Geosmithia species in southeastern USA and their affinity to beetle vectors and tree hosts

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ABSTRACT

The fungal genus *Geosmithia* is best known due to one species, *G. morbida,* which is vectored by the walnut twig beetle (WTB) and contributes to Thousand Cankers Disease (TCD) on walnut and wingnut trees. However, the genus is globally very diverse and abundant, and dominates a ubiquitous but understudied niche — the twig-infesting, phloem-feeding bark beetle mycobiome. The *Geosmithia* community in North America is only now beginning to be described. Very limited information is available for the South East, despite the region's potential to be a *Geosmithia* diversity hotspot. To survey the *Geosmithia* community in the subtropical USA, to assess their beetle and tree associations, and to test for the presence of *G. morbida*, we systematically deployed branch sections of nine tree species, including three Juglandaceae, in North Florida. We recovered 55 *Geosmithia* isolates from 195 beetle specimens from 45 exposed branch units. Neither *G. morbida* nor its beetle vector were detected. We identified 14 *Geosmithia* species; those in the *G. pallida* species complex were the most prevalent. Four undescribed phylogenetic species were recovered, indicating that the *Geosmithia* diversity in North America remains under-documented. Analysis of the association of *Geosmithia* with beetles and trees suggested that most species are generalists, five display preference for certain tree species, and none is specific to any beetle species.

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1. Introduction

Members of *Geosmithia* (Ascomycota: Bionectriaceae) are globally distributed, ubiquitous fungi that are commonly associated with bark and ambrosia beetles (Coleoptera: Scolytinae), especially with the phloem-feeding species (Kolařík et al., 2007, 2017; Lin et al., 2016; Pitt, 1979). Other wood-boring insects such as the Bostrichidae and Curculionidae may also vector *Geosmithia* species (Juzwik et al., 2015; Kolařík et al., 2017). *Geosmithia* species are predominantly isolated from beetles from woody materials, although they have been documented from a few other substrates including soil (Kolařík et al., 2004), seed-feeding beetles (Huang et al., unpublished), animal skin (Crous et al., 2018), indoor environment (Crous et al., 2018), insect-free plant tissues (McPherson et al., 2013), and food materials (Pitt and Hocking, 2012). Spores of *Geosmithia* are presumably transmitted by adhering to the exterior surfaces of their beetle vectors and are not known to be carried in specialized fungal transport organs (mycangia) as is known for many mutualistic fungal associates of bark and ambrosia beetles. Despite their associations with bark beetles, the ecological roles of most *Geosmithia* species in the symbiosis remain obscure. Some species serve as a food source or supplementary nutrition for the beetles (Kolařík and Kirkendall, 2010; Machingambi et al., 2014), but most are probably commensals with minimal or no benefit to the beetle. Some *Geosmithia* species exhibit extracellular antimicrobial metabolites but without a known ecological implication (Stodůlková et al., 2009). *Geosmithia* species are found almost exclusively on branch- and twig-dwelling bark beetles but rarely on trunk-infesting bark beetles (Kolařík and Jankowiak, 2013; Jankowiak et al., 2014). Given the fact that trunk-infesting beetles behave as pests more commonly than twig boring beetles, their fungal flora, i.e. the ophiostomatoid fungi, has received much more research attention. Branch- and twig-infesting bark beetles are equally common and diverse, but the intriguing mycobiota associated with them remains understudied.

One *Geosmithia* species is known to contribute to a significant tree disease: the canker-causing *G. morbida* (Kolařík et al., 2011). Following high density colonization by its beetle vector, the walnut twig beetle (WTB, *Pityophthorus juglandis*), in the phloem of walnut (*Juglans* spp.) or wingnut (*Pterocarya* spp.) trees, the fungus causes numerous small lesions and the disease is termed Thousand Cankers Disease (TCD) (Tisserat et al., 2009; Kolařík et al., 2011; Hishinuma et al., 2016). TCD has been reported in western and northeastern USA and recently in Europe (Tisserat et al., 2009; Grant et al., 2011; Hadziabdic et al., 2013; Montecchio et al., 2014). While originally *G. morbida* was considered an invasive species in most of the USA, the population structure of the fungus suggests that it is a native and a widespread species, albeit rare (Zerillo et al., 2014). The emergence and the disappearance of the Thousand Cankers Disease in the eastern US is, therefore, most likely a result of environmental stress on the trees, not of a pathogen invasion. Another species, *Geosmithia* sp. 41, was reported to induce dieback symptom on coast live oak (*Quercus agrifolia*) (Kolařík et al., 2017; originally reported as *G. pallida* by Lynch et al., 2014). These two mildly pathogenic species were thought to assist the colonization of beetle vectors by suppressing the defense system of tree hosts, however, this "immunosuppressing hypothesis" has been challenged (see Six and Wingfield, 2011).

Fungal communities associated with phloem-infesting bark beetles are shaped by multiple biotic and abiotic factors. The tree host is one of the most important factors. Several studies have shown that beetle species infesting the same tree species share similar fungal assemblages of ophiostomatoid fungi (Kirisits, 2004; Linnakoski et al., 2012; Jankowiak et al., 2017a). Other factors affecting the fungal community structure include beetle ecology, the surrounding host tree community, and climatic factors (Six and Bentz, 2007; Jankowiak et al., 2017b). These factors also influence the communities of *Geosmithia*, most notably by the fact that different beetles co-infesting the same host tree have similar *Geosmithia* assemblages (Kolařík et al., 2008; Machingambi et al., 2014). Several *Geosmithia* species inhabit living tree as endophytes, but their effect on the resulting *Geosmithia* community has not been evaluated (McPherson et al., 2013).

The specificity of the association between *Geosmithia*, the beetle vectors and the host trees is variable. *Geosmithia* species range from generalists to specialists for both beetle vectors and host trees (Kolařík et al., 2008, 2017; Kolařík and Jankowiak, 2013). For example, *Geosmithia ulmacea* is vectored solely by bark beetles infesting *Ulmus* species, *Geosmithia* sp. 12 is vectored by *Hylesinus* spp. from *Fraxinus* spp., *Geosmithia morbida* is vectored by *Pityophthorus juglandis* from *Juglans* and *Pterocarya* spp., and *G.* sp. 34 and 44 occurring exclusively on beetles from *Calocedrus decurrens* and *Pinus* spp. (Kolařík et al., 2017). In contrast, some generalist *Geosmithia*, e.g. members in the *G. pallida* species complex (GPSC), can be recovered from varied beetle vectors from varied hosts. It remains unclear whether the host tree specialist *Geosmithia* are also specific to particular beetle vectors. Some *Geosmithia* species are found almost exclusively on beetles that are specific to a limited range of tree species. The specificity observed could be an artefact of specificity of some bark beetles to host trees, or beetle-selected microenvironment.

The question of vector specificity is important for our understanding of the economically important Thousand Cankers Disease. Surveys of the *G. morbida* in North America have revealed that *P. juglandis* is the predominant vector, but some generalist beetles such as *Xylosandrus crassiusculus* (Curculionidae, Scolytinae), *Xyleborinus saxesenii* (Curculionidae, Scolytinae), and *Stenomimus pallidus* (Curculionidae, Cossoninae) emerging from *J. nigra* can also harbor *G. morbida* propagules (Juzwik et al., 2015, 2016). A broad, systematic survey of alternative vectors of *G. morbida* is therefore needed, considering the possibility of spread of the fungus beyond the original vector.

Geosmithia studies in North America have focused on the causal agents of TCD (i.e. *G. morbida*) and mostly conducted in the West and Northeast, where black walnuts are prevalent (Burns and Honkala, 1990). The community of *Geosmithia* species in the Southeast, however, has never before been systematically addressed. The two *Geosmithia* community surveys in North America have hinted at what appears to be a large species diversity with many undocumented species and new *Geosmithia*-beetle-tree associations (Kolařík et al., 2017; Huang et al., 2018). The Southeastern region hosts the highest diversity of tree species and the highest diversity of bark beetles (Atkinson, 2018), and therefore it may be the center of the *Geosmithia* diversity in North America.

We conducted a culture-based survey of *Geosmithia* associated with bark beetles in North Florida and Georgia. Our replicated and phylogenetically informed sampling design allowed us to ask three questions: (1) Are the causal agents of TCD (the fungus and the beetle) present in the surveyed region? (2) If *G. morbida* is present, are there any alternative beetle vector or tree hosts that accommodate this fungus? and (3) What is the specificity of the *Geosmithia* association with its beetle vectors and tree hosts?

