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ORIGINAL RESEARCH PAPER

Geosmithia species associated with fir-infesting beetles in Poland

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Abstract
Geosmithia species (Ascomycota: Hypocreales) are common ectosymbionts of scolytine bark and ambrosia beetles that feed on coniferous and deciduous trees in different forest ecosystems. *Geosmithia morbida* is the canker pathogen that causes extensive mortality of *Juglans nigra*. Because little is known regarding the *Geosmithia* species on European silver fir (*Abies alba*), we have investigated the diversity and abundance of these fungi associated with insects infesting European silver fir in Poland. Samples associated with eight beetle species were collected from three fir forests. Fungi were isolated from beetles and galleries. Isolates were identified based on morphological characteristics, DNA sequence comparison for three gene regions (ITS, ßT, TEF1-α), and phylogenetic analyses. *Geosmithia* was detected in 33% of the total 531 beetle samples obtained from *A. alba*. Two undescribed species of *Geosmithia* were distinguished, *Geosmithia* sp. 9 and *Geosmithia* sp. 16. Associations of *Pityokteines* spp. with *Geosmithia* fungi were recorded for the first time. *Pityokteines vorontzowi* and *Pityophthorus pityographus* appear to be regular vectors for *Geosmithia* sp. 9 and *Geosmithia* sp. 16, respectively. *Pityokteines curvidens* and *Cryphalus piceae* were associated with *Geosmitha* sp. 9 at lower frequencies.

Keywords
Abies alba; ambrosia beetles; bark beetles; *Geosmithia*, weevil

Introduction

Bark- and wood-boring beetles (Coleoptera) are commonly associated with various species of ascomycetes in three orders: Ophiostomatales, Microascales, and Hypocreales [1,2]. Members of the Hypocreales are common in all types of moist forests [3], however, only *Geosmithia* fungi that produce *Penicillium*-like conidiophores are associates of bark beetles on hardwood and conifer trees worldwide [4–16]. The bark beetle–*Geosmithia* relationships vary, ranging from specialists with a limited range of vectors to generalists recorded with numerous vectors [4,6,9,11]. On Pinaceae, the typical vectors of *Geosmithia* are bark beetles infesting *Pinus sylvestris* L. The pine-associated *Geosmithia* are a group of specialists living only on the Pinaceae [9,12,17]. Among *Geosmithia* species, only *G. morbida* M. Kolařík, Freeland, C. Utley & Tisserat has been considered a highly aggressive pathogen of walnuts in the USA [7] and Europe [18]. Other *Geosmithia* species appear to be nonpathogenic to conifers [13,19].

Knowledge of conifer bark beetle-associated fungi in Poland remains incomplete. The Ophiostomatales associated with many conifer-feeding beetles have been well characterized in Poland (e.g., [20–28]). Relationships between *Geosmithia* fungi and pine- and spruce-infesting bark beetles are also relatively well known in Poland [9,12,17,19]. The results of these studies show that *Geosmithia* fungi develop stable symbiotic relationships with different bark beetle species, and resemble ophiostomatoid fungi in their host and vector affinities and life strategy evolution. Little is currently known about

fir-infesting beetles and their associated *Geosmithia*. Hitherto, only *Cryphalus piceae* (Ratz.) has been screened for *Geosmithia* spp. [19].

In this survey, eight beetle species and their galleries were collected from three different sites in the silver fir stands of Poland. The main objective of this study was to provide insight into the diversity of *Geosmithia* species associated with fir-infesting beetles in Poland.

Material and methods

Study area and sampling of bark beetles and galleries

The beetles and galleries were collected during the study of Jankowiak et al. [28]. The bark beetles and weevils were collected at three sites in Poland. These research sites were composed mainly of European silver fir (Tab. 1).

Tab. 1 Features of the study areas, list of samples, and characteristics of the beetle species.

