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Symbiosis and pathogenicity of *Geosmithia* and *Talaromyces* spp. associated with the cypress bark beetles *Phloeosinus* spp. and their parasitoids

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Summary

Fungi associated with cypress bark beetles are practically unknown in the Eastern Mediterranean. Our study focused on the fungi associated with the body parts and galleries of two indigenous cypress bark beetles, *Phloeosinus armatus* and *P. bicolor*, sampled from *Cupressus sempervirens* trees in different regions in Israel. Arbitrarily primed PCR, performed on genomic DNA of 302 isolates, clustered the fungal population into five distinct groups. Multilocus phylogeny, split-network analyses and morphological characterization identified the isolates as *Geosmithia omnicola*, *Geosmithia langdonii*, *Geosmithia* sp. 708b, *Geosmithia cupressina* sp. nov. CBS147103 and *Talaromyces cupressi* sp. nov. CBS147104. Of these fungal isolates, *G. cupressina* and *T. cupressi* are newly described, and their morphological features and phylogenetic designations are presented. Inoculation of intact cypress saplings in an outdoor net-house revealed that only the representative isolate *T. cupressi* sp. nov. CBS147104 causes 100% disease incidence, whereas *Geosmithia* spp. isolates are not pathogenic. A number of these fungi were isolated from parasitoids that emerged from branch and stem sections colonized by *P. armatus*. This study suggests a long and stable association between *Phloeosinus* and *Geosmithia* species, and a possible role for additional associated fungal species as pathogens or endophytes of *C. sempervirens* trees in Israel.

Introduction

A striking characteristic of the bark beetle (Curculionidae, Scolytinae) is its widespread association with fungi, mainly members of the Ascomycetes (Six, 2012). These diverse and complex relationships are well-documented for bark beetle development on trees of the Pinaceae (Six and Wingfield, 2011; Dohet *et al*., 2016). However, knowledge of the interactions between bark beetles and fungi associated with members of the Cupressaceae family is scarce. Roughly 115 bark beetle species, belonging to six genera, are associated with the Cupressaceae, compared to approximately 610 belonging to ca. 50 genera affiliated with the Pinaceae (Wood, 1986; Wood and Bright, 1992); nearly 90% of those associated with the Cupressaceae belong to the tribe *Phloeosinini*. The two presently studied bark beetles belong to the genus *Phloeosinus* Chapuis. Nine *Phloeosinus* spp. are known from the Mediterranean area; five of them breed on several genera of the Cupressaceae, three on *Cedrus* (Pinaceae) and one on *Pinus* (Wood and Bright, 1992; Pfeffer, 1995; Faccoli and Sidoti, 2013). However, global climate change is reshaping the distribution of *Phloeosinus* spp. in the Mediterranean region and Europe. For example, there are extensive reports of *P. bicolor*, *P. rudis* and *P. thujae* in Western and Central Europe (Moraal, 2010; Fiala and Holuša, 2019), whereas in Israel, the activity of *P. armatus* has markedly increased while that of *P. bicolor* has become uncommon (Z. Mendel, unpublished data).

Information on the association of particular fungal species with *Phloeosinus* spp. is limited; nevertheless, *Geosmithia* spp. have often been recovered from various congeners, i.e. *P. dentatus*, *P. thujae*, *P. fulgens*, *P. cupressi*, *P. sequoia*, *P. canadensis*, *P. punctatus*, *P. deleoni* and *P. serratus* in North America (Huang *et al*., 2017, 2019; Kolařík *et al*., 2017; Hernández-García *et al*., 2020), and *P. henschi* and *P. thujae* in Western Europe and the Mediterranean region (Kolařík *et al*., 2007, 2008). Kolařík *et al*. (2007) suggested that *Geosmithia* spp. have a limited area of

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distribution, probably due to their dependency on the geographical location of their vectors. However, information regarding the role of *Geosmithia* spp. with bark beetles (Scolytinae) in general is limited; Kolařík *et al*. (2008) proposed that associations between *Geosmithia* spp. and bark beetles may have been very stable and that symbioses became a fundamental factor in the speciation of *Geosmithia*.

Geosmithia Pitt (1979) is a highly diverse, globally distributed, polyphyletic genus of mitosporic filamentous fungi that is found in close association with subcortical insects (Kolařík *et al*., 2005; Kolařík *et al*., 2008). The genus was established by Pitt in 1979 for fungi formerly placed in the genus *Penicillium*. *Geosmithia* is characterized by long *Penicillium*-like smooth to rough-walled conidiophores with cylindrical phialides and globose to ellipsoidal or cylindrical conidia arranged in long chains (Pitt, 1979; Kolařík *et al*., 2004; Kolařík *et al*., 2005; Kolařík *et al*., 2008; Kolařík and Kirkendall, 2010). Members of *Geosmithia* produce dry and hydrophobic conidia, in contrast to other entomochoric species such as *Ophiostoma* which form sticky conidia (Kolařík *et al*., 2017). Jankowiak and Rossa (2008) hypothesized that bark beetles breeding in drier substrates are unable to maintain mutualism with ophiostomatoid fungi, and are thus involved in symbioses with *Geosmithia* spp., indicating that these latter fungi are very well-adapted to colonization of dry tree tissues. *Geosmithia* spp. are predominantly associated with insects, including bark and ambrosia beetles (Kolařík *et al*., 2005; Kolařík and Kirkendall, 2010; Dori-Bachash *et al*., 2015; Huang *et al*., 2017). Apart from insect colonization, they survive and proliferate on other substrates such as plant debris, soil and cereals, and as endophytes (Kolařík *et al*., 2004; Kolařík *et al*., 2008; Pitt and Hocking, 2009; Kolařík and Jankowiak, 2013; McPherson *et al*., 2013). *Geosmithia* and their bark beetle vectors colonize various hosts, including pines (Jankowiak and Rossa, 2008; Dori-Bachash *et al*., 2015), oaks (McPherson *et al*., 2013), junipers (Hernández-García *et al*., 2020) and walnuts (Kolařík *et al*., 2011). Several bark beetles have been associated as vectors of *Geosmithia* spp. in Asia, Australia, and North and South America, with over 32 species detected in Europe (Kirschner, 2001; Kolařík *et al*., 2004; Kubatova *et al*., 2004; Kolařík *et al*., 2005; Kolařík *et al*., 2007; Kolařík *et al*., 2008; Kolařík *et al*., 2011; Dori-Bachash *et al*., 2015). The structure of *Geosmithia* communities in Europe suggests a high fidelity in association by both the fungal and beetle partners (Pepori *et al*., 2015). The relationship between *Geosmithia* and its associated beetles ranges from obligatory to incidental, and from mutualism to commensalism or antagonism (Huang *et al*., 2017; Jankowiak and Bilański, 2018). Most species are presumably commensals, because their beetle associates do not display any obvious morphological adaptations for *Geosmithia* vectoring, or any nutritional dependence. However, some *Geosmithia* spp. have evolved into morphologically modified nutritional ambrosial species (Kolařík and Kirkendall, 2010), with traits that are convergent with many other ambrosia fungi (Kasson *et al*., 2013; Hulcr and Stelinski, 2017). Furthermore, *Geosmithia* congeners exhibit various degrees of specificity with their beetle vectors, ranging from specialists with limited distribution on vectors that feed on a single plant genus/family, to generalists, i.e. associated with numerous vectors, to species that are not associated with insects at all (e.g. living as saprophytes and/or endophytes) (Kolařík *et al*., 2004; Pitt and Hocking, 2009; McPherson *et al*., 2013; Kolařík *et al*., 2017). Although most of the *Geosmithia* spp. are saprophytic in nature, *G. morbida* (Kolařík, Freeland, Utley and Tisserat) (Tisserat *et al*., 2009) and *Geosmithia* sp. 41 (Lynch *et al*., 2014; Kolařík *et al*., 2017) are pathogenic on black walnut (*Juglans nigra*) and coast live oak (*Quercus agrifolia*) respectively. However, both of these *Geosmithia* species live saprophytically in association with bark beetles and other tree hosts. The mechanism determining pathogenicity of these *Geosmithia* species to new hosts, as opposed to other members of the genus that survive as saprophytes, remains unclear. Genome-sequencing analyses of *G. morbida*, the causal agent of thousand canker disease of walnut and wingnut in the USA and Europe respectively, revealed a smaller genome compared to those of several of its closely related non-pathogenic species of the order. In comparison to other species, it is also characterized by its ability to degrade the lignocellulose complex (Veselská *et al*., 2019). Thus, it is plausible to assume that over the course of evolutionary adaptation, *G. morbida* may have emerged as a pathogen, although this modification has not yet been characterized (Schuelke *et al*., 2016, 2017).

Bark beetles play a vital role in *Geosmithia* conidial dispersion (Pepori *et al*., 2015). Several hypotheses have been suggested regarding the effect and ecological role of *Geosmithia* on host trees, but no substantial evidence has been provided. Some studies have suggested that these fungi serve as nutritional symbionts for ambrosia beetles (Kolařík and Kirkendall, 2010), whereas others suggest that the plant-pathogenic *Geosmithia* spp. increase the overall fitness of the vector, as found in the case of *G. morbida* vectored by *Pityophthorus juglandis* (Tisserat *et al*., 2009; Montecchio *et al*., 2014). *Geosmithia pallida* (Kolařík, Kubátová and Pažoutová) isolated from *Scolytus intricatus* produces toxins that inhibit root formation in *Lepidium sativum* (Cízková *et al*., 2005). Similarly, *G. pallida* vectored by *Pseudopityophthorus pubipennis* has been reported as a pathogen of oak trees in California (Lynch *et al*., 2014). In contrast, *G. langdonii* (Kolařík, Kubátová and Pažoutová) was

identified as a non-pathogenic associate of the bark beetle, and also as an endophyte of coast live oaks in California (McPherson *et al*., 2013). Another study reported isolation of *G. langdonii* and other *Geosmithia* spp. from *Ulmus minor* afflicted with Dutch elm disease in Switzerland, although their role was not described (Hänzi *et al*., 2016). Furthermore, *G. langdonii* and *G. levendula* produce bioactive compounds with antimicrobial and antileishmanial activities (Stodulkova *et al*., 2010; Malaka *et al*., 2013). Several species of *Geosmithia* can also inhibit phoretic mites (Machingambi *et al*., 2014). Although the above studies reflect several roles for *Geosmithia*, beneficial aspects of the association with bark beetles remain unclear.

The objective of this study was to identify and characterize fungi associated with the cypress bark beetles *P. armatus* and *P. bicolor* and their hymenopteran parasitoids, and isolated from the beetle gallery zone in the host tree *C. sempervirens*. We also describe and illustrate two new fungal species, *G. cupressina* and *T. cupressi*, characterize their morphology and infer their molecular phylogenetic designations. Interaction of the isolated fungi with the host tree was also assessed. Special attention was paid to the association of *Talaromyces cupressi* sp. nov. with the studied cypress bark beetles, as it is recorded for the first time in the present study.

Results

Isolation of beetle-associated fungi

In total, 167 samples from 10 locations infested by two species of bark beetle, *P. armatus* and *P. bicolor*, were collected during the study. Altogether, 302 fungal strains were isolated from the adult bark beetle species (163), their larvae (19) and their galleries (120) (Table 1). Specifically, 28.5% and 25.5% of the fungal strains were isolated from adult beetles of *P. armatus* and *P. bicolor* respectively, 6.3% from their larvae, and 24.2% and 15.6% originated from the galleries of *P. armatus* and *P. bicolor* respectively. Almost all of the fungal isolates exhibited characteristic *Geosmithia* colony morphologies (floccose to velutinous and/or powdery, moderately growing mycelial colonies with penicillius subtending conidial chains), categorizing them into five groups. Among them, four groups produced white mycelia with cylindrical to ellipsoidal conidia, whereas the fifth group produced white mycelia that turned green upon sporulation, containing rod-shaped conidia.