2. Materials and methods

2.1. Sampling and isolation

We sampled host trees that represent the local diversity of Juglandaceae: black walnut (*J. nigra*), pignut hickory (*Carya glabra*), and pecan (*C. illinoinensis*), and six tree species that are phylogenetically divergent but common in the Southeast: red cedar (*Juniperus virginiana*), loblolly pine (*Pinus taeda*), laurel oak (*Quercus laurifolia*), American sweetgum (*Liquidambar styraciflua*), sugarberry (*Celtis laevigata*), and white ash (*Fraxinus americana*). To characterize the *Geosmithia*-beetle-tree association, we applied a sampling design that allowed for tree species replication and equal sampling effort among various locations (Fig. 1). From March 2017 to April 2018, we deployed freshly cut branches of the targeted tree species as a lure for bark beetles. One lure unit was represented by two to five branches (40–50 cm long × 3–15 cm diameter) of a targeted host severed from a healthy tree and wrapped in a bundle. For each tree species, five units were deployed. The branch units were hung for 1–2 months approximately 1 m above ground in natural forests in which each given tree species was abundant. For black walnut, branch sections were severed from a landscape stand from the UF campus. Given the small population of black walnut trees in surveyed regions, the lure units were hung around the closely related tree species such as pignut hickory and pecan.

We investigated the occurrences of *Geosmithia* species from up to five beetle individuals for each beetle species from each of the tree species where that beetle occurred. Beetle specimens were identified based on their morphology (Wood, 2007). Fungal isolation was performed by vortexing a whole beetle specimen in a 1.5 mL tube containing 1 mL sterilized distilled water and a drop of Tween 80 for 1 min. The vortexed fluid was serially diluted into concentrations of 1:10, 1:100, and 1:1000 and 100 μL for each concentration was then plated on 2% Malt Extract Agar media (MEA, BD Difco). Plates were incubated in the dark at 25 °C for 7–14 d with examination at intervals for the *Geosmithia* fruiting structures. The *Geosmithia* colonies were determined based on their morphotypes and then CFUs (Colony Forming Units) were counted for each plate to determine the frequencies and proportion of each of the *Geosmithia* isolates. Colonies of other fungal species and bacteria were neither quantified nor isolated. Pure cultures of *Geosmithia* species were obtained by using a sterilized scalpel to cut
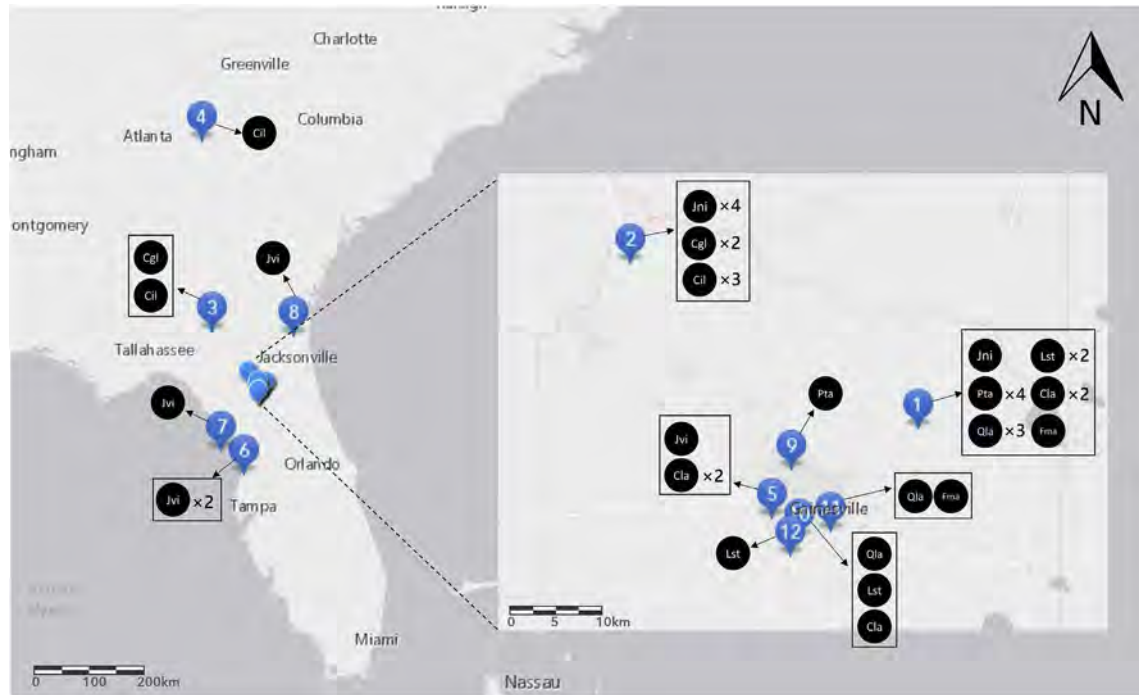
Fig. 1. Map of sampling sites in present study. Site codes: 1 - Austin Cary Forest, FL; 2 - Eewl pecan farm, FL; 3 - Shiloh pecan Farm, GA; 4 - Athens, GA; 5 - Gainesville 1, FL; 6 - Salt Marsh Trails, Homosassa, FL; 7 - Cedar Key State Park, FL; 8 - Cumberland, GA; 9 - Agricultural Experimental Station, UF, FL; 10 - Lake Alice, UF, FL; 11 - Sweetwater Wetlands Park, FL; 12 - Gainesville 2, FL. Tree codes: Jni - *Juglans nigra*; Cgl - *Carya glabra*; Cil - *Carya illinoensis*; Jvi - *Juniperus virginiana*; Pta - *Pinus taeda*; Qla - *Quercus laurifolia*; Lst - *Liquidambar styraciflua*; Cla - *Celtis laevigata*; Fam - *Fraxinus americana*.

the margin of each *Geosmithia*-suspected colony.

2.2. DNA extraction, amplification and sequencing

DNA of *Geosmithia* isolates was extracted from pure cultures by using a DNeasy Tissue and Blood DNA extraction kit (Qiagen, Inc., Valencia, California, USA) following the manufacturer's protocol; or using Extract-N-Amp Plant PCR kit (Sigma-Aldrich, St. Louis MO.) with the following steps: incubating ca. 15 μg mycelia in a tube containing 20 μL extraction solution at 96 °C for 30 min; following incubation, adding 20 μL of 3% BSA (bovine serum albumin), vortexing, and centrifuging at 5000 g; using the upper 15 μL of the supernatant as the PCR template. The complete nuc rDNA region of the ITS1-5.8S-ITS2 (ITS) and the partial translation elongation factor 1-α gene (*TEF*-1α) of fungal isolates were amplified for molecular identification. The ITS region was amplified by using the primer pair ITS-1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993) and the *TEF*-1α gene was amplified using EF1-983F/EF1-2218R (Rehner and Buckley, 2005). PCR amplification was carried out in a final 15 μL PCR reaction mixture consisting of 50—100 ng template DNA, 1.25 U Taq polymerase (Takara Bio Inc), 200 μM dNTP, 0.5 μM of each primer, and 5% DMSO (V/V). The PCR conditions were as follows: 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 40 s at 50—55 °C, and 1 min at 72 °C. The final extension step was 10 min at 72 °C. Amplification products were purified and sequenced on an ABI 3130XL at Genewiz, Inc. (South Plainfield, NJ, USA) and at Eurofins, Inc. (Louisville, KY, USA).

2.3. DNA sequences analyses

The recovered DNA sequences of *Geosmithia* species are listed in Table 2. *Emericellopsis pallida* CBS 490.71 was chosen as the phylogenetic outgroup because it is a closely related genus, and both its ITS region and *TEF*-1α were available on NCBI. Sequences

were aligned by using the online version of MAFFT (Katoh and Standley, 2013). Phylogenetic relationships were inferred from both individual partitions and the concatenated multilocus dataset combined using supermat() function in phytools package in R (Revell, 2011). The best nucleotide substitution model for each partition was determined in jModelTest 2.1.1 (Darriba et al., 2012) on the University of Florida HiPerGator 2.0 and selected based on the lowest Akaike Information Criterion (AIC) value. Maximum likelihood (ML) phylogenetic analyses were conducted by using RAxML 8.2.2 (Stamatakis, 2014) with recommended partition parameters to assess the tree topology and bootstrap values from 1000 pseudoreplicate searches. Bayesian inference (BI) were estimated by using MrBayes 3.2.5 (Ronquist et al., 2012) with recommended partition parameters. Two MCMC runs of four chains were executed simultaneously from a random starting tree for 1000000 generations, every 100 generations were sampled resulting in 10000 trees, and 2500 trees were discarded during burn-in. Posterior probabilities were estimated from the retained 7501 trees. The NEXUS file containing concatenated two-loci sequences and ML-inferred phylogeny was deposited in TreeBASE (study no. S22946) Dendrogram trees were visualized and edited by using TreeGraph2 (Stöver and Müller, 2010) and modified using Inkscape (https://inkscape.org/en/).

2.4. Community analysis

To evaluate our sampling completeness and total species richness of *Geosmithia* species diversity within our sampling area, we calculated rarified and extrapolated species richness with 95% confidence intervals obtained using a bootstrap method with 200 replications (Chao et al., 2014). The analysis was conducted using the iNEXT R package (Hsieh et al., 2016).

