Ophiostomatoid fungi associated with three spruce-infesting bark beetles in Slovenia

Andreja Repe, Thomas Kirisits, Barbara Piškur, Maarten Groot, Bojka Kump, Maja Jurc

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ORIGINAL PAPER

Ophiostomatoid fungi associated with three spruce-infesting bark beetles in Slovenia

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Abstract
• ***Context*** Ophiostomatoid fungi can severely affect the health and economic value of Norway spruce trees (*Picea abies*). Although the diversity of ophiostomatoid species and their associations with insects have been well-investigated in central and northern Europe, little is known about the conditions in south-eastern Europe.
• ***Aim*** This study aims to study the assemblages of ophiostomatoid fungi associated with three bark beetle species (*Ips typographus*, *Ips amitinus*, and *Pityogenes chalcographus*) that infect Norway spruce in Slovenia.
• ***Methods*** Bark beetles were sampled in four phytogeographic regions in Slovenia. The fungi found on the bark beetles were identified based on morphology, DNA sequence comparisons of ITS regions and phylogenetic analysis. The species compositions of the fungal associates of the three insect species were compared and the pairwise associations of the occurrence of the fungal species were analysed.
• ***Results*** Thirteen different species were found. The most commonly encountered fungal associates of the beetles were *Ophiostoma bicolor*, *Ophiostoma brunneo-ciliatum*, *Grosmannia piceiperda*, *Ophiostoma ainoae*, *Ceratocystiopsis minuta*, and *Grosmannia penicillata*. The composition of the fungal associates differed among the bark beetle species, but not among the phytogeographic regions.
• ***Conclusions*** This study confirms that ophiostomatoid species are common associates of the investigated bark beetle species. Many ophiostomatoid species have strong host associations. *I. typographus* and *P. chalcographus* can act as effective vectors for *O. bicolor*, *O. ainoae*, *G. piceiperda* and *O. brunneo-ciliatum*, whereas *I. amitinus* often carries *G. piceiperda* and *C. minuta* in Slovenian forests.

Keywords *Picea abies* · *Ophiostoma* · *Grosmannia* · *Ceratocystis* · *Ceratocystiopsis* · Forestry · Ophiostomatoid fungi · Bark beetle · Fungal associations · Phytogeographic regions · Blue stain fungi · Forest pathology · Forest entomology · Scolytinae

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A. Repe (✉) · M. Jurc
Department of Forestry and Renewable Forest Resources, Biotechnical Faculty, University of Ljubljana, Večna pot 83, 1000 Ljubljana, Slovenia
e-mail: andreja.repe@bf.uni-lj.si

T. Kirisits
Institute of Forest Entomology, Forest Pathology and Forest Protection Department for Forest and Soil Sciences, BOKU—University of Natural Resources and Applied Life Sciences, Hasenauerstrasse 38, 1190 Vienna, Austria

B. Piškur · M. de Groot
Department of Forest Protection, Slovenian Forestry Institute, Večna pot 2, 1000 Ljubljana, Slovenia

B. Kump
Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

1 Introduction

Slovenia, with approximately 60 % forest cover, is one of the most forested countries in Europe. Nearly half of the growing stock consists of conifers, 31.5 % of which are Norway spruce (*Picea abies* [L.] Karst.), although their share in natural forests is significantly lower (Babuder and Pohleven 1995). In Slovenia, *P. abies* is frequently found on sites where it does

not occur naturally and is therefore often susceptible to various abiotic and biotic factors. As a consequence, the health of the forest collapses and the number of sanitary fellings increases. In Slovenia, a common cause for sanitary felling of *P. abies* trees is damage caused by insects, mostly bark beetles (Coleoptera, Scolytinae), followed by diseases such as pathogenic fungi and wind throw (Forest Report of Slovenia 2011). The most damaging bark beetles on *P. abies* trees in Slovenia are *Ips typographus* (Linnaeus 1758), *Pityogenes chalcographus* (Linnaeus 1761), *Ips amitinus* (Eichhoff 1871) and *Polygraphus poligraphus* (Linnaeus 1758) (Jurc 2006). *I. typographus*, which is the bark beetle species that is most aggressive toward *P. abies*, develops on weakened or freshly harvested trees. *P. chalcographus* may also be a quite aggressive bark beetle species, attacking *P. abies* particularly when the trees are drought-, snow- or wind throw-stressed (Jurc 2006). Both insects become primary pests under outbreak conditions (Jurc 2006). *I. amitinus* and *I. typographus* frequently colonise trees together, causing high levels of host mortality (Jurc and Bojović 2004). In Slovenia, *I. typographus* and *P. chalcographus* are widely distributed inside and outside the natural distribution range of *P. abies*, whereas *I. amitinus* is particularly common at high elevations in the Slovenian Alps (Jurc and Bojović 2004).

Bark beetles are well-known vectors of ophiostomatoid fungi. These fungi are introduced into a new host tree during bark beetle attacks and the construction of their egg galleries (Paine et al. 1997; Harrington 2005; Jankowiak et al. 2009). The larvae that hatch from the eggs later pupate at the end of larval galleries (Jurc 2006). Sporulation of fungi in the pupal chambers is particularly important for later fungi dissemination (Harrington 2005). After pupation, the young adults emerge. They carry the fungal spores on specialised structures called mycangium or most commonly, freely on their bodies. The spores can also be eaten and passed through the digestive tract (Harrington 1993; Paine et al. 1997).

