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SHORT COMMUNICATION

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First report of *Geosmithia langdonii* and *Geosmithia* spp. isolated from a decaying elm (*Ulmus minor*) in Geneva, Switzerland

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Abstract

The mortality of a young elm *Ulmus minor* in 2014 in Geneva prompted a search for the microorganisms potentially involved. Symptoms included foliar chlorosis and wilting followed by defoliation of branches. Wood symptoms included a brown streaking of sap wood and brown stains in trunk and branches. The comparison of the resulting ITS rDNA sequences to the NCBI Nucleotide database allowed to identify 10 different organisms. The genus *Geosmithia* represented 48% of the isolates belonging to three species: *Geosmithia langdonii* (7 isolates) and 2 unknown morphologically and genetically different *Geosmithia* sp. 1 and sp. 2 (4 isolates). *Geosmithia* species are very little known ascomycetes, which have been recently shown to be opportunistic pathogens on broadleaved trees and conifers, living as saprobes in galleries of many bark beetle species. In the case described here, *Geosmithia langdonii*, and the unknown *Geosmithia* species were found in symptomatic wood while bark beetle galleries were found in close regions of the symptomatic wood. *Geosmithia langdonii* was the major fungus retrieved from the symptomatic wood and could have contributed, along with other identified fungal species, to a pathogenic complex producing symptoms similar to the ones of the Dutch Elm Disease and led to the dieback of this elm tree. *Geosmithia langdonii* and 2 yet unknown *Geosmithia* species (sp. 1 and sp. 2), different from any other reported *Geosmithia* species are reported from an elm tree in Switzerland for the first time.

Key words

Dutch elm disease *Geosmithia langdonii*, *Geosmithia* spp., elm, *Ulmus minor*, endophytes

Dutch elm disease (DED) caused by *Ophiostoma ulmi* and *O. novo-ulmi* is a matter of concern for city corporations, using elm trees in urban plantations. The death occurrence of a young elm in Sept. 2014, in Geneva (lat. 46.1984, long. 6.1423) in an urban plantation of several *Ulmus minor* trees, after a few months of decay symptoms, which appeared end of May 2014 (Fig. 1), prompted a search for the microorganisms potentially involved. Symptoms included foliar chlorosis and wilting followed by defoliation of branches. No symptoms were observed on the other elms trees of this plantation. The tree was felled 2 weeks after death. A large

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Figure 1. Wilting symptoms on an elm tree on 20th June 2014

branch and a small branch were cut in sections. One section of each was surface disinfected with ethanol 70% and sampled by coring, with a Pressler hand auger, in symptomatic regions as shown on Figure 2A. Wood symptoms included a brown streaking of sap wood and brown stains in trunk and branches. Three wood cores (5 mm diam.) were cultivated in Petri dishes on Potato Glucose Agar (PGA) medium (Roth AG, Switzerland) plus ampicillin (50 µg/mL) (Roth AG) at room temperature (Fig. 3) and 23 fungal organisms were isolated in pure cultures (3 isolates from the trunk section sample, 19 isolates from the large branch section and 1 from the small branch section) on PGA medium. All isolates were observed and classified morphologically with the help of a dissecting microscope.

Subcultures for each morphologically different organism were grown in 50 mL Potato Glucose Broth (PGB) medium (Roth AG) plus ampicillin (50 µg/mL) in 150 mL sterile Erlenmeyer flasks, under shaking (100 rpm) at room temperature for one week. Fungal DNA of 14 selected isolates was extracted following a modified protocol adapted from Lefort and Douglas (1999) and assayed in a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA) in order to adjust the final DNA concentration to 50 ng/µl in ultrapure water. PCR of the internal transcribed spacer rDNA (ITS1-5.8S-ITS2) was carried out with the primers pair ITS 4 and ITS 5 (White et al 1990). PCR reactions were performed in a Thermocycler Biometra (Göttingen, Germany) using the Taq polymerase and buffer from Biotools (Madrid, Spain) according to the following programme: initial denaturation for 3 min at 95°C, 38 cycles of 60 s at 95°C, 45 s at 56°C, 60 s at 72°C terminated by a final elongation of 6 min at 72°C. Reactions were carried out in 50 µL final volume at a final concentration of 1.5 mM $MgCl_2$, 100 µM each primer and 0.8 mM dNTPs. After amplification, PCR products quality was assessed by gel electrophoresis in a 1% agarose gel in 1x TBE (Tris