Genetic diversity of the fungal isolates

Amplification products were obtained for all 302 isolates collected in this study using three arbitrarily primed (Ap)-PCR primers: $(CAG)_5$, $(GACA)_4$ and $(GACAC)_3$ (Supporting Information Fig. S1a). A high level of genetic diversity was observed, categorizing the isolates into five distinct genetic groups (304a, 516a, 701a, 701c, 708b) (Supporting Information Fig. S1b). Fourteen representative isolates were then selected from the five genetically distinct groups for further identification and characterization based on multigene phylogenetic analyses, pathogenicity testing and morphological characterization.

Multigene phylogenetic analysis of the representative isolates

Maximum parsimony and split-network analyses for the Geosmithia isolates. The multilocus sequence alignment was comprised of 58 nucleotide sequences and 2394 characters [nuclear ribosomal internal transcribed spacer

Table 1. Sample collection sites, collection dates and isolate frequencies.

Collection site	Collection date	Geographical coordinates	Number of fungal isolates from each beetle species					Total
			P. armatus	*P. bicolor*	*P. bicolor*#	*P. armatus**	*P. bicolor**	
1. Nahsholim	Aug. 2017	32°36′51″N; 34°55′17″E	5	10	15	13	6	49
2. Eshtaol	Sept. 2017	31°46′51″N; 35°36′36″E	10	10	0	8	4	32
3. Gan Shmuel	Oct. 2017	32°27′11″N; 34°57′01″E	0	0	4	2	2	8
4. Natur	Oct. 2017	32°51′12″N; 35°45′13″E	6	8	0	0	0	14
5. Sharon	Oct. 2017	32°34′62″N; 34°89′54″E	21	10	0	5	11	47
6. Givat Haim Meuhad	Nov. 2017	32°23′30″N; 34°55′48″E	7	8	0	7	4	26
7. Or HaNer	May 2018	31°33′27″N; 34°36′7.19″E	15	14	0	22	12	63
8. Merom Golan	Jun. 2018	33°08′00″N; 35°46′33″E	11	9	0	11	3	34
9. Volcani Centre, ARO	Jul. 2018	31°59′25″N; 34°49′84″E	5	0	0	0	0	5
10. Cabri (western Galilee)	Mar. 2019	33°15′23″N; 35°8′56″E	11	8	0	0	5	24
Total number of fungal isolates			86	77	19	73	47	**302**

#,*Fungal isolates originating from larvae# and galleries* of beetle species.

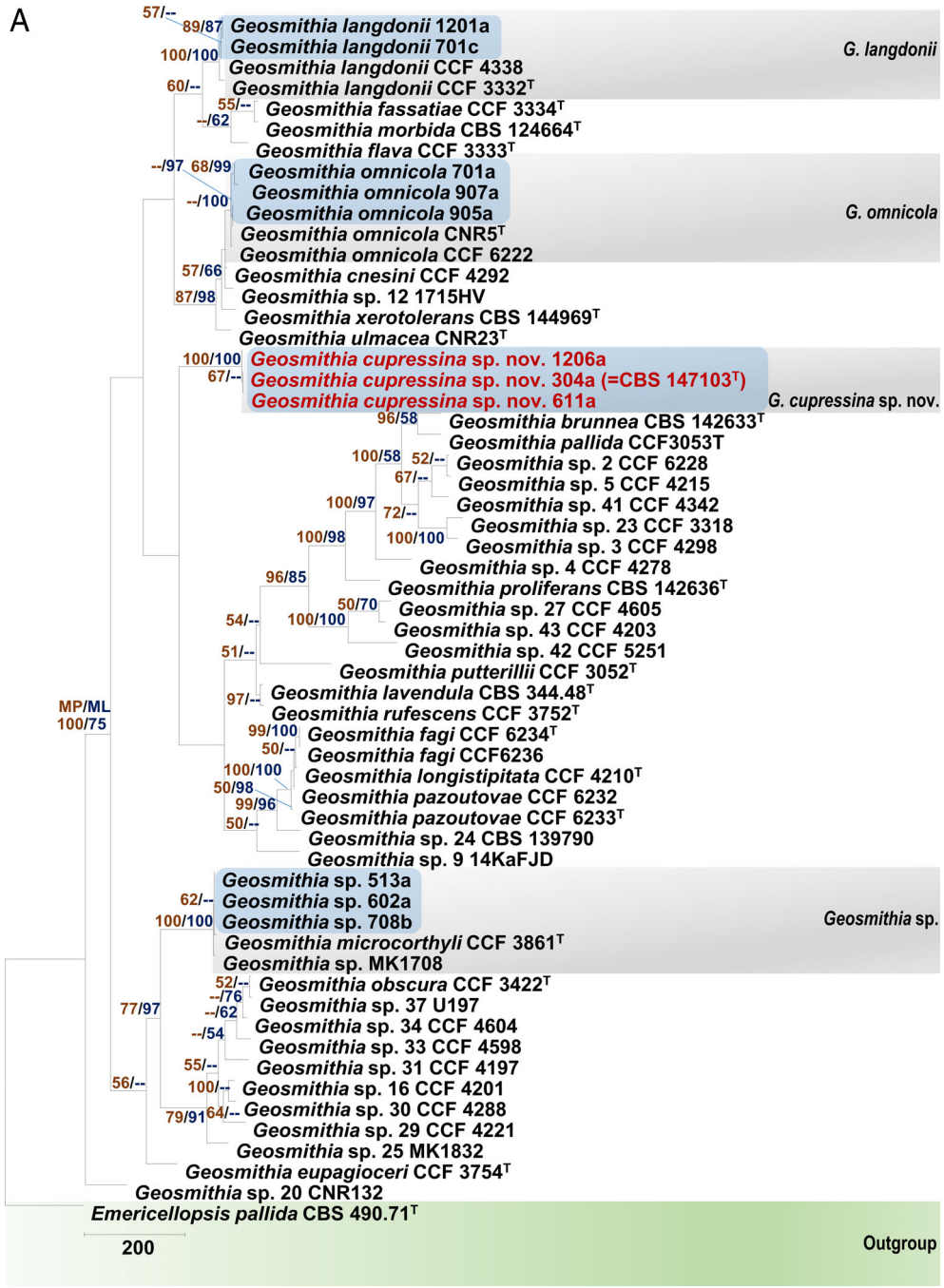

Fig. 1. A. Maximum parsimony tree showing phylogenetic affinities of *Geosmithia* isolates from this study (highlighted in blue rectangles with rounded corners), obtained from heuristic search of the ITS, RPB2 and Tub2 dataset. *Emericellopsis pallida* is the outgroup taxon and bootstrap support values (MP/ML) >50% are shown at the nodes (^{T} = ex-type strains).
B. SplitsTree Neighbour-Net graph of *Geosmithia* isolates from this study (^{T} = ex-type strain).

(ITS) region, 1–593; RNA polymerase II second largest subunit (RPB2), 594–1494; β-tubulin (Tub2), 1495–2394], including gaps. Translation elongation factor 1-alpha (EF1α) sequences were not used in the analysis due to an incomplete dataset for reference sequences. A total of 76 characters from the ambiguously aligned regions were excluded from the maximum parsimony (MP) analysis and of the remaining 2318 characters processed, 800 were parsimony-informative, 308 parsimony-uninformative and 1210 constant (Treebase 28 461). The number of rearrangements assessed in the heuristic search was 121 281 192, and the resulting tree is presented in Fig. 1A

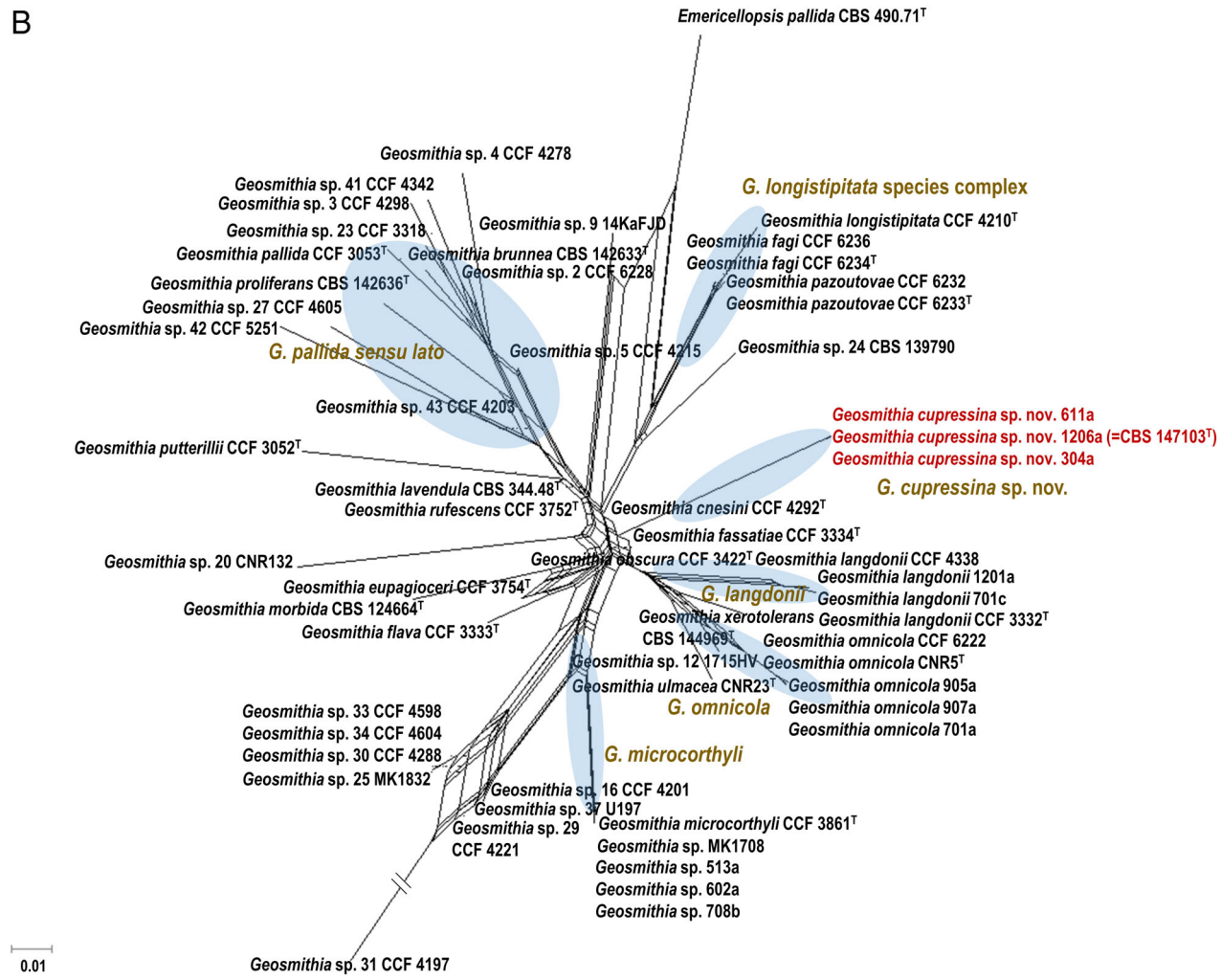

Fig. 1. (*Continued*)

[tree length (TL) = 3603, consistency index (CI) = 0.514, retention index (RI) = 0.766, rescaled consistency index (RC) = 0.394, homoplasy index (HI) = 0.486]. *Emericellopsis pallida* was included as an outgroup taxon in the analysis. The observed bootstrap support of the branches as obtained by MP and maximum likelihood (ML) analyses is shown next to the branches. All of the observed clades were well-supported. Based on the MP and ML phylogenetic analyses, three *Geosmithia* isolates – 701a, 905a and 907a – clustered with the ex-type isolate of *G. omnicola*; two isolates – 701c and 1201a – clustered with the ex-type isolate of *G. langdonii*; and three isolates – 513a, 602a and 708b – clustered with the ex-type isolate of *G. microcorthyli*. In addition, another strongly supported clade [bootstrap value = 100% (both MP/ML)] was obtained, which did not cluster with any of the reference ex-type strains of *Geosmithia*. This novel clade was comprised of three isolates: 304a (=CBS147103)^T, 611a and 1206a, and is described in this study as *Geosmithia cupressina* sp. nov. CBS147103. Neighbour-Net analysis showed similar phylogenetic relationships (Fig. 1B), and no evidence of recombination within the species groups was found based on pairwise homoplasy index (PHI) test ($p = 1.0$). This further validates the novelty of *Geosmithia cupressina* sp. nov. CBS147103.