Location	Mucharz	Rozpucie	Nawojowa
Phytogeographic region	Beskid Makowski Mountains	Sanocko-Turczańskie Mountains	Beskid Wyspowy Mountains
Geographic coordinates	49°48′7.98″ N	49°34′59.92″ N	49°35′19.38″ N
	19°29′19.50″ E	22°25′18.28″ E	20°52′28.49″ E
Altidude m a.s.l.	425	427	500
Forest stand composition (%)	*A. alba* 100	*A. alba* 90	*A. alba* 100
		F. sylvatica 10	
Tree age	110	122	90
Year sampling	2009	2014	2013
No. of examained samples	100	599	447
Beetle species	*Pityokteines vorontzowi*	**	***

** *Cryphalus piceae*, *Orthotomicus laricis*, *Pissodes piceae*, *Pityokteines curvidens*, *Pityophthorus pityographus*, *Xyleborinus saxesenii*, *Trypodendron lineatum*; *** *Cryphalus piceae*, *Pissodes piceae*, *Pityokteines curvidens*, *Trypodendron lineatum*.

Fungi were isolated from beetles or galleries. The adult beetles of *C. piceae*, *Orthotomicus laricis* (Fabr.), *Pissodes piceae* (IIIig.), *Pityokteines curvidens* (Ger.), *Pityophthorus pityographus* (Ratz.), *Trypodendron lineatum* (Oliv.), and *Xyleborinus saxesenii* (Ratz.) were collected from May to July in the years 2013 and 2014. The beetles of *Pityokteines vorontzowi* (Jacob.) were collected from May to June in 2009.

Newly emerged beetles were collected according to the procedure described by Jankowiak et al. [28]. The adults feeding on fir were excised from their galleries with sterilized forceps about 2–4 weeks after the main flight period and 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, and each collected beetle was placed individually into a sterile Eppendorf tube.

The beetle galleries, except *P. vorontzowi*, were collected from windblown trees naturally infested by insects, and from trapping logs (2 m long, 0.2 m in diameter) or branches lying stacked in stands from May–July in 2013 and 2014. The galleries of *P. vorontzowi* were collected from May till June 2009. Complete galleries, including sapwood up to 2 cm away from the tunnel, were removed from the wood and placed in separate paper bags.

Due to differences in the number of collected beetles and their galleries, the galleries and the beetles were treated as similar substrates (e.g., one beetle of *P. curvidens* + one gallery of *P. curvidens* = two beetle samples of *P. curvidens*). A total of 531 beetle samples were collected.

Fungal isolations and morphological grouping

For fungal isolations, each beetle was removed from its storage microtube with sterilised tweezers, morphologically identified with taxonomical keys [29,30], and squashed onto the surface of malt extract agar (MEA; 20 g Biocorp malt extract, 20 g Biocorp agar, and 0.2 g tetracycline, per liter of distilled water) in Petri dishes. Fragments of discolored sapwood underneath each gallery up to a depth of 10 mm into the sapwood (two to six fragments per gallery; 4 × 4 mm size) were collected without disinfection, and plated on MEA medium in Petri dishes. This was performed according to the isolation procedures described by Jankowiak et al. [28].

The plates were than incubated at 25°C for 2–6 weeks and observed daily for fungal growth. When necessary, 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 by using a Nikon Eclipse 50i microscope (Nikon Corporation, Tokyo, Japan) and an Invenio 5S digital camera (DeltaPix, Maalov, Denmark) with Coolview 1.6.0 software (Precoptic, Warsaw, Poland). Representative isolates of fungi were deposited in the culture collection of the Department of Forest Pathology, Mycology and Tree Physiology, Hugo Kołłątaj University of Agriculture, Krakow, Poland (Tab. 2).

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

Geosmithia species*	Isolate No.**	Substrate/origin	GenBank accesion No.		
			ITS1-5.8S-ITS2	β-tubulin	TEF 1- α
Geosmithia sp. 9	14KaFJD	*Cryphalus piceae* / beetle	KY568172	KY568478	KY568678
	14KcFJD	*Cryphalus piceae* / beetle	KY568173	KY568479	KY568679
	59KaFJD	*Pityokteines curvidens* / gallery	KY568174	KY568480	KY568680
	32KFJD	*Cryphalus piceae* / gallery	KY568175	KY568481	KY568681
	59KbFJD	*Pityokteines curvidens* / gallery	KY568176	KY568482	
	63KcFJD	*Pityokteines curvidens* / gallery	KY568177	KY568483	KY568682
	63KdFJD	*Pityokteines curvidens* / gallery	KY568178	KY568484	KY568683
	11KFJD	*Cryphalus piceae* / beetle	KY568179	KY568485	KY568684
	12KFJD	*Cryphalus piceae* / beetle	KY568180	KY568486	KY568685
	5KaFJD	*Pityophthorus pityographus* / beetle	KY568181	KY568487	KY568686
Geosmithia sp. 16	5KbFJD	*Pityophthorus pityographus* / beetle	KY568182	KY568488	KY568687
	4KFJD	*Pityophthorus pityographus* / beetle	KY568183	KY568489	KY568688
	2KFJD	*Pityophthorus pityographus* / beetle	KY568184	KY568490	KY568689
	14KbFJD	*Cryphalus piceae* / beetle	KY568185	KY568491	
	1KFJD	*Pityophthorus pityographus* / beetle	KY568186	KY568492	KY568690
	21KFJD	*Pityophthorus pityographus* / gallery	KY568187	KY568493	KY568691

