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Widespread horizontal transfer of the *cerato-ulmin* gene between *Ophiostoma novo-ulmi* and *Geosmithia* species

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ABSTRACT

Previous work had shown that a sequence homologous to the gene encoding class II hydrophobin cerato-ulmin from the fungus *Ophiostoma novo-ulmi*, the causal agent of Dutch Elm Disease (DED), was present in a strain of the unrelated species *Geosmithia* species 5 (*Ascomycota*: *Hypocreales*) isolated from *Ulmus minor* affected by DED. As both fungi occupy the same habitat, even if different ecological niches, the occurrence of horizontal gene transfer was proposed. In the present work we have analysed for the presence of the *cerato-ulmin* gene 70 *Geosmithia* strains representing 29 species, isolated from different host plants and geographic locations. The gene was found in 52.1 % of the strains derived from elm trees, while none of those isolated from nonelms possessed it. The expression of the gene in *Geosmithia* was also assessed by real time PCR in different growth conditions (liquid culture, solid culture, elm sawdust, dual culture with *O. novo-ulmi*), and was found to be extremely low in all conditions tested. On the basis of these results we propose that the *cerato-ulmin* gene is not functional in *Geosmithia*, but can be considered instead a marker of more extensive transfers of genetic material as shown in other fungi.

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Introduction

Horizontal gene transfer (HGT) involves the exchange of genetic information between phylogenetically distant organisms across the normal reproductive barriers. This phenomenon is common in prokaryotes where it is involved in the acquirement of traits such as antibiotic resistance, virulence, and metabolic abilities, enabling the colonization of new ecological niches and leading to the appearance of new species (Ochman *et al.* 2000). Because of the magnitude of HGT some authors have questioned the validity of the species concept in prokaryotes and have proposed that bacterial evolution should be described by means other than the standard tree of life (Ochman *et al.* 2000; Gogarten & Townsend 2005; Boto 2010).

In recent years the increasing availability of whole genome sequences has allowed to appreciate HGT also in eukaryotes. Most documented cases involve the transfer of bacterial genes to unicellular eukaryotes where they have been proposed to play a role in adaptation processes (Andersson *et al.* 2003; Gojkovic *et al.* 2004; Ricard *et al.* 2006; Bowler *et al.* 2008; Schönknecht *et al.* 2013), but the transfer of genes from viral, bacterial or eukaryotic donors to plants and animals has also been described (reviewed in Keeling & Palmer 2008; Bock 2010; Dunning Hotopp 2011). In filamentous fungi, in particular, HGT has been demonstrated in several instances and is involved in the acquirement of important characters such as virulence, leading in some cases to the emergence of new pathogens or of new host specificities for existing ones (Oliver & Solomon 2008; Mehrabi *et al.* 2011; Gardiner *et al.* 2013). This is the case for the fungus *Pyrenophora tritici-repentis* whose ability to infect wheat has been related to the transfer of the gene encoding the host-selective toxin ToxA from the wheat pathogen *Stagonospora nodorum* (Friesen *et al.* 2006). In addition to the moving of single genes there is now evidence of more extensive transfers, involving gene clusters and whole chromosomes. A 23-gene cluster comprising the biosynthetic pathway for the production of the toxic metabolite sterigmatocystin has been horizontally transferred from *Aspergillus nidulans* to *Podospora anserina*, thus enriching the secondary metabolite repertoire of the recipient (Slot & Rokas 2011). The transfer of whole chromosomes was also involved in the acquirement of pathogenicity by the tomato pathogens *Alternaria alternata* and *Fusarium oxysporum* f. sp. *lycopersici* (Akagi *et al.* 2009; Ma *et al.* 2010). Finally, Richards *et al.* (2011) showed how interkingdom gene transfer from filamentous fungi to oomycetes of at least 21 genes with roles in nutrient acquisition, degradation of plant surface components, and suppression of plant defences led to the appearance of plant pathogenic oomycetes.

Previous work demonstrated the presence in the *Geosmithia* species 5 (*Ascomycota*: *Hypocreales*) strain IVV7, isolated from *Ulmus minor* affected by Dutch Elm Disease (DED), of an 827 bp genomic fragment comprising the gene encoding the hydrophobin *cerato-ulmin* (*cu*) from *Ophiostoma novo-ulmi* (Bettini *et al.* 2010). The fungi of the genus *Geosmithia* are saprobes vectored by bark beetles infesting hardwoods and conifers worldwide. In particular, *Geosmithia* spp. cooccur regularly with phytopathogenic *Ophiostoma* species on elms or other trees (Kolařík *et al.* 2007, 2008; Kolařík & Jankowiak 2013), occupying the same habitat even if different ecological niches. Continuous physical contact between organisms, as a consequence of habitat sharing, was suggested to be a favourable condition for the transfer of genetic material (Aguileta *et al.* 2009; Fitzpatrick 2012), therefore horizontal transfer of the *cu* gene between *Geosmithia* and *Ophiostoma* was proposed.

Hydrophobins are small hydrophobic proteins secreted by filamentous fungi. They are divided in two classes having similar functional characteristics: class I hydrophobins have been found in ascomycetes and basidiomycetes, while class II hydrophobins are present only in ascomycetes (Wösten 2001; Whiteford & Spanu 2002). Both class I and class II hydrophobins have multiple roles in fungal growth and development, by taking part in the formation of aerial hyphae, fruiting bodies, microsclerotia, and microconidial chains (Fuchs *et al.* 2004; Yamada *et al.* 2005; Karlsson *et al.* 2007; Klimes *et al.* 2008), and in the maturation of the fungal cell wall (van Wetter *et al.* 2000). They are also important players in the establishment of relationships between fungi and other organisms, as in lichen-forming-ascomycetes (Scherrer *et al.* 2000), in ectomycorrhizal fungi (Mankel *et al.* 2002), and in host–pathogen interactions, where they can be the primary determinants of pathogenesis (Talbot 2003; Kim *et al.* 2005; Aimanianda *et al.* 2009).

