A NON-*OPHIOSTOMA* FUNGUS EXPRESSES THE GENE ENCODING THE HYDROPHOBIN CERATO-ULMIN

A. Scala[1], C. Comparini[1], S. Tegli[1] and F. Scala[2]

[1] Department of Agricultural Biotechnology, Section of Plant Pathology, University of Florence, Via della Lastruccia 10, 50019 Sesto Fiorentino, Italy
[2] Department Ar.Bo.Pa.Ve., Section of Plant Pathology, University of Naples "Federico II", Via Università 100, 80055 Portici, Italy

SUMMARY

Strain IVV7 isolated from an elm tree showing typical symptoms of Dutch elm disease was identified as *Geosmithia* sp. by colony morphology and sequencing of the internal transcribed spacers (ITS1 and ITS2) and 5.8S region of the nuclear rDNA. UPGMA analysis revealed that IVV7 was taxonomically related to the species *G. pallida*, *G. putterillii* and *G. lavendula*. IVV7 was shown to express the gene of the hydrophobin cerato-ulmin by accumulating the protein in the cell walls and in the culture medium. This is the first report of the cerato-ulmin presence in a non-*Ophiostoma* fungus.

Key words: Cerato-ulmin, ITS region, *Geosmithia* sp., *Ophiostoma* sp., Dutch elm disease.

INTRODUCTION

Cerato-ulmin (CU) is a class II hydrophobin protein of about 8000 Da (Yaguchi *et al.*, 1993; Scala *et al.*, 1994), produced by the Ascomycota *Ophiostoma ulmi* (Buisman) Nannf., *O. novo-ulmi* Brasier and *O. himal-ulmi* Brasier et M. D. Mehrotra. *O. ulmi* and *O. novo-ulmi* are responsible for the Dutch elm disease (DED) that in the 20th century destroyed most of the elms native to Europe and North America (*Ulmus minor* Miller, *U. glabra* Huds., *U. procera* Salisb., *U. americana* L., *U. rubra* Muhl.) (Holmes and Heybroek, 1990; Brasier, 1991). *O. himal-ulmi* is endemic in the western Himalayas in apparent natural balance with the native elms, whereas it is very aggressive to European elms (Brasier and Mehrotra, 1995).

CU accumulates in the cell walls of DED fungi (Svircev *et al.*, 1988; Scala *et al.*, 1997;) and is released in the liquid culture medium (Takai, 1974; Brasier *et al.*, 1990; Brasier, 1991; Tegli *et al.*, 1994; Brasier and Mehrotra, 1995). It has been suggested that CU plays a key role in DED pathogenesis and in parasitic fitness of DED pathogens (Takai, 1974; Richards, 1993; Scala *et al.*, 1997; Temple *et al.*, 1997). The relationship between CU production and virulence of DED pathogens is still debated (Del Sorbo *et al.*, 2002). In another ophiostomatoid species, *O. quercus*, non-pathogenic towards elm trees, a protein immunologically related to CU was present in the mycelial cell wall, but not in the culture medium (Scala F. *et al.*, 1997). Moreover, Del Sorbo *et al.* (2000) detected in *O. quercus* a DNA sequence that cross-hybridized with an *O. novo-ulmi cu* gene fragment, thus suggesting the presence of a *cu*-orthologous gene in this species. Cloning and sequencing of the *cu* gene from *O. quercus* is in progress; attempts to find the CU protein in other *Ophiostomas* and some related genera (i.e. *Ceratocystis*) were unsuccessful (A. Scala *et al.*, unpublished information).

In this paper we report for the first time that a non-*Ophiostoma* fungus isolated from an elm tree showing typical DED symptoms has the *cu* gene and secretes the CU protein.