* According to [9]. ** All isolates originated from *Abies alba* at Rozpucie site.

DNA extraction, amplification, and phylogenetic analyses

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 determined for the internal transcribed spacer regions (ITS1 and ITS2) including the

5.8S gene (ITS), β-tubulin (βT), and the elongation factor 1-α (TEF1-α) using the following primers: ITS: ITS1-F [31] and ITS4 [32], βT: T10 [33] and Bt2b [34], TEF1-α: F-728F and F-986R [35].

Gene fragments were amplified according to the procedure described by Jankowiak et al. [28]. The sequences (Tab. 2) were deposited in NCBI GenBank and compared with those in the GenBank using the BLASTn algorithm.

All sequences were aligned online with the MAFFT v6 [36], using the E-INS-i option with a gap-opening penalty of 1.53 and an offset value of 0.00. Phylogenetic analyses were performed using the maximum likelihood (ML) and Bayesian inference (BI) methods according to the procedures of Jankowiak and Bilański [27] and Jankowiak et al. [37]. For the ML and BI analyses, the best fitting substitution models for each data set were estimated using the corrected Akaike's information criterion (AICc) in jModelTest 0.1.1 [38]. The best evolutionary substitution models for ITS and βT datasets were respectively GTR+I+G and HKY+I+G.

ML searches were conducted in PhyML 3.0 [39] 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 carried out with MrBayes v3.1.2 [40]. 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 in Tracer v1.4.1 [41]. All sequences generated in this study were deposited in NCBI GenBank (Tab. 2) and are presented in the phylogenetic trees (Fig. 1 and Fig. 2). *Acremonium alternatum* Link was used as an outgroup.

Results

Collections of bark beetles and fungal isolations

In the collected material eight subcortical beetle species were found (Tab. 1 and Tab. 3). A total of 218 *Geosmithia* isolates were obtained from 531 bark beetle individuals and their galleries. Altogether, 16 were selected for molecular identification (Tab. 2). The presence of *Geosmithia* in galleries of *P. curvidens* is presented in Fig. 3.

Fungal identification and DNA sequence comparisons

Based on our preliminary morphological investigation, the obtained isolates were separated into two *Geosmithia* groups. Both groups presented penicillium-like asexual stages. Genotype analyses confirmed that groups corresponding to the genera *Geosmithia* were in the Hypocreales (Fig. 1).

The partial βT gene was used to identify isolates at the species level (Fig. 2). Due to the lack of reference sequences for the TEF1-α gene for *Geosmithia* in GenBank, the phylogenetic analysis for this gene was not been performed in this study.

Analyses of the ITS sequences revealed that our isolates belong to the *Geosmithia* genus. ITS sequence analysis showed also that two morphological groups of *Geosmithia* could be divided into two clades: the first clade containing *Geosmithia* sp. 16, 25, and 29–31, and the second clade represented *Geosmithia* sp. 9 (Fig. 1). The ßT tree for isolates from the first clade supported the identification of *Geosmithia* sp. 16, while the remaining isolates grouped with the reference sequences of *Geosmithia* sp. 9 (Fig. 2).

Prevalence of different fungal species

The frequencies of occurrence of the *Geosmithia* species varied between beetle species (Tab. 3). Several bark beetle species were commonly found in association with *Geosmithia* fungi, which were isolated from ≥70% of the sampled beetles and galleries. The following beetle species appeared to have rather strict associations with *Geosmithia* fungi: *P. pityographus* and *P. vorontzowi*. *Geosmithia* fungi were also present in 24–37% of the

Fig. 1 Phylogram obtained from analyses of the ITS data for the *Geosmithia* spp. Sequences obtained during this study are presented in bold type. Presented phylograms were obtained from maximum likelihood (ML) analyses. The bootstrap values (>75%) for ML and posterior probabilities (>75%) that were obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. * Bootstrap values <75%.