To test for the effects of tree host and beetle vector species on the probability of recovering each *Geosmithia* species, we

Table 1

The sampling overview: localities, tree hosts, and beetle vectors and the presence/absence of *Geosmithia* species from each beetle individual. Each "X" represents an isolation of *Geosmithia* species. Multiple lines per beetle species represent multiple specimens from which isolation was attempted, up to 5 per host and locality combination.

Columns under *Geosmithia species*[b]: *brunnea*, *pallida*, sp.2, sp.23, sp.41, *ominicola*, sp.12, *obscura*, sp.21, *lavendula*, sp.45, sp.46, sp.47, sp.48

Trees sp.	Localities	Beetles species	brunnea	pallida	sp.2	sp.23	sp.41	ominicola	sp.12	obscura	sp.21	lavendula	sp.45	sp.46	sp.47	sp.48
Juglans nigra	Austin Cary Forest (1)[a] a, FL	*Hypothenemus eruditus*														
	Eewl pecan farm (2) a, FL	*Xylosandrus crassiusculus*														
		Hypothenemus eruditus												X		
	Eewl pecan farm (2) b, FL	*Hypothenemus eruditus*												X		
	Eewl pecan farm (2) c, FL	*Hypothenemus eruditus*														
												X				
		Hypothenemus dissimilis														
		Xylosandrus compactus														
Carya glabra	Eewl pecan farm (2) d, FL	*Hypothenemus eruditus*														
	Eewl pecan farm (2) a, FL	*Hypothenemus eruditus*														
	Shiloh pecan Farm (3), GA	*Xylobiops basilaris*														
	Eewl pecan farm (2) b, FL	*Hypothenemus eruditus*														
Carya illinoensis	Eewl pecan farm (2) a, FL	*Pseudopityophthorus minutissimus*			X											
	Athens (4), GA	*Chramesus hicoriae*														
		Hypothenemus rotundicollis							X							
									X							
									X							
							X		X							
							X									
		Xylobiops basilaris							X							
												X				
	Eewl pecan farm (2) b, FL	*Xylosandrus crassiusculus*														
		Hypothenemus eruditus														
		Hypothenemus dissimilis					X		X							
	Shiloh pecan Farm, GA	*Hypothenemus eruditus*														
	Eewl pecan farm (2) c, FL	*Xylosandrus compactus*														
		Xylobiops basilaris														

(*continued on next page*)

Table 1 (*continued*)

Trees sp.	Localities	Beetles species	Geosmithia species[b]													
			brunnea	pallida	sp.2	sp.23	sp.41	ominicola	sp.12	obscura	sp.21	lavendula	sp.45	sp.46	sp.47	sp.48
Juniperus virginiana	Gainesville (5) 1, FL	*Hypothenemus dissimilis*		X											X	
		Ambrosiodmus lecontei														
		Phloeosinus dentatus														
	Salt Marsh Trails (6) a, Homosassa, FL	*Phloeosinus dentatus*								X						
					X											
	Salt Marsh Trails (6) b, Homosassa, FL	*Phloeosinus dentatus*									X					
					X											
	Cedar Key Museum State Park (7), FL	*Phloeosinus dentatus*			X											
					X											
	Cumberland (8), GA	*Phloeosinus dentatus*			X						X					
					X											
																X
																X
Pinus taeda	Austin Cary Forest (1) a, FL	*Pityophthorus pulicarius*														
		Pityophthorus confusus														
	Austin Cary Forest (1) b, FL	*Pityophthorus annectens*											X			
													X			
	Austin Cary Forest (1) c, FL	*Pityophthorus annectens*														
	Austin Cary Forest (1) d, FL	*Pityophthorus pulicarius*														
	Agricultural Experimental Station (9), UF, FL	*Ips avulsus* *Pityophthorus pulicarius*											X			
		Pityophthorus annectens														
Quercus laurifolia	Austin Cary Forest (1) a, FL	*Pseudopityophthorus minutissimus*					X							X		
	Austin Cary Forest (1) b, FL	*Hypothenemus dissimilis*	X													X

	Austin Cary Forest (1) c, FL	*Micracisella nanula*			X	X	X	X
		Pseudopityophthorus minutissimus	X		X			
			X	X	X			
				X				
	Lake Alice (10), UF, FL	*Pseudopityophthorus minutissimus*	X					
			X					
	Sweetwater Wetlands Park (11), FL	*Pseudopityophthorus minutissimus*			X			
					X			
Liquidambar styraciflua	Austin Cary Forest (1) a, FL	*Pityophthorus liquidambarus*						
		Hypothenemus interstitialis						
		Xylosandrus compactus						
	Austin Cary Forest (1) b, FL	*Pseudopityophthorus minutissimus*						
	Lake Alice (10), UF, FL	*Xylosandrus compactus*	X					
			X					
		Pseudopityophthorus minutissimus						
	Gainesville (12) 2, FL	*Hypothenemus eruditus*						
		Pseudopityophthorus minutissimus						
Celtis laevigata	Austin Cary Forest (1) a, FL	*Xylosandrus germanus*						
		Xyleborinus saxeseni						
		Xylosandrus compactus						
		Hypothenemus eruditus						

Table 1 (*continued*)

Trees sp.	Localities	Beetles species	Geosmithia species[b]													
			brunnea	pallida	sp.2	sp.23	sp.41	ominicola	sp.12	obscura	sp.21	lavendula	sp.45	sp.46	sp.47	sp.48
		Xylosandrus crassiusculus				X										
	Gainesville (5) 1, FL	Chramesus chapuisii				X										
		Pseudopotyopthorus asperulus														
		Hypothenemus eruditus														
	Lake Alice (10), UF, FL	Chramesus chapuisii			X											
		Phloeotribus texanus														
		Hypothenemus brunneus														
	Gainesville (5) 1, FL	Phloeotribus texanus				X										
		Hypothenemus eruditus														
	Austin Cary Forest (1) b, FL	Xylobiops basilaris														
		Xylosandrus crassiusculus														
		Hypothenemus eruditus														
		Xylosandrus compactus														
	Sweetwater Wetlands Park (11), FL	Xylosandrus compactus														
	Austin Cary Forest (1) a, FL	Pseudothysanoes dislocatus														

[a] Numbers in brackets refer to site codes in Fig. 1.
[b] Species numeric codes Kolarik et al. (2017); G. sp.45–48 are assigned in present study as putative new species.

Table 2

Host ranges, beetle vectors, and GenBank accession numbers of Geosmithia species included in the phylogenetic analyses. Geosmithia species recovered in the present study were shown in bold.

