Annals of Forest Science (2013) 70:391–402
DOI 10.1007/s13595-013-0266-z

ORIGINAL PAPER

Diversity of ophiostomatoid fungi associated with the large pine weevil, *Hylobius abietis*, and infested Scots pine seedlings in Poland

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Received: 12 July 2012 / Accepted: 23 January 2013 / Published online: 15 February 2013

Abstract
• ***Context*** Bark beetles are known to be associated with fungi, especially the ophiostomatoid fungi. However, very little is known about role of pine weevils, e.g., *Hylobius abietis*, as a vector of these fungi in Europe.
• ***Aims*** The aims of our study were to demonstrate the effectiveness of *H. abietis* as a vector of ophiostomatoid fungi in Poland and to identify these fungi in Scots pine seedlings damaged by weevil maturation feeding.
• ***Methods*** Insects and damaged Scots pine seedlings were collected from 21 reforestation sites in Poland. The fungi were identified based on morphology, DNA sequence comparisons for two gene regions (ITS, β-tubulin) and phylogenetic analyses.
• ***Results*** Sixteen of the ophiostomatoid species were isolated and identified. In all insect populations, *Leptographium procerum* was the most commonly isolated fungus (84 %). *Ophiostoma quercus* was also found at a relatively high frequency (16 %). Other ophiostomatoid fungi were found only rarely. Among these rarer fungi, four species, *Leptographium lundbergii*, *Ophiostoma floccosum*, *Ophiostoma piliferum* and *Sporothrix inflata*, were isolated above 3 %. *L. procerum* was isolated most frequently and was found in 88 % of the damaged seedlings. *S. inflata* was isolated from 26 %, while *O. quercus* occurred in 10 % of the seedlings.
• ***Conclusion*** This study confirmed that *L. procerum* and *O. quercus* were common associates of *H. abietis*, while others species were found inconsistently and in low numbers, indicating causal associations. *H. abietis* also acted as an effective vector transmitting ophiostomatoid species, especially *L. procerum* and *S. inflata*, to Scots pine seedlings.

Keywords *Hylobius abietis* · Insect–fungus interactions · Ophiostomatoid fungi · *Pinus sylvestris* · Weevil

Handling Editor: Francois Lieutier

Contribution of the co-authors Robert Jankowiak: writing the paper, collecting of samples, identification of fungi and analysing the data. Piotr Bilański: collecting of samples and analysing the data.

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1 Introduction

The large pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae), is a serious pest in managed stands of young Scots pine and Norway spruce in Poland (Skrzecz and Moore 1997). This weevil is found throughout Europe, across northern Asia, and into Japan, and it causes heavy economic losses, especially in Scandinavia and central Europe (Grégoire and Evans 2004). In Europe, damage caused by *H. abietis* is a major problem for conifer forests in which cutting is an aspect of the silvicultural management system and in those with even-aged, young monocultures (Örlander et al. 1997). In recent years (1991–2010), *H. abietis* damage has affected new clear-cutting areas of approximately 20,000 ha in Poland (Tarwacki 2011). The high risk of *H. abietis* damage seen in many reforested sites is likely due to the optimal breeding conditions for larvae that develop in the stumps and root systems of trees after clear-felling. The adult weevils that emerge from the stumps then feed on the stem-bark of young seedlings from the root collar up. The extensive weevil attack reduces plant growth and often leads to death when the stems of seedlings are completely ring-barked (Wallertz et al. 2005).

Bark beetles live in a close association with ophiostomatoid fungi (Ascomycota: Ophiostomatales) (Wingfield et al. 1993). The majority of ophiostomatoid species include the sexual genera *Ophiostoma* and the asexual genera *Pesotum*, *Sporothrix* and *Hyalorhinocladiella*, and *Grosmannia* with *Leptographium* anamorphs (Zipfel et al. 2006). The association between bark beetles and fungi may provide benefits to both parties. Fungi associated with bark beetles play very important roles in the degradation of difficult-to-decompose plant structures and provide the nutrient sources for the insects' development. They may protect bark beetle larvae against detrimental fungi and balance moisture in bark beetle galleries, and due to their phytopathogenicity, they may kill or accelerate the decline of stressed host trees. In turn, beetles act as vectors carrying fungal propagules with them to the nutrient-rich inner bark (Six 2003; Six and Wingfield 2011). Although the existence of a mutualistic relationship between phloem-feeding weevils and fungi remains unproven, Pestaña and Santolomazza-Carbone (2010) recently demonstrated that *Pissodes castaneus* (De Geer) benefits from the presence of *Leptographium serpens* (Goid.) M.J. Wingf. in *Pinus pinaster* Aiton in Spain. This provides evidence that weevils with a similar life strategy as bark beetles (development under the bark of trees) can be consistently associated with various microbes. We certainly know that phloem-feeding weevils act as effective transmission vectors of root-rot fungi, such as *Heterobasidion annosum* Fr. (Bref.) (Kadlec et al. 1992) and *Armillaria* spp. (Livingstone and Wingfield 1982), the rust pathogen *Peridermium pini* (Willd.) Lév. (Pappinen and von Weissenberg 1994) and the ophiostomatoid fungi (Viiri 2004). Most weevils are contaminated with fungal spores both internally (within the digestive tract) and externally (Viiri 2004). According to Lévieux et al. (1994), adults of *H. abietis* disperse fungal spores on the surface of their bodies, especially around the anterior part of the dorsal and lateral sides of the pronotum. Spores are located in cuticular depressions associated with the pronotal setae. These authors also speculated that the spores are additionally protected by secretions of the pronotal grandular apparatus.