Cerato-ulmin (CU) is a class II hydrophobin of about 8 kDa present on the cell surface and/or excreted in culture by the phytopathogenic fungi *Ophiostoma ulmi*, *O. novo-ulmi*, and *Ophiostoma himal-ulmi*, and by the nonpathogen *Ophiostoma quercus* (Svircev *et al.* 1988; Scala *et al.* 1997; Carresi *et al.* 2008). *Ophiostoma ulmi* and *O. novo-ulmi* are responsible, respectively, for the first and the second DED pandemics that decimated elm populations in Europe, North America, and Asia since the last century. The first pandemic was caused in the early 1900s by the nonaggressive, weakly pathogenic species *O. ulmi*, and the second, more severe, was caused since the early 1940s by the highly aggressive species *O. novo-ulmi* (Brasier & Buck 2001). *Ophiostoma novo-ulmi* exists in two subspecies with different geographical origin: the typical form, also known as Eurasian race (hereafter as *O. novo-ulmi* only) and the North American race described as *O. novo-ulmi* subspecies *americana* (Brasier & Kirk 2001). Both forms, including their hybrids, are present in Europe (Brasier & Kirk 2010). The other DED species, *O. himal-ulmi*, was isolated in 1990s in the western Himalayas (Brasier & Mehrotra 1995). It can be highly pathogenic on european elms, however no DED symptoms were detected on himalayan elms thus suggesting that the disease was at an endemically low level in this region. *Ophiostoma quercus*, on the other hand, is a widely distributed sapwood-staining fungus occurring on different hardwood hosts (Grobbelaar *et al.* 2009). When inoculated on elm trees it is able to colonize the plant without causing any appreciable alterations or disease symptoms (Sutherland *et al.* 1995; Del Sorbo *et al.* 2000).

For many years CU had been regarded as a virulence factor in DED pathogenesis (Takai 1974; Richards 1993; Del Sorbo *et al.* 2000), a view that has been challenged by the finding that pathogenicity of *O. novo-ulmi* mutants unable to produce

CU did not differ with respect to wild type strains (Brasier *et al.* 1995; Bowden *et al.* 1996; Tegli & Scala 1996), and that overexpression of the *O. novo-ulmi cu* gene in the less aggressive species *O. ulmi* did not increase the virulence of the latter (Temple *et al.* 1997). These results prompted some authors to propose that CU could be a factor involved in the fitness of DED pathogens, improving their ability to colonize and infect elm trees (Temple *et al.* 1997; Temple & Horgen 2000). In fact, CU renders the *Ophiostoma* cell surface more hydrophobic, favouring the adhesion of the fungus to the insect vector and hence its dissemination and the spread of the disease; it also protects the fungus from dehydration (Temple *et al.* 1997; Temple & Horgen 2000; Whiteford & Spanu 2002).

In order to evaluate the magnitude of the HGT between *O. novo-ulmi* and *Geosmithia* spp., we performed an extensive search for the presence of the *cu* gene in a number of isolates of the genus *Geosmithia* derived from different host plants and geographic locations. The expression of the *cu* gene in *Geosmithia* was also assessed in varying growth conditions.

Materials and methods

Fungal strains and culture

Ophiostoma novo-ulmi isolate 182 (Carresi *et al.* 2008) and the *Geosmithia* strains, representing 29 different species, used in this study (Table 1), were maintained on Potato Dextrose Agar medium (BD Difco™). Plates were incubated in the dark at 24 ± 1 °C. For liquid culture, an agar plug was transferred to 100 ml flasks containing 20 ml of modified Takai medium (Scala *et al.* 1994). Flasks were wrapped in aluminium foil and incubated on a rotary shaker at 100 rpm at 24 ± 1 °C. To recover the mycelium cultures were centrifuged (2500 rcf, 20 min, room temperature) and pellets stored at −20 °C.

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA extraction from mycelium was carried out with the NucleoSpin® Plant II kit (Macherey–Nagel GmbH & Co. KG). DNA concentration was evaluated with a Qubit® 2.0 fluorometer (Invitrogen by Life Technologies) and PCR amplifications for both the *cu* gene and the ITS region of the rDNA cistron were carried out as described (Bettini *et al.* 2012). For the amplification of the *cu* gene the following primers, designed on the sequence of *Ophiostoma novo-ulmi* isolate 182 (GenBank accession no. KF725663), were used: 5′-AAATCTT CAAAATGCAGTTCTC-3′ (forward) and 5′-AGAAGAATCGAAT GAAAACTTGATG-3′ (reverse). Primers ITS1 and ITS4 (White *et al.* 1990) were used for the amplification of the ITS region of rDNA cistron.

DNA sequencing and sequence analysis

Sequencing of the amplified fragments was performed by Eurofins MWG Operon (Ebersberg, Germany) on either purified PCR products or on bands extracted from agarose gels (NucleoSpin® Gel and PCR Clean-up kit, Macherey–Nagel GmbH & Co. KG). Sequences of the *cu* gene from *Geosmithia* were deposited in GenBank under accession numbers KF484882–KF484905.

GenBank homology searches were done with either blastn or blastx (Altschul *et al.* 1990) with default parameters. Alignments of the *Geosmithia cu* sequences with the *cu* gene sequences from *Ophiostoma ulmi* (GenBank accession nos. U23203 and Z80081), *Ophiostoma novo-ulmi* (GenBank accession nos. AJ295751, Z80082, Z80085, EU006082–EU006087), *Ophiostoma himal-ulmi* (GenBank accession nos. Z80083 and Z80084), *Ophiostoma quercus* (GenBank accession nos. EF447294, EF447295, EF447296), and *Geosmithia* sp. 5 strain IVV7 (GenBank accession no. DQ377561) were obtained using MAFFT version 6 (http://mafft.cbrc.jp/alignment/software/) (Katoh & Toh 2008). The final dataset had 41 sequences and 459 characters, of which 179 were variable, 244 conservative and 23 singletons. Maximum Likelihood (ML) analyses were performed in PhyML 3.0 (Guindon *et al.* 2010), using Kimura-2 parameter model and bootstrap support was obtained using 500 replicates. Evolutionary models were determined on datasets using MEGA 5.05 (Tamura *et al.* 2011). The *cu* gene sequences from *O. quercus* were selected as outgroup based on a preliminary Neighbour-Joining unrooted tree constructed using the most similar sequences in MEGA 5.05.

