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Ophiostomatatoid fungi (*Ascomycota*) associated with *Ips acuminatus* (*Coleoptera*) in eastern Poland

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Abstract: *Ips acuminatus* (*Coleoptera*, *Scolytinae*) is a pine-infesting bark beetle that occurs throughout Europe and Asia. Recently, the insect has killed numerous Scots pines (*Pinus sylvestris*) in eastern Poland. Several species of ophiostomatoid fungi are associated with *I. acuminatus* in Europe, but no research has been done on the fungi associated with this bark beetle in Central Europe specifically. The aim of this study was to identify the ophiostomatoid fungal associates of *I. acuminatus* in eastern Poland, where tree mortality caused by this beetle species has recently increased. Field surveys in Puławy and Mircze Forest Districts yielded a total of 2 269 fungal isolates from 237 beetles and 204 beetle galleries. Isolates were grouped based on morphology and representatives of each group were identified based on DNA sequences of the ITS, LSU, β-tubulin, calmodulin and elongation factor 1-α gene regions. A total of seven previously described species of ophiostomatoid fungi were identified. The dominant species were *Graphilbum acuminatum* and *Sporothrix pseudoabietina*. This study revealed that the community of ophiostomatoid fungi associated with *I. acuminatus* in Poland is different from those reported in other regions of Europe. In addition, molecular data suggest that *S. pseudoabietina* is a synonym of *S. villosa* in the *Sporothrix gossypina* & *S. stenoceras* species complexes.

Keywords: blue-stain fungi, *Microascales*, *Pinus sylvestris*, *Ophiostomatales*, sharp-dentated bark beetle

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Introduction

European bark beetles in the genus *Ips* (*Coleoptera*: *Curculionidae*: *Scolytinae*) are mainly secondary pests that infest weakened, injured, felled or windthrown conifer hosts. For this reason, most members of *Ips* have only minor economic importance, although *Ips typographus* (Linnaeus, 1758), the most destructive species in the genus, can cause extensive damage of Norway spruce (*Picea abies* (L.) H. Karst.) in Europe and Asia (Wermelinger, 2004). However, recently Scots pines (*Pinus sylvestris* L.) were observed to have been killed by the sharp-dentated bark beetle *Ips acuminatus* (L. Gyllenhal, 1827) in different regions

of Europe (Wermelinger et al., 2008; Colombari et al., 2012, 2013; Siitonen, 2014; Plewa & Mokrzycki, 2017).

The biology and ecology of *I. acuminatus* are relatively well understood. The insect has a wide geographical distribution across Europe and Asia, which is mainly determined by the range of its principal host tree, Scots pine (Löbl & Smetana, 2011). The beetle is usually found in the upper parts of trunks and thick branches of mature pines but it is not uncommon to also find it in young stands (Siitonen, 2014; Plewa & Mokrzycki, 2017). This insect is generally known for low levels of aggressiveness, infesting mainly weakened or dead standing trees as well as fallen trees or logging residues (Altenkirch et al., 2002; Siitonen, 2014). However, in recent years, outbreaks of *I. acuminatus* have been observed in Alpine forests in Italy, Switzerland, Austria (Wermelinger et al., 2008; Krehan, 2011; Colombari et al., 2012, 2013), and Finland (Siitonen, 2014). In Poland, *I. acuminatus* was considered for many years to be of minor significance. However, recently its population has rapidly grown, necessitating an increase in sanitary cuttings of Scots pines (Plewa & Mokrzycki, 2017; Skrzecz & Perlińska, 2018). In 2019, there was a 26,000 hectare forest attacked by *I. acuminatus* in Poland (Zajączkowski et al., 2020). The extensive damage caused by *I. acuminatus* has been linked to hot and dry summers that have increased the susceptibility of pine trees to bark beetle infestations (Rebetez & Dobbertin, 2004; Dobbertin et al., 2007; Wermelinger et al., 2008; Colombari et al., 2012; Siitonen, 2014). *Ips acuminatus* infestations are most common in pines growing in highly fertile soil due to their plate-like root system, which leads to an increase in susceptibility to attacks by bark and wood boring insects during periods of drought or other abiotic stressors (Sierota et al., 2019). Because of climate change, several other species that had minor economic importance in the past (e.g., *T. piniperda* (Linnaeus, 1758), *T. destruens* (T.V. Wollaston, 1865), *T. minor* (G.L. Hartig, 1834), *Phaenops cyanea* (Fabricius, 1775), and *Orthotomicus erosus* (T.V. Wollaston, 1857)) have also recently become more aggressive, and caused severe losses to European pine forests (Pernek et al., 2019; Hlávková & Doležal, 2022).

Ips beetles live in close association with various fungi, most notable with species in the *Ophiostomatales* and *Microascales* (*Ascomycota*, *Sordariomycetes*) (Kirisits, 2004; Linnakoski et al., 2012; Six, 2012; Wingfield et al., 2017). These fungi are also referred to as so-called ophiostomatoid fungi, a polyphyletic group characterized by the production of sticky spore masses at the apices of flask-shaped sexual fruiting structures (de Beer & Wingfield, 2013; de Beer et al., 2013 a, b, 2016, 2022). Ophiostomatoid fungi are tree- or wood-infecting, and cause a dark bluish discoloration in the sapwood (Kirisits, 2004). Some ophiostomatoid fungi are responsible for serious tree diseases, such as *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf., which is involved in *P. abies* mortality (Wermelinger, 2004). Other ophiostomatoid fungi have been shown to be slightly or moderately virulent when inoculated in living host trees (Kirisits, 2004). These fungi may exhaust and overcome the tree's chemical defenses which consequently accelerates the decline of stressed host trees and ultimately helps their beetle vectors reproduce in the phloem (Lieutier et al., 2009). However, suggestions that the beetles depend on these fungi to kill trees have been challenged by Six and Wingfield (2011). These researchers suggested that the virulence ofophiostomatoid fungi did not usually have an important role in ecology of bark beetles.

The *Ophiostomatales* accommodates the single family *Ophiostomataceae*, comprising 16 accepted genera, including *Ceratocystiopsis, Grosmannia, Graphilbum, Hawksworthiomyces, Leptographium, Ophiostoma, Raffaelea* and *Sporothrix* (de Beer et al., 2022). Currently, the *Microascales* includes seven clearly defined families (Hyde et al., 2020), three of which (*Ceratocystidaceae*, *Gondwanamycetaceae* and *Graphiaceae*) are considered to be ophiostomatoid species (Wingfield et al., 1993).

There are several reports of fungi associated with *I. acuminatus* in Europe. In Fennoscandia, this beetle species is commonly associated with *Graphilbum acuminatum* (R. Jankowiak & H. Solheim), *Ophiostoma macrosporum* (Francke-Grosm.) Z.W. de Beer & M.J. Wingf., *O. clavatum* Math. and *O. minus* (Hedgc.) Syd. & P. Syd. (Mathiesen, 1950, 1951; Rennerfelt, 1950; Mathiesen-Käärik, 1953; Waalberg, 2015). *Ophiostoma brunneo-ciliatum* Math.-Käärik, *O. clavatum* and unindetified *Ophiostoma* species are known to be associated with *I. acuminatus* in France and Italy (Lieutier et al., 1991; Guérard et al., 2000; Villari, 2012), although recently Linnakoski et al. (2016) used molecular phylogenetics to provide evidence that isolates identified as *O. brunneo-ciliatum* in France rather represent *O. clavatum*. However, research on the association between ophiostomatoid fungi and *I. acuminatus*, in Central and Eastern Europe is limited, with only *Graphilbum cf. rectangulisporium* (Ohtaka, Masuya &Yamaoka) Z.W. de Beer & M.J. Wingf., *Ophiostoma ips* (Rumbold) Nannf. and *O. minus* having been reported from Ukraine as associates of the beetle to date (Davydenko et al., 2017). For this reason and because *I. acuminatus* was previously of minor significance but has become a major pest, we decided to study the ophiostomatoid fungal associates of this beetle in eastern Poland. Detailed research on bark beetle ecology are important to develop new and improve existing methods of pest management.

In this study, we surveyed the ophiostomatoid fungal associates of *I. acuminatus* in Poland. The aim of the present study was to: 1) explore the diversity of ophiostomatoid species associated with *I. acuminatus* on *P. sylvestris* in eastern Poland, where *I. acuminatus* is most abundant; and 2) morphologically characterize *Sporothrix pseudoabetina* H. Wang, Q. Lu & Z. Zhang, a fungal species new to Poland.

Materials and methods

Study area and sampling of bark beetles

Ips acuminatus was sampled in September 2016 in the Mircze Forest District (Witków), and in October 2018 in the Puławy Forest District (Sadłowice, Skoki, Wronów) (Fig. 1). All research sites were in Scots pine stands that contained at least 60% *P. sylvestris* (Table 1). Our study areas were in one of the most severely damaged pine forests in eastern Poland (Zajączkowski et al., 2020).

Fungi were isolated from the beetles and their galleries (Sadłowice, Witków, Wronów) or only from their galleries (Skoki). Beetles and galleries were collected from trees naturally infested by *I. acuminatus*. Trees were characterized by yellow to brown discolouration of the needles and dying of branches and crown thinning exceeding 50% (Fig. 2a). When we sampled, beetle and larvae were present in the galleries.

Adult beetles were collected from the galleries of colonized trees. The beetles were collected with sterile forceps, placed individually in sterile Eppendorf tubes (1.5 ml), and stored at 4 °C for 1–2 days until the fungal isolations were performed. One to two beetles were collected from each gallery. Altogether, 237 beetle specimens were obtained from the

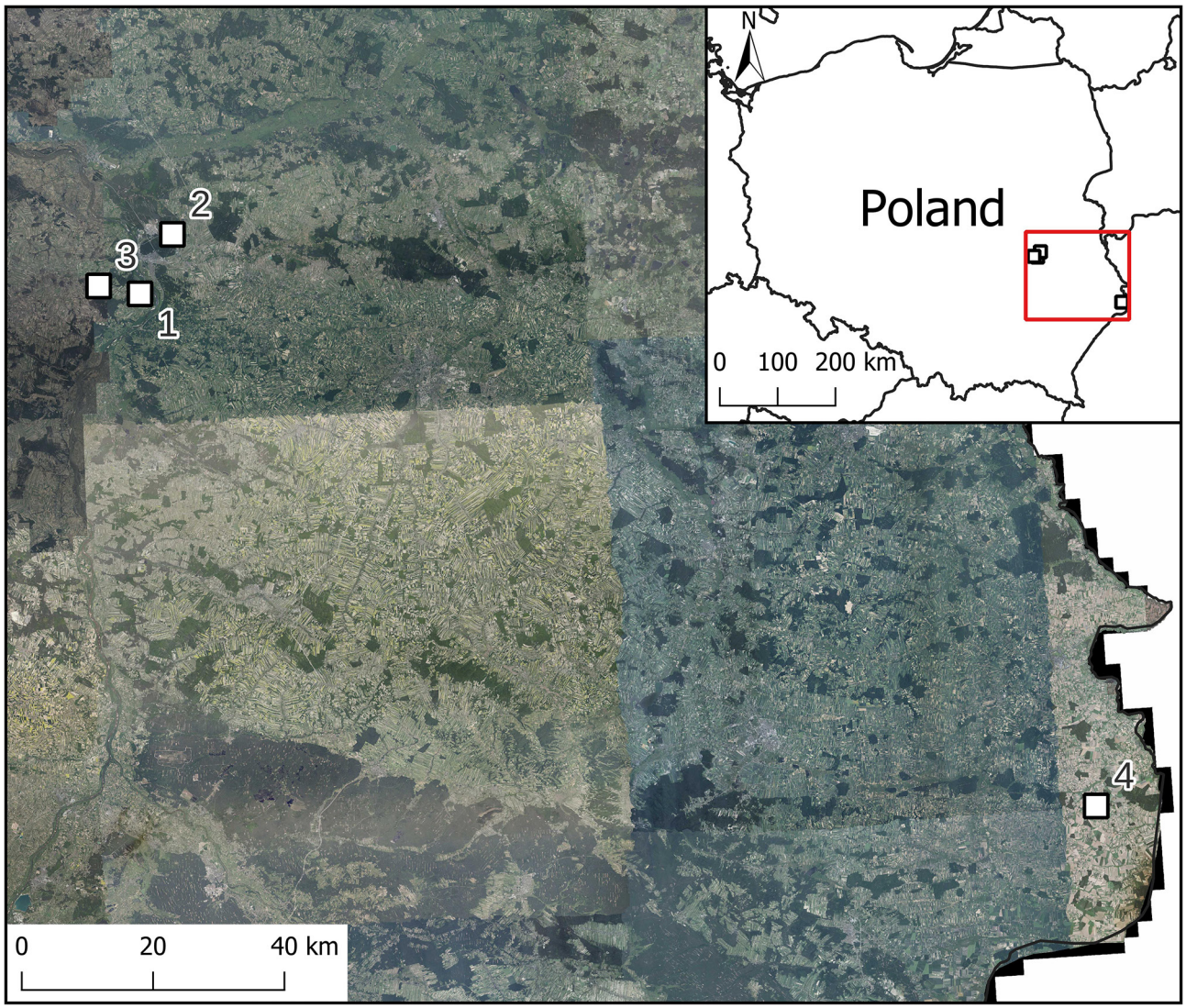

Fig. 1. Map of sample sites in eastern Poland: 1 – Sadłowice, 2 – Wronów, 3 – Skoki, 4 – Witków. Source of orthophotos https://mapy.geoportal.gov.pl/wss/service/PZGIK/ORTO/WMTS/StandardResolution

three sites. Complete galleries, including stained or unstained sapwood up to 2 cm away from the tunnels (Fig. 2 b–d), were removed from the wood and placed in separate paper bags. A total of 204 *I. acuminatus* galleries were collected.

Table 1. Characteristics of the study sites

Location	Sadłowice	Wronów	Skoki	Witków
Administrator	Puławy Forest District	Puławy Forest District	Puławy Forest District	Mircze Forest Distict
Forest compartment	211a	137d	248h	230k
Geographic coordinates	51.3695 N, 21.9478 E	51.4490 N, 22.0241 E	51.3825 N, 21.8582 E	50.5993 N, 23.9594 E
Altidude m a.s.l.	130	136	175	221
Forest stand composition in %	*Pinus sylvestris* 60 *Quercus robur* 40	*P. sylvestris* 100	*P. sylvestris* 90 *Q. robur* 10	*P. sylvestris* 90 *Q. robur* 10
Tree age (*P. sylvestris*)	95	138	55	105
Year sampling	2018	2018	2018	2016
No. of examained samples	140 (100 beetles + 40 galleries)	62 (32 beetles + 30 galleries)	44 galleries	198 (108 beetles + 90 galleries)

Fig. 2. (a) Dead and dying *Pinus sylvestris* trees in the Puławy Forest District infested by *Ips acuminatus*; (b) Exposed galleries of *I. acuminatus* on the sapwood surface of *P. sylvestris* colonized by *Graphilbum acuminatum* and *Sporothrix pseudoabietina*; (c, d) Extensive blue-stain development in the sapwood of *P. sylvestris* caused by *Leptographium procerum* (c) and *Ophiostoma minus* (d); (e, f) *Pesotum*-like asexual morphs formed by *G. acuminatum* in *I. acuminantus* galleries

Fungal isolations and morphological grouping

For fungal isolations from insects, each beetle was removed from its storage microtube with sterile forceps, morphologically identified with taxonomical keys (Nunberg, 1981), and squashed onto 2% malt extract agar (MEA; 2% malt extract from Biocorp Polska Sp. z.o.o., Poland and 2% agar from Biocorp Polska Sp. z.o.o , Poland) in Petri dishes containing 0.2 g/l tetracycline HCL (BioShop®, Canada Inc., Burlington). For fungal isolations from gallery pieces, 4×4 mm fragments of sapwood were collected at a depth of 10 mm into the sapwood and plated on 2% MEA medium in Petri dishes. Six fragments were plated per gallery.

The Petri dishes were incubated at 25 °C for 2–6 weeks and observed daily for fungal growth. Cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh 2% MEA. Purified cultures were grouped according to culture morphology with the aid of a Nikon Eclipse

Table 2. Cultures examined in this study and their GenBank accession numbers

Taxon	Isolate no.[a]	Origin	Site	GenBank accesion no.[b, c]				
				ITS	LSU	*TUB2*	*CAL*	*TEF1*
				***Graphilbum* species**				
Graphilbum acuminatum (Taxon 1)	KFL68816IA-=CBS145825 =CMW54767	beetle	Witków	**MN548900**	OQ344779	**MN548936**	**MN548989**	**MN548950**
	KFL72016IA	gallery	Witków	MW540753	OQ344780	OQ352132		OQ352133
	KFL99116IA-=CBS145827 =CMW54768	gallery	Witków	**MN548901**	OQ344781	**MN548937**	**MN548990**	**MN548951**
	KFL100816IA-=CBS145828 =CMW54769	beetle	Witków	**MN548902**	OQ344782	MN548938	MN548991	MN548952
	KFL20518IA	beetle	Sadłowice	MW540754	OQ344783			
	KFL22118IA	beetle	Sadłowice	MW540755	OQ344784			
	KFL22718IA	beetle	Sadłowice	MW540756				
	KFL24518IA	gallery	Sadłowice	MW540757				
Graphilbum fragrans (Taxon 2)	KFL27018IA	gallery	Wronów	MW540752		MW540769		MW540783
				***Leptographium* species**				
Leptographium procerum (Taxon 3)	KFL29118IA	gallery	Skoki		MW540765	MW540770		MW540784
	KFL29218IA	gallery	Skoki		MW540766	MW540771		MW540785
	KFL29318IA	gallery	Skoki		MW540767	MW540772		MW540786
Leptographium sosnaicola (Taxon 4)	KFL74216IA	gallery	Witków		MW540768	MW540773		MW540787
				***Ophiostoma* species**				
Ophiostoma minus (Taxon 5)	KFL99016IA	gallery	Witków	MW540758		MW540774		
				***Sporothrix* species**				
Sporothrix pseudoabietina (Taxon 6)	KFL70716IA[d] =CBS 147969 =CMW57302	gallery	Witków	MW540759		MW540775	MW540779	MW540788
	KFL71316IA	gallery	Witków	MW540760		MW540776	MW540780	MW540789
	KFL71516IA[d] = CMW57303	gallery	Witków	MW540761		MW540777	MW540781	MW540790
	KFL29018IA	gallery	Skoki	MW540762		MW540778	MW540782	MW540791
				Microascales				
				***Graphium* species**				
Graphium pseudormiticum (Taxon 7)	KFL20618IA	beetle	Sadłowice	MW540750	MW540763			
	KFL20218IA	beetle	Sadłowice	MW540751	MW540764			

[a] CBS=Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMW=Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; KFL=Culture collection of the Department of Forest Ecosystems Protection, University of Agriculture in Krakow, Poland;

[b] ITS=internal transcribed spacer region of the nuclear ribosomal DNA gene; LSU=internal transcribed spacer region 2 and the 28S large subunit of the nrDNA gene; *TUB2*=β-tubulin; CAL= calmodulin; *TEF1*=Translation elongation factor 1-alpha.

[c] sequences obtained in previously survey (Jankowiak et al., 2020) are indicated in bold.

[d] Isolates used in growth and morphological studies.

50*i* microscope (Nikon® Corporation, Tokyo, Japan) and Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) with Coolview 1.6.0 software (Precoptic®, Warsaw, Poland). Depending on the size of the morphological group, between one and eight isolates from each group were chosen for molecular identification. Representative isolates of fungi were deposited in the culture collection of Department of Forest Ecosystems Protection, Hugo Kołłątaj University of Agriculture, Cracow, Poland. Some representative isolates of *Graphilbum acuminatum* and *S. pseudoabietina* were deposited in the culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands and in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa (Table 2).

DNA extraction, amplification and phylogenetic analyses

All the isolates were initially grouped based on morphological characters and only selected isolates of these groups were sequenced. DNA was extracted using the Genomic Mini AX Plant Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. Partial gene sequences were obtained for the internal transcribed spacer regions (ITS1 and ITS2), including the 5.8S gene (ITS), the 28S large subunit (LSU), the β-tubulin gene (*TUB2*), the translation elongation factor 1-α gene (*TEF1*) and the calmodulin gene (*CAL*) using the primers listed in Table 3.

Gene fragments were amplified in 25 μL reactions 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). Amplification of the gene regions was performed under the following conditions: a denaturation step at 98 °C for 30 s followed by 35 cycles of 5 s at 98 °C, 10 s at 52–64 °C (depending on the optimal Tm of the primers and fungal species) and 30 s at 72 °C, and a final chain elongation step at 72 °C for 8 min. The amplification reactions were performed using a LabCycler thermocycler (SensoQuest Biomedical Electronics GmbH, Germany).

The amplified products were sequenced using the BigDye® Terminator v 3.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA 94404, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA) at the DNA Research Centre (Poznań, Poland) using the same primers as those used for the PCR. The sequences (Table 2) were deposited in NCBI GenBank and compared with those in GenBank using the BLASTn algorithm.

All the sequences were aligned online using MAFFT v 6 (Katoh & Toh, 2008) with the E-INS-i option, a gap-opening penalty of 1.53 and an offset value of 0.00. The datasets were analysed using maximum likelihood (ML) and Bayesian inference (BI). For the ML and Bayesian analyses, the best-fitting substitution models for each dataset were estimated using the corrected Akaike information criterion (AIC) in jModelTest 0.1.1 (Posada, 2008). The selected models are listed in Table 3.

Maximum likelihood (ML) searches were conducted in PhyML 3.0 (Guindon et al., 2010) via the Montpelier online server (http://www.atgc-montpellier.fr/phyml/) with 1000 bootstrap replicates. In addition, BI analyses based on Markov chain Monte Carlo (MCMC) were carried out with MrBayes v 3.1.2 (Ronquist & Huelsenbeck, 2003). The MCMC chains were run for 10 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

Table 3. Loci examined in the phylogenetic analyses

Locus	Primers	Fungi	Nucleotide substitution models Maximum Likehood (ML) and Bayesian inference (BI)
ITS	ITS1-F (Gardes & Bruns, 1993), ITS4 (White et al., 1990)	All genera	*Graphilbum* spp. or *Graphium* spp. GTR+G, *Ophiostoma* spp. or *Sporothrix* spp. GTR+I+G
LSU	ITS3 (White et al., 1990), LR3 (Vilgalys & Hester, 1990)	*Leptographium* spp., *Graphium* spp.	GTR+I+G
TUB2	Bt2a/Bt2b (Glass & Donaldson, 1995) or T10 (O'Donnell & Cigelnik, 1997)	All fungal species except *Graphium* spp.	*Leptographium procerum* complex GTR+I *Leptographium lundbergii* complex HKY+I *Ophiostoma* spp. HKY+I *Sporothrix* spp. HKY+G
TEF1	F-728F (Carbone & Kohn 1999), EF2 (O'Donnell et al. 1998)	*Graphilbum* spp., *Sporothrix* spp.	GTR+G
	EF1F, EF2R (Jacobs et al. 2004)	*Leptographium* spp.	*Leptographium procerum* complex HKY+G *Leptographium lundbergii* complex GTR+I
CAL	CL1, CL2a (O'Donnell et al. 2000) or CL3F, CL3R (Duong et al. 2012)	*Sporothrix* spp.	GTR+I+G

ITS: the internal transcribed spacer region and intervening 5.8SnrRNA; LSU: the internal transcribed spacer (ITS2), part of the large subunit (28S) of the rDNA operon, *TUB2*: β-tubulin, *TEF1*: translation elongation factor 1-alpha, *CAL*: calmodulin.

in Tracer v 1.4.1 (Rambaut & Drummond, 2007). All sequences generated in this study were deposited in NCBI GenBank (Table 2) and are presented in the phylogenetic trees (Figs 3–6, S1–S11).

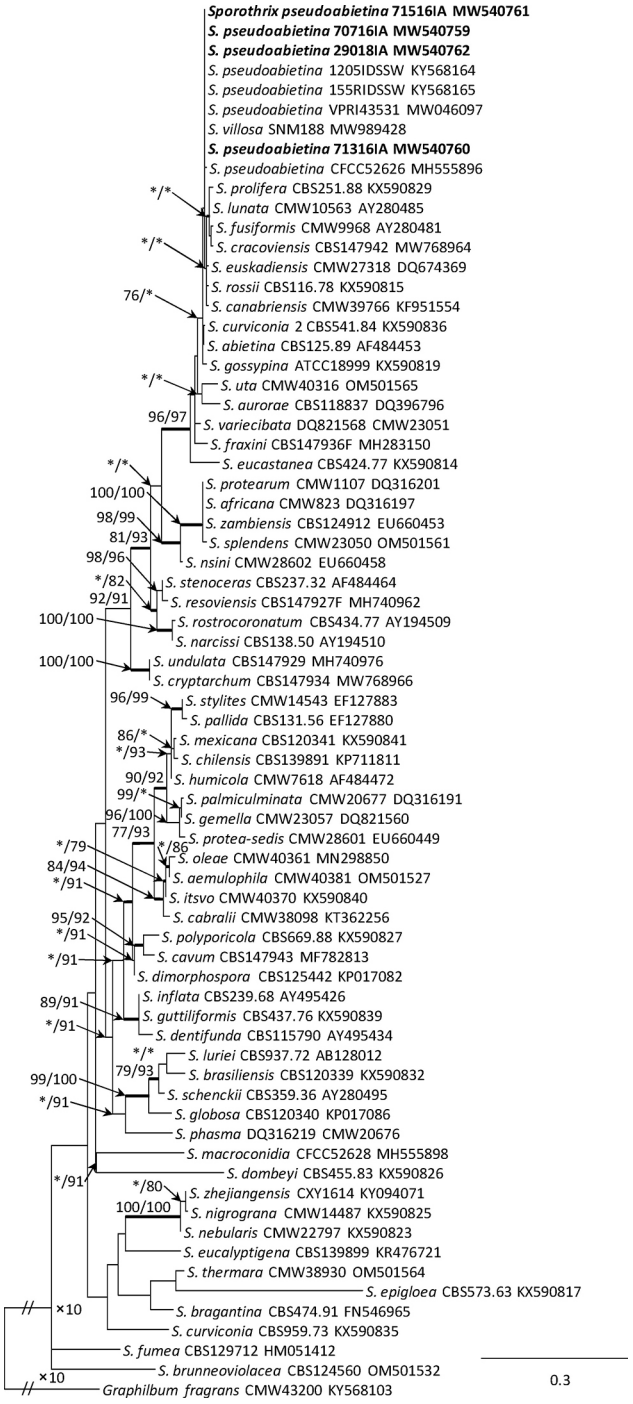

Fig. 3. Phylogram from Maximum Likelihood (ML) analyses of ITS data for *Sporothrix* spp. Sequences obtained in this study are in bold. Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values <75%. The tree is drawn to scale (see bar) with branch lengths measured in the number of substitutions per site. *Graphilbum fragrans* represents the outgroup

Morphological characters of *Sporothrix pseudoabietina*

Cultures were grown on 2% MEA with or without pine twigs to induce ascocarp formation. The autoclaved pine twigs with bark were placed in the middle of the agar plates. Fungal cultures were grown starting with a single spore, and all isolates were crossed in all possible combinations following the technique described by Grobbelaar et al. (2010). Cultures were incubated at 25 °C for 14–21 d and inspected frequently for the formation of fruiting structures.

Morphological characteristics were examined by mounting the asexual fruiting structures in 80% lactic acid on glass slides, and these were observed using a Nikon Eclipse 50*i* microscope (Nikon® Corporation, Tokyo, Japan) with an Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) to capture photographic images. Fifty measurements were made for each significant taxonomically relevant structure whenever possible with Coolview 1.6.0 software (Precoptic®, Warsaw, Poland). Averages, ranges and standard deviations were computed for the measurements, and these are presented in the format "(min–)(mean–SD)–(mean+SD)(–max)".

Growth characteristics were determined by analysing the radial growth of two representative isolates (Table 2). Agar disks 5 mm in diameter were cut from actively growing margins of colonies of each isolate to be tested and placed at the centre of plates containing 2% MEA. Four plates for each isolate were incubated at each of the following temperatures: 5, 10, 15, 20, 25, 30 and 35 °C. Radial growth (two measurements per plate) was determined 7 d after inoculation, and growth rates were calculated as mm/d.