Ophiostomatoid fungi are known to be associated with many regeneration weevils (Coleoptera: Curculionidae) in North America (Eckhardt et al. 2007; Hanula et al. 2002; Jacobs and Wingfield 2001; Nevill and Alexander 1992; Wingfield 1983; Zanzot et al. 2010). In the southeastern USA, loblolly pine (*Pinus teada* L.) decline is caused by *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., *Leptographium terebrantis* S.J. Barras & T.J. Perry and *L. serpens* and their curculionid vectors, including *Hylobius pales* (Herbst) and *Pachylobius picivorus* Germar (Eckhardt et al. 2007). In a recent study in Georgian longleaf pine (*Pinus palustris* Miller) stands, the same vectors were found to be infested with *L. procerum*; *L. terebrantis*; *Grosmannia huntii* (Rob.-Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf.; *Ophiostoma ips* (Rumbold) Nannf. and other *Ophiostoma* and *Pesotum* species (Zanzot et al. 2010). In the northeastern USA, the fungal symbionts (*L. procerum* and *L. terebrantis*) of *H. pales* and *Pissodes nemorensis* Germar were found to be an important mortality factor in white pine (*Pinus strobus* L.) root decline (Nevill and Alexander 1992). The same species of *Leptographium* and other ophiostomatoid fungi, including *O. ips* and *G. huntii*, are vectored by *H. pales*, *P. picivorus* and *Hylobius radicis* Buchanan and have been implicated as factors in the decline of red pine (*Pinus resinosa* Aiton) (Klepzig et al. 1991). In addition, a recently described species, *Leptographium bhutanense* X.D. Zhou, K. Jacobs & M.J. Wingf., has been found in association with *Hylobitelus chenkupdorjii* Osella on *Pinus wallichiana* A.B. Jacks in Bhutan (Zhou et al. 2008).

The ecology of regeneration weevils and their associated fungi has already been well characterised in North America; however, information about European root-feeding weevil-associated ophiostomatoid fungi is very limited (Lévieux et al. 1994; Piou 1993). In France, *H. abietis* has been found carrying *L. procerum*, *Leptographium wingfieldii* M. Morelet, *Ophiostoma canum* (Münch) Syd. & P. Syd. and *Ophiostoma piliferum* (Fr.) Syd. & P. Syd. (Piou 1993). Of all the fungi reported by Piou (1993), *L. procerum* was the most commonly observed. This species had also been isolated from dead Scots pine seedlings that were damaged by large pine weevil feeding. *H. abietis* was also shown to be associated with *Leptographium alethinum* K. Jacobs, M.J. Wingf. & Uzunovic in England (Jacobs et al. 2001). The objectives of this study were (1) to determine the distribution and frequency of the ophiostomatoid species associated with *H. abietis* in Poland and (2) to determine whether adults of *H. abietis* introduce ophiostomatoid fungi to young live Scots pine seedlings during maturation feeding.

2 Materials and methods

2.1 Beetle and seedling sampling

Newly emerged *H. abietis* adults were collected from trap logs placed in 21 reforested stands of Scots pine in Poland during 2009–2011 (Fig. 1). The sites did not differ in tree species distribution before clear felling and were established on formerly Scots pine-dominated sites with a small mix of *Quercus robur* L. and *Betula pendula* Roth. All reforested areas were surrounded by managed Scots pine forests in various age classes. To obtain weevils, the trap logs (approximately 40 per site), 1.0 m long and 0.2 m in diameter, were laid on the soil in each reforestation site. Between April and May, 1,050 weevils (50 adults per site) were

caught with sterile forceps and then stored individually in sterile microtubes (1.5 mL) for later fungal isolations. The adults were collected during maturation feeding when they walked upon or bit into the bark surface of trap logs. To perform the isolation, each weevil was removed from the storage microtube with sterilised tweezers and squashed onto the surface of a selective medium for *Ophiostoma* spp. (CMEA: 20 g malt extract, 20 g agar, 0.2 g tetracycline and 0.2 g cycloheximide; all per litre of distilled water).

The Scots pine seedlings were collected in early May 2011 from four reforested sites (Fig. 1). The 1-year-old bare-rooted seedlings were planted in mid-April and had not been treated with insecticide. All seedlings were collected during the period of weevil maturation feeding (typically on the bark of seedlings stems) and were living specimens with green-yellow needles. The areas damaged by weevils were located on the main stems and reached to the xylem of seedlings. Scots pine seedlings damaged by weevils were cut with a sterile secateur and placed in separate, clean paper bags. Then, seedlings were stored for a maximum of 48 h in a cool room at 4 °C until they were used for the isolation of fungi. Wound samples were cut into 6-cm-long sections and surface-disinfected by immersion in 95 % ethyl alcohol. After drying, small pieces of wounds (approximately 5×5 mm) were removed from each wound section (six pieces per one wound) and placed in Petri dishes containing CMEA medium. Altogether, 1,392 samples of wounds were collected in this study. Fragments were taken from a total of 232 seedlings damaged by *H. abietis* (28–93 seedlings/site).