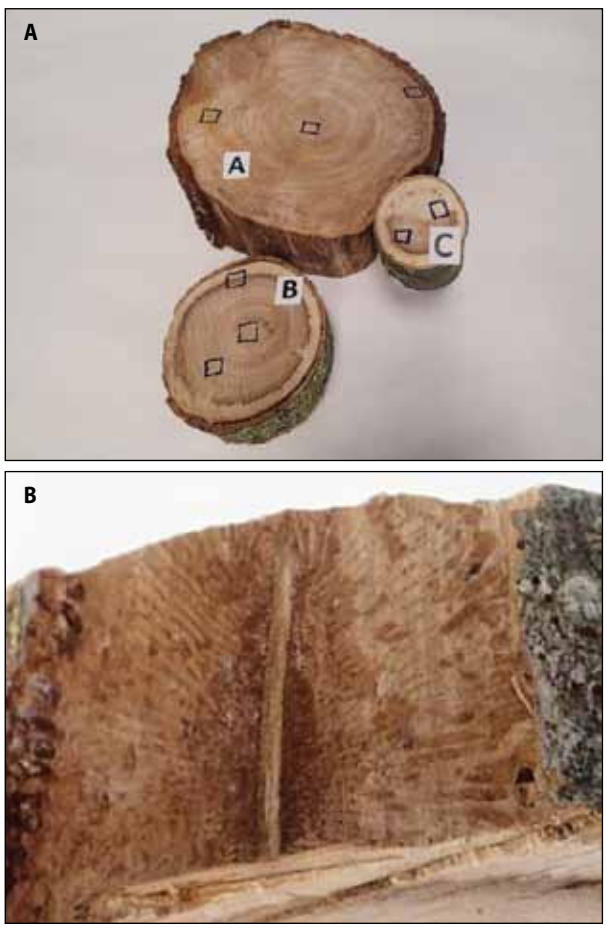

Figure 2. Symptomatic regions sampled on the trunk and branch sections

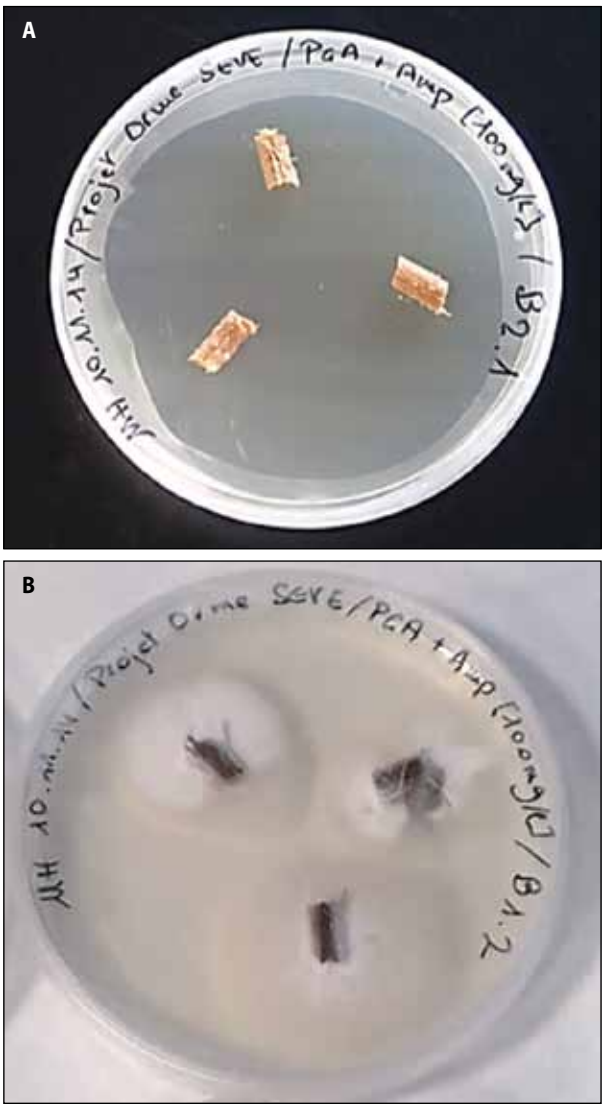

Figure 3. Wood cores on PGA medium (A); Fungal growth on cores on PGA medium (B)