Species[a]	Isolation no.[c]	Beetle vectors	Tree host/substratum	GenBank accession no.		References
				ITS	TEF1-α	
G. proliferans	CBS 142636 T	*Phloeotribus frontalis*	*Acer negundo*	KY872744	KY872749	Huang et al. (2018)
G. proliferans	CBS 142637	*Phloeotribus frontalis*	*Acer negundo*	KY872745	KY872750	Huang et al. (2018)
G. langdonii	CCF 3332 T	*Scolytus intricatus*	*Quercus robur*	KF808297	HG799876	Kolarik et al. (2005, 2017)
G. langdonii	CCF 4338	*Cryphalus pubescens*	*Sequoia serpervirens*	HF546245	HG799877	Kolarik et al. (2017)
G. putterillii	CCF 3052 T	N/A	discoloured timber of Beilschmiedia tawa	AF033384	HG799853	Kolarik et al. (2004, 2017)
G. putterillii	U307	Pityophthorus sp.	*Pinus ponderosa*	HF546306	MH580529	Kolarik et al. (2017)
G. pallida	CCF 3053 T	N/A	cotton yarn	AJ578486	NA	Kolarik et al. (2004, 2017)
G. pallida	CCF 3324	N/A	Soil	NA	HG799846	Kolarik et al. (2004, 2017)
G. flava	CCF 3333 T (=MK101)	Xiphydria sp.	*Castanea sativa*	AJ578483	MH580541	Kolarik et al. (2004)
G. flava	CCF 4337 (=U56)	Cerambycidae sp.	*Pseudotsuga menziesii*	HF546244	MH580542	Kolarik et al. (2004)
G. obscura	CCF 3422 T	*Scolytus intricatus*	*Quercus robur*	AJ784999	MH580539	Kolarik et al. (2005)
G. obscura	CCF 3425 = MK616	*Scolytus carpini*	*Carpinus betulus*	AM181460	MH580540	Kolarik et al. (2005)
G. lavendula	CCF 3051	NA	Laboratory contamination	AF033385	NA	Kolarik et al. (2004)
G. lavendula	CCF 3394	*Carphoborus vestitus*	*Pistacia terebinthus*	AM421098	NA	Kolarik et al. (2007)
G. morbida	CCF 4576 (=U173)	*Pityophthorus juglandis*	*Juglans nigra*	HF546282	MH580544	Kolarik et al. (2007)
G. morbida	CCF 3881 = CBS 124663	*Pityophthorus juglandis*	*Juglans nigra*	FN434082	MH580543	Kolarik et al. (2010)
G. microcorthyli	CCF 3861 T	Microcorthylus sp.	*Cassia grandis*	FM986798	MH580560	Kolařík et Kirkendall (2010)
G. rufescens	CCF 3751	*Cnesinus lecontei*	*Croton draco*	AM947667	NA	Kolarik et al. (2010)
G. rufescens	CCF 4524	*Cnesinus lecontei*	*Croton draco*	AM947668	NA	Kolarik et al. (2010)
G. fassatiae	AK31/98	*Scolytus intricatus*	Quercus sp.	AM421039	MH580557	Kolarik et al. (2008)
G. fassatiae	CCF 3334 T	NA	*Quercus pubescens*	AJ578482	MH580530	Kolarik et al. (2005)
G. sp. 2	U107	*Scolytys rugulosus*	Prunus sp.	HF546256	HG799855	Kolarik et al. (2017)
G. sp. 2	MK642	*Hylesinus orni*	*Fraxinus ornus*	NA	HG799852	Kolarik et al. (2017)
G. sp. 2	CCF 3319	N/A	ex tree in apple orchard	NA	HG799840	Kolarik et al. (2017)
G. sp. 2	CCF 3320	N/A	ex Cucumis melo	NA	HG799841	Kolarik et al. (2017)
G. sp. 3	CCF 4298	*Scolytus intricatus*	*Quercus dalechampii*	AM181436	HG799891	Kolarik et al. (2008, 2017)
G. sp. 3	CCF 3481	*Scolytus carpini*	*Carpinus betulus*	AM181467	HG799842	Kolarik et al. (2017)
G. sp. 3	CCF 3344	*Scolytus intricatus*	*Quercus robur*	NA	HG799848	Kolarik et al. (2008, 2017)
G. sp. 4	CCF 4278	*Pteleobius vittatus*	*Ulmus laevis*	AM181466	HG799850	Kolarik et al. (2008, 2017)
G. sp. 5	CCF 3341	*Scolytus intricatus*	*Quercus petraea*	AJ578487	HG799837	Kolarik et al. (2004, 2017)
G. sp. 5	CCF 4215	*Pityophthorus pityographus*	*Picea abies*	HE604117	HG799854	Kolarik and Jankowiak (2013)
G. sp. 5	AK192/98	*Scolytus intricatus*	*Quercus robur*	NA	HG799835	Kolarik et al. (2017)
G. sp. 8	CCF 3358	*Scolytus intricatus*	*Quercus petraea*	AM181421	MH580559	Kolařík et Kirkendall (2010)
G. sp. 9	RJ0266	*Ips cembrae*	Larix decidua	HE604123	MH580551	Kolarik and Jankowiak (2013)
G. omnicola10	MK1707	*Hylesinus orni*	Fraxinus sp.	AM181452	MH580558	Kolařík et al. (2008)
G. omnicola10	IMI 194089	NA	Air	AM181450	NA	Kolařík et al. (2008)
G. sp. 11	CCF 3555 = MK551	*Scolytus intricatus*	*Quercus pubescens*	AM181419	MH580545	Kolařík et al. (2008)
G. sp. 11	CCF 3556	*Scolytus intricatus*	*Quercus pubescens*	AM181418	NA	Kolařík et al. (2008)
G. sp. 12	CCF 4320 = U164	*Hylesinus oregonus*	Fraxinus sp.	HF546229	MH580532	Kolarik et al. (2017)
G. sp. 12	CCF 3557 = MK661	*Leperisinus orni*	*Fraxinus excelsior*	AM181431	MH580531	Kolařík et al. (2008)
G. ulmacea 13	CCF 3559	*Scolytus multistriatus*	Ulmus sp.	AM181439	MH580535	Kolařík et al. (2008)
G. ulmacea 13	1226	*Scolytus schevyrewi*	Ulmus sp.	KJ716463	NA	Zerillo et al. (2014)
G. sp. 16	CCF 4201	*Pityophthorus pityographus*	*Picea abies*	HE604146	HE604206	Kolarik and Jankowiak (2013)
G. sp. 16	RJ34m	*Pityophthorus pityographus*	*Picea abies*	NA	HE604207	Kolarik and Jankowiak (2013)
G. sp. 19	CCF 3658 = MK1085a	*Hypoborus ficus*	*Ficus carica*	AM421085	MH580546	Kolarik et al. (2007)
G. sp. 19	CCF 3655	*Hypoborus ficus*	*Ficus carica*	AM421075	NA	Kolarik et al. (2007)
G. sp. 20	CCF 4316 = MK119b	*Phloesinus fulgens*	*Calocedrus decurrens*	HF546226	MH580547	Kolarik et al. (2017)
G. sp. 20	U193	*Scolytus schevyrewi*	*Ulmus pumila*	HF546287	MH580548	Kolarik et al. (2017)
G. sp. 21	CCF 5270	*Pseudotsuga menziesii*	Pityophthorus sp., Scolytus oregoni, Cryphalus	HF546289	MH580534	Kolarik et al. (2017)
G. sp. 21	CCF 4280	*Hypoborus ficus*	*Ficus carica*	AM421049	MH580533	Kolarik et al. (2007)
G. sp. 22	CCF 3645	*Phloetribus scarabeoides*	*Olea europaea*	AM421061	MH580552	Kolarik et al. (2007)
G. sp. 22	CCF 3652	*Phloetribus scarabeoides*	*Olea europaea*	AM421062	MH580553	Kolarik et al. (2007)
G. sp. 23	CCF 3318	scolytid beetles	*Persea gratissima*	AJ578489	HG799845	Kolarik et al. (2004, 2017)
G. sp. 23	CCF 3639	*Scolytus rugulosus*	*Prunus armeniaca*	AM421068	HG799838	Kolarik et al. (2004, 2017)

Table 2 (continued)