Maximum parsimony and split-network analyses for the Talaromyces isolates. The multilocus sequence alignment was comprised of 33 nucleotide sequences and 2623 characters [β-tubulin (BenA), 1–549; calmodulin (CaM), 550–1153; ITS, 1154–1807; RPB2, 1808–2623], including gaps. A total of 68 characters from the ambiguously aligned regions were excluded from the MP analysis and of the remaining 2555 characters processed, 1073 were parsimony-informative, 265 parsimony-uninformative and 1217 constant. The number of rearrangements assessed in the heuristic search was 290 954 and five trees were generated with no significant

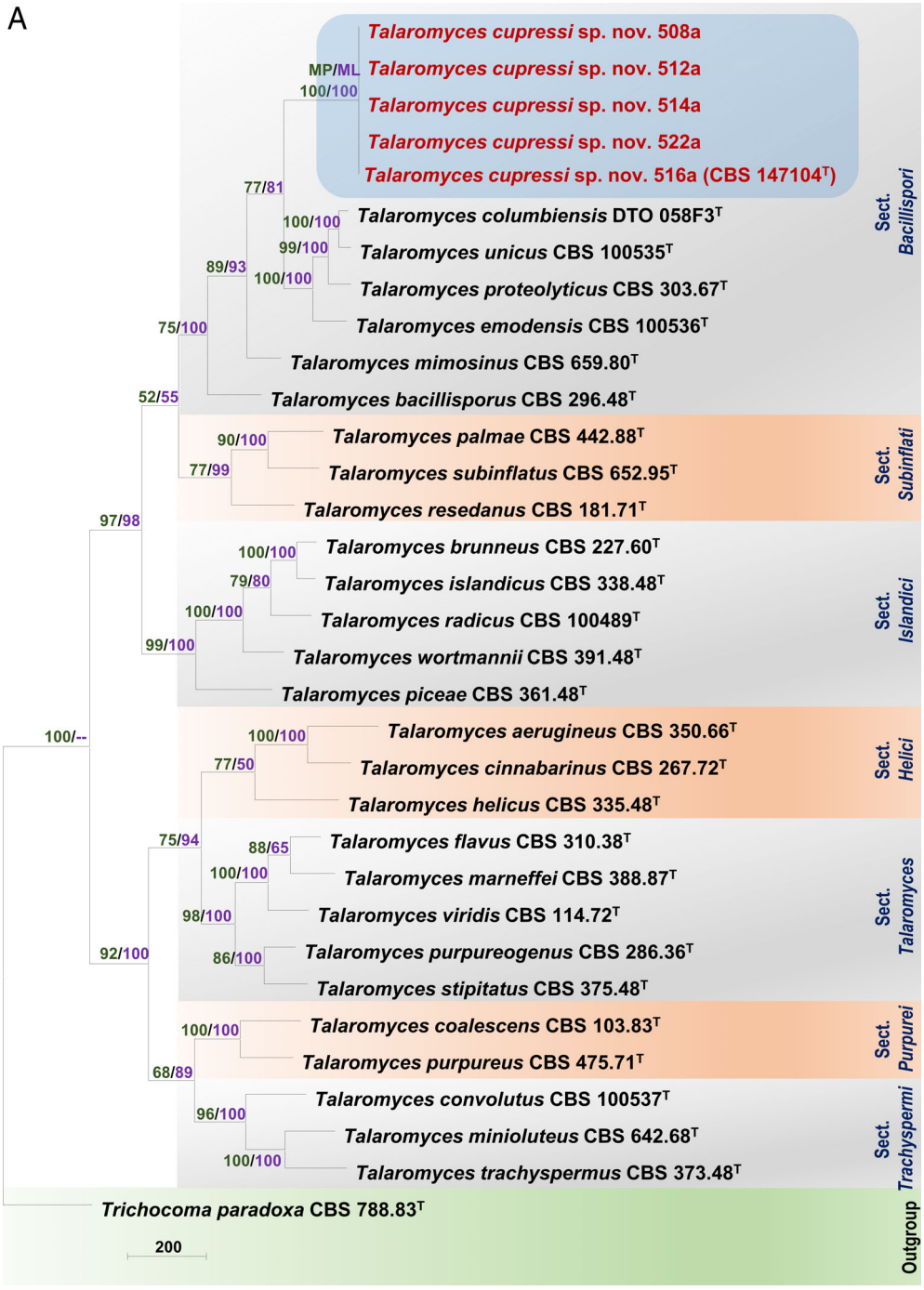

Fig. 2. A. Maximum parsimony tree showing phylogenetic affinities of *Talaromyces* isolates from this study (highlighted in blue rectangles with rounded corners), obtained from heuristic search of the ITS, BenA, CaM and RPB2 datasets. *Trichocoma paradoxa* is the outgroup taxon and bootstrap support values (MP/ML) >50% are shown at the nodes (^{T} = ex-type strains).
B. SplitsTree Neighbour-Net graph of *Talaromyces* isolates from this study (^{T} = ex-type strain).

difference in tree topologies (data not shown) (Treebase 28 461). One of the resulting trees is presented in Fig. 2A (TL = 6277, CI = 0.399, RI = 0.559, RC = 0.223, HI = 0.601). The phylogenetic tree was comprised of seven clades belonging to *Talaromyces* section *Trachyspermi*, *Purpurei*, *Talaromyces*, *Helici*, *Islandici*, *Subinflati* and *Bacillispori*. *Trichocoma paradoxa* was included as an outgroup taxon in the analysis. The observed bootstrap support of most branches was high (>70%). Based on the MP and ML phylogenetic analyses, all five

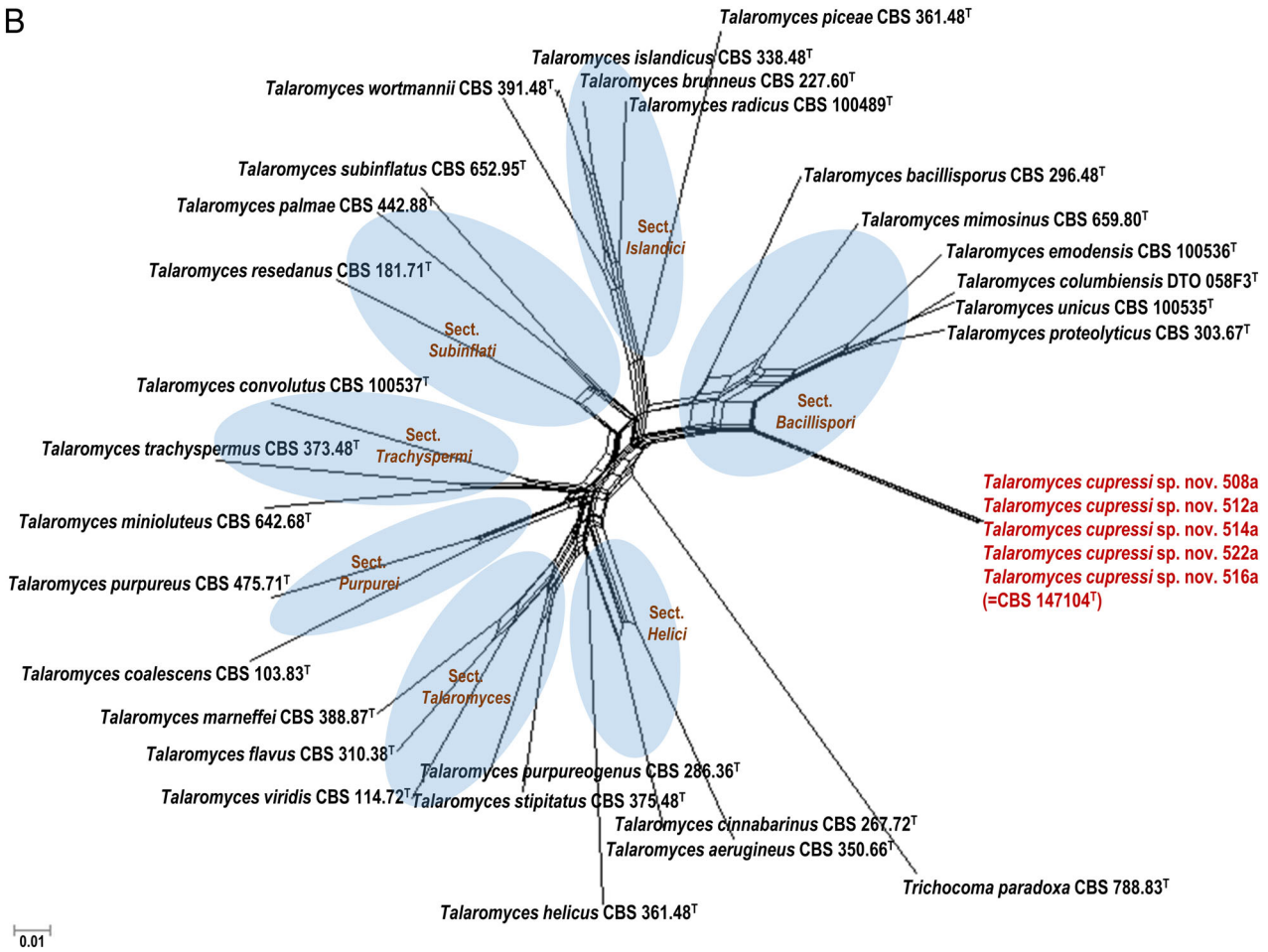

Fig. 2. (*Continued*)

representative *Talaromyces* isolates in this study formed a strongly supported clade (MP/ML bootstrap value = 100%) within section *Bacillispori* and did not cluster with any of the existing ex-type strain sequences of this section. Neighbour-Net analysis showed similar phylogenetic relationships (Fig. 2B), and no evidence of recombination within the species groups was found based on the PHI test ($p = 0.9988$). Phylograms obtained based on single gene sequences (data not shown), in addition to the multilocus sequence dataset, supported the novelty of the five isolates [508a, 512a, 514a, 516a (=CBS147104)^T, 522a]. Consequently, based on morphology and genetic characters, the new species was described in this study as *Talaromyces cupressi* sp. nov. (CBS147104).

Taxonomy

Geosmithia cupressina. V. Meshram, M. Maymon, G. Sharma, A. Protasov, Z. Mendel and S. Freeman sp. nov. – MycoBank: MB837645, Fig. 3.