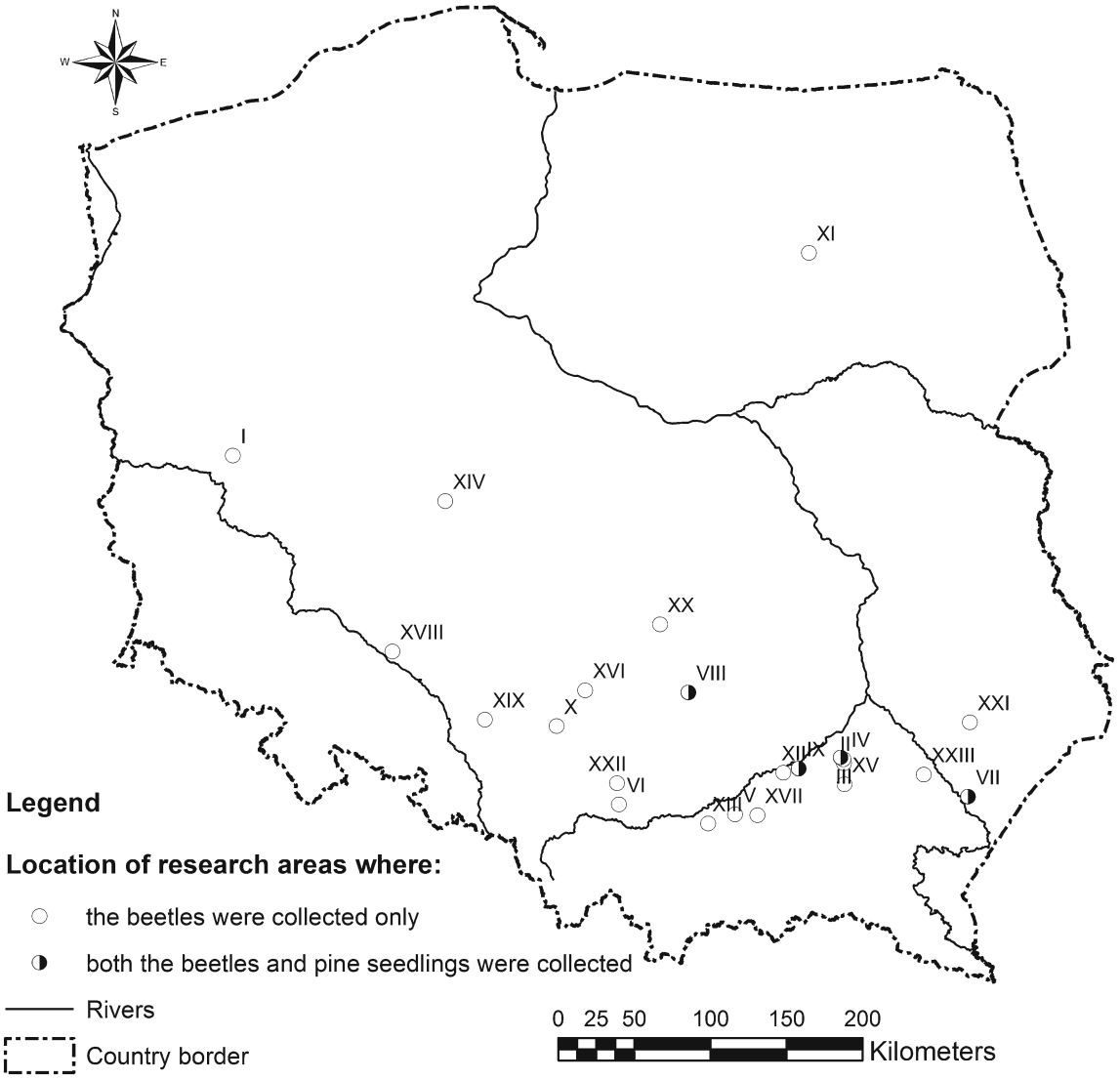

Fig. 1 Location of sites where adults of *H. abietis* and seedlings damaged by insects were collected: *I* Babimost, *II* Pateraki, *III* Czajkowa 1, *IV* Czajkowa 2, *V* Dąbrowa Tarnowska, *VI* Dulowa, *VII* Jarosław, *VIII* Jędrzejów, *IX* Szczurowa, *X* Koszęcin, *XI* Myszyniec, *XII* Lubasz, *XIII* Stanisławice, *XIV* Taczanów, *XV* Tuszyma, *XVI* Złoty Potok, *XVII* Wierzchosławice, *XVIII* Oława, *XIX* Strzelce Opolskie, *XX* Przedbórz, *XXI* Biłgoraj

2.2 Culture procedures, fungal identification and sequences analyses

All isolations were made on 2 % CMEA medium. When necessary, the cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh 2 % MEA. The cultures were incubated at room temperature in the dark. Purified cultures were grouped according to culture morphology using a Nikon Eclipse 50*i* microscope (Nicon® Corporation, Tokyo, Japan) and an Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) with the Coolview 1.6.0 software (Precoptic®, Warsaw, Poland). Fungal structures (conidiophores, conidia) and colony characteristics were compared with the descriptions of species given in the literature (de Hoog 1974; Jacobs and Wingfield 2001; Kim et al. 2005; Linnakoski et al. 2010; Roets et al. 2008; Upadhyay 1981). Isolates were selected for DNA sequencing from each morphological group, and these cultures 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 the PrepMan Ultra Sample preparation reagent (Applied Biosystems, Foster City, CA, USA) using the manufacturer's protocol. The ITS rDNA region (ITS1-5.8S-ITS2) was amplified using the primers ITS 1F (Gardes and Bruns 1993) and ITS 4 (White et al. 1990). Part of the β-tubulin gene region was amplified using primers Bt2a and Bt2b (Glass and Donaldson 1995). Gene fragments were amplified in a 25-μL reaction mixture containing 0.25 μL of Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), 5 μL Phusion HF buffer (5×), 0.5 μL dNTPs (10 mM), 0.75 μL DMSO (100 %) and 0.5 μL of each primer (25 μM). The amplification reactions were performed using a Biometra T-Personal 48 Thermocycler (Biometra GmbH, Goettingen, Germany). The PCR conditions were an initial denaturation step at 98 °C for 30 s followed by 35 cycles of 5 s at 98 °C, 10 s at 57 °C and 30 s at 72 °C, and a final chain elongation at 72 °C for 8 min. The PCR products were visualised under UV light on a 2 % agarose gel stained with 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 that were used for the PCR reaction. Sequences (Table 1) were compared with the data from GenBank using a BLAST similarity search. All sequences were aligned online with MAFFT v6 (Katoh and Toh 2008), using the E-INS-i option with a gap opening penalty of 1.53 and an offset value of 0.00.

Datasets were analysed using maximum likelihood (ML) and Bayesian inference (BI). For ML and Bayesian analyses, the best-fit substitution models for each data set were established using the corrected Akaike information criterion in jModelTest 0.1.1 (Posada 2008). The selected model for the ITS was GTR+I+G; for the β-tubulin gene, it was TrN+G (*Grosmannia*/*Leptographium* and *Ophiostoma piceae* and *Ophiostoma minus* complexes), GTR+G (*Sporothrix schenckii–Ophiostoma stenoceras* complex). ML searches were conducted in PhyML 3.0 (Guindon et al. 2010), via the Montpelier online server (http://www.atgc-montpellier.fr/phyml/) with 1,000 bootstrap replicates. BI analyses based on a Markov Chain Monte Carlo (MCMC) were performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). The MCMC chains were run for ten million generations using the best fitting model. Trees were sampled every 100 generations, resulting in 100,000 trees from both runs. The burn-in value for each dataset was determined in Tracer v1.4.1 (Rambaut and Drummond 2007).

All sequences generated in this study were deposited in the NCBI GenBank (Table 1) and are presented in the phylogenetic tree (Fig. 2).

2.3 Statistical analyses

The Simpson diversity index (Simpson 1949) was used to indicate fungal diversity. The index is defined as:

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

where P_i is the probability of sampling a species, i is the frequency of species i/total frequency for all species and S is species richness, the number of species per sample. The Shannon–Weaver diversity index was also used to compare the diversity of fungal taxa of *H. abietis* from different sites. This index, $H=-\sum(P_i \times \ln P_i)$, combines measurements of richness with those of evenness, so that rare species carry less weight. P_i is the proportion of the total sample represented by species i (Hill et al. 2003). Evenness (E), a measure of the relative abundance of species, is expressed as $E=H/H_{max}$, where $H_{max}=-\ln(S)$. Fungal dominance was determined by Camargo's (1993) index ($1/S$), where S represents species richness. A species was defined as dominant if $P_i>1/S$.

3 Results

3.1 Identification of ophiostomatoid species

Ophiostomatoid fungi were recovered from 86 % of the adult weevils and from 90 % of the seedlings. The isolations from adults of *H. abietis* and pine seedling yielded a total of 1,564 fungal isolates. Morphological investigation showed that 16 ophiostomatoid groups that produced *Leptographium*, *Pesotum*, *Hyalorhinocladiella* and *Sporothrix* anamorph structures in culture were collected. Four groups presented

Table 1 Cultures used in this study and GenBank accession numbers for sequences

Fungi	Strain	Site	GenBank accession no.	
			ITS rDNA	β-Tubulin
Leptographium procerum	630RJ	Jarosław	JX028559	JX046803
	18RJ	Jędrzejów	JX028560	
	434RJ	Myszyniec	JX028561	
Leptographium lundbergii	603RJ	Dulowa	JX028557	
	593RJ	Dulowa	JX028558	JX046802
Leptographium truncatum	585aRJ	Dulowa	JX028562	
	721RJ	Jędrzejów	JX028563	JX046804
Pesotum fragrans	619aRJ	Jarosław	JX028587	
	770RJ	Czajkowa I	JX028588	JX046816
Ophiostoma piliferum	739RJ	Stanisławice	JX028573	JX046811
Ophiostoma quercus	2RJ	Jędrzejów	JX028574	
	626RJ	Jarosław	JX028575	
	619bRJ	Jarosław	JX028576	JX046813
	278RJ	Dąbrowa Tarnowska	JX028580	
	777RJ	Czajkowa II	JX028577	
	749RJ	Taczanów	JX028581	
	534pRJ	Czajkowa I	JX028578	
	597aRJ	Dulowa	JX028579	
	624RJ	Jarosław		JX046812
Ophiostoma piceae	686RJ	Złoty Potok	JX028569	
	989pRJ	Jędrzejów	JX028571	
	601RJ	Jarosław		JX046810
	28RJ	Jędrzejów	JX028570	
	442RJ	Myszyniec	JX028572	JX046809
Ophiostoma floccosum	682RJ	Złoty Potok	JX028566	JX046806
	688RJ	Złoty Potok	JX028567	
Ophiostoma minus	733aRJ	Stanisławice	JX028585	
	734RJ	Stanisławice	JX028586	JX046807
Grosmannia radiaticola	535RJ	Koszęcin	JX028554	JX046800
	585bRJ	Dulowa	JX028556	JX046801
	597bRJ	Dulowa	JX028555	
Sporothrix inflata	581RJ	Koszęcin	JX028589	
	577aRJ	Koszęcin	JX028590	JX046817
Ophiostoma cf. *abietinum*	764RJ	Czajkowa II	JX028564	JX046805
	673pRJ	Jędrzejów	JX028565	
Ophiostoma sp.	479RJ	Czajkowa I	JX028582	JX046814
Sporothrix variecibatus	577bRJ	Koszęcin	JX028591	JX046818
Ophiostoma stenoceras	986pRJ	Jędrzejów	JX028584	JX046815
	968pRJ	Jędrzejów	JX028583	
Ophiostoma pallidulum	767RJ	Czajkowa II	JX028568	JX046808

Leptographium-like anamorphs while 12 groups formed *Pesotum*, *Hyalorhinocladiella* and *Sporothrix* anamorphs (Tables 2 and 3).

The PCR resulted in fragments of 320–1,145 bp for the ITS region and 222–489 bp for the β-tubulin gene regions in the subset of isolates sequenced. Further analyses of the ITS and β-tubulin sequences confirmed that the 15 morphological groups could be separated into two distinct phylogenetic groups within the *Ophiostomatales*. Groups with *Leptographium*-like anamorphs were related to *Grosmannia* and/or *Leptographium* species, while the remaining groups correspond well to species of *Ophiostoma*. However, *Pesotum fragrans* (Math.-Käärik) G. Okada & Seifert with *Pesotum* anamorph formed a

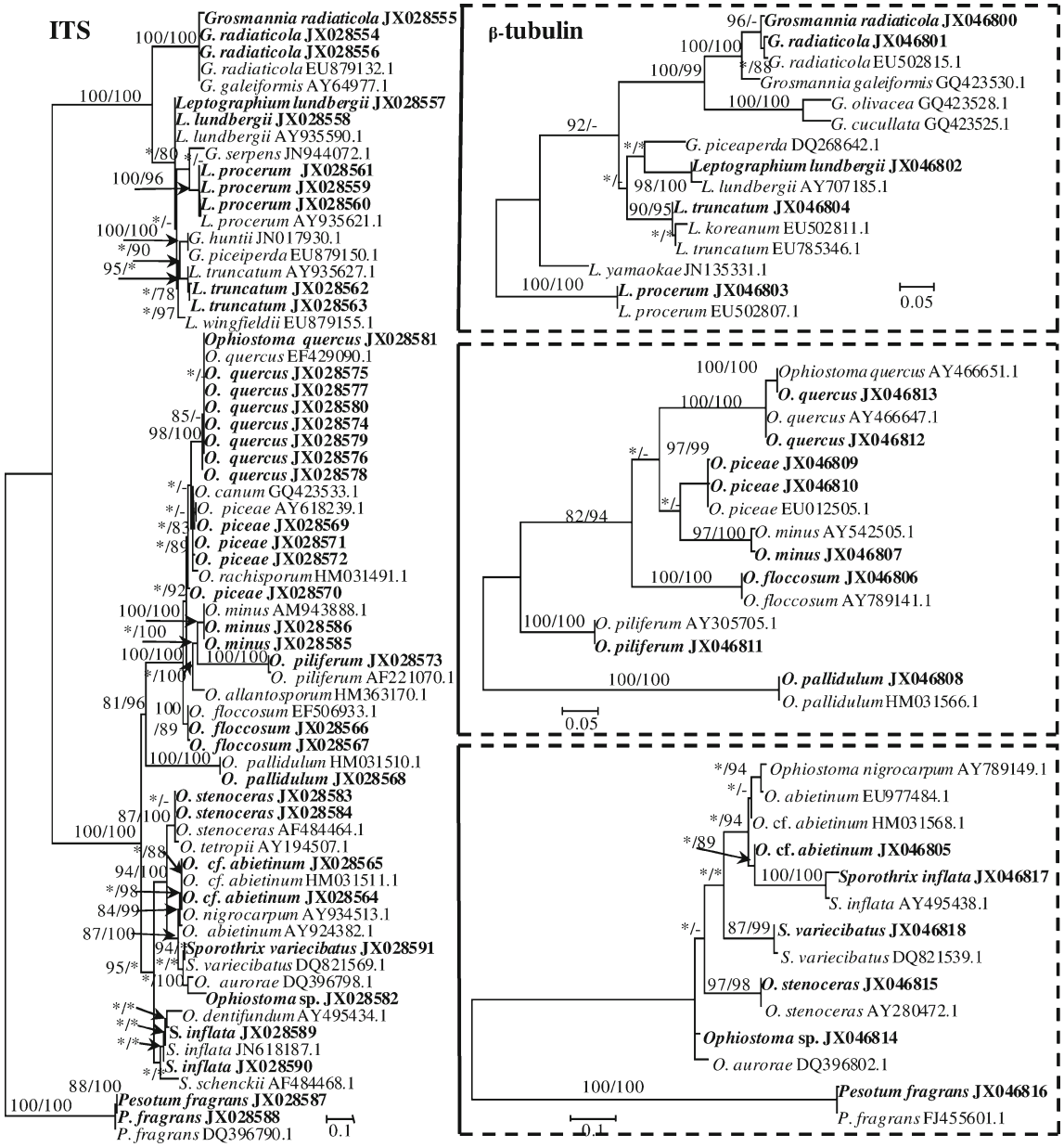

Fig. 2 Phylograms obtained from analyses of ITS sequence data, revealing the identity of ophiostomatoid fungi isolated from *H. abietis* infesting pine forests in Poland. Phylograms of partial β-tubulin data are shown for species groups where ITS sequences did not resolve the identity of Polish isolates. The β-tubulin data sets were analysed separately from each other because of differences among the three groups in the presence and absence of introns 3, 4 and 5. Sequences obtained during this study are presented in *bold type*. All phylograms presented were obtained from maximum likelihood (ML) analyses. Bootstrap values >75 % for ML and posterior probabilities >75 % obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI

distinct third lineage in the *Ophiostomatales* (Fig. 2). The ITS and β-tubulin data confirmed that groups with *Leptographium*-like anamorphs represented four species, including *Grosmannia radiaticola* (J.J. Kim, Seifert & G.H. Kim) Zipfel, Z.W. de Beer & M.J. Wingf., *Leptographium lundbergii* Lagerb. & Melin, *L. procerum* and *Leptographium truncatum* (M.J. Wingf. & Marasas) M.J. Wingf. Groups with affinity to the genus *Ophiostoma* represented ten known species (*Ophiostoma* cf. *abietinum* (Peck) M.J. Wingf.; *Ophiostoma floccosum* Math.-Käärik; *O. minus* (Hedgc.) Syd. & P. Syd.; *Ophiostoma pallidulum* Linnakoski, Z.W. de Beer & M.J. Wingf.; *O. piceae* (Münch) Syd. & P. Syd.; *O. piliferum*; *Ophiostoma quercus* (Georgev.) Nannf.; *O. stenoceras* (Robak) Nanf.; *Sporothrix inflata* de Hoog 1974; *Sporothrix variecibatus* Roets, Z.W. de Beer & Crous and one unknown species named here as *Ophiostoma* sp. The isolate of *Ophiostoma* sp. produced *Sporothrix* anamorph in culture, and in phylogenetic analyses, it was most

Table 2 Isolation number and frequencies (in parentheses) of ophiostomatoid fungi associated with *H. abietis* adults collected at 21 study sites in Poland

Fungi	Locations[a]																				
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	Total
G. radiaticola			1 (2)	1 (2)		11 (22)[b]				8 (16)	1 (2)											22 (2.1)
L. lundbergii	3 (6)	2 (4)	3 (6)			9 (18)	5 (10)			1 (2)	4 (8)	4 (8)	1 (2)					4 (8)		3 (6)		39 (3.7)
L. procerum	29 (58)[b]	50 (100)[b]	50 (100)[b]	50 (100)[b]	36 (72)[b]	43 (86)[b]	46 (92)[b]	39 (78)[b]	50 (100)[b]	49 (98)[b]	47 (94)[b]	45 (90)[b]	46 (92)[b]	44 (88)[b]	26 (52)[b]	35 (70)[b]	39 (78)[b]	45 (90)[b]	45 (90)[b]	40 (80)[b]	26 (52)[b]	880 (83.8)[b]
L. truncatum						2 (4)		1 (2)														3 (0.3)
O. cf. *abietinum*			2 (4)	3 (6)		3 (6)	3 (6)			7 (14)		4 (8)	1 (2)	3 (6)								26 (2.5)
O. floccosum				4 (8)			6 (12)				10 (20)			3 (6)		10 (20)		3 (6)		3 (6)		39 (3.7)
O. minus								1 (2)					2 (4)			1 (2)						4 (0.4)
O. pallidulum				1 (2)												1 (2)						2 (0.2)
O. piceae						1 (2)										2 (4)						3 (0.3)
O. piliferum						3 (6)	4 (8)	2 (4)	4 (8)		2 (4)		7 (14)			13 (26)[b]						35 (3.3)
O. quercus	1 (2)	5 (10)	4 (8)	9 (18)	8 (16)	4 (8)	34 (68)[b]	14 (28)[b]	4 (8)	4 (8)	10 (20)	16 (32)[b]	12 (24)[b]	8 (16)	1 (2)	16 (32)[b]		18 (36)[b]				168 (16.0)[b]
P. fragrans							4 (8)				2 (4)			1 (2)								7 (0.7)
S. inflata		4 (8)	1 (2)	4 (8)			3 (6)			14 (28)[b]		7 (14)										33 (3.1)
S. variecibatus										1 (2)												1 (0.1)
Ophiostoma sp.			1 (2)																			1 (0.1)
No. of total fungal isolates	33	61	62	72	44	76	105	57	58	84	76	76	69	59	27	78	39	70	45	46	26	1263
Species richness (S)	3	4	7	7	2	8	8	5	3	7	7	5	6	5	2	7	1	4	1	3	1	15
D	0.219	0.316	0.341	0.494	0.298	0.638	0.693	0.470	0.247	0.613	0.579	0.591	0.514	0.420	0.071	0.704	0.000	0.516	0.000	0.235	0.000	0.492
H	0.437	0.659	0.807	1.086	0.474	1.417	1.487	0.864	0.497	1.295	1.234	1.168	1.032	0.862	0.158	1.402	0.000	0.932	0.000	0.478	0.000	1.173
Total no. of samples	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	1050

Frequency of occurrence: $F=(NS/NTs)\times 100$, where F represents the frequency of occurrence (%) of the fungus, NS represent the number of beetles from which a particular fungus was isolated and NTs represent the total number of beetles

D the Simpson diversity index, *H* the Shannon–Weaver diversity index

[a] Location number

[b] Dominant species

Table 3 Isolation number and frequencies (in parentheses) of ophiostomatoid fungi isolated from seedlings attacked by *H. abietis*

Fungi	Locations				
	III	VII	VIII	IX	Total
Leptographium procerum	85 (91.4)	28 (100.0)	68 (98.5)	24 (57.1)	205 (88.4)
Ophiostoma floccosum	3 (3.2)				3 (1.3)
Ophiostoma piceae				1 (2.4)	1 (0.4)
Ophiostoma quercus	16 (17.2)	2 (7.1)	1 (1.4)	5 (11.9)	24 (10.3)
Ophiostoma stenoceras	2 (0.2)		2 (2.9)	2 (4.8)	6 (2.6)
Sporothrix inflata	51 (54.8)	1 (3.6)	5 (7.2)	3 (7.1)	60 (25.7)
Sporothrix variecibatus				1 (2.4)	1 (0.4)
Ophiostoma cf. *abietinum*			1 (1.4)		1 (0.4)
Total no. of isolates	157	31	77	36	301
Number of seedlings (%)	93	28	69	42	232

Percentages given in parentheses were calculated as no. of individual seedlings colonising by the respective fungus/total number of seedlings)×100

closely related to *Ophiostoma aurorae* X.D. Zhou & M.J. Wingf. (Fig. 2).

3.2 Fungal isolation from *H. abietis* beetles

From the crushed adults of *H. abietis*, we obtained 1,263 isolates comprising 15 species (Table 2). The most dominant species was *L. procerum*, which was isolated with an average isolation frequency of 84 %. This species was dominant at all sites and was isolated from every beetle sampled at four sites (Pateraki, Czajkowa I, Czajkowa II, Szczurowa). The second-most dominant isolate was the *O. quercus*, which was isolated from 16 % of the beetles (from 0 % at Wierzchosławice, Strzelce Opolskie, Przedbórz and Biłgoraj to 68 % at Jarosław). In contrast to *L. procerum*, *O. quercus* was dominant at six sites (Table 2). Other species were isolated only rarely. Among these, four species, *L. lundbergii*, *O. floccosum*, *O. piliferum* and *S. inflata*, were isolated above 3 % of the time. However, *O. piliferum*, *S. inflata* and *G. radiaticola* dominated one site each. *L. truncatum O.* cf. *abietinum*, *O. pallidulum*, *O. minus*, *O. piceae*, *P. fragrans* and *S. variecibatus* were found occasionally (Table 2).

The fungal biodiversity and species-richness values varied among the 21 sites (Table 2). The fungal community associated with *H. abietis* in Jarosław, Dulowa and Złoty Potok had the highest biodiversity ($D>0.64$; $H>1.4$) and species-richness values (Jarosław—eight species, Dulowa and Złoty Potok—seven species). The lowest biodiversity and species richness were found in Wierzchosławice, Strzelce Opolskie and Biłgoraj (D, $H=0$; $S=1$ species) (Table 2).

3.3 Fungal isolation from seedlings damaged by *H. abietis*

A total of 301 fungal isolates were obtained from the 232 live seedlings sampled in this study. Eight ophiostomatoid species were isolated: *L. procerum*, *O.* cf. *abietinum*, *O. floccosum*, *O. piceae*, *O. quercus*, *O. stenoceras*, *S. inflata* and *S. variecibatus* (Table 3). *L. procerum* was isolated most frequently and was found in 88 % of the seedlings (ranging from 57 % at Szczurowa to 100 % at Jarosław). *S. inflata* was isolated from 26 % (4 % at Jarosław to 55 % at Czajkowa 1), while *O. quercus* was isolated from 10 % (1 % at Jędrzejów to 17 % Czajkowa I) of the seedlings. The remaining species were isolated less frequently (Table 3).

4 Discussion

This survey of adult beetles showed that ophiostomatoid species were vectored by *H. abietis* in pine stands in Poland. This association was also confirmed by the isolation of these fungi from pine seedlings during the maturation feeding of *H. abietis*. In this study, in which identification was based on morphological characteristics and DNA comparisons, 16 species of ophiostomatoid fungi (including *Grosmannia*, *Ophiostoma*, *Leptographium*, *Pesotum* and *Sporothrix* spp.) were isolated from the beetles. The most commonly encountered fungal associates of *H. abietis* were *L. procerum* and *O. quercus*.

The results of this study show that the fungal diversity associated with *H. abietis* in Poland is significantly greater than the diversity that was previously recognised in France (Piou 1993). In France, *H. abietis* beetles were only found to be associated with *L. procerum*, *L. wingfieldii*, *O. canum* and *O. piliferum*, while we isolated 14 additional ophiostomatoid species that had not previously been reported in association with *H. abietis*. We did not isolate *L. wingfieldii* and *O. canum*, which were found in the French study. However, the first species is very rarely detected in Poland (Jankowiak 2006a), although *O. canum* is a common associate of *Tomicus* spp. in Poland (Jankowiak 2008) and in

other parts of Europe (Kirisits 2004; Linnakoski et al. 2012a). A similarly high fungal diversity has been recently described for *H. pales* on *P. palustris* in Georgia (Zanzot et al. 2010). These authors found that *L. procerum*, *L. terebrantis*, *L. serpens*, *G. huntii*, *O. ips*-like, *Ophiostoma* spp. and *Pesotum* spp. were associated with *H. pales*.

Due to the biology of *H. abietis*, the majority of ophiostomatoid species vectored by this weevil represent species regarded as soil-borne fungi (e.g., *S. inflata*) or as associates of root-feeding bark beetles (e.g. *Leptographium* spp.). In contrast to bark beetles, the *H. abietis* adults remain active for 2 to 3 years, their feeding occurs on the stems and twigs of seedlings or on roots of trees and the adults spend most of their lives on the ground (Nordenhem 1989). In view of these differences, an important question can be raised: How constant and specific are the symbiotic relationship of ophiostomatoid fungi with this weevil? We consider *H. abietis* to be intimately associated with ophiostomatoid fungi, in particular with *Leptographium* spp. The fact that spores of fungi carried by the adults of *H. abietis* are probably protected by secretions of the pronotal grandular apparatus (Lévieux et al. 1994) supports this view. Additionally, the majority of ophiostomatoid fungi, which were found on adults of *H. abietis* in this study, were also isolated from the galleries of *H. abietis* (Jankowiak and Bilański, unpublished data).

Consistent with the findings of Piou (1993), the most commonly encountered fungal associate of *H. abietis* was *L. procerum*. This fungus was isolated from the adults with very high frequency and was dominant at all sites, suggesting a specific relationship. The association of *L. procerum* with *H. abietis* found in this study was not surprising because this fungus is known to be a coloniser of conifer roots (Barnard et al. 1991; Eckhardt et al. 2007; Otrosina et al. 1999) and an associate of pine-infesting bark beetles (Kirisits 2004; Linnakoski et al. 2012b). *L. procerum* is likely the species in the genus *Leptographium* that occurs most frequently on Scots pine in Poland. To date, this fungus has been recorded in Poland in association with *Tomicus* spp. (Jankowiak 2006a, 2008), *Ips sexdentatus* (Börn.) (Jankowiak 2012), *Trypodendron lineatum* (Oliv.), *Hylastes* ssp., *Hylurgus ligniperda* (Fabr.) (Jankowiak and Kolařík, unpublished data) and cerambycid beetles (Jankowiak and Rossa 2007). Recently, Jankowiak et al. (2012) also reported *L. procerum* as a common coloniser of pine roots in Poland.

The frequency of *O. quercus* on *H. abietis* adults was unexpectedly high; this fungus dominated the fungal community of weevils in some sites. According to Harrington et al. (2001), *O. piceae* is most often isolated from conifer tree hosts, while *O. quercus* mostly colonises tissues of hardwood trees. However, recent studies have shown that *O. quercus* is much more widely distributed on woody substrates than had previously been recognised. The fungus has been reported on pine plantations (Reay et al. 2005; Thwaites et al. 2005; Zhou et al. 2006). *O. piceae* has been indentified in Poland in several earlier studies (Jankowiak 2006a, 2008) that were based only on morphology. However, the DNA sequence comparisons used in this study suggest that *O. quercus* can be more widespread in *Pinus 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 2012; Jankowiak and Bilański, unpublished data). Detailed ecological studies are needed to define the host range for *O. quercus* and *O. piceae*.

In contrast to *O. quercus*, the presence of *S. inflata* on *H. abietis* adults was expected because this fungus is frequently isolated from soil and the other substrates associated with soil environments (de Hoog 1974). Recently, *S. inflata* was also isolated from the roots of dying and dead young Scots pine in Poland (Jankowiak et al. 2012). This study indicates that *H. abietis*, in addition to being a vector for *L. procerum* and *O. quercus*, is also a carrier of the soil-borne fungus *S. inflata*.

Similarly, *L. lundbergii*, *O. floccosum* and *O. piliferum* were also isolated from the adults of *H. abietis* at a relatively low and variable frequency. These fungi are known agents of blue stain on *P. sylvestris*, and they are also associated with pine-infesting bark beetles (Kirisits 2004; Linnakoski et al. 2012a, b). *L. lundbergii*, for example, is commonly associated with root-feeding beetles (*Hylastes* spp. and *H. ligniperda*; Jankowiak and Bilański, unpublished data) and *Hylurgops palliatus* (Gyll.) (Jankowiak 2006b), while *O. floccosum* was found in association with *I. sexdentatus* in Poland (Jankowiak 2012). These fungi seem to occur as infrequent associates of *H. abietis*. The results of this study also showed that similar ecological areas may contain *O.* cf. *abietinum*, which was found at a low frequency in the present work. It was also recently recorded in association with *Tomicus minor* (Hart.) in Russia (Linnakoski et al. 2010) and with *I. sexdentatus* in Poland (Jankowiak 2012).

Another species found in the present study was *G. radiaticola* (anamorph *Pesotum pini* (L.J. Hutchison & J. Reid) G. Okada & Seifert). This fungus was originally described on *Pinus radiata* D. Don. in New Zealand (Kim et al. 2005) with *Leptographium*-like anamorph. In our study, isolates of *G. radiaticola* produced a similar type of conidial structures in culture, but none of these isolates produced teleomorphs, even with mating and the addition of wooden blocks to the medium. This species is morphologically and phylogenetically similar to *Grosmannia galeiformis* (B.K. Bakshi) Zipfel, Z.W. de Beer & M.J. Wingf. (Kim et al. 2005), which is a relatively well-known species in Scandinavia and others parts of Europe (Bakshi 1951; Mathiesen-Käärik 1953; Zhou et al. 2004). According to Kim et al.