Species[a]	Isolation no.[c]	Beetle vectors	Tree host/substratum	GenBank accession no.		References
				ITS	TEF1-α	
G. sp. 23	U160	Scolytus multistriatus	Ulmus pumila	HF546284	HG799859	Kolarik et al. (2017)
G. sp. 24	CCF 4294 = MK1837	Pityogenes quadridens	Pinus sylvestris	HE604165	MH580555	Kolarik and Jankowiak (2013)
G. sp. 24	MK1772	Pityophthorus pityographus	Pinus sylvestris	HE604164	MH580556	Kolarik and Jankowiak (2013)
G. sp. 25	MK1832	Cryphalus abietis	Abies alba	HE604128	HE604218	Kolarik and Jankowiak (2013)
G. sp. 25	CCF 4205	Cryphalus piceae Pityophthorus pityographus	Abies alba	HE604127	HE604219	Kolarik and Jankowiak (2013)
G sp. 26	CCF 4222 = RJ26	Pinus sylvestris	Pityogenes bidentatus	HE604158	LN907595	Kolařík et Jankowiak (2016)
G sp. 26	CCF 4223 = MK1796	Pinus sylvestris	Pityophthorus pityographus	HE604112	LN907596	Kolařík et Jankowiak (2016)
G. sp. 27	CCF 4605	Pityophthorus sp.	Pinus ponderosae	HF546309	HG799867	Kolarik and Jankowiak (2013)
G. sp. 27	CCF 4206	Pityogenes bidentatus	Pinus sylvestris	HE794978	HG799839	Kolarik et al. (2017)
G. sp. 28	RJ278m	Pityophthorus pityographus	Picea abies	HE604124	NA	Kolarik and Jankowiak (2013)
G. sp. 28	RJ279m	Pityophthorus pityographus	Picea abies	HE604154	MH580554	Kolarik and Jankowiak (2013)
G. sp. 29	CCF 4221	Cryphalus piceae Pityophthorus pityographus	Abies alba	HE604125	HE604233	Kolarik and Jankowiak (2013)
G. sp. 30	CCF 4288	Ips cembrae	Larix decidua	HE604132	HE604216	Kolarik and Jankowiak (2013)
G. sp. 30	CCF 4219	Cryphalus abietis	Abies alba	NA	HE604221	Kolarik and Jankowiak (2013)
G. sp. 31	CCF 4197	Pityogenes bidentatus	Pinus sylvestris	NA	HE604229	Kolarik and Jankowiak (2013)
G. sp. 31	CCF 4196	Pityophthorus pityographus	Pinus sylvestris	NA	HE604230	Kolarik and Jankowiak (2013)
G. sp. 32	CCF 3554	Phloeosinus thujae	Chamaecyparis pisifera	AM181426	HG799874	Kolarik et al. (2008, 2017)
G. sp. 32	CCF 5242	Phloesinus sequiae	Sequoia serpervirens	HF546265	HG799873	Kolarik et al. (2008, 2017)
G. sp. 33	CCF 4598	Scolytus praeceps	Abies concolor	HF546331	HG799869	Kolarik et al. (2017)
G. sp. 34	CCF 4604	Ips plastographus	Calocedrus decurrens	HF546295	HG799866	Kolarik et al. (2017)
G. sp. 34	U417	Scolytus praeceps	Abies concolor	HF546330	HG799868	Kolarik et al. (2017)
G. sp. 35	U196	Pityophthorus sp. Scolytus oregoni Cryphalus pubescens	Pseudotsuga menziesii	HF546231	NA	Kolarik et al. (2017)
G. sp. 36	CCF 4328 = U316	Pityophthorus sp.	Pinus muricata	HF546236	NA	Kolarik et al. (2017)
G. sp. 36	MK1814	NA	Cedrus atlantica	NA	MH580538	present study
G. sp. 37	U197	Pityophthorus sp. Scolytus oregoni Cryphalus pubescens	Pseudotsuga menziesii	HF546288	HG799862	Kolarik et al. (2017)
G. sp. 38	U79	Pseudopityophthorus pubipennis	Notholithocarpus densiflorus	HF546346	MH580537	Kolarik et al. (2017)
G. sp. 38	CCF 5241 (=U95)	Pseudopityophthorus pubipennis	Quercus acrifolia	HF546251	MH580536	Kolarik et al. (2017)
G. sp. 39	U323	Pityophthorus juglandis	Juglans hindsii	HF546314	NA	Kolarik et al. (2017)
G. sp. 40	CCF 5250 (=U143)	Pityophthorus sp.	Pinus ponderosa	HF546273	MH580550	Kolarik et al. (2017)
G. sp. 40	CCF 5245 (=U306a)	Ips plastographus	Pinus radiata	HF546304	MH580549	Kolarik et al. (2017)
G. sp. 41	U215	Cossoninae sp.	Artemisia arborea	HF546292	HG799865	Kolarik et al. (2017)
G. sp. 41	CCF 4342	Bostrichidae	Toxicodendron diversilobum	HF546249	HG799871	Kolarik et al. (2017)
G. sp. 41	U64	Scobicia declivis	Umbellularia californica	HF546342	HG799870	Kolarik et al. (2017)
G. sp. 42	U166	Phloesinus canadensis	Chamaecyparis sp.	HF546279	HG799860	Kolarik et al. (2017)
G. sp. 42	CCF 5251	Scolytus rugulosus	Prunus sp.	HF546285	HG799861	Kolarik et al. (2017)
G. sp. 43	CCF 4203	Pityogenes knechteli Pityophthorus sp.	Pinus ponderosae	HF546223	HG799864	Kolarik et al. (2017)
G. sp. 44	CCF 4333 (=U410)	Phloeosinus fulgens	Phloeosinus fulgens	HF546241	LN907598	Kolarik et al. (2017)
G. sp. 44	CCF 4332 (=U408)	Pinus sabiniana	Pityophthorus sp.	HF546240	LN907599	Kolarik et al. (2017)
G. brunnea	CBS 142634	Xylosandrus compactus	Liquidambar styraciflua	KY872741	KY872746	present study
G. brunnea	CBS 142635	Xylosandrus compactus	Liquidambar styraciflua	KY872742	KY872747	present study
G. brunnea	CBS 142633 T	Hypothenemus dissimilis	Quercus sp.	KY872743	KY872748	present study
G. ominicola	Hulcr 17349	Micracisella nanula	Quercus laurifolia	MH426757	MH580485	present study
G. pallida	Hulcr 17003	Xylobiops basilaris	Carya illinoinensis	MH426751	MH580481	present study
G. pallida	Hulcr 17350	Pseudopityophthorus minutissimus	Quercus laurifolia	MH426758	MH580486	present study
G. pallida	Hulcr 17353	Pseudopityophthorus minutissimus	Quercus laurifolia	MH426761	MH580488	present study
G. pallida	Hulcr 18164	Pseudopityophthorus minutissimus	Carya illinoinensis	MH426775	MH580500	present study
G. pallida	Hulcr 18777	Pseudopityophthorus minutissimus	Quercus laurifolia	MH426778	MH580503	present study
G. pallida	Hulcr 18778	Pseudopityophthorus minutissimus	Quercus laurifolia	MH426779	MH580504	present study
G. obscura	Hulcr 18146	Xylobiops basilaris	Carya illinoinensis	MH426774	MH580499	present study

G. obscura	Hulcr 18775	Phloeosinus dentatus	*Juniperus virginiana*	MH426777	MH580502	present study
G. obscura	Hulcr 19181	*Hypothenemus eruditus*	*Juglans nigra*	MH426788	MH580509	present study
G. lavendula	Hulcr 17347	*Micracisella nanula*	*Quercus laurifolia*	MH426755	MH580484	present study
G. sp. 2	Hulcr 10913	*Chramesus chapuisii*	*Celtis laevigata*	MH426746	MH580477	present study
G. sp. 2	Hulcr 17352	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426760	MH580521	present study
G. sp. 2	Hulcr 17357	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426763	MH580489	present study
G. sp. 2	Hulcr 18903	*Juniperus virginiana*	*Phloeosinus dentatus*	MH426781	MH580519	present study
G. sp. 2	Hulcr 18904	*Juniperus virginiana*	*Phloeosinus dentatus*	MH426782	MH580520	present study
G. sp. 2	Hulcr 19183	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426790	MH580516	present study
G. sp. 2	Hulcr 19184	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426791	MH580517	present study
G. sp. 2	Hulcr 19185	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426792	MH580518	present study
G. sp. 2	Hulcr 19187	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426794	MH580512	present study
G. sp. 2	Hulcr 19189	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426795	MH580513	present study
G. sp. 12	Hulcr 17348	*Micracisella nanula*	*Quercus laurifolia*	MH426756	MH580492	present study
G. sp. 12	Hulcr 18136	*Hypothenemus rotundicollis*	*Carya illinoinensis*	MH426767	MH580491	present study
G. sp. 12	Hulcr 18137	*Hypothenemus rotundicollis*	*Carya illinoinensis*	MH426768	MH580493	present study
G. sp. 12	Hulcr 18138	*Hypothenemus rotundicollis*	*Carya illinoinensis*	MH426769	MH580494	present study
G. sp. 12	Hulcr 18139	*Hypothenemus rotundicollis*	*Carya illinoinensis*	MH426770	MH580495	present study
G. sp. 12	Hulcr 18145	*Xylobiops basilaris*	*Carya illinoinensis*	MH426773	MH580498	present study
G. sp. 12	Hulcr 19079	*Hypothenemus dissimilis*	*Carya illinoinensis*	MH426787	MH580508	present study
G. sp. 21	Hulcr 18907	*Juniperus virginiana*	*Phloeosinus dentatus*	MH426785	MH580506	present study
G. sp. 21	Hulcr 19186	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426793	MH580511	present study
G. sp. 23	Hulcr 14582	*Phloeotribus texanus*	*Celtis laevigata*	MH426750	MH580526	present study
G. sp. 23	Hulcr 17358	*Xylosandrus crassiusculus*	*Celtis laevigata*	MH426764	MH580527	present study
G. sp. 23	Hulcr 17359	*Xylosandrus crassiusculus*	*Celtis laevigata*	MH426765	MH580528	present study
G. sp. 41	Hulcr 11574	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426747	MH580478	present study
G. sp. 41	Hulcr 17346	*Micracisella nanula*	*Quercus laurifolia*	MH426754	MH580483	present study
G. sp. 41	Hulcr 17351	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426759	MH580524	present study
G. sp. 41	Hulcr 17354	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426762	MH580525	present study
G. sp. 41	Hulcr 18143	*Hypothenemus rotundicollis*	*Carya illinoinensis*	MH426771	MH580496	present study
G. sp. 41	Hulcr 18144	*Hypothenemus rotundicollis*	*Carya illinoinensis*	MH426772	MH580497	present study
G. sp. 41	Hulcr 18905	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426783	MH580522	present study
G. sp. 41	Hulcr 18906	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426784	MH580523	present study
G. sp. 41	Hulcr 19078	*Hypothenemus dissimilis*	*Carya illinoinensis*	MH426786	MH580507	present study
G. sp. 45	Hulcr 17004	*Pityophthorus annectens*	*Pinus taeda*	MH426752	MH580482	present study
G. sp. 45	Hulcr 17006	*Pityophthorus annectens*	*Pinus taeda*	MH426753	MH580487	present study
G. sp. 45	Hulcr 18823	*Pityophthorus pulicarius*	*Pinus taeda*	MH426780	MH580505	present study
G. sp. 46	Hulcr 11575	*Pseudopityophthorus minutissimus*	*Quercus laurifolia*	MH426748	MH580479	present study
G. sp. 46	Hulcr 18077	*Hypothenemus eruditus*	*Juglans nigra*	MH426766	MH580490	present study
G. sp. 46	Hulcr 18201	*Hypothenemus eruditus*	*Juglans nigra*	MH426776	MH580501	present study
G. sp. 47	Hulcr 11904	*Hypothenemus dissimilis*	*Quercus laurifolia*	MH426749	MH580480	present study
G. sp. 47	Hulcr 19182	*Hypothenemus dissimilis*	*Carya illinoinensis*	MH426789	MH580510	present study
G. sp. 48	Hulcr 19190	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426796	MH580514	present study
G. sp. 48	Hulcr 19192	*Phloeosinus dentatus*	*Juniperus virginiana*	MH426797	MH580515	present study
Emericellopsis pallida b	CBS 490.71 T	NA	NA	NR_145052	KC998998	Grum-Grzhimaylo et al. (2013)

a Species numeric codes corresponding to Kolarik et al. (2007, 2008), Kolarik and Jankowiak (2013), and Kolarik et al. (2017).
b E. pallida selected as outgroup of phylogenies.
C Superscript T denote type strain.