RNA extraction and real time PCR

Geosmithia sp. 5 strain IVV7 was grown as described in 100 ml flasks containing 20 ml of liquid modified Takai medium for 4, 8, 12, 16, and 20 d. At each time-point cultures were centrifuged and pellets frozen at −80 °C. Three 4 d-old *Ophiostoma novo-ulmi* isolate 182 liquid cultures were used as control. Strains from *Geosmithia* spp. 2, 5, 10, 13, 20, and *Geosmithia langdonii* were grown in the same conditions for 8 d. For the induction experiments, *Geosmithia* sp. 5 strain IVV7 was grown for 4, 6, 8, and 12 d on 90 mm diameter Petri dishes either on elm sawdust medium (Baccelli *et al.* 2012) or in dual culture with *O. novo-ulmi*. To this aim 2×10^7 conidia of *O. novo-ulmi* in 50 µl were inoculated on modified Takai medium and incubated at 24 ± 1 °C in the dark. After 4 d of growth a sterile cellophane disk was placed onto each plate and a 6 mm diameter agar plug from a *Geosmithia* sp. 5 strain IVV7 solid culture was cut with a sterile cork-borer and placed in the centre of the plate. For each time-point at least six plates were prepared and incubated as described. Mycelium collected at each time-point was frozen at −80 °C. As a control, *Geosmithia* sp. 5 strain IVV7 was grown on cellophane disks placed onto modified Takai solid medium plates.

Total RNA was extracted using the NucleoSpin® RNA Plant kit (Macherey–Nagel GmbH & Co. KG). After the extraction a DNase digestion was performed (RQ DNase, Promega) followed by LiCl precipitation. RNA concentration was evaluated with a Qubit® 2.0 fluorometer (Invitrogen by Life Technologies) and a PCR was carried out to ensure that the contaminant DNA had been removed by subjecting 500 ng of total RNA to amplification with primers for the ITS region of the rDNA cistron. Conditions for PCR were as described in Section 2.2. Five-hundred nanogrammes of total RNA were then reverse transcribed (TaqMan Reverse Transcription Reagents, Applied Biosystems by Life Technologies). The amount of *cu* transcript was evaluated by means of real time PCR with TaqMan®

Table 1 – *Geosmithia* species and strains used in the present study. Isolates identified by CCF code have been deposited in the Culture Collection of Fungi (Prague, Czech Republic), while the others are deposited in the personal collections of A.L. Pepori (IPP-CNR, Sesto Fiorentino, Italy) and M. Kolařík (Institute of Microbiology of the ASCR, Prague, Czech Republic). Species numbering is from Kolařík *et al.* (2007, 2008) and Kolařík & Jankowiak (2013).

Species	Strain N° (CCF code)	Source	Geographic origin	Reference	Presence of *cu* gene, GenBank Acc. N°
Geosmithia species 1	MK1724 (CCF3660)	*Xylocleptes bispinus* on *Clematis vitalba*	Břeclav, Bulhary, Czech Republic	Kolařík *et al.* 2007, 2008	No
Geosmithia species 2	MK1623 (CCF4273)	*Scolytus kirschii* on *Ulmus minor*	Jorairatar, Andalusia, Spain	Kolařík *et al.* 2004, 2008	Yes, KF484882
Geosmithia species 2	MK1638	*Scolytus multistriatus* on *Ulmus minor*	Aracena, Andalusia, Spain	Kolařík *et al.* 2004, 2008	No
Geosmithia species 2	CNR39	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	No
Geosmithia species 2	CNR40	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484883
Geosmithia species 2	CNR42	*Ulmus glabra*	Rapatice, Pilsen, Czech Republic	Pepori 2012	Yes, KF484884
Geosmithia species 2	CNR45	*Ulmus laevis*	Dobřiň, Ústí nad Labem, Czech Republic	Pepori 2012	No
Geosmithia species 2	CNR46	*Ulmus minor*	Jílové u Prahy, Žampach, Sazava river, Czech Republic	Pepori 2012	Yes, KF484885
Geosmithia species 2	CNR54	*Ulmus glabra*	Hostenice, Usti nad Labem, Czech Republic	Pepori 2012	Yes, KF484886
Geosmithia species 3	MK134 (CCF3336)	*Scolytus rugulosus* on *Malus domestica*	Opočno, Louny, Czech Republic	Kolařík *et al.* 2004, 2008	No
Geosmithia species 4	MK1722 (CCF4278)	*Pteleobius vittatus* on *Ulmus laevis*	Břeclav, Kančí obora, Czech Republic	Kolařík *et al.* 2008	No
Geosmithia pallida	CNR136	Elm clone U'FL634' (IPP-CNR)	Ugnano (FI), Italy	Pepori 2012	No
Geosmithia species 5	IVV7	*Ulmus minor* tree affected by DED	Vibo Valentia (RC), Italy	Bettini *et al.* 2010	Yes, DQ377561
Geosmithia species 5	MK445a (CCF3477)	*Scolytus multistriatus* on *Ulmus* sp.	Louny, Czech Republic	Kolařík *et al.* 2004, 2008	Yes, KF484887
Geosmithia species 5	MK971	*Pteleobius vittatus* on *Ulmus minor*	Milovický les, Bulhary, Czech Republic	Kolařík *et al.* 2004, 2008	No
Geosmithia species 5	MK980	*Pteleobius vittatus* on *Ulmus laevis*	Kančí obora forest, Břeclav, Czech Republic	Kolařík *et al.* 2004, 2008	Yes, KF484888
Geosmithia species 5	MK1550a (CCF4271)	*Scolytus multistriatus* on *Ulmus laevis*	Kančí obora forest, Břeclav, Czech Republic	Kolařík *et al.* 2004, 2008	No
Geosmithia species 5	CNR28	*Ulmus minor*	Středokluky, Czech Republic	Pepori 2012	No
Geosmithia species 5	CNR31	*Ulmus glabra*	Milá, Usti nad Labem, Czech Republic	Pepori 2012	Yes, KF484889
Geosmithia species 5	CNR33	*Ulmus minor*	Žabokliky, Usti nad Labem, Czech Republic	Pepori 2012	Yes, KF484890
Geosmithia species 5	CNR36	*Ulmus minor*	Jílové u Prahy, Žampach, Czech Republic	Pepori 2012	Yes, KF484891
Geosmithia species 5	CNR48	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484892
Geosmithia putterillii	U131a (CCF4202)	*Phloeosinus sequoiae* on *Sequoia sempervirens*	Bohemian river, CA, USA	M.K., unpubl.	No
Geosmithia flava	MK264 (CCF3354)	*Leperisinus fraxini* on *Fraxinus excelsior*	Muráň plain, Slovakia	Kolařík *et al.* 2004	No
Geosmithia species 8	MK263 (CCF4258)	*Scolytus intricatus* on *Quercus dalechampii*	Šiance hill, Muráňská planina, Slovakia	Kolařík *et al.* 2008	No
Geosmithia species 9	RJ113k (CCF4311)	*Cryphalus piceae* on *Abies alba*	Czajowice, Poland	Kolařík & Jankowiak 2013	No
Geosmithia species 10	MK1788 (CCF4286)	*Hypoborus ficus* on *Ficus carica*	Suvalan, Azerbaijan	Kolařík *et al.* 2007, 2008	No
Geosmithia species 10	MK441 (CCF3553)	*Scolytus multistriatus* on *Ulmus minor*	Louny, Czech Republic	Kolařík *et al.* 2007, 2008	No
Geosmithia species 10	MK544 (CCF4301)	*Pteleobius vittatus* on *Ulmus* species	Kőris-hegy, Bakony range, Hungary	Kolařík *et al.* 2007, 2008	No
Geosmithia species 10	MK989 (CCF3560)	*Scolytus pygmaeus* on *Ulmus minor*	Milovický les, Bulhary, Czech Republic	Kolařík *et al.* 2007, 2008	No

Table 1 – (*continued*)

Species	Strain N° (CCF code)	Source	Geographic origin	Reference	Presence of *cu* gene, GenBank Acc. N°
Geosmithia species 10	MK1508 (CCF4269)	*Scolytus kirschii* on *Ulmus minor*	Termoli (CB), Italy	Kolařík *et al.* 2007, 2008	No
Geosmithia species 10	MK1703	*Scolytus multistriatus* on *Ulmus laevis*	Břeclav, Kančí obora forest, Czech Republic	Kolařík *et al.* 2007, 2008	No
Geosmithia species 10	CNR8	*Ulmus laevis*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484894
Geosmithia species 10	CNR10	*Ulmus minor*	Jílové u Prahy, Žampach, Czech Republic	Pepori 2012	Yes, KF484895
Geosmithia species 10	CNR16	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484896
Geosmithia species 10	CNR17	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484897
Geosmithia species 10	CNR20	*Ulmus laevis*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484898
Geosmithia species 10	CNR21	*Ulmus glabra*	Nemetice, Czech Republic	Pepori 2012	Yes, KF484899
Geosmithia species 10	CNR32	*Ulmus laevis*	Středokluky, Czech Republic	Pepori 2012	Yes, KF484900
Geosmithia species 10	CNR41	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484901
Geosmithia species 10	CNR69	*Ulmus glabra*	Hostenice, Czech Republic	Pepori 2012	Yes, KF484902
Geosmithia species 10	CNR71	*Ulmus glabra*	Březno u Loun, Czech Republic	Pepori 2012	Yes, KF484903
Geosmithia species 10	CNR72	*Ulmus laevis*	Dobřiň, Ústí nad Labem, Czech Republic	Pepori 2012	No
Geosmithia species 11	MK551 (CCF3555)	*Scolytus intricatus* on *Quercus pubescens*	Villányi hegy Mts., Vokány, Hungary	Kolařík *et al.* 2008	No
Geosmithia species 12	MK661 (CCF3557)	*Leperisinus orni* on *Fraxinus excelsior*	Balaton region, Szent György hegy hill, Hungary	Kolařík *et al.* 2008	No
Geosmithia species 13	MK1515a	*Pteleobius vittatus* on *Ulmus minor*	Milovický les, Bulhary, Czech Republic	Kolařík *et al.* 2008	Yes, KF484904
Geosmithia species 13	MK924	*Scolytus multistriatus* on *Ulmus minor*	Hodonín, Bulhary, Milovický les forest, Czech Republic	Kolařík *et al.* 2008	No
Geosmithia species 13	MK963	*Pteleobius vittatus* on *Ulmus laevis*	Kančí obora forest, Břeclav, Czech Republic	Kolařík *et al.* 2008	No
Geosmithia species 13	MK977 (CCF3559^{T})	*Pteleobius vittatus* on *Ulmus minor*	Milovický les, Bulhary, Czech Republic	Kolařík *et al.* 2008	No
Geosmithia species 13	MK1856	*Scolytus multistriatus* on *Ulmus minor*	Sušice nad Otavou, Czech Republic	Pepori 2012	No
Geosmithia langdonii	MK1619 (CCF4272)	Bostrichid beetle on *Pistacia lentiscus*	Sesimbra, Portugal	Kolařík *et al.* 2005	No
Geosmithia langdonii	MK1646	*Scolytus multistriatus* on *Ulmus laevis*	Neratovice, Cerninovsko, Czech Republic	Kolařík *et al.* 2005	No
Geosmithia langdonii	CNR11	*Ulmus minor*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	Yes, KF484905
Geosmithia langdonii	CNR26	*Ulmus laevis*	Libický luh, Velký Osek, Czech Republic	Pepori 2012	No
Geosmithia langdonii	CNR93	*Ulmus minor*	Žabokliky, Usti nad Labem, Czech Republic	Pepori 2012	No
Geosmithia obscura	MK616 (CCF3425)	*Scolytus carpini* on *Carpinus betulus*	Bakony range, Vinye near of Fodöfö, Hungary	Kolařík *et al.* 2005	No
Geosmithia lavendula	MK1781 (CCF4285)	*Hypoborus ficus* on *Ficus carica*	Baki Sahari, Baku, Azerbaijan	Kolařík *et al.* 2007	No
Geosmithia species 20	CNR132	Elm clone U'FL634' (IPP-CNR)	Ugnano (FI) Italy	Pepori 2012	Yes, KF484893
Geosmithia species 21	MK1761 (CCF4280)	*Hypoborus ficus* on *Ficus carica*	Wadi al Furiáh, West Bank, Israel	Kolařík *et al.* 2007	No

(*continued on next page*)

Table 1 – (continued)