MATERIALS AND METHODS

Fungal strains and culture conditions. The non-*Ophiostoma* strain, named IVV7, together with a number of *O. novo-ulmi* strains named IVV*n* (with $n \geq 1$) were isolated from *U. minor* trees showing DED symptoms in the province of Vibo Valentia, Southern Italy, during a survey of the genetic variability of the Italian population of DED fungi (Casu, 1998). In the present study we used strains IVV1, IVV4, IVV11 and IVV13 of *O. novo-ulmi* and IBR1, IBR9 and IMC3 of *O. ulmi* (Casu, 1998), and strains 182 of *O. novo-ulmi* and E2 and R21 of *O. ulmi* (Scala F. *et al.*, 1997).

Geosmithia pallida (G. Sm.) Kolařík, Kubátová and Pažoutová, comb. nov., strain CCM8281 was from the Czech Collection of Microorganisms, Czech Republic; *G. lavendula* (Raper et Fennell) Pitt strains No. 582.67 and No.868.70 and *G. putterillii* (Thom) Pitt strains no. 179.92 and no. 233.61 were from the Centraalbureau voor Schimmelcultures, The Netherlands.

Single-spore-derived fungal colonies were routinely cultivated on Sigma (St. Louis, MO, USA) Potato Dex-

Corresponding author: A. Scala
Fax: +39.055.4573232
E-mail: aniello.scala@unifi.it

trose Agar (PDA) and Oxoid (Basingstoke, UK) Malt Extract Agar (MEA), or in liquid shake culture using the so-called Takai medium prepared according to Takai and Richards (1978) with some modifications as described by Scala *et al.* (1994). For long-term storage, conidia collected from 3-day-old liquid shaken minicultures (3 ml) in Takai medium were resuspended in 20% (v/v) glycerol and stored at -70°C.

Fungal morphology, basal physiological characterization and cerato-ulmin detection. To observe IVV7 colony morphology, the mycelium was grown for 14 days on MEA. Growth was determined according to the method of Tegli and Scala (1996) and expressed as daily radial growth (mm day^{-1}).

Production of CU in culture filtrates was determined by the turbidimetric method described by Takai and Richards (1978) as refined by Del Sorbo *et al.* (2000), with microtiter plates, and expressed as the cerato-ulmin production index (c.p.i.). The CU production of each isolate was determined on samples collected from five independent flasks. Each determination was performed in triplicate wells, and the final data were expressed as mean ± standard error (SE).

The CU concentration in culture filtrates was also determined by ELISA according to the procedure described in Scala *et al.* (1997), with an anti-CU antiserum raised in rabbit against purified CU from culture filtrates of *O. novo-ulmi* H328 (Scala *et al.*, 1994).

The presence of CU on the surface of fungal conidia and hyphae was revealed by an Immuno-Fluorescence (IF) assay as described by Scala F. *et al.* (1997) and observed with a Leitz Orthoplan microscope (Germany) with an incidence light excitation system, equipped with UV filters and a 75-W Leitz 100Z Xenon lamp.

Elm inoculations, pathogenicity index and reisolation of pathogens. Pathogenicity field trials were conducted at the "Azienda Agricola di Montepaldi" of the University of Florence, San Casciano Val di Pesa, Italy. Four-year-old plants of a commercial, DED-susceptible elm clone of *U. glabra*, whose heights varied from 2.0 to 2.5 m, were inoculated at about 1.4 m above ground level on June 1, 2005. To minimize environmental risks, a 10-m-wide, vegetation-free zone was created around inoculated plants. On each tree, a cut was made in the sapwood of the main stem with a sterile scalpel followed by the application of 150 µl of a suspension containing 10^8 conidia ml^{-1} according to the method described by Scala *et al.* (1997). Before inoculation, conidia were washed three times with sterile distilled water to avoid contamination with residual CU present in the culture filtrate. IVV7, *O. novo-ulmi* strain 182 or *O. ulmi* strain E2 were each inoculated in four plants, whereas one plant was inoculated with each of the *Geosmithia* strains (G. *lavendula* 582.67 and 868.70, and *G. putterillii* 179.92 and 233.61). The drops containing the conidia were immediately absorbed by the trees, and the wounds were then sealed with tape. Disease symptoms, including determination of percentage of foliar symptoms (chlorosis and wilting, or defoliation) and vascular discoloration length, were assessed on August 24, after 85 days. In order to determine if any alteration was caused by the presence of these fungi, 2-cm-thick stem cylinders were collected from each elm plant at the inoculation site as well as in intervals from 5 to 30 cm above and below it. From each cylinder, some fragments of xylem and adjacent tissues were aseptically removed underneath the cortex and placed on PDA adjusted to pH 3.5 with a sterile tartaric acid solution according to the manufacturer's instructions. If necessary, to confirm the identity of reisolated fungi, DNA was extracted and subjected to ITS analysis, as described below.

DNA sequencing and phylogenetic analysis. For DNA preparation, agar plugs were taken from cultures grown on MEA for 4-5 days at 23°C, and spread onto sterile sheets of cellophane overlaid on MEA plates. After 3-7 days of growth at 23°C in the dark, about 0.2 g (fresh weight) of mycelium was harvested from the cellophane sheet by scraping the surface with a scalpel. Genomic DNA was extracted from the mycelium using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The DNA quality and concentration was evaluated on 1% agarose gel in presence of High DNA Mass Ladder (Invitrogen Corporation, Carlsbad, CA, USA).

The *cu* gene was isolated according to the procedure of Bowden *et al.* (1994). The ITS1-5,8S-ITS2 region was amplified using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') as described by White *et al.* (1990) according to the procedure reported by Kolařík *et al.* (2004). The PCR products were purified with QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA) and stored at -20°C until used for sequencing, performed by MWG (AG Biotech, Ebersberg, Germany).

Sequences were analysed using the BLASTn 2.2.13 program (www.ncbi.nlm.nih.gov/BLAST). Sequences other than those of IVV7 were obtained from GenBank. Multiple sequence alignments were done with CLUSTAL W (http://clustalw.genome.jp). Phylogenetic analysis was conducted using MEGA version 3.0 (Kumar *et al.*, 2004), and the phylogenetic trees were constructed from the evolutionary distance data calculated using the Unweighted Pair Group Method with Arithmetic Averaging (UPGMA), nucleotide model (number of differences) (Nei and Kumar, 2000). The robustness of branches was assessed by a bootstrap analysis with 1,000 replicates.

RESULTS

Strain IVV7 was isolated from an *U. minor* tree showing DED symptoms, such as leaf chlorosis and

Fig. 1. Colony of IVV7 grown for 14 days on Malt Extract Agar at 23°C in the dark.

wilting, defoliation and xylem discoloration. Colony morphology on MEA (Fig. 1) indicated that it was not an *Ophiostoma* fungus. The colony had a central zone with a white, rough mycelial overgrowth, whose contour was rich in hyphal cords forming arborescent tufts. The growth rates of IVV7 on MEA at 23°C and 32°C, two temperatures used to characterize and differentiate the DED Ophiostomas, were 1.34 ± 0.11 mm day^{-1} and 0.60 ± 0.18 mm day^{-1}, respectively, and differed from the average values obtained for *O. novo-ulmi* strains 182, IVV1, IVV4, IVV11, IVV13 (3.91 ± 0.14 mm day^{-1} and 0.18 ± 0.04 mm day^{-1}, respectively). In the same experiment the strains E2, R21, IBR1, IBR9, IMC3 of *O. ulmi* gave a growth of 1.86 ± 0.25 mm day^{-1} and 2.00 ± 0.29 mm day^{-1} at 23°C and 32°C, respectively. The Tukey test revealed that IVV7 behaved in a manner not statistically different from *O. ulmi* at 23°C and *O. novo-ulmi* at 32°C.

When grown in the Takai shaken medium at 23°C, IVV7 caused turbidity suggesting the presence of a substance able to aggregate. The turbidity of the culture filtrate was measured by the turbidimetric assay usually used for CU determination and expressed as CU production index (c.p.i.) (Table 1). The c.p.i. of IVV7 (189 ± 22) was lower than the index of *O. novo-ulmi* (628 ± 49) ($P < 0.05$), but much higher than that of *O. ulmi* (8 ± 5) ($P < 0.001$). To verify if the substance produced *in vitro* by IVV7 was the CU protein, an ELISA using a CU-specific antiserum was done. The reaction was positive. The amount of CU produced by IVV7 (2.7 ± 0.5 mg ml^{-1}) was not statistically different from that of the high CU producer *O. novo-ulmi* (3.9 ± 0.4 mg ml^{-1}), and was significantly higher than that of *O. ulmi* (0.9 ± 0.4 mg ml^{-1}) ($P < 0.05$) (Table 1). Ten single-spore-derived colonies of IVV7 were subcultured monthly for a period of eight months on MEA, and conserved the ability to excrete CU in culture (data not shown). The immunofluorescence assay indicated the presence of CU on the surface of IVV7 hyphae and conidia (Table 1). However, the results showed that the CU fluorescence index of IVV7, *O. novo ulmi* and *O. ulmi* did not correlate with the CU production in culture. In previous work, Scala F. *et al.* (1997) reported that also in *O. novo ulmi*, *O. ulmi* and *O. quercus* there is no correlation between the amount of CU present on the cell wall and the CU produced in culture or present in cells.

Using *cu*-specific primers, a single DNA fragment was amplified from IVV7 and then sequenced. It consisted of 827 bp (Accession No. DQ377561), with 317 nucleotides before the ATG codon and 56 after the stop codon TAA. The sequence showed best scores and e-values with the *cu* genes of *O. novo-ulmi*: for example, the score was of 1608 with the *cu* gene Accession No. Z80085, and the e-value = 0.0. UPGMA analysis confirmed the very strong relationship between the IVV7 *cu* amplicon and the *cu* genes of the DED Ophiostomas (Fig. 2). Sequence analysis with BLASTp software showed only one conservative substitution (asp→glu) in position 75 in the deduced sequence of the IVV7 CU protein.

To classify strain IVV7, the ITS region was analysed. A 514 bp fragment containing the internal transcribed spacers (ITS1 and ITS2) and the 5.8S region of the nuclear rDNA was sequenced (Accession No. DQ377560) and the sequence analysed using BLASTn software.

The IVV7 fragment was closely related to the ITS re-

Table 1. Cerato-ulmin (CU) production in the culture filtrates and presence in the cell walls of IVV7, *Ophiostoma novo-ulmi* and *O. ulmi.*

Strain	CU production in the culture filtrate[w]		CU surface presence
	c.p.i.	ELISA	IF[z]
IVV7	189 ± 22b	2.7 ± 0.9a	+
Ophiostoma novo-ulmi	628 ± 49a	3.9 ± 0.6a	++
Ophiostoma ulmi	8 ± 5c	0.9 ± 0.7b	++

Results of CU production were means ± SE of five values, which were obtained by five replicates in the case of IVV7, and by one replicate for each of five strains in the case of *O. novo-ulmi* (182, IVV1, IVV4, IVV11, IVV13) and of *O. ulmi* (E2, R21, IBR1, IBR9, IMC3). Means in the column followed by the same letter did not differ significantly at $P \geq 0.05$, according to the Tukey test.

[w] Expressed as cerato-ulmin production index (c.p.i.) ± SE, assayed by the turbidimetric assay, and as ng of CU mg^{-1} lyophilized fungal biomass ± SE, assayed by ELISA.

[z] Expressed as fluorescence index, determined on a scale, where - = no visible fluorescence; + = faint fluorescence; ++ = moderate-to-intense fluorescence. Data were based on observations of 30 microscope fields in two independent experiments.

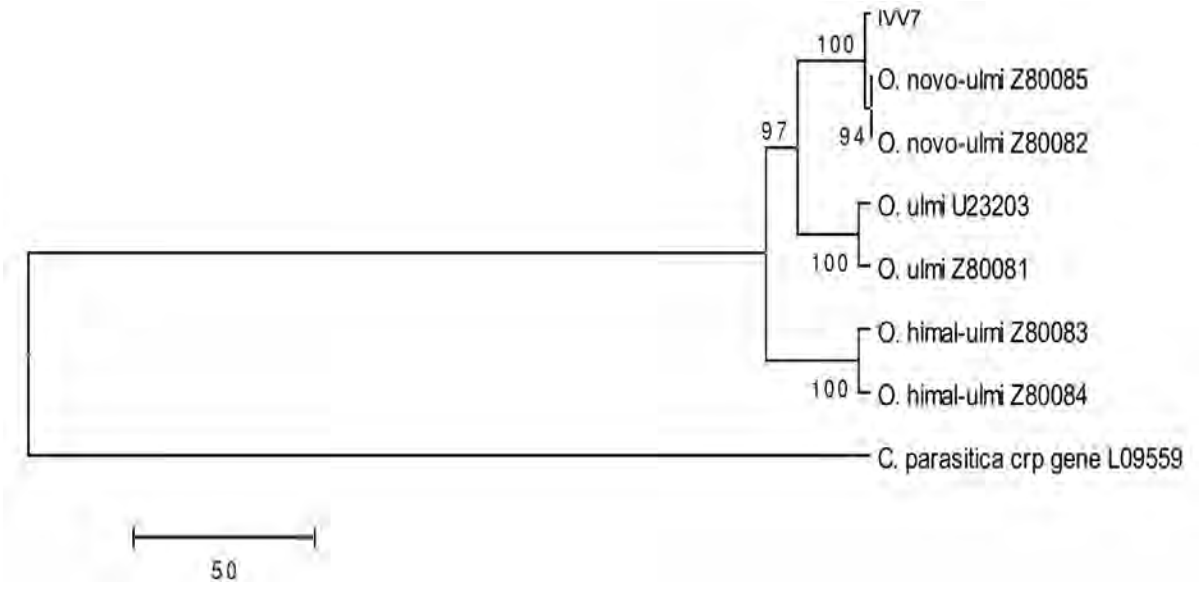

Fig. 2. Phylogenetic tree generated by UPGMA analysis based on *cu* gene sequences describing the relationships of IVV7 with the CU-producing Ophiostomas. Bootstrap values (percentages of 1,000 replications) are presented at the node. Reference sequences were retrieved from the NCBI GenBank under the accession numbers indicated. The scale bar indicates the distance for 50 nucleotides substitutions.

gion of various species of the genus *Geosmithia*, such as *G. pallida* (Accession No. GPA578485: score = 981; e-value = 0.0), *G. lavendula* (Accession No. AF033385: score = 704, e-value = 0.0) and *G. putterillii* (Accession No. AJ628350: score = 624, e-value = 2e-177). UPGMA analysis showed that the genera *Geosmithia* and *Ophiostoma* were well separated, and that IVV7 clearly belonged to the *Geosmithia* cluster (Fig. 3). In addition, the morphological characters of the IVV7 colony strongly resemble those of *Geosmithia* spp. used in this work and in the studies carried out by Kolařík *et al.* (2004; 2005).

G. pallida CCM8281, *G. lavendula* 582.67 and 868.70, and *G. putterillii* 179.92 and 233.61 did not produce CU in Takai medium and were negative in the immunofluorescence assay. Moreover, attempts to amplify the *cu* gene by PCR from these fungi were all unsuccessful (data not shown).

When artificially inoculated in *U. glabra* trees (this species has the same susceptibility to DED as *U. minor*), the *Geosmithia* strains did not produce any leaf chlorosis or defoliation (Table 2). However, they caused slight vascular discoloration around the inoculation site, which extended about 4 cm in length. IVV7 caused negligible leaf chlorosis (about 2% of leaves), while the vascular discoloration (about 8 cm in length) was not statistically different from that of *Geosmithia* spp. (4 ± 2). Eighty five days after inoculation, IVV7 was reisolated from the discoloured sections of the plant. These results show that IVV7 is was not responsible for the DED symptoms shown by the elm tree from which it was isolated. As expected, *O. novo-ulmi* 182 and *O. ulmi* E2 caused abundant leaf chlorosis and wilt (90 and 16% of leaves, respectively); both fungi also produced an intense browning in the vascular zone.

Table 2. Pathogenicity towards *Ulmus glabra* trees of IVV7, *Ophiostoma novo-ulmi*, *O. ulmi*, *Geosmithia pallida* and *G. putterillii.*

Strain	Pathogenicity towards elm trees[w]	
	Vascular discoloration length (cm)	Leaf chlorosis and wilting or defoliation (% of the total crown)
IVV7	8 ± 2c	2 ± 1b
O. novo-ulmi 182	45 ± 7a	80 ± 14a
O. ulmi E2	25 ± 3b	4 ± 1b
Geosmithia spp.	4 ± 2c	0 ± 0b
Distilled water	0 ± 0c	0 ± 0b

Results were means ± SE of four values, which were obtained by four replicates in the case of IVV7, *O. novo-ulmi* and *O. ulmi* and distilled water, and by one replicate for each of four strains in the case of *Geosmithia* spp. (*G. lavendula* 582.67 and 868.70, *G. putterillii* 179.92 and 233.61).
Means in the column followed by the same letter did not differ significantly at $P \geq 0.05$, according to the Tukey test. Date of inoculation: June 1, 2005; disease symptoms were assessed on August 24, 2005.
[w] Expressed as cm of discoloured tissue from the inoculation point ± SE, and the mean percentage of cholorotic, wilted leaves and/or defoliation ± SE.

DISCUSSION

Strain IVV7, isolated from an elm tree showing typical Dutch elm disease symptoms, was identified as *Geo-*

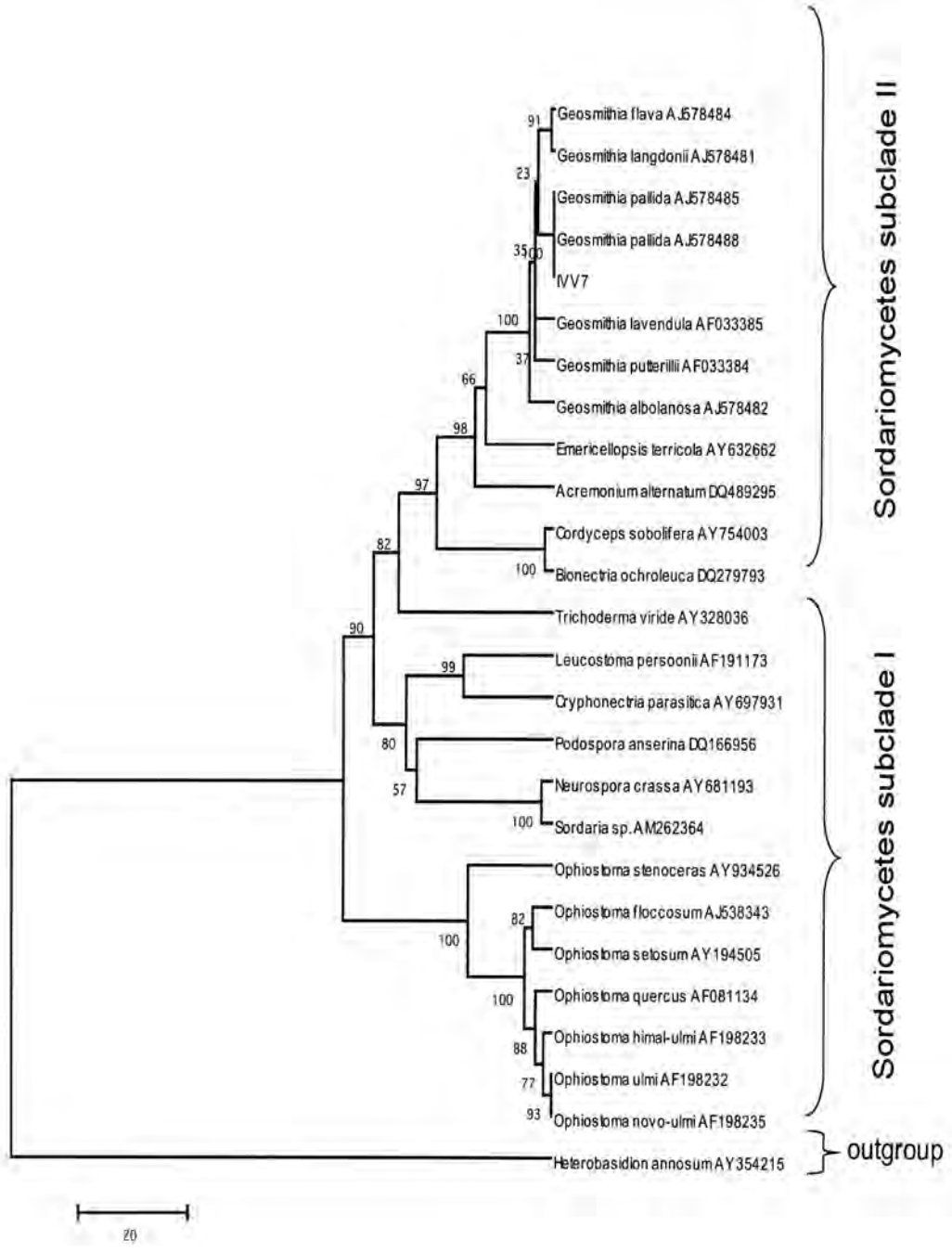

Fig. 3. Phylogenetic tree generated by UPGMA analysis based on sequences of the ITS region (including 5.8S rDNA), showing the relationships of IVV7 with various species belonging to the euascomycete group *Sordariomycetes* containing the genera *Geosmithia* and *Ophiostoma*. Bootstrap values (percentages of 1,000 replications) are presented at the node. Reference sequences were retrieved from the NCBI GenBank under the accession numbers indicated. The scale bar indicates the distance for 20 nucleotides substitutions.

smithia sp. by colony morphology and ITS region analysis. IVV7 was shown to express the CU hydrophobin gene by accumulating the protein in the cell wall and in the culture medium. However, expression of the *cu* gene in IVV7 did not confer ability to cause DED symptoms in susceptible elm trees, differently from what occurred in *O. quercus*. Del Sorbo *et al.* (2000) observed that eight CU producing transformants of *O. quercus* caused an increase in severity of vascular and/or foliar symptoms; moreover, host leaves displaying typical DED symptoms contained amounts of the toxin similar to those determined in leaves of plants inoculated with DED pathogens. Although the positive influence of *cu* gene insertion on the pathogenicity of *O. quercus* could derive from interaction with other genes, it has been suggested that this hydrophobin may function as a virulence factor when introduced into *O. quercus*, a species strictly related to the DED Ophiostomas (Del Sorbo *et al.*, 2002).

The hydrophobins are small, moderately hydrophobic proteins with eight cysteines ordered in a particular manner in their primary structure (Kershaw and Talbot, 1998; Whiteford and Spanu, 2002; Linder *et al.*, 2005). Many hydrophobins have been described, and despite an apparent structural similarity, amino acid homology among them is limited. Wessels (1997) observed a very low identity and similarity (<10%) among all hydrophobins described. Even among class II hydrophobins, the most uniform subgroup of these proteins, high sequence identity was observed only when strictly related fungal species were considered. These results support the hypothesis that the hydrophobin CU is present only in the genus *Ophiostoma*, but not in "distant" taxa such as the genus *Geosmithia*.

Ogawa *et al.* (1997) showed that *Geosmithia* is a polyphyletic taxon with affinities to the Hypocreales and Eurotiales. According to Tehler *et al.* (2003) *Geosmithia* together with other genera, i.e. *Beauveria*, *Bionectria* and *Trichoderma*, belong to "subclade II" of the euascomycete group *Sordariomycetes*, while "subclade I" contains the genera *Ophiostoma*, *Podospora*, *Neurospora*, *Cryphonectria* and *Magnaporthe*. Recently, interest towards *Geosmithia* has grown, and numerous authors have revised this multifaceted genus. Kolařík *et al.* (2004) studied the genetic variability of many isolates traditionally grouped in *G. putterillii* and *G. lavendula* by RAPDs, ITS sequencing, and morphological characters. They showed that the newly recognized species *G. pallida* and *G. flava* are stable in culture and have a worldwide distribution. Kolařík *et al.* (2004) provided a key based on colony features and micromorphology in order to define the hypocrealean species of the genus *Geosmithia*.

Moreover, some isolates of *Geosmithia* spp. are associated with elm bark beetles, like the ophiostomatoid fungi (Kolařík *et al.*, 2004; 2005; Čižkova *et al.*, 2005). In the present work IVV7 has been shown to be close to the hypocrealean species *G. pallida*, *G. putterillii* and *G. lavendula*, known to be typical fungi inhabiting galleries of many phloem-feeding bark beetles. Therefore, it is possible that isolates of *Geosmithia* and *Ophiostoma* come into prolonged physical contact within elm trees. Despite the undoubted taxonomical distance between these genera, it cannot be excluded that a chromosome or a DNA fragment containing the *cu* gene of *O. novo-ulmi* may have been exceptionally introgressed in the *Geosmithia* genome by a tentative cross or hyphal anatomosis. In this regard, IVV7 has been shown to stimulate the production of sterile perithecia when crossed with *O. novo-ulmi* strains of sexual compatibility type B (data not shown).

Differently from prokaryotes in which an extensive amount of horizontal gene transfer (HGT) is considered a major factor for the evolution of genomes (Jain *et al.*, 1999), in multicellular eucaryotes HGT is generally assumed to play a minor role. In fungi, HGT has been invoked to justify the evidence of unusual features of genetic elements such as single genes or gene clusters (Rosewich and Kistler, 2000). Kimura *et al.* (1998) found that in *Fusarium graminearum* the trichothecene 3-O-acetyltransferase gene, that plays a pivotal role for the well-being of the type B trichothecene producers, is not located in the biosynthetic gene cluster as expected, but it is between the UTP-ammonia ligase and the phosphate permease genes which are not related to trichothecene biosynthesis. The authors suggested that this result may be attributed to a horizontal gene transfer. More interestingly, the genes for the biosynthesis of host-selective toxins, known to be clustered in *Alternaria alternata*, *Cochliobolus carbonum* and *C. heterostrophus*, and the chromosomal regions containing these clusters are entirely absent in the non-pathogenic strains of these species. This discontinuous cluster distribution together with other features such as the distinctive codon usage and GC content may be good indications of a horizontal transfer (Rosewich and Kistler, 2000; Walton, 2004). Other genetic materials are reported to be horizontally transferred between fungi, including virus-like dsRNAs between *O. ulmi* and *O. novo-ulmi* (Hong *et al.*, 1999), or between *S. sclerotiorum* and *S. minor* (Brasier *et al.*, 1998, Melzer *et al.*, 2002), and a mitochondrial plasmid from *Ascobolus immerses* to *Podospora anserine* (Kempken, 1995).

In some cases, transitory hyphal fusions appeared to be sufficient to transmit dsRNA elements, as with a hypovirulence-associated dsRNA from *Sclerotinia homoeocarpa* that is conspecific with the mitovirus 3a-Ld from *Ophiostoma novo-ulmi* (Deng *et al.*, 2003).

To date, we do not know if IVV7 contains other genes from *O. novo-ulmi*. Moreover, even if isolates of *Ophiostoma* and *Geosmithia* have been reported to come in contact frequently in elm trees, one does not know whether horizontal transfer of genetic material between the two fungi is common.

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