(2005), *G. radiaticola* is heterothallic with two mating types, but *G. galeiformis* appeared to be homothallic. Zhou et al. (2004) provided evidence that *G. galeiformis* is heterothallic, although some of their isolates were also homothallic. *G. radiaticola* can be distinguished from *G. galeiformis* only by the sizes of the ascospores (Kim et al. 2005). The ITS sequences of these species are very similar, but their β-tubulin sequences differ substantially (Fig. 2). Despite these differences, the taxonomic status of both species remains unclear, especially given that Thwaites et al. (2005) considered *G. radiaticola* to be the same species as *G. galeiformis*. Up to the present study, *G. radiaticola* was known only outside Europe. However, recently published data using β-tubulin and EF 1-α genes for fungal identification show that *G. radiaticola* is distributed throughout Europe and in other continents, while the *G. galeiformis* occurs only in association with *Hylastes brunneus* Erchison, *H. palliatus* Gyll. and *T. lineatum* in northern Europe (Linnakoski et al. 2012b). DNA sequence comparisons for two gene regions of isolates obtained from *H. abietis* confirmed that *G. radiaticola* occurs in Europe. This study represents the first reports of *G. radiaticola* from central Europe and from Scots pine.

Although it has been recovered only rarely from the *H. abietis* and infested pine seedlings, *S. variecibatus* was found in the present study. This fungus has previously been described by Roets et al. (2008), where it was found in South African mites occurring in *Protea* spp. infructescences and *Eucalyptus* leaf litter. It has also been detected as a dominant fungal species in a mixed microbial culture obtained from activated petrochemical refinery sludge (Rene et al. 2010), soil in Spain and *Eucalyptus* tree cankers in Australia (de Beer, personal communication). Our study has shown that *S. variecibatus* also occurs in pine forest habitats, suggesting that the species may be distributed throughout the world.

The ecology of *O. pallidulum*, which was occasionally isolated from adult *H. abietis* in this study, is poorly understood. This species has been described by Linnakoski et al. (2010), who isolated it from several different bark beetles infesting pine and spruce trees in Finland, where it was found mainly in association with *H. brunneus*. Recently, *O. pallidulum* was also isolated from the roots of dying and dead young Scots pine in Poland (Jankowiak et al. 2012). This may indicate that *O. pallidulum* is closely associated with the soil environment.

The other four ophiostomatoid species, *O. minus*, *L. truncatum*, *P. fragrans* and *Ophiostoma* sp., were isolated at very low frequencies from adults of *H. abietis*. The very low frequency of *O. minus* occurrence was surprising because it causes blue stain on pine timber and is a very common associate of various pine-infesting bark beetles in Europe (Kirisits 2004; Linnakoski et al. 2012a). The lower frequency of *O. minus* on beetles could be due to its ecological niches. It appears to be specifically associated mainly with the *Tomicus* spp. that infest middle and upper parts of trees in Poland (Jankowiak 2006a, 2008). In contrast to *Tomicus* spp., *H. abietis* beetles lives mainly on the ground of the forest (*Hylobius* hibernates in the upper layers of the soil). Another species, *P. fragrans*, has been occasionally collected from several bark beetles in Europe (Kirisits 2004; Linnakoski et al. 2012a; Romón et al. 2007). It has been reported in only one study in Poland. Jankowiak and Kolarik (2010) isolated the species from galleries of *Cryphalus piceae* (Ratz.) on *Abies alba* Mill. *L. truncatum* was detected for the first time in Poland in this study. In Europe, this species is associated with root-feeding bark beetles on Scots pine (Gibbs and Inman 1991; Linnakoski 2011; Linnakoski et al. 2012b; Wingfield and Gibbs 1991).

In this study, the species spectrum and frequency of fungal associates of *H. abietis* differed from site to site. This variation in fungal population composition is well-known from the literature and could be due to many different factors (Linnakoski et al. 2012a). The geographical and climatic differences seem to be a main factor that affects the assemblages of fungi. Other factors that could be important include sampling and isolation methodology. However, despite application of the same sampling and isolation methods and the similar characteristics of sampling sites (e.g. the same reforestation technology) in this study, the fungal communities differed among sites. We suspect that geographical variation, different environmental conditions (temperature, humidity) and microsites (degree of sunlight trap logs) could have the largest influence on the fungi associated with *H. abietis*.

The results show a strong relationship between fungi transported by *H. abietis* adults and the presence of these fungi in pine seedlings. All of the fungal species isolated from seedlings (except *O. stenoceras*) were also found on adult weevils, suggesting that *H. abietis* very effectively introduces the ophiostomatoid species into the fresh tissues of pine seedlings during maturation feeding. This pattern is similar to the scenario described by Reay et al. (2002, 2005) for *P. radiata* seedlings damaged by *Hylastes ater* Payk. As in the results obtained from our adult weevils, *L. procerum* was the most frequently isolated species from seedlings (88 %). This finding is also in agreement with the results of Piou (1993), although he found *L. procerum* on only 18 % of seedlings. The different health conditions of pine seedlings in each study might have affected the species found. In this work, live pine seedlings were used for the fungal isolations, while in previous studies the seedlings were dead. We also isolated fungi from dead seedlings, but we did not detect the ophiostomatoid species in these seedlings. For this reason, Piou (1993) probably did not find other ophiostomatoid species in seedlings. In contrast to the

results from adults of *H. abietis*, *S. inflata* was isolated more frequently than *O. quercus* from seedlings. As a soil-borne fungus, *S. inflata* might also infect seedlings from the surrounding soil following damage caused by *H. abietis*. According to Piou (1993), the presence of *L. procerum* in seedlings damaged by *H. abietis* could increase the mortality of seedlings damaged by weevil feeding. A similar relationship between the severity of *H. ater* feeding damage and the presence and number of species of *Ophiostoma* were also noted by Reay et al. (2005) in New Zealand.

In conclusion, in contrast to previous studies, we show that considerably more diverse fungal species were associated with the large pine weevil. This study confirms that *L. procerum* is a common associate of *H. abietis. O. quercus* seems to also be a common associate of the pine weevil, but its frequency in different sites was variable. Other ophiostomatoid species were found inconsistently and in low numbers, indicating causal associations. All ophiostomatoid species recovered in this study (with the exception of *L. procerum*) had never before been isolated from *H. abietis*. This is also the first report of *G. radiaticola* in central Europe and *L. truncatum* and *S. variecibatus* in Poland. In this study, *H. abietis* also acted as an effective vector carrying ophiostomatoid species to *P. sylvestris* seedlings, especially in the cases of *L. procerum* and *S. inflata*.

Funding The work was supported by grants from the Polish Ministry of Education and Science NN309 049037.

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