicated that *L.* cf. *truncatum*, and *O. minus* were more virulent than other fungi associated with *I. sexdentatus*.

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