The ophiostomatoid fungi are a diverse, polyphyletic group of morphologically similar genera of ascomycetes ubiquitously found on coniferous and deciduous tree species (Kirisits 2004). These fungi have similar teleomorph structures (long-necked perithecia containing evanescent asci) but different anamorphs (Jacobs and Wingfield 2001). The morphological similarity among the ophiostomatoid fungi can be explained by convergent evolution resulting from their association with insects and other arthropods (Kirisits 2004). They include the following teleomorph genera within the order Ophiostomatales: *Ophiostoma* Syd. & P. Syd., *Ceratocystiopsis* H.P. Upadhyay & W.B.Kendr., *Grosmannia* Goid. (= *Ophiostoma* sensu lato [s. l.]), *Fragosphaeria* Shear 1923, *Pesotum fragrans* complex and related anamorphic fungi (Zipfel et al. 2006). Another blue stain genus is *Ceratocystis* Ellis & Halst., which belongs to the order Microascales. Ophiostomatoid fungi are mostly competitive saprotrophs or weak parasites colonising the phloem and sapwood of gymnosperms and angiosperms, usually at the early stages of fungal succession (Jacobs and Wingfield 2001). Wood colonisation by ophiostomatoid fungi can lead to sapstain, a blue to grey discoloration of the sapwood, which is therefore also referred to as blue stain (Kirisits 2004). Some ophiostomatoid fungi are aggressive tree pathogens that cause serious vascular wilt and vascular stain diseases (e.g. Dutch elm disease, black stain root disease, canker stain disease of plane trees and oak wilt), resulting in the disruption of water transport and tree death (Kirisits 2004). Both sapstain and vascular stain diseases may affect *P. abies* and result in economic losses for forestry and the wood industry.

In Slovenia, bark beetles have been studied extensively, but research on bark beetle-associated fungi has been only preliminary to date or has not included the beetles investigated in this study, e.g. *Trypodendron lineatum* (Babuder and Pohleven 1993, 1995). The aim of this study was thus to contribute to the knowledge of *Scolytus*–fungus interactions in Slovenia as well as in Europe as the southernmost study of the mycobiota associated with spruce bark beetles. The major aim of the present study was to investigate the composition of ophiostomatoid fungi associated with three bark beetle species, including *I. typographus*, *I. amitinus* and *P. chalcographus*, in four different phytogeographic regions of Slovenia. Regarding the interactions among the fungi, the aim was to determine whether certain fungal species are more likely to appear simultaneously. The interactions can be either positive or negative. Some fungi might appear together more commonly or inhibit the growth of others.

2 Materials and methods

2.1 Study areas, sample collection and fungal isolation

The bark beetles were collected at six sites in four phytogeographic regions (Alpine, Dinaric, Predinaric, Subpanonic) in Slovenia. All of the collection sites (Table 1) were located in secondary *P. abies* stands. These are forest stands that were created by a disorder, in our case anthropogenic (felling of natural forests and reforestation with non-indigenous species). The beetles were collected in the main swarming period of the first generation, from May to July in the years 2008–2010. The collection dates were dependent on the temperatures (which differed according to the year of collection and with the altitude). Between March and April during the first 2 years, healthy looking *P. abies* trees were felled on each site (one to two trees per site). In 2010, recently felled *P. abies* trees not infested with beetles were used as trap trees at the Košenjak site. Any of the three investigated insect species, if present, were collected from the trap trees. *I. typographus* was sampled in five locations in the Alpine, Predinaric, Dinaric and Subpanonic regions (Table 1). The lowest number of individuals was collected at the Vučja jama site in the Subpanonic

Table 1 Features of the study areas, list of samples and characteristics of the bark beetles

Location	Boršt	Pugled	Ig	Mokerc	Košenjak	Vučja jama
Phytogeographic region	Alpine	Predinaric	Dinaric	Dinaric	Alpine	Subpanonic
Geographic coordinates	46°24′07″N 14°05′07″E	45°40′43″N 14°51′14″E	45°57′23″N 14°31′05″E	45°40′18″N 14°44′33″E	46º38′45″N 15º2′10″E	46º40′19″N 15º44′34″E
Altitude	850 m a.s.l.	475 m a.s.l.	325 m a.s.l.	800 m a.s.l.	1270 m a.s.l.	370 m a.s.l.
Parent material	Limestone	Limestone	Dolomite	Dolomite	Siliceous metamorphic rocks	Marl
Forest community	*Luzulo–Fagetum*	*Omphalodo–Fagetum*	*Omphalodo–Fagetum*	*Omphalodo–Fagetum*	*Avenello flexuosae–Piceetum*	*Querco–Fagetum*
% of *Picea abies* in the stand	>80	>80	15	15	>80	3
Tree age (approximate)	80	80	80	80	80	80
Year of sampling	2008	2008, 2009	2008	2009	2010	2009
Sampling period	May	June (2008), May (2009)	July	May	June and July	June
Bark beetle	I.t., P.c.	I.t., P.c.	I.t., P.c.	I.t., P.c.	I.a.	I.t.
N of examined beetles	30	30, 40	30	40	45, 80	20
Beetles' population phase	Outbreak	Transition	Endemic	Endemic	Transition	Endemic
Average annual temperature	6–8 °C	8–10 °C	6–8 °C	6–8 °C	4–6 °C	8–10 °C
Average annual precipitation	1,800–2,000 mm	1,500–1,600 mm	1,300–1,400 mm	1,300–1,400 mm	1,300–1,400 mm	1,000–1,100 mm

I.t. Ips typographus, *P.c. Pityogenes chalcographus*, *I.a. Ips amitinus*, *Outbreak* >50 % of the mature *P. abies* were colonised by bark beetles, the beetles were colonising vigorous trees, *Transition* <10 to 50 % tree mortality, *Endemic* <10 % cumulative tree mortality, *Post-outbreak* near 100 % mortality (Aukema et al. 2005)

region. *P. chalcographus* was found at four locations within the Alpine, Predinaric and Dinaric regions (Table 1). *I. amitinus* was found only in the Košenjak site in the Alpine region (Table 1).