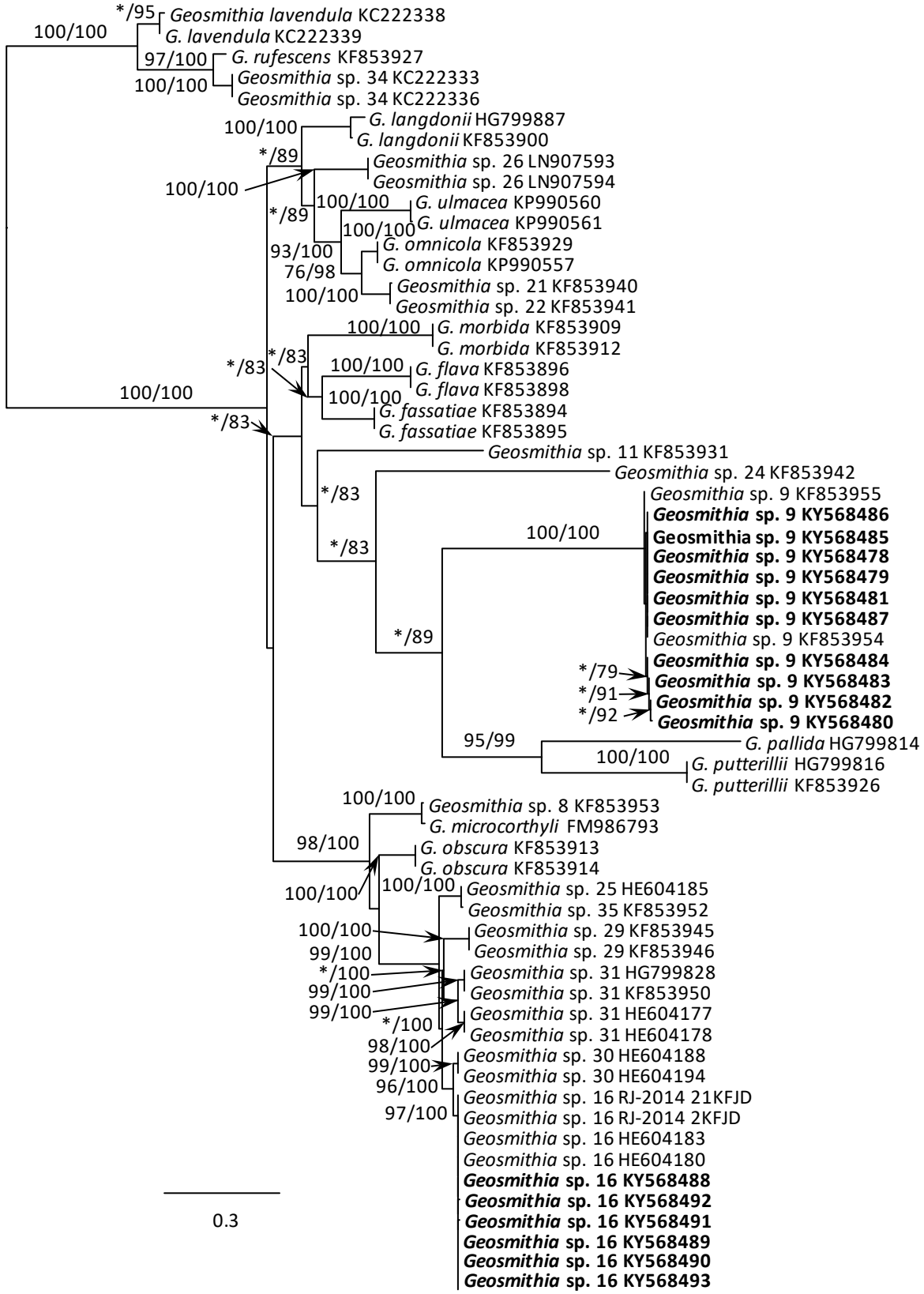

Fig. 2 Phylogram obtained from analyses of β-tubulin sequence data for the *Geosmithia* spp. Sequences obtained during this study are presented in bold type. Presented phylograms were obtained from maximum likelihood (ML) analyses. The bootstrap values (>75%) for ML and posterior probabilities (>75%) that were obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. * Bootstrap values <75%.