conducted a linear model-based analysis of the recovered *Geosmithia* assemblages. We used the manyglm() function of the mvabund package of R (Wang et al., 2012) to fit individual binomial linear models for the presence/absence of each *Geosmithia* species, with tree host, beetle vector, and tree/beetle interaction term as predictors. This approach provides an overall multivariate test of the effect of beetle and tree species on the composition of the recovered *Geosmithia* species assemblages, as well as individual tests of tree and beetle species on the occurrence of each *Geosmithia* species with adjusted *P*-values to account for multiple comparisons (see details in Wang et al., 2012). We chose to exclude samples from *C. glabra* (n = 4) and *F. americana* (n = 2) from the community analyses because these tree species yielded only 4 and 2 beetles, respectively, and *Geosmithia* was not recovered from any of these individuals. We also excluded *Geosmithia* species that were recovered in fewer than three isolates (*G.* sp. 10, *G.* sp. 21, *G. lavendula*, *G.* sp. 47, *G.* sp. 48).

3. Results

3.1. Identification of Geosmithia species and their occurrence on trees and beetles

In total, 195 beetle specimens were excised from 45 exposed branch units (Table 1), of which 23.1% (n = 45) yielded *Geosmithia* species resulting in 55 isolates. We did not find *Pityophthorus juglandis*, the known vector of TCD, in the assayed branch baits.

Among the 55 *Geosmithia* isolates, 14 species were determined based on the morphological characteristics and molecular makers (Fig. 2). We did not recover *G. morbida* in our isolates. Species in the *G. pallida* species complex (GPSC, i.e. *pallida*, *brunnea*, sp. 2, sp. 23, and sp. 41) accounted for 56% of the isolates (n = 31), in which *G.* sp. 2 (n = 10) and *G.* sp. 41 (n = 9) had the highest incidence, followed by *G. pallida* (n = 6), *G. brunnea* (n = 3), and *G.* sp. 23 (n = 3). For species other than the GPSC (n = 24), *G.* sp. 12 was the most frequently isolated species (n = 7), whereas other species were isolated less frequently, i.e. *G.* sp. 10 (n = 1), *G. obscura* (n = 3), *G.* sp. 21 (n = 2), and *G. lavendula* (n = 1). Ten *Geosmithia* isolates, which clustered separately into four phylogenetic species, did not conform to any previously reported *Geosmithia* species and are considered putatively to be new species (Fig. 2). These putative new species were assigned numbers following Kolařík et al. (2017), namely *G.* sp. 45, *G.* sp. 46, *G.* sp. 47, *G.* sp. 48. Three isolates of *G.* sp. 45 were isolated from *Pityophthorus annectens* and *Pityophthorus pulicarius* from *P. taeda*. Three isolates of *G.* sp. 46 were isolated from *Hypothenemus dissimilis* from *Q. laurifolia* and *Hypothenemus eruditus* from *J. nigra*. Two isolates of *G.* sp. 47 were isolated from *H. dissimilis* from *C. illinoinensis* and *Q. laurifolia*. Two isolates of *G.* sp. 48 were isolated from *Phloeosinus dentatus* from *J. virginiana*.

Occurrences of *Geosmithia* species were highly varied among tree and beetle species (Figs. 3 and 4). In general, beetle specimens from *Q. laurifolia* (73.1%), *J. virginiana* (54.5%), and *C. illinoinensis* (46.4%) had the highest rate of *Geosmithia* species recovery. Other tree species had much lower recovery rates; *P. taeda* (15.4%), *J. nigra* (13%), *C. laevigata* (10.5%), and *L. styraciflua* (6.1%). Beetles from *C. glabra* and *F. americana* had no *Geosmithia* associated.

3.2. Community analysis

We estimated a total *Geosmithia* species richness of 14.66 ± 1.3 species in the sampling area. Our estimated sampling coverage was 96%, indicating that our samples were representative of the *Geosmithia* species diversity on the studied substrates in the assayed regions (Fig. 5).

Recovery rates were calculated as the percentage of individual

beetles in each tree species from which each *Geosmithia* species was recovered. From the perspective of tree hosts, *G.* sp. 2 had the highest recovery rate from *J. virginiana* (33.3%), followed by *G.* sp. 41 from *Q. laurifolia* (28.5%), *G.* sp. 12 from *C. illinoensis* (21.4%), and *G. pallida* from *Q. laurifolia* (19%); other *Geosmithia* species showed lower recovery rates ranging from 2% to 11% (Fig. 3). We recovered *Geosmithia* species from 13 of 24 beetle species sampled, with a range of 1—4 *Geosmithia* species per beetle vector (Fig. 4). *Hypothenemus rotundicollis* had the highest recovery rate of *G.* sp. 12 (80%), though this fungus was also isolated from three other beetle species (*H. dissimilis*, *Micracisella nanula*, and *Pseudopityophthorus minutissimus*). *Micracisella nanula* vectored *G.* sp. 41, *G.* sp. 12, *G. ominicola*, and *G. lavendula* (all 50%); the latter two were found exclusively in association with this beetle. However, we only recovered two specimens of *M. nanula*, which greatly limited our ability to make robust inferences about the consistency and/or specificity of these relationships. The other beetle species showed variable association with *Geosmithia* species ranging from 3% to 35% (Fig. 4).

We found evidence for tree host preferences among *Geosmithia* species, but no evidence for preference or specificity to beetle vectors. General linear model-based analysis indicated a significant effect of tree species on the multivariate composition of the recovered *Geosmithia* assemblages, but no significant effect of beetle species or interaction between tree and beetle (Table 3). Individual species-level tests showed that tree species was a significant predictor of the presence of five *Geosmithia* species: *G. pallida*, *G.* sp. 2, *G.* sp. 41, *G.* sp. 12, and *G.* sp. 45 (all *P* < 0.05). *Geosmithia pallida* was found most frequently in *Q. laurifolia*, *G.* sp. 2 in *J. virginiana*, *G.* sp. 41 in *Q. laurifolia* and *Carya illinoensis*, *G.* sp. 12 in *C. illinoensis*, and *G.* sp. 45 in *P. taeda*. Beetle vector was not a significant predictor for the presence of any *Geosmithia* species, nor was the interaction between beetle and tree species a significant predictor for *Geosmithia* species present (Table 3). This suggests that *Geosmithia* prevalence is determined by the substrate (i.e. tree species) and that the fungi are able to disperse on multiple non-specific vectors.

4. Discussion

This is the first study to quantitatively examine the symbiotic relationship of *Geosmithia* species with their beetle vectors and host trees in the southeastern USA. We systematically deployed branch sections from targeted species to be colonized by bark beetles that potentially carry *Geosmithia* species, and quantitatively described the *Geosmithia* community in relation to their beetle vectors and host trees.

We considered beetle individuals as a quantitative unit for studying their association with *Geosmithia* species. Therefore, the recovery rate of *Geosmithia* species in the present study could also be extrapolated, to some degree, to the effectiveness of these beetle species to transmit *Geosmithia* propagules among tree hosts in the environment.

The species richness analysis suggested that our sampling effort was sufficient to accumulate a representative sample of regional *Geosmithia*. The uniform, factorial sampling effort also allowed us to quantitatively analyze the *Geosmithia* specificity, and it appeared that the fungi were more responsive to the host trees than to the identity of their beetle vectors. Therefore, additional *Geosmithia* fungi are likely to be found in surveys of additional tree species, but sampling different beetles from the trees already sampled is less likely to recover many new fungal taxa.