Etymology – Latin '*cupressina*' refers to the host plant *Cupressus sempervirens*

Type – ISRAEL, Givat Haim Meuhad, Cabri isolated from *P. bicolor* (Coleoptera; Scolytinae), colonizing *C. sempervirens* L. (Cupressaceae), 3 Oct. 2017, isolated by Vineet Meshram, leg. Stanley Freeman (Holotype: 304a, ex-type culture: CBS147103, HUJIHERB-913460)

Teleomorph – not observed

Gene sequences – ex-holotype: MT955332 (ITS), MT991505 (EF1α), MT991483 (RPB2), MT991494 (Tub2)

Description – Colony diameter, 7 d (mm): Czapek yeast autolysate agar (CYA) 37–40; CYA 37°C 10–14; CYA 40°C no growth; CYA + NaCl 36–38; Blakeslee's malt extract agar (MEAbi) 35–37; malt extract agar (MEA) 34–38; oatmeal agar (OA) 25–27; creatine sucrose agar (CREA) 34–37

Colony characters – CYA 25°C, 7 d: colonies low, plane, sulcate, sunken at centre, velutinous, moderately growing, margins low, entire (1 mm), mycelia white, sporulation moderate, white, soluble pigment absent, droplets of exudate absent, reverse pale yellow. CYA + NaCl

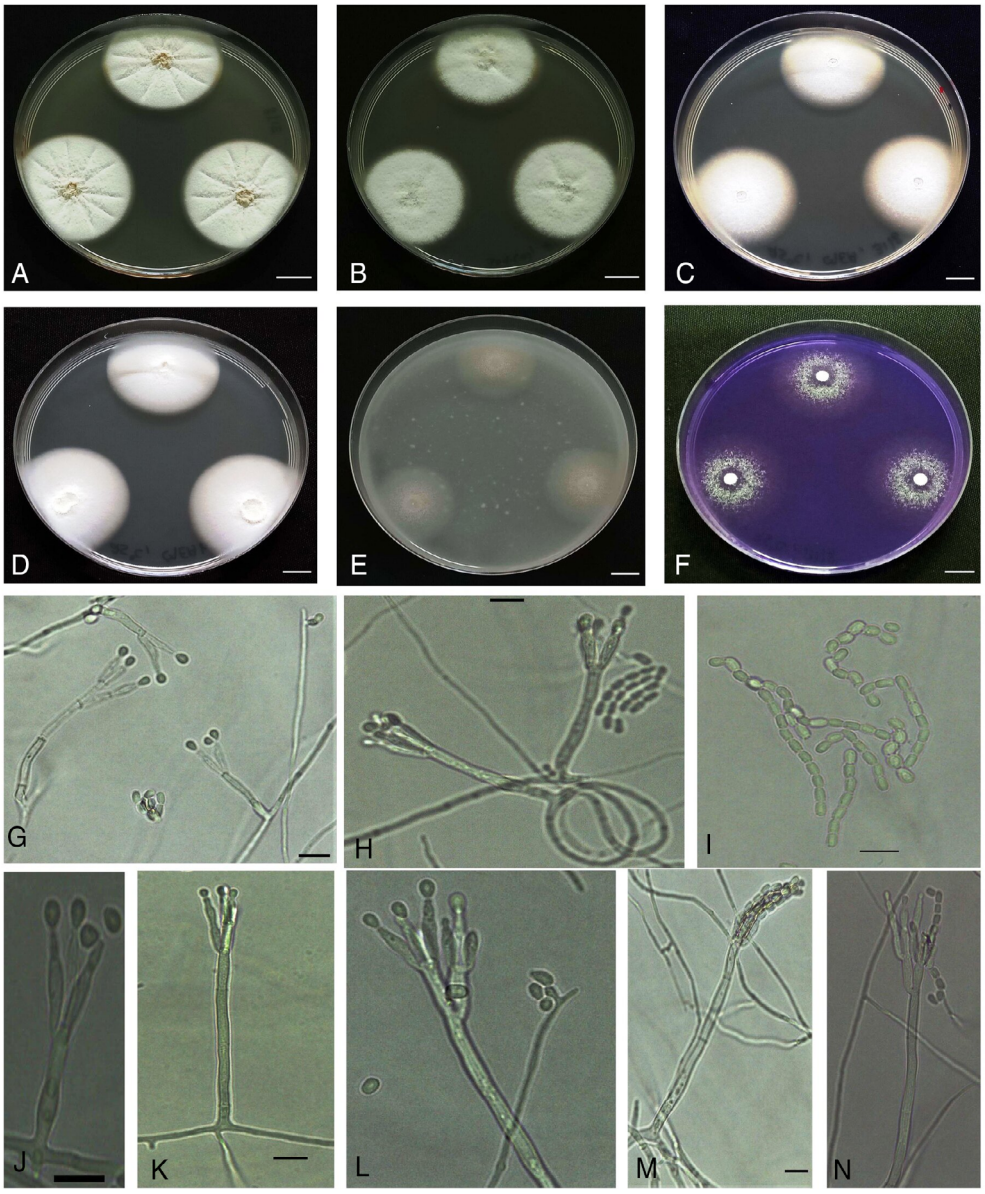

Fig. 3. *Geosmithia cupressina* sp. nov. CBS147103.
A–F. Colonies at 25°C after 7 d. (A) CYA, (B) CYA + NaCl, (C) MEAbi, (D) MEA, (E) OA, (F) CREA.
G–K. Monoverticillate conidiophore.
M. Monoverticillate and (L, N) biverticillate conidiophore, conidiophore forming conidial chains.
I. Conidia. Bars, A–F = 90 mm, G–N = 10 μm.

25°C, 7 d: colonies low, slightly sulcate, sunken at centre, texture velutinous to loosely funiculose, margins low, entire (1–2 mm), mycelia white, sporulation moderate, white, soluble pigment absent, droplets of exudate absent, reverse pale yellow. MEAbi 25°C, 7 d: colonies low, plane, sunken at centre, margins entire (1–2 mm), mycelia white, velutinous, sporulation dense, white, soluble pigment absent, droplets of exudate absent, reverse pale yellow at centre, white at margins. MEA 25°C, 7 d: colonies low, plane, margin entire, mycelia white, velutinous to loosely funiculose, sporulation dense, white, soluble pigment absent, droplets of exudate absent, reverse pale yellow. OA 25°C, 7 days: colonies low, plane, moderately growing, margins low, entire, mycelia white, sporulation sparse to moderate, white, pigment absent, droplets of exudate absent, reverse white. CREA 25°C, 7 d: colonies low, plane, moderately growing, margins low, entire, no acid production.

Micromorphology – Hyphae branched, hyaline, septate (1.6)- 2.61 ± 0.67 -(3.9) μm thick, conidiophore smooth-walled, mostly monoverticillate to biverticillate with minor portions having subterminal branching, stipes

smooth-walled, (21.4)- 42.84 ± 14.29 -(73.7) × (2.3)- 3.61 ± 0.65 -(4.9) μm; metulae 2–4, divergent, (7.5)- 10.18 ± 1.4 -(12.9) × (1)- 2.8 ± 0.74 -(4.1) μm; phialides cylindrical, 3–6 per metula, (7.4)- 12.21 ± 2.73 -(16.2) × (1.5)- 2.46 ± 0.49 -(3.7) μm; conidia smooth, ellipsoidal to cylindrical, (3.1)- 4.02 ± 0.67 -(5.1) × (2.24)- 2.93 ± 0.49 -(4.1) μm; ascomata not observed.

Distinguishing features – *Geosmithia cupressina* sp. nov. CBS147103 is characterized by white, velutinous to loosely funiculose colonies on most media and by growth at 37°C. Among all described species, only *G. carolliae* (Cunha *et al*., 2018) and *Geosmithia lavendula* Pitt (Pitt, 1979) tolerate 37°C, but they can be distinguished by distinct reddish colour of sporulation. *Geosmithia cupressina* sp. nov. CBS147103 produces mono-biverticillate conidiophores, whereas *G. langdonii*, *G. morbida*, *G. omnicola* and *G. flava* produce biverticillate to hexaverticillate conidiophores. Furthermore, *G. cupressina* sp. nov. CBS147103 produces ellipsoidal to cylindrical conidia, whereas species like *G. eupagioceri*, *G. microcorthyli* and *G. flava* produce globose, subglobose or doliiform conidia and *G. fassatiae* forms subglobose or barrel-shaped conidia (Supporting Information Table S1).

Talaromyces cupressi. V. Meshram, M. Maymon, G. Sharma, A. Protasov, Z. Mendel and S. Freeman sp. nov. – MycoBank; MB837646, Fig. 4.

Etymology – Latin '*cupressi*' refers to the host plant *Cupressus sempervirens*

In: *Talaromyces* section *bacillispori*

Type – ISRAEL, Sharon, isolated from *P. bicolor* (Coleoptera; Scolytinae), colonizing *C. sempervirens* L. (Cupressaceae), 18 Oct. 2017, isolated by Vineet Meshram, leg. Stanley Freeman (Holotype: 516a, ex-type culture: CBS147104, HUJIHERB-913461)

Teleomorph – not observed

Gene sequences – ex-holotype: MT955352 (ITS), MT991517 (CaM), MT991522 (RPB2), MT991527 (BenA)

Description – Colony diameter, 7 d (mm): CYA 24–26; CYA 37°C 15–18; CYA + NaCl 9–12; CYA + NaCl 37°C no growth; MEAbi 29–31; MEA 26–28; OA 19–22; CREA 11–13

Colony characters – CYA 25°C, 7 d: colonies low, slightly raised at centre, floccose to funiculose, moderately growing, margins low, entire (1–2 mm), mycelia white, sporulation moderate, white, soluble pigment absent, droplets of exudate absent, reverse pale yellow. CYA + NaCl, 25°C, 7 d: colonies low, slow growing, texture floccose, margins low, entire (1 mm), mycelia white, both front and reverse, sporulation moderate, white, pigment absent, droplets of exudate absent. MEAbi 25°C, 7 d: colonies low, plane, slightly sulcate, sunken at centre, margins entire (1–2 mm), mycelia white, floccose, sporulation dense, white, pigment absent, droplets of exudate absent. MEA 25°C, 7 d: colonies low, slightly raised at the centre, margin entire (1–2 mm), mycelia light orange, floccose, sporulation dense, white, pigment absent, droplets of exudate absent, reverse pale orange at centre, white at margins. OA 25°C, 7 d: colonies low, plane, moderately growing, margins low, entire, mycelia white, sporulation sparse to moderate, pigment absent, droplets of exudate absent, reverse white. CREA 25°C, 7 d: colonies low, plane, slow growing, margins low, entire, strong acid production.

Micromorphology – Hyphae long, branched, hyaline, coenocytic (2.1)- 3.24 ± 0.55 -(4.3) μm thick; conidiophores with solitary phialides or biverticillate; stipes smooth-walled (13.4)- 21.06 ± 7.26 -(36.1) × (1.8)- 3.02 ± 0.51 -(3.9) μm, phialides acerose, 1–2, (27.7)- 36.5 ± 11.33 -(60.2) × (1.4)- 2.52 ± 0.59 -(3.4) μm; conidia arranged in chain over phialides, sometimes intertwined, rod-shaped, (3.1)- 4.55 ± 0.68 -(5.8) × (1.1)- 2.06 ± 0.45 -(2.9) μm; ascomata not observed.

Distinguishing features – *Talaromyces cupressi* sp. nov. CBS147104 is characterized by the presence of conidiophores with solitary phialides over which rod-shaped conidia lie that sometimes intertwine to form long conidial chains. Unlike most of the members (*T. bacillisporus*, *T. emodensis*, *T. mimosinus* and *T. unicus*) of the *Bacillispori* section, *T. cupressi* sp. nov. CBS147104 does not produce ascomata. *T. cupressi* sp. nov. CBS147104 grows faster than *T. columbiensis*, *T. emodensis*, *T. mimosinus*, *T. unicus* and *T. proteolyticus* at 37°C on CYA. Furthermore, *T. cupressi* sp. nov. CBS147104 differs from *T. emodensis*, *T. mimosinus* and *T. bacillisporus* by acid production on CREA (Supporting Information Table S2).