Tab. 3 Frequencies* (%) of *Geosmithia* species obtained from eight beetle species collected from *Abies alba* in Poland.

Geosmithia species**	*Pityokteines curvidens*[A]	*Pityokteines vorontzowi*[A]	*Trypodendron lineatum*[A]	*Cryphalus piceae*[A]	*Pityophthorus pityographus*[A]	*Pissodes piceae*[A]	*Orthotomicus laricis*[B]	*Xyleborinus saxesenii*[B]
Geosmithia sp. 9	23.5	70		36.7	2.2			
Geosmithia sp. 16				0.6	77.8			
Total No. of isolates	385	66		253	40			
Species richness (S)	1	1		2	2			
Total No. of samples with *Geosmithia* species (%)	23.5 (38)	70 (35)		37.2 (67)	80 (36)			
Total No. samples	162	50	70	180	45	12	8	4

[A] Samples included beetles and galleries. [B] Samples included only beetles. * The frequency of occurrence was calculated according to the following formula: $F = (NS/NTs) \times 100$; 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. ** According to [9].

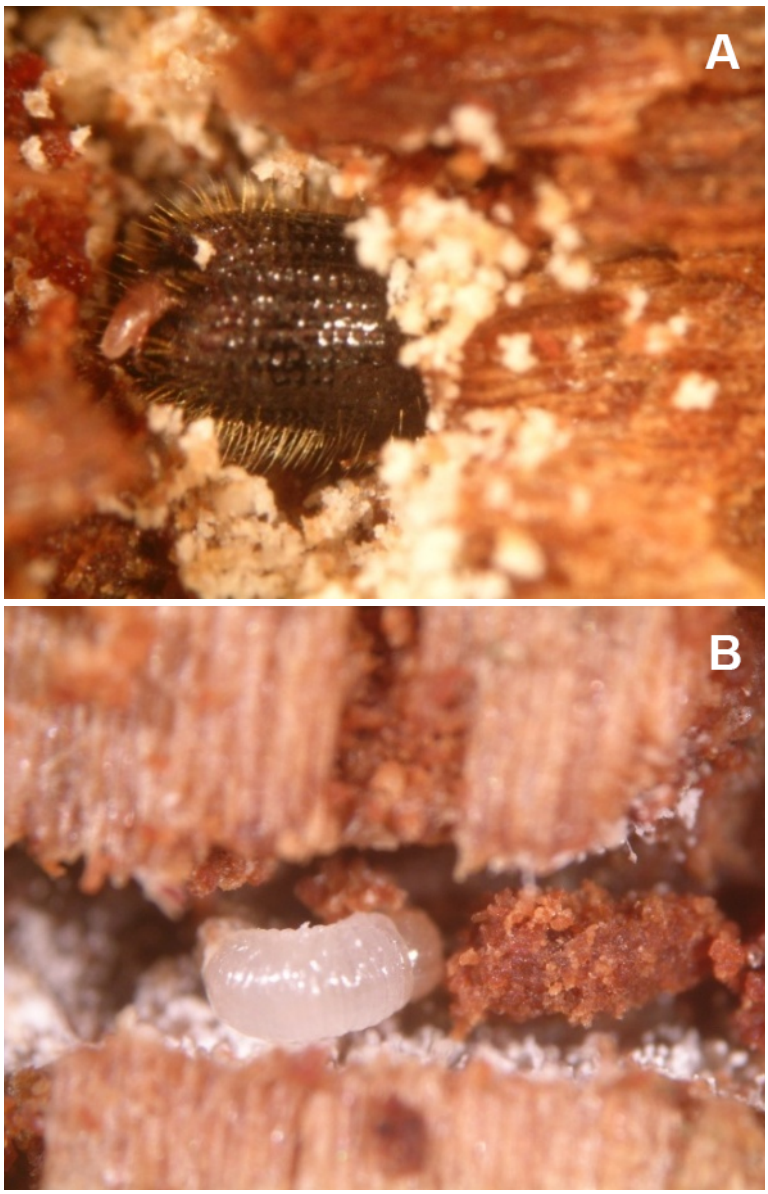

Fig. 3 Example of the association between *Pityokteines curvidens* and *Geosmithia* sp. 9. (**A**) Adult (with attached mite, white arrow) walking in a gallery containing conidiophores of *Geosmithia* sp. 9. (**B**) Larva in a gallery colonized by *Geosmithia* sp. 9.

sampled beetles and galleries: *P. curvidens* and *C. piceae*. Beetle species, where *Geosmithia* fungi were not isolated from beetles and galleries, included *O. laricis*, *P. piceae*, *T. lineatum*, and *X. saxesenii* (Tab. 3).

The *Geosmithia* species that was found in association with the broadest vector spectrum (four beetle species) was *Geosmithia* sp. 9. This fungus was the most frequently encountered fungus on *P. vorontzowi*. *Geosmithia* sp. 9 was also commonly found on *C. piceae* and *P. curvidens* (Tab. 3). Less widespread species included *Geosmithia* sp. 16 isolated from two vectors. This fungus was commonly found on *P. pityographus* and sporadically on *C. piceae* (Tab. 3).

Discussion

Associations of *Geosmithia* spp. with various tree hosts and vectors are relatively well known in Europe. In general, *Geosmithia* is more frequent on hardwoods than on conifers, although *Geosmithia* is commonly found in association with pine-infesting bark beetles in Central Europe [2,4,5,6,9,12,15,17,18]. Our survey demonstrated that *Geosmithia* fungi are also regular associates of phloem-breeding bark beetles on European silver fir. This association was previously documented for *C. piceae* in Poland [19].

The European silver fir weevil (*P. piceae*) and the striped ambrosia beetle (*T. lineatum*) were not associated with *Geosmithia* in our study. Similar results have recently been reported from the Western USA [11]. According to a study conducted by Kolařík et al. [11], *Geosmithia* spp. are carried by insects that enter the phloem and xylem as adults, but not by woodborers (e.g., weevils)

where adults do not enter these tissues. Although, there are many studies confirming associations between ambrosia beetles and *Geosmithia* fungi [8,10,11,14,16], our study showed that *T. lineatum* does not transmit *Geosmithia*. There was also no evidence that this ambrosia beetle can transmit *Geosmithia* fungi in pine habitats [9].

The genus *Geosmithia* currently includes 44 recognized taxa with only 16 described to date, most of which are associated with phloem-breeding bark beetles [4,5,7–16]. In the present study, we identified two distinct *Geosmithia* operational taxonomic units (OTUs) based on morphological and molecular characterizations. They were closely related to previously revealed species, and included *Geosmithia* sp. 9 and *Geosmithia* sp. 16 [9,12]. Although *Geosmithia* sp. 9 is known from *P. pityographus* on *Picea abies* (L.) H. Karst. and *Pityogenes chalcographus* (L.) on *P. sylvestris* and *P. abies* [9,12], there was evidence that this fungus is mainly associated with *C. piceae* on *A. alba* [19]. Our results, therefore, support those of Jankowiak and Kolařík [19] who often found *Geosmithia* sp. 9 from *C. piceae*. In addition, *Geosmithia* sp. 9 was especially associated with *Pityokteines* species, especially *P. vorontzowi*. This suggests that *Geosmithia* sp. 9 is a specialist that is mainly limited to *A. alba* and fir-infesting bark beetles. Other examples of *Geosmithia* specialists include *G. ulmacea* (*Ulmus*) or *G. morbida* (*Juglans*) [7,15,18]. *Geosmithia* sp. 16 is mainly known from *Pityogenes bidentatus* (Herbst) and *P. pityographus* on *P. sylvestris* [9,12,17]. *Geosmithia* sp. 16 was also found to be associated with *P. pityographus* in the present study. Our findings suggest that this fungus has a high affinity for *P. pityographus*. However, the host range of *Geosmithia* sp. 16 appears to be wider than *Geosmithia* sp. 9, taking into account that *P. pityographus* as a polyphagous species colonizes many conifers.

According to Jankowiak et al. [12], *Geosmithia* spp. seem to be associated mainly with insects breeding under thin bark, such as on branches. In contrast, beetles that feed under thick bark (e.g., the trunk) more frequently transmit ophiostomatoid fungi. Our results support this hypothesis because *P. vorontzowi* and *P. pityographus* had the strongest relationship with *Geosmithia*, while *C. piceae* and *P. curvidens* have shown less frequent association with *Geosmithia*. *Pityokteines vorontzowi* and *P. pityographus* colonize thin branches as compared to *C. piceae* and *P. curvidens*, which attack trunks or thicker branches [42].

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