The beetle galleries under the bark were opened with a knife and the beetles (regardless of the gender) were collected using forceps. One to two beetles were collected from each gallery, and the number of collected beetles varied (Table 1). Each collected beetle was placed individually in a sterile Eppendorf tube (Eppendorf, Hamburg, Germany) and stored at 4 °C for 1–2 days until the fungal isolations were performed. After each extraction, the tools were dipped in absolute ethanol (Carlo Erba Reagents, Milano, Italy) and flame-sterilised to avoid contamination. Altogether, 624 specimens of *P. chalcographus*, *I. typographus* and *I. amitinus* were obtained at the six sites.

For the fungal isolation, the beetles (which were not disinfected before the fungal isolation) were carefully transferred to and squashed directly on 2 % malt extract agar (MEA; 2 % Bacto malt extract, 1.5 % Difco technical agar; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). In half of the plates, the medium was supplemented with the antibiotics cycloheximide (which is selective for *Ophiostoma* s. l.; Zipfel et al. 2006) and streptomycin sulphate (1 % Bacto malt extract, 1.5 % Difco agar, 0.02 % cycloheximide (Sigma-Aldrich, St. Louis, MO, USA), 0.01 % streptomycin (Sigma-Aldrich)), as described by Jacobs and Wingfield (2001). Both antibiotics were added to the media to prevent contamination. The plates were then incubated in the dark for 2 to 3 weeks at temperatures between 20 and 25 °C. Pure cultures were obtained by transferring small pieces of MEA with the mycelia of the fungi growing from spores in the bark beetle bodies to new 1.5 % MEA plates. We added 4-cm-long autoclaved pieces of *P. abies* sapwood to the medium and/or placed the cultures under UV light to stimulate the growth of fruiting bodies.

2.2 Morphological identification

The fungi were identified based on the micro-morphological characteristics of their sexual and asexual structures observed using an Olympus SZX 12 lens and an Olympus BX51 dissecting microscope. Ascocarps, synnemata and conidiophores were mounted on glass slides in water or in 1 % lactophenol cotton blue (Fluka, Buchs, Switzerland). The fungal isolates were initially grouped based on their colony morphology, with each group representing a putative species. The morphological keys and descriptions by Solheim (1986), Jacobs and Wingfield (2001) and Linnakoski et al. (2010) were used for the species identification. Reference strains of all of the isolated ophiostomatoid fungi were deposited in the culture collection of the Laboratory for Forest Protection (ZLVG) at the Slovenian Forestry Institute, Ljubljana, Slovenia. In

addition, representative isolates were deposited in the Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands (Table 2).

2.3 DNA sequencing and phylogenetic analysis

Altogether, 71 isolates were included in the molecular studies to confirm the species identification resulting from the morphological examinations. Isolates representing different putative species based on their morphology were grown in a liquid malt extract medium (2 % Bacto malt extract) for approximately 2 weeks. Approximately 10 mg of mycelium was taken from the actively growing cultures. DNA was extracted using the 2 % (*w*/*v*) hexadecyltrimethylammonium bromide protocol (Rogers and Bendich 1985). The extracted DNA was resuspended in 30 μl of Tris–EDTA (Sigma-Aldrich). The ITS ribosomal DNA (rDNA) region (ITS1-5.8S-ITS2) was amplified using the primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The reaction mixture (25 μl final volume) contained 6.85 μl of ddH_2O, 0.5 μl of a dNTP mixture (250 U), 5 μl of reaction buffer (×5), 2.5 μl of $MgCl_2$ (25 nM), 0.15 μl of Go Taq DNA polymerase (Promega, WI, USA) and 2.5 μl of each primer (Applied Biosystems, Cheshire, UK). The PCR reactions were performed using an AB Applied Biosystems 2720 Thermal cycler (Applied Biosystems, CA, USA). The cycling parameters followed the procedure described by Linnakoski et al. (2010): an initial 2-min denaturation step at 95 °C, followed by 40 cycles of 30 s of denaturation at 95 °C, 30 s of primer-annealing at 54 °C and 1 min of DNA extension at 72 °C, with a final DNA extension for 8 min at 72 °C. The PCR products were separated on a 1 % (*w*/*v*) agarose gel that was stained with SYBR Safe, and the amplicons were visualised under UV light. The PCR products were purified using a High Pure PCR Product Purification kit (Roche, Germany). Both strands were sequenced using the PCR primers at the Macrogen (Korea)

Table 2 List of the representative strains, their features and the results of the BLASTn searches

Fungal species	Collection site	Vector	Conidiophores[a]	Anamorph[b]	Representative strain no. CBS[c]	EMBL accession no.[d]	ZLVG no.[e]
Ophiostoma ainoae	Mokerc	*I. typographus*	Synnematous	*Pesotum*	CBS134051	HE866690	ZLVG341
Ophiostoma bicolor	Boršt	*P. chalcographus*	Mononematous	*Sporothrix*	CBS134052	HE86691	ZLVG358
Ophiostoma brunneo-ciliatum	Košenjak	*I. amitinus*	Discrete, synnematous	*Pesotum*	CBS134050	HE866692	ZLVG357
Grosmannia cucullata	Mokerc	*P. chalcographus*	Discrete, synnematous	*Pesotum*	CBS134056	HE866693	ZLVG344
Ophiostoma piceae	Boršt	*I. typographus*	Discrete, synnematous and mononematous	*Pesotum* and *Sporothrix*	CBS134312	HE866694	ZLVG343
Grosmannia penicillata	Vučja jama	*I. typographus*	Discrete, mononematous	*Leptographium*	CBS134055	HE866695	ZLVG347
Grosmannia piceiperda	Ig	*P. chalcographus*	Discrete, mononematous	*Leptographium*	CBS134057	HE866696	ZLVG345
Ceratocystis polonica	Pugled	*I. typographus*	Mononematous	*Thielaviopsis*	CBS134045	HE866697	ZLVG349
Graphium fimbriisporum	Pugled	*I. typographus*	Discrete, synnematous	*Graphium*	CBS134054	HE866699	ZLVG353
Leptographium sp.	Mokerc	*I. typographus*	Discrete, mononematous	*Leptographium*	CBS134053	HE866700	ZLVG351
O. fuscum	Boršt	*P. chalcographus*	Mononematous and synnematous	*Hyalorhinocladiella* and *Pesotum*	CBS134049	HE866698	ZLVG352
Pesotum sp.	Boršt	*I. typographus*	Synnematous and mononematous	*Pesotum* and *Sporothrix*	CBS134048	HE866701	ZLVG350
Ceratocystiopsis cf. *minuta*	Košenjak	*I. amitinus*	Mononematous	*Hyalorhinocladiella*	CBS134047		ZLVG355