4.1. TCD causal agents

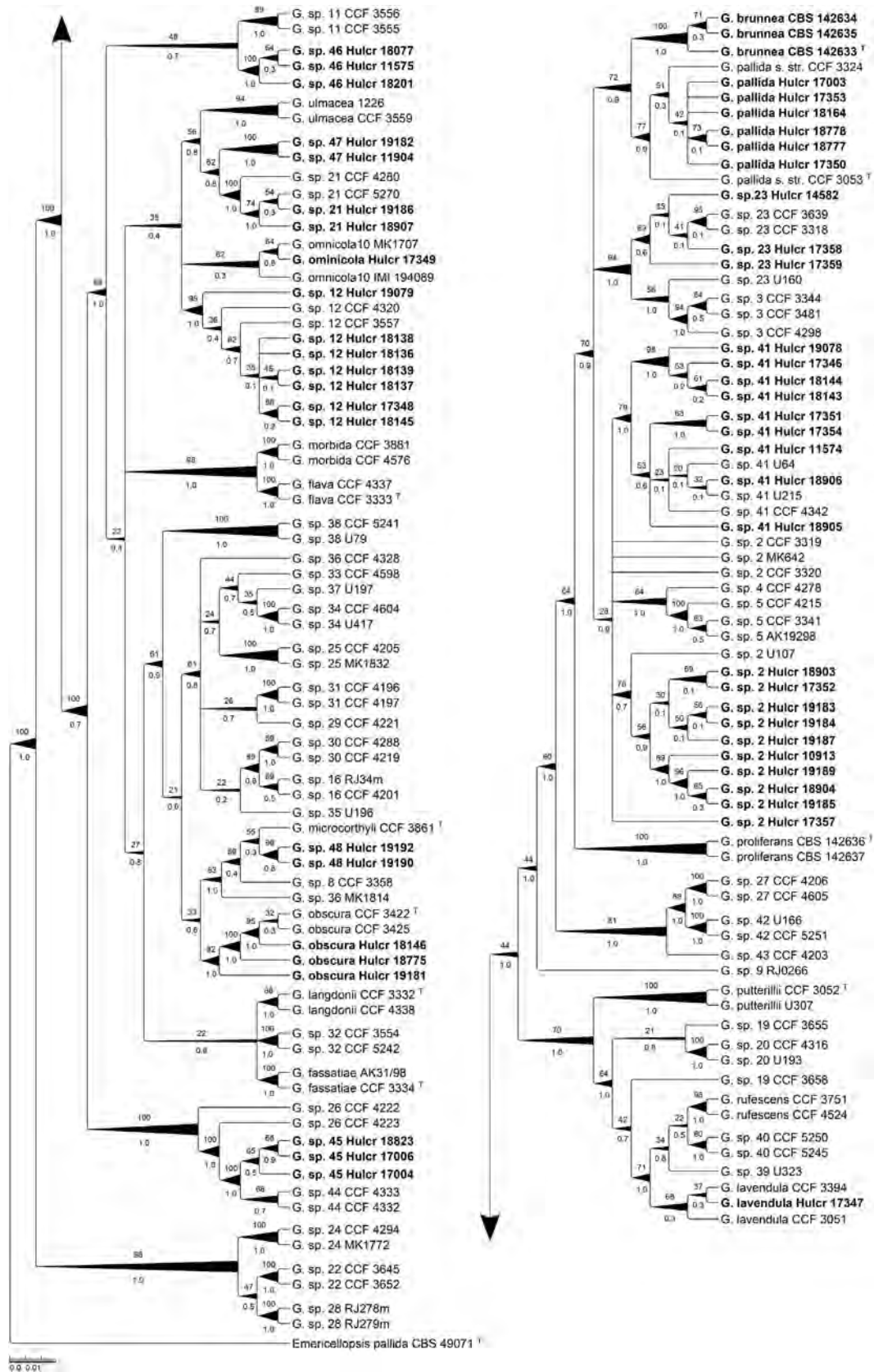We found neither *P. juglandis* nor *G. morbida* in our assayed

Fig. 2. Phylogenetic relationships between the *Geosmithia* isolates, and their position within the genus. The tree was obtained using Maximum Likelihood analysis of the combined ITS and TEF1-α sequences dataset. *Geosmithia* species recovered in the present study are shown in bold. Bootstrap values/Bayesian posterior probabilities were shown above/below nodes. *Emericellopsis pallida* was selected as outgroup. Type strains are indicated with superscript T. Species numeric codes correspond to Kolařík et al. (2017).
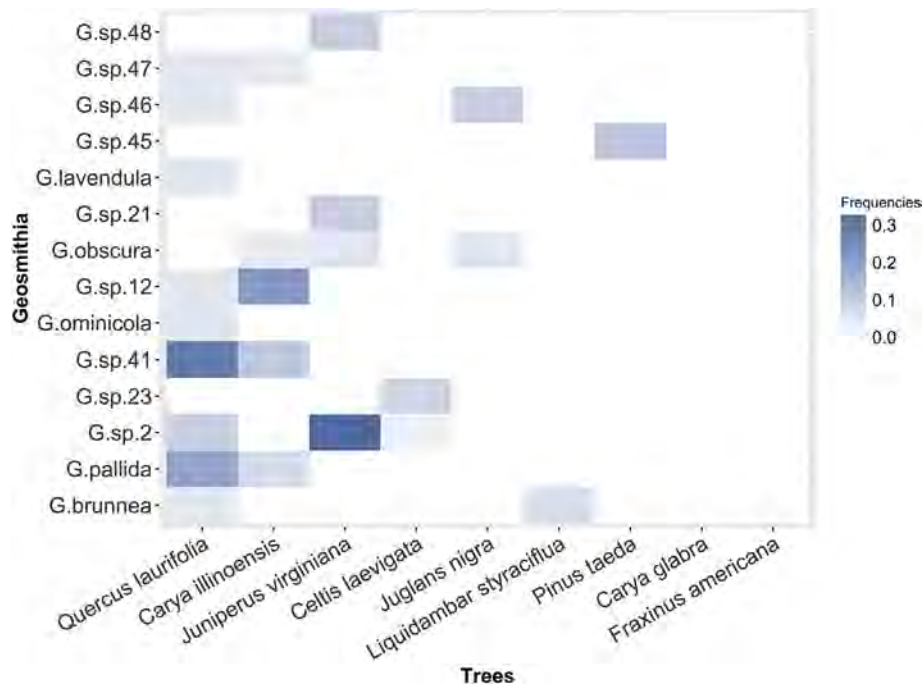
Fig. 3. Heat map showing the occurrence frequencies of *Geosmithia* species from tree species.
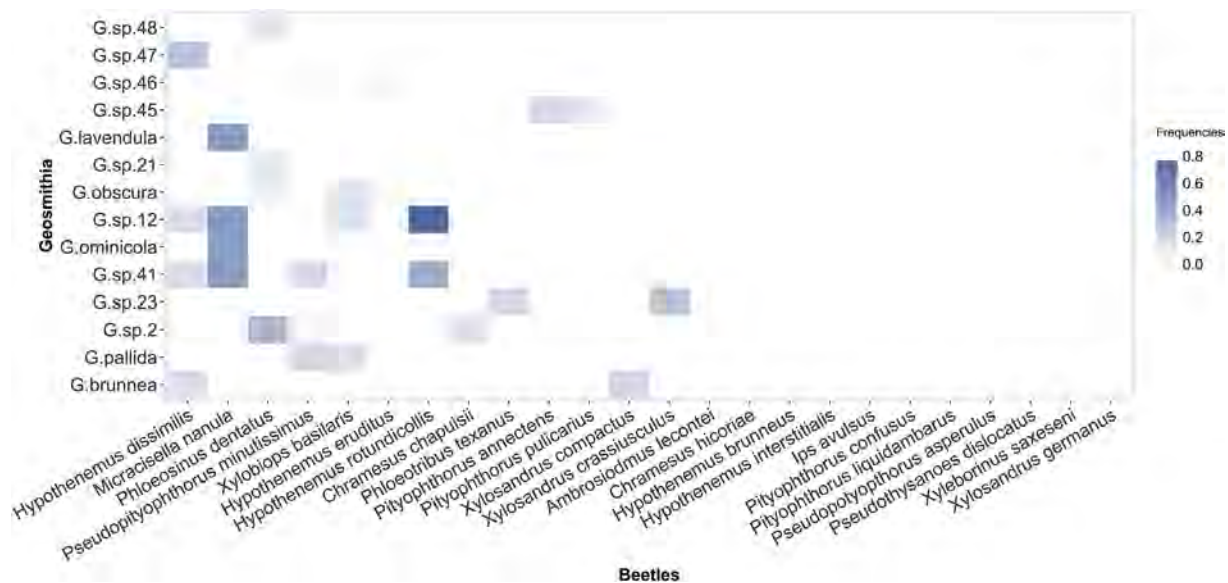
Fig. 4. Heat map showing the occurrence frequencies of *Geosmithia* species from beetle species.

samples. Beetle species excised from the bait branches of three tree species in the Juglandaceae were primarily polyphagous bark or ambrosia beetles (*Hypothenemus eruditus*, *H. dissimilis*, *H. rotundicollis*, *X. crassiusculus*, *X. compactus*, *P. minutissimus*), one Juglandaceae-specific species (*Chramesus hicoriae*), and one bostrichid beetles (*Xylobiops basilaris*). These beetles carried diverse *Geosmithia* species but appeared not to carry *G. morbida* in this region. The absence of *G. morbida* and its vector (*P. juglandis*) from our survey is probably a reflection of the rarity of *Juglans* spp. in surveyed regions. Both appear to require *Juglans* or *Pterocarya* spp. for their development. *G. morbida* colonizes multiple *Juglans* and *Pterocarya* species (Utley et al., 2013; Hishinuma et al., 2016), but it does not affect other closely related genera such as *Carya* spp.