Results

Collections of fungal isolates

Ophiostomatoid fungi were recovered from 98.3% of the beetles and 100% of the galleries. A total of 2269 fungal isolates were obtained from 441 beetle individuals and their galleries. Of these, 413 isolates were obtained from beetles and 1856 from galleries (Table 3). The following fungal groups were obtained: 26 isolates of *Graphium* (1.1% of the total isolates), 1331 isolates of *Graphilbum* (58.7%), 5 isolates of *Leptographium* (0.2%), 2 isolates of *Ophiostoma* (0.1%), and 905 isolates of *Sporothrix* (39.9%).

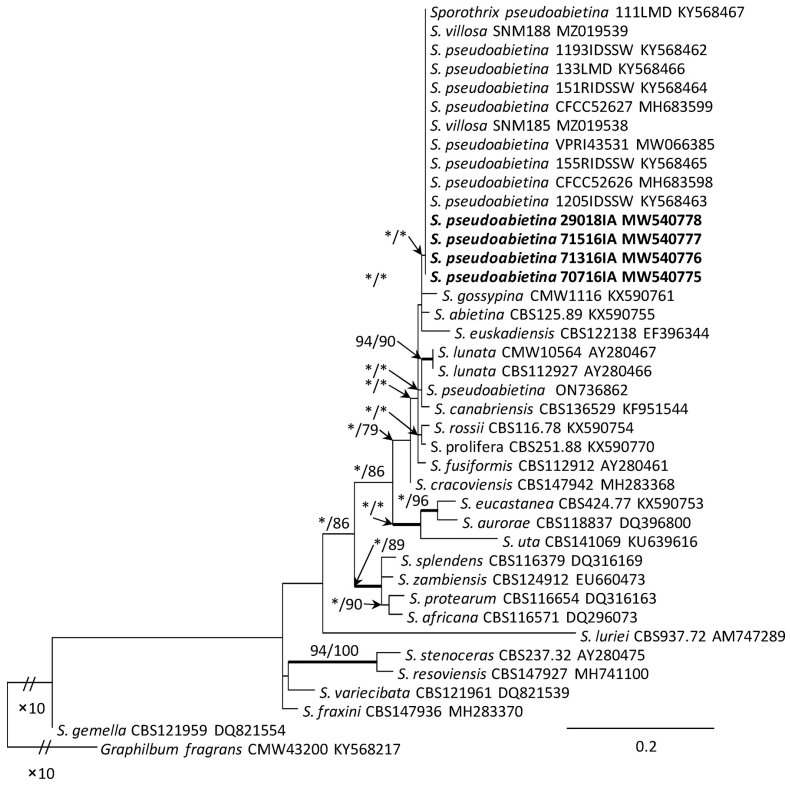

Fig. 4. Phylogram from Maximum Likelihood (ML) analyses of *TUB2* data for *Sporothrix stenoceras* & *S. gossypina* species complexes. Sequences obtained in this study are in bold. Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values <75%. The tree is drawn to scale (see bar) with branch lengths measured in the number of substitutions per site. *Graphilbum fragrans* represents the outgroup

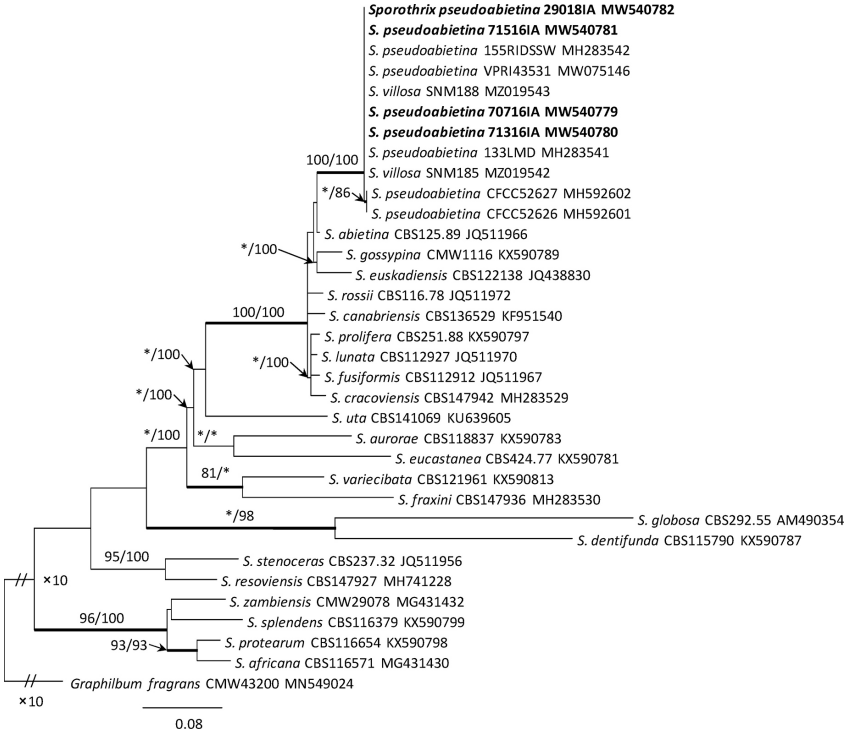

Fig. 5. Phylogram from Maximum Likelihood (ML) analyses of *CAL* data for *Sporothrix stenoceras* & *S. gossypina* species complexes. Sequences obtained in this study are in bold. Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values <75%. The tree is drawn to scale (see bar) with branch lengths measured in the number of substitutions per site. *Graphilbum fragrans* represents the outgroup

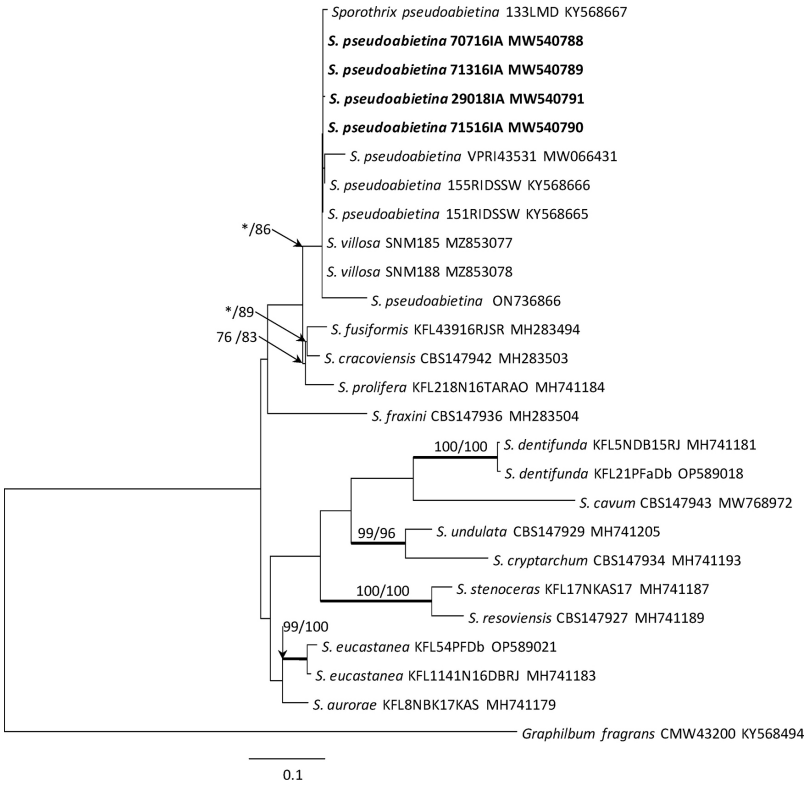

Fig. 6. Phylogram from Maximum Likelihood (ML) analyses of *TEF1* data for *Sporothrix stenoceras* & *S. gossypina* species complexes. Sequences obtained in this study are in bold. Bootstrap values ≥ 75% for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values <75%. The tree is drawn to scale (see bar) with branch lengths measured in the number of substitutions per site. *Graphilbum fragrans* represents the outgroup

DNA sequencing and phylogenetic analyses

DNA sequence data was obtained for 20 isolates that were selected as representatives of the different morphological groups (Table 2). BLAST analyses of the ribosomal DNA sequences placed the majority of the isolates in the *Ophiostomatales* (Taxa 1–6), while two belonged to the *Microascales* (Taxon 7). Based on phylogenetic analyses of the ITS (Figs 3, S1, S3, S10) and LSU (Figs S4, S5) regions, the fungi belonged to six species in the *Ophiostomatales*, including two species each of *Graphilbum* (Taxa 1–2) and *Leptographium* (Taxa 3-4), and one species each of *Ophiostoma* (Taxon 5) and *Sporothrix* (Taxon 6). Taxon 7 was *Graphium pseudormiticum* M. Mouton & M.J. Wingf. (Figs S3–S4).

Analyses of the ITS and *TEF1* data for *Graphilbum* revealed that the isolates labelled as Taxon 1 represented *G. acuminatum* (Figs S1–S2). The ITS sequence from Taxon 2 (27018IA) was identical to an ex-type isolate of *Graphilbum fragrans* (Math.-Käärik) Z.W. de Beer, Seifert & M.J. Wingf. (CBS 279.54) from Sweden and to isolate CBS 138720 from South Africa (Fig. S1), while the *TEF1* sequence was identical to an isolate of *G. fragrans* (CMW 44159) obtained from *Trypodendron lineatum* (Olivier) that infested *Abies alba* Mill. in Poland (Fig. S2). Taxon 3 grouped with *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. (Fig. S5) in the LSU tree, and grouped with an ex-epitype isolate of *L. procerum* (CBS 1138288) from the USA (Figs S6–S7) based on the *TUB2* and *TEF1* trees. Taxon 4 grouped with *Leptographium sosnaicola* R. Jankowiak in the LSU tree (Fig. S5), and specifically grouped with an ex-type isolate of *L. sosnaicola* (CBS 147023) from Poland based on the *TUB2* and *TEF1* trees (Figs S8–S9). Taxon 5 was *O. minus* based on the ITS and *TUB2* sequences (Figs S10–S11), and was identical or nearly identical to many other European and Chinese *O. minus* isolates. Taxon 6 grouped in the *S. gossypina* & *S. stenoceras* species complexes based on ITS sequences (Fig. 3), while it grouped with isolates of *S. pseudoabietina* from China, Croatia and Australia, *Sporothrix villosa* R.L. Chang & X.Y. Zhang from China, and *Sporothrix* isolates labelled as *Sporothrix* sp. 1 from Poland and Czechia (Jankowiak et al., 2017) based on the *CAL*, *TUB2* and *TEF1* trees (Figs 4–6).

Frequencies of isolation

In total, 2243 (98.8%) isolates belonged to the *Ophiostomatales*, while 26 (1.2%) isolates belonged to the *Microascales*. *Graphilbum acuminatum* was the most

commonly isolated fungus, as it was found in 96.6% of beetles and 98.7% of galleries (Fig. 2e, f). This was followed by *S. pseudoabietina*, which was isolated from 64.6% of beetles and 79.8% of galleries. The remaining species were rarely isolated (Table 4).

Six of seven species were isolated from the galleries, while three species were isolated from the beetles. One species (*Gr. pseudormiticum*) was found only on beetles and four species (*G. fragrans*, *L. procerum*, *L. sosnaicola*, *O. minus*) were found only in galleries.

Table 4. Frequencies (%)* of ophiostomatoid fungi obtained from *Ips acuminatus* beetles (B) and their galleries (G) collected from Scots pines in Poland

Fungus species	Sadłowice		Wronów		Skoki	Witków		Total	
	B	G	B	G	G	B	G	B	G
Graphilbum acuminatum	96	92.5	100	100	100	98.1	87.8	98.7	96.6
Graphilbum fragrans				3.3					0.5
Graphium pseudormiticum	26							10.1	
Leptographium procerum					6.8				1.5
Leptographium sosnaicola							1		0.5
Ophiostoma minus							2		1
Sporothrix pseudoabietina	76	92.5	78.1	100	100	48.1	53.5	64.6	79.8
Total no. isolates	198	432	57	217	532	158	675	413	1856
Total no. of samples with ophiostomatoid species (%)	98	100	96.9	100	100	99.1	100	98.3	100
Species richness (S)	3	2	2	3	3	2	4	3	6
Total no. samples	100	40	32	30	44	108	90	237	204

* The frequency of occurrence was calculated according to the following formula: F=(NS/NTs) × 100, where F represents the frequency of occurrence (%) of the fungus, NS represents the number of samples from which a particular fungus was isolated, and NTs represents the total number of samples.

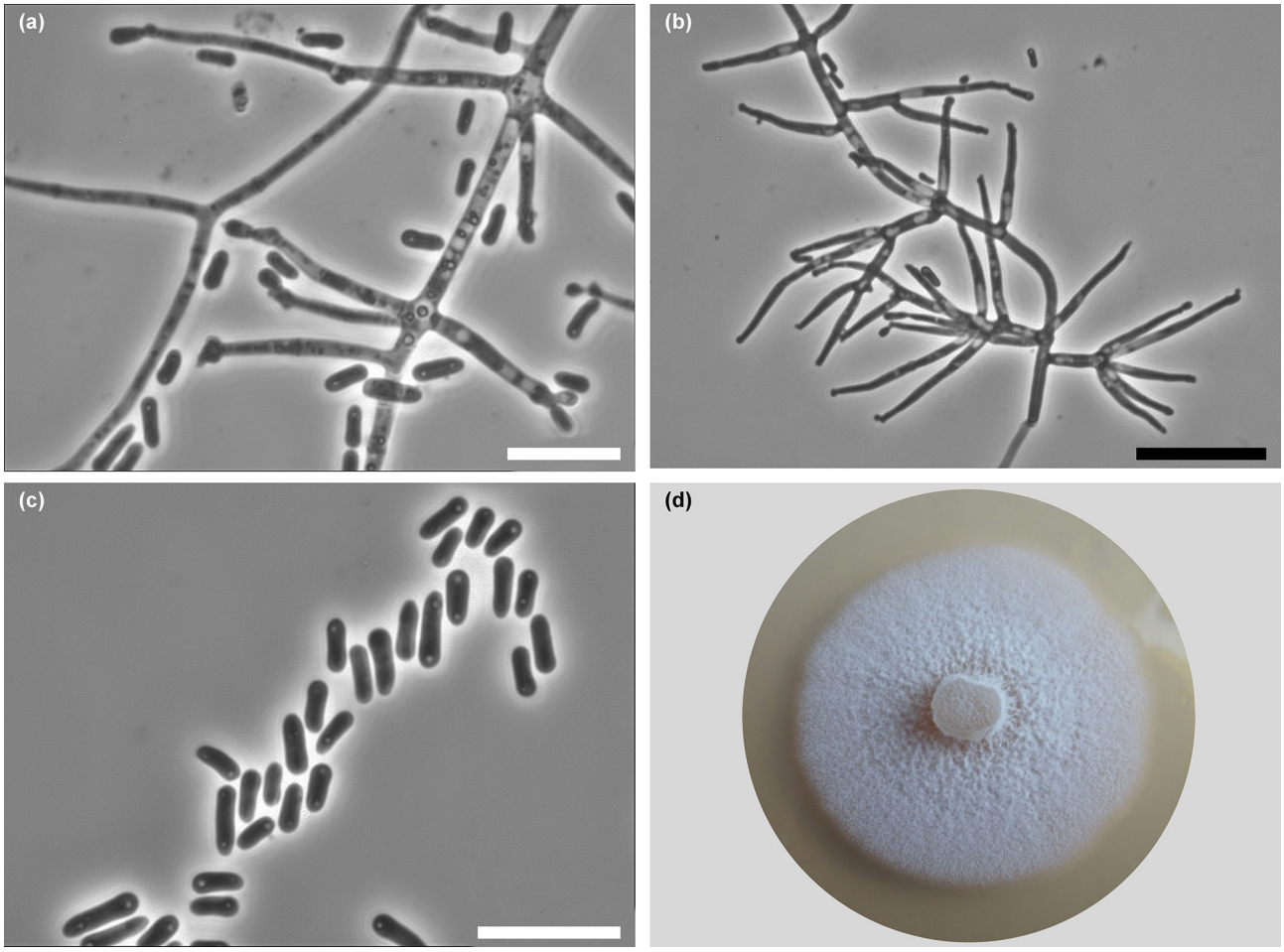

Fig. 7. *Sporothrix pseudoabietina* (CBS 147969) (a) Simple conidiophores with conidiogenous cell with denticles at the apex; (b) Monoverticillate branched conidiophores; (c) Conidia; (d) Fourteen-day-old culture on MEA. Scale bars: a, c = 10 µm, b = 25 µm

Graphilbum acuminatum and *S. pseudoabietina* were found in all survey locations, while the other species occurred in only one sampling site (Table 4).

Taxonomy of *Sporothrix pseudoabietina*

In this study, molecular data were generated for *S. pseudoabietina* from Poland. The description of the fungus based on Polish isolates is as follows:

***Sporothrix pseudoabietina* H. Wang, Q. Lu & Z. Zhang**

Sexual morph not observed. Asexual structures produced on sterilized pine twigs placed on the surface of malt agar in Petri dishes. *Conidiophores* hyaline, micronematous, simple, straight, simple or monoverticillate branched (Fig. 7 a, b). *Conidiogenous cells* blastics, cylindrical, terminal, straight or curved, slightly tapering toward the apex, swollen apical part forming conidia by sympodial proliferation on not-well visible denticles, (11.7–)14.6–30.6(–42.9) µm long, (0.9–)1.2–1.9(–2.5) µm wide at the base. Apical part with denticles (0.6–)0.9–2.5(–4.8) µm long and (0.5–)0.8–1.5(–2) µm wide. *Conidia* hyaline, unicellular, smooth, clavate or oblong, slightly curved, with pointed bases, (2.6–)3.1–5.9(–8.6) × (0.6–)0.9–1.3(–1.6) µm, formed on terminal denticles (Figure 7 c). Culture characteristics on MEA: cultures having optimum growth at 25 °C (1.2 mm/d) followed by at 30 °C (1.1 mm/d), white, growing in a circular pattern with smooth margins, with abundant aerial mycelium (Fig. 7d).

Specimen examined: on *Pinus sylvestris* – POLAND, Lubelskie District, Mircze Forest District, Witków, Sep. 2016, leg. Z. Kołodziej, CBS 147969=CMW 57302=70716IA.

Distribution: Australia (Trollip et al., 2021), south-western China (Wang et al., 2019), Croatia (Kovač et al., 2022); Poland, Czechia (Jankowiak et al., 2017, this study).

Host: *Pinus radiata* D. Don., *P. caribaea* × *elliottii*, *P. taeda* L., *P. elliottii* Engelm. (Trollip et al., 2021); *P. yunnanensis* Franch. (Wang et al., 2019); *P. halepensis* Mill. (Kovač et al., 2022), *P. sylvestris* (this study), *Picea abies*, *Larix decidua* Mill. (Jankowiak et al., 2017).

Insect vectors: *Ips grandicollis* (W. Eichhoff, 1868), *Xyleborus nr. ferrugineus* (J.C. Fabricius, 1801) (Trollip et al., 2021), *Tomicus yunnanensis* Kirkendall & Faccoli and *T. minor* (G.L. Hartig, 1834) (Wang et al., 2019); *Orthotomicus erosus* (T.V. Wollaston, 1857) (Kovač et al., 2022), *Hylastes ater* (G. Paykull, 1800), *I. amitinus* (W. Eichhoff, 1872), *I. cembrae* (O. Heer, 1836), *I. duplicatus* (C.R. Sahlberg, 1836) (Jankowiak et al., 2017), *I. acuminatus* (this study).

Discussion

In the present study, we collected *I. acuminatus* and its galleries from multiple pine forests in eastern Poland. From these beetles and galleries, we recovered 2269 isolates of ophiostomatoid fungi which represented seven well-defined and previously described taxa. This is the first study focused exclusively on the fungi associated with *I. acuminatus* in Central Europe.

Most of these isolates resided in the *Ophiostomatales* (de Beer & Wingfield, 2013; de Beer et al., 2022). Among them, the most numerous were those from *G. acuminatum* and *S. pseudoabietina*, which appear to be strictly associated with this beetle species in Poland. A comparison of the present study with published studies from Fennoscandia, France, Italy and Ukraine showed that the fungal assemblages of *I. acuminatus* from Poland are distinct from those elsewhere in Europe. In terms of species diversity, *I. acuminatus* from Fennoscandia has the largest number of species (i.e., 17): 13 species, namely *Ceratocystiopsis minuta* (Siemaszko) H.P. Upadhyay & W.B. Kendr., *Endoconidiophora coerulescens* Münch, *Graphium pycnocephalum* Grosmann, *Hyalorhinocladiella macrospora*, *Leptographium lundbergii* Lagerb. & Melin, *Ophiostoma canum* (Münch) Syd. & P. Syd., *O. clavatum*, *O. floccosum* Math.-Käärik, *O. ips*, *O. minus*, *O. piceae* (Münch) Syd. & P. Syd. and *O. piliferum* (Fr.) Syd. & P. Syd. were found in Sweden (Mathiesen, 1950, 1951; Rennerfelt, 1950; Mathiesen-Käärik, 1953), while only four species, *G. acuminatum, Graphium* sp., *O. minus* and *O. macrosporum* (Francke-Grosm.) Z.W de Beer & M.J. Wingf. occurred in Norway (Waalberg, 2015). Four species (*O. clavatum*, *O. macrosporum*, *O. piceae* and *O. piliferum*) were found in Germany and the former nation of Yugoslavia (Francke-Grosmann, 1963), while four additional species (*O. brunneo-ciliatum*, *O. ips*, *O. minus*, and *Ophiostoma* sp.) were found in France (Lieutier et al., 1991), although Villari recently (2012) provided evidence that the fungus identified as *O. bruuneo-cilliatum* in France was probably *O. clavatum* instead. Finally, six species, namely *Graphilbum* cf. *rectangulisporium, Grosmannia olivacea* (Math-Käärik) Zipfel, Z.W de Beer & M.J. Wingf., *O. ips, O. minus, O. pallidulum* Linnak., Z.W. de Beer & M.J. Wingf. and *O. piceae* are known from *I. acuminatus* in Ukraine (Davydenko et al., 2017). From the above listing it is evident that *G. acuminatum* and *O. minus* were the only fungal species associated with *I. acuminatus* in both Poland and other European regions. In this study, associations between *I. acuminatus* and the ophiostomatoid fungi *Graphium pseudormiticum*, *G. fragrans*, *L. procerum* and *L. sosnaicola* were reported the first time. Our findings combined with previous reports showed that different assemblages of ophiostomatoid fungi are associated with *I. acuminatus* throughout its Eurasian range, although there

is some overlap in species reported in different locations. Recently, Chang et al. (2017) surveyed the mycobiota of *I. acuminatus* in China on *Pinus kesiya* Rolye & Gordon and reported only two species (*O. ips* and *G. pseudormiticum*) that were also found in association with this beetle in Europe. Five other species, *Graphilbum puerense* R. Chang & Z.W. de Beer, *Grosmannia yunnanensis* Yamaoka, Masuya & M.J. Wingf., *Ophiostoma acororum* R. Chang & Z.W. de Beer, *O. quercus* (Georgv.) Nannf. and *Sporothrix nebularis* Romón, Z.W. de Beer & M.J. Wingf. were exclusively associated with *I. acuminatus* in China (Chang et al., 2017). Similar patterns were also found across the ranges of other bark beetles, such as *I. typographus* (Kirisits, 2004; Linnakoski, 2012). These differences may be explained by climatic and host tree variation across study sites, as well as differences in survey methods (sampling methods, time of year of surveys, methods of fungal identification, etc.).

Of all the species collected in this study, *G. acuminatum* was the most commonly and consistently isolated from *I. acuminatus*. At all locations, this fungus was isolated from between 96% and 100% of beetles and between 92.5% and 100% of galleries. This fungus was also the most common species associated with *I. acuminatus* in Norway (Waalberg, 2015; Jankowiak et al., 2020). *Graphilbum puerense* has been reported as a fungal associate of *I. acuminatus* in China (Chang et al., 2017), while other *Graphilbum* species (labeled as *G. cf. rectangulisporium*) was found in association with *I. acuminatus* in Ukraine (Davydenko et al., 2017). The Ukrainian isolates appear to be closely related to *G. crescericum* Romón & M.J. Wingf., *G. niveum* R.L. Chang & X.Y. Zhang, and *G. sexdentatum* R. Jankowiak & H. Solheim based on ITS data. In addition, *G. fragrans* was found in this study. This species has previously been reported from Poland, in association with several other bark beetles, especially *T. lineatum* (Jankowiak et al., 2017). These results confirmed a close association between *Graphilbum* species and *I. acuminatus* although the populations of the beetle in different geographic areas have different compositions of *Graphilbum* spp. The differences in fungal species compositions associated with bark beetles at the different locations were also found in *I. typographus* (Kirisits, 2004; Chang et al., 2019).

Another species found in frequent association with *I. acuminatus* was *S. pseudoabetina*. This fungus was previously isolated from *H. ater* and *I. duplicatus* on *P. abies,* and *I. amitinus* and *I. cembrae* on *L. decidua* in Poland and Czechia (Jankowiak et al., 2017), as well as from *T. yunnanensis* and *T. minor* on *P. yunnanensis* in China (Wang et al., 2019). Recently, it has been also found in association with pine-infesting bark beetles in Australia (Trollip et al., 2021) and Croatia (Kovač et al., 2022). Our study and other findings suggest that *S. pseudoabietina* is broadly associated with numerous tree and insect species worldwide. In addition, DNA sequences suggested that *S. villosa* R.L. Chang & X.Y. Zhang, which was described from *Cryphalus piceae* (Ratzeburg) on *Pinus thunbergii* Parlatore in China (Chang et al., 2021) is a synonym of *S. pseudoabietina*. However, despite the identical phylogenetic placements, the fungal species differ morphologically. *Sporothrix pseudoabietina* produces conidia that are twice as large as *S. villosa* (3–9 × 1–4.8 µm vs. 1.2–4.1 × 0.7–1.4 µm). In addition, the conidia lengths of Polish isolates of *S. pseudoabietina* (2.6–8.6 µm) resemble the conidia lengths of *S. pseudoabietina* from China, while their widths (0.6–1.6 µm) corresponded to the conidia of *S. villosa* rather than *S. pseudoabietina* from China. In addition, only Chinese isolates of *S. pseudoabietina* produced sexual morphs. More detailed morphological and molecular studies are needed to resolve the status of both *Sporothrix* species.

The other species reported in this study, namely *L. procerum*, *O. minus* and *Graphium pseudormiticum* have wide distributions in Europe, where they are associated with several species of pine bark beetles and weevils (Kirisits, 2004; Linnakoski, 2012; Jankowiak & Bilański, 2013 a, b, c). These fungi were found inconsistently and in low numbers, suggesting that the associations between the fungi and *I. acuminatus* are causal or incidental. *Leptographium sosnaicola* was also rarely observed, although it has been reported as an associate of *Ips sexdenatus* (I.K.H. Börner, 1766) in Poland (Jankowiak, 2012; 2021). It is possible that *I. acuminatus* galleries are sometimes contaminated by fungi transmitted by *I. sexdentatus*, despite these beetle species preferring different parts of the trunk.

Of all the species reported in this study, *O. minus* is the only fungus known to have a relatively high level of pathogenicity to Scots pine (Lieutier et al., 1989; Solheim & Långström, 1991; Solheim et al., 1993; Jankowiak, 2006, 2011, 2012, 2013), while *L. procerum* is a weak pathogen (Lu et al., 2009). These fungi are unlikely to play a major role in the decline of trees in this study because they were rarely isolated. However, there is information available about the potential pathogenicity of *G. acuminatum* and *S. pseudoabietina*, which were isolated at high frequencies in this study. Other *Graphilbum* species, such as *G. cf. rectangulisporium*, have been implicated as causal agents of the decline of Scots pine seedlings (Jankowiak 2012; Davydenko et al., 2017), although Dori-Bachash et al. (2015) reported that *G. rectangulisporium* did not cause lesions or mortality on seedlings of *P. halepensis* and *P. brutia* Ten. in Israel. Future studies are needed to investigate the pathogenicity of the fungi associated with *I. acuminatus* in Poland.

Conclusion

The results of this study showed that *I. acuminatus* was primarily associated with only two ophiostomatoid fungi in Poland: *G. acuminatum* and *S. pseudoabietina*. This fungal community was different from those reported in other regions of Eurasia. Molecular data suggests that *S. pseudoabietina* is synonym of *S. villosa* in the *S. gossypina* & *S. stenoceras* species complexes. However, comparisons of morphological characters suggest that the two fungi may still be different species. Because ophiostomatoid fungi are potential agents of stain diseases and vascular wilt, research on the phytopathogenicity of ophiostomatoid fungi associated with *I. acuminatus* is recommended.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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