Resolving the complex of *Diaporthe* (*Phomopsis*) species occurring on *Foeniculum vulgare* in Portugal

Santos, J.M.[1*] and Phillips, A.J.L.[1]

[1]Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

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Diaporthe species and their *Phomopsis* anamorphs are endophytes and pathogens on a wide range of plant hosts, and are responsible for several diseases, some of which are of economic importance. Species in this genus have been described mainly on the basis of host association and to a lesser extent on their micromorphology. However, studies have revealed that host association is of minor taxonomic importance, due to the wide host ranges of some species. Moreover, more than one species can occur on a single host. On the other hand, morphological characters are not always suitable for species definition because of their plasticity and overlap between different species. *Foeniculum vulgare* is an herbaceous plant known to harbour a complex of *Diaporthe* and *Phomopsis* species. In this study, this complex is resolved and several taxa are delineated. An intensive sampling of *Diaporthe* species and *Phomopsis* anamorphs found on *F. vulgare* in Portugal was done and a collection of isolates was established in culture. Isolates were characterised and grouped according to their microsatellite-primed PCR (MSP-PCR) profiles. Representative isolates were subsequently selected for a molecular phylogeny based on the rDNA ITS region (ITS1–5.8S–ITS2). Combining morphological, cultural and molecular data, four species were distinguished from *F. vulgare*. *Diaporthe angelicae* is shown to be the most common pathogen of this host in Portugal. *Diaporthe lusitanicae* is newly described, whereas the teleomorph of *Phomopsis theicola* was revealed to be distinct from *Diaporthe theicola*, and is described as *Diaporthe neotheicola*.

Key words: *Phomopsis*, MSP-PCR, ITS, phylogeny, systematics, taxonomy

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*Corresponding author: Jorge Santos; e-mail: jmsantos@fct.unl.pt

Introduction

The ascomycete genus *Diaporthe* Nitschke includes plant pathogens, endophytes and species of unknown affinity that are most commonly seen as their *Phomopsis* (Sacc.) Bubák anamorphs (Sánchez Márquez *et al*., 2007; Duong *et al*., 2008; Kodsueb *et al*., 2008; Thongkantha *et al*., 2008). Many of the species incite cankers, diebacks, rots, spots and wilts on a wide range of plants species, some of which are of economic importance.

Species in *Diaporthe* were characterised largely on host association until Wehmeyer's (1933) revision placed many names in synonymy, thus reducing 650 species to about 70. The synonymies he proposed were based on morphology of herbarium specimens of the teleomorph, and no anamorph characters were considered. As a result, some of his synonymies have now been rejected (e.g. Linders and van der Aa, 1995; Phillips, 2003).

Species names in *Phomopsis* were also based largely on host association and more than 1000 species have been described (Uecker, 1988). However, it is now recognised that host is of minor importance in the taxonomy of *Phomopsis* (Rehner and Uecker, 1994; Mostert *et al*., 2001). Not only are species non-host specific but more than one species can occur on a single host.

Correct identification of a pathogen is an essential step towards meaningful studies on epidemiology and control of a plant disease. It is also essential for the implementation of international phytosanitary measures. There-

fore there is an urgent need to formulate robust species concepts and clear, unambiguous descriptions of the species in *Diaporthe* and *Phomopsis*.

In the present work, the *Diaporthe* and *Phomopsis* species associated with wild fennel (*Foeniculum vulgare*) were studied. *Foeniculum vulgare* is a common herbaceous plant belonging to the Apiaceae that is widespread throughout Portugal and all Mediterranean regions. Besides its economic importance in food and cosmetic industries (Zeller and Rychlik, 2006), weeds like wild fennel can act as alternative hosts for pathogens of other economic important crops.

In a preliminary study (Phillips, 2003) at least two *Diaporthe* spp. and two *Phomopsis* spp. were found on *F. vulgare*. Thus, this host clearly harbours a complex of *Diaporthe* and *Phomopsis* species. Therefore the aim of this study was to fully characterise the species of *Diaporthe* and *Phomopsis* that occur on *F. vulgare* in Portugal and to connect the *Diaporthe* species with their respective *Phomopsis* anamorphs. In view of the proposal to move away from dual nomenclature (Rossman and Samuels, 2005), and using a single name for each genus, we propose to give preference to the older *Diaporthe* (1870) rather than the younger anamorph genus, *Phomopsis* (1905). For this reason, we declined to introduce separate names for the anamorphs of the new species.

Materials and methods

Isolates and morphology

Foeniculum vulgare plants showing symptoms of infection by *Diaporthe* or *Phomopsis* were collected from 10 localities in Portugal. Conidia or ascospores were spread onto half strength potato-dextrose agar (½PDA) plates prepared from 20 g/L Difco Potato Dextrose Agar (Difco Laboratories, Detroit, MI, EUA), plus 10 g/L agar. After incubating for 24 h at room temperature (RT), individual germinating conidia or ascospores were transferred to fresh ½PDA plates. A total of 128 strains were isolated. The isolates selected and used in the present work are summarised in Table 1. Reference isolates were deposited in the public culture collection at Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

To induce anamorphic sporulation, isolates were inoculated on 2% water agar (WA) plates containing pieces of healthy autoclaved (20 min, 121°C, 1 bar) fennel stems and incubated at RT under diffuse daylight. To study the morphology of the teleomorph the method of Brayford (1990) was used. In attempts to induce formation of the teleomorph, ten single ascospore or single conidium isolates of a species were paired in every possible combination. Plates were incubated under the same conditions as used for anamorphic sporulation. Anamorphic structures were mounted in 100% lactic acid while asci and ascospores were mounted in Shear's medium (Booth, 1971) without any stain. Observations on micromorphological features were made with Leica MZ95 and Leica DMR microscopes and digital images were recorded with Leica DC300 and Leica DFC320 cameras, respectively. Measurements were made with the measurement module of the Leica IM500 image management system (Leica Microsystems GmbH, Wetzlar, Germany). Mean, standard deviation (S.D.) and 95% confidence intervals were calculated for asci, ascospores, alpha conidia and beta conidia. Minimum and maximum dimensions are given in parenthesis. In the case of asci, these values were rounded to the closest half micron. *n* = total of measured structures.

Growth rates were determined on PDA plates incubated in darkness at 25°C for up to 120 h. Two isolates per species were inoculated in triplicate. Isolates were pre-inoculated to ensure they were in an active state of growth. To standardise measurements, circular inocula 5 mm diam. were placed in the centre of 86 mm Petri dishes. For each replicate, two perpendicular diameters were measured at 24 h intervals.

DNA isolation

A DNA extraction protocol optimised for *Phomopsis* and *Diaporthe* strains was developed. The isolates were grown on PDA plates in darkness at 25°C until they completely covered the medium surface. The mycelium was then scraped off and collected in a 2 mL Eppendorf tube with 50 µL of auto-

Table 1. Isolates used in this study.

