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Morphological and molecular characterisation of *Geosmithia* species on European elms

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ABSTRACT

Species of the genus *Geosmithia* are associated with insect species, mainly bark beetles. On *Ulmus* spp., the same beetles are also vectors of *Ophiostoma ulmi* s.l., the agent of Dutch elm disease (DED), a worldwide elm disease. Aim of this paper is to characterise *Geosmithia* species associated with elms and/or elm beetles in Europe. Seventy-two strains representative of all morphological taxonomic units were used to build a phylogenetic tree based on ITS, β-tubulin and elongation factor 1-α gene regions. On the basis of molecular and morpho-physiological traits, seven taxonomic entities were identified. In addition to the species previously known our results assigned strains previously identified as *Geosmithia pallida* to two separate taxa: *Geosmithia* sp. 2 and *Geosmithia* sp. 5. Two new species, *Geosmithia omnicola* and *Geosmithia ulmacea*, are described. Two strains were assigned to the partially described species *Geosmithia* sp. 20. *Geosmithia* species living on *Ulmus* do not discriminate between elm species, but between different environments. The association between *Ulmus* and *Geosmithia* is common, stable, and seems to be related to specific vectors. The relationship between *Geosmithia* and *Ophiostoma* would deserve further investigation, as these fungi share the same vectors and habitat for a significant part of their life cycles.

Introduction

Geosmithia Pitt is a monophyletic morphogenus of anamorphic hyphomycetes that currently includes 32 published and at least 20 unpublished species of mitosporic fungi (Kolařík *et al.* 2004, 2005, 2007, 2008, 2011; Kolařík & Kirkendall 2010; Hulcr & Dunn 2011; Kolařík & Jankowiak 2013). These fungi were initially grouped in the genus *Penicillium*, until Pitt (1979) distinguished them as a separate genus on the basis of the morphology of phialides, the lack of the distinctive narrowed necks of *Penicillium* species, and the different colour of the mass of conidia, which are typically green in some

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Penicillium species, but never in *Geosmithia*. *Penicillium pallidum*, *Penicillium cylindrosorum* G. Smith, and *Penicillium emersonii* were originally aggregated to genus *Penicillium* by Raper & Thom (1949), thence into the genus *Geosmithia* (Pitt 1979).

Geosmithia fungi have worldwide distribution and are mainly associated to phloem-feeding bark beetles (*Coleoptera: Curculionidae*, *Scolytinae*) on broadleaved and conifer trees. Many *Geosmithia* isolates were also collected and described from other substrates such as soil, cereals, wood, foodstuffs, and some of them have been found as true plant endophytes (Pitt 1979; Kolařík *et al.* 2004, 2005, 2007, 2011; Pitt & Hocking 2009). A new *Geosmithia* species, *Geosmithia morbida*, was identified as the agent of thousand cankers disease of black walnut in North America (Kolařík *et al.* 2011), and recently also in Europe (Montecchio & Faccoli 2014). The fungus is spread by feeding and reproductive activities of the walnut twig beetle, *Pityophthorus juglandis*. Moreover, two species, *Geosmithia eupagioceri* and *Geosmithia microcorthyli*, have been described among ambrosia fungi of beetle species in Costa Rica (Kolařík & Kirkendall 2010).

In *Geosmithia* – bark beetles association, insects have a fundamental role in ensuring the dispersal of conidia: dry-spored *Geosmithia* were found on more than 20 bark beetle species in Europe, Africa, North and Central America (Kolařík *et al.* 2004, 2005, 2007, 2008, 2011; Kubátová *et al.* 2004). The structure of *Geosmithia* communities isolated from bark beetles living in central Europe suggests high fidelity in association by both partners (Kolařík *et al.* 2007).

Similar to other fungi that are regular bark beetle associates, also in the case of *Geosmithia*, the benefit of the association for beetles still remains vague (Kolařík *et al.* 2007, 2008). Fungi could play a role in insect feeding (Kolařík & Kirkendall 2010), or affect their fitness through the production of secondary metabolites that could inhibit detrimental microbes or act as repellents towards predators (Stodulková *et al.* 2009). Kolařík & Kirkendall (2010) have proposed that the association of fungi with phloeophagous bark beetles is evolutionarily ancestral, followed by at least three independent shifts to obligate association with ambrosia beetles and by fundamental morphological adaptations. There is growing evidence that this association is consistent and evolutionarily stable. The most convincing proof is the discovery in the genus *Geosmithia* of true ambrosia species, the presence of the beetle-vectored pathogen *G. morbida*, and the fact that some *Geosmithia* species are specialists restricted to few insect vectors and host plants over large geographic areas (Kolařík *et al.* 2007, 2008; Kolarık & Jankowiak 2013).

Host trees of bark beetles associated with *Geosmithia* species, i.e. *Quercus*, *Prunus* and *Ulmus*, are abundant, widely distributed, present in temperate areas for a long time and not taxonomically isolated in their area (Brändle & Brandl 2001). In particular, *Geosmithia* species on *Ulmus* share the same habitat, although different ecological niches, with ascomycete fungi of the genus *Ophiostoma*, causal agents of Dutch elm disease (DED). DED, one of the most destructive diseases of woody trees ever known in plant pathology, is caused by *Ophiostoma ulmi*, *Ophiostoma novo-ulmi* and *Ophiostoma himal-ulmi*. Spread of the fungus and successful infection of elms are mainly due to the synchrony between the life cycles of host-tree, pathogen, and elm bark beetles such as *Scolytus scolytus* F. and *Scolytus multistriatus* Marsham. Such a synchrony enables the insect vectors to spread the pathogen when host plants are more prone to infection and when temperatures are favourable to fungal growth, enhancing the pathogen aggression (Webber & Brasier 1984; Webber & Gibbs 1989; Webber 1990; 2000; Faccoli *et al.* 1998; Faccoli 2001, 2004; Santini & Faccoli 2015). The same insects are also vectors of *Geosmithia* species on elm.

Bettini *et al.* (2014) have recently demonstrated the transfer of a genomic fragment containing the gene encoding the hydrophobin cerato-ulmin between *O. novo-ulmi* and *Geosmithia* spp., suggesting a closer relationship between the two species beyond simple coexistence inside elm trees. Continuous physical contact between organisms, as a consequence of habitat sharing, has been in fact identified as a favourable condition for the horizontal transfer of genetic material (Aguileta *et al.* 2009; Fitzpatrick 2012).

Several *Geosmithia* species have been previously reported on bark beetles associated with elm: *Geosmithia pallida*, *Geosmithia flava*, *Geosmithia langdonii*, *Geosmithia* spp. 10, 13 and 20 were isolated in beetles galleries into elm trees (Kolařík *et al.* 2004, 2005, 2007, 2008). However, a systematic study of *Geosmithia* species associated with European species of the genus *Ulmus* is still lacking. The main aim of this paper was to clarify the taxonomy of *Geosmithia* species on elm trees in the Czech Republic and in Central Italy in order to explore their origin and evolution, to identify the species most frequently associated with *O. novo-ulmi*, and to investigate the possible relationship between these fungi.