(Utley et al., 2013; Sitz et al., 2016). Similarly, the beetle can carry other *Geosmithia* species (Kolařík et al., 2017), but is not known to develop in hosts other than *Juglans* and *Pterocarya* spp. (Hefty et al., 2018). The strict association between *G. morbida* and its vector may therefore be a consequence of the specificity of both to *Juglans* and *Pterocarya* spp., not a consequence of an exclusive mutual relationship.

4.2. Beetle vector and tree host affinities with Geosmithia spp.

Based on our association analysis, three species in the *G. pallida* species complex (GPSC) showed affinities to certain tree hosts, i.e. *G. pallida* s str. and *G.* sp. 41 to *Q. laurifolia* and *G.* sp. 2 to
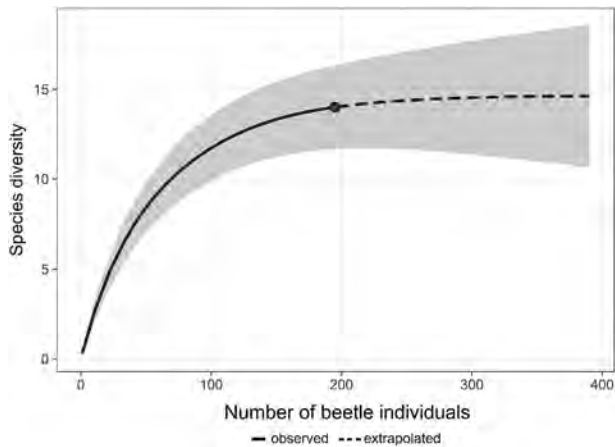
Fig. 5. Rarefaction curve of *Geosmithia* species isolated from 195 beetle specimens. Observed samples are denoted by a solid line and extrapolated segment extended to 390 samples by a dashed line. The 95% confidence intervals (shaded areas) were obtained by a bootstrap method based on 200 replications.

Table 3
Geosmithia specificity test using linear-model-based analysis showing the alpha-corrected p values of the occurrence differences of Geosmithia species on trees, beetles, and the interaction term. Note that the trees C. glabra and F. americana, and Geosmithia species with fewer than three isolates, were not included in the analysis (see methods).

	Trees	Beetles	Trees:Beetles
Multivariate	0.001*	0.891	0.135
G. brunnea	0.313	1	0.812
G. pallida	0.004*	0.961	0.812
G. sp.2	0.001*	0.974	0.812
G. sp.23	0.167	0.846	0.812
G. sp.41	0.001*	0.961	0.142
G. sp.12	0.001*	1	0.745
G. obscura	0.372	0.994	0.812
G.sp.45	0.041*	0.999	0.812
G.sp.46	0.167	0.999	0.812

* significant, P < 0.05.

J. virginiana. However, this was a statistical association, and whether this pattern is a reflection of true metabolic specificity will require future experimental tests. These three *Geosmithia* species were recovered occasionally from other tree than the one noted above, suggesting that these affinities for tree host species reflect some degree of preference, but not strict physiological specificity. Other studies also documented a broad host breath of most *Geosmithia*, particularly in the *pallida* species complex, which were recovered from various beetle vectors from diverse tree hosts including angiosperms and gymnosperms (Kolařík et al., 2008; Kolařík and Jankowiak, 2013). In our sample, the three cases of fungus-tree association that are statistically significant, are supported also by the fact that the three *Geosmithia* species were recovered from the same trees across multiple locations, indicating that their affinities for these trees is not a local phenomenon.

G. sp. 12 was reported to be specific to *Hylesinus* spp. from Oleaceae trees (e.g. *Fraxinus* spp.) as its almost exclusive recoveries from such beetle/tree combination (Kolařík et al., 2008, 2017). Similarly, a survey in the Southeastern USA had found *G.* sp. 12 were abundantly associated with *H. aculeatus* from *Fraxinus* sp. (Huang et al., 2018). We demonstrated that *G.* sp. 12 has a broader niche, having recovered it from diverse beetle species (*H. rotundicollis*, *H. dissimilis*, *M. nanula*, and *X. basilaris*) from two unrelated trees (*C. illinoinensis* and *Q. laurifolia*). The lack of specificity of *G.* sp. 12 to *Hylesinus* spp. with *Fraxinus* spp. in our sample may be a result of a

tree host switch of *G.* sp. 12 in this region.

Beetle species that infested the three Juglandaceae trees (*J. nigra*, *C. glabra*, and *C. illinoinensis*) were mostly polyphagous beetles. Except for the beetles from *C. glabra*, which had no associated *Geosmithia*, the *Geosmithia* community composition of beetles from *J. nigra* and *C. illinoinensis* were considerably different, even for those from the same geographical range. These results suggest that wood substrata of different tree species or the competitive capacity of fungi themselves might play a pivotal role in determining the *Geosmithia* community occurring on a given tree species. Some species in the Juglandaceae are known to produce toxic compounds, such as juglone, that displays inhibitory effect on other plants (Hejl et al., 1993). The chemical content of wood substrata might, therefore, play a role in selecting fungal colonizers (Lyr, 1962; Tsuneda and Kennedy, 1980; Dix, 2012).

We unveiled four putative new species, i.e. *G.* sp. 45—48, confirming the under-documented diversity of *Geosmithia* in the USA. *Geosmithia* sp. 46 and *G.* sp. 47 were both isolated from polyphagous beetles (*Hypothenemus* spp. and *P. minutissimus*) from various trees, and are therefore probably generalists. *Geosmithia* sp. 48 was found on the beetle *P. dentatus* which is specific to *J. virginiana*. While *P. dentatus* was also the vector of other *Geosmithia* species in our survey, *G.* sp. 48 might also be an opportunist vectored by *P. dentatus* on *J. virginiana*. *Geosmithia* sp. 45 was recovered exclusively from *P. taeda*. Three isolates of *G.* sp. 45 were recovered from the pine-specific bark beetles *P. annectens* and *P. pulicarius*. Interestingly, the pinaceous specificity was also suggested in *G.* sp. 44 and *G.* sp. 26 (Kolařík et al., 2013, 2017), the sister taxa of *G.* sp. 45. The distinct chemical environment in *Pinus* spp., resulting from its diverse defensive compounds such as terpenes (Bridges, 1987; Zulak and Bohlmann, 2010) and other defensive mechanisms (Ralph et al., 2006) may be the reason for ecological speciation and evolution of specificity of several *Geosmithia* on hosts in the Pinaceae (Kolařík and Jankowiak, 2013). Host preferences for either hardwoods or for conifers have also been demonstrated in many ophiostomatoid fungi (Harrington et al., 2001; Grobbelaar et al., 2009; De Beer et al., 2014; Jankowiak et al., 2017b). Although *G.* sp. 45 showed a similar specificity to Pinaceae as the closely related *G.* sp. 44, its recovery rate was significantly lower than that of *G.* sp. 44 in a survey of the western USA (Kolařík et al., 2017). The discrepancy might represent the low abundance of this fungus in the Southeast or it might be a consequence of sampling differences between the two studies.

5. Conclusion

The association among fungi, bark beetle vectors, and host trees is often thought to be complicated, but continued sampling is beginning to explain the patterns in these relationships. This dataset suggests that the distribution of *Geosmithia* among beetle vectors is primarily driven by the encounters of the beetles and the fungi in the hosts tree substrates, and in some cases by phylogenetic relatedness between the fungi, but the fungus-beetle associations are flexible. Moreover, as the beetles transmit the fungi among trees passively, a large part of the resulting community patterns may be due to neutral processes. The subcortical fungal genus *Geosmithia* is a system superbly suitable for studying the tripartite tree-vector-fungus dynamics due to the ease of its sampling, increasingly better taxonomy, and a convenient level of diversity: even in a limited region of the southeastern USA, the community includes specialists and generalists, commensals, mutualists and parasites. By implementing proper sampling strategies, analytical methods, and molecular tools, the *Geosmithia* system can help us unveil the true dynamics behind the relationship of this tripartite fungus-vector-host symbiosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2019.02.005.

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