Species	Isolate no.[1]	Origin	Collector	GenBank accession no. (ITS)[2]
D. ambigua	Di-C002/1	Vale Andeiro, Azeitão	Jorge M. Santos	
	Di-C002/2	Vale Andeiro, Azeitão	Jorge M. Santos	
	Di-C002/8	Vale Andeiro, Azeitão	Jorge M. Santos	
	CBS 123211; Di-C002/9	Vale Andeiro, Azeitão	Jorge M. Santos	EU814478
	Di-C003/2	Vale Andeiro, Azeitão	Jorge M. Santos	
	Di-C003/6	Vale Andeiro, Azeitão	Jorge M. Santos	
	CBS 123210; Di-C003/10	Vale Andeiro, Azeitão	Jorge M. Santos	EU814479
D. angelicae	Ph-C003/2	Qta. Torre, Caparica	Jorge M. Santos	
	Ph-C005/2	Qta. Torre, Caparica	Jorge M. Santos	
	Ph-C006/1	Qta. Torre, Caparica	Jorge M. Santos	
	Ph-C032/1	EAN, Oeiras	Jorge M. Santos	EU814462
	Ph-C068/2	EAN, Oeiras	Jorge M. Santos	EU814463
	Ph-C094/2	Qta. Torre, Caparica	Jorge M. Santos	EU814464
	Ph-C098/2	Qta. Torre, Caparica	Jorge M. Santos	EU814465
	Ph-C118/1	Monte de Caparica	Jorge M. Santos	EU814466
	Ph-C121/1	Monte de Caparica	Jorge M. Santos	EU814467
	CBS 123215; Ph-C133/1	Malveira da Serra	Alan J. L. Phillips	EU814468
	Ph-C135/1	Malveira da Serra	Alan J. L. Phillips	EU814469
	Ph-C142/2	Torre, Caparica	Jorge M. Santos	EU814470
	Ph-C150/1	Vale Andeiro, Azeitão	Jorge M. Santos	EU814471
	CBS 123214; Ph-C159/1	Qta. Sol, Alenquer	Jorge M. Santos	EU814472
	Ph-C165/2	Qta. Sol, Alenquer	Jorge M. Santos	EU814473
	Ph-C166/1	Faro	Alan J. L. Phillips	EU814474
	Ph-C168/1	Évora	Alan J. L. Phillips	EU814475
D. lusitanicae	**Ph-C086/2**	EAN, Oeiras	Jorge M. Santos	EU814476
	Ph-C170/1	Aveiro	Artur Alves	EU873551
	Di-C001/1	EAN, Oeiras	Jorge M. Santos	
	CBS 123213; **Di-C001/3**	EAN, Oeiras	Jorge M. Santos	
	Di-C001/4	EAN, Oeiras	Jorge M. Santos	
	CBS 123212; **Di-C001/5**	EAN, Oeiras	Jorge M. Santos	EU814477
	Di-C001/10	EAN, Oeiras	Jorge M. Santos	
D. neotheicola	**Di-C004/1**	Évora	Alan J. L. Phillips	
	CBS 123209; **Di-C004/4**	Évora	Alan J. L. Phillips	
	CBS 123208; **Di-C004/5**	Évora	Alan J. L. Phillips	EU814480
	Di-C004/9	Évora	Alan J. L. Phillips	

[1]CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; Ph-C & Di-C: Culture collection housed at Centro de Recursos Microbiológicos, Caparica, Portugal.
[2] ITS: internal transcribed spacer.
EAN: Estação Agronómica Nacional
Ex-type cultures are in bold.

claved glass micro spheres (230–320 µm diam.). The tubes were then placed in liquid nitrogen for 5 min and transferred to ice. To separate organic and aqueous phases, 250 µL of phenol and 250 µL of chloroform were added, together with 500 µL of lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% Triton X-100, 1% SDS). Tubes were vortexed for 20 minutes and then centrifuged (19274 × g, 4°C, 25 minutes). The aqueous phase was transferred to a new 1.5 mL tube and the nucleic acids precipitated with an equal volume of cold absolute isopropanol. The tubes were centrifuged again (19274 × g, 4°C, 10 minutes), the supernatants discarded and the pellets washed with 1 mL of cold 70% ethanol. After a further centrifugation (19274 × g, 4°C, 5 minutes), the supernatants were discarded and the pellets dried at RT with the tubes open in an inverted position. RNA was digested by incubating the pellets with 50 µL of TE (10 mM Tris, 1 mM EDTA) + RNAse A (Sigma®) (50 $\mu g.mL^{-1}$) at 55°C for 15 minutes.

MSP-PCR

MSP-PCR profiles were generated following the protocol of Uddin and Stevenson (1997), using M13 (5' – GAG GGT GGC GGT TCT – 3') (Meyer *et al*., 1993) and $(GTG)_5$ primers (20 pmol per 25 µL reaction) and extending the elongation step (72°C) to 2 min. 10 µL of reaction mixture were applied in 1.5% (w/v) agarose gels in TBE (Tris Borate EDTA) 0.5X and the amplicons separated by electrophoresis at 80 V for 3 h 30 minutes. The gels were then stained with ethidium bromide (EtBr) and photographed under ultraviolet light. The isolates were clustered on the basis of their profiles in a consensus dendrogram built with Bionumerics version 4.61 (Applied Maths, Kortrijk, Belgium, 1998), using Pearson's correlation coefficient and UPGMA. The reproducibility level was calculated as the mean value of the reproducibility obtained for each primer independently. For this purpose, and for each primer, 10% of the isolates were chosen randomly and their profiles redone.

Sequence analysis

The primers ITS1 (5' – TCC GTA GGT GAA CCT GCG G – 3') (White *et al*., 1990) and NL-4 (5' – GGT CCG TGT TTC AAG ACG G – 3') (O'Donnell, 1993) were used to amplify the ITS-D1/D2 rDNA region. The PCR reaction mixture consisted of 50–100 ng of genomic DNA, 15 pmol of each primer, 200 µM of each dNTP, 3 mM $MgCl_2$, 1% DMSO, 1 U *Taq* DNA polymerase (recombinant) (Fermentas Life Sciences, Ontario, Canada) and was made up to a total volume of 50 µL. The cycling conditions were: 5 min at 95°C, followed by 40 cycles of 1 min at 94°C, 30 s at 50°C and 1 min at 72°C, and a final step of 10 min at 72°C. The amplicons were purified with illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. The ITS region was sequenced in both directions by STAB VIDA, Lda. using ITS1 and ITS4 (5' – TCC TCC GCT TAT TGA TAT GC – 3') (White *et al*., 1990) primers. Sequences were edited with BioEdit v. 7.0.9.0 (Hall, 1999) and aligned with ClustalX (1.83) (Thompson *et al*., 1997). Additional sequences were obtained from GenBank using BLAST (Basic Local Alignment Search Tool) (Altschul *et al*., 1990) and included in the alignment. Sequences of *Valsa ceratosperma* (Tode) Maire (DQ241769) and *Leucostoma persoonii* (Nitschke) Höhn. (DQ996042) were used as outgroups. Terminal regions, with missing data in some of the isolates, were excluded from the analysis. All molecular characters were unordered and given equal weight during the analysis. Phylogenetic trees were inferred in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2002) by Neighbour-Joining (NJ) using Kimura 2-parameter distance and Maximum Parsimony (MP) using the heuristic search option with random addition of sequences (1000 replications), tree bisection-reconnection (TBR) and MULTREES options ON. Gaps were regarded as a fifth character in MP. Bootstrap support values with 1000 replications (Felsenstein, 1985) were calculated for tree branches in both phylogenetic methods. MP bootstrap analysis was done with MULTREES option off and 10 random sequence additions in each of 1000 pseudoreplicates. Sequences obtained from GenBank are listed by their accession numbers and taxon names in the tree (Fig. 2), while newly generated sequences are listed by their isolate number. Newly generated sequences