Diversity analysis of fungal isolates

A total of 302 fungal isolates from 10 different collection sites were analysed from five different host tissues including the beetles, their larvae and galleries, and were further classified into five different fungal species. Among these five, four were *Geosmithia* spp. and the remaining one was associated with *Talaromyces* species. *Geosmithia omnicola* was the most dominant species, although it showed a distinctly different relative abundance (RA) (4%–100%) at each of the sampling sites. *Geosmithia* sp. 708b (11.11%–75%) was the second-most dominant species, isolated from eight sampling sites, followed by *G. cupressina* sp. nov. CBS147103 (10.2%–48.6%) and *G. langdonii* (20.83%–32.35%) isolated from seven and three sampling sites respectively. *Talaromyces cupressi* sp. nov. CBS147104 was the least frequent species and was collected from a single

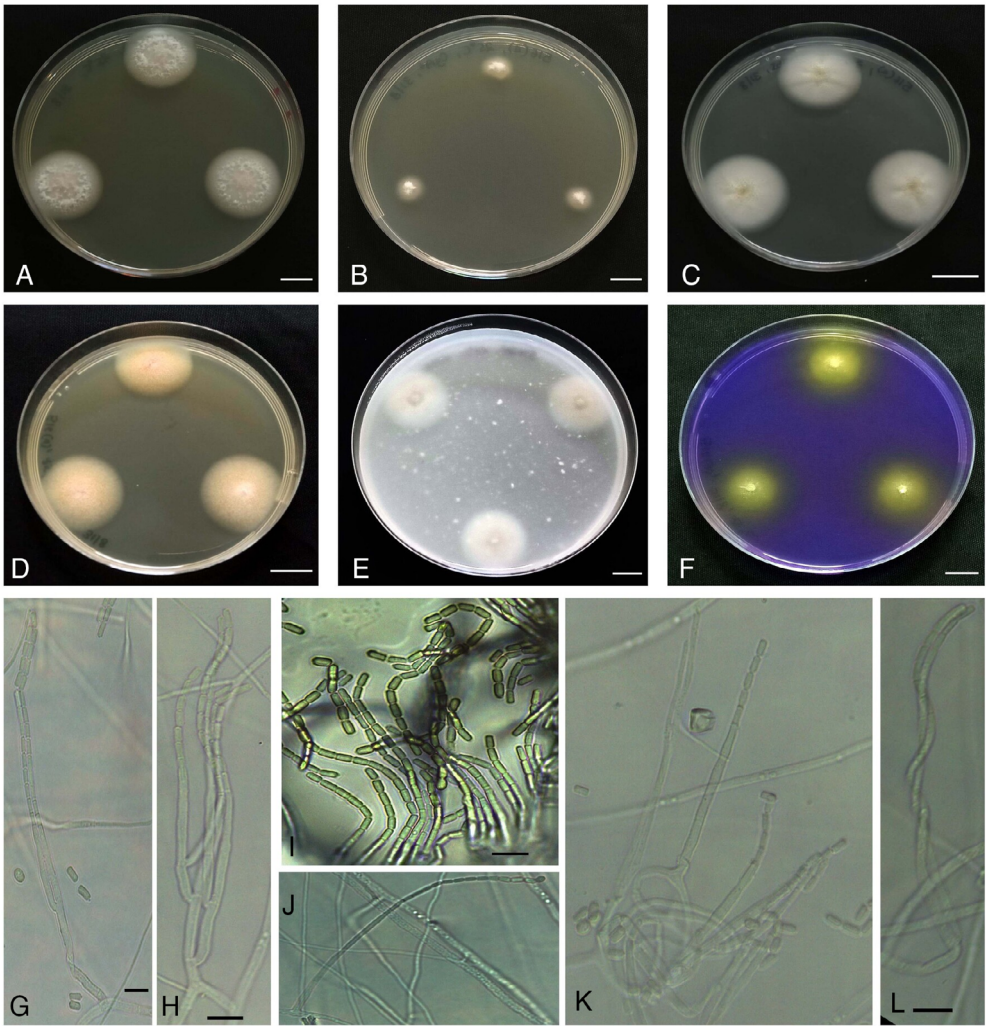

Fig. 4. *Talaromyces cupressi* sp. nov. CBS147104.
A–F. Colonies at 25°C after 7 d. (A) CYA, (B) CYA + NaCl, (C) MEAbi, (D) MEA, (E) OA, (F) CREA.
G, H. Biverticillate conidia.
I. Conidia.
J–L. Solitary phialides with conidia. Bars, A–F = 90 mm, G–L = 10 μm.

sampling site – the Sharon area (Supporting Information Fig. S2a).

The isolation recovery (%) of fungal species from different hosts was analysed (Supporting Information Fig. S2b). *Geosmithia omnicola* was the most dominant and commonly isolated fungal species, found in every host, with the highest isolation recovery percentage rates. It accounted for 41.6% in *P. bicolor*, 39.5% in *P. armatus*, 63.2% in larvae of *P. bicolor* and 46.8% and 56.2% in galleries associated with *P. bicolor* and *P. armatus* respectively. *Geosmithia* sp. 708b and *G. cupressina* sp. nov. CBS147103 were the second-most commonly isolated fungal species present in all host tissues, whereas *G. langdonii* was isolated from host beetles with a RA of 14.29% in *P. bicolor* and 11.63% in *P. armatus*, and 4.26%–17.81% in their galleries respectively. *Talaromyces cupressi* sp. nov. CBS147104 was the least isolated fungal species with a RA of 3.5% in *P. armatus*, 6.5% in *P. bicolor* and 12.8% in the respective galleries.

The diversity of fungal isolates in different hosts was evaluated using various indices (Shannon–Wiener index and Simpson's diversity index) and their components, i.e. species richness and evenness. As summarized in Table 2, the species richness of fungal isolates was highest (5) in *P. bicolor* and its galleries, followed by *P. armatus* (5) and its galleries (4), while the lowest species richness was observed in larvae of *P. bicolor* (3). Margalef's richness index (*D′*) was highest in galleries of *P. bicolor* (1.039), followed by *P. bicolor* (0.921) and *P. armatus* (0.898). Shannon–Wiener index (*H′*) was slightly higher in the fungal isolates of *P. bicolor* (1.447) than in *P. armatus* (1.403) and galleries of *P. bicolor* (1.365).

Table 2. Diversity indices of fungal isolates colonizing beetles, larvae and galleries.

	Biodiversity indices (α)							
Source	Species richness (*S*)	Pielou's evenness index (*E*)	Simpson's index of dominance (λ)	Simpson's index (*D*)	Simpson's diversity index (1 − *D*)	Simpson's reciprocal index (1/*D*)	Shannon–Wiener index (*H*′)	Margalef's richness index (*D*′)
P. bicolor	5	0.899	0.064	0.259	0.741	3.855	1.447	0.921
P. armatus	5	0.874	0.081	0.267	0.733	3.745	1.403	0.898
P. bicolor[#]	3	0.827	0.003	0.438	0.561	2.281	0.909	0.679
*P. bicolor**	5	0.848	0.024	0.289	0.711	3.45	1.365	1.039
*P. armatus**	4	0.828	0.058	0.377	0.623	2.65	1.143	0.729

[#,*]Fungal isolates originating from larvae[#] and galleries* of beetle species.

Table 3. Bray–Curtis and Jaccard's indices for fungal isolates of *Phloeosinus* spp. and their galleries.

	Biodiversity indices (β)	
Source	Bray–Curtis dissimilarity index	Jaccard's similarity index
Adult beetles	0.1 (10%)	0.33 (33%)
Galleries embedded in host plant tissue	0.34 (34%)	0.31 (31%)

Similarly, Simpson's diversity index (1-D) followed the same pattern, where it was highest in *P. bicolor* (0.741), followed by *P. armatus* (0.733) and galleries of *P. bicolor* (0.711), compared to galleries of *P. armatus* (0.623) and larvae of *P. bicolor* (0.561). Furthermore, a higher degree of fungal species dominance was observed between both host beetles (0.064–0.081), whereas a comparatively low level of dominance was observed in larvae of *P. bicolor* (0.003) and its galleries (0.024) respectively. Species evenness (0.82–0.89) was uniform in all beetle hosts (Table 2). Furthermore, a moderate level of similarity (33%) and a low level of dissimilarity (10%) were observed among fungal species isolated from the two beetles. However, in galleries, 34% of the fungal isolates were dissimilar and 31% of the isolates were similar according to Bray–Curtis and Jaccard's indices respectively (Table 3).

Pathogenicity on Cupressus sempervirens *saplings*

In outdoor net-house experiments, pathogenicity of the five representative isolates (*G. omnicola* 701a, *G. langdonii* 701c, *Geosmithia* sp. 708b, *G. cupressina* sp. nov. CBS147103 and *T. cupressi* sp. nov. CBS147104) was assayed by inoculating wounded and non-wounded cypress stems. Among the five tested representative isolates, only *T. cupressi* sp. nov. CBS147104 caused severe disease symptoms on stems, with 100% disease incidence and 96 ± 8.3% disease severity. Inoculation with *T. cupressi* sp. nov. CBS147104 at the wounded sites resulted in typical disease symptoms: infected stems exhibited browning and necrosis within 6–7 days post-inoculation (dpi) (Fig. 5A). Inoculation of non-wounded stems did not result in disease symptoms with any of the tested isolates. Furthermore, inoculation with *T. cupressi* sp. nov. CBS147104 also exhibited severe disease symptoms on pine stems, with 100% disease incidence and 92 ± 8.1% disease severity (Fig. 5B). Disease symptoms in pine were similar to those observed in cypress stems. No disease symptoms developed in control treatments. To validate Koch's postulates, the fungi were re-isolated from the infected host tissues and their identity was confirmed using morphological, microscopic and molecular (Ap-PCR) methods.

Isolation and characterization of fungi from parasitoids associated with cypress bark beetles

A total of 14 fungal isolates (*Geosmithia* spp.) were obtained from the three tested parasitoid species. Among them, eight fungal isolates were isolated from *Dendrosoter protuberans* Nees (Braconidae), one from *Eurytoma morio* Bohemann (Eurytomidae) and five from *Metacolus unifasciatus* Foerster (Pteromalidae). According to Ap-PCR banding patterns, fungal isolates were grouped into three categories. Two of the fungal isolates showed similar banding patterns to those of the representative fungal strains (*G. langdonii* and *G. cupressina* sp. nov.) isolated from beetles (Supporting Information Fig. S1a,b). Molecular taxonomy analyses using multilocus sequence typing (MLST) and network analysis coupled with morphological and microscopic characters substantiated these findings (Fig. 1A and B).

Discussion

This is the first study of mycobiota associated with the cypress bark beetles *P. armatus* and *P. bicolor* in Israel, and their role in Italian cypress trees colonized by these

bark beetles. Morphological and phylogenetic analyses suggested that these isolates belong to four distinct *Geosmithia* species and one *Talaromyces* species; three of these – *G. langdonii*, *Geosmithia* sp. 708b and *G. omnicola* – are formally described species, whereas *G. cupressina* sp. nov. CBS147103 and *T. cupressi* sp. nov. CBS147104 are newly described in this study. This is the first documentation of a symbiotic relationship between *Geosmithia* species and a bark beetle vector on cypress in Israel. This research, together with that of Dori-Bachash *et al*. (2015), considers pine bark beetles as a rich and diverse guild of *Geosmithia* associated with bark beetles in Israel.

Geosmithia species are non-ophiostomatoid, dry spore-producing fungi that currently include 60 phylogenetic species with 18 formally described taxa (Strzałka *et al*., 2021). The infraspecific variations in species of *Geosmithia*, e.g. colony appearance and microscopic characters, make it difficult to identify them based on their morphological features alone. The most crucial morphological characters that accurately characterize *Geosmithia* species are penicillate conidiophores with centate dry conidia. Most of the *Geosmithia* species also produce peg foot and substrate conidia. The newly described *G. cupressina* exhibits similar features; however, it is characterized by its unique thermotolerant nature, which is useful in species identification. Apart from *G. cupressina*, only *G. lavendula*, *G. carolliae*, *G. proliferans* and *G. morbida* are able to grow at 37°C. However, they can be distinguished by the production of red to vinaceous and/or yellow pigment and restricted growth on various media respectively (Pitt, 1979; Kolařík *et al*., 2011; Huang *et al*., 2017; Cunha *et al*., 2018). Furthermore, other important features for distinguishing *G. cupressina* from previously described species are colony growth rate, conidiophore structure, size and number of metulae and phialides, and shape of the conidia (Supporting Information Table S1). The thermotolerant nature of *G. cupressina* suggests that the fungus originates from the Mediterranean area; in addition, both species of *Phloeosinus* host beetles are native to this region.