[a] Conidiophores for the respective species found in this study

[b] Anamorphs for the respective species found in this study

[c] Representative strain no. CBS = Culture collection at Centraalbureau voor Schimmelcultures

[d] EMBL accession no. = nucleotide sequences, sequenced in this study and deposited in the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database

[e] ZLVG no. = Culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute

sequencing facilities using an Automatic Sequencer 3730XL (Applied Biosystems). The sequence traces were inspected visually, and indistinct nucleotides were clarified by comparing the sequences from both strands using BioEdit 7.0.0 software. Each sequence was used to perform an individual nucleotide–nucleotide search with the BLASTn algorithm in the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

The rDNA sequences obtained in this study and selected sequences from GenBank were aligned using the ClustalW program. The phylogenetic relationships were inferred by the neighbour-joining (NJ) method using MEGA5 with all of the parameters set to default. The evolutionary distances were computed using the maximum composite likelihood method. The analysis involved 35 nucleotide sequences. Bootstrapping (1,000 replicates) was performed to assess the confidence level at each branch. There were a total of 644 positions in the final data set.

2.4 Data analysis

The dissimilarity in the composition of the fungal species isolated from *I. typographus*, *I. amitinus* and *P. chalcographus*, as well as the dissimilarity of the fungal assemblages from different phytogeographic regions (for *I. typographus* and *P. chalcographus* only), were tested using a permutational multivariate analysis of variance (PerMANOVA) with the Jaccard dissimilarity index. When a significant difference was found, the bark beetle species were tested pairwise with a PerMANOVA using the Holm correction.

The mean number of ophiostomatoid species per bark beetle was calculated. The Kruskal–Wallis test was used to test whether the medium number of ophiostomatoid fungi carried by an individual beetle differed significantly by beetle species. When a significant difference was detected, a post hoc pairwise comparison among the insect species was conducted using the Games–Howell test.

For each bark beetle species, the percentage of specimens from which at least one ophiostomatoid fungus was isolated was calculated. The data were analysed using a Ryan's multiple comparison test of proportions to determine whether there were differences among the three beetle species.

The joint occurrence of pairs of fungal species on a beetle specimen was calculated using Kendall's Tau-b test. This test was used to test if some species are more likely than others to occur together. The results of the test ranged from −1 (species never occur together) to +1 (species are always found together).

Most of the tests were performed using the SPSS for Windows 17.0 program (SPSS Inc., Chicago, IL, USA). The PerMANOVA was conducted using the vegan package in the statistical program R (R Development Core Team 2011).

3 Results

3.1 Occurrence and identification of fungal associates

Fungi were isolated from 59.6 % of the examined beetles, resulting in 454 strains of various ophiostomatoid fungi and 136 strains of other fungi (most frequently isolated were *Penicillium* sp., *Aspergillus* sp., *Acremonium* sp., *Alternaria* sp. and *Fusarium* sp.) as well as yeasts, which occurred frequently but were not recorded in detail. Ophiostomatoid fungi were isolated from 40.0 % of the investigated beetles. *I. amitinus* was the species associated with the highest frequency of appearance of all the fungi species followed by *I. typographus* and *P. chalcographus* (75.5, 65.5, and 37.8 %, respectively). The beetles were placed in the same order considering the frequencies of ophiostomatoid fungi (Table 3).

The groupings of the fungal entities based on their morphology were fully consistent with groupings based on their ITS rDNA sequences. Thirteen species of ophiostomatoid fungi were identified (Table 2). The phylogenetic placement of the isolates collected during this investigation, as well as those of the reference sequences obtained from GenBank, are in agreement with the results of recent European studies (Linnakoski et al. 2010, 2012; Jankowiak and Kolařík 2010). The phylogenetic analysis distinguished between species in the order Microascales and those in the order Ophiostomatales and resulted in the clear resolution of the monophyletic lineages described by Zipfel et al. (2006) (Fig. 1).

Eleven ophiostomatoid fungi were assigned to known species. These species included *Ophiostoma ainoae* H. Solheim, *Ophiostoma bicolor* R. W. Davidson & D. E. Wells, *Ophiostoma brunneo-ciliatum* Math.-Käärik, *Grosmannia cucullata* (H. Solheim) Zipfel, Z.W. de Beer & M.J. Wingf, *Ophiostoma piceae* (Münch) Syd. & P. Syd., *Grosmannia penicillata* (Grosmann) Goid., *Grosmannia piceiperda* (Rumbold) Goid., *Ceratocystis polonica* (Siemaszko) C. Moreau and *Graphium fimbriisporum* (M. Morelet) K. Jacobs, Kirisits & M.J. Wingf. (Table 2). Sequencing of the ITS rDNA region of *Ceratocystiopsis minuta* (Siemaszko) H.P. Upadhyay & W.B. Kendr. failed in eight repetitions. Consequently, this fungus was not included in the phylogenetic analyses and thus isolates of *C. minuta* were assigned to *C.* cf. *minuta* based solely on morphology. Two taxa obtained in this study could not be unambiguously determined, most likely because they belong to hitherto undescribed species. *Sporothrix* and *Pesotum* anamorphic states, but no teleomorph structures, were observed in the isolates identified as *Pesotum* sp. The cultured organisms were dark with no aerial mycelium The BLASTn searches showed that these isolates were most closely related to a *Grosmannia* sp. Similarly, *Leptographium* sp. also formed only anamorph structures and the cultured organisms were dark with aerial mycelium. Analyses of the rDNA ITS sequences of *Leptographium* sp. indicated that this species was