Species	Strain N° (CCF code)	Source	Geographic origin	Reference	Presence of *cu* gene, GenBank Acc. N°
Geosmithia species 22	MK739 (CCF3645)	*Phloetribus scarabeoides* on *Olea europaea*	Wadi al Mujib, Jordan	Kolařík *et al.* 2007	No
Geosmithia species 23	MK781 (CCF3639)	*Scolytus rugulosus* on *Prunus armeniaca*	Demircili, Silifke, Içel Province, Turkey	Kolařík *et al.* 2007	No
Geosmithia species 25	MK1829a (CCF4211)	*Cryphalus piceae* on *Abies alba*	Pašínovice, Czech Republic	Kolařík & Jankowiak 2013	No
Geosmithia species 26	MK1828 (CCF4293)	*Pityophthorus pityographus* on *Pinus sylvestris*	Sedlčany, Czech Republic	Kolařík & Jankowiak 2013	No
Geosmithia species 28	RJ279m (CCF4210)	*Polygraphus polygraphus* on *Picea abies*	Chyszówki, Poland	Kolařík & Jankowiak 2013	No
Geosmithia species 29	MK1809b (CCF4199)	*Cryphalus piceae*, *Pityophthorus pityographus* on *Abies alba*	Příběnice, Czech Republic	Kolařík & Jankowiak 2013	No
Geosmithia species U316	U316 (CCF4328)	Bark beetle on *Pinus muricata*	Monterey, CA, USA	M.K., unpubl.	No
Geosmithia eupagioceri	CCF3754	*Eupagiocerus dentipes* on *Paullinia renesii*	Heredia, Birrí, Costa Rica	Kolařík & Kirkendall 2010	No
Geosmithia microcorthyli	CCF3861	*Microcorthylus* species on *Cassia grandis*	Heredia, Birrí, Costa Rica	Kolařík & Kirkendall 2010	No
Geosmithia rufescens	MK1821 (CCF4524)	*Cnesinus lecontei* on *Croton draco*	Heredia, Birrí, Costa Rica	Kolařík & Kirkendall 2010	No
Geosmithia morbida	1259	*Pityophthorus juglandis* on *Juglans* species	Oregon, USA	Kolařík *et al.* 2011	No

MGB probes. Probe and primers were synthesized by the Custom TaqMan® Gene Expression Assay Service (Applied Biosystems by Life Technologies). The sequences of the primers and of the probe, which spanned the boundary between the first and the second exon, were as follows: *cu*-fwd 5′-TTGGTGTGGCCAATCTTGACT-3′, *cu*-rev 5′-GGAACTGGCTGG GAGACGTA-3′, *cu*-probe 5′-CCATGGCCCCCCAAGCGTG-3′. A reference calibration curve was set up with serial dilutions of cloned *O. novo-ulmi cu* cDNA. Total RNA was extracted from *O. novo-ulmi* isolate 182 mycelium, treated with DNase and the presence of contaminating genomic DNA assessed as described. A single-step RT-PCR (AccessQuick™ RT-PCR System, Promega Corporation) was then carried out on 1 μg of RNA with the following *cu* gene-specific primers: 5′-AAATCTTCAAAATGCAGTTCTC-3′ (forward) and 5′-CGAGCT-TAAATGCCGACGGGGT-3′ (reverse). Reactions were run on an agarose gel to check for the presence of a single band of the desired size (318 bp), which was cloned in the pCR®II plasmid (TA Cloning Kit, Invitrogen by Life Technologies). The identity of the fragment was finally verified by sequencing.

Amplification reactions were run in triplicate in a 7300 Real Time PCR System (Applied Biosystems by Life Technologies) and contained 100 ng cDNA, 1× TaqMan® Gene Expression Assay mix, and 1× TaqMan® Universal PCR Master Mix. Thermocycling conditions were as recommended by the manufacturer. Each experiment was repeated at least twice. For all the experiments the threshold cycles were plotted against the $\log_{10}$ of the known standard amounts. Regression analysis was carried out to determine the equation of the line that best fitted the data and the regression coefficient R^2 values, which were comprised between 0.995 and 0.998.

Results and discussion

Occurrence of the cu *gene in* Geosmithia *species*

Seventy *Geosmithia* strains representing 29 species (Table 1) were analysed for the presence of the *cu* gene by PCR with gene-specific primers as described in Section 2.2. Forty-six strains representing seven species were derived from insect vectors infesting elm trees or were isolated directly from decaying elm trees and 24 strains were obtained from insect vectors on plants other than elms. Fragments of the expected size (678 bp), corresponding to the entire sequence of the *cu* gene (454 bp) and including 224 bp downstream to the translation stop codon, were obtained in 24 strains derived from elm trees (52.1 %) and sequenced. Amplification from fungi isolated from plants other than elms failed or gave bands of size different than expected. Some of these fragments were randomly chosen for sequencing and subjected to blastx searches, but in no case similarity to the *cu* gene was detected (Supplementary Material Table 1).

On the other hand, blastn searches for the *Geosmithia cu* sequences showed 100 % identity to the *Ophiostoma novo-ulmi cu* gene (Accession nos. EU006082–EU006086,), and 99 % identity to both *O. novo-ulmi cu* gene accession no. AJ295751 and the *cu* gene sequence from *Geosmithia* IVV7 (*E*-values 0.0). This was due, respectively, to the presence of a point mutation at an intron splice site in the *O. novo-ulmi cu* sequence (Pipe *et al.* 2000) and of two nucleotide substitutions in the IVV7 *cu* sequence: a C to G transversion at position +301 in the second exon and an A to G transition at position +384 in the second intron. The substitution in the coding region resulted in a Glu to

Asp conservative change at position 75 in the primary sequence of the deduced protein.

Identity values between the *cu* gene sequences from *Geosmithia* spp. and the *cu* gene sequences from the other *Ophiostoma* spp. are reported in Supplementary Material (Table 2).

Ophiostoma cu sequences obtained from GenBank and the *cu* sequences from *Geosmithia* spp. were aligned and PhyML 3.0 was used to construct a phylogenetic tree, which showed that the *Ophiostoma* spp. formed distinct clusters and that the *cu* sequences from *Geosmithia* clustered with *O. novo-ulmi* (Fig 1). Taken together, these results demonstrated that the HGT event occurred between *Geosmithia* spp. and *O. novo-ulmi*. On this basis we propose that the HGT between the two fungi is very recent and ongoing around Europe. In fact, the strains harbouring the *cu* gene were isolated in Czech Republic, Italy, and Spain and belonged to *Geosmithia* spp. 2, 5, 10, 13, 20, and *Geosmithia langdonii* (Table 1), coherently with the known distribution pattern of these species that are associated with phloeophagus bark beetles infesting broad-leaved trees in both the Mediterranean area and temperate Europe (Kolařík *et al.* 2007, 2008).

Our hypothesis of a very recent transfer of the *cu* gene between *Geosmithia* and *O. novo-ulmi* seemed in contradiction with the presence of two nucleotide substitutions in the *cu* gene sequence from *Geosmithia* sp. 5 strain IVV7, resulting in a slightly longer branch length in the phylogenetic tree (Fig 1). If the observed substitutions occurred in IVV7 after the transfer of the gene from *O. novo-ulmi* we must suppose this event to be much more ancient, 70 y since the appearance of *O. novo-ulmi* in Europe being too short a time span for substitutions to occur. However, data on the appearance of *O. novo-ulmi* in Europe allowed to rule out the possibility that the transfer of the *cu* gene happened earlier than 1940s, since all *Ophiostoma* strains isolated before 1940s are *Ophiostoma ulmi* (Brasier 1991). An alternative scenario could be that the observed substitutions were already present in the *cu* gene in the *O. novo-ulmi* isolate involved in the HGT event with IVV7. The possibility of the existence of variant forms of the

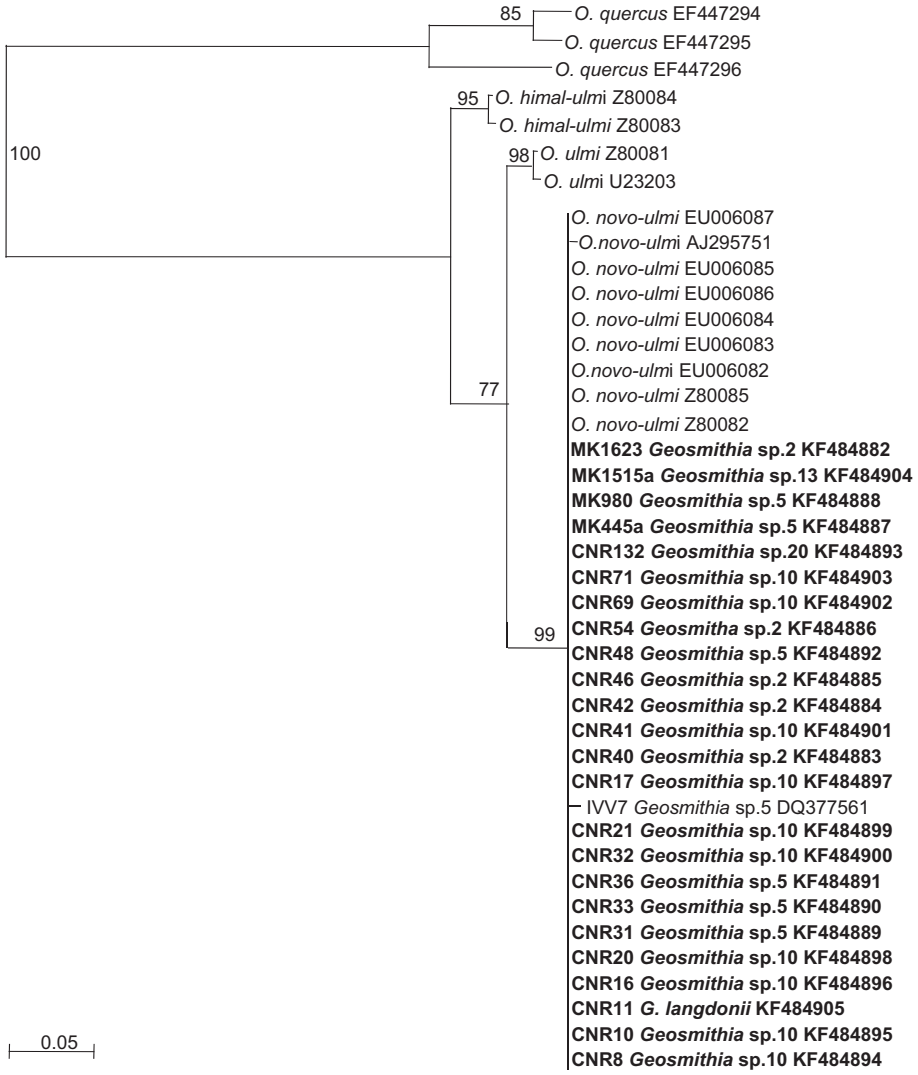

Fig 1 – ML tree based on the aligned *cu* gene sequences from *O. ulmi*, *O. novo-ulmi*, *O. himal-ulmi*, *O. quercus*, and *Geosmithia* spp. (in bold). GenBank accession numbers are indicated for each sequence. Phylogenetic tree was constructed with PhyML 3.0 performing bootstrap analysis with 500 replicates. The *cu* gene sequences from *O. quercus* were used as outgroup.

cu gene is sustained by the report by Pipe *et al.* (2000) of a *O. novo-ulmi* strain whose *cu* gene had a mutation in an intron splice site.

Finally, intraspecies variability was observed in *Geosmithia* spp. in terms of the presence or absence of the *cu* gene, which was found in five out of eight strains of *Geosmithia* sp. 2, six out of nine strains of *Geosmithia* sp. 5, ten out of 17 strains of *Geosmithia* sp. 10, one out of five strains of *Geosmithia* sp. 13, and *G. langdonii*. In order to explain this observation we propose that multiple and independent HGT events occurred between *O. novo-ulmi* and *Geosmithia* spp., favoured by the close proximity of the two fungi as a consequence of habitat sharing. These HGT events could involve different *O. novo-ulmi* genomic fragments, not necessarily comprising the *cu* gene. The absence of the *cu* gene in some *Geosmithia* strains could therefore be accounted for by two possibilities: (i) these strains could harbour other fragments of the *O. novo-ulmi* genome, even if the very high frequency of the *cu* gene in *Geosmithia* suggests that the transferred region is not completely random, or (ii) they were not involved in HGT. The lack of information concerning *Geosmithia* genomes does not allow at present to verify this hypothesis.

Expression of the cu *gene in* Geosmithia *species*

In order to assess if the *cu* gene was functional in *Geosmithia*, its expression level was evaluated by real time PCR. As a first step a time-course was set up on our reference species *Geosmithia* sp. 5 strain IVV7 by growing the fungus in liquid culture for 4, 8, 12, 16, and 20 d. Results showed that the *cu* mRNA was present in strain IVV7, *albeit* in an extremely low amount, from day 8 to the end of the experiment, the maximum being reached after 8 d of growth (73.4 ag of *cu* mRNA μg^{-1} total RNA) (Fig 2A). The quantity of *cu* mRNA in *Ophiostoma novo-ulmi* isolate 182 after 4 d of growth in the same conditions was 67 fg μg^{-1} total RNA, thus about 1000-fold higher than the highest level reached in strain IVV7. The same time-point was therefore used to analyse *cu* gene expression in eight strains belonging to the species where the gene was found, i.e. *Geosmithia* sp. 2 (CNR46), *Geosmithia* sp. 5 (MK980 and CNR36), *Geosmithia* sp. 10 (CNR32 and CNR71), *Geosmithia* sp. 13 (MK1515a), *Geosmithia* sp. 20 (CNR132), and *Geosmithia langdonii* (CNR11). *cu* mRNA was detected in all the strains tested, its amount being variable with respect to the reference species (Fig 2B). The amount of *cu* mRNA in all the strains tested was always in the same quantitative range as in strain IVV7, thus showing that the nucleotide substitutions present in the IVV7 *cu* gene did not affect its expression. However, in the strain CNR36 the quantity of *cu* mRNA was so low as to be negligible.

The fungi of the genus *Geosmithia* used in this study were endophytic organisms that lived in the galleries built by subcorticolous bark beetles inside decaying elm trees showing DED symptoms. *Ophiostoma novo-ulmi* is known to be present in the microbiota associated with insect galleries (Kolařík *et al.* 2011), as shown also in other instances (Kolařík *et al.* 2007, 2008). To test if growing *Geosmithia* in conditions more similar to its natural habitat could increase the expression of the *cu* gene, *Geosmithia* sp. 5 strain IVV7 was grown for 4, 6, 8, and 12 d on medium containing elm sawdust and in dual culture with *O. novo-ulmi* isolate 182. Results obtained

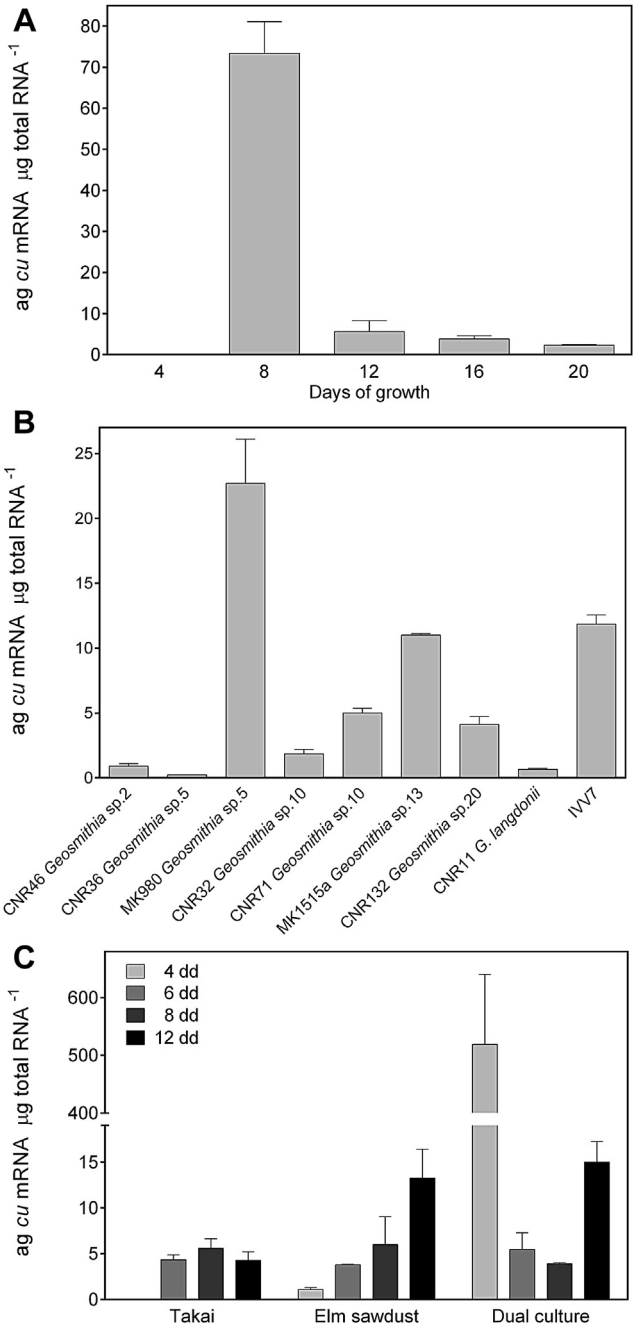

Fig 2 – *cu* gene expression in *Geosmithia* spp. as determined by real time PCR. (A) *cu* mRNA in *Geosmithia* sp. 5 strain IVV7 after 4, 8, 12, 16, and 20 d of growth in liquid culture; (B) *cu* mRNA in *Geosmithia* spp. 2 (strain CNR46), 5 (strains MK980 and CNR36), 10 (strains CNR32 and CNR71), 13 (strain MK1515a), 20 (CNR132), and *G. langdonii* (strain CNR11) after 8 d of growth in liquid culture. The reference species *Geosmithia* sp. 5 strain IVV7 was used as a control; (C) *cu* mRNA in *Geosmithia* sp. 5 strain IVV7 after 4, 6, 8, and 12 d of growth on solid Takai medium, on medium containing elm sawdust, and in dual culture with *O. novo-ulmi*. Gene expression was indicated as attograms (ag) of *cu* mRNA μg^{-1} of total RNA.

showed that on elm sawdust the amount of *cu* mRNA was increased by about three-fold, and by 100-fold after 4 d of dual culture, in comparison to the average value for control grown on solid Takai medium (Fig 2C). The level of *cu* mRNA remained in all cases very low also with respect to *O. novo-ulmi* grown for 4 d on solid Takai medium (0.72 pg *cu* mRNA μg^{-1} total RNA). To exclude any contamination of the RNA samples from strain IVV7 by *O. novo-ulmi*, the one having the highest *cu* mRNA content (4 d of dual culture) was reverse transcribed and the ITS region of the rDNA cistron was amplified with the universal primers ITS1 and ITS4 (White *et al.* 1990). The amplicon size difference allowed to distinguish the two species, being approximately 650 bp and 560 bp for *O. novo-ulmi* and *Geosmithia pallida*, respectively. No band corresponding to the size of *Ophiostoma* ITS was found in the IVV7 sample after 50 cycles of amplification (Fig 3). Moreover, sequencing of the RT-PCR product showed that the ITS sequence amplified was only that of strain IVV7, demonstrating that no *Ophiostoma* RNA was present in the original sample.

The *cu* expression levels detected in *Geosmithia* were much lower than those reported for *O. novo-ulmi* (Tadesse *et al.* 2003), thus raising the question of the functional significance of *cu* mRNA in the former species. The genomic fragment transferred between *O. novo-ulmi* and *Geosmithia* comprised 317 bp upstream to the coding sequence (Bettini *et al.* 2010), a region where several putative regulatory motifs are present (Carresi *et al.* 2008). On this basis it could be hypothesized that the *Geosmithia* transcriptional machinery recognized the regulatory region of the *Ophiostoma* gene, even if with scarce efficiency. The conservation of the *cu* gene sequence in *Geosmithia* could therefore be a consequence of the very recent transfer rather than reflect the presence of selective constraints on a functional sequence.

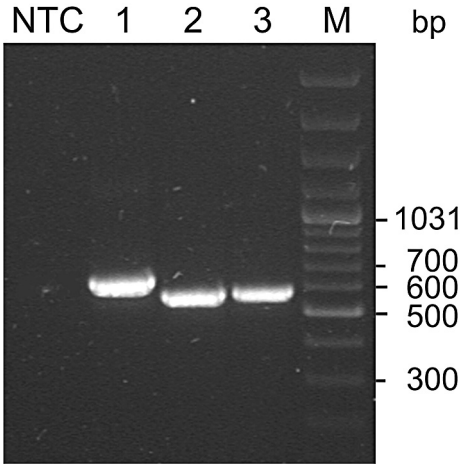

Fig 3 – Test for contamination of *Geosmithia* sp. 5 strain IVV7 samples from dual culture with *O. novo-ulmi*. RNA extracted from strain IVV7 after 4 d of cocultivation was reverse transcribed and PCR was carried out with universal primers ITS1 and ITS4. Amplifications with *O. novo-ulmi* and strain IVV7 DNA were carried out as controls. NTC, no template control; lane 1, *O. novo-ulmi* DNA; lane 2, *Geosmithia* sp. 5 strain IVV7 DNA; lane 3, *Geosmithia* sp. 5 strain IVV7 cDNA, 4 d of dual culture; M, GeneRuler 100 bp DNA Ladder Plus (Fermentas, Thermo Scientific Molecular Biology).

The HGT event involving the *cu* gene happened with very high frequency, as 52.1 % of the *Geosmithia* strains isolated from elm trees were found to possess the gene. A similar frequency was reported by Coelho *et al.* (2013) for the horizontal transfer of the fructose transporter gene *FSY1*, that was present in 109 fungal genomes over 241 analysed. However in this case, as in most reports of HGT, there is an immediate advantage for the recipient species from the acquirement of the foreign gene. In our case this is less evident, also because strain IVV7 had recently been shown to possess a previously unidentified class II hydrophobin, GEO1 (Bettini *et al.* 2012). In conclusion, the *cu* gene could be a marker of more extensive transfers of genetic material, as documented in other fungi.

In spite of the growing number of HGTs described in fungi the mechanisms underlying the transfer and the integration of genetic material in the recipient genomes remain obscure, except for a few cases. Formation of transient hyphal or conidial anastomoses, direct uptake of DNA, presence of mobile elements in the vicinity of the transferred sequence and recombination have been proposed (Mehrabi *et al.* 2011; Richards *et al.* 2011; Fitzpatrick 2012). Richards *et al.* (2009) showed that in two gene transfers from a fungus to the bryophyte moss *Physcomitrella patens*, the HGT was located near to a putative transposable element. In other instances the transferred sequence was found in the subtelomeric regions of chromosomes, characterized by frequent rearrangements and rich in transposable elements (Coelho *et al.* 2013). On the other hand, homologous recombination was involved in the transfer of genetic material between the yeast wine strain *Saccharomyces cerevisiae* EC1118 and *Zygosaccharomyces bailii* (Novo *et al.* 2009) and of the penicillin biosynthetic gene cluster in *Penicillium chrysogenum* (Rosewich & Kistler 2000).

In order to assess if transposable elements were present in the regions flanking the *cu* gene, that could account for its transfer between *O. novo-ulmi* and *Geosmithia* spp., we took advantage of the recently published genome sequence of *O. novo-ulmi* (Forgetta *et al.* 2013). Firstly, a blastn search was performed to identify the sequence containing the *cu* gene, which was found in the genomic scaffold scaffold00002 (GenBank acc. no. KB209922). A second blastn search on the scaffold00002 revealed two sequences with 99 and 95 % identity (*E*-values 0.0), respectively, to *O. novo-ulmi* transposons OPHIO1 and OPHIO3 (Bouvet *et al.* 2007) located about 500 and 700 kbp from the 3′ end of the *cu* gene. However, due to the large distance between these elements and the *cu* gene, their involvement in the gene transfer event could be excluded.

The high frequency of *cu* gene transfer between *O. novo-ulmi* and *Geosmithia* spp. suggested the establishment of a closer relationship between the two species beyond simple habitat sharing inside elm trees. Interestingly, rare interspecific sexual crosses were demonstrated in *O. novo-ulmi* to be involved in the acquirement of mating type and vegetative incompatibility genes from *Ophiostoma ulmi* (Paoletti *et al.* 2006), and the induction of sterile perithecia production was stimulated in dual culture of our reference species *Geosmithia* sp. 5 strain IVV7 and *O. novo-ulmi* cultures mating type B

strains (Pepori 2012). Further work is underway in our laboratories to assess the possible mechanism for gene transfer between *Geosmithia* spp. and *O. novo-ulmi*.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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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.2014.04.007.

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