The ambiguous morphology highlights the importance of incorporating sequence data for identification of *Geosmithia* at the species level (Pepori *et al*., 2015; Huang *et al*., 2017; Strzałka *et al*., 2021). ITS barcodes have previously been used to identify several *Geosmithia* species, including *G. flava* (Kolařík, Kubátová and Pažoutová), *G. putterillii* (Thom) Pitt, *G. fassatiae* (Kolařík, Kubátová and Pažoutová), *G. langdonii* (Kolařík, Kubátová and Pažoutová) and *G. obscura* (Thom) Pitt (Kolařík *et al*., 2004; Kolařík *et al*., 2005; Kolařík *et al*., 2007; McPherson *et al*., 2013; Hernández-García *et al*., 2020). However, ITS sequence analysis has failed to determine the phylogenetic relationships among several *Geosmithia* species, such as *Geosmithia* sp. 32, *Geosmithia* sp. 36, *G. microcorthyli* (Kolařík) (Kolařík and Kirkendall, 2010), and the *Geosmithia* sp. 24 complex (Dori-Bachash *et al*., 2015), due to lack of effective phylogenetic characters or potential occurrences of paralogues. Therefore, additional partial gene fragments, such as RPB2, EF1α and Tub2, are recommended for reliable species-level identification of *Geosmithia* (Kolařík and Kirkendall, 2010). *Geosmithia cupressina* sp. nov. CBS147103 reported from Israel in this study shared 100% identity with the ITS sequences of *Geosmithia* sp. 46 associated with *Juglans* and *Quercus* spp., reported from the USA (Huang *et al*., 2019). However, because we lack the strain in our collection and are missing reference sequences (Tub2 and RPB2), we were unable to compare *Geosmithia* sp. 46 with the novel *G. cupressina* sp. nov. CBS147103. In any event, further studies are required to explore more phylogenetically related strains of *G. cupressina* sp. nov. CBS147103. In the present study, phylogenetic relationships of the *Geosmithia* isolates were established using multigene-sequence datasets (ITS, Tub2, RPB2) and *G. cupressina* sp. nov. CBS147103 was subsequently delineated as a novel species and formally described. In addition, the multigene phylogeny was supported by the Neighbour-Net graph generated by SplitsTree network analysis, indicating no evidence for possible recombination among the existing *Geosmithia* species and *G. cupressina* sp. nov. CBS147103. This is the first study incorporating the use of the SplitsTree network for *Geosmithia* phylogeny. Previously, a multigene phylogenetic approach was used to identify *G. eupagioceri* (Kolařík), *G. microcorthyli*, *G. omnicola* (Pepori, Kolařík, Bettini, Vettraino and Santini), *G. ulmacea* (Pepori, Kolařík, Bettini, Vettraino and Santini), *G. pazoutovae* (Strzałka, Jankowiak and Kolařík) and *G. longistipitata* isolated from different sources, such as *Ulmus* spp. and from several Scolytinae species, i.e. *Eupagiocerus dentipes*, *Microcorthylus* sp., *Polygraphus poligraphus* and *Scolytus intricatus* (Kolařík and Kirkendall, 2010; Pepori *et al*., 2015; Strzałka *et al*., 2021).

Previous surveys have documented strains of *G. omnicola* and *G. langdonii* in new areas, from beetle vectors and host tree species (Kolařík *et al*., 2005; Kolařík *et al*., 2007; Kolařík *et al*., 2008; Machingambi *et al*., 2014; Pepori *et al*., 2015; Kolařík *et al*., 2017; Strzałka *et al*., 2021); those studies described them as generalist fungi isolated from bark and ambrosia beetles, their galleries, and endophytes of their host trees. The new records of *Geosmithia* from Israel indicate that the distribution of these fungi is substantially wider, located from the western Galilee, northwest of Hof HaCarmel, to central and southern Israel. This is the first record of the presence of this globally distributed *Geosmithia* spp. in the Eastern Mediterranean region. These findings, along with records from the Czech Republic, Slovakia Republic, Bulgaria, Poland, Portugal, South Africa, Mexico and the USA (Kolařík *et al*., 2005; Kolařík *et al*., 2007;

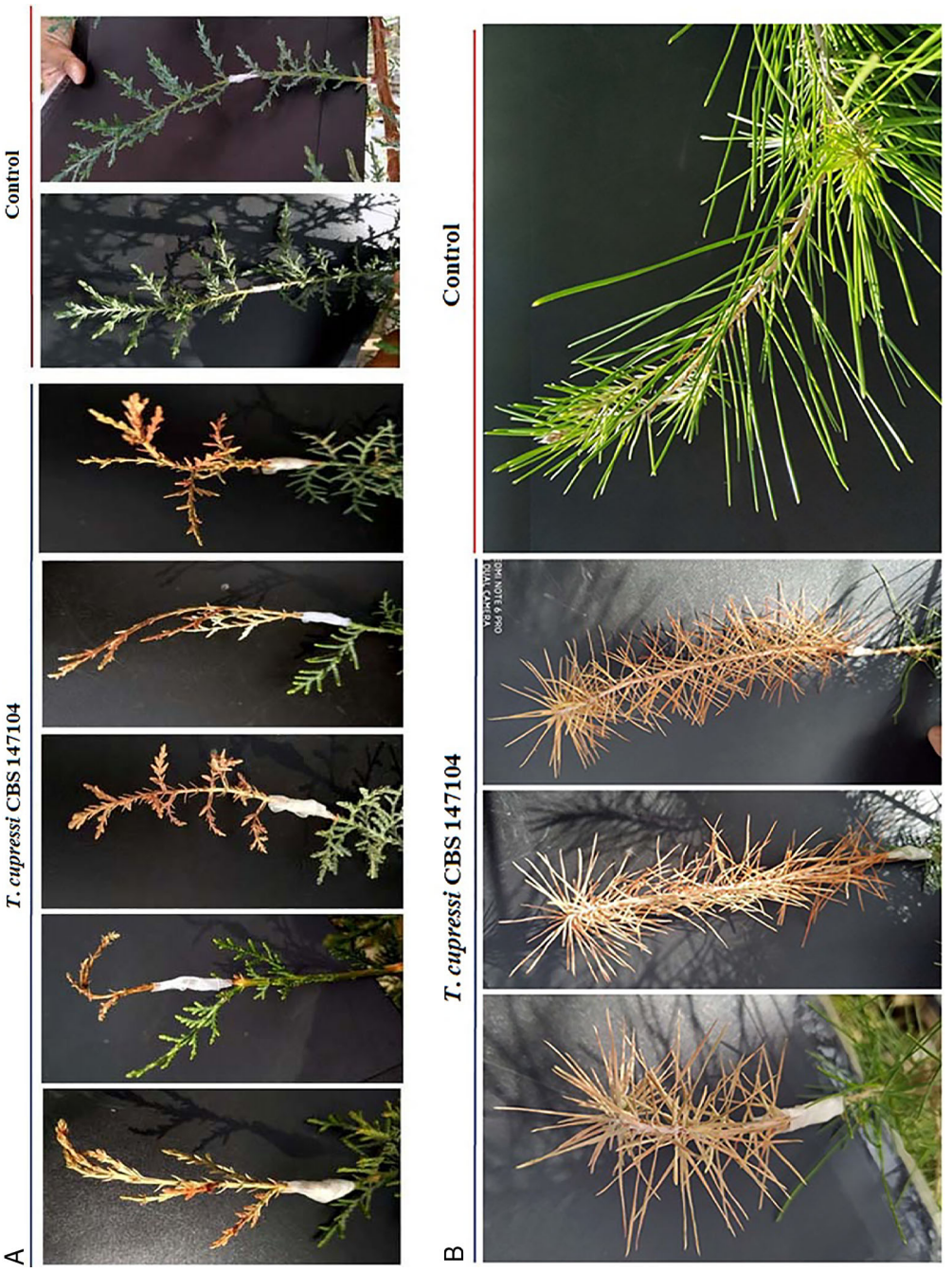

Fig. 5. Pathogenicity assay conducted under natural net-house conditions with *Talaromyces cupressi* CBS147104, 21 dpi on (A) *Cupressus sempervirens* and (B) *Pinus brutia*.

Kolařík *et al*., 2008; Machingambi *et al*., 2014; Pepori *et al*., 2015; Kolařík *et al*., 2017; Hernández-García *et al*., 2020; Strzałka *et al*., 2021), support the wide geographic distribution of *G. omnicola* and *G. langdonii*.

In our study, *G. omnicola* was the most frequent *Geosmithia* species isolated among both studied congeners, found in close and stable association with cypress bark beetles and their galleries. This fungal species was found on bark beetles and in their galleries from all the 10 sampling sites in Israel. *Geosmithia omnicola* is a generalist and has been collected from various conifers, hardwood trees and shrubs in Poland, Czech Republic, Hungary and the USA (Kolařík *et al*., 2007; Pepori *et al*., 2015; Kolařík *et al*., 2017; Strzałka *et al*., 2021). The fungus was also isolated as an endophyte during our study from the cortex of healthy cypress trees (data not shown). Similar observations were made by McPherson *et al*. (2013), where a *Geosmithia* sp. was isolated as an associate of bark beetles and also as an endophyte. However, Pepori *et al*. (2015) reported contrasting observations in elm trees, where *Geosmithia* spp. were only isolated from bark beetle galleries and not from the beetles themselves. *Geosmithia omnicola* shows a high degree of variability in its association with beetle vectors (*Carphoborus perrisi*, *Chaetoptelius vestitus*, *Cryphalin* sp., *Hypoborus ficus*, *Ips typographus*, *Liparthrum* sp., *Phloeosinus* spp., *Phleotribus scarabaeoides*, *Pteleobius vittatus*, *Scolytus* spp.), their host trees (*Ficus carica*, *Juniperus* spp., *Picea abies*, *Pistacia lentiscus*, *Prunus domestica*, *Olea europaea*, *Ulmus* spp., *Virgilia oroboides*) and habitat range (Central Europe, Eurasia, Mediterranean, Africa), and for this reason, it was named 'omnicola' (Kolařík *et al*., 2007; Machingambi *et al*., 2014; Pepori *et al*., 2015).

Geosmithia langdonii, another generalist congener, was identified in the present study from beetles and their galleries. Our findings are in agreement with Strzałka *et al*. (2021), who isolated this fungus from other Scolytinae and their galleries. It was also isolated from the symptomatic wood affected by Dutch elm disease in a survey of fungi in Switzerland, and as an endophyte of *Quercus agrifolia* in California (McPherson *et al*., 2013; Pepori *et al*., 2015). In previous surveys, this fungus was frequently associated with *P. thujae* and *P. serratus* colonizing *Chamaecyparis* and *Juniperus* spp., indicating specificity for this group of insects, although it may also be found in association with a wide range of beetle vectors such as *Phloeotribus rhododactylus*, *Scolytus* spp. and *Ernoporicus fagi*, and a wide tree range including *Sarothamnus scoparius*, *Carpinus betulus*, *Quercus* spp., *Ulmus* spp., *Fagus sylvatica* and *Prunus domestica* growing in European, Mediterranean, Mexican and Eurasian regions (Kolařík *et al*., 2005; Kolařík *et al*., 2008; Hernández-García *et al*., 2020; Strzałka *et al*., 2021). *G. microcorthyli* has been relatively less reported, having only been found on beetles isolated from *Cassia grandis* (Kolařík and Kirkendall, 2010).

Inoculation of 2-year-old cypress and pine saplings with *Geosmithia* spp. did not produce disease symptoms, corroborating the findings of the present study with previous reports by Jankowiak and Kolařík (2010), Dori-Bachash *et al*. (2015) and Strzałka *et al*. (2021) based on conifer and hardwood seedlings inoculated with bark beetles associated with *Geosmithia* spp. (including *G. omnicola* and *G. langdonii*). According to our findings, it is evident that *Geosmithia* spp. are mostly saprophytic; however, *Geosmithia* sp. 41 and *G. morbida* are pathogenic in nature (Tisserat *et al*., 2009; Lynch *et al*., 2014) and therefore, their ecological role remains unclear.