Table 3 Ophiostomatoid fungi isolated from *I. typographus*, *P. chalcographus* and *I. amitinus* in this study

	I. typographus		*P. chalcographus*		*I. amitinus*		Total	
	N[a]	%[b]	*N*[a]	%[b]	*N*[a]	%[b]	*N*	%
Ophiostoma ainoae	21a	9.1	26a	13.8	0b	0	47	7.5
Ophiostoma bicolor	56a	24.1	14b	7.5	38a	18.6	108	17.3
Ophiostoma brunneo-ciliatum	0a	0.0	0a	0.00	71b	34.8	71	11.4
Grosmannia cucullata	8a	3.5	3a	1.6	8a	3.9	19	3.0
Ophiostoma piceae	7a	3.0	4a	2.1	19b	9.3	30	4.8
Grosmannia penicillata	3a	1.3	0a	0.0	38b	18.6	41	6.6
Grosmannia piceaperda	11a	4.7	10a	5.3	27b	13.2	48	7.7
Ceratocystis polonica	6b	2.6	0a	0.0	10b	4.9	16	2.6
Ceratocystiopsis minuta	0a	0.0	0a	0.0	43b	21.1	43	6.9
Graphium fimbriisporum	4b	1.7	0a	0.0	5ab	2.5	9	1.4
Leptographium sp.	3a	1.3	1a	0.5	0a	0	4	0.6
Ophiostoma fuscum	0a	0.0	5b	2.7	8b	3.9	13	2.1
Pesotum sp.	2a	0.9	3a	1.6	0a	0	5	0.8
N of beetles[c]	232		188		204			
N of isolates	121		66		267		454	
N of taxa	10		8		10		13	
Mean number of ophiostomatoid species per beetle	0.5		0.4		1.3		0.7	
Beetles carrying at least one ophiostomatoid species (%)	31.5a		25.0a		64.2b		40.0	

The data were analysed with Ryan's multiple comparison test of proportions, separately for each fungus, to determine whether the fungal frequencies differed among the three beetle species. Values followed by different letters were significantly different at $P<0.05$.

[a] Number of isolates of the respective fungal species obtained from the three beetle species

[b] Frequency in percentage of the respective fungal species carried by the three bark beetle species (= *N* fungi/*N* beetles × 100)

[c] Number of beetles per species that were investigated

most closely related to other *Grosmannia* and *Leptographium* sequences (Fig. 1).

3.2 Fungi associated with the three spruce bark beetle species

All three bark beetle species were associated with several ophiostomatoid fungi. Ten fungal species were found to be associated with *I. typographus*, eight with *P. chalcographus*, and ten with *I. amitinus*. Four species, including *O. bicolor*, *G. cucullata*, *O. piceae* and *G. piceiperda* were isolated from all three bark beetles species. In Table 3, the mean numbers of ophiostomatoid fungi per bark beetle specimen are presented for the three scolytine species. The Kruskal–Wallis test indicated a significant difference in the mean numbers of ophiostomatoid species per beetle individual for the three bark beetle species ($\chi^2=135.2$, $df=2$, $P<0.001$). The Games–Howell test detected a difference between *I. amitinus* and *P. chalcographus* as well as between the two *Ips* species ($P<0.001$ for both pairwise comparisons), whereas the difference between *P. chalcographus* and *I. typographus* was not significant.

The composition of the ophiostomatoid fungal associates differed significantly between the bark beetle species ($F=22.5$, $P<0.001$, $R^2=0.13$). The post hoc tests revealed that the fungal species composition differed between all three species pairs (*I. typographus*–*P. chalcographus*: $F=8.1884$, $P<0.01$; *I. typographus*–*I. amitinus*: $F=30.967$, $P<0.01$; *P. chalcographus*–*I. amitinus*: $F=24.9$, $P<0.01$). Notably, specimens of *I. amitinus* had a much more similar fungal composition than did *I. typographus* and *P. chalcographus* individuals.

The comparisons showed that the frequencies of some fungal species differed significantly between the insects (Table 2). Overall, the most frequently isolated species was *O. bicolor* (17.3 %), more often from *I. typographus* and *I. amitinus* than *P. chalcographus*. *O. brunneo-ciliatum* was the second most frequently isolated fungus, although it was isolated only from *I. amitinus*. This species was followed in frequency by *G. piceiperda*, *O. ainoae*, *C. minuta*, *G. penicillata*, *O. piceae*, *G. cucullata*, *C. polonica*, *Ophiostoma fuscum*, *G. fimbriisporum*, *Leptographium* sp. and *Pesotum* sp. *C. minuta* was isolated from only *I. amitinus*. *O. ainoae*, the most frequent associate of *P. chalcographus*, was not obtained from *I. amitinus*. Moreover, *G. piceiperda* and *G. penicillata* were more frequently isolated from *I. amitinus* than from the two other scolytines. *C. polonica* was obtained only from *I. typographus* and *I. amitinus*; however, its isolation frequencies were low.

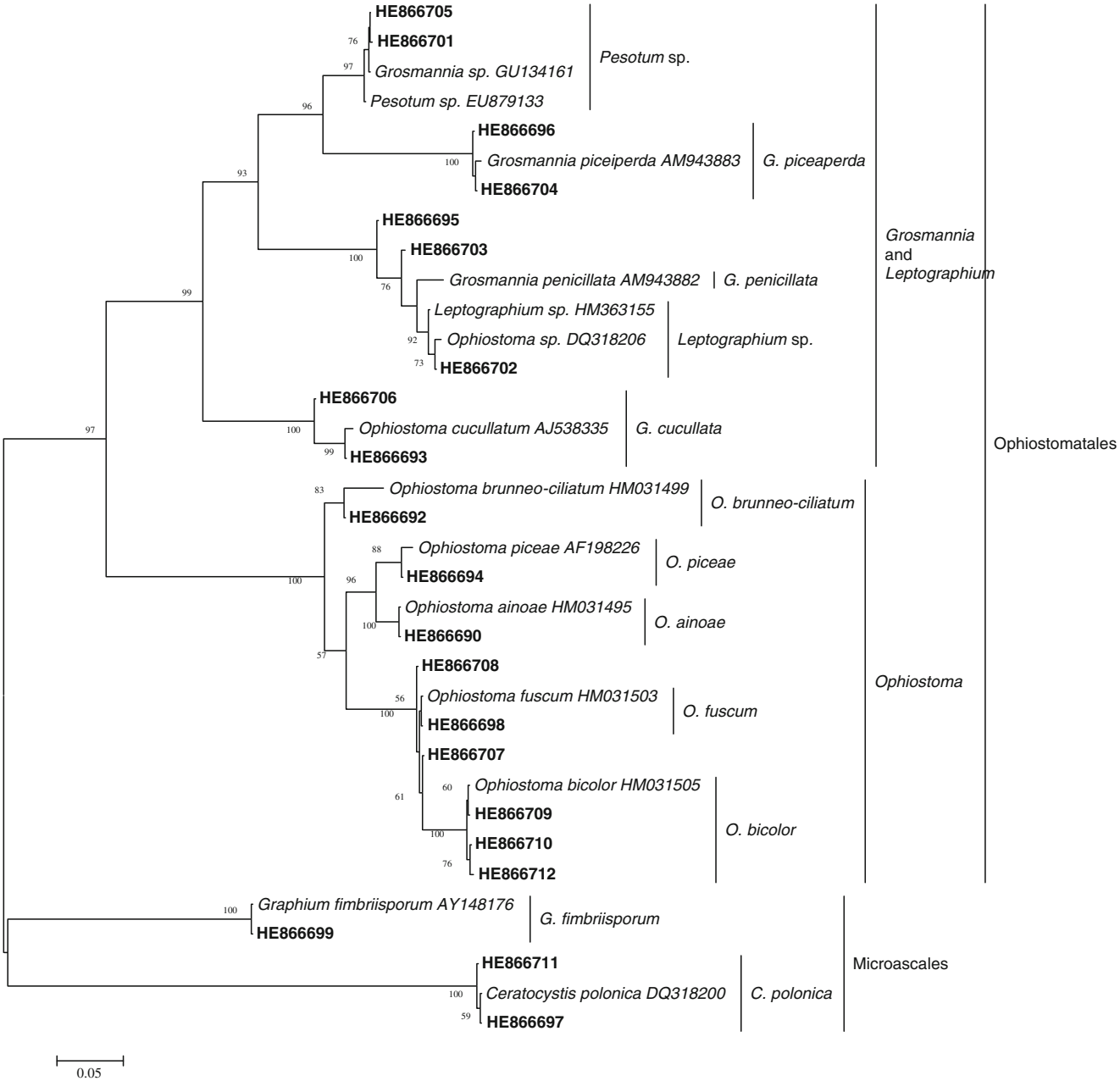

Fig. 1 Phylogram obtained using NJ based on the ITS rDNA sequences. Novel sequences obtained during this study are printed in *bold type*. The tree is unrooted. NJ bootstrap support values above 55 % are indicated at the nodes

3.3 Fungi in different phytogeographic regions

A total of 308 isolates were obtained in the Alpine region, 64 in the Dinaric region, 27 in the Subpanonic region and 55 in the Predinaric region. No difference was found in the assemblage of ophiostomatoid fungi associated with *I. typographus* or *P. chalcographus* among the phytogeographic regions (F=1.6422, P=0.06, R^2=0.04 and F=1.7307, P=0.07, R^2=0.06, respectively). In all four regions, *O. bicolor* was the most frequently isolated fungus.

In the Alpine region, all of the fungi except *Leptographium* sp. were found. Nine different fungal species were obtained from the other three regions. *C. minuta* and O. *brunneo-ciliatum* were found only in the Alpine region. This result is because *I. amitinus*, with which they were found to be exclusively associated, was present only in the Alpine region, unlike *I. typographus* and *P. chalcographus*, which were collected in all three phytogeographic regions. *G. penicillata*, *O. fuscum*, *Leptographium* sp. and *Pesotum* sp. were found at relatively low frequencies in the regions where they were

detected. *G. penicillata* was not found in the Dinaric region, whereas *Leptographium* sp. was found only there. *O. fuscum* was not found in the Subpanonic region, and *Pesotum* sp. was not found in the Predinaric region.

3.4 Associations among the fungi

A total of 105 pairs of ophiostomatoid and non-ophiostomatoid fungi were found. The correlations among the fungi were calculated to detect statistically significant associations in the occurrence of certain pairs of fungi. In most cases, there were no correlations between species pairs or the correlations were weakly positive or negative. The correlations that were statistically significant ($P<0.05$) included *O. brunneo-ciliatum* in each of the species: *C. minuta* ($\tau_b=0.530$, $P<0.001$), *O. piceae* ($\tau_b=0.207$, $P<0.001$), *G. penicillata* ($\tau_b=0.343$, $P<0.001$), *G. fimbriisporum* ($\tau_b=0.128$, $P<0.001$), *Hyalorhinocladiella* sp. ($\tau_b=0.120$, $P=0.001$) and yeasts ($\tau_b=0.161$, $P<0.001$). *C. minuta* also showed a positive association with *G. fimbriisporum* ($\tau_b=0.128$, $P<0.001$), *O. piceae* ($\tau_b=0.180$, $P<0.001$), *G. penicillata* ($\tau_b=0.132$, $P<0.001$) and yeasts ($\tau_b=0.135$, $P<0.001$). *O. piceae* showed a positive association with *G. fimbriisporum* ($\tau_b=0.102$, $P<0.01$) and *G. piceiperda* ($\tau_b=0.110$, $P<0.01$). Furthermore, *G. piceiperda* showed a positive association with *G. cucullata* ($\tau_b=0.240$, $P<0.001$). There was a slightly negative association between *O. ainoae* and yeasts ($\tau_b=-0.104$, $P=0.05$). Other pairs of fungi did not show any statistically significant negative associations.

4 Discussion

All three of the investigated bark beetles were found to be associated with a diverse assemblage of ophiostomatoid fungi. The present study is the first in which the ophiostomatoid fungi associated with these three spruce bark beetle species in Slovenia were extensively studied. Most of the ophiostomatoid species identified in this study are reported here for the first time in Slovenia and all are reported for the first time in connection with these three bark beetles.

Similar investigations of bark–beetle-associated fungi on *P. abies* have been conducted throughout Europe, particularly of the fungal associates of *I. typographus*. The present study was the southernmost investigation of fungi associated with *I. typographus*, *I. amitinus* and *P. chalcographus*. The present results concerning the ophiostomatoid mycobiota of bark beetles on *P. abies* are comparable to those conducted in various other parts of Europe and show that these three insects are associated with similar assemblages of fungi across the continent, although differences between bark beetle species and between certain studies occurred (e.g. Solheim 1986; Krokene and Solheim 1996; Kirschner 2001; Viiri and Lieutier 2004; Kirisits 2004, 2010; Sallé et al. 2005; Jankowiak 2005; Jankowiak et al. 2009; Linnakoski et al. 2010).

The composition of the fungal associates in this study depended on the bark beetle species. Among the three insects, individuals of *I. amitinus* were associated with almost the same fungal species, whereas there was a large variation in the fungal species composition among individual specimens of *I. typographus* and *P. chalcographus*. However, comparisons of the fungal composition in this study have methodological limitations: all three bark beetles were not collected every year, and they were not collected at all of the localities because some species did not occur at some of the sites. Particularly, *I. amitinus* was collected from a much smaller area compared to the other two species because it has a smaller range and occurs in only a few regions of Slovenia, specifically at high elevations in the Alps. This range limitation may explain the high similarity in the fungal composition among *I. amitinus* individuals. It is likely that the composition of the mycobiota of this scolytine species would have been more variable if collections had been conducted in a wider geographic area. We are confident, however, that the most frequent fungal associates of *I. amitinus* in Slovenia were recorded in this study. The number of detected ophiostomatoid species associated with *I. amitinus* (10) was identical to that of *I. typographus* and higher than that of *P. chalcographus*.

DNA sequences of the ITS, β-tubulin and the large subunit of ribosomal RNA (LSU) are commonly used to resolve phylogenetic relationships among ophiostomatoid fungi within the Ophiostomatales (Zipfel et al. 2006; Linnakoski et al. 2010). In this study, the phylogram based on the rDNA ITS gene region supported previous findings regarding the delineation of different phylogenetic groups, namely *Ophiostoma* senso stricto and *Grosmannia* (Jankowiak and Kolařík 2010; Linnakoski et al. 2010). The molecular analyses generally confirmed the initial designation of the fungal species based on their morphological characteristics. Although the rDNA ITS is the most extensively used region for DNA barcoding purposes and phylogenetic analyses (Jankowiak and Kolařík 2010; Linnakoski et al. 2010), complexes of closely related taxa within the ophiostomatoid fungi can be resolved only by analysing additional gene regions and constructing multi-gene phylogenies (Zipfel et al. 2006; Linnakoski et al. 2010). Two taxa from Slovenia, *Leptographium* sp. and *Pesotum* sp., most likely represent species that have not yet been described. Therefore, their β-tubulin and LSU sequences should be analysed for further characterisation of these ophiostomatoid species.

The most frequently and consistently occurring fungus isolated from the spruce bark beetles in this study was *O. bicolor*, which is in concordance with previous investigations conducted in various parts of Europe (Solheim 1986; Kirisits 2004; Viiri and Lieutier 2004; Jankowiak and Hilszczański

2005; Sallé et al. 2005). *O. brunneo-ciliatum* and *C. minuta*, the species most commonly associated with *I. amitinus*, were not isolated from the other two insects during our investigation. *O. ainoae* was not found in connection with *I. amitinus*, which is consistent with the results of other similar investigations (Kirisits 2004; Jankowiak et al. 2009). It was obtained at higher frequencies in association with *P. chalcographus* in this study than in investigations in Poland (Jankowiak et al. 2009) and Austria (Kirisits 2004). Linnakoski et al. (2010) reported *O. brunneo-ciliatum* to be associated with both *I. typographus* and *P. chalcographus*. However Jankowiak et al. (2009) and Kirisits (2001) speculated that on *P. abies*, *O. brunneo-ciliatum* may be specifically associated only with *I. amitinus*. The second main host species of *I. amitinus* is *Pinus cembra*. *O. brunneo-ciliatum* was the species most frequently isolated from this host, both directly from *I. amitinus* beetles and from infected sapwood (Kirisits 2004; Kirisits unpublished data). It appears that *O. ainoae* is closely related to vectors colonising *P. abies* trees, whereas *O. brunneo-ciliatum* has most often been found as a fungal associate of various bark beetle species on pine trees (Kirisits 2004; Linnakoski et al. 2010), with *I. amitinus* on *P. abies* (Kirisits 2004; Jankowiak et al. 2009) and with *Ips cembrae* on larch trees (Kirisits 2004). Thus, it appears that *O. brunneo-ciliatum* (replacing *O. ainoae* on *I. typographus* and *P. chalcographus*) reflects the association of *I. amitinus* with *P. cembra* as second major host.

In contrast to our findings, *C. minuta* has been documented as commonly associated with *I. typographus* and *P. chalcographus* in other studies (Viiri and Lieutier 2004; Jankowiak and Hilszczański 2005; Jankowiak et al. 2009; Kirisits 2004).