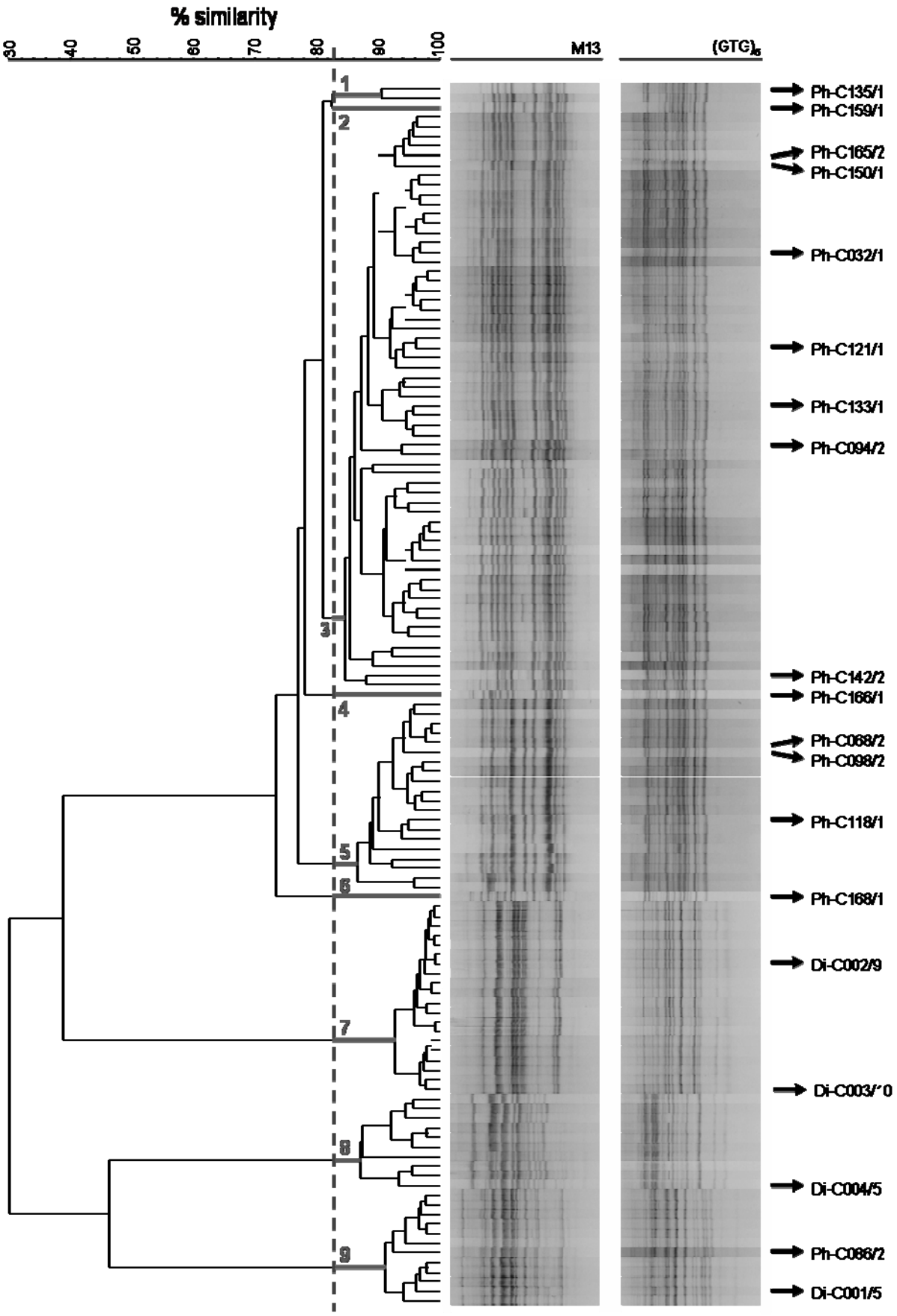

Fig. 1. Consensus dendrogram from M13 and $(GTG)_5$ MSP-PCR profiles performed in Bionumerics using Pearson's correlation coefficient and UPGMA. The vertical dashed line corresponds to the reproducibility level (82.7%) from which nine groups of isolates are formed (numbered grey horizontal lines). Isolates shown with their respective numbers were selected for the construction of a phylogeny.

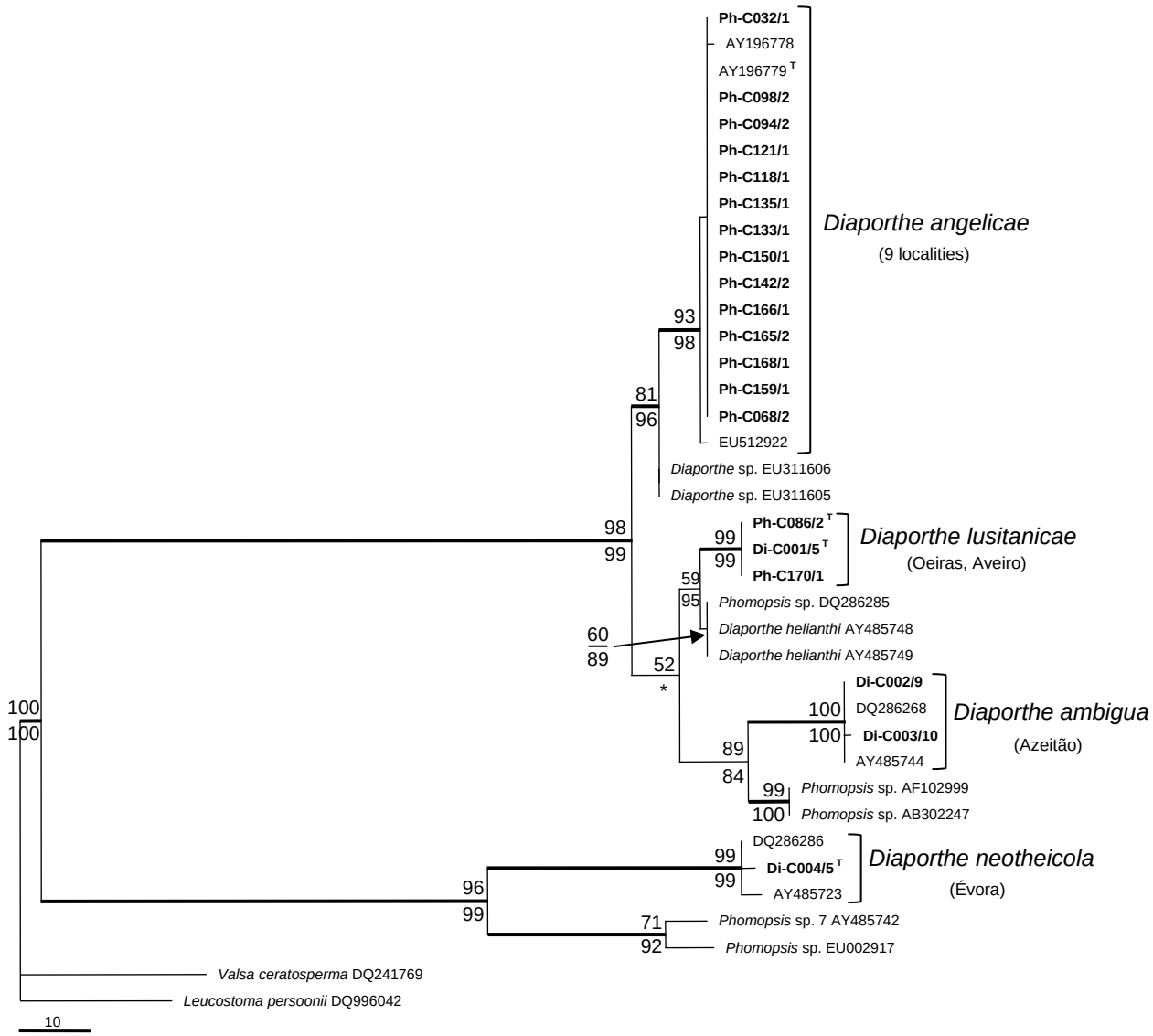

Fig. 2. One of 11 equally parsimonious trees resulting from the alignment of 561 characters of ITS rDNA region. Length = 255; consistency index (CI) = 0.816; retention index (RI) = 0.922; rescaled consistency index (RC) = 0.752; homoplasy index (HI) = 0.184. Newly generated sequences are listed in bold. Bootstrap values with 1000 replications are shown above the branches for Maximum Parsimony and below the branches for Neighbour-Joining (NJ). Branch marked with * is not present in NJ tree. Thickened lines indicate branches that are present in the strict consensus tree. Bar represents 10 changes. *Valsa ceratosperma* (DQ241769) and *Leucostoma persoonii* (DQ996042) were included as outgroups. Locality names indicate the origin, within each clade, of the strains isolated in the present work. ^{T} Ex-type or ex-epitype culture. Phylogeny deposited in TreeBASE (S2209).

have been deposited in GenBank (Table 1) and the alignment and phylogeny in TreeBASE (Matrix M4195; Study S2209).

Results

A variability analysis was done to assess genetic diversity within the collection of isolates of *Diaporthe* and *Phomopsis* from *Foeniculum vulgare*. The strains were clustered in a consensus dendrogram based on M13 and $(GTG)_5$ MSP-PCR profiles (Fig. 1). Nine meaningful groups were formed from the reproducibility level (82.7%). Representative isolates from each group and, when possible, isolates from different geographic regions of the country within the same group were selected for the phylogenetic analysis. A further isolate (Ph-C170/1) from Aveiro was also included in this analysis.

The ITS rDNA region of the selected isolates was sequenced and BLAST searches were done to select closely related sequences. An alignment containing 561 characters (including alignment gaps) and spanning ITS1, 5.8S and ITS2 complete sequences as well as 18S and 28S partial sequences was included in the phylogenetic analysis using Neighbour-

Joining (NJ) and Maximum Parsimony (MP). Of the 561 characters, 116 were parsimony informative and included in the analysis resulting in 11 equally parsimonious trees. One of the trees is shown in Fig. 2 with thickened lines indicating branches present in the strict consensus tree. The NJ analysis resulted in a tree with the same topology as Fig. 2, except for only one branch. MP bootstrap values are shown above the branches and NJ bootstrap values are shown below the branches. Four clades were formed, corresponding to four different species sampled from *F. vulgare*. The first clade consists of *Diaporthe angelicae* (Berk.) Farr & Castlebury, present in nine of the ten localities sampled, with bootstrap values of 93% (MP) and 98% (NJ). The second clade consists of *Diaporthe lusitanicae* A.J.L. Phillips & J.M. Santos, a new species sampled in Oeiras and Aveiro, with bootstrap values of 99% for both MP and NJ. The third clade consists of *Diaporthe ambigua* Nitschke, isolated from two distinct plants from Azeitão, with bootstrap values of 100% (MP and NJ). The fourth clade (NJ and MP bootstrap values of 99%) consists of *Diaporthe neotheicola* A.J.L. Phillips & J.M. Santos, the newly described teleomorph of *Phomopsis theicola* Curzi, sampled from a plant from Évora.

Taxonomy

Diaporthe ambigua Nitschke, Pyrenomycetes Germanici: 311 (1867).

Anamorph: *Phomopsis ambigua* (Sacc.) Traverso, Flora Italica Cryptogama, Pars 1. Fungi. *Pyrenomycetae*. *Xylariaceae*, *Valsaceae*, *Ceratostomataceae* 2(1): 266 (1906).

≡ *Phoma ambigua* Sacc. Sylloge Fungorum 2: 91 (1880).

Habitat: reported on *Aspalathus linearis* (van Rensburg *et al*., 2006), *Foeniculum vulgare*, *Malus domestica* (Smit *et al*., 1996), *Malus sylvestris* (Crous *et al*., 2000), *Malus* sp. (Dennis, 1986), *Prunus salicina* (Smit *et al*., 1996), *Prunus* sp. (Mostert *et al*., 2001), *Pyrus communis* (Nitschke, 1867), *Pyrus ussuriensis* (Tai, 1979) and *Vitis vinifera* (van Niekerk *et al*., 2005).

Known distribution: China, Cuba (Tai, 1979), Germany (Nitschke, 1867), Netherlands (Myburg *et al*., 1999), South Africa (Smit *et al*., 1996), United Kingdom (Dennis, 1986) and United States (Washington) (Shaw, 1973).

Material examined: See Table 1 for isolates studied.

Notes: van Rensburg *et al*. (2006) gave a detailed description and proposed an epitype specimen for this species.

Perithecia formed in all single ascospore cultures studied. Therefore, this species is self-fertile.

Diaporthe angelicae (Berk.) Farr & Castlebury, Mycoscience 44: 204 (2003). (Fig. 3)

≡ *Sphaeria angelicae* Berk., Mag. Zool. Bot. 28 (1837).

Anamorph: *Phomopsis* sp.

Pycnidia subglobose to discoid, 220–320 µm in its widest diam., superficial, appearing as black dots scattered on the host epidermis. Pycnidial neck not evident. *Conidiomata* on *Foeniculum vulgare* stems in culture pycnidial, cone-shaped, 180–580 µm wide, 190–400 µm high, embedded just beneath the plant epidermis. Yellowish translucent conidial drop exuded from the ostiole. Conidiophores cylindrical, single- to multi-septate, hyaline, smooth, sinuous and branched, 7.8–18.0 × 1.6–2.9 µm. Conidiogenous cells phialidic, cylindrical, tapering towards the apex. Periclinal thickening present. Collarette not seen. Conidiogenous cells terminal, attached to the apex of conidiophores, 10.0–14.9 × 1.7–2.5 µm, and lateral, belonging to the main axis of conidiophore but showing a lateral prolongation, 4.7–14.0 × 1.4–2.4 µm. Alpha conidia unicellular, fusiform, tapering towards both ends, (6.5–)8.6–8.8(–11.0) × (2.2–)2.8–2.9(–3.9) µm, mean ± S.D. = 8.7 ± 0.9 × 2.9 ± 0.3 µm (n = 600), hyaline, biguttulate, very often with several additional smaller guttules. Beta conidia hyaline, aseptate, eguttulate, filiform, curved or hamate, with obtuse ends, present in higher number and previously to alpha conidia, (15.3–)24.2–25.9(–30.6) × (0.8–)1.0–1.1(–1.3) µm, mean ± S.D. = 25.1 ± 3.0 × 1.0 ± 0.1 µm (n = 50).

Cultures on PDA at 25°C, in darkness, increasing in diameter by ≈ 16 mm diam. per day. Colony entirely white on surface and reverse, feathery, with slightly fringed margin. At day 5 of incubation, colony centre

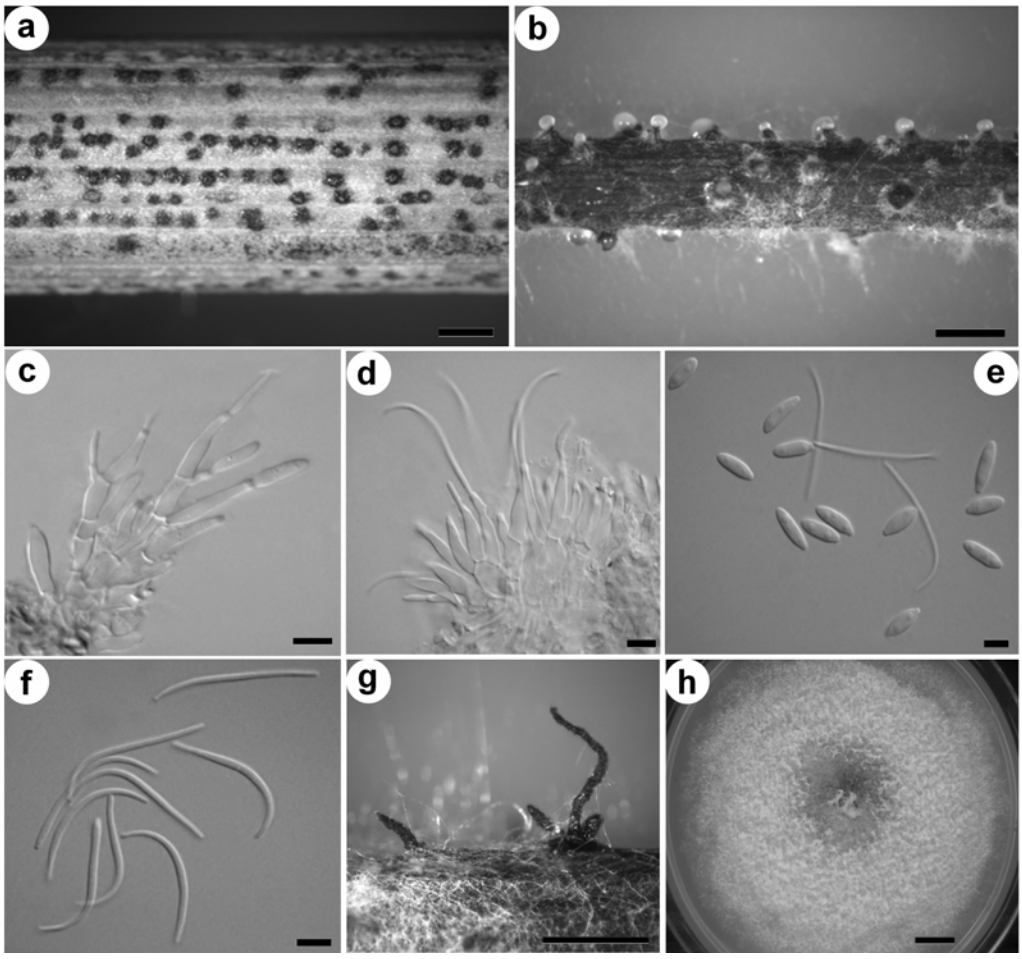

Fig. 3. *Diaporthe angelicae*. Pycnidia on *F. vulgare* stem *in vivo* (**a**, Ph-H105) and in culture (**b**, Ph-C159/1). **c** and **d**, conidiophores (Ph-C005/2). **e**, α conidia (Ph-C098/2). **f**, β conidia (Ph-C003/2). **g**, perithecia on *F. vulgare* stem in culture (Ph-C005/2 × Ph-C006/1). **h**, 5 days old culture (Ph-C159/1). Bars: a, b, g = 1 mm; c, d, e, f = 5 µm; h = 1 cm. Ph-H: specimen collection housed at Centro de Recursos Microbiológicos, Caparica, Portugal.

becoming brown to black. Greenish brown colour as a reaction to light exposure.

Habitat: on decaying stems of *Angelica sylvestris*, *Angelica* sp., *A. atropurpurea*, *Anthriscus* sp., *Daucus carota*, *Eryngium campestre*, *Heracleum sphondylium* (Castlebury *et al*., 2003), *Anthriscus sylvestris* (Eriksson, 1992), *Carum carvi* (Stoykow and Denchev, 2006), *Foeniculum vulgare*, *Oenanthe crocata* (Kirk and Spooner, 1984), *Pastinaca sativa* (Munk, 1957), and *Peucedanum oreoselinum* (Wehmeyer, 1933).

Known distribution: Austria, Germany, Switzerland, United States (Wisconsin) (Castlebury *et al*., 2003), Belgium, France, Portugal (Wehmeyer, 1933), British Columbia (Barr, 1978), Bulgaria (Stoykow and Denchev, 2006), Czech Republic (Sukova, 2001), Denmark (Munk, 1957), Holland (Steketee, 2002), Ireland (Muskett and Malone, 1983), Sweden (Eriksson, 1992), England (Kirk and Spooner, 1984; Dennis, 1995) and Scotland (Hebrides) (Dennis, 1986).

Material examined: See Table 1 for isolates studied.

Notes: When Castlebury *et al*. (2003) transferred this species to *Diaporthe* they did not find alpha conidia. Since we found such conidia, we have provided an emended description of this species.

The teleomorph was not found in the field. Crossings between some isolates resulted in infertile perithecia and this species is considered to be non self-fertile.

Diaporthe lusitanicae A.J.L. Phillips & J.M. Santos, **sp. nov.** (Fig. 4)

MycoBank: 512256.

Etymology: Named after the country where it was first found, namely Portugal.

Entostromatibus effusis, circumcinctis cum zona nigricanti. Peritheciis immersis, globosis, 220-410 µm diam., aggregatis, collo apicali erumpenti usque 430 µm

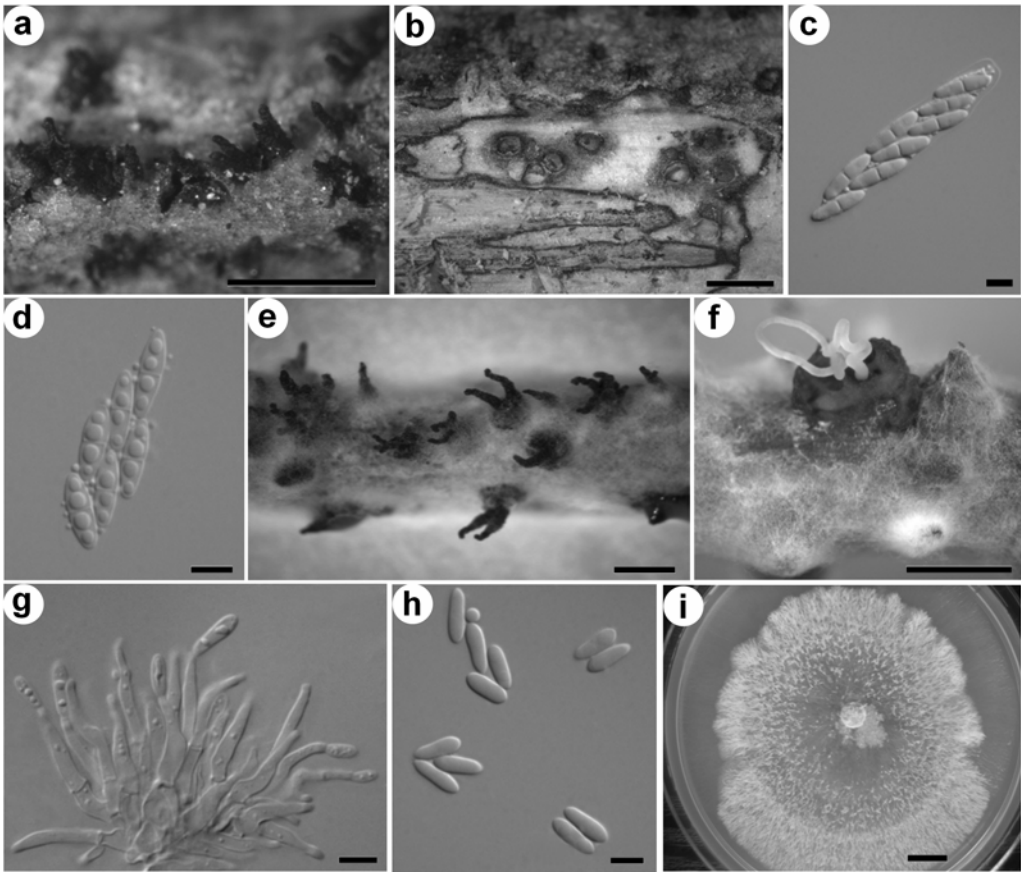

Fig. 4. *Diaporthe lusitanicae*. Perithecia at the base of *F. vulgare* stem (CBS-H 20130): **a**, perithecial necks emerging from host surface; **b**, transversal cut of perithecia and stroma. **c**, ascus with ascospores (CBS-H 20130). **d**, ascospores (CBS-H 20130). **e**, pycnidia on *F. vulgare* stem in culture (**Di-C001/3**). **f**, α conidia cirri (**Di-C001/10**). **g**, conidiophores (**Di-C001/5**). **h**, α conidia (**Di-C001/4**). **i**, 3 days old culture (**Di-C001/3**). Bars: a, b, e, f = 1 mm; c, d, g, h = 5 µm; i = 1 cm. Ex-type cultures are in bold.

longo praeditis. Ascis unitunicatis, 42–52.5 × 6.5–8 µm, cylindricis, hyalinis, octosporis, cum annulis apicalibus distingentibu, refractis, inamyloideis. Ascosporis 9–12 × 2.5–3 µm, biseriatis, hyalinis, bicellularibus, ellipsoidies, apice utrinque rotundatis, ad septum constrictis, 4-guttulatis.

Anamorphosis in agar cum *Foeniculum vulgare* stipes: *conidiomata* eustromatica, ad pycnidia, globosis, 410–870 µm diam. Conidiophoris cylindricis, 4–9 × 2–2.5 µm. Cellulae conidiogenae hyalinae, phialidicae, enteroblasticae, 11–15.0 × 1.5–3 µm. Alpha conidiis hyalinis, ovatis vel fusoideis, unicellularibus, 7–9 × 2–3 µm. Beta conidiis non vidis.

Perithecia globose, 220–410 µm diam., deeply embedded in the substrate, aggregated in more or less cohesive clusters with variable number of perithecia. Black ectostroma, surrounding the entire entostroma and visible as a dark line; dorsal, ventral and lateral surfaces evident. Dorsal ectostroma effuse, not dipping at all, or only very slightly into the bark tissues between the perithecial clusters, punctuated by groups of protruding ostioles, 310–430 µm above the host surface. Asci unitunicate, (42.0–)44.5–46.5(–52.5) × (6.5–)7.5–8.0(–8.0) µm, mean ± S.D. = 45.5 ± 2.5 × 7.6 ± 0.4 µm (*n* = 20), cylindrical, widest at centre and rounded towards the apexes. Conspicuous refractive apical ring present. Ascospores hyaline, more or less biseriate, rarely uniseriate, (9.1–)10.0–10.4(–12.1) × (2.4–)2.7–2.8(–3.2) µm, mean ± S.D. = 10.2 ± 0.7 × 2.8 ± 0.2 µm (*n* = 50), smooth, ellipsoid, with rounded ends, bicellular, constricted at the septum, basal cell slightly longer and apical cell slightly wider. Four-guttules, two in each cell, central ones widest.

Anamorph: *Phomopsis* sp.

Conidiomata on *Foeniculum vulgare* stems in culture pycnidial, globose, 410–870 µm diam., scattered on the substrate surface. Pycnidial necks hairy, 310–750 µm long, coated with short hyphae. One to seven necks forming from a single pycnidium. Conidiophores unicellular, cylindrical, hyaline, smooth, 4.0–9.2 × 2.0–2.3 µm, bearing a phialidic conidiogenous cell, cylindrical,

filiform, straight or slightly curved, tapering towards the apex, 11.1–14.8 × 1.6–2.7 µm. Collarette not seen. Conidia released as filiform cirri. Alpha conidia unicellular, oval to fusoid, rounded at both ends, (6.8–)7.6–7.8(–9.0) × (2.0–)2.4–2.5(–2.9) µm, mean ± S.D. = 7.7 ± 0.4 × 2.4 ± 0.2 µm (*n* = 100), hyaline, biguttulate, sometimes bearing a basal conidiogenous scar. Beta conidia not seen.

Cultures on PDA at 25°C, in darkness, with a linear growth of ≈ 24 mm diam. per day. Colony entirely white on surface and reverse, cottony, with slightly lobate margin. At day 3 of incubation, colony centre becoming pale brown, the reverse dark cream.

Habitat: on senescent stems of *Foeniculum vulgare*.

Known distribution: Portugal.

Material examined: PORTUGAL, Estação Agronómica Nacional (Oeiras), 14 August 2007, J.M. Santos, on *F. vulgare*, (CBS-H 20130, **HOLOTYPE**; cultures ex-type Ph-C086/2, Di-C001/1, Di-C001/3 = CBS 123213, Di-C001/4, Di-C001/5 = CBS 123212 and Di-C001/10).

Notes: This species is similar to *Diaporthe prunicola* (Peck) Wehm. but differs in having narrower perithecia, shorter asci and ellipsoid ascospores that are shorter, wider and considerably constricted at the septum. Another similar species is *Diaporthe caryigena* Ellis & Everh., which has wider perithecia, longer and wider clavate asci and larger ascospores with only one guttule in each cell.

Crossings between some single ascospore isolates of *D. lusitanicae* resulted in fertile perithecia. Therefore, this species is considered to be non self-fertile.

Diaporthe neotheicola A.J.L. Phillips & J.M. Santos, **sp. nov.** (Fig. 5)

MycoBank: MB 512257

Etymology: Named after its anamorph, *Phomopsis theicola* Curzi, with the suffix neo to distinguish it from *Diaporthe theicola* Curzi, once thought to be the teleomorph of *P. theicola*.

Entostromatibus artus, circumcinctis cum zona nigricanti. Peritheciis immersis, globosis, 370–450 µm diam., separatus, collo apicali brevis. Ascis unitunicatis, 50–64.5 × 7–9 µm, clavulatis, hyalinis, octosporis, cum annulis apicalibus distingentibu, refractis, inamyloideis. Ascosporis 9–11.5 × 3–4.5 µm, ± biseriatis, hyalinis, bicellularibus, ellipsoideis vel ovatis, interdum ad septum constrictis, 0-2-4-guttulatis.

Perithecia slightly embedded in the plant tissues, globose, 370–450 µm in its widest diam., enclosed within small, restricted entostroma completely surrounded by a black ectostromatic line, normally containing only one or two perithecia. Perithecial necks not observed in the field specimen. Perithecia of identical dimensions on *Foeniculum vulgare* stems in culture, with black, prominent and very long necks, 980–3400 × 120–170 µm, hairy, filiform, curved over itself and frequently branched. Asci with remains of epiplasm inside, unitunicate, (50.0–)54.0–57.5(–64.5) × (7.0–)7.5–8.0(–9.0) µm, mean ± S.D. = 56.0 ± 4.0 × 7.9 ± 0.6 µm (*n* = 20), clavate, with visible refractive apical ring, (7-)8-spored, sometimes showing an aborted ascospore when 7-spored. Ascospores barely biseriate, hyaline, smooth, (8.9–)10.3–10.6(–11.6) × (2.9–)3.4–3.6(–4.3) µm, mean ± S.D. = 10.4 ± 0.6 × 3.5 ± 0.3 µm (*n* = 50), ellipsoid to oval, with thick cell wall, medianly septate, widest just above the septum, sometimes constricted at the septum, eguttulate, bi or four-guttulate. When four-guttulate, the two central guttules, closer to the septum, are the largest.

Anamorph: *Phomopsis theicola* Curzi, Atti dell'Istituto Botanico della Università e Laboratorio Crittogamico di Pavia 3(3): 64 (1927).

Conidiomata on *Foeniculum vulgare* stems in culture pycnidial, of variable morphology and dimensions, globose to subglobose, cone-shaped, 420–730 µm diam. × 280–1130 µm high, more or less protruding. Pycnidia without evident neck, naked or covered with dense layer of hyphae, embedded in host just beneath the epidermis, sometimes inside stromatic areas, surrounded by a dorsal black ectostromatic line. Alpha and beta conidiophores identical and intermingled with long, septate and highly branched paraphyses. Conidiophores cylindrical, hyaline, smooth, single- to multi-septate, 6.7–23.5 × 1.6–3.1 µm. Alpha and beta conidiogenous cells phialidic, cylindrical to filiform, tapering towards the apex, with periclinal thickening present. Collarette not seen. Conidiogenous cells of two types: terminal, attached to the apex of conidiophores, 10.6–19.1 × 1.4–2.4 µm, and lateral, belonging to the main axis of the conidiophore but with a lateral prolongation, 2.6–9.8 × 1.0–1.6 µm, giving a branched aspect to the conidiophore. Conidia

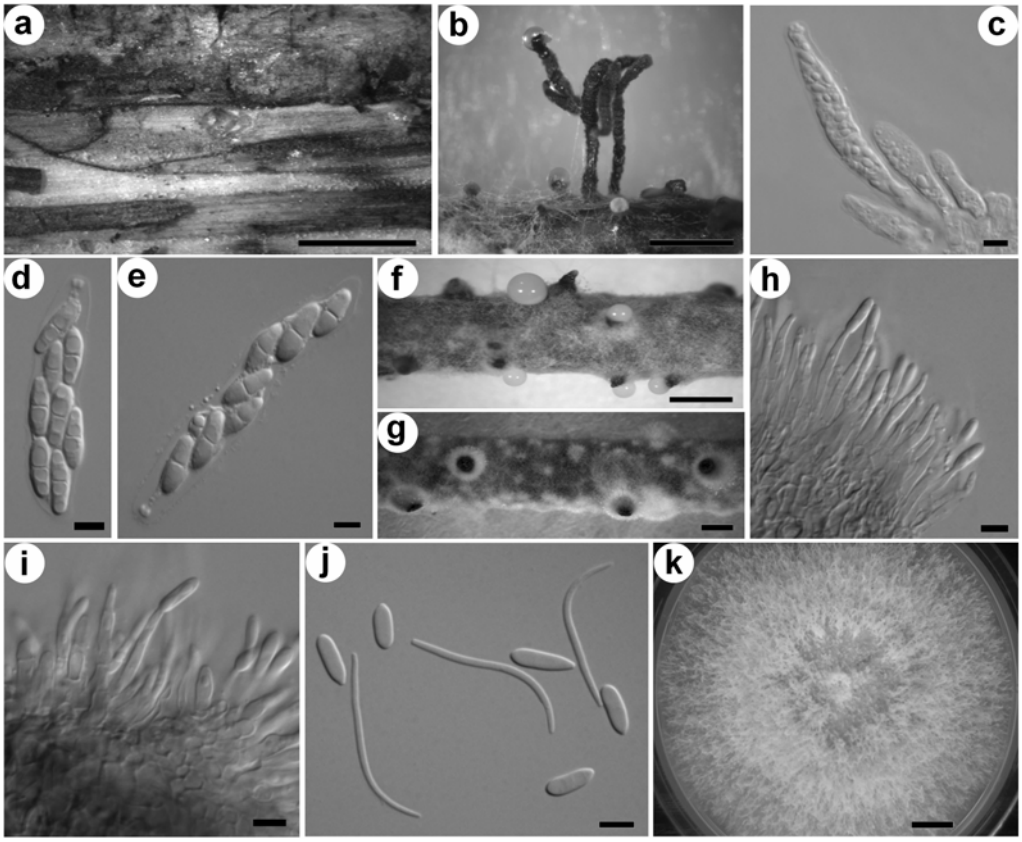

Fig. 5. *Diaporthe neotheicola*. **a**, perithecium and stroma at the base of *F. vulgare* stem *in vivo* (CBS-H 20131). **b**, perithecia on *F. vulgare* stem in culture (**Di-C004/1** × **Di-C004/4**). **c**, ascogenesis (**Di-C004/4** × **Di-C004/5**). **d**, ascus with 8 ascospores (**Di-C004/1** × **Di-C004/4**). **e**, ascus with 7 ascospores (**Di-C004/4** × **Di-C004/5**). **f** (**Di-C004/5**) and **g** (**Di-C004/9**), pycnidia on *F. vulgare* stems in culture. **h** and **i**, conidiophores (**Di-C004/5**). **j**, α and β conidia (**Di-C004/5**). **k**, 5 days old culture (**Di-C004/4**). Bars: a, b, f, g = 1 mm; c, d, e, h, i, j = 5 µm; k = 1 cm. Ex-type cultures are in bold.

exuded in a translucent yellow drop. Alpha conidia unicellular, fusoid, with obtuse ends and basal conidiogenous scar, (6.6–)7.6–8.0(–9.5) × (1.9–)2.2–2.3(–2.6) µm, mean ± S.D. = 7.8 ± 0.6 × 2.2 ± 0.2 µm (n = 50), hyaline, mostly biguttulate in water, although this is difficult to see in lactic acid mounts. Beta conidia hyaline, aseptate, filiform, curved, eguttulate, with rounded ends, (21.5–)25.7–26.7(–30.3) × (0.8–)1.0(–1.1) µm, mean ± S.D. = 26.2 ± 1.9 × 1.0 ± 0.1 µm (n = 50). Both types of conidia are produced in the same pycnidium although some pycnidia produce mostly one type.

Cultures on PDA at 25°C, in darkness, increasing in diameter by ≈ 16 mm per day. Colony entirely white on surface and reverse, cottony, with slightly fringed margin. At day 5 of incubation, colony reverse centre with pale olivaceous green pigmentation.

Habitat: reported on *Aspalathus linearis* (van Rensburg *et al*., 2006), *Camellia sinensis* (Uecker, 1988), *Foeniculum vulgare* and *Protea*, *Prunus*, *Pyrus* and *Vitis vinifera* (Mostert *et al*., 2001; van Niekerk *et al*., 2005).

Known distribution: Australia, Portugal, South Africa (Mostert *et al*., 2001) and Italy (Uecker, 1988).

Material examined: PORTUGAL, Évora, November 2007, A.J.L. Phillips, on *F. vulgare* (CBS-H 20131, **HOLOTYPE**; cultures ex-type Di-C004/1, Di-C004/4 = CBS 123209, Di-C004/5 = CBS 123208 and Di-C004/9).

Notes: Mostert *et al*. (2001) described the anamorph of this species under the name of *Phomopsis* sp. 1. However, since we report some additional characters, an emended description is given above.

This species is similar to *Diaporthe eumorpha* (Durieu & Mont.) Maire but has wider perithecia and longer asci and ascospores. Another similar species is *Diaporthe perjuncta* Niessl, which has no dorsal ectostromatic layer, its ascospores are fusoid-ellipsoid, sometimes inequilateral and are longer.

Crossings between some single ascospore isolates of *D. neotheicola* resulted in fertile perithecia. Therefore, this species is considered to be non self-fertile.

The unusual length of perithecial necks of *D. neotheicola* is most probably due to high levels of humidity during incubation.