89 mM, boric acid 89 mM, EDTA 2 mM, pH 8) and revealed by GelRed™ visualized through a UV transilluminator. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA). The sequencing of PCR products was carried out at Microsynth AG facilities (Balgach, Switzerland) and sequenced finally edited with Finch TV v 1.4.0 (Geospiza Inc., Seattle, WA, USA).

The comparison of the 14 resulting ITS rDNA sequences to the NCBI Nucleotide database (National Center of Biotechnology Information, Bethesda, USA) allowed identifying 10 different organisms, for which sequences have been registered in NCBI under the accessions KP790123-KP790136. The diversity of identified fungi is shown in Table 1. A number of morphologically identical isolates was not sequenced but are reported in Table 1. The genus *Geosmithia* represented 48% of the isolates belonging to three species: *Geosmithia langdonii* (7 isolates) and 2 unknown morphologically and genetically different *Geosmithia* sp. 1 (2 isolates) and *Geosmithia* sp. 2 (2 isolates). All these isolates were retrieved from the brown sap wood regions in the small and large branches, but not from the trunk as shown on Table 1. The 3 *Geosmithia* species were present in the large branch and one *Geosmithia* sp. was present in the small branch. Three representative isolates, *Geosmithia langdonii* UASWS1324, *Geosmithia* sp. 1 UASWS1325 and *Geosmithia* sp. 2 UASWS1334, have been deposited in the collection DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) under the reference numbers DSM 100090, DSM 100091 and DSM 100360, respectively. The appearance of these three *Geosmithia* species is shown on Figure 4 and conidia on a conidiophore of *G. langdonii* are shown on Figure 5. Most noticeably, none of the two fungal species responsible for DED, *Ophiostoma ulmi* or *Ophiostoma novo-ulmi* were isolated from symptomatic wood samples. The other identified fungi were potential endophytes such as *Cladosporium sphaerospermum*, 1 unknown *Cladosporium* sp., 1 *Aspergillus* sp., *Penicillium brevicompactum* and 2 unknown *Sordariomycetes* sp. genetically close to *Aureobasidium* sp. or *Coniochaeta* sp. It is unclear if some of them could be part of a phytopathogenic complex in association with *Geosmithia* species, able to lead to the observed tree's decay.

Geosmithia species are very little known ascomycetes, which have been recently shown to be opportunistic pathogens on broadleaved trees and conifers, living as saprobes in galleries of many bark beetle species (Jankowiak et al. 2014; Kolařík et al. 2005, 2007). Unknown and undescribed *Geosmithia* species were already reported on elm (Kolařík et al. 2005, 2007). Among the 50 described *Geosmithia* species, nine have been found on elm (Kolařík et al. 2008). More recently Pepori et al. (2015) isolated 72 strains of 6 *Geosmithia* species associated with elms and elm beetles, 3 of these species remaining undescribed. One up to four different species could be retrieved from a single tree. This is

Table 1. Identification of isolated fungi from wood samples (sample origin, sample type, isolates codes, isolates accession in the collection of the Plants and Pathogens research group, GenBank accession and isolate identities)