The taxonomy of *Geosmithia* was studied by phylogenetic analysis based on ITS rDNA, elongation factor 1-α and β-tubulin gene sequences, combined with the analysis of morpho-physiological traits.

Materials and methods

Sampling and isolation of Geosmithia *fungi*

During 2009–2010, 47 elm trees infested by subcorticolous insects were sampled from 17 sites in the Czech Republic and three sites in central Italy (Table 1). Most of the sampled elm trees showed DED symptoms caused by *Ophiostoma novo-ulmi*. Parts (mostly branches) of the infested host plants were collected and immediately transferred to the laboratory. Larvae, ova, frass, and wood dust from each gallery system (larval galleries belonging to the same mother gallery) were plated directly onto Petri dishes containing malt extract isolation medium (2 % MEA, Oxoid, Basingstoke, UK) and cultivated at 24 °C for 7–14 d. Twenty gallery systems were processed from each sample.

Identification of Geosmithia *fungi*

Pure cultures obtained as described above were sorted into morphotypes using cultural and micro-morphological observations (Pitt 1979; Kolařík *et al.* 2004, 2005). In total, over 200 *Geosmithia* spp. isolates were grouped into six morphotypes

Table 1 – *Geosmithia* strains used in the present study and GenBank accession numbers of their rDNA sequences.

Host	Location	Isolate number	*Species*	ITS	βTub	EF1-α
				GenBank accession n.		
U. minor	Strèdokluky, Central Bohemia, Czech Rep.	**CNR19**	***G. omnicola***	KR229878	KP990557	KR135483
		CNR28	***G. sp. 5***	KR229885	KP990565	KR135492
		CNR23	***G. ulmacea***	KR229881	KP990560	KR135487
U. minor	Strèdokluky, Central Bohemia, Czech Rep.	**CNR5**	***G. omnicola***	KR229867	KP990546	KR135473
		CNR18	***G. omnicola***	KR229877	KP990556	KR135482
U. laevis	Strèdokluky, Central Bohemia, Czech Rep.	**CNR13**	***G. omnicola***	KR229872	KP990551	KR135478
		CNR15	***G. omnicola***	KR229874	KP990553	KR135480
		CNR32	***G. omnicola***	KR229888	KP990568	
U. minor	Libick Luh Velky Osek, Vilegia, Central Bohemia, Czech Rep.	**CNR25**	***G. langdonii***	KR229882	KP990562	KR135489
		CNR38	***G. omnicola***	KR229891	KP990571	KR135498
		CNR17	***G. omnicola***	KR229876	KP990555	KR135481
		CNR21	***G. omnicola***	KR229880	KP990559	KR135485
		CNR40	***G. sp. 2***	KR229893	KP990573	KR135500
		CNR33	***G. sp. 5***	KR229889	KP990569	KR135495
U. minor	Libick Luh Velky Osek, Vilegia, Central Bohemia, Czech Rep.	**CNR11**	***G. langdonii***	KR229871	KP990550	KR135477
		CNR16	***G. omnicola***	KR229875	KP990554	
		CNR43	***G. omnicola***	KR229894	KP990575	KR135502
		CNR39	***G. sp. 2***	KR229892	KP990572	KR135499
		CNR42	***G. sp. 2***		KP990574	KR135501
		CNR24	***G. ulmacea***		KP990561	KR135488
U. minor	Libick Luh Velky Osek, Vilegia, Central Bohemia, Czech Rep.	**CNR7**[a]	***G. langdonii***	KR229869	KP990548	KR135475
		CNR27	***G. langdonii***	KR229884	KP990564	KR135491
		CNR14	***G. omnicola***	KR229873	KP990552	KR135479
		CNR48	***G. sp. 5***	KR229897	KP990579	KR135506
		CNR31	***G. sp. 5***	KR229887	KP990567	KR135494
U. laevis	Libick Luh Velky Osek, Vilegia, Central Bohemia, Czech Rep.	**CNR6**[a]	***G. langdonii***	KR229868	KP990547	KR135474
		CNR26	***G. langdonii***	KR229883	KP990563	KR135490
		CNR8	***G. omnicola***	KR229870	KP990549	KR135476
		CNR20	***G. omnicola***	KR229879	KP990558	KR135484
		CNR30	***G. sp. 5***	KR229886	KP990566	KR135493
		CNR36	***G. sp. 5***	KR229890	KP990570	KR135496
U. minor	Marsovice, Czech Rep.	**CNR120**	***G. flava***	KR229927	KP990611	KR135539
		CNR117[a]	***G. langdonii***	KR229924	KP990608	KR135537
U. glabra	Marsovice, Czech Rep.	**CNR49**	***G. sp. 5***			KR135507
U. laevis	Libis, Central Bohemia, Czech Rep.	**CNR104**[a]	***G. langdonii***	KR229920	KP990603	KR135532
		CNR51	***G. ominola***	KR229898	KP990580	KR135508
		CNR61	***G. sp. 2***	KR229905	KP990587	KR135516
		CNR102	***G. sp. 5***	KR229919	KP990602	KR135531
U. laevis	Libis, Central Bohemia, Czech Rep.	**CNR76**	***G. langdonii***		KP990595	KR135524
		CNR111[a]	***G. langdonii***	KR229922	KP990605	KR135534
		CNR112	***G. langdonii***		KP990606	KR135535
		CNR44	***G. omnicola***		KP990576	KR135503
		CNR119	***G. omnicola***	KR229926	KP990610	KR135538
		CNR60	***G. sp. 2***	KR229904	KP990586	KR135515
		CNR78	***G. sp. 2***	KR229914	KP990597	KR135526
U. minor	Libis, Central Bohemia, Czech Rep.	**CNR118**	***G. omnicola***	KR229925	KP990609	
U. laevis	Dobrin, Usti nad Labem, Czech Rep.	**CNR72**	***G. omnicola***	KR229910	KP990592	
		CNR45	***G. sp. 2***	KR229895	KP990577	KR135504
		CNR46	***G. sp. 2***	KR229896	KP990578	KR135505
U. glabra	Hostenice, Usti nad Labem, Czech Rep.	**CNR73**	***G. omnicola***	KR229911	KP990593	KR135522
		CNR124	***G. sp. 2***	KR229928	KP990612	KR135540
		CNR59	***G. sp. 5***			KR135514
		CNR63	***G. sp. 5***	KR229906	KP990588	KR135517
U. glabra	Hostenice, Usti nad Labem, Czech Rep.	**CNR105**[a]	***G. langdonii***	KR229921	KP990604	KR135533
		CNR84	***G. omnicola***	KR229915	KP990598	KR135527
		CNR86	***G. omnicola***	KR229916	KP990599	KR135528
		CNR74	***G. omnicola***	KR229912	KP990594	KR135523
		CNR99	***G. sp. 2***	KR229918	KP990601	KR135530
		CNR54	***G. sp. 2***	KR229901	KP990583	KR135511
		CNR52	***G. sp. 2***	KR229899	KP990581	KR135509
		CNR69	***G. sp. 2***	KR229907	KP990589	KR135519
		CNR53	***G. sp. 5***	KR229900	KP990582	KR135510

(*continued on next page*)

Table 1 – (continued)

Host	Location	Isolate number	*Species*	ITS	βTub	EF1-α
				GenBank accession n.		
U. glabra	Milà, Usti nad Labem, Czech Rep.	**CNR77**	***G. omnicola***	KR229913	KP990596	KR135525
		CNR56	***G. sp. 5***	KR229902	KP990584	KR135512
		CNR57	***G. sp. 5***	KR229903	KP990585	KR135513
U. glabra	Milà, Usti nad Labem, Czech Rep.	**CNR70**[a]	***G. langdonii***	KR229908	KP990590	KR135520
U. glabra	Milà, Usti nad Labem, Czech Rep.	**CNR98**[a]	***G. langdonii***	KR229917	KP990600	KR135529
		CNR71	***G. omnicola***	KR229909	KP990591	KR135521
		CNR115	***G. omnicola***	KR229923	KP990607	KR135536
Ulmus FL364	Ugnano, Florence, Italy	**CNR132**	***G. sp. 20***	KR229929	KP990614	KR135541
Ulmus FL364	Ugnano, Florence, Italy	**CNR126**	***G. sp. 2***		KP990613	
		CNR134	***G. sp. 20***	KR229930	KP990615	KR135542

a Strains of *G. langdonii* with the same nucleotide sequences.

(Table 2). 72 representative strains were selected, grown on 2 % MEA at 25 °C for five days, and then maintained at 4 °C. Only monosporic isolates were used for further analyses. Ex-type and other representative strains were freeze-dried in slants of 2 % MEA and deposited in the Institute for Sustainable Plant Protection-CNR Florence collection facility (ID numbers are reported in Table 1).

In order to characterise two new species, previously known with their provisional names G. sp. 10 and G. sp. 13 (Kolařík *et al.* 2007, 2008), a representative strain of each morphotype was cultured on MEA 2 %, Czapek Yeast Autolysate Agar (CYA, Oxoid, Basingstoke, UK) (Pitt 1979) and Czapek Dox Agar (CZD, Oxoid, Basingstoke, UK) (Raper & Thom 1949). Cultures were incubated in the dark at 25 °C and 37 °C and the micromorphology was studied on 7–14 d old colonies.

Growth rate

Mycelial plugs 6 mm in diameter were taken from the margins of actively growing, 7 day-old cultures of monosporic strains and placed onto a Petri dish with 20 ml of 2 % MEA. Plates were incubated at 15, 25, and 30 °C. For each temperature, three replicates per strain were used. Radial growth was measured after 2, 7, 10, and 14 d of growth. Differences among *Geosmithia* species in daily growth rate at different temperatures were analysed by means of ANOVA.

DNA extraction and analysis

Genomic DNA was isolated from mycelium grown on 2 % MEA for 3 d at 25 °C in darkness, according to the protocol by Lee & Taylor (1990). The concentration and purity of the extracted nucleic acid were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

The DNA of all the samples was used as template for the amplification of the following regions:

The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) cistron (480 bp) was amplified using the primers (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.* 1990). A 540 bp fragment of the β-tubulin gene was amplified using universal primers Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass & Donaldson 1995) and T10 (5′-ACGATAGGTTCACCTCCA-GAC-3′) (O'Donnell & Cigelnik 1997). A 280 bp fragment of the elongation factor 1-α (EF1-α) gene was amplified using the primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) (Carbone & Kohn 1999, modified).

The amplification protocol used to amplify the ITS and β-tubulin regions was as follows: reaction mixtures (25 µl) contained 50 ng of genomic DNA, 5 µM of each primer, 200 µM dNTPs (GeneSpin, Milan, Italy), 1 × PCR Buffer (GeneSpin,

Table 2 – *Geosmithia* OTU Colony characters (growth on MEA, 14 d, 25 °C) (Kolarik *et al.* 2007 mod.).

M1. *G. pallida*: Colony low, texture funiculose sometimes with velutinous areas at the colony margin, obverse yellowish brown, light brown, reverse dull yellow to light brown or olive green, soluble pigment absent.
M2. *G. langdonii*: Colony low or slightly raised, texture velutinous with a deep crust of conidia, or almost floccose, obverse white to cream sometimes with orange shades, reverse pale yellow to amber, sometimes with shades of rose or orange, soluble pigment absent.
M3. *G. flava*: Colony low or slightly raised centrally, texture velutinous with a crust of conidial chains, often with floccose or funiculose areas or wholly strongly funiculose, obverse light yellow to dull yellow or slightly yellow, soluble pigment absent.
M4. *G. omnicola*: Colony low, texture velutinous or slightly funiculose with apparent overgrowth of aerial mycelium, obverse white to cream, sometimes with slightly orange or yellowish shades, soluble pigment absent.
M5. *G. ulmacea*: Colony low, surface and texture plane or raised centrally and wringled; substrate mycelium dense, forming tough basal felt; aerial mycelium hyaline; sporulation weak to moderate, pale cream to yellowish; reverse yellowish to ochre; soluble pigment absent.
M6. *G. sp. 20*: Colony low, texture plane or velutinous with a deep crust of conidia, obverse white, reverse dull light yellow or white, soluble pigment absent.

Milan, Italy), 25 mM $MgCl_2$, and five units of Taq polymerase (TaqPol, GeneSpin, Milan, Italy). Amplifications were carried out in a Mastercycler (Eppendorf, Hamburg, Germany) for one cycle at 95 °C for 5 min, 30 cycles at 95 °C for 5 min, 56 °C for 90 s, 72 °C for 2 min, and one cycle at 72 °C for 10 min. The conditions for the amplification of EF1-α were: 20 ng of genomic DNA, 10 μM of each primer, 200 μM dNTPs (GeneSpin, Milan, Italy), 1X PCR Buffer (GeneSpin, Milan, Italy), 25 mM $MgCl_2$, five units of Taq polymerase (TaqPol, GeneSpin, Milan, Italy). Reaction mixtures were subjected to 30 cycles as follows: one cycle at 95 °C for 8 min, 35 cycles at 95 °C for 15 s, 55 °C for 20 s, 72 °C for 60 s, and one cycle at 72 °C for 5 min.

Sequencing and phylogenetic analysis

PCR products were visualized on a 1 % agarose gel and purified using the NucleoSpin Extract II kit (Macherey–Nagel, KG, Germany). All PCR products were sequenced by Eurofins MWG Operon (Ebersberg, Germany). GenBank accession numbers for all sequences from this study are reported in Table 1.

The ITS sequences were compared with those of *Geosmithia* species present in GenBank, with a particular focus on those isolated from elm.

One dataset for each gene and a combined dataset of three gene regions were aligned with MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/) (Katoh & Toh 2010) and optimized manually with BioEdit (Hall 1999) to allow maximum alignment. Gaps were treated as missing data. Ophiostoma novo-ulmi ITS (GenBank accession no. KF854009 and EF429091), β-tubulin (GenBank accession no. AY305712) and EF1-α (GenBank accession no. KF899885) were used as outgroup sequences.

The aligned datasets were then analysed with PAUP (Phylogenetic Analyses Using Parsimony) v. 4.0b10 as described by Swofford (2003). Maximum parsimony (MP) was performed using the heuristic research option with random stepwise addition in 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithm and random taxon addition of sequences for the construction of Maximum Parsimony trees. All characters were weighted equally, and character state transitions were treated as unordered. Parameters measured for parsimony included tree length (TL), consistency index (CI), rescaled consistency index (RC), retention index (RI) and homoplasy index (HI). Bootstrap support values were evaluated using 1000 bootstrap replicates (Hillis & Bull 1993). The resulting trees were saved in Phylip format and viewed with FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

In addition, datasets were analysed using Bayesian inference (BI) with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), with a General Time Reversible (GTR) model and gamma-distributed rate variation across sites. For the Bayesian inference, a Markov chain Monte Carlo (MCMC) (Larget & Simon 1999) method was performed to confirm the topology of the tree, by running four chains simultaneously starting from a random tree topology and run for ten million cycles, sampling a tree every 100 generations. The stationary phase was reached when the average standard deviation of split frequencies approached 0.01. Trees that preceded the stabilization of the likelihood value (the burn-in) were discarded (about 25 %), and the remaining trees were used to calculate a 50 % majority-rule consensus phylogram that was viewed and edited with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html), with Bayesian posterior probabilities (PP) values for the internal tree nodes.

Results

Morphological and cultural characteristics

Tissue explants from elm samples gave rise to 200 isolates that produced penicillia and conidia typical of the genus *Geosmithia*. Six groups were identified on the basis of culture morphology. Two groups were identified to the species level, *Geosmithia langdonii* (morphotype 2) and *Geosmithia pallida* (morphotype 1). The main morphological characteristics of the six morphotypes (according to Kolařík *et al.* 2007) are reported in Table 2. From 18 tree samples no *Geosmithia* spp. was obtained. Fungi belonging to other genera (*Bionectria* spp. [7 %] and other *Sordariales* [1 %]) were also isolated.

The optimal temperature for growth was 25 °C for all *Geosmithia* samples. Growth was in general faster at 30 °C than at 15 °C (Fig 1). In particular, *Geosmithia* sp. 20 had significantly higher growth rate at 30 °C than all other *Geosmithia* spp. in this work. On the other hand, the growth rate of *Geosmithia* sp. 13 (Kolařík *et al.* 2008) was the lowest both at 25 °C and 30 °C.

DNA sequencing and analysis

High quality genomic DNA was obtained from 90 % of the samples using the protocol reported by Lee & Taylor (1990). PCR resulted in fragments of 480 bp (ITS rDNA), 540 bp (β-tubulin) and 280 bp (EF1-α). DNA barcodes produced in this study can be accessed through GenBank (Table 1).

Maximum parsimony and Bayesian inference produced nearly identical topologies for all datasets (ITS, β-tubulin, EF1-α and the combined dataset with three genes).

Aligned ITS dataset consisted of 113 in-group sequences of which 66 represent isolates obtained in this study and 47 known *Geosmithia* spp. obtained from GenBank. For sake of brevity, one of the most parsimonious trees (length = 121, CI = 0.760, RI = 0.981, RC = 0.778,

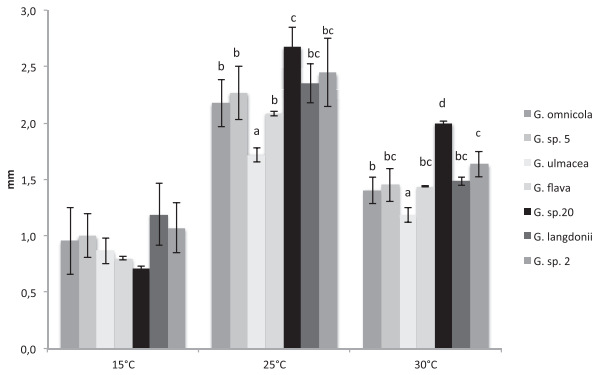

Fig 1 – Mean daily radial mycelial growth rate of different *Geosmithia* species, cultured on MEA 2 % at 15, 25, 30 °C and measured at 2, 7, 10 and 14 d.

HI = 0.693) is shown in Supplementary Fig 1. Seven terminal clades were observed for all isolates considered in the phylogenetic analyses of ITS sequence data (Supplementary Fig 1). *Geosmithia langdonii* (14 GenBank isolates and 12 isolates from this study) set up clade I; clade II represents *Geosmithia flava* with one wild strain, and clade IV consisted of one wild strain and one isolate of *Geosmithia* sp. 13 (Kolařík *et al.* 2008). Twenty-five of the isolates obtained in this study grouped to clade III referable to *Geosmithia* sp. 10 (Kolařík *et al.* 2007), while two isolates from Italy clustered to clade V, referable to *Geosmithia*. sp. 20 (Kolařík *et al.* 2007). Clade VI and clade VII contain *Geosmithia* sp. 5 and sp. 2, respectively (Kolařík *et al.* 2008).

The phylogenetic analyses of β-tubulin and EF1-α sequence data resulted in the same clades. One of the most parsimonious trees for either the β-tubulin (length = 252, CI = 0.771, RI = 0.980, RC = 0.762, HI = 0.745) and the EF1-α gene data (length = 233, CI = 0.775, RI = 0.978, RC = 0.777, HI = 0.692), is shown in Supplementary Fig 2 and Supplementary Fig 3, respectively.

Combined data for the ITS, β-tubulin and EF1-α gene regions produced a dataset containing 63 in-group sequences of 1250 characters of which 478 characters were parsimony informative (CI = 0.687, RI = 0.928, RC = 0.788, HI = 0.723). Phylogenetic analysis revealed some well-supported clades (Fig 2): all clades have a distinct and unique phenotype and represent clearly defined and easily recognizable morphotypes. Clade I corresponds to *Geosmithia* sp. 10 (Kolařík *et al.* 2007); clade II completely matches to *G. langdonii*; clade III represents *Geosmithia* sp. 13 (Kolařík *et al.* 2008); clade IV corresponds to *G. flava* and clade V to *Geosmithia* sp. 20 with Italian samples. Clade VI and VII contain isolates referable to *Geosmithia* sp. 2 and *Geosmithia* sp. 5, respectively (Kolařík *et al.* 2008).

Taxonomy

On the basis of morphological and phylogenetic analysis supported by robust bootstrapping results, *Geosmithia* sp. 10 and *Geosmithia* sp. 13 are described as new species.

Geosmithia omnicola sp. nov. (Fig 3).

MycoBank: MB812584, Holotype: CNR5.

Geosmithia OTU10 in Kolařík *et al.* (2007).

Etymology: '*omnicola*' (from Latin '*omnis*' = every) refers to the high plasticity in growing on many different host vectors.

Colony diam (14 d): MEA at 25 °C: 25–60 mm; CYA at 25 °C: 38–60 mm; CZD at 25 °C: 28–60 mm; MEA at 37 °C: no growth or microcolonies only, microcolonies only or 3–5 mm; CYA at 37 °C: not growth or microcolonies only, microcolonies only or 3–8 mm; CZD at 37 °C: not growth or microcolonies only, microcolonies only or 2–6 mm.

Colony characteristics MEA: margin narrow, entire; substrate mycelium white, velutinous with floccose and funicolose areas or wholly strongly funicolose in the inner part (old part of mycelium); aerial mycelium hyaline overgrowth of aerial mycelium; sporulation moderate to heavy, pale cream; vegetative mycelium hyaline; reverse lighter; soluble pigment – absent.

Colony characteristics CYA: plane, low or slightly raised centrally, aerial mycelium less than on MEA, pale to dull yellow. (MK1768 and CNR72 colour brown to orange).

Colony characteristics CZD: plane, low with surface texture velutinous or floccose, mycelium pale to dull yellow in the inner of colony, low aerial mycelium.

Yeast-like colonies: absent.

Conidiophore: Penicillium-like, arising from surface mycelium, verrucose, septate; base often foot cell like or typically curved (peg foot); stipe 70–130 × 2–3.5 μm; penicillus asymmetric often with divaricate branch, sometimes with phialides arising on the different levels, 3–5 (6) × branched, 20–70 × 15–30 μm, 1. branch 11–28 μm × 1.4–3.5 μm, 2–4 per cluster; 2. branch 9–15 × 1.3–2.5 μm, 2–3 per cluster, metulae 5–15 × 2.0–2.0, 2 per cluster; phialides cylindrical without distinct neck, 5–10 × 1.7–2.4, 2–3 per cluster.

Conidial chains: not persistent, not forming crust.

Conidia: cylindrical to ellipsoid, (2.8–) 3.5–3.8 (–4.8) × (1.2–) 1.7–1.9 (–2.7) μm.

Substrate conidia: absent. Teleomorph: unknown.

Distribution: temperature Europe and Mediterranean area.

Ecology: in galleries and surrounding (phloem and sapwood) of bark beetles.

Specimens examined: MK1915, MK1508, MK989, MK544, MK1788, MK1516, MK768, CNR5, CNR8, CNR14, CNR15, CNR44, CNR19, CNR69, CNR72, CNR77, CNR115.

Holotype: CNR5 (dried culture on MEA, Fungal Collection Institute for Sustainable Plant Protection – CNR, Florence, Italy). Isolated from *Ulmus laevis*, Libick Luh Velky Osek, Vilegia, Central Bohemia, Czech Rep.

Diagnostic features and similar species: G. omnicola is characterized by substrate mycelium white velutinous with floccose and funicolose areas or wholly strongly funicolose in the inner part. *G. omnicola* is morphologically similar to *Geosmithia* sp. 21 (Kolařík *et al.* 2007), but it can be distinguished from this species based on ITS rDNA data.

Notes: All strains exhibited almost identical morphology, differing in colony growth rate only. The strain MK1788 grows less than the other. MK989 grows at 37 °C faster than the others.

Geosmithia ulmacea sp. nov. (Fig 4).

MycoBank: MB812582, Holotype: CNR23.

Geosmithia OTU13 in Kolařík *et al.* (2008).

Etymology: typically associated to bark beetles of the species of genus *Ulmus*.

Colony diam (14 d): MEA at 25 °C: 15–25 mm; CYA at 25 °C: 22–45 mm; CZD at 25 °C: 12–28 mm; MEA at 37 °C: no growth.

Colony characteristic MEA: margin lobate even in freshly isolated strains; substrate mycelium dense, forming tough basal felt; aerial mycelium hyaline; surface and texture plane or raised centrally and wringled, velutionus; sporulation weak to moderate, pale cream to yellowish; vegetative mycelium hyaline; reverse yellowish to ochre; soluble pigment – absent.

Colony characteristic CYA: similar to MEA, sporulation low.

Colony characteristic CZD: similar to MEA, colour of conidia *en masse* more intensive, pale to dull yellow, sometimes with shades of brown, reverse lighter.

Yeast-like colonies: absent.

Conidiophore: Penicillium-like, arising from surface mycelium, verrucose; base often foot cell like or typically curved (peg foot); stipe 100–250 × 2.5–3.5 μm; penicillus asymmetric often with divaricate branch, sometimes with phialides arising on the different levels, 3–5 (6) × branched,