Our results clearly indicated that *Geosmithia* spp. are frequent associates of bark beetles on cypress in Israel. The number of *Geosmithia* spp. recovered per sample was influenced by the size of the sample, with a minimum of one fungal species to a maximum of four in the largest sample (Kolařík and Jankowiak, 2013). Similar isolation patterns were observed in the present study. Furthermore, previous studies have also indicated that the size of the beetle to which the conidia adhere affects the number of recovered isolates (Lieutier *et al*., 2016). The latter observation is in agreement with our study, in which an increased number of *Geosmithia* isolates were recovered from *P. armatus* and their galleries compared to its smaller-sized counterpart *P. bicolor*. This beetle–*Geosmithia* relationship has already been documented for several trees associated with bark beetles in the Mediterranean region (Kolařík *et al*., 2004; Kolařík *et al*., 2007; Kolařík *et al*., 2008; Dori-Bachash *et al*., 2015). A stable and specific association between cypress-associated bark beetles and *Geosmithia* species may result from the type of substrata preferred by these insects. *Phloeosinus* species, which preferentially breed on drier substrata, are able to maintain mutualism with *Geosmithia* species which are better adapted to survival in branches of cypress trees that are exposed to elevated temperatures. Furthermore, *Phloeosinus* species did not carry any ophiostomatoid fungi, a frequent associate of bark beetles. This is likely due to susceptibility of ophiostomatoid fungi to desiccation in the dryer substrates compared to *Geosmithia* species, able to maintain mutualism for an extended period under dry conditions. The expected distribution of the newly described *Geosmithia* species can be inferred from the distribution of the beetle vector. These bark beetles, indigenous to Mediterranean and Middle Eastern countries, have also been introduced in other countries, such as Italy and the USA, where the host tree grows and is susceptible to infection (Lieutier *et al*., 2016).

The Margalef index can reflect richness of fungal species. The larger the values of *S* and *D′*, the richer the *Geosmithia* species are in a particular host tissue. Species diversity was analysed by the Shannon–Wiener (*H′*) and Simpson diversity indices. These indices take into account the heterogeneity/homogeneity of the species frequencies. In general, the higher the Shannon's diversity index (commonly ranging between 1 and 4) and the closer the Simpson's diversity index is to 1, the more intensified the heritable variation and the stronger the adaptive capacity for microenvironmental changes, such as distribution range and entrance into new environments. As observed in the present study, the species richness and Simpson's diversity index were comparable to those of *Geosmithia* species associated with bark beetles, such as *Pityophthorus bidentatus*, *Pityophthorus pityographus* and *Pityogenes chalcographus*, but higher than those in association with *Dryocoetes autographus*, *Ips typographus*, *Ips amitinus* and *Hylurgops palliatus* infesting *Picea abies* and *Pinus sylvestris* in Poland (Jankowiak *et al*., 2014). *Geosmithia* species colonizing *P. bicolor* and its galleries showed the highest species richness and diversity. The varied trends of Shannon–Wiener and Simpson's diversity indices should be kept consistent. However, there were slight differences for *Geosmithia* species colonizing various host tissues which might be attributed to the significant interaction of the number, isolation frequency and species richness of the isolates. There were no specific *Geosmithia* species restricted to a particular beetle vector. We expected to find a more obligatory relationship between the fungi and beetles; however, it appears that the *Geosmithia* species are not very specific, but are generalist in nature. Similar observations were made in the case of fungi associated with pine bark beetles where we found the same fungal species in different bark beetles (Dori-Bachash *et al*., 2015). *Geosmithia* species associated with ambrosia beetles exhibit a distinct level of vector- or host-specificity worldwide; however, in this study, we looked at non-ambrosia beetle species, thus may be generalists as previously reported (Kolařík and Kirkendall, 2010; Strzałka *et al*., 2021).

The genus *Phloeosinus* is comprised of more than 60 formally described species, nine of them residing in the Mediterranean region, of which five (44%) have been studied for the presence of *Geosmithia*, exhibiting an incidence of 100% with at least one *Geosmithia* strain isolated per bark beetle species, as in the case of *P. henschi* and *P. thujae* (Kolařík *et al*., 2007; Kolařík *et al*., 2008). Similar *Phloeosinus–Geosmithia* association patterns were observed in previous studies carried out in Mexico and the USA (Huang *et al*., 2017; Kolařík *et al*., 2017; Hernández-García *et al*., 2020). The high diversity of *Geosmithia* species concentrated in the niches formed in bark beetle-colonized cypress trees raises some intriguing questions. All of these fungal members are non-specifically associated with the studied *Phloeosinus* spp. as well as with other bark beetles. This suggests specific fungal adaptation to certain conditions among the wide range of physiological deteriorating niches of the cypress tree cortex tissues.

One of the most interesting findings of the study was the association of *Talaromyces* spp. with *Phloeosinus* spp. Generally, *Talaromyces* (Benj) species are cosmopolitan in nature; they have been isolated from various substrates, including soil, air, leaf litter, food products, humans and animals (Samson *et al*., 2011; Yilmaz *et al*., 2014, 2016) but to the best of our knowledge, this is the first report of isolation from bark beetles or their galleries. A multigene phylogenetic approach based on the ITS, BenA, CaM and RPB2 gene regions was applied to study the relationship of the newly described *Talaromyces* species. A split-network analysis also supported the multigene phylogeny. Split-network analysis has been previously applied for the description of *Talaromyces amestolkiae* (Yilmaz, Houbraken, Frisvad and Samson) (Yilmaz *et al*., 2016). Phylogenetic analysis of the combined ITS, BenA, CaM and RPB2 sequences indicated that *T. columbiensis* (Yilmaz, López-Quint., Vasco-Pal., Frisvad and Houbraken), *T. emodensis* (Udagawa), *T. unicus* (Tzean, Chen and Shiu) and *T. proteolyticus* [(Kamyschko) Samson, Yilmaz and Frisvad] are closely related, whereas *T. cupressi* sp. nov. CBS147104 is evolutionarily distinct from previously described *Talaromyces* species within the *Bacillispori* section. *Talaromyces cupressi* sp. nov. CBS147104 is supported by similarities in its morphological characters, including solitary phialides, rod-shaped conidia and acid production. Despite these similarities, *T. cupressi* sp. nov. CBS147104 could be distinguished from *T. emodensis*, *T. mimosinus* (Hocking) and *T. unicus* by distinct phenotypic characters, conidial arrangement and shape, and its acid production. The reverse-side colony colour of *T. cupressi* sp. nov. CBS147104 on CYA is pale yellow, whereas that of *T. bacillisporus* is dark green and that of *T. emodensis* is brownish red. Another notable feature of *T. cupressi* sp. nov. CBS147104 is that it produces solitary phialides and rod-shaped conidia (similar to *T. bacillisporus*), whereas *T. emodensis* and *T. unicus* produce mono- to biverticillate conidiophores with ovoid to ellipsoidal conidia, and *T. proteolyticus* produces biverticillate conidiophores with globose to subglobose conidia. Furthermore, *T. cupressi* sp. nov. CBS147104 lacks ascoma formation, similar to *T. proteolyticus* (Supporting Information Table S2). Thus, our data show that this strain represents a new species of *Talaromyces* in the section *Bacillispori*. To the best of our knowledge, the present study is the first to record the association of a

Talaromyces species with bark beetles. *Talaromyces* species produce thermotolerant conidia which enable them to survive on branches of cypress trees that are exposed to the elevated temperatures and drought conditions typical of a Mediterranean climate. This may indicate why it was found in association with *Phloeosinus* species. Furthermore, unlike anamorphic counterparts from the genus, *T. cupressi* sp. nov. CBS147104 exhibited a high degree of pathogenicity in both inoculated cypress and pine host trees, in contrast to the selected *Geosmithia* spp. isolates used in our study.

Knowledge regarding the role and relationship between fungi and associated beetles is scant. Insects may be carriers of fungi, and because the beetles and their parasitoids predominantly develop within the cypress tree, a complex relationship exists among all involved. Furthermore, the role of fungi in beetle nutrition is an important component, as is their role in the process of preparing or improving the cortex for fungal establishment. They may also serve as part of the insect's diet, either facultatively or obligatorily. Because interactions among the respective fungi and their beetle associates have not been studied, these are nascent areas for further research.

The gallery zones of *Phloeosinus* species in cypress trees are very different from that of bark beetles associated with Pinaceae such as pine or spruce. Pine bark beetles occur in the Mediterranean Basin, which is rich in ophiostomatoid ascomycetes. This fungal flora is the main food for a large and diverse population of mites and nematode species present in the pine bark beetle galleries. The scarcity and usually, absence of the latter two groups in the gallery areas of both *Phloeosinus* spp. in Cupressaceae host trees (Lieutier *et al*., 2016) may be explained by the limited appearance of ophiostomatoid ascomycetes in the galleries; e.g. lack of nematodes associated with bark beetles (Scolytinae) has been observed for *P. armatus* (*P. bicolor* was not examined) in Israel (Xue *et al*., 2019). The fungal population in bark beetle-infested pine trees which support diverse insect predators is comprised of approximately 47 species belonging to four orders (Lieutier *et al*., 2016). *Aulonium ruficorne* (Colydiidae) and *Corticeus* spp. (Tenebrionidae) occur frequently in Scolytinae-infested pine trees, but these species are rarely associated with cypress trees in Israel (Halperin and Holzschuh, 1984).

Nine species of hymenopteran parasitoids belonging to five families were recovered from both *Phloeosinus* spp. in Israel; a few of the species, particularly *Dendrosoter protuberans*, *Metacolus unifasciatus* and *Eurytoma morio*, are frequent and responsible for high parasitism of the beetle species (Mendel, 1986). It is not surprising that many of the studied *Geosmithia* spp. in the present study were also isolated from the three frequently occurring parasitoids that attack these *Phloeosinus* species (Mendel, 1986). These parasitoids attack larvae and pupae of both cypress bark beetles, while the emerging wasps are in close contact with the gallery system where the fungi are located. The wasps' transmission capability is unknown; although theoretically at least, they may transfer the fungi between different tree groups because *D. protuberans* may attack bark beetles of broad-leaved trees; *M. unifasciatus* also parasitizes pine bark beetles; and *E. morio* attacks bark beetles of both pine and broad-leaved trees (Mendel, 1986).

In conclusion, the present study indicates that of the five fungal species carried by the two bark beetles *P. bicolor* and *P. armatus*, only *T. cupressi* sp. nov. CBS147104 is pathogenic to healthy saplings of *C. sempervirens*, whereas the other four fungal species – *G. langdonii*, *Geosmithia* sp. 708b, *G. omnicola* and *G. cupressina* sp. nov. CBS147103 – did not cause disease symptoms under the tested *in vivo* conditions. The present study is also the first to report on a *Geosmithia* assemblage with two *Phloeosinus* species colonizing *C. sempervirens*. Our findings suggest a long and stable association between both *Phloeosinus* and *Geosmithia* spp., a possible role for certain associated fungal species, as pathogens or endophytes, of cypress trees, and species richness potentially reflecting adaptation to different degrees of deterioration of the cortex of cypress trees. None of the studied fungi was specific to bark beetle species. Several *Geosmithia* spp. were also isolated for the first time from hymenopteran parasitoid species that develop and attack bark beetles.

Experimental procedures

Study area and collection of bark beetles, larvae and galleries

Adult beetles, larvae and active galleries of *P. armatus* and *P. bicolor* were sampled from *C. sempervirens* adult and mature trees colonized by the beetles. The sampling was conducted at 10 different sites in Israel from August 2017 to March 2019 (Table 1; Fig. 6). The samples were taken from naturally infested standing trees and others after induced beetle attack, by leaving freshly felled trap trees in semi-shaded sites during the warm months (May to October). A number of the side branches were removed and placed on the trunk to minimize direct solar radiation. The cut trees remained on the forest floor for 3–4 weeks during the warm months (June to September), and for 4 months during the winter months (November to February). Stem sections colonized with beetles and typical galleries were then brought to the laboratory for examination. In addition, stem sections from healthy cypress trees with no visual symptoms of bark beetle colonization were collected to serve as control samples.

Isolation of fungi from bark beetles, larvae and galleries

Fungi were isolated by plating beetles and larvae on potato dextrose agar (Difco, Becton, Dickinson and Company, MD, USA) in Petri dishes (MiniPlast, Ein Shemer, Israel), supplemented with 250 μg ml^{-1} chloramphenicol (Arcos Organics, NJ, USA) (PDAC) without surface sterilization. Fungal strains were single-spored from growing cultures after incubating at 25 ± 2°C for 7 days. Isolates were then stored in 15% (vol./vol.) glycerol as axenic cultures at −80°C. For isolation of fungi from galleries, 1.5-cm^2 sections of cortex (bark) or wood with conspicuous galleries were placed in Eppendorf tubes containing 1.5 ml saline solution [0.85% (wt./vol.) NaCl] and centrifuged at 10 000*g* for 10 min. Then, 1 ml of the saline solution was decanted and 100 μl was serially diluted (10^{-1} to 10^{-4}), single-spored, plated on PDAC Petri dishes and incubated at 26 ± 2°C for 7 d. Growing cultures were prepared and stored as previously described (Freeman *et al*., 2013; Dori-Bachash *et al*., 2015).

Fungal DNA extraction

Genomic DNA was extracted from all collected fungal isolates according to Dori-Bachash *et al*. (2015). The extracted DNA was visualized under UV light (Enduro GDS, Labnet International, NJ, USA) after separation in 1.2% agarose gels (SeaKem LE Agarose, ME, USA) stained with ethidium bromide. The purity and quantity of DNA were determined using an ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) at 260 and 280 nm (Freeman *et al*., 2013; Dori-Bachash *et al*., 2015).

Arbitrarily primed PCR amplification of fungal DNA

Ap-PCR was performed on DNA of all 302 isolates with three of the repeat-motif primers – $(CAG)_5$, $(GACA)_4$ and $(GACAC)_3$ (Integrated DNA Technologies, IA, USA) – according to Dori-Bachash *et al*. (2015) (Supporting Information Table S3). The amplification products were separated in 1.8% agarose gels (SeaKem LE Agarose) in 1× Tris-acetate-EDTA buffer and run at a constant voltage of 80 V for 1.5 h. Representative isolates were chosen from those that had identical banding patterns after Ap-PCR amplification. Ap-PCR was repeated twice for reference isolate DNA to verify reproducibility of the results (Freeman *et al*., 1993; Dori-Bachash *et al*., 2015; Sharma *et al*., 2017).

Molecular taxonomy of the representative fungal isolates

Evolutionary relationships and speciation were established by employing MLST as described by Dori-Bachash *et al*. (2015), Yilmaz *et al*. (2016) and Strzałka *et al*. (2021). *Geosmithia* species were identified using four nuclear gene fragments chosen for the present MLST study: ITS (ITS1 and ITS4), Tub2 (T1 and T2), RPB2 (5F2 and 7cr) and EF1α (EF1-728F and EF1-986R). *Talaromyces* species were identified using MLST of β-tubulin (BenA, 2a and 2b), CaM (CMD 5 and 6), ITS and RPB2 primer-pair combinations (Integrated DNA Technologies) (Supporting Information Table S3). The PCRs were carried out as described by Freeman *et al*. (2013), Dori-Bachash *et al*. (2015), Yilmaz *et al*. (2016) and Strzałka *et al*. (2021). Approximately 300- to 1200-bp PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH, Germany). The amplified products were sequenced at Macrogen (The Netherlands).

Multilocus phylogeny and network analyses

Neighbour-Net and multilocus sequence analyses were used to predict the phylogenetic affiliations of *Geosmithia* and *Talaromyces* isolates. The raw sequence files were analysed using MEGA-X v.10.1.07 (Kumar *et al*., 2018) to generate assembled sequences. Although >20 species and >30 phylogenetic species have been molecularly established in *Geosmithia* (Kolařík *et al*., 2017; Strzałka *et al*., 2021), only 20 accepted *Geosmithia* species and 31 other phylogenetic *Geosmithia* species were used in the sequence-based delimitation analyses as listed in Supporting Information Table S4. EF1α sequences were not used due to missing reference sequences and shorter sequences (~200 bp) generated in this study. Similarly, for the *Talaromyces* genus, reference ex-type strain sequences from each of the accepted sections (Houbraken *et al*., 2020) were incorporated into the sequence-based delimitation analyses as listed in Supporting Information Table S5. Reference sequences from the ex-type strains of *Geosmithia* spp. (Strzałka *et al*., 2021) and *Talaromyces* spp. (Houbraken *et al*., 2020) for phylogenetic analyses were retrieved from NCBI GenBank. The GenBank accession numbers for the sequences generated in this study along with reference ex-type strain sequences used in the phylogenetic analyses are listed in Supporting Information Tables S4 and S5. The multilocus concatenated dataset was generated using SequenceMatrix v.1.7.8 (Vaidya *et al*., 2011), and MP analyses were performed in PAUP version 4.0b10 (Swofford and Sullivan, 2002). The gaps were treated as missing data and any ambiguous regions in the alignment were excluded from the dataset. The phylogenetic trees were generated using the heuristic search option with Tree Bisection Reconnection branch swapping and 20 random sequence additions. Maxtrees were set to 1000 and zero-length branches were collapsed. Tree statistics TL, CI, RI, RC and HI were recorded. ML

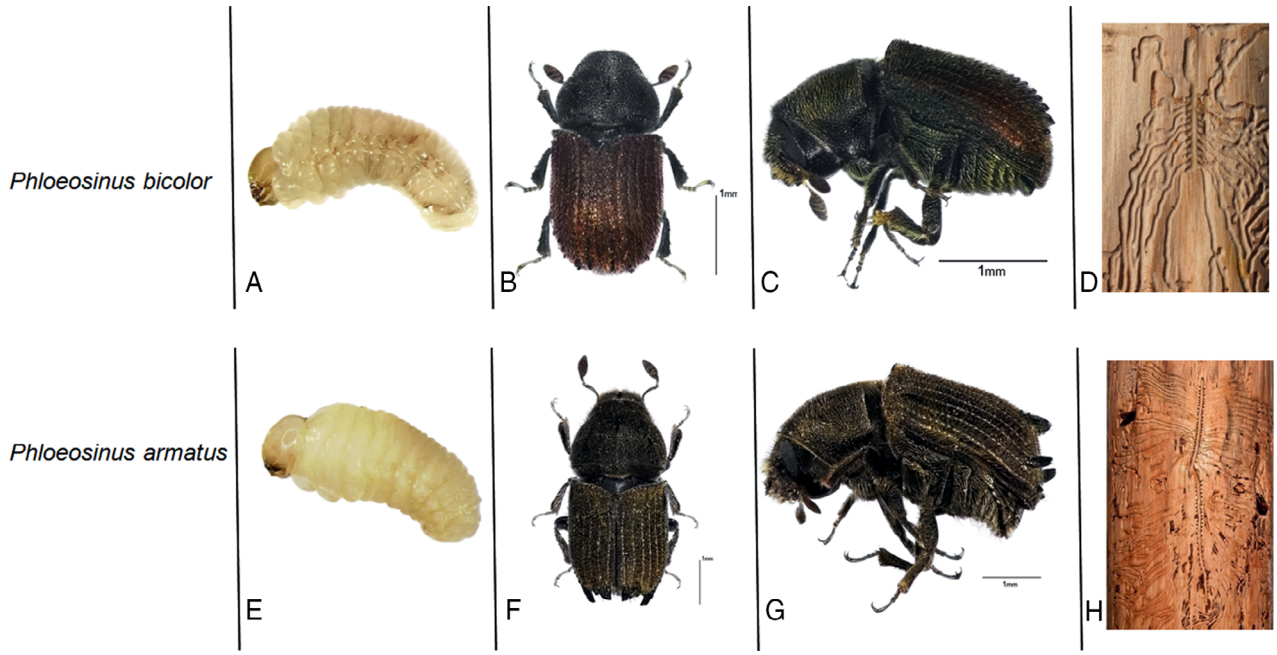

Fig. 6. Bark beetles and their galleries collected during the study.
A–D. *Phloeosinus bicolor*: (A) larvae, (B, C) adult beetles, (D) galleries.
E–H. *Phloeosinus armatus*: (E) larvae, (F, G) adult beetles, (H) galleries, Bar = 1 mm.

analysis was performed with MEGA-X using the General Time Reversible substitution model with gamma distribution. Individual gene trees were also generated for *Geosmithia* and *Talaromyces* spp. respectively, using the ML method (data not shown). A bootstrap analysis of 100 replicates was conducted to establish the nodal support in both MP and ML analyses. The final tree images were edited using Microsoft PowerPoint version 2016 and the bootstrap values (>50%) for the observed branching pattern are shown alongside the branch nodes. The multilocus datasets for *Geosmithia* spp. and *Talaromyces* spp. were also respectively analysed using SplitsTree v.4.16.1 (https://uni-tuebingen.de/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/informatik/lehrstuehle/algorithms-in-bioinformatics/software/splitstree/) to construct an unrooted, equal angle, Neighbour-Net splits network with uncorrected *p*-distance. To determine possible recombination between the determined species groups, PHI test was conducted in SplitsTree (Huson and Bryant, 2006). Species delimitation was also verified using the Genealogical Concordance Phylogenetic Species Recognition criterion. For each genus, the topological congruencies were verified between multigene and individual-gene trees, for the identified *Geosmithia* and *Talaromyces* species.

Taxonomy

Colony characters of the two novel fungal isolates were studied on different media under different growth conditions. Cultures were inoculated on CYA, CYA supplemented with 5% NaCl, MEA, MEAbi, OA and CREA by three-point inoculation on 90-mm Petri plates. Media preparations, incubation conditions and microscopic preparations followed the recommendations of Visagie *et al*. (2014). Colony colour codes refer to Kornerup and Wanscher (1967). Microscopic observations were performed with an Olympus BX61 microscope equipped with DP-25 camera and Cell Sense Dimension 1.6 software (Olympus, Japan). Cultures of the putative new *Geosmithia* and *Talaromyces* species isolated during the study were deposited in the culture collection of Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and in the herbarium (HUJIHERB) of the National Natural Collections of the Hebrew University of Jerusalem, Israel.

Diversity analysis of fungal isolates

Using species as the statistical unit, the number of isolates and their RA for each fungal species isolated from beetles, larvae or galleries was calculated. Percent RA was calculated according to the formula suggested by Li *et al*. (2016). Similarly, diversity of the fungal species isolated from the beetles, larvae and their galleries was evaluated using seven alpha diversity indices and two beta-diversity indices (Kumar and Hyde, 2004; Jankowiak *et al*., 2014; Li *et al*., 2016; Jankowiak and Bilański, 2018).

Pathogenicity of Cupressus sempervirens *saplings*

Pathogenicity of the representative fungal isolates (*G. omnicola* 701a, *G. langdonii* 701c, *Geosmithia* sp. 708b, *G. cupressina* sp. nov. CBS147103 and *T. cupressi* sp. nov. CBS147104) was tested on 2- to 3-year-old cypress plants located under outdoor screenhouse conditions between April and July 2019 (no precipitation occurred during that period, with mean recorded temperature measurements of 30 ± 2/25 ± 2°C day/night). Inoculation was conducted using two different methods: in the first, a 1 cm strip of cortex was removed and an actively growing mycelial plug (5 mm) was placed over the wounded area with the mycelia facing the cambium. The wounded region was then covered with sterile moist filter paper and sealed with Parafilm; in the second method, a mycelial plug was placed directly on non-wounded stems as described for wounded stems. PDAC plugs without mycelia were inoculated as described for both methods and served as controls. Symptoms were evaluated and rated 21 dpi. Each representative isolate was inoculated on five stems per plant containing three replicates; the experiment was conducted twice. Fungi were re-isolated and identified from infected segments of the plant by Ap-PCR and microscopy, to verify Koch's postulates (Dori-Bachash *et al*., 2015). Disease severity was calculated using the 0–5 point ranking at 21 dpi, as described by Sharma *et al*. (2017).

Collection, isolation and characterization of fungal isolates from parasitoids of the studied cypress bark beetles

Parasitoids that emerged from branch and stem sections colonized by *P. armatus* were collected in Cabri forest, western Galilee in late April 2019. The parasitoids that emerged on 13 June 2019 were identified as *Dendrosoter protuberans*, *Eurytoma morio* and *Metacolus unifasciatus*. Isolation and characterization of the fungal isolates from these parasitoids were conducted as described in sections Isolation of fungi from bark beetles, larvae and galleries to Multilocus phylogeny and network analyses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.