Sample origin	Sample type	Isolates codes	Isolates accessions	GenBank accessions	Isolate identity
A Trunk section	Core A1	A1.1.1	UASWS1321	KP790123	*Cladosporium sphaerospermum*
	Core A2	A2.1.1	UASWS1322	KP790124	*Aspergillus* sp.
	Core A3	A3.1.1	UASWS1323	KP790125	*Penicillium brevicompactum*
B Large branch section	Core B1	B1.1.1	UASWS1324	KP790126	*Geosmithia langdonii*
		B1.1.2			*Geosmithia langdonii*
		B1.1.3	UASWS1325	KP790127	*Geosmithia* sp. 1
		B1.1.4			*Geosmithia langdonii*
		B1.2.1	UASWS1326	KP790128	*Geosmithia langdonii*
		B1.2.2	UASWS1327	KP790129	*Geosmithia* sp. 1
		B1.2.3a	UASWS1328	KP790130	*Geosmithia langdonii*
		B1.2.3b	UASWS1329	KP790131	*Cladosporium* sp.
		B1.2.3c			*Geosmithia langdonii*
		B1.2.4			*Geosmithia* sp. 2
		B1.2.5	UASWS1330	KP790132	*Cladosporium* sp.
		B1.2.6	UASWS1331	KP790133	*Geosmithia langdonii*
	Core B3	B3.1.1	UASWS1332	KP790134	*Sordariomycetes* sp. 1
		B3.1.2			*Sordariomycetes* sp. 1
		B3.1.3			*Sordariomycetes* sp. 1
		B3.1.4	UASWS1333	KP790135	*Sordariomycetes* sp. 2
		B3.2.1			*Sordariomycetes* sp. 2
		B3.2.2			*Sordariomycetes* sp. 2
		B3.2.3			*Sordariomycetes* sp. 2
C Small branch section	Core C2	C2.2.1	UASWS1334	KP790136	*Geosmithia* sp.

therefore congruent with the observations of three different *Geosmithia* species reported here in a single tree. The comparison of the ITS DNA sequences of these 3 undescribed species, *Geosmithia* sp. 2, *Geosmithia* sp. 5 and *Geosmithia* sp. 20 (Pepori et al. 2015) to the species *Geosmithia* sp. 1 and *Geosmithia* sp. 2 of the present study yielded 91%, 91%, and 99% identity over 83% of the sequence length, respectively for *Geosmithia* sp. 1 93% identity, over 79% of the sequence length with each for *Geosmithia* sp. 2, suggesting that the diversity of *Geosmithia* species in elms is even greater. In the present case, galleries of bark beetles were observed in the wood of the trunk section (Fig. 2B) but not in the branches. An isolate of an undescribed *Geosmithia* sp. was found associated once with Dutch elm disease (Scala et al. 2007) in Italy. *G. langdonii* was also found on elm in a survey of fungi associated to elms (Pepori 2012) and recently identified as a bark-beetle associate and an endophyte of *Quercus agrifolia* in California (McPherson et al. 2013). Similarly to *Ophiostoma ulmi* (or *O. novo-ulmi*) in DED, the pathogenic *Geosmithia* species have most of the time been shown to be associated with bark beetle species. As stated by Kolařík et al. (2008), *Geosmithia* species would be dispersed by *Phloeophagus* bark beetles, with which the association might be very stable and specific, leading to symbiosis and speciation of *Geosmithia* species in some cases. That was the case for *Geosmithia morbida*, the agent of the Thousand Cankers Disease of walnut tree species in Europe and the USA (Kolařík et al. 2011; Zerillo et al. 2014; Montecchio et al. 2014), which has been shown to be associated with the bark beetle *Pityophtorus juglan-*