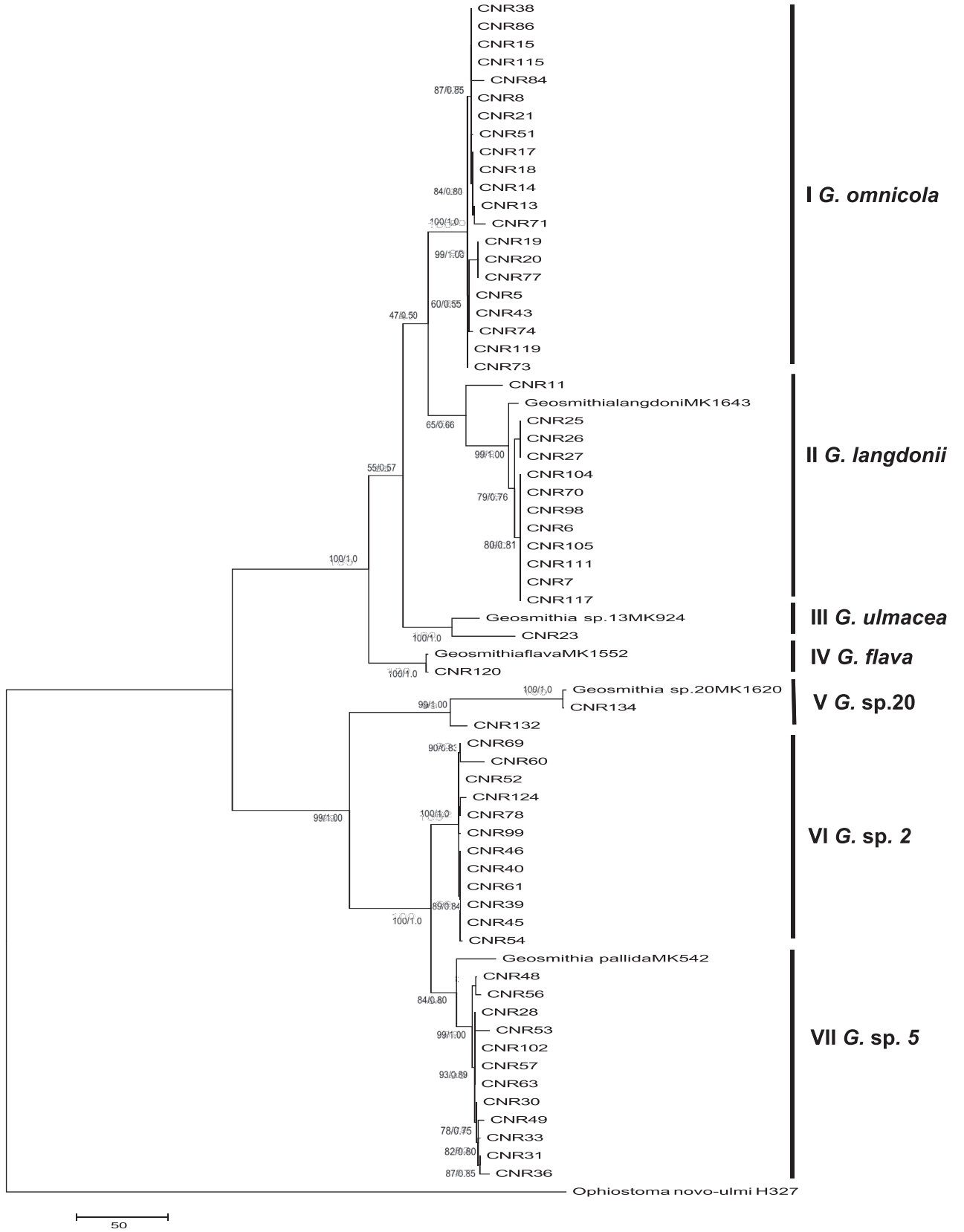

Fig 2 – One of the most parsimonious trees (length = 788) from the combined sequence datasets of the ITS rDNA, β-tubulin and EF1-α loci is shown (CI = 0.687, RI = 0.928, RC = 0.788, HI = 0.723). The MP and Bayesian posterior probability are indicated next to the branches. *Ophiostoma novo-ulmi* ITS, β-tubulin and EF1-α sequences are used as outgroup.

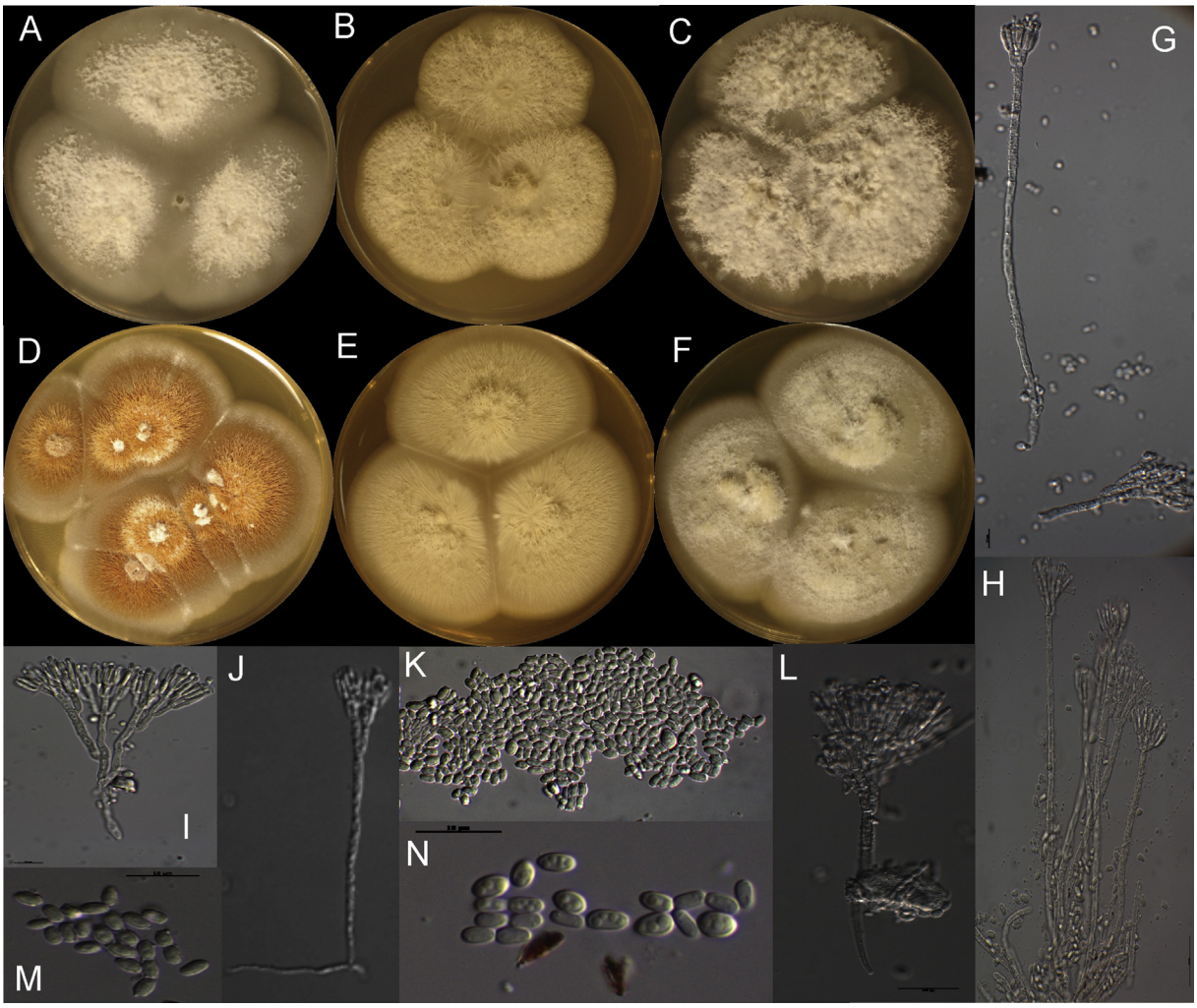

Fig 3 – *Geosmithia omnicola*. Two-week-old colonies grown at 25 °C on MEA (A–C) and CYA (D–F): A MK1516, B CNR19, C CNR8, D MK1768, E CNR19, F CNR15. Conidiophores: G CNR15, H CNR14, I MK1516, J CNR8, L MK989. Conidia: K CNR8, M CNR15, N MK1516. Bars: I, G, K, L, M, N 10 μm; H–J 20 μm.

35–125 × 25–50 μm, 1. branch 13–33 μm × 2.5–3.5 μm, 2–4 per cluster; 2. branch 10–15 × 2.0–2.8 μm, 2–3 per cluster, metulae 10–15 × 2.0–2.0, 2 per cluster; phialides cylindrical without distinct neck, 9–14 × 1.7–2.4, 2–3 per cluster.

Conidial chains: not persistent, not forming crust, 100–200 μm long.

Conidia: cylindrical to ellipsoid, (3.7–) 4.2–4.5 (–5.5) × (2.0–) 2.1–2.4 (–5.0) μm.

Substrate conidia: absent.

Teleomorph: unknown.

Distribution: Czech Republic, Colorado (USA).

Ecology: Galleries of elm bark beetles.

Specimens examined: CCF3559 (=MK977), MK1515a, MK1519, MK924, MK963, MK1701, CNR23, CNR24.

Holotype: CNR23 (dried culture on MEA, Fungal Collection Institute for Sustainable Plant Protection – CNR, Florence, Italy). Isolated from *Ulmus minor*, Strèdokluky, Central Bohemia, Czech Rep.