Discussion

The *Diaporthe* and *Phomopsis* species present on *Foeniculum vulgare* were identified by association of the isolate's ITS sequences with others available in GenBank except for *Diaporthe lusitanicae*, which was shown to be phylogenetically distinct from the isolates with the most similar available sequences. *Diaporthe angelicae* was identified by association to the ex-epitype strain of this species, CBS 111592 (AY196779). *Diaporthe ambigua* was also identified by association to ITS sequences of this species, namely from the CBS 117374 isolate (DQ286268), identified by van Rensburg *et al*. (2006) because of its close phylogenetic relationship to the ex-epitype culture CBS 114015 (AF230767). This last sequence was not included in the present phylogeny because of its poor coverage (86%) of the analysed region, in spite of having 99% identity with the newly generated sequences. The teleomorphic isolates from Évora were phylogenetically the same species as *Phomopsis theicola* STE-U 5567 isolate (AY485723), under the name of *Phomopsis* sp. 1 (van Niekerk *et al*., 2005), and CBS 117166 (DQ286286), which was identified by van Rensburg *et al*. (2006) to species level because of its phylogenetic relationship to the ex-type strain CBS 187.27 (Fig. 2). Thus, it was determined that the *Diaporthe* sp. from Évora is the teleomorph of *P. theicola*. The ITS sequence of CBS 187.27 was not included in our phylogenetic analyses because it was not available in GenBank. *Phomopsis* sp. 1 was later shown be to *P. theicola* (van Rensburg *et al*., 2006).

Curzi (1927) described *Diaporthe theicola* as the teleomorph of *P. theicola*. However, he based this on the close proximity of the teleomorph with the anamorph on the host tissue and did not make any connections through culture of ascospores. Furthermore, Curzi (1927) stated that the morphologies do not completely match and that the relationship of *P. theicola* to *D. theicola* should be reappraised, something that has not been done until now. A search was made for specimens and cultures of *D. theicola*. However, no specimens could be found in PAV and it is thus considered that the type specimen is no longer extant. Furthermore, no cultures could be traced. However, the morphology of *D. theicola* as described by Curzi (1927) in the protologue differs sufficiently from that of the teleomorph found in the present work for these two species to be considered as distinct. The perithecia in *D. theicola* are narrower than in *D. neotheicola*, as are the asci. Ascospores are fusoid, typically not constricted at the septum and longer and narrower than in *D. neotheicola*. For this reason we conclude that *D. theicola* is not the teleomorph of *P. theicola*. *Diaporthe neotheicola* as described in this paper was unambiguously connected (culture of ascospores, production of teleomorph in culture and ITS phylogeny) to the ex-type culture of *P. theicola*.

To confirm that *D. lusitanicae* represents an undescribed species in *Diaporthe*, its morphology was compared with all the other *Phomopsis* and *Diaporthe* species described until now on Apiaceae hosts as listed by Farr *et al*. (2008). None of these species correspond to *D. lusitanicae*. Moreover, it is phylogenetically distinct from any other species with available ITS sequences in GenBank. Therefore, we decided to describe this as a new species. *Diaporthe neotheicola* was also compared morphologically with the same Apiaceae-associated species and other species listed by Wehmeyer (1933) to exclude the possibility it has been described previously under a different name.

Comparing Figs 1 and 2 it is possible to see that group 7 of the dendrogram consists of *D. ambigua* isolates from Azeitão, group 8 includes *D. neotheicola* isolates from Évora and group 9 consists of *D. lusitanicae* strains from Oeiras and Aveiro. Isolates in groups 1 to 6 belong to *D. angelicae*, totalling 86 strains from nine of the 10 localities sampled. Judging from these results it seems reasonable to speculate that *D. angelicae* might be the

main pathogen on *F. vulgare*, at least in Portugal. However, pathogenicity studies have to be done in order to clarify this. In fact, this species has been reported on several Apiaceae hosts throughout North America and Europe, including Portugal (Wehmeyer, 1933), namely *Angelica* and *Anthriscus* species (Eriksson, 1992; Castlebury *et al*., 2003) and *Carum carvi* (Stoykow and Denchev, 2006), *Daucus carota*, *Eryngium campestre*, *Heracleum sphondylium* (Castlebury *et al*., 2003), *Oenanthe crocata* (Kirk and Spooner, 1984), *Pastinaca sativa* (Munk, 1957) and *Peucedanum oreoselinum* (Wehmeyer, 1933). Besides the ubiquity of *D. angelicae* on *F. vulgare*, the teleomorphic state of this species was not found in the field. However, isolates from a single host plant produced perithecia when crossed *in vitro*, although these were infertile. The epitype specimen of *D. angelicae* is teleomorphic and was collected in Austria. Therefore, it would seem that the inability to form the teleomorph is not a common trait in this species. The reason why the Portuguese isolates did not mate successfully remains to be determined. It is possible that this inability is the reason why the teleomorph was never found in the field during this work.

In contrast to *D. angelicae*, *D. lusitanicae* seems to be a minor pathogen of wild fennel, being present in only two of the 10 localities sampled.

Diaporthe ambigua and *D. neotheicola* showed geographic specificity. An example is the presence of the well known apple pathogen, *D. ambigua*, in two different plants in Azeitão that were separated by approximately 500 m. This observation linked to the fact that these species were found at the base of the stems, next to the soil, raises the possibility of a saprophytic behaviour of these species on wild fennel. Not being able to colonise this host when it is alive, they could take advantage of senescence to grow over dead stems. A purely saprophytic behaviour of these species on *F. vulgare* may not present any risk to this host but it can act as reservoir for the fungus to grow and sporulate until it finds its primary host. The same may be true for *D. lusitanicae*, but at present we do not know if this species is a pathogen or is purely saprobic.

Diaporthe ambigua was initially described on *Pyrus communis* in Germany (Nitschke, 1867) and later on apple, pear and plum trees in South Africa (Smit *et al*., 1996). More recently van Rensburg *et al*. (2006) described this species on *Aspalathus linearis*. Besides the wide host range of this species, none of them are related to *F. vulgare*, supporting the idea that it may have only a saprophytic behaviour on this host.

Phomopsis theicola was first described on *Camellia sinensis* (tea plant), in Italy (Uecker, 1988). It was later isolated from *Vitis vinifera*, *Protea* and *Pyrus* (Mostert *et al*., 2001; van Niekerk *et al*., 2005), and more recently from *Aspalathus linearis* (van Rensburg *et al*., 2006). This wide host range has now been further broadened with the first description of its teleomorph on an Apiaceae host.

Besides the species presently described, other *Phomopsis* and *Diaporthe* species have previously been described from *F. vulgare*. They are *Diaporthe foeniculacea* Niessl (von Thümen, 1880), *Phomopsis foeniculina* (Sacc.) Câmara (Câmara, 1947), *Phomopsis foeniculi* Du Manoir & Vegh (Du Manoir and Vegh, 1981) and *Phomopsis brunaudiana* (Sacc.) B. Sutton (Sutton, 1996). Phillips (2003) established the teleomorph-anamorph connection between *D. foeniculacea* and *P. foeniculina*. A surprising result was the absence of *D. foeniculacea* among the species found in this host. However, although Phillips (2003) described this species and its anamorph *P. foeniculina* from fennel plants in Portugal, he based this on morphological data alone. Given the difficulties in discriminating species of *Diaporthe* and *Phomopsis* morphologically, these identifications cannot be regarded as fully reliable.

The considerable overlap of morphological features between the species presently described makes it possible to conclude that the phylogenetic species concept is the one that best fits these fungi. The need to link anamorphs with their teleomorphs using phylogeny has been emphasised by Shenoy *et al*. (2007). The present resolution of

Diaporthe and *Phomopsis* species on *Foeniculum vulgare*, with its biodiversity and ecological implications, restate the need for further studies on the taxonomy of these phytopathogenic fungi, reconsidering their classification and building a bridge between the classical and molecular approaches on species identification.

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