The pathogenic species *C. polonica* was only occasionally isolated from *I. typographus* and *I. amitinus* in the present investigations. The infrequent association of this fungus with these two bark beetle species is in agreement with the results of studies conducted in Austria (Kirisits 2004) and Poland (Jankowiak et al. 2009). Kirisits (2004) suggested that *C. polonica* was associated mainly with those two *Ips* species as well as *Ips duplicatus* (Krokene and Solheim 1996), but only sporadically or not at all with other scolytines. In numerous studies in different European countries, the abundance of *C. polonica* in association with *I. typographus* varied from rare to frequent, or the fungus was not recorded at all (Kirisits 2004). The occasional and casual association between the pathogenic *C. polonica* and the aggressive bark beetles in our investigation is in agreement with the results of studies by Sallé et al. (2005) but is not consistent with findings from Norway (Solheim 1986; Krokene and Solheim 1996), Poland (Jankowiak 2005; Kirisits 2010) and some areas in Austria (Kirisits 2004), where *C. polonica* was consistently associated with *I. typographus*. In the present research, the rare occurrence of the *C. polonica* may be influenced by another methodological aspect: that is half of our plates were treated with cycloheximide to which *C. polonica* is sensitive.

The rare occurrence of *C. polonica* may support the view of Six and Wingfield (2011), who recently challenged the classic paradigm that phytopathogenic fungal associates of tree-killing bark beetles are responsible for overwhelming the tree defence mechanisms, are primary causes of tree mortality and are a prerequisite for aggressive bark beetle species to colonise live trees. Host tree defence is another reason for lack of *C. polonica* in the collections, although this finding does not necessarily confirm the paradigm. The rare occurrence of *C. polonica* may be partly explained by the fact that it is difficult to isolate this species directly from beetles, particularly because there is no selective medium available for *Ceratocystis* spp. (Kirisits 2010). The same applies to *C. minuta*, which is also sensitive to cycloheximide and grows slowly. Both are difficult to isolate directly from beetles and are better isolated from galleries. The different methods used to isolate the fungi from different niches (Viiri and Lieutier 2004; Persson et al. 2009) may also explain why these species were obtained only sporadically. The fungal associates of bark beetles in this study were identified only on the basis of being isolated from beetles, not also from galleries. The number of examined beetles was not large (Table 1), which could also affect the results.

G. piceiperda and *G. penicillata* were not found commonly associated with *I. typographus* and *P. chalcographus* in Slovenia, which is in contrast to the results of other studies (Kirisits 2004; Sallé et al. 2005). However, these species were consistently associated with *I. amitinus*, similar to the findings of investigations conducted in Poland (Jankowiak et al. 2009), where *G. piceiperda* was frequently encountered as a fungal associate of *I. amitinus*, and in Austria (Kirisits 2004).

O. piceae was found associated with *I. amitinus* at higher frequencies than in the study from Poland (Jankowiak et al. 2009) and it occurred less frequently with the other two insect species examined in the present study. The other ophiostomatoid fungi (*G. cucullata*, *G. fimbriisporum*, *O. fuscum*, *Leptographium* sp. and *Pesotum* sp.) were isolated at low frequencies in our investigation. *O. fuscum* was recently described by Linnakoski et al. (2010), who found it at low frequencies in association with *I. typographus* and *P. chalcographus*. In the present study, this fungus was rarely isolated from *P. chalcographus* and *I. amitinus*, which extends the known distribution range of this species to south-central Europe. Moreover, for the first time, *O. fuscum* is reported as a fungal associate of *I. amitinus*. *G. fimbriisporum* was not isolated from *P. chalcographus* in Slovenia, although it was commonly found as associate of this insect in some European studies (Kirisits 2004).

In studies performed in different European countries (Viiri and Lieutier 2004; Jankowiak 2005; Jankowiak and Kolařík 2010), differences in the fungal communities among sites were detected. The assemblage of fungi is most likely influenced by many factors. Although the bark beetles in

this study were collected at six sites in four phytogeographic regions and the beetles' population phases (Table 1) differed among the sites, there were no differences among the sites with regard to *I. typographus* and *P. chalcographus*. This result could be attributed to the fact that the host plant species was the same (*P. abies*) and, that regardless of the beetles' population phase or the region, the samples were collected in secondary (planted or even damaged) *P. abies* stands, which renders the environment for all of the collection sites similar. The second possibility is that the fungal assemblage did not deviate between localities because the samples of beetles were limited, as noted earlier.

The interactions between fungi and bark beetles differ with time, location and resources, a phenomenon referred to as the 'context dependency' of the association (Klepzig et al. 2001). These associations may also depend on the relationship between the fungal associates and other organisms, such as yeast and bacteria. Nevertheless, most of the fungal pairs found in the present study appeared to occur in association only occasionally. There were pairs of fungi that occurred slightly more frequently together than independently from each other. This was particularly true for fungal associates of *I. amitinus*. For example, *O. brunneo-ciliatum* was often isolated from the beetles together with other fungi, and the same is true for *C. minuta* and *O. piceae*. The occurrence of *G. piceiperda* and *G. penicillata* on individual beetles did correlate significantly in France (Viiri and Lieutier 2004), but this was not the case in present study.

In conclusion, all three bark beetle species were frequently associated with ophiostomatoid fungi. Although our research was limited to the three scolytine species that are most harmful to the economically most important forest tree species in Slovenia, *P. abies*, the variety and number of fungal species encountered in this study suggest that the diversity of ophiostomatoid fungi in this part of Europe is high. Because ophiostomatoid fungi are potential agents of sapstain and vascular wilt and stain diseases, which decrease the quality and value of timber, further investigations of the fungal associates of other bark beetles in Slovenia as well as investigations of the phytopathogenicity of ophiostomatoid fungi are recommended.

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