Diagnostic features and similar species: Restricted growth on MEA, small number of elements in the conidiophore, short and broad conidia. *G. ulmacea* grows faster on CYA than on the other substrates and it does not grow at 37 °C.

Notes: All strains exhibited almost identical morphology, differing in colony growth rate only. An isolate obtained from *Scolytus sheeveriie* sampled from *Ulmus* sp. in Fort Collins (USA) has an identical ITS rDNA sequence to *G. ulmacea* (N. Tisserat pers. comm.). This finding illustrates a broad distribution and high host specificity of *G. ulmacea*.

Discussion

The sampling work amassed a collection of 72 isolates identified as *Geosmithia* spp. whose phylogenetic relationships were determined through morphological, cultural and molecular characterization. Since the use of just ITS data is not fully reliable for identification of many species of *Geosmithia* (Kolařík & Kirkendall 2010; Kolařík *et al.* 2011; Kolařík & Jankowiak 2013; Machingambi *et al.* 2014), phylogenetic characterisation was based on three genes: ITS rDNA, β-tubulin and EF1-α nucleotide sequences. These analyses enabled us to identify

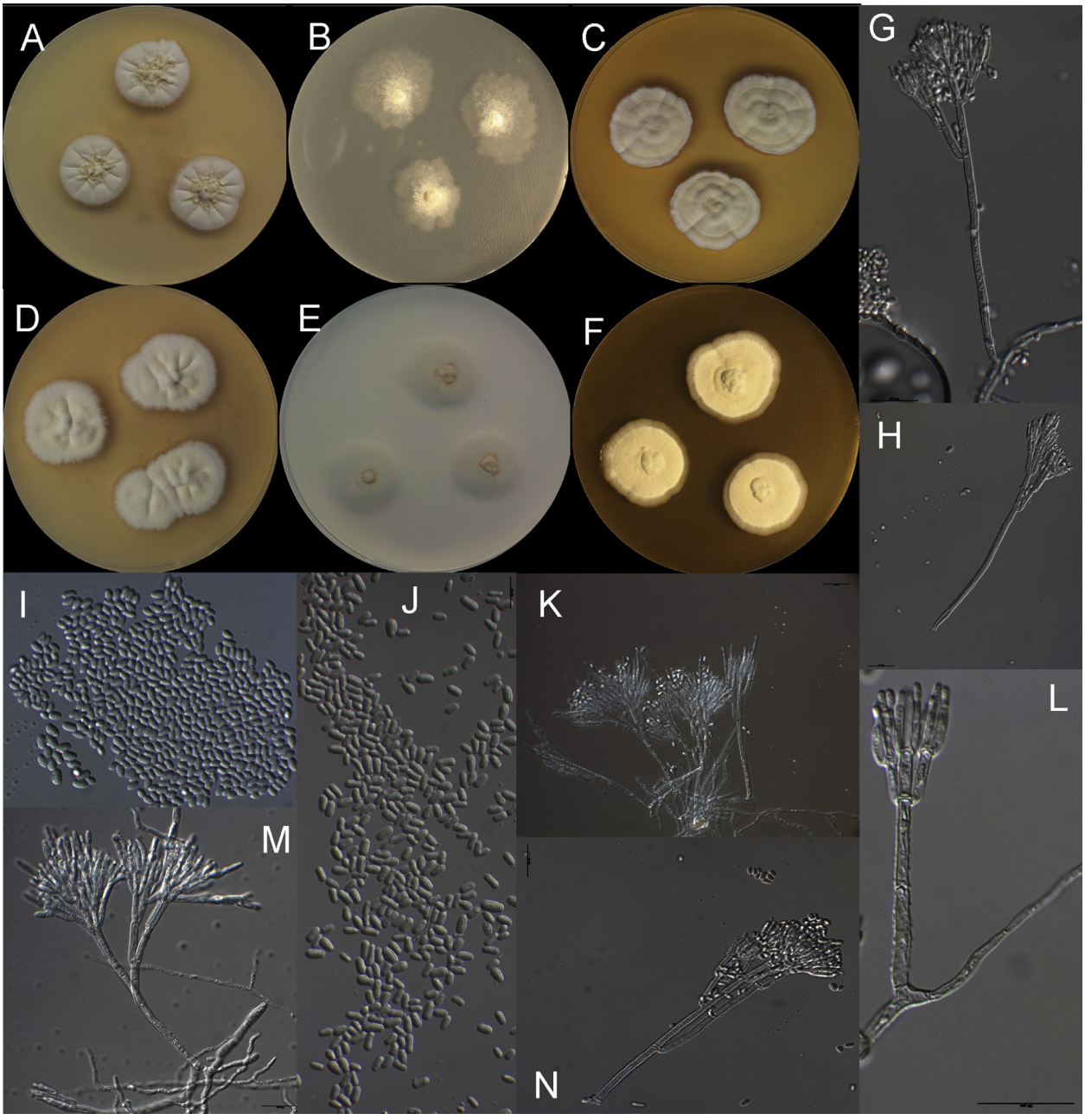

Fig 4 – *Geosmithia ulmacea*. Two-week-old colonies grown at 25 °C on MEA (A, D), CZD (B, E) and CYA (C–F): A-C CNR23, D-E MK977, F MK924. Conidiophores: G MK977, H CNR23, K MK924, L MK977, M MK1515, N MK1519. Conidia: I CNR23, J MK977. Bars: G, H, K, L, M, N 20 μm; I–J 10 μm.

seven *Geosmithia* species living on European species of the genus *Ulmus* from larvae, ova, frass and wood dust in bark beetle galleries of dying, stressed or weakened elm trees. The wild strains in this study were assigned to *Geosmithia* sp. 2 and sp. 5 (previously described as an unique species *Geosmithia pallida*), *Geosmithia langdonii*, and to some previously undescribed (*Geosmithia omnicola*, *Geosmithia ulmacea*) or partially described (*Geosmithia* sp. 20, Kolařík *et al.* 2007) species. All *Geosmithia* spp. isolates collected on elm in this work belong to the *Hypocreales* lineage, as reported by Kolařík *et al.* (2011).

G. pallida is reported as one of the most variable *Geosmithia* species, exhibiting a high genotypic and morphological diversity probably owing to recombination events or range overlaps of previously allopatric populations (Kolařík *et al.* 2004, 2007). *G. pallida* is in fact a species complex including several different types (Kolařík *et al.* 2004). The population analysed in this paper had highly variable morphological and molecular features, confirming the hypothesis of a complex of species (M. Kolařík, unpublished results). This species complex is neither exclusive on elms nor on elm's vectors (Kolařík *et al.* 2004, 2007): its extreme variability for both morphological and genetic traits may be attributed to the fact that the species is in its centre of origin (McDonald & Linde 2002; Dutech *et al.* 2007). The founder population within a species' endemic range is generally expected to portray higher genetic diversity than introduced populations (Nei *et al.* 1975; Dlugosch & Parker 2008).

Strains clustering to clade V (Fig 4) form a new taxonomic entity closely related to the partially described *Geosmithia* sp. 20 (Kolařík *et al.* 2007). This clade is closely related to both *G. pallida* complex sp. 2 and sp. 5 collected in this study. This result suggests the existence of a common ancestor species from which both species (*G. pallida* sp. 2–5 and *Geosmithia* sp. 20) were derived and diversified depending on the colonised environment. Based on the growth rate trial, *Geosmithia* sp. 20 is the most thermophilic: at higher temperature (30 °C), it shows in fact the highest growth rate, while at low temperature (15 °C), growth was slower with respect to the other species analysed. *Geosmithia* sp. 20 was only isolated in Tuscany (Italy) in this study, and in Andalusia (Southern Spain) from a dying field elm (Kolařík *et al.* 2007). This suggests that this previously unknown species might represent a Mediterranean species of the *G. pallida* complex, or a species *per se* adapted to a Mediterranean/sub-tropical climate, associated with bark beetles. Interestingly, all the strains isolated in Italy were obtained from elm trees unaffected by DED.

Geosmithia langdonii, previously reported on *Quercus robur*, *Quercus petreae*, *Quercus delechampii*, *Malus domestica*, *Carpinus betulus*, and *Fagus sylvatica* (Kolařík *et al.* 2005), was also recently identified both as a bark beetle associate and as an endophyte of coast live oak in California (McPherson *et al.* 2013). In the present study this species has been found quite frequently: 12 isolates from different elm species and geographical locations were obtained. Stimulatingly, no sequence variation was found in the three genomic regions analysed (over 1250 bp) for eight out of twelve isolates. These eight strains were isolated in five different sites in Czech Republic, at least 130 km from each other, and from all three elm species native to this region: *Ulmus minor*, *Ulmus glabra* and *Ulmus laevis*. These findings support the hypothesis that this cluster is a clonal population, vegetatively incompatible with the others, recently introduced and therefore showing low gene and allelic diversity (McDonald & Linde 2002; Dutech *et al.* 2007), as expected for a small founder population in a new region (Andjic *et al.* 2011). Genetic uniformity of such a widely distributed population cannot be completely explained by the reproductive features of *Geosmithia* spp. (even if it is thought to be completely asexual like *Penicillium*) (Tuthill 2004), or by the high mobility of its conidia, or by its dispersal by bark beetles, that fly just over short distances (no longer than 400 m) (Lanier 1989). Similar effects have also been attributed to anthropogenic movement of plant material (Rivas *et al.* 2004; Andjic *et al.* 2011), or to the spread of a genotype with higher fitness which tends to replace the existing population (Brasier *et al.* 2004).

G. ulmacea, previously known as *Geosmithia* sp. 13 (Kolařík *et al.* 2007; 2008), was isolated from all European elm species, but never reported on any other plant genus. It is, therefore, the first host-specific *Geosmithia* species to be described, at least on hardwoods. A specific association between two organisms generally derives from a long coevolution and specialisation. Kolařík *et al.* (2007) suggested that the mixture of *Geosmithia* species is mostly determined by the associated insect species. Hence, the association between *G. ulmacea* and elm might be considered secondary, given that elm bark beetles are specialist on elms.

G. omnicola, previously described as *Geosmithia* sp. 10 (Kolařík *et al.* 2007, 2008), was collected from conifers, broad-leaved trees and shrub species in the Mediterranean area and in Central Europe. This species was found on elm at all the collection sites in Czech Republic, but it may be present in other parts of Europe with similar environmental conditions, as it was previously reported in Hungary, Spain, and Italy (Kolařík *et al.* 2007, 2008). It shows a high plasticity in growth temperatures, and in host vector, host plants and habitat range (Kolařík *et al.* 2007, 2008); for this reason it was named '*omnicola*' (from Latin '*omnis*' = every).

Unlike McPherson *et al.* (2013), who found *Geosmithia* strains both on live and dead trees and on beetles in the trees, we were able to isolate strains of *Geosmithia* only from bark beetle galleries in the aftermath of the colonization and oviposition in the bark of dying, stressed, or otherwise weakened elms. No *Geosmithia* strains were ever successfully isolated in the present study on healthy or wilting trees in the absence of colonization by beetles. This result is in contrast with the general assumption that the *Geosmithia* species reported here are true endophytes (Kolařík *et al.* 2004, 2005, 2007, 2011).

Moreover, in the present study *Geosmithia* species were mostly isolated from DED-infected elm trees. As both *Geosmithia* and *Ophiostoma novo-ulmi* share the same habitat (even if they occupy different ecological niches), are transported to the host by the same vectors, and exchange at least one gene at high frequency (Bettini *et al.* 2014), it is possible that their coexistence within tissues of dying elms is not accidental, but the consequence of a recent association. In future research, special attention will be devoted to study *G. ulmacea*, for which the association with *O. novo-ulmi* seems to be more plausible, being this species an elm-specialist species. In addition, at least in one case, horizontal gene transfer of the cerato-ulmin gene from *Ophiostoma novo-ulmi* to *G. ulmacea* was observed. For these reasons, the association with *O. novo-ulmi* could be more probable.

Two different isolates of *Geosmithia* sp. 20 were isolated from a dying plant of the Euro-Asian hybrid elm FL364, a clone selected by the Institute for Sustainable Plant Protection (CNR) for resistance to DED. The plant did not show any DED symptom and it was not possible to isolate *Ophiostoma novo-ulmi* from its tissues. It is worth noticing that at least one of the isolates described in the present paper should have been in contact with *O. novo-ulmi*, since it harbours the *cu* gene (Bettini *et al.* 2014). This result suggests that the association between *Geosmithia* spp. and *Ophiostoma novo-ulmi* is not mandatory, and that the presence of *Geosmithia* on elms is strictly related to the presence of galleries of subcorticolous insects.

Since different European elm species host the same beetle species, the present results seem to confirm that the association with beetle vector species is the main determinant of the composition of *Geosmithia* communities, as previously reported by Kolařík *et al.* (2007). Clustering of samples in ordination analysis is mainly correlated with the material used for isolations (bark beetles and their respective feeding plant), but not with geographical origin. Our results also suggest that *Geosmithia* populations do not differ between elm species, but that markedly different environments encourage different species, at least when the *G. pallida* complex is considered.

During the last century, elm bark beetles have become the main vectors of DED, spreading DED pandemics in Europe. As a consequence of these pandemics, the number of suitable

habitats for both DED fungi and *Geosmithia* species, i.e. dying, stressed or weakened trees colonised by beetles, has tremendously increased. The higher frequency of these habitats in the past few decades has probably favoured the recent discovery and study of *Geosmithia* species by the scientific community.

The present results will serve as a platform for further studies that will be aimed at clarifying the ecological niche and life cycle of *Geosmithia* species on elm, to advance the knowledge on the interactions among *O. novo-ulmi* and *Geosmithia*, and to investigate whether this association might play a role in the regulation of the pathogenesis of DED.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.08.003.

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