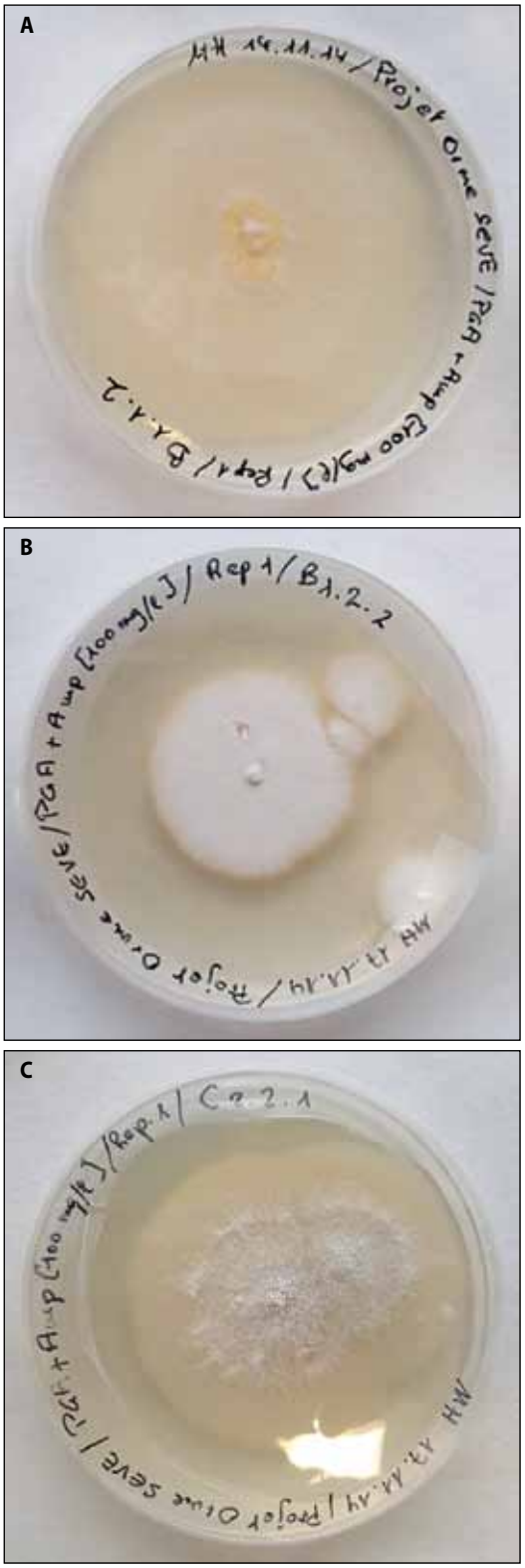

Figure 4. Relative proportions of fungal isolates

Figure 5. Colonies of *Geosmithia langdonii* isolate UASWS1324 v, *Geosmithia* sp. 1 isolate UASWS1325 (B) et *Geosmithia* sp. 2 isolate UASWS1334 (C) grown on PGA medium

dis. Geosmithia pallida was found associated with the bark beetle *Pseudopityophthorus pubipennis* in foamy bark canker in *Quercus agrifolia* in California (Lynch et al. 2014). Other species such as *Geosmithia fassatiae, G. langdonii* and *G. obscura* were discovered in association with *Scolytus intricatus* on several oak species (Kolařík et al. 2005, 2007). *Geosmithia langdonii* was also described as associated to *Platypus cylindrus* in the dieback of cork oaks (*Quercus suber*) in Algeria (Belhoucine et al. 2011). If pathogenicity of *Geosmithia langdonii* has not yet been proved, its effects on the growth of broadleaved trees and on roots have been demonstrated by Cízková et al. (2005). *Geosmithia langdonii* has also been often isolated in association with *Ophiostoma ulmi* and *O. novo-ulmi* in dying elms affected by DED (Pepori 2012; Bettini et al. 2014; Pepori et al. 2014). Besides these reported cases of plant pathogenicity, one *Geosmithia* species, *Geosmithia argillacea* has been shown to cause an invasive mycosis in humans associated to a cystic fibrosis (Giraud et al. 2010; De Ravin et al. 2011).

In the case described here, *Geosmithia langdonii*, and 2 other unknown *Geosmithia* species. *Geosmithia* sp. 1 and sp. 2 were found in symptomatic wood while bark beetle galleries are found in close regions of the symptomatic wood. *Geosmithia langdonii* was the major fungus retrieved from the symptomatic wood and could

have taken part to a pathogenic complex contributing to DED symptoms and dieback of this elm tree. The hypothesis that this latter species, or the two unknown *Geosmithia* species, could not be part of a pathogenic complex cannot be completely withdrawn in absence of inoculations experiments confirming their pathogenicity. It cannot either be excluded that these species could be common endophytes of elm trees.

The main finding of this study is to report, for the first time, *Geosmithia langdonii* and 2 unknown species of *Geosmithia,* different from any known species, from an elm tree in Switzerland.

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http://www.ncbi.nlm.nih.gov/nuccore/814603658 Geosmithia langdonii isolate UASWS1324 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.

http://www.ncbi.nlm.nih.gov/nuccore/814603659 Geosmithia sp. 1 UASWS1325 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

http://www.ncbi.nlm.nih.gov/nuccore/814603668 Geosmithia sp. 2 UASWS1334 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence.