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RESOLVING CRYPTIC SPECIES COMPLEXES IN *DIPLODIA*

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Aos meus pais.

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Resumo

Enquanto que a taxonomia e patologia dos géneros *Botryosphaeria* e *Neofusicoccum* têm sido estudados intensivamente, o género *Diplodia* apresenta inúmeros problemas por resolucionar a nível taxonómico. Ao género *Diplodia* pertencem espécies patogénicas como *D. pinea* (*blight* em pinheiros), *D. corticola* (cancro e dieback do carvalho), *D. mutila* e *D. seriata,* podendo causar doenças em diversos hospedeiros. Este facto sugere que estas duas espécies possam ser afinal complexos de espécies.

O objectivo deste trabalho foi tentar resolver estes complexos de espécies crípticas, isto é, espécies que são morfologicamente semelhantes e podem apenas ser distinguidas a nível filogenético.

Os isolados aqui estudados foram caracterizados com base na sua micromorfologia, incluindo a dimensão, forma, cor e septação dos conídios, morfologia das colónias e taxa de crescimento micelial. As relações filogenéticas entre os isolados foram determinadas, primeiramente, através da utilização de microssatélites e posteriormente pela análise das sequências de nucleótidos da região ITS do rDNA e dos genes EF1-α (factor de elongação e translação 1-α) e β-tubulina. Através da integração das características morfológicas com os dados filogenéticos, foi possível resolver diversas espécies pertencentes ao complexo de *Diplodia mutila*, duas novas espécies foram descritas (*D. bulgarica* and *D. intermedia*), a posição filogenética das espécies *D. fraxini* e *D. malorum* foi clarificada e verificou-se que o grupo de espécies de *D. pinea* poderá ser igualmente um complexo de espécies.

Palavras-chave: *Botryosphaeria, Diplodia,* complexos de espécies, morfologia, filogenia, taxonomia.

Abstract

While the taxonomy and pathology of *Botryosphaeria* and *Neofusicoccum* has been studied intensively, *Diplodia* has several taxonomic problems that need to be resolved. This genus contains well-known plant pathogens including *D. pinea* (blight on pine trees), *D. corticola* (canker and dieback of cork oak) and *D. mutila* and *D. seriata,* which occur on a wide range of hosts. This suggests that these two species may in fact represent species complexes.

The aim of this work was to resolve these cryptic species complexes - species that are morphologically indistinguishable and can be only separated at the phylogenetic level.

Isolates were characterized on the basis of their micromorphology, including conidial dimensions, shape, pigmentation and septation, colony morphology and mycelial growth rate. Phylogenetic relationships were determined, firstly by microsatellite fingerprinting and then through analysis of ITS nucleotide sequences, supplemented with partial sequences of the translation elongation factor 1-α and β-tubulin genes. By integrating morphological characters with phylogenetic data several species were resolved within the *Diplodia mutila* complex, two new species (*D. bulgarica* and *D. intermedia*) were described, the status of *D. fraxini* and *D. malorum* was clarified and it was revealed that the *D. pinea* group may also be a complex of species.

Keywords: *Botryosphaeria, Diplodia,* species complexes, morphology, phylogeny, taxonomy.

Resumo alargado

Um estudo recente de revisão filogenética do fungo *Botryosphaeria* revelou que este género é composto por inúmeras linhagens que correspodem cada uma a géneros individuais. Assim, o género *Botryosphaeria* compreende, actualmente, apenas duas espécies - *B. dothidea* e *B. corticis* – enquanto que os géneros restantes ficam conhecidos pelos nomes genéricos das respectivas formas anamórficas, incluíndo os géneros *Neofusicoccum, Lasiodiplodia, Dothiorella* and *Diplodia*.

Durante os últimos anos, a taxonomia e patologia dos géneros *Botryosphaeria* e *Neofusicoccum* têm sido estudados intensivamente, ao contrário do género *Diplodia* que apresenta inúmeros problemas por resolucionar a nível taxonómico. Ao género *Diplodia* pertencem diversas espécies tais como *D. pinea* (*blight* em pinheiros), *D. corticola* (cancro e dieback do carvalho) e *D. mutila* e *D. seriata* que podem causar doenças em diversos hospedeiros. O extenso leque de hospedeiros que *D. mutila* e *D. seriata* apresentam, sugere que estas duas espécies possam ser afinal complexos de espécies.

O objectivo deste trabalho foi tentar resolver estes complexos de espécies crípticas, isto é, espécies que são morfologicamente semelhantes e podem apenas ser distinguidas a nível filogenético.

Os isolados aqui estudados foram caracterizados com base na sua micromorfologia, incluindo a dimensão, forma, cor e septação dos conídios, bem como a morfologia das colónias e a taxa de crescimento micelial. As diversas espécies apresentaram pequenas diferenças a nível morfológico, permitindo a sua identificação e distinção. As relações filogenéticas dos isolados foram determinadas, primeiramente, através da utilização de microssatélites e posteriormente pela análise das sequências de nucleótidos da região ITS do rDNA e também de sequências parciais dos genes EF1-α (factor de elongação e translação 1-α) e β-tubulina. Foram desenhadas árvores filogenéticas com base nos genes referidos e nestas foram distinguidos três grupos filogenéticos de espécies de conídios hialinos, sendo o primeiro constituído por *D. mutila*, *D. olivarum*, *D. africana*, *D. rosulata*, *D. malorum* e *D. fraxini*, o segundo por *D. cupressi*, *"B". tsugae* e *D. bulgarica* e o último representado unicamente pela espécie *D. corticola*; e um grupo de espécies de conídios castanhos, nomedamente, *D. pinea*, *D. scrobiculata*, *D. seriata* e *D. intermedia.*

Através da integração das características morfológicas com os dados filogenéticos, foi possível resolver diversas espécies pertencentes ao complexo de *Diplodia mutila*. Com base nas análises morfológica e filogenética duas espécies foram descritas como novas, nomeadamente, *D. bulgarica* sp. nov. e *D. intermedia* sp. nov., enquanto que outras duas espécies (*D. malorum* e *D. fraxini*) cujos nomes já existiam apesar de raramente serem

utilizados, foram clarificadas e reconhecidas como distintas e válidas no presente trabalho. Apesar da grande semelhança morfológica aparente entre as espécies relacionadas com *Diplodia mutila,* as espécies *D. bulgarica, D. malorum e D. fraxini* diferenciaram-se com base nas dimensões e rapidez de coloração dos seus conídios. Assim, enquanto que os conídios de *D. malorum* são os mais longos, os de *D. faxini* são os mais largos do grupo. *D. bulgarica* é distinguida facilmente pelo rápido desenvolvimento de pigmentação dos seus conídios após libertação do picnídio. Relativamente ao grupo de espécies com conídios castanhos, *D. seriata* é aquela que apresenta os conídios de menores dimensões enquanto que em *D. pinea* residem os conídios mais compridos e mais largos. *D. intermedia* caracteriza-se morfologicamante por uma posição intermédia entre as espécies *D. pinea* e *D. scrobiculata*, mas é facilmente distinguida pela presença de microconídios. Esta espécie apresenta conídios de dimensões médias inferiores aos das espécies *D. pinea* e *D. scrobiculata*, mas superiores aos de *D. seriata*.

De um modo geral, a existência de especialização de algumas espécies por determinados hospedeiros foi aparente, especialmente nas espécies pertencentes ao complexo *D. mutila*, como por exemplo nos casos *D. olivarum* (*Olea* sp.), *D. fraxini* (maioritariamente *Fraxinus* sp.), *D. africana* (*Prunus persica*), *D. rosulata* (*Prunus africana*), *D. malorum* (*Malus* sp.), *D. bulgarica* (*Malus* sp.), *D. cupressi* (*Cupressus* sp. and *Juniperus* sp.) and *"B." tsugae* (*Tsuga* sp.). Esta especialização de algumas espécies relativamente a determinados hospedeiros poderá ser de grande relevância no controlo das doenças provocadas por estas espécies, especialmente na redução da utilização de fungicidas e outros produtos químicos tóxicos aquando dos tratamentos de determinadas culturas que poderão não estar realmente infectadas.

Neste trabalho, foi também possível verificar que o grupo *D. pinea* poderá ser também um complexo de espécies, havendo necessidade de, futuramente, se realizarem estudos mais aprofundados relativamente a estas espécies.

Palavras-chave: *Botryosphaeria, Diplodia,* complexos de espécies, morfologia, filogenia, taxonomia.

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Abbreviations and Acronyms

bp	base pair
CAA	A. Alves, Universidade de Aveiro, Portugal
CAP	A. J. L. Phillips, Universidade Nova de Lisboa, Portugal
CBS	Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
cm	centimetre
CMW	M.J. Wingfield, FABI, University of Pretonia, South Africa
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EF	translation elongation factor
g	gram(s)
g	gravity (centrifugal)
GenBank	Sequence database from National Centre for Biotechnology Information (NCBI)
h	hour(s)
ITS	Internal Transcribed Spacer
l	litre
JL	J. Luque, IRTA, Spain
LSU	Large subunit
M	molar (mol l^{-1})
max.	maximum
mg	milligram
$MgCl_2$	Magnesium chloride
min	minute
ml	millilitre (10^{-3} dm^3)
mM	millimolar
MP	Maximum parsimony (phylogeny)
MSP-PCR	Microsatellite-Primed Polymerase Chain Reaction
ng	nanogram
NJ	Neighbour joining (phylogeny)
PCR	polymerase chain reaction
pH	hydrogen ion concentration (negative log of)
rDNA	Deoxyribonucleic acid that codifies for rRNA

RNA	ribonucleic acid e.g. mRNA = messenger RNA; rRNA = ribosomal RNA; tRNA = transfer RNA
RNase	ribonuclease
rpm	revolutions per minute
s	second (time)
S.D.	standard derivation of samples (statistical)
SDS	sodium dodecylsulphate
SSR	Simple Sequence Repeat
STE-U	Stellenbosch University, Department of Plant Pathology, South Africa
STR	Herbarium of the Institut de Botanique, Strasbourg, France
TAE	Tris buffer, acetic acid and EDTA
***Taq* polymerase**	thermostable DNA polymerase (*Thermus aquaticus*)
TBE	Tris buffer, boric acid and EDTA
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	units
UV	ultraviolet
v	volume (physical measure)
V	Volts
v:v	volume per volume (concentration)
w/v	weight/volume
µl	microlitre
µm	micrometre
µM	micromolar

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Chapter 1
Introduction

1.1. The family *Botryosphaeriaceae*

Species in the *Botryosphaeriaceae* are cosmopolitan and occur on a wide range of hosts including monocotyledons, dicotyledons and gymnosperms, on woody branches, herbaceous leaves, stems and haulms of grasses, on twigs and in the thalli of lichens (Barr, 1987). Species are saprobic or parasitic (Smith *et al.*, 1996, Denman *et al.*, 2000), and can cause die-back and canker diseases of numerous woody hosts (von Arx, 1987).

The genus *Botryosphaeria* Ces & De Not. was introduced in 1863 by Cesati and De Notaris who included 12 species, but did not provide a detailed morphological description of them. Saccardo (1877) emended the initial generic description of Cesati and De Notaris (1863) to exclude hypocreaceous species, which he transferred to *Gibberella* and *Lisea*. Since Cesati and De Notaris (1863) did not designate a type species for the genus, von Höhnel (1909) suggested *B. berengeriana* De Not. as lectotype, while Theissen and Sydow (1915) suggested *B. quercuum* (Schwein.) Sacc. The latter species was accepted by von Arx and Muller (1954, 1975) and von Arx (1981). However, neither of these two species was suitable as the type since they were not included in the original description of the genus. Therefore, Barr (1972) proposed *B. dothidea* (Moug. : Fr.) Ces & De Not. because it was one of the original species described and it conforms with Saccardo's (1877) emendation. Barr's (1972) proposal has been generally accepted (Slippers *et al.,* 2004a). To stabilize the name, Slippers *et al.* (2004a) proposed a neotype and an epitype for *B. dothidea* and made ex-epitype cultures available.

Since it was introduced in 1863, the position of *Botryosphaeria* within the higher classification of Ascomycetes has been the subject of many taxonomic rearrangements, as explained by Denman *et al.* (2000) and Crous *et al.* (2006). Currently, it is widely accepted that *Botryosphaeria* is included in the family *Botryosphaeriaceae* erected by Theissen and Sydow (1918). This family has been discussed in detail by von Arx & Müller (1954). For many years the *Botryosphaeriaceae* was regarded either as a member of the Pleosporales (Barr, 1972; Eriksson, 1981) or the Dothideales (von Arx & Müller, 1975; Hawksworth *et al.*, 1995). In a recent classification of ascomycetes, Eriksson (2006) accepted the family *Botryosphaeriaceae* in the class Dothideomycetes, but listed it as a family *incertae sedis* due to the lack of support for the ordinal placement of the family. Schoch *et al.* (2006) showed through a multigene phylogeny that the *Botryosphaeriaceae* reside in a distinct clade separate from both the Pleosporales and Dothideales. For this reason they introduced the Botryosphaeriales to accommodate the *Botryosphaeriaceae*.

The genus has been described as forming uni- to multilocular ascomata with multi-layered walls, occurring singly or in clusters, often intermixed with conidiomata, which are

pycnidial. Asci are bitunicate, with a thick endotunica, stalked or sessile, clavate, with a well-developed apical chamber, forming in a basal hymenial layer, intermixed among hyaline pseudoparaphyses that are frequently constricted at the septa, usually disintegrating when the asci are mature. Ascospores are hyaline and aseptate, but they can become brown and septate with age. Because of this feature (ascospores that become brown with age), von Arx & Müller (1954) combined *Dothidea visci* (with brown ascospores) into *Botryosphaeria* as *B. visci*. Later, von Arx & Müller (1975) also placed the dark-spored *Neodeightonia subglobosa* in *Botryosphaeria*. Since this is the type species of *Neodeightonia*, they thus reduced this genus to synonymy under *Botryosphaeria*. In making these synonymies, von Arx & Müller (1954, 1975) effectively broadened the concept of *Botryosphaeria* to include species with brown ascospores. Phillips *et al.* (2005) and Luque *et al.* (2005) followed this broad concept and described *B. iberica* and *B. viticola* (both with dark ascospores) as new species in *Botryosphaeria*.

Conidiogenesis is holoblastic, indeterminate with subsequent conidia formed enteroblastically with proliferation at the same level to form typical phialides *sensu* Sutton (1980) with periclinal thickenings, or proliferating percurrently resulting in annellations. Conidia can be hyaline or brown, one-celled or one-septate, with either a thin or a thick wall. (von Arx & Müller, 1954; Shoemaker, 1964; Eriksson, 1981; Sivanesan, 1984; Denman *et al.,* 2000; Alves *et al.,* 2004).

Crous *et al.* (2006) were of the opinion that the wide range of morphologies displayed in both the anamorphs and the teleomorphs of *Botryosphaeria* species was too diverse for it to be regarded as a single genus. Through a study of partial sequences of the D1/D2 domain of the ribosomal large subunit (LSU) gene, Crous *et al.* (2006) showed that *Botryosphaeria* is composed of 10 phylogenetic lineages corresponding to different genera. To avoid unnecessary introduction of new generic names, they opted to use existing anamorph generic names for most of the lineages, and restricted the use of *Botryosphaeria* to *B. dothidea* and *B. corticis* (Demaree & M.S. Wilcox) Arx & E. Müll. To avoid any confusion, species that were previously included in *Botryosphaeria* will be referred to in this thesis as *Botryosphaeriaceae*.

The classification and identification of species in the *Botryosphaeriaceae* and related anamorphs have traditionally been based on morphology and host association. For the description of the genus *Botryosphaeria* and until the 1950's the general morphology of the ascospores and stromatal and ascomatal morphology was considered taxonomically and phylogenetically informative (Denman *et al.,* 2000). Besides the fact that this morphological species concept can be useful in some situations, it tends to underestimate the true diversity among the species (Taylor *et al.,* 1999). Nevertheless, while morphological characters of the teleomorph clearly define the genus (von Arx & Müller, 1954; Denman *et al.*, 2000; Alves *et*

al., 2004) they are extremely conserved and provide insufficient diversity to allow inequivocable differentiation of many of the species. Moreover, the teleomorphic state is uncommon in nature and rarely forms in culture. In contrast, the anamorphs form relatively easily in culture and display a wide range of morphologies that separate the species. Thus, the taxonomy of the genus and species differentiation is largely dependent on the morphology of the asexually reproducing state (Pennycook & Samuels, 1985; Denman *et al.*, 2000; Jacobs *et al.,* 1998; Crous *et al.,* 2006).

Through time, identification of species has relied greatly on morphological features of the conidia, including size, shape, color, septation, texture and wall thickness. Certain cultural aspects such as colony morphology, chromogenicity and temperature effects on mycelia growth rate have also been used to differentiate species (Shoemaker, 1964; Laundon, 1973; Pennycook & Samuels, 1985; Denman *et al.,* 2000; Phillips, 2002; Lazzizera *et al.,* 2008; Alves *et al.,* 2008). However, some morphological characters used to circumscribe species within anamorphic genera associated with *Botryosphaeria* can be extremely flexible. Thus, size ranges of conidia of different species overlap, whereas age and state of maturity affect conidial pigmentation and septation. Morphological characters can also be influenced by the substrate on which the fungus is growing. Moreover, a single species is able to colonize a wide range of hosts, and multiple species may be found on the same host (Slippers *et al.*, 2006; de Wet *et al.*, 2008).

Since species may differ in minor morphological features and consequent overlapping may occur, this has emphasized the utility of other means of characterization and, therefore, there is a need for tools that can provide accurate and reproducible identifications of species of *Botryosphaeriaceae*.

In recent years, advances in molecular techniques, like DNA sequencing, and particularly the analysis of nucleotide sequences of ribosomal RNA genes (rDNA) have been used increasingly to distinguish taxa in the *Botryosphaeriaceae* and made a great contribution to fungal taxonomy and systematic (Seifert *et al.,* 1995; Taylor *et al.,* 2000). In this way, nucleotide sequences of the 5.8S nuclear ribosomal DNA gene and the flanking internal transcribed spacers, ITS1 and ITS2, have been widely used (e.g. Jacobs & Rehner, 1998; Denman *et al.,* 2000; Zhou & Stanosz, 2001b). Differences in DNA sequences have also been successfully combined with morphological characteristics to identify and describe *Botryosphaeriaceae* taxa (e.g. Denman *et al.,* 2003; Phillips *et al.,* 2005; present work). Jacobs and Rehner (1998) employed molecular methods to reveal phylogenetic relationships among species of *Botryosphaeria* and were the first to use ITS rDNA sequences in this analysis confirming that there was some congruence between morphological and cultural characters and ITS sequences, at least for some taxa. Later, Denman *et al.* (2000) reviewed the anamorph genera associated with *Botryosphaeria* and on the basis of ITS phylogenies

recognized that the species studied were separated into two main clades corresponding to *Fusicoccum* and *Diplodia*. These taxa were delineated as having typically hyaline, narrower conidia with thin walls (*Fusicoccum*), or conidia that are wider with thicker walls and often become pigmented with age (*Diplodia*) (Jacobs & Rehner, 1998; Denman *et al.*, 2000; Zhou & Stanosz, 2001b; Alves *et al.,* 2004). More recently, *Dothiorella* was re-instated to accommodate the anamorphs of a group of *Botryosphaeriaceae* with pigmented one-septate conidia, which develop these features at an early stage of their development and before they are discharged from the pycnidia (in contrast to *Diplodia*) (Phillips *et al.,* 2005). The problems involving *Botryosphaeriaceae* taxonomy could be the result of the relationships of some groups to the *Botryosphaeriaceae* that were not clearly based on morphology, or because cultures were not available for molecular studies (Slippers & Wingfield*,* 2007).

Polymerase chain reaction (PCR) - based genomic fingerprinting, such as the microsatellite-primed polymerase chain reaction (MSP-PCR), is a good alternative to methods that rely on specifically targeted primers (Gadanho *et al.,* 2003; Alves *et al.,* 2007). These techniques, which analize the whole genome, have been shown to be relatively robust and discriminatory (Olive & Bean, 1999; Alves *et al.,* 2007). Microsatellites are polymorphic loci present in non-coding DNA that consist of repeating units of 1–5 nucleotides (Bennett, 2000). Molecular typing by MSP-PCR allows separation between different taxa of fungi, at inter- or intra-specific levels. Microsatellites owe their variability to an increased rate of mutation compared to other neutral regions of DNA. They can be amplified for identification by the PCR with complementary primers generating, after electrophoresis in agarose gel, a band profile revealing polymorphisms in the genome of different strains or species (Thanos *et al.,* 1996; Meyer *et al.,* 1997; Gadanho *et al.,* 2003).

Ribosomal RNA genes (rDNA) have been widely used in phylogenetic studies of fungi (Knutzen *et al.,* 2004).The low rate of polymorphism in the rDNA transcription unit in addition to the fact that the different coding regions of the rDNA repeats usually show distinct evolution rates allows this DNA to provide information at almost any systematic level (Hillis *et al.,* 1991; Bruns *et al.,* 1991).

The ITS (Internal Transcribed Spacers) region is one of the most widely sequenced DNA region in fungi (Figure 1.1) and is useful for taxonomic comparisons of closely related species and genera (Berbee & Taylor, 2001). Internal transcribed spacers are sequences located in eukaryotic rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 25S rRNA coding regions (ITS2). These spacer sequences have a high evolution rate and are present in all known nuclear rRNA genes of eukaryotes (White *et al.,* 1991)

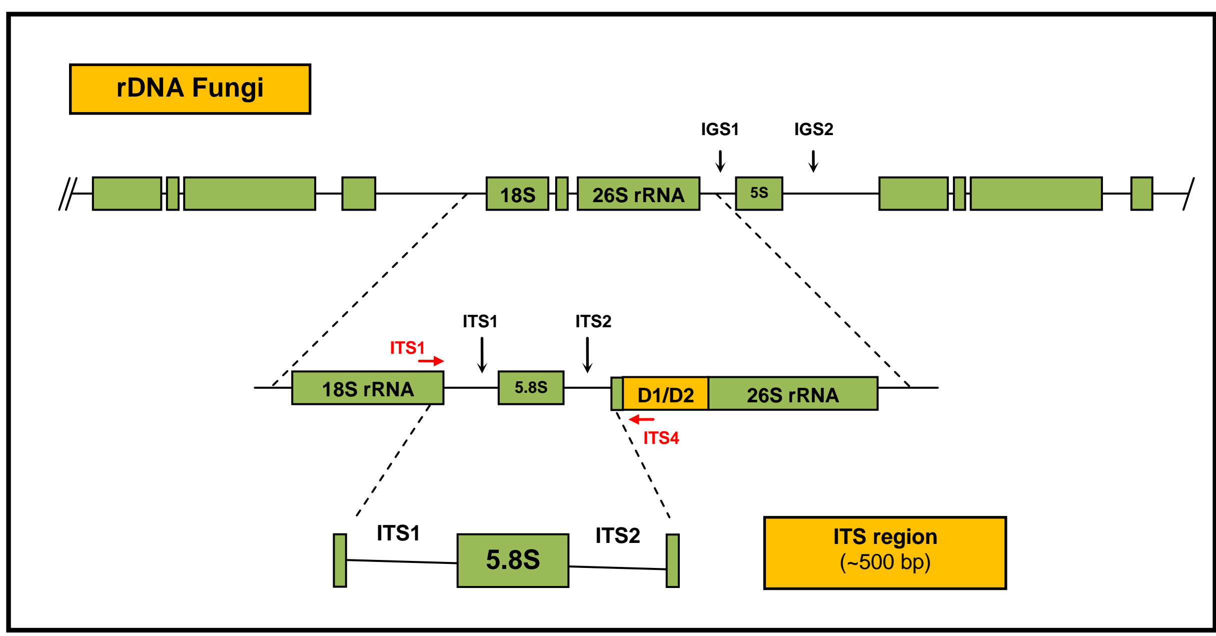

Figure 1.1. Schematic representation of the most frequent organization of rRNA genes in fungi and location of the ITS region and the primers (in red) used in its sequencing (Adapted from: *Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA - http://www.biology.duke.edu/fungi/mycolab/primers.htm*).

Most taxonomic studies on *Botryosphaeriaceae* using DNA sequence differences have used ITS rDNA phylogenies, but this single gene can underestimate the true species diversity among complexes of cryptic species (Taylor *et al.,* 2000). In this regard, multiple gene sequence phylogenies have been successfully applied to identify cryptic species in the *Botryosphaeriaceae*, previously disregarded or of uncertain identity (e.g. Smith *et al.,* 2001; Zhou & Stanosz, 2001b; Phillips *et al.,* 2002; Denman *et al.,* 2003; Alves *et al.,* 2004; Slippers *et al.,* 2004a; Alves *et al.,* 2008; present work). Most commonly data from sequences of protein coding genes, such as partial sequence of the translation elongation factor 1- alpha (EF1-α) as well as β-tubulin gene, have been combined with ITS sequences and contributed to the taxonomy of *Botryosphaeriaceae* (de Wet *et al.,* 2003; Slippers *et al.,* 2004a; van Niekerk *et al.,* 2004; Phillips *et al.,* 2005).

The translation elongation factor 1-α (EF1-α) is a highly conserved ubiquitous protein involved in translation and known to be a useful gene for resolving phylogenetic relationships at the species level due to differentiation at silent nucleotide sites (Cho *et al.,* 1995; Roger *et al.,* 1999; Hedin & Maddison, 2001), as well as in deeper divergences where amino acid substitutions provide phylogenetic resolution (Regier & Shultz, 1997, 1998; Regier *et al.,* 2004, 2005). The EF1-α gene facilitates GTP dependent binding of tRNAs to the acceptor site of ribosomes. Although initially reported to be single-copy in a variety of organisms, EF1-α has increasingly been found to occur in multiple copies in the nuclear genome (Goetze, 2006). It includes more than 1300bp of coding sequence; hence it contains many potential

characters. Furthermore, it is free of internal repeats and other obvious features that may complicate analysis (Cho *et al.,* 1995).

Another protein coding gene of major importance is the β-tubulin gene. The tubulins (α and β) are clearly not ideal markers for fungal phylogeny, due to the obvious rate heterogeneity between different taxa. However, a number of important features of fungal evolution are supported by both tubulin phylogenies. Currently, tubulins are among the more widely sampled protein-coding genes from fungi, and provide some interesting insights into deep fungal evolution and fungal gene structure (Keeling, 2002). Thus, it is clear that in fungal phylogeny each gene has its strengths and weaknesses, and no believable comprehensive phylogeny will emerge from the analysis of a single gene (Keeling, 2002).

In the past, host association was often used to distinguish or describe species of the *Botryosphaeriaceae*. However, it has become clear that host association is not always a good indication for species description in this family. Certain *Botryosphaeriaceae* are clearly generalist species, able to infect a wide range of unrelated hosts (e.g. *B. dothidea*, *L. theobromae* (Pat.) Griffon & Maubl. and *D. seriata* De Not.). Others are more specialized and appear to infect only a specific host genus or group of related host genera (e.g. *D. pinea* (Desm.) J. Kickx f., *N. eucalyptorum* and *N. eucalypticola* Slippers, Crous & M.J. Wingfield). The difficulties associated with identifying many members of the *Botryosphaeriaceae* on the basis of morphological characteristics has, however, made it difficult to study host association patterns in the group. Such host association patterns are important when seeking to understand the driving forces of evolution in the group, patterns of co-evolution with specific hosts, as well as, for pathology and epidemiology studies. Host association patterns that are apparent amongst the *Diplodia*-like anamorphs of the *Botryosphaeriaceae* remain uncertain and are largely unexplored. This is partly due to the taxonomic problems that have been associated with the *Botryosphaeriaceae* and particularly a reliance on morphology to identify species.

As mentioned earlier (page 2), a recent phylogenetic study by Crous *et al.* (2006) separated multiple lineages within the family *Botryosphaeriaceae*, and these were shown to correlate with morphology and were thought to represent individual genera. While this required numerous name changes, it provided a more natural classification for this group. The new nomenclature should allow for a more stable and accurate taxonomic framework in the future and this will strongly influence our understanding of the ecology of the *Botryosphaeriaceae* (Slippers & Wingfield, 2007). The new lineages within what was known as *Botryosphaeria* consist of the anamorph genera *Diplodia* (including *Sphaeropsis*), *Lasiodiplodia*, *Neofusicoccum*, *Pseudofusicoccum*, *Macrophomina*, *Neoscytalidium* and *Dothiorella.* Thus, *Botryosphaeria* is now considered to be a small genus consisting of only *B. dothidea* (the type species of the genus) and *B. corticis* (Crous *et al.,* 2006). Where the

taxonomy remains uncertain the name "*Botryosphaeria*" is used in the broad sense, as in the case of "*Botryosphaeria*" *quercuum. Neofusicoccum* and *Pseudofusicoccum* were described to accommodate other *Botryosphaeriaceae* with *Fusicoccum*-like conidia. *Botryosphaeriaceae* with *Diplodia*-like anamorphs (including *Sphaeropsis*), were retained in *Diplodia*, but the teleomorph name, *Botryosphaeria*, is no longer suitable for them.

While the study of Crous *et al.* (2006) brought new clarity to the taxonomy of the *Botryosphaeriaceae*, it also highlighted many remaining taxonomic problems. Particularly, the identity and phylogenetic relationships of genera with *Diplodia*-like anamorphs of the *Botryosphaeriaceae* that either belong in *Diplodia*, *Dothiorella* and *Lasiodiplodia*, which in the past have been unclear. Their conidia are similar in size and shape (mostly ovoid with a length:width ratio of 2–3:1), thick-walled, and often become pigmented as they age. These characters make the *Diplodia*-like anamorph genera distinctly different from other anamorph genera of the *Botryosphaeriaceae* such as those with *Fusicoccum*-like anamorphs.

1.2. The genus *Diplodia*

The genus *Diplodia*, based on *D. mutila* Fr. and previously considered one of the anamorphic genera of *Botryosphaeria* (teleomorph), is now regarded as a distinct genus, accommodating both teleomorph and anamorph states. Since the genus name *Botryosphaeria* is no longer available for this fungus, *B. stevensii* is not a suitable name for the teleomorph of *D. mutila.*

The history of *B. stevensii* has been very confused. In 1936, Stevens discovered a *Physalospora* species, single ascospore cultures of which produced conidia typical of *D. mutila.* Because no described species had been referred to the teleomorph of *D. mutila,* he made a new combination in *Physalospora* as *P. mutila* (Fr.) N.E. Stevens. However, because of the lack of ascomycete elements in the type of *D. mutila,* he in fact described a new species. Because this was published after the 1st January 1935 without a Latin diagnosis, the name was not valid. Shoemaker (1964) provided a Latin description for *P. mutila,* thus validating the name, but considered it to be better accommodated in *Botryosphaeria.* Because of the earlier name, *Botryosphaeria mutila* (Schwein.) Cooke, Grevillea 13: 101 (1885), Shoemaker proposed a new one, *Botryosphaeria stevensii* Shoemaker, typified by the type of *P. mutila* N.E. Stevens ex Shoemaker. When Luque and Girbal (1989) reported *B. stevensii* from *Quercus suber* they mentioned that conidia of the strains they examined were larger than normal for this species but they regarded this as natural variation in the fungus. In 2001, Zhou and Stanosz suggested that the name *B. stevensii* might have been applied to

more than one species, thus raising the possibility that the fungus reported on oak in fact was not *B. stevensii.* More recently Alves *et al.* (2004) recognized the suggestion by Zhou and Stanosz and revealed that *B. stevensii* was not a homogeneous species.

The genus *Diplodia* is typified by *D. mutila* which has hyaline, aseptate conidia that can become brown and septate with age (Alves *et al.,* 2004) and has been reported from numerous host plants worldwide (Sutton, 1980). The taxonomic history of *D. mutila* was reviewed by Stevens (1933) and Sutton (1980). Briefly, Fries (1823) described *Sphaeria mutila* and distributed two exsiccati under that name, which were later reported to be devoid of spores (Sutton, 1980; Alves *et al.*, 2004). Montagne sent Fries a fungus that was identified as *S. mutila.* The record was listed under *S. mutila* Fr. by Montagne (1834) with the note that this species would become the type of a new genus, *Diplodia,* later characterized by Fries (1849). Therefore, the name of the pycnidial fungus dates from Montagne (1834) and it is typified by his material and the correct citation is *Diplodia mutila* Fr. in Montagne (1834). In the original description, Montagne (1834) described the conidia as "*Asci* [conidia] *elliptico-oblongi, didymi, sporidiis binis referti*." Stevens (1933) studied slides of Montagne's exsiccatus in STR and described the conidia as hyaline and aseptate with a thick smooth, glassy wall, although pale brown and one-septate conidia were sometimes present. Both Shoemaker (1964) and Laundon (1973) agreed with Stevens' concept. Sutton (1980), however, described the conidia as hyaline at first, with a large central guttule, but becoming dark brown and one-septate when mature. In his illustration of this species he depicts a predominance of dark conidia. Nevertheless, Alves *et al.* (2004), examined Montagne's specimen of *D. mutila* in Kew, K(M)99664 (isotype) (Figure 1.2) and found that the vast majority of conidia were hyaline and aseptate, although pale brown and one- or two-septate conidia were seen rarely. In these respects, Alves *et al.* (2004) findings from this specimen corresponded more closely to Stevens' (1933) description of this species than to Sutton's (1980) account.

Figure 1.2. Isotype of *Diplodia mutila* Fr., K(M)99664. France, Ardennes, on bark of *Populus nigra*. ex herb. Montagne. **a)** Bark of *Poppulus nigra*, **b)** Conidiomata of *D. mutila* on the host, **c)** Pycnidia of *D. mutila*, **d)** Hyaline conidia of *D. mutila* (Images by A.J.L. Phillips).

According to the Index Fungorum[1], approximately 1300 species have been described for the genus *Diplodia* over the years (Figure 1.3). Of these, however, few of the names are in regular use in the mycological and phytopathological literature. In the last 10 years several *Diplodia* species were described including *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf. (de Wet *et al*., 2003), *D. corticola* A.J.L. Phillips, Alves & Luque (Alves *et al.,* 2004), *D. rosulata* Gure, Slippers & Stenlid (Gure *et al*., 2005), *D. cupressi* A.J.L. Phillips & A. Alves (Alves *et al*., 2006), *D. africana* Damm & Crous (Damm *et al*., 2007) and *D. olivarum* A.J.L. Phillips, Frisullo & Lazzizera (Lazzizera *et al*., 2008). These species were distinguished on minor morphological differences in the conidia and on phylogenical data. In the case of *D. scrobiculata* it was necessary to include sequence data from six protein coding genes and six SSR loci to differentiate this species from *D. pinea* (de Wet *et al*., 2003).

[1] http://www.indexfungorum.org/

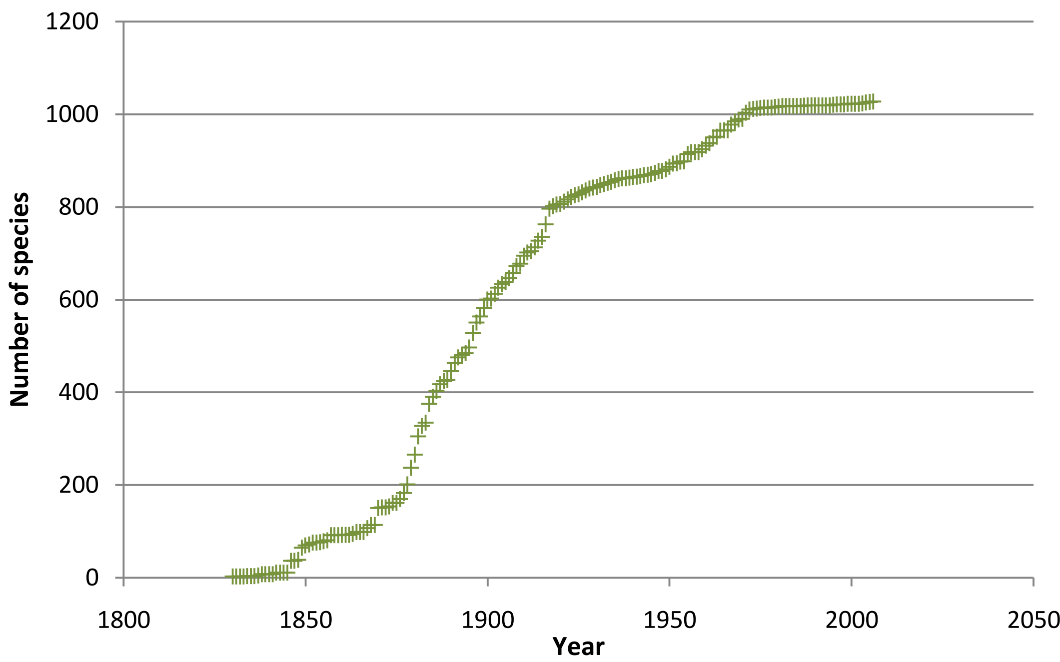

Figure 1.3. Number of species in *Diplodia* described over the years (Source: Index Fungorum, 2008)

Based on the type species, *Diplodia mutila*, and according to Phillips *et al.* (2005), the genus *Diplodia* can be described as follows:

"Mycelium immersed or superficial, branched, septate, melanized, dark brown. *Conidiomata* pycnidial, ostiolate, formed in uni- or multiloculate stromata containing up to 20 pycnidial locules each with a prominent ostiole, immersed, becoming erumpent at maturity. *Ostiole* central, circular, papillate. *Paraphyses* lacking. *Conidiophores* (when present) hyaline, simple, occasionally septate, rarely branched, cylindrical, arising from the cells lining the pycnidial cavity. *Conidiogenous cells* holoblastic, hyaline, cylindrical, determinate or proliferating at the same level giving rise to periclinal thickenings, or proliferating percurrently and forming two or three annellations. *Conidia* initially hyaline, thick-walled, becoming brown when mature but sometimes the coloration is delayed or never occurs, occasionally becoming one-euseptate. Both young and mature conidia can occur concurrently in the same pycnidium, resulting in a mixture of hyaline and dark conidia".

Aims of the work

As revealed by Alves *et al.* (2004), *Diplodia mutila* (as *Botryosphaeria stevensii*) is not a homogeneous species, confirming the suggestion by Zhou and Stanosz (2001a, b) that this name has been applied to more than one species. Due to the widespread occurrence of this species, the wide range of hosts it colonizes and its recognized morphological variability (Alves *et al.,* 2004) it is possible that *Diplodia mutila* is composed of a number of cryptic species.

This work was carried out with the overall objective of contributing to the clarification of the taxonomy and phylogeny of the genus *Diplodia* occurring on woody hosts. Specific aims were:

- To collect and characterize species of *Diplodia* from a number of woody hosts
- To determine suitable molecular techniques to clarify the taxonomy of *Diplodia* and help differentiate and resolve complexes of species within the genus
- To assess phylogenetic diversity within *Diplodia*

Chapter 2

Materials and Methods

2.1. Collection of isolates

A collection of isolates of species of *Diplodia* and related species was established from a variety of hosts collected in different regions of Portugal and also other countries (Table 2.1). Some of the isolates considered in this collection were already in culture, and stored on Difco (Becton, Dickinson and Company, Sparks, USA) potato-dextrose agar (PDA) for later studies, while others were obtained directly from the infected host (Figure 2.1).

Material from diseased branches was collected from *Malus sylvestris* at Monte da Caparica, Portugal.

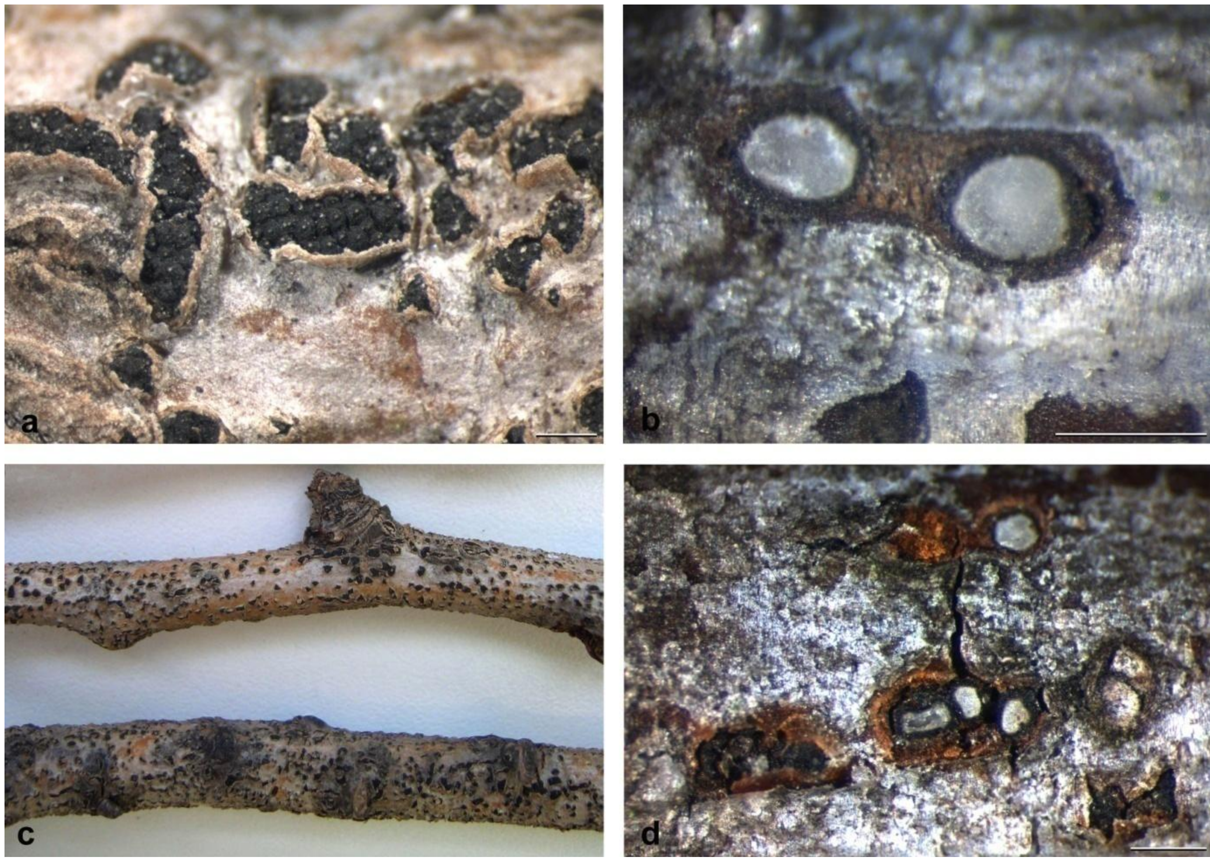

Figure 2.1. Fruiting structures of *Diplodia* sp. on branches of *Populus alba* (a) and *Malus* sp. (b – d). Scale bars = 0.5 mm.

Single spore isolates were established from conidia and ascospores on PDA. A portion of the contents of the fruiting structures was taken and spread over the surface of a Petri dish of PDA, which was subsequently incubated at 25 ºC overnight. The following day individual germinating spores were transferred to fresh plates of PDA. A slide was prepared from the remaining portion of the fruit-body. In this way, the single spore isolates could be linked to the semi-permanent preparation and their identity verified by microscopy (see 2.2. Morphology).

2.1.1. Storage of isolates

Isolates were maintained on half-strength PDA in Petri dishes and test tubes at 4ºC. A method for long-term storage was used. This technique involves growing the fungi on filter paper, overlying half-strength PDA. When the mycelium covers most of the filter paper's surface, it is taken out gently, transferred to a glass Petri dish and dried in a dessicator at 25 ºC. The filter paper is then cut into several pieces of, approximately 5 mm × 5 mm, and put inside sterile paper envelopes, appropriately labelled and stored in hermetic plastic bags at -20 ºC (Figure 2.2). In this way, the physical volume of the stock of isolates is reduced greatly and cultures are easily reactivated by transferring a piece of filter paper to an appropriate medium. This procedure was tested with all isolates and the mycelial growth was normal and occurred within 24 h–48 h of transferring the filter paper to agar plates, depending on the isolates' particular cultural features. This method was generally successful and the viability of the isolates was maintained.

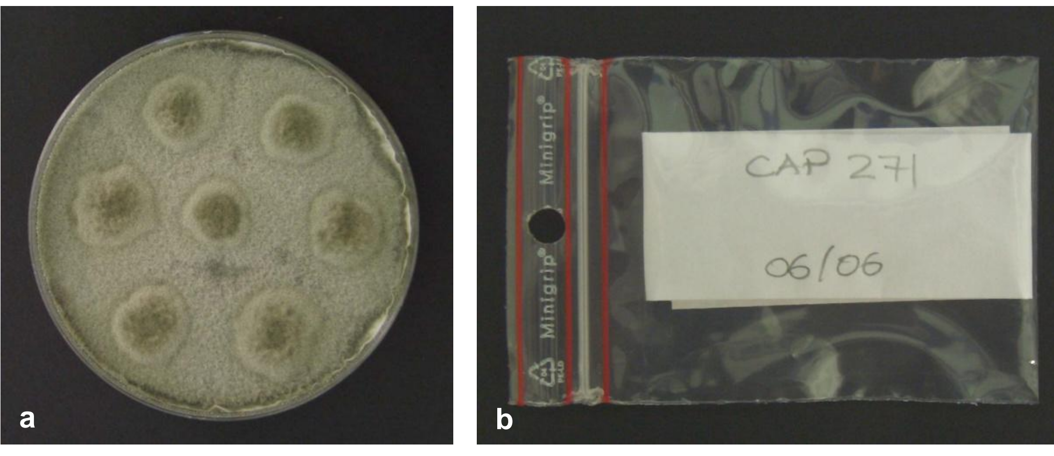

Figure 2.2. Long term storage technique: a) Fungi growth on filter paper over PDA medium; b) Storage in paper envelopes and hermetic plastic bags at -20ºC.

For the identification of the isolates the letters CAP and the number of the order of acquisition were used. For each isolate a passport was constructed including data on the host, identification of the collector, location of the collected material, date of isolation, and fungus species. The collection of isolates of *Diplodia* sp. consisted of 54 isolates, either acquired during this work or collected previously and studied in other works, is presented in Table 2.1.

Table 2.1. Isolates of "*Botryosphaeria*" and *Diplodia* species considered in this study.

Isolate number	Species	Host	Collector	Date	Location	GenBank		
						ITS	EF1-α	β-tubulin
CAP 331	*Diplodia bulgarica* sp. nov.	*Malus sylvestris*	S. Bobev	04.2005	Plovdiv, Bulgaria	-	-	-
CAP 332	*Diplodia bulgarica* sp. nov.	*Malus sylvestris*	S. Bobev	05.2005	Plovdiv, Bulgaria	-	-	-
CAP 333	*Diplodia bulgarica* sp. nov.	*Malus sylvestris*	S. Bobev	06.2005	Plovdiv, Bulgaria	-	-	-
CAP 265	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 266	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 267	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 268	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 269	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 270	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 271	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 272	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 274	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 275	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 277	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 278	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 340	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2007	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 341	*Diplodia malorum*	*Malus sylvestris*	A.J.L. Phillips	02.2007	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 129 - CBS112554	*Diplodia malorum*	*Malus* sylvestris	A.J.L. Phillips	02.2002	Monte da Caparica, Setúbal, Portugal	*AY259095*	*DQ458870*	*DQ458851*
CAP 062 - CBS112553	*Diplodia mutila*	*Vitis vinifera*	A.J.L. Phillips	07.1996	Montemor-o-Novo, Portugal	*AY259093*	*AY573219*	*DQ458850*
CAP 302	*Diplodia fraxini*	*Fraxinus ornus*	A. Sidoti	2006	Sicily, Italy	-	-	-
JL 375	*Diplodia fraxini*	-	J. Luque	-	Cataluña, Spain	*DQ458887*	*DQ458887*	*DQ458852*
JL 453	*Diplodia fraxini*	*Lonicera niger*	J. Luque	-	Cataluña, Spain	-	-	-
CAP 301	*Diplodia olivarum*	*Ceratonia siliqua*	A. Sidoti	2006	Sicily, Italy	-	-	-

CAP 222	*Diplodia olivarum*	*Olea europaea*	S.Frisullo	12. 2004	Cutrofiano, Lecce, Puglia, Italy	*EU392295*	*EU392272*	-
CAP 224	*Diplodia olivarum*	*Olea europaea*	S.Frisullo	12. 2004	Salice Salentino, Lecce, Puglia, Italy	*EU392296*	*EU392273*	-
CAP 225	*Diplodia olivarum*	*Olea europaea*	S.Frisullo	12. 2004	Campi Salentino, Lecce, Puglia, Italy	*EU392297*	*EU392274*	-
CAP235	*Diplodia olivarum*	*Olea europaea*	S.Frisullo	11. 2004	San Pietro Vernotico, Brindisi, Puglia, Italy	*EU392301*	*EU392278*	-
CAP 257	*Diplodia olivarum*	*Olea europaea*	S.Frisullo	11. 2004	Montesano Salentino, Lecce, Puglia, Italy	-	-	-
CAP 166	*Diplodia pinea*	*Olea europaea*	S.Frisullo	01. 2001	Scanzano, Matera, Basilicata, Italy	*EU392284*	*EU392261*	-
CAP 168	*Diplodia pinea*	*Olea europaea*	S. Frisullo	11. 2001	Scorano, Lecce, Puglia, Italy	*EU392285*	*EU392262*	-
CAP 169	*Diplodia pinea*	*Olea europaea*	S.Frisullo	11. 2001	Cutrofiano, Lecce, Puglia, Italy	*EU392286*	*EU392263*	-
CAP 339	*Diplodia pinea*	*Pinus* sp.	S. Bobev	09.2006	Belgium	-	-	-
CAP 130 - CBS112556	*Diplodia intermedia* sp. nov.	*Pyrus communis*	A.J.L. Phillips	02.2002	Monte da Caparica, Setúbal, Portugal	*AY259096*	-	-
CAP 150	*Diplodia intermedia* sp. nov.	*Cydonia* sp.	S. Santos	09.2003	Torres Vedras, Portugal	-	-	-
CAP 273	*Diplodia intermedia* sp. nov.	*Malus sylvestris*	A.J.L. Phillips	02.2006	Monte da Caparica, Setúbal, Portugal	-	-	-
CAP 163	*Diplodia scrobiculata*	*Olea europaea*	S. Frisullo	11.2000	Supersano, Lecce, Puglia, Italy	*EU392283*	*EU392260*	-
CAP 063 - CBS112555	*Diplodia seriata*	*Vitis vinifera*	A.J.L. Phillips	07.1996	Montemor-o-Novo, Portugal	*AY259093*	*AY573219*	-
CAP 148	*Diplodia seriata*	*Vitis vinifera*	L. Mugnai	2002	Italy	*DQ458889*	*DQ458874*	-
CAP 154	*Diplodia seriata*	*Vitis vinifera*	P. Larignon	2002	France	*EU392303*	*EU392280*	-
CAP 160	*Diplodia seriata*	*Vitis vinifera*	L. Mugnai	2002	Italy	*EU392303*	*EU392280*	-
CAP 171	*Diplodia seriata*	*Olea europaea*	S.Frisullo	01.2002	Ruffano, Lecce, Puglia, Italy	*EU392305*	*EU392282*	-
CAP 172	*Diplodia seriata*	*Olea europaea*	S.Frisullo	01. 2002	Ruffano, Lecce, Puglia, Italy	*EU392287*	*EU392264*	-
CAP 207	*Diplodia seriata*	*Olea europaea*	S.Frisullo	12. 2004	Matino, Lecce, Puglia, Italy	*EU392291*	*EU392268*	-
CAP 208	*Diplodia seriata*	*Olea europaea*	S.Frisullo	12. 2004	Ugento, Lecce, Puglia, Italy	*EU392292*	*EU392269*	-
CAP 228	*Diplodia seriata*	*Olea europaea*	S.Frisullo	12. 2004	Supersano, Lecce, Puglia, Italy	*EU392298*	*EU392275*	-
CAP 229	*Diplodia seriata*	*Olea europaea*	S.Frisullo	12. 2004	Brindisi, Brindisi, Puglia, Italy	*EU392299*	*EU392276*	-
CAP 230	*Diplodia seriata*	*Olea europaea*	S.Frisullo	12. 2004	Spongano, Lecce, Puglia, Italy	*EU392300*	*EU392277*	-
CAP 255	*Diplodia seriata*	*Olea europaea*	S.Frisullo	11.2004	Brindisi, Brindisi, Puglia, Italy	-	-	-
CAP 276	*Diplodia seriata*	*Malus sylvestris*	A.J.L. Phillips	02. 2006	Monte da Caparica , UNL, Portugal	-	-	-

CAP 328	*Diplodia seriata*	*Prunus domestica*	S. Bobev	06.2004	Plovdiv, Bulgaria	-	-	-
CAP 335	*Diplodia seriata*	*Malus sylvestris*	S. Bobev	05.2006	Plovdiv, Bulgaria	-	-	-
CAP 337	*Diplodia seriata*	*Cotoneaster bullatus*	S. Bobev	11.2006	Plovdiv, Bulgaria	-	-	-
CAP 338	*Diplodia seriata*	*Vitis vinifera*	S. Bobev	12.2006	Plovdiv, Bulgaria	-	-	-
CAP 330	*Diplodia* sp.	*Pyracantha coccinea*	S. Bobev	01.2005	Plovdiv, Bulgaria	-	-	-

[1] Acronyms of culture collections: **CAP** - Alan J.L. Phillips, Universidade Nova de Lisboa, Portugal; **CBS** - Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CMW** - M.J. Wingfield, FABI, University of Pretonia, South Africa; **JL** - J. Luque, IRTA, Spain.

[2] Sequence numbers in *italics* were retrieved from GenBank. All other sequences were obtained in this study and will be submitted to CBS as well as respective cultures.

2.2. Molecular Characterization of the isolates

2.2.1. DNA extraction

The following methods were tested for isolation of genomic DNA from fresh mycelium.

Method 1. Laboratory Protocols for Molecular Techniques, Swedish University of Agricultural Sciences, Uppsala, Sweden (1998).

Fungal isolates were grown on half strength PDA (potato dextrose agar) for 1–2 weeks to ensure adequate development of the mycelium.

1. Firstly, the mycelium was scraped from the surface of the medium and transferred to a 1.5 ml Eppendorf tube (up to a volume of approximately 0,1 ml).
2. To each tube was added 600 µl of 2 % CTAB lysis buffer (2 % CTAB; 100 mM 1 M Tris-HCl, pH 8; 1400 mM 5 M NaCl; 20 mM 0.5 M EDTA, pH 8) and ground thoroughly with a micro-pestle until the solution was homogeneous.
3. The Eppendorf tubes were then placed in a heating block at 65 °C, for approximately one hour and then centrifuged at 19000 × *g* for 5 min, at room temperature.
4. With a pipette, the upper phase was transferred to a new marked Eppendorf tube to which 1 vol of chloroform was added. The tubes were mixed well by hand until the suspension was colloid.
5. After centrifugation at 19000 × *g* for 5 min at room temperature, the upper phase was transferred into a new marked Eppendorf tube.
6. DNA was precipitated with 1.5 vol of isopropanol and then the tubes were agitated and put at -20 °C for 30 minutes or left overnight.
7. Samples were centrifuged at 14500 rpm for 30 min, at 4 °C and the upper phase was discarded.
8. To wash the DNA, 200 µl of ice-cold 70 % ethanol (-20 °C) were added and the, samples were centrifuged at 19000 × *g* for 5 min, at 4 °C.
9. Finally, the upper phase was discarded and the DNA-pellet was dried for approximately 5 minutes on the heating block.
10. The pellet was then resuspended in 50 µl TE buffer (10 mM 1 M Tris-HCl, pH 8; 1 mM 0.5 M EDTA, pH 8).

Method 2. According to Möller *et al.* (1992).

Fungal isolates were grown on half strength PDA for 1–2 weeks to ensure adequate development of the mycelium.

1. Firstly, the mycelium was scraped from the surface of the medium and added to 500 µl of TES buffer (100 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; 2 % SDS) in a 2 ml Eppendorf tube, until the solution was very turbid.
2. The tubes were then mixed by inversion, boiled in a heating block at 100–110 °C for 3 minutes and placed on ice for 10 minutes.
3. Subsequently, 10 µl of Proteinase K (20 mg/ml) were added and the tubes were incubated for 30 minutes at 65 °C and swirled occasionally.
4. Salt concentration was increased by adding 140 µl of 5 M NaCl. After adding 65 µl of 10 % CTAB the tubes were incubated again for 30 minutes at 65 °C and swirled occasionally.
5. Then, 1 ml of chloroform:isoamylalcohol (24:1) was added, the tubes were mixed carefully by inversion for 1 minute, incubated for 30 minutes on ice and centrifuged for 10 minutes at 19000 × *g* at 4 °C.
6. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube and 225 µl of 5 M NH_4O acetate was added and the tubes were incubated for 30 minutes on ice.
7. After centrifugation at 19000 × *g* for 10 minutes at 4 °C, the supernatant was transferred to a fresh 1.5 ml Eppendorf tube.
8. Nucleic acids were precipitated by adding 0.5 vol of ice-cold isopropanol and the tubes were mixed carefully, kept for 30 minutes on ice and centrifuged directly at 19000 × *g* for 10 minutes at 4 °C.
9. The supernatant was discarded and the DNA pellet washed twice with ice-cold 70 % ethanol and centrifuged for 10 minutes at 19000 × *g* at 4 °C.
10. Finally, the supernatant was discarded, the DNA pellet was dried at room temperature and after complete evaporation of the ethanol the pellet was resuspended in 50 µl of TE buffer.

For both methods, DNA dilutions were made in the proportion of 1:500 with sterile bi-destiled water. The original stocks and DNA dilutions were labelled appropriately and stored at -20 °C.

To check for the presence of DNA 5 µl of the preparation was loaded onto a 1 % agarose gel in 1 × TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA, pH 8) and

developed at 80 V for 45 minutes. The extracted DNA was used as template in the subsequent amplification reactions.

2.2.2. DNA amplification

All information about the primers used in the molecular characterization of this study is presented in Table 2.2.

Table 2.2. Primers used in the molecular characterization studies

Region / Gene	Primer name	Sequence (5'→3')	Reference
Internal transcribed spacer	M13	GAG GGT GGC GGT TCT	-
	$(GTG)_5$	GTG GTG GTG GTG GTG	-
	ITS1	TCC GTA GGT GAA CCT GCG G	White *et al.*, 1990
	ITS4	TCC TCC GCT TAT TGA TAT GC	White *et al.*, 1990
Translation elongation factor 1-α	EF1-728F	CAT CGA GAA GTT CGA GAA GG	Carbone and Kohn, 1999
	EF1-986R	TAC TTG AAG GAA CCC TTA CC	Carbone and Kohn, 1999
	EF1-688F	CGG TCA CTT GAT CTA CAA GTG C	Alves, 2008
	EF1-1251R	CCT CGA ACT CAC CAG TAC CG	Alves, 2008
β-tubulin	Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC	Glass & Donaldson, 1995
	Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	Glass & Donaldson, 1995

All PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania) while the primers were supplied by STAB Vida, Lda (Oeiras, Portugal), except M13 and $(GTG)_5$, which were supplied by MWG Biotech AG (Ebersberg, Germany).

2.2.2.1. Determination of polymorphisms of microsatellite regions (MSP-PCR)

After DNA isolation, a preliminary molecular characterization of the isolates was carried out by Microsatellite-Primed PCR (MSP-PCR) fingerprinting. The core sequence of the phage M13 and the synthetic oligonucleotide $(GTG)_5$, which is specific to simple repetitive DNA sequences, were tested as single primers for MSP-PCR fingerprint. The reaction mix of 25 µl of final volume contained contained 1× PCR buffer (1:1 v:v PCR buffer without $MgCl_2$: PCR buffer with $(NH_4)_2SO_4$), 3 mM $MgCl_2$; 200 µM of each nucleotide; 15 pmol of each primer; 1U *Taq* polymerase and 50–100 ng of the template DNA dilution referred to in 2.2.1. *DNA extraction*. The amplification conditions were as follows in Table 2.3:

Table 2.3. Amplification conditions for MSP-PCR.

Stages	Temperature	Duration	Number of cycles
Pre-denaturation	95ºC	5 minues	
Denaturation	94ºC	1 minute	35 cycles
Annealing	45ºC	1 minute	
Elongation	72ºC	2 minutes	
Final Elongation	72ºC	10 minutes	

The amplified products were separated by electrophoresis in 2 % (w/v) agarose (Gibco, BRL) gels, in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1mM EDTA, pH 8) for 3 hours and 30 minutes at 80 V. A mixture of size markers was used to estimate the molecular weight of the DNA fragments and consisted of DNA from phage λ cleaved with *Hin*dIII and DNA from φX174 cleaved with *Hae*III (MBI Fermentas). The DNA fingerprint was visualized under UV illumination (Molecular Imager Gel Doc XR System, Bio-Rad) after staining with ethidium bromide (2 mg/ml). The DNA fingerprint was analyzed with both visual analysis and GelCompar V.4.1 (Applied Maths 1998, Belgium). In this program, similarity between all banding patterns were estimated by Pearson correlation and grouped in a dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering method (Sneath *et al.*, 1973).

2.2.2.2. Amplification of the ITS regions from rDNA

PCR amplification of the nuclear 5.8S ribosomal RNA gene and its flanking ITS regions was performed with ITS1 and ITS4 (White *et al.*, 1990) primers, as described by Alves *et al.* (2004). The 5.8S ribosomal DNA gene and its flanking ITS region was amplified in a Biometra - T Gradient Thermblock thermal cycler using the primers ITS1 and ITS4. PCR reaction mixtures contained 1× PCR buffer (1:1 v:v PCR buffer without $MgCl_2$: PCR buffer with $(NH_4)_2SO_4$), 3 mM $MgCl_2$; 200 µM of each nucleotide; 15 pmol of each primer; 5 % DMSO (Dimethyl Sulfoxide) to improve the amplification of some difficult DNA templates.; 1U *Taq* polymerase and 50-100 ng of template DNA. Each reaction volume was made up to 50µl with sterile water. Negative controls with sterile water instead of template DNA were used in every PCR reaction. The amplification conditions for ITS regions were as follows in Table 2.4:

Table 2.4. Amplification conditions for the ITS region.

Stages	Temperature	Duration	Number of cycles
Pre-denaturation	95ºC	7 minutes	
Denaturation	94ºC	1 minute	35 cycles
Annealing	50ºC	1 minute	
Elongation	72ºC	1 minute	
Final Elongation	72ºC	10 minutes	

After amplification, 5 µl of each PCR product were separated by electrophoresis in 1% (w/v) agarose gels, in 1X TAE (40 mM Tris, 40 mM NaAc, 2 mM EDTA, pH 8), for 45 minutes at 80 V. Gels were stained with ethidium bromide and visualized in a UV transilluminator Molecular Imager Gel Doc XR System (Bio-Rad) to assess PCR amplification. GeneRuler - DNA Ladder Mix (MBI Fermentas) was run at both sides of each gel to estimate the size of the DNA fragments. DNA was quantified by the software program Quantity One® 1-D Analysis Software V.4.5.0 (Bio-Rad), by comparison with the range of standard DNA concentrations from the molecular size marker (GeneRuler - DNA Ladder Mix).

2.2.2.3. Amplification of the translation elongation factor 1-alpha (EF1- α) gene

The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-alpha (EF1- α) gene as described by Phillips *et al.* (2005). For some isolates, amplification of the EF1-α region was impossible with the primer set EF1- 728F and EF1-986R. Therefore, the primers EF1-688F and EF1-1251R were used according to Alves *et al.* (2008). The PCR reaction mixture was identical to the one used to amplify the ITS region, except for the $MgCl_2$ concentration, which was increased to 4 mM providing better amplification of this gene. The amplification conditions for the EF1-α region were as follows in Table 2.5:

Table 2.5. Amplification conditions for the EF1-α gene.

Stages	Temperature	Duration	Number of cycles
Pre-denaturation	95ºC	5 minutes	
Denaturation	94ºC	30 seconds	35 cycles
Annealing	55ºC	45 seconds	
Elongation	72ºC	1 minute and 30 seconds	
Final Elongation	72ºC	10 minutes	

All the subsequent processes, such as electrophoresis and DNA quantification conditions, were similar to those described above for the ITS region.

2.2.2.4. Amplification of the β-tubulin gene

To amplify part of the β-tubulin gene, the primers Bt2a and Bt2b (Glass & Donaldson, 1995) were used, as described by Alves *et al* (2006). Procedures and conditions were the same as for amplification of EF1- α.

2.2.3. DNA purification and sequencing

The amplified PCR fragments were purified with the GFX PCR DNA - Gel Band Purification Kit (GE Healthcare) before DNA sequencing, as explained by the manufacturer. Both strands of all the PCR products (ITS region, EF1- α gene and β-tubulin gene) were sequenced by STAB Vida, Lda. The complete sequences of the ITS region (partial 18S, ITS1, 5.8S gene, ITS2 and partial 28S sequences) and partial EF1-α and β-tubulin genes were read and edited with BioEdit Sequence Alignment Editor v.7.0.4.1 and Finch TV v.1.4.0 softwares. All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences. Additional nucleotide sequences for all three DNA regions were retrieved from GenBank (Table 2.7).

2.2.4. Phylogenetic analysis

All the sequences obtained for this study were aligned with ClustalX version 1.83 (Thompson *et al.*, 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences= 25%). Alignments were checked and manual adjustments were made where necessary. Phylogenetic

information contained in indels (gaps) was incorporated into the analyses using simple indel coding as implemented by GapCoder (Young and Healy, 2003).

Phylogenetic analyses of sequence data were done using PAUP* (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2003) for Maximum-parsimony (MP) and Neighbour joining (NJ) analyses. The outgroup taxa selected for rooting the trees were *Lasiodiplodia theobromae* and *Lasiodiplodia gonubiensis* and trees were visualized with TreeView (Page, 1996).

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1000 bootstrap replications (Hillis and Bull, 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

The validity of the partition homogeneity test in PAUP* for determining whether multiple datasets should be combined has been questioned (Cunningham, 1997; Barker and Lutzoni, 2002). In this study, the possibility of combining the ITS, EF1-α and β-tubulin datasets was assessed by comparing highly supported clades among trees generated from the different datasets to detect conflict. High support typically refers to bootstrap support values ≥ 70% (Alfaro *et al*., 2003). If no conflict exists between the highly supported clades in trees generated from these different datasets, it is likely that the genes share similar phylogenetic histories and phylogenetic resolution and support could ultimately be increased by combining the datasets (Miller and Huhndorf, 2004).

Table 2.6. Isolates of *Diplodia* and "*Botryosphaeria"* species previously studied or retrieved from GenBank and included in the phylogenetic analyses of the present work.

Accession number	Species	Host	Collector	Date	Locality	GenBank		
						ITS	EF1-α	B-tubulin
CBS 418.64	*"Botryosphaeria" tsugae*	*Tsuga heterophylla*	A. Funk	11.1964	British Columbia, Lake Cowichan, Canada	*DQ458888*	*DQ458873*	*DQ458855*
CBS 110302	*Botryosphaeria dothidea*	*Vitis vinifera*	A.J.L. Phillips	01.1996	Montemor-o-Novo, Portugal	*AY259092*	*AY573218*	*EU673106*
CBS 112547	*Diplodia corticola*	*Quercus ilex*	M.E. Sánchez	01.2001	La Rozuela. Córdoba	*AY259110*	*DQ458872*	-
CBS 112549	*Diplodia corticola*	*Quercus suber*	A. Alves	02.2002	Requeixo, Aveiro, Portugal	*AY259100*	*AY573227*	*DQ458853*
STE-U 5908	*Diplodia africana*	*Prunus persica*	U. Damm	06.2004	Paarl, Western Cape, South Africa	*EF445343*	*EF445382*	-
STE-U 5946	*Diplodia africana*	*Prunus persica*	U. Damm	06.2004	Paarl, Western Cape, South Africa	*EF445344*	*EF445383*	-
CBS 168.87	*Diplodia cupressi*	*Cupressus sempervirens*	Z. Solel	03.1987	Bet Dagan, Israel	*DQ458893*	*DQ458878*	*DQ458861*
CBS 261.85	*Diplodia cupressi*	*Cupressus sempervirens*	Z. Solel	05.1985	Bet Dagan, Israel	*DQ458894*	*DQ458879*	*DQ458862*
CBS 230.30	*Diplodia mutila*	*Phoenix dactylifera*	LL. Huillier	1930	California, USA	*DQ458886*	*DQ458869*	*DQ458849*
CBS 393.84	*Diplodia pinea* "A"	*Pinus nigra*	H.A. van der Aa	06.1984	Putten, Netherlands	*DQ458895*	*DQ458880*	*DQ458863*
CBS 109727	*Diplodia pinea* "A"	*Pinus radiata*	W.J. Swart	09.2001	Stellenbosch, South Africa	*DQ458897*	*DQ458882*	*DQ458865*
CBS 109725	*Diplodia pinea* "C"	*Pinus patula*	M.J. Wingfield	09.2001	Habinsaran, Indonesia	*DQ458896*	*DQ458881*	*DQ458864*
CBS 109943	*Diplodia pinea* "C"	*Pinus patula*	M.J. Wingfield	01.2002	Indonesia	*DQ458898*	*DQ458883*	*DQ458866*
CBS 116472	*Diplodia rosulata*	*Prunus africana*	A. Gure	07.2001	Gambo, forest Enterprise, Ethiopia	*EU430266*	*EU430268*	*EU673131*
CBS 116470	*Diplodia rosulata*	*Prunus africana*	A. Gure	07.2001	Gambo, forest Enterprise, Ethiopia	*EU430265*	*EU430267*	*EU673132*
CBS 109944	*Diplodia scrobiculata*	*Pinus greggii*	M.J. Wingfield	01.2002	Mexico	*DQ458899*	*DQ458884*	*DQ458867*
CBS 113423	*Diplodia scrobiculata*	*Pinus greggii*	M.J. Wingfield	01.2002	Mexico	*DQ458900*	*DQ458885*	*DQ458868*
CBS 121884	*Diplodia seriata*	*Olea europaea*	S. Frisullo	12.2004	Lucugnano, Lecce, Puglia, Italy	*EU392288*	*EU392265*	-
CBS 121885	*Diplodia seriata*	*Olea europaea*	S. Frisullo	12.2004	Casarano, Lecce, Puglia, Italy	*EU392289*	*EU392266*	-
CAP 206	*Diplodia seriata*	*Olea europaea*	S. Frisullo	12.2004	Ruffano, Lecce, Puglia, Italy	*EU392290*	*EU392267*	-
CAP 217	*Diplodia seriata*	*Olea europaea*	S. Frisullo	12.2004	Ruffano, Lecce, Puglia, Italy	*EU392293*	*EU392270*	-
CAP 220	*Diplodia seriata*	*Olea europaea*	S. Frisullo	12.2004	Felline, Lecce, Puglia, Italy	*EU392294*	*EU392271*	-

CBS 115812	*Lasiodiplodia gonubiensis*	*Syzygium cordatum*	D. Pavlic	-	South Africa	*DQ458892*	*DQ458877*	*DQ458860*
CBS 124.13	*Lasiodiplodia theobromae*	Unknown	J.J. Taubenhaus	11.1913	USA	*DQ458890*	*DQ458875*	*DQ458858*
CAA 006	*Lasiodiplodia theobromae*	*Vitis vinifera*	T.J. Michailides	-	USA	*DQ458891*	*DQ458876*	*DQ458859*
CBS 110299	*Neofusicoccum luteum*	*Vitis vinifera*	A.J.L. Phillips	01.1996	Oeiras, Portugal	*AY259091*	*AY573217*	*DQ458848*
CBS 110301	*Neofusicoccum parvum*	*Vitis vinifera*	A.J.L. Phillips	03.1998	Palmela, Portugal	*AY259098*	*AY673221*	*EU673095*
CBS 115475	*Neofusicoccum ribis*	*Ribes sp.*	B. slippers/ G. Hudler		New York, USA	*AY236935*	*AY236877*	*AY236906*

[1] Acronyms of culture collections: **CAA** - A. Alves, Universidade de Aveiro, Portugal; **CAP** - Alan J.L. Phillips, Universidade Nova de Lisboa, Portugal; **CBS** - Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CMW** - M.J. Wingfield, FABI, University of Pretonia, South Africa; **STE-U** – Stellenbosch University, Department of Plant Pathology, South Africa.

[2] Sequence numbers in *italics* were retrieved from GenBank.

2.3. Morphological Characterization of the isolates

All the isolates in this study were characterized on the basis of their micromorphological features, type of conidiomata, mode of conidiogenesis, morphology of the conidiophores and conidiogeneous cells and dimensions, shape, texture, pigmentation and septation of the conidia. Characters derived from conidial dimensions, such as length/width ratio were also taken into account. Other characters, such as colony morphology, pigmentation and mycelia growth rate were recorded. The media used as well as incubation conditions were standardized to ensure repeatable and reliable results.

Microscope preparations were made by cutting horizontally through the conidioma or ascoma and transferring the contents to a drop of sterile water in a microscope slide. The water on the slide was allowed to evaporate at room temperature before applying a drop of 100% lactic acid and covering with a coverslip. These preparations were examined with a Leica DMR HC microscope and photographs were recorded with a Leica DFC 320 digital camera.

2.3.1. Measurement of conidia

Measurements were made from conidia taken directly from the host (as explained previously in 2.1. *Collection of isolates*) and from conidia formed in culture. To induce sporulation in culture, five autoclaved pine needles, each approximately 4 cm long, were placed on a Petri dish of 2% water agar. Thus, with a low nutritive medium, the fungus develops fruiting structures over the solid substrate, namely the pine needles (Figure 2.3).

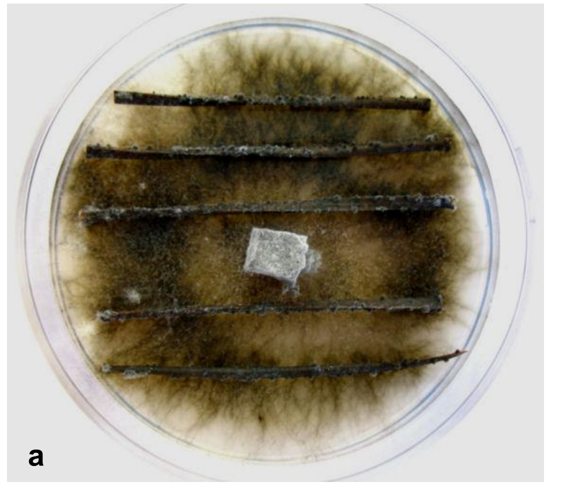

Figure 2.3. Sporulation in culture method (a) and pine needles with conidiomata (b).

To prepare microscope slides, samples were collected from either host or culture, placed on a glass slide and mounted in 100% lactic acid and covered with a coverslip. All observations on micromorphological features were made with a Leica DMR HC microscope with Nomarski differential interference contrast illumination and images were recorded with a Leica DFC 320 digital camera. Measurements were made with the Leica IM500 Image Manager measurement module, of 50 conidia for each isolate, from images taken with the 100X objective lens. Mean, standard deviation and 95% confidence intervals were calculated. Conidial dimensions are presented as 95% confidence intervals with minimum and maximum dimensions in parenthesis, followed by the mean and standard deviation values.

2.3.2. Mycelial growth rate

Mycelial growth rate was measured in terms of fungus growth in 90 mm diameter Petri dishes of PDA, incubated at 25 °C in darkness. Three plates were used for each isolate and the radial mycelial growth was measured perpendicularly for each plate and the mean calculated to determine the growth rates for each isolate. Measurements were made after 24, 48 and 72 hours as shown in Figure 2.4.

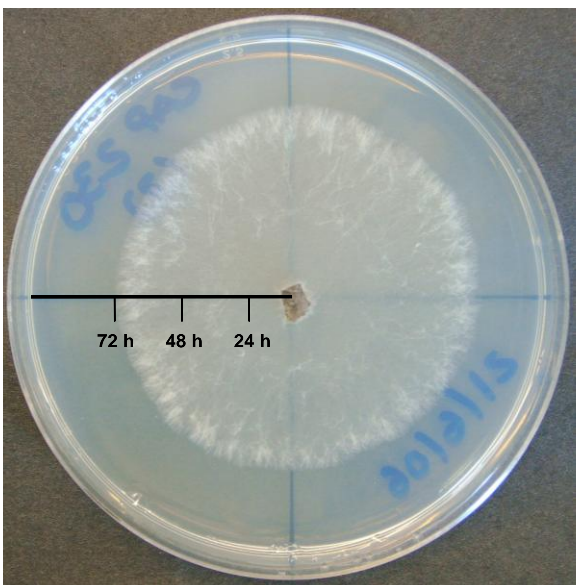

Figure 2.4. Mycelial growth measurements in PDA.

To ensure the acquisition of real values, due to the fact that mycelia growth is slower in the first 24 hours, the mean of the sum of the last 48 hours was used, to obtain the daily growth rate.

Chapter 3
Results

3.1. Molecular Characterization of the isolates

3.1.1. Determination of polymorphisms of microsatellite regions (MSP-PCR)

The preliminary analysis of the collection of isolates by MSP-PCR was performed with the aim of determining groups of isolates from which representative isolates could be selected for in-depth studies. The set of strains used in this MSP-PCR characterization included just some of the isolates from the collection of this study in order to correlate the positions of the main species present in the collection. Both primers yielded diverse and complex genomic fingerprints, with several bands. The banding profiles varied according to the strain and also with the primer used (Figure 3.1). The ability for both primers to produce specific fingerprints for the strains belonging to the same species was generally apparent. However, some strains were not clearly discriminated and appear to be incorrectly assigned by the fingerprints. The minor variability between the fingerprints of a given MSP-PCR class can be the result of variability caused by the method, or simply reflect intra-species variation.

Two dendrograms were produced, one for each of the primers used, by GelCompar V.4.1, based on the relationship between the various fingerprints. The dendrograms obtained from the combined numerical analysis of the fingerprints are shown in Figure 3.2. In general, a good inter-species discrimination was obtained since strains of the same species had similar banding patterns, whereas strains that represented different species gave rise to distinct PCR fingerprints.

The main groups of species defined in clusters by the dendrogram were *D. mutila, D. olivarum, D.seriata, D. pinea* and *D. scrobiculata*, and three unknown species that were *D. malorum*-like, *D. mutila*-like*, D. pinea*-like. Each of these groups of isolates are highlighted by a different color in the dendrogram. Instead of setting a threshold similarity value for cluster definition, the clusters obtained were established by combining the numerical analysis shown in the dendrogram with a visual comparison of the fingerprints. Therefore, the clusters correspond to groups of isolates showing visually indistinguishable or highly similar profiles.

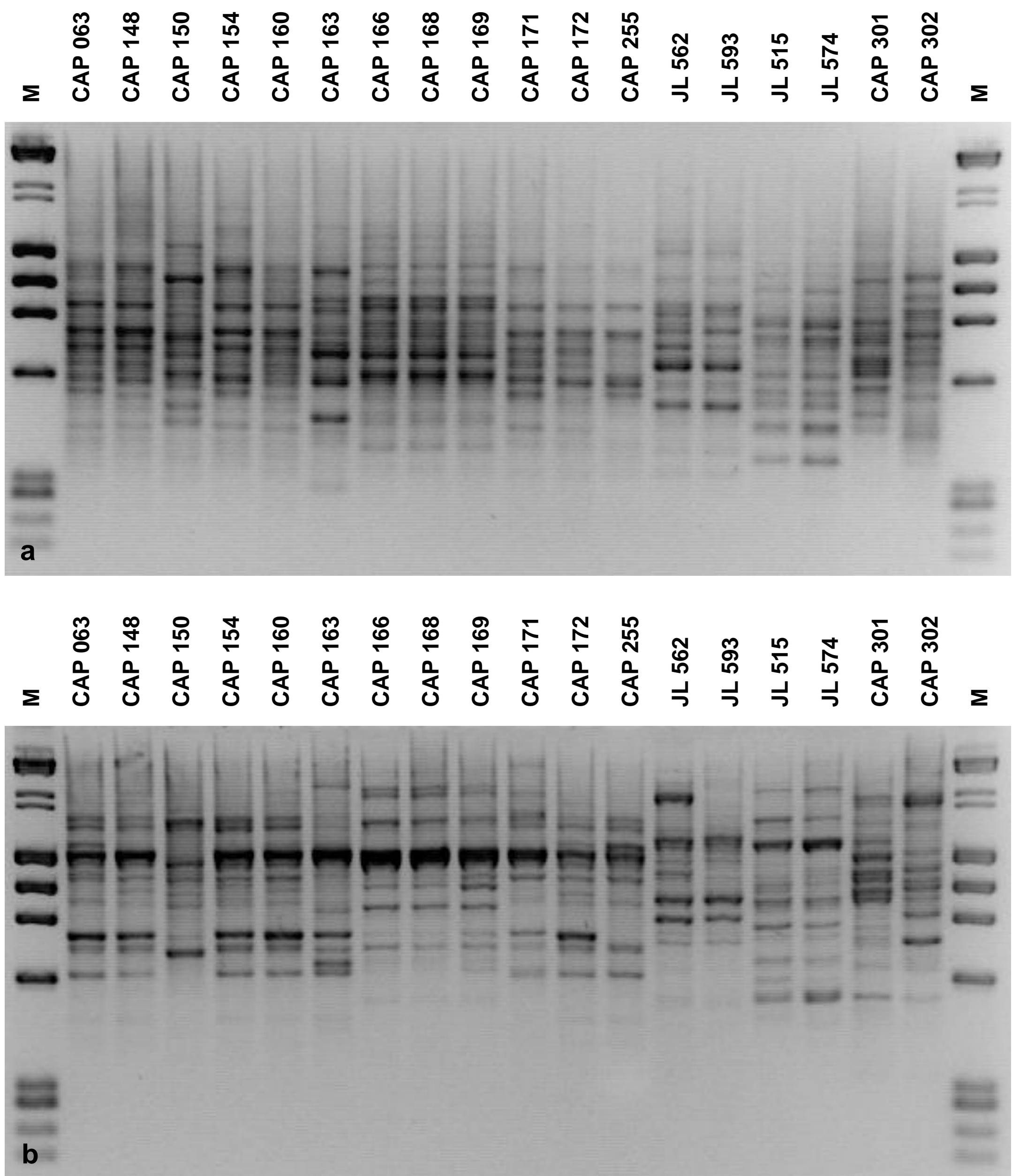

Figure 3.1. Examples of MSP-PCR fingerprints obtained with primers (a) $(GTG)_5$ and (b) M13 after separations by electrophoresis in a 2% agarose gel, at 80V for 3.5 hours; (M) Mixture of markers "Lambda DNA/*Hin*dIII" and "ΦX174 DNA/*Bsu*RI (*Hae*III)".

From the analysis of the $(GTG)_5$ dendrogram, a good resolution was generally clear for both dark and hyaline-spored species and different species were well separated into clusters. In contrast, the M13 dendrogram was not well resolved for some of the species, especially the *D. seriata* - *D. pinea* complex. Not all the strains of the *Diplodia* species obtained in these dendrograms were clearly discriminated by MSP-PCR fingerprinting. Isolate CAP062, representing *D. mutila*, in the M13 fingerprint, lays at one end of the dendrogram. Furthermore, isolates JL375 and JL453 clustered with the dark-spored isolates instead of

with the hyaline ones where they would be expected. In this way, MSP-PCR analysis was, in general, not suitable for species discrimination. Nevertheless, it was possible to establish groups of isolates for in-depth study of morphology and for phylogenetic analysis.

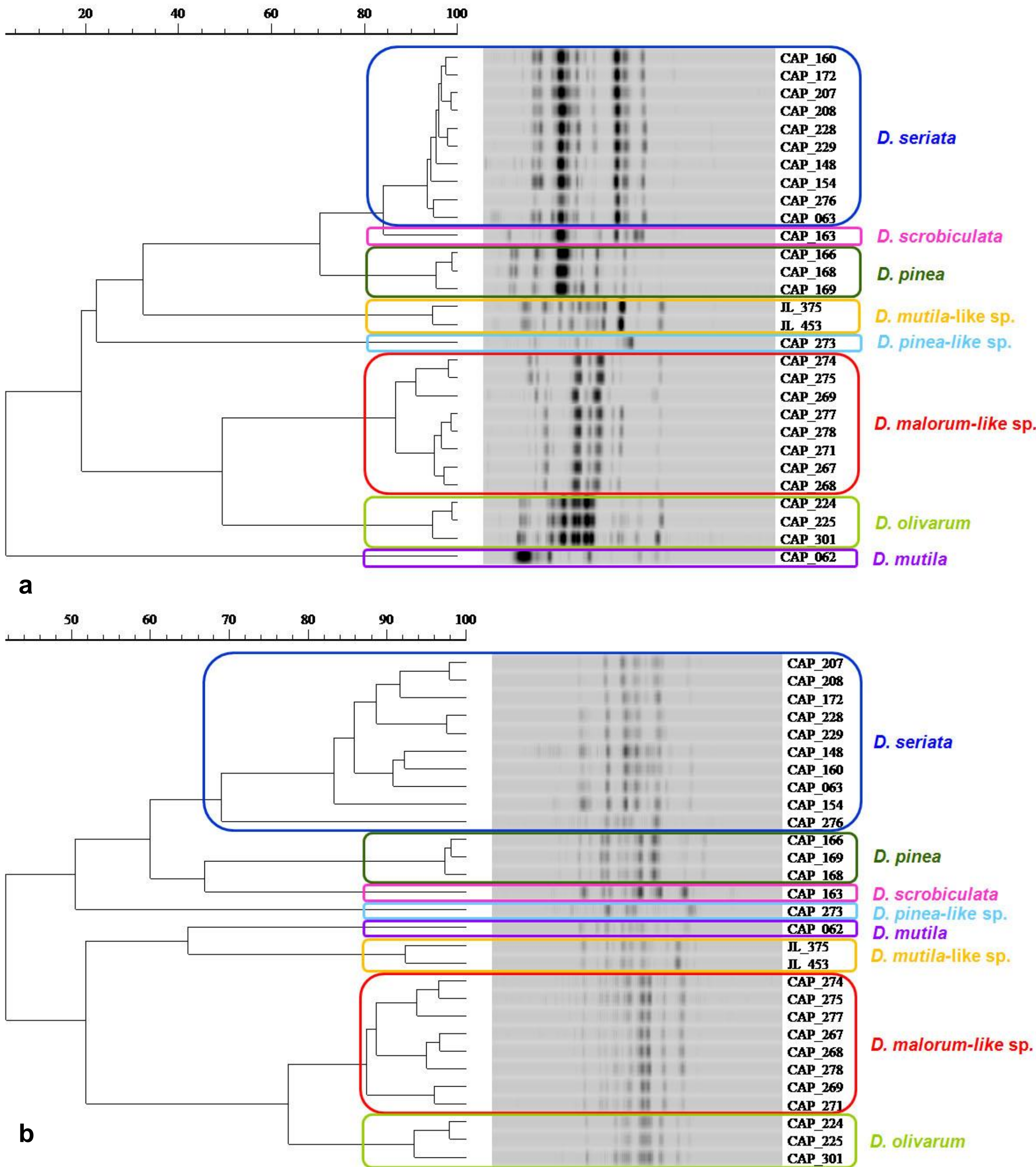

Figure 3.2 . MSP-PCR dendrograms and fingerprints of a collection of isolates of *Diplodia* sp. generated with the primers **a)** M13 and **b)** $(GTG)_5$.

3.1.2. Phylogeny

3.1.2.1. ITS and EF1-α phylogeny

For the ITS and EF1-α sequence dataset, either sequenced in this study or retrieved from GenBank, the alignment of 68 isolates, including the three outgroup isolates, consisted of 545 characters for the ITS region and 314 characters for EF1-α gene, including alignment gaps. Sequences of the two genes were aligned and analysed separately by Maximum Parsimony and Neighbor Joining analyses, and the resulting trees were compared. The single gene trees reflected the same underlying phylogeny, indicating that the ITS and EF1-α datasets could be combined. The start of the EF1-α sequences were incomplete in some of the GenBank accessions and the corresponding parts of all isolates were excluded from the analyses. Furthermore, on account of the different primers used to generate some of the sequences, the bases corresponding to the primers at the end of the sequences were also excluded. In the analyses, all alignment gaps were treated as fifth character. The tree was rooted to *Lasiodiplodia theobromae* and *L. gonubiensis*.

The combined dataset contained 859 characters. Of the 859 characters, 601 were constant, 20 were variable and parsimony uninformative and 238 were parsimony informative. The 32 equally parsimonious trees generated from the heuristic search exhibited low levels of homoplasy as indicated by a consistency index of 0.799, a retention index of 0.954, a homoplasy index of 0.201 and tree length of 428 steps. Tree topologies resulting from MP and NJ analyses were similar and only the former is shown.

The tree resulting from Maximum Parsimony analysis is presented in Figure 3.3 with MP and NJ bootstrap values above and below the branches, respectively. Two major clades were resolved. One clade (MP and NJ bootstrap = 100 %) corresponded to *Diplodia corticola* (Clade IV) and the second clade (MP bootstrap = 57 %; NJ bootstrap = 69 %) was resolved into three other well supported clades (Clades I, II and III). Clades I and II (MP bootstrap = 99 %; NJ bootstrap = 96 % and MP bootstrap = 73 %; NJ bootstrap = 91 %) corresponded to species with conidia that remain hyaline for a considerable time before darkening and becoming generally one-septate only after discharge, and Clade III (MP bootstrap = 100 %; NJ bootstrap = 99 %) corresponded to species with conidia that darken before discharge and remain mostly aseptate.

Thus, within Clade I, six sub-clades supported by high bootstrap values and corresponding to six species could be distinguished. These sub-clades were identified as *D. olivarum* (MP bootstrap = 99 %; NJ bootstrap = 97 %), *D. mutila* (MP bootstrap = 100 %; NJ bootstrap = 95 %), *D. africana* (MP bootstrap = 96 %; NJ bootstrap = 97%), *D. rosulata* (MP

bootstrap = 100 %; NJ bootstrap = 100 %), *D. malorum* (MP bootstrap = 99 %; NJ bootstrap = 99 %) and *D. fraxini* (MP bootstrap = 87 %; NJ bootstrap = 82 %).

Clade II consisted of three species, namely *D. cupressi* (MP bootstrap = 100 %; NJ bootstrap = 100 %), *"Botryosphaeria" tsugae*, represented by a single isolate, and an undescribed species represented by three isolates from Bulgaria and described here as *Diplodia bulgarica* sp. nov. (MP bootstrap = 99 %; NJ bootstrap = 100 %).

Four distinct sub-clades could be identified within Clade III and they were *D. pinea* (MP bootstrap = 62 %; NJ bootstrap = 63 %), an undescribed species, for which we introduce the name *D. intermedia* (MP bootstrap = 80 %; NJ bootstrap = 74 %), *D. scrobiculata* (MP bootstrap = 97 %; NJ bootstrap = 95 %) and *D. seriata* (MP bootstrap = 91 %; NJ bootstrap = 96 %). Isolate CAP330, which frequently clustered close to the isolates of *D. intermedia*, did not have a stable position within this clade.

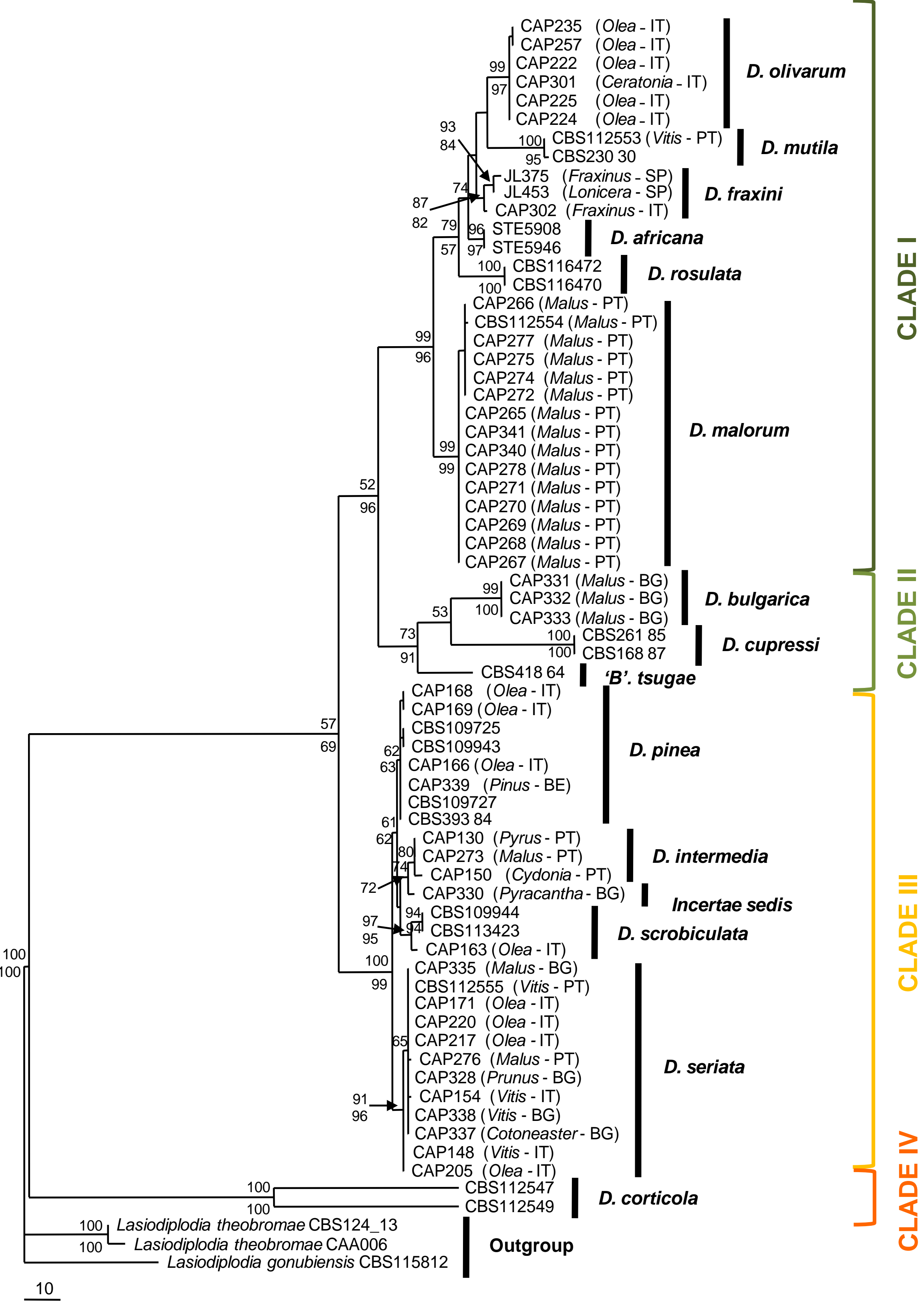

Figure 3.3. One of the 32 equally parsimonious trees, resulting from maximum parsimony analysis of combined ITS and EF1-α sequence data for 68 isolates in the *Botryosphaeriaceae*. Support for the branches is given above (MP bootstrap) and below (NJ bootstrap) the nodes. The tree is rooted to *Lasiodiplodia theobromae* and *L. gonubiensis*. The host species followed by the location of collection are shown for some isolates. Legend: BE - Belgium; BG - Bulgaria; IT - Italy; PT - Portugal; SP - Spain.

3.1.2.2. ITS, EF1-α and β-tubulin phylogeny

The aim of the multi-gene phylogeny was to provide better resolution of some of the groups of species obtained in the latter phylogenetic tree, namely the clade containing the species with dark conidia. The dataset included a selection of isolates of the dark-spored *Diplodia*, specifically *D. seriata*, *D. pinea*, *D. scrobiculata* and the presumed new species.

Nucleotide sequences of the ITS region and part of the EF1-α and β-tubulin genes were determined. Other sequences included in the analyses were retrieved from GenBank (Table 2.7). Sequences of the three genes were aligned and analysed separately by MP and NJ analyses, and the resulting trees were compared. No conflicts were detected between the single gene phylogenies indicating that the datasets could be combined.

For the multi-gene dataset (ITS, EF1-α and β-tubulin) the alignment of 25 isolates, including the two outgroup species, consisted of 526 characters for the ITS region, 284 characters for the EF1-α region, and 436 characters for the β -tubulin region, including alignment gaps. In the analyses, indels were coded separately and added to the end of the alignment as characters 1255 to 1271 and all alignment gaps were treated as missing data. Exclusions were made in positions 820, 821 and 1269 due to incomplete sequences in two of the GenBank accessions. The combined dataset contained 1257 characters, of which 10 were variable and parsimony uninformative and 1152 were constant. Maximum parsimony analysis of the remaining 95 characters resulted in two equally most parsimonious trees (tree length = 113 steps; CI = 0.956; RI = 0.968; HI = 0.044). The MP and NJ phylogenetic analyses generated trees with similar topologies. The NJ tree is presented in Figure 3.4 with NJ and MP bootstrap supports above and below the branches, respectively.

The clades observed in this tree are similar to those obtained in the two-gene tree. Within the ingroup taxa, four distinct clades could be identified, which correspond to the known species *D. seriata* (NJ bootstrap = 91 %; MP bootstrap = 90 %), *D. pinea* (NJ bootstrap = 81 %; MP bootstrap = 64 %), *D. scrobiculata* (NJ bootstrap = 98 %; MP bootstrap = 99 %) and the undescribed species represented by CAP 130, 150 and 273 (NJ bootstrap = 85 %; MP bootstrap = 74 %). Isolate CAP330 clustered apart from the other groups, in contrast to the two-gene analysis, and therefore could not be considered as belonging to any of the other species studied.

In this dataset, addition of partial nucleotide sequence of β-tubulin gene improved the resolution within this group of species. The clade containing the undescribed species was clearly separated from all the other clades included in the analyses. In both NJ and MP analyses, this clade was very well supported by bootstrap values of 85 % between isolates CAP150 and CAP273 and 85 % including isolate CAP130, in the NJ analysis and 88 % and 74 %, respectively, in the MP analysis.

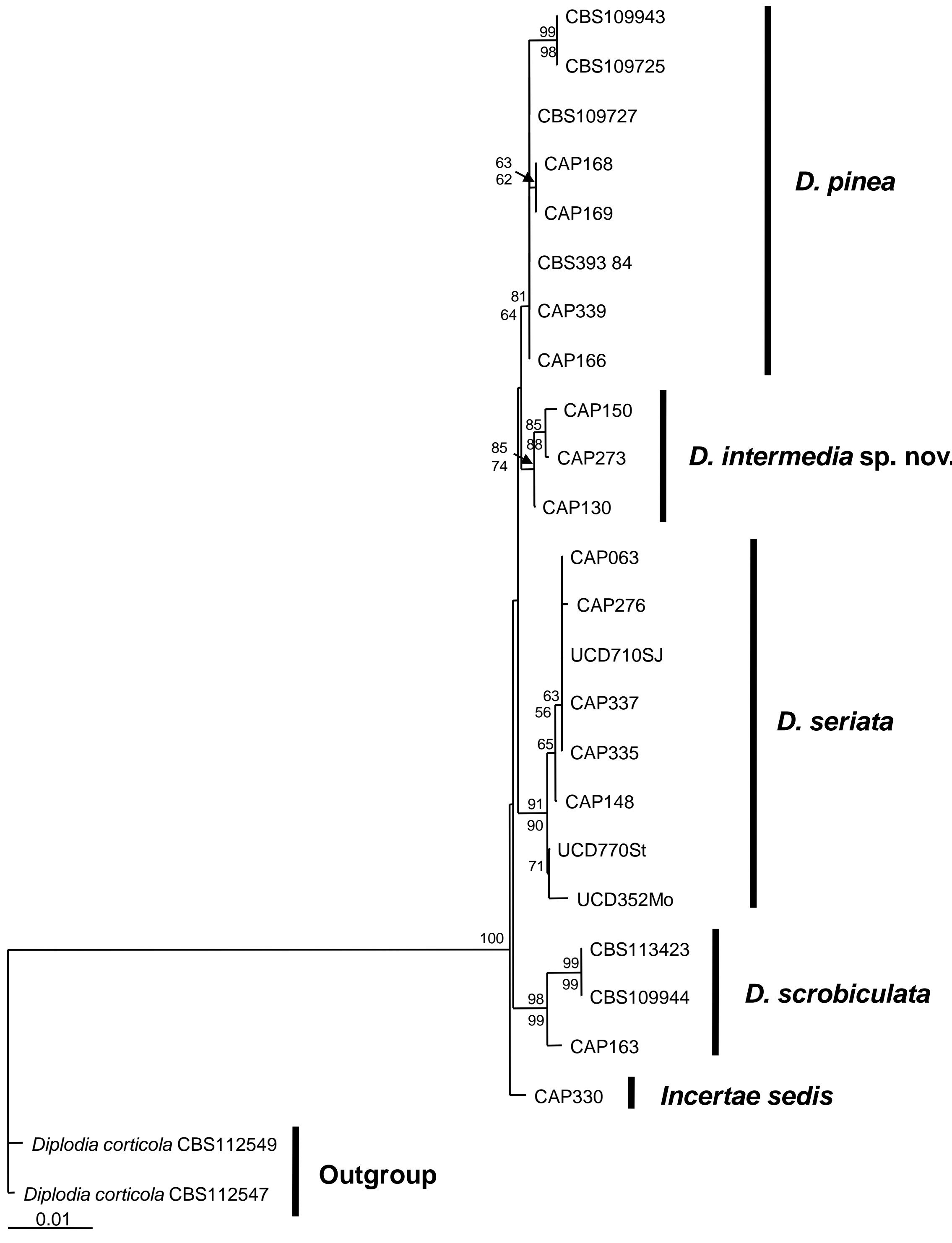

Figure 3.4 . Phylogenetic relationships among the *Diplodia seriata* and *Diplodia pinea* complexes of species, based on neighbour joining analysis of combined ITS, EF1-α and β–tubulin sequence data**.** Support for the branching points are given above (NJ bootstrap) and below (MP bootstrap) the nodes. The tree is rooted to *Diplodia corticola.*

3.2. Morphological characterization

Morphology of all the isolates corresponded with that commonly associated with the genus *Diplodia*.

3.2.1 Conidiomata

Most of the isolates formed conidiomata with mature conidia within 14–21 days of incubation on pine needles on water agar, at 25 °C.

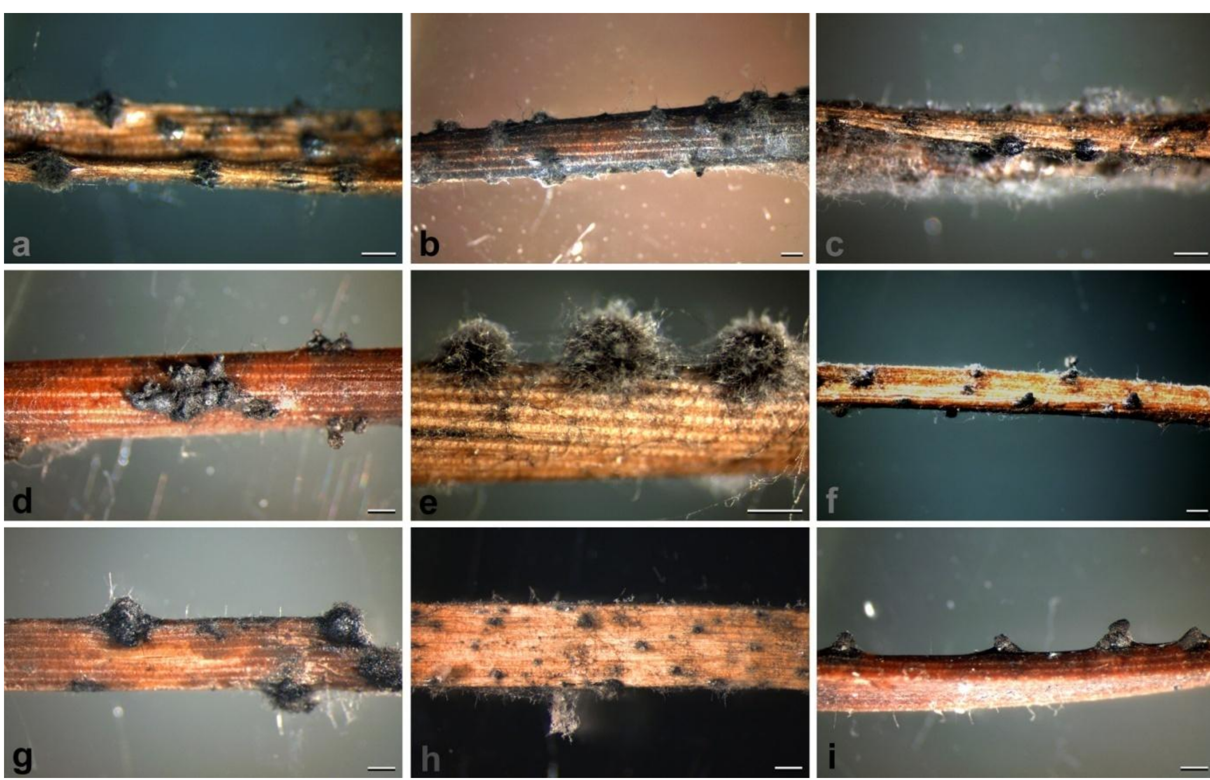

Figure 3.5. Conidiomata formed on pine needles of a) *D. fraxini* (JL375); b) *D. seriata* (CAP338); c) *D. pinea* (CAP168); d) *Diplodia* sp. (CAP330) e) *D. malorum* (CAP274); f) *D. intermedia* (CAP273); g) *D. olivarum* (CAP224); h) *D. scrobiculata* (CAP163); i) *D. bulgarica* (CAP333). Scale bars= 0,5 mm.

The conidiomata differed morphologically between the different species (Figure 3.5) displaying various sizes, shapes, degree of aggregation, color, amount of superficial mycelium, or whether they were superficial or immersed in the host tissue.

3.2.2. Conidia

Conidia from all the isolates that sporulated in culture, varied in shape, dimensions, color, texture and septation as shown in Figure 3.6.

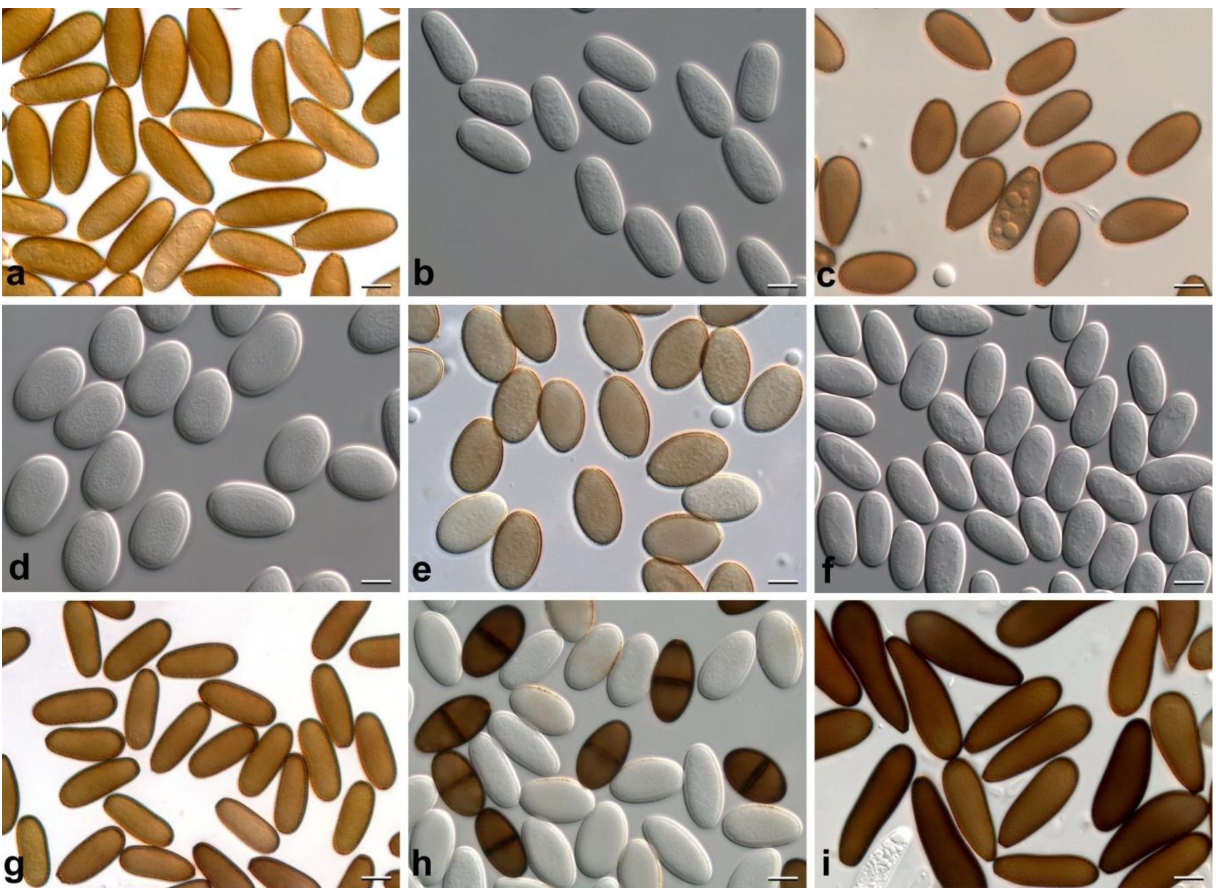

Figure 3.6. Conidia of the studied species a) *D. scrobiculata*; b) *D. mutila*; c) *D. intermedia*; d) *D.* fraxini; e) *D. bulgarica*; f) *D. olivarum*; g) *D. seriata*; h) *D. malorum* i) *D. pinea.* Scale bars: 10 μm.

All conidial measurements determined in this study, hyaline and dark, originated from host and culture, are presented, respectively, in Appendices I and II. Conidia displayed a wide range of dimensions between the different species studied.

For some isolates, a comparison of the dimensions of conidia obtained by sporulation in culture and directly from the host was also performed as in Appendix III. In Figure 3.7 are shown the mean values for length, width and length/width ratio of conidia from this selection of isolates.

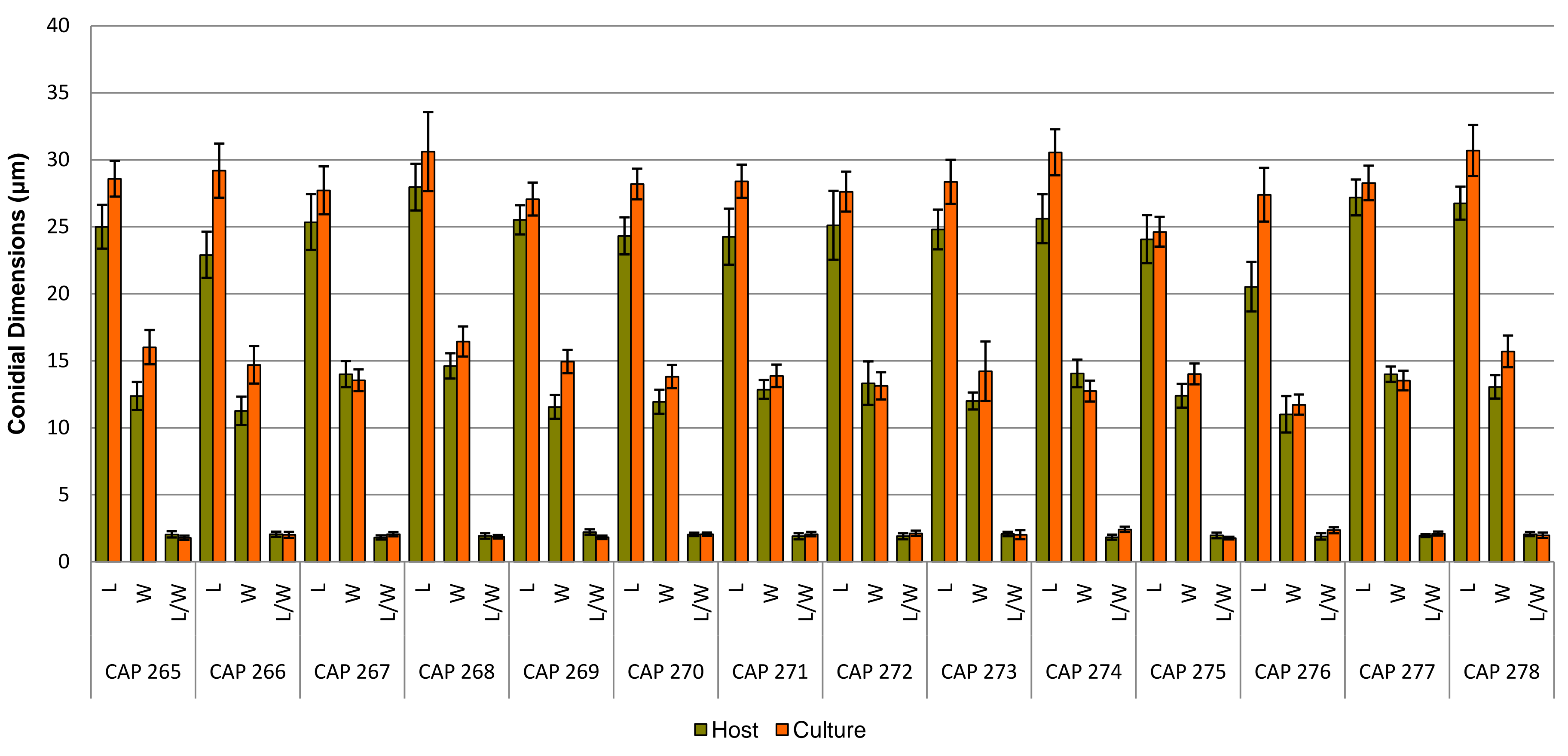

Figure 3.7. Mean values of conidial dimensions obtained from host and from culture, of some isolates of *D.intermedia* (CAP273), *D. seriata* (CAP276) and *D. malorum* (other isolates), collected from *Malus sylvestris*. L - length; W - width; L/W - length/width ratio. Error bars represent standard deviation.

This group of isolates, acquired from both host and culture, were obtained from the same host tree - *Malus sylvestris*. Some differences were observed between the isolates, especially on the mean values for both conidial length and width individually. It is apparent that the conidia produced in culture are always longer than the ones taken from the host, though nothing can be inferred for the width of the conidia as it is generally higher in most of the isolates, but lower in some.

The higher mean values of conidial length (in culture and in host) are found in isolates CAP268, CAP274 and CAP278 which are *D. malorum*. In contrast, *D. seriata* isolate CAP276 had the shortest conidia. The length/width ratio is very similar in all the isolates. Moreover, no other conclusions can be made regarding the different species in this group (*D. malorum*, *D. seriata* - CAP276 and *D. intermedia* - CAP273).

Figure 3.8 illustrates, for some of the studied isolates, the differences in conidial dimensions of conidia obtained directly from the host and from pure culture.

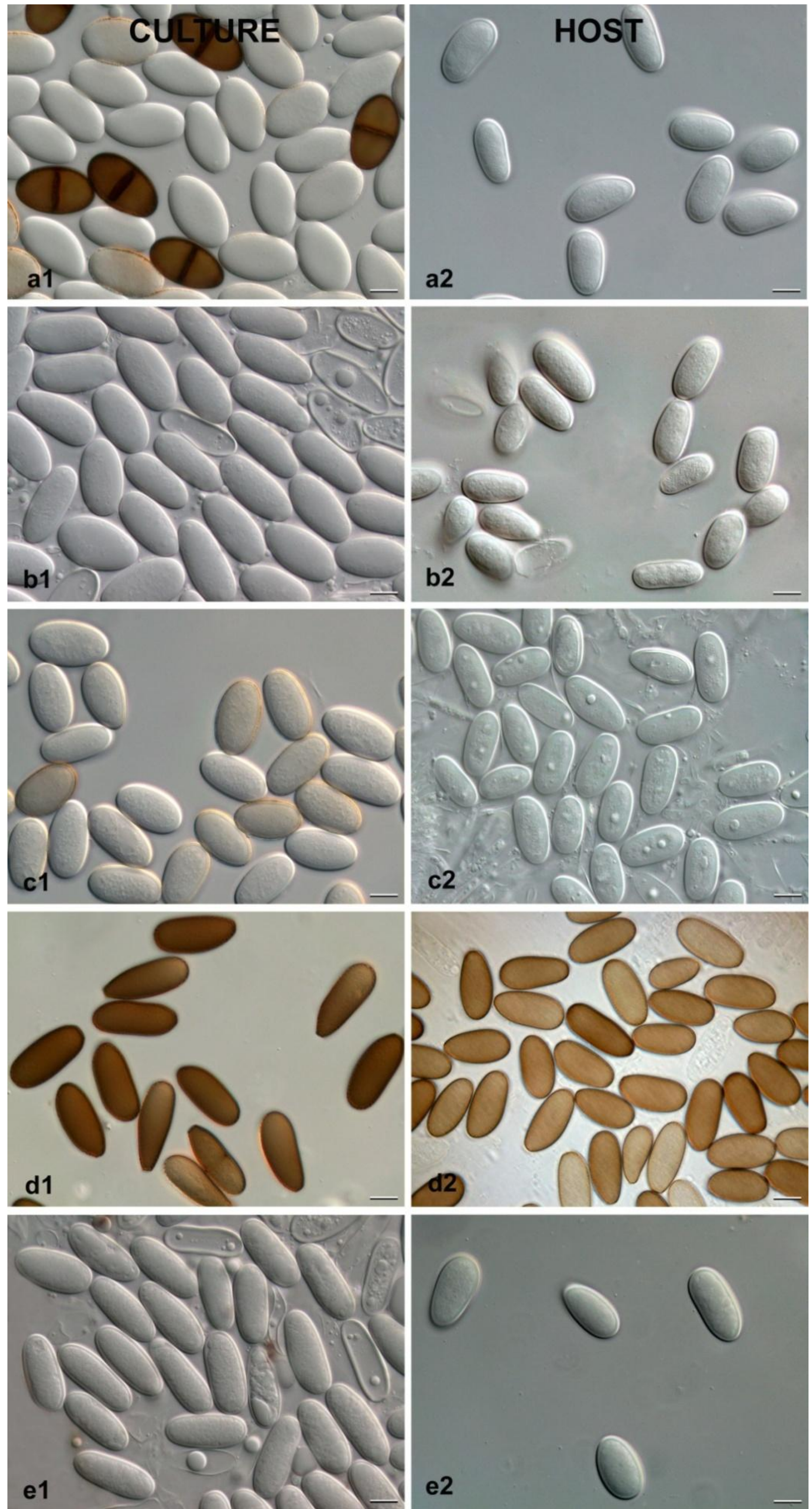

Figure 3.8. Comparison between the conidia of some isolates of *Diplodia* sp. obtained from culture (1) and from host (2). a) CAP265 (*D. malorum*); b) CAP266 (*D. malorum*); c) CAP270 (*D. malorum*); d) CAP273 (*D. intermedia*); e) CAP274 (*D. malorum*).

Regarding the nine species of *Diplodia* considered in this work, the mean values of conidial dimensions were studied, taking into account the variety of isolates belonging to each group. It is important to consider that the number of isolates studied for each species differed and, consequently, the number of conidia measured for each species was variable (Appendix VI). The same analysis is depicted in Figure 3.9.

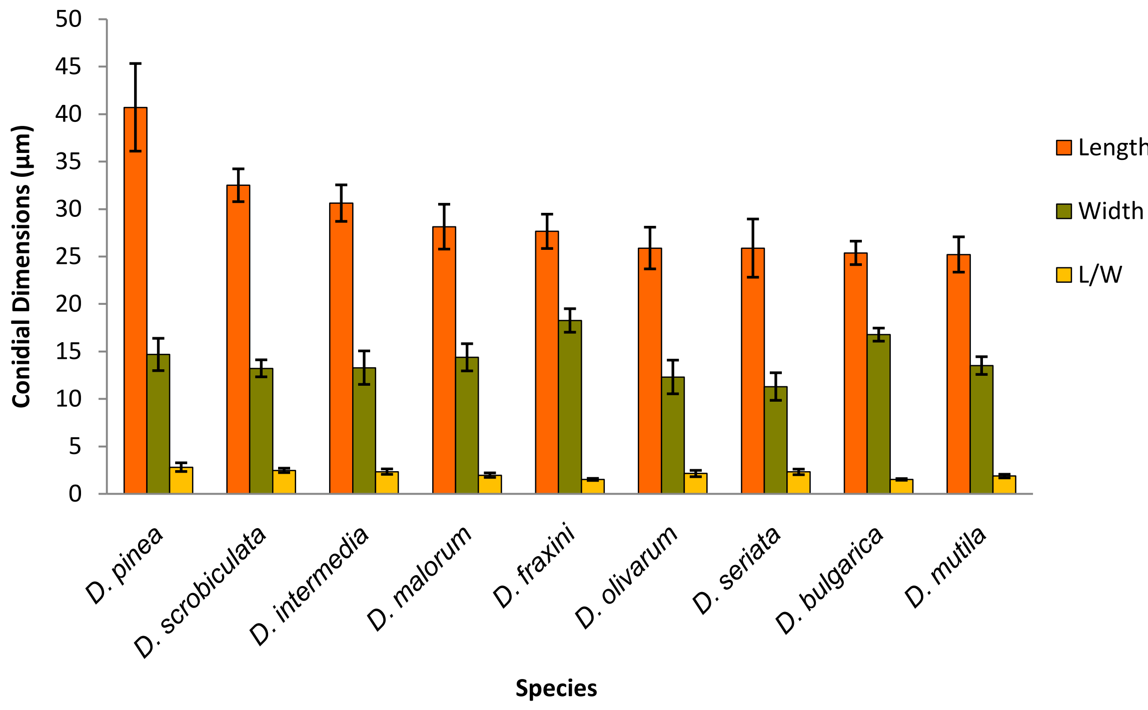

Figure 3.9. Comparison of the mean conidial dimensions of the studied species. Error bars represent standard deviation.

From the analysis of Fig. 3.9, a difference between the mean values of the dimensions of the isolates belonging to the *D. pinea* complex and the other species is noticeable. In this way, the longest conidia are seen in *D. pinea* isolates, which were approximately 41 µm long, followed by *D. scrobiculata* and *D. intermedia* with 32.5 and 30.6 µm, respectively. Conidia of *D. seriata* are the shortest, overlapping with species in the *D. mutila* group. Species belonging to the *D. mutila* group have short conidia, ranging between 25.2 µm in *D. mutila* and 28.1 µm in *D. malorum*. In terms of widths, *D. fraxini* has the widest conidia (18.3 µm) followed by *D. bulgaria* (16.8 µm). In contrast, the conidia of *D. olivarum* are much narrower (12.3 µm). Regarding the length/width ratios *D. bulgarica* and *D. fraxini* are the species with the lowest average values of 1.5 while *D. pinea* and *D. scrobiculata* have the highest ratios, 2.8 and 2.5, respectively.

3.2.3. Mycelial growth rate

Due to the intraspecific variability within this group of fungi and to ensure accurate, representative and informative results, all measurements were made under standardized conditions of media as well as the incubation conditions of the cultures.

Mycelial growth rates were determined for all isolates in this study and are presented in Appendix IV and in Table 3.1.

Table 3.1. Daily mycelial growth rates (mm) of the isolates in study.

Growth Rate in 24 hours (mm)					
ISOLATES	**G.R.**	**ISOLATES**	**G.R.**	**ISOLATES**	**G.R.**
CAP 062	11.2	**CAP 255**	15.2	**CAP 332**	7.5
CAP 063	15.2	**CAP 257**	13.3	**CAP 333**	9.0
CAP 129	14.3	**CAP 265**	13.8	**CAP 334**	8.7
CAP 148	16.8	**CAP 266**	12.8	**CAP 335**	13.5
CAP 150	9.0	**CAP 267**	14.3	**CAP 336**	19.7
CAP 154	14.2	**CAP 268**	13.8	**CAP 337**	12.0
CAP 160	14.7	**CAP 269**	13.3	**CAP 338**	13.8
CAP 163	5.2	**CAP 270**	12.8	**CAP 339**	11.2
CAP 166	12.7	**CAP 271**	15.3	**CAP 340**	11.0
CAP 167	8.3	**CAP 272**	12.3	**CAP 341**	11.5
CAP 168	14.7	**CAP 273**	10.0	**CAP 342**	14.7
CAP 169	14.3	**CAP 274**	13.8	**CAP 343**	7.0
CAP 170	15.8	**CAP 275**	13.5	**CAP 344**	5.7
CAP 171	16.5	**CAP 276**	14.8	**CAP 345**	13.3
CAP 172	13.7	**CAP 277**	13.2	**CAP 346**	15.5
CAP 207	13.7	**CAP 278**	4.0	**CAP 347**	14.2
CAP 208	14.0	**CAP 279**	15.7	**CAP 348**	6.3
CAP 221	12.2	**CAP 301**	13.2	**CAP 257**	15.3
CAP 222	13.7	**CAP 302**	10.8	**JL 375**	16.0
CAP 224	12.5	**CAP 326**	8.0	**JL 453**	17.0
CAP 225	14.3	**CAP 327**	6.2	**JL 515**	9.0
CAP 228	13.5	**CAP 328**	13.3	**JL 562**	17.2
CAP 229	15.0	**CAP 329**	6.2	**JL 574**	9.2
CAP 230	16.5	**CAP 330**	7.7	**JL 593**	7.5
CAP 235	14.5	**CAP 331**	9.8		

Figure 3.10 illustrates the daily mycelial growth rates for the nine species of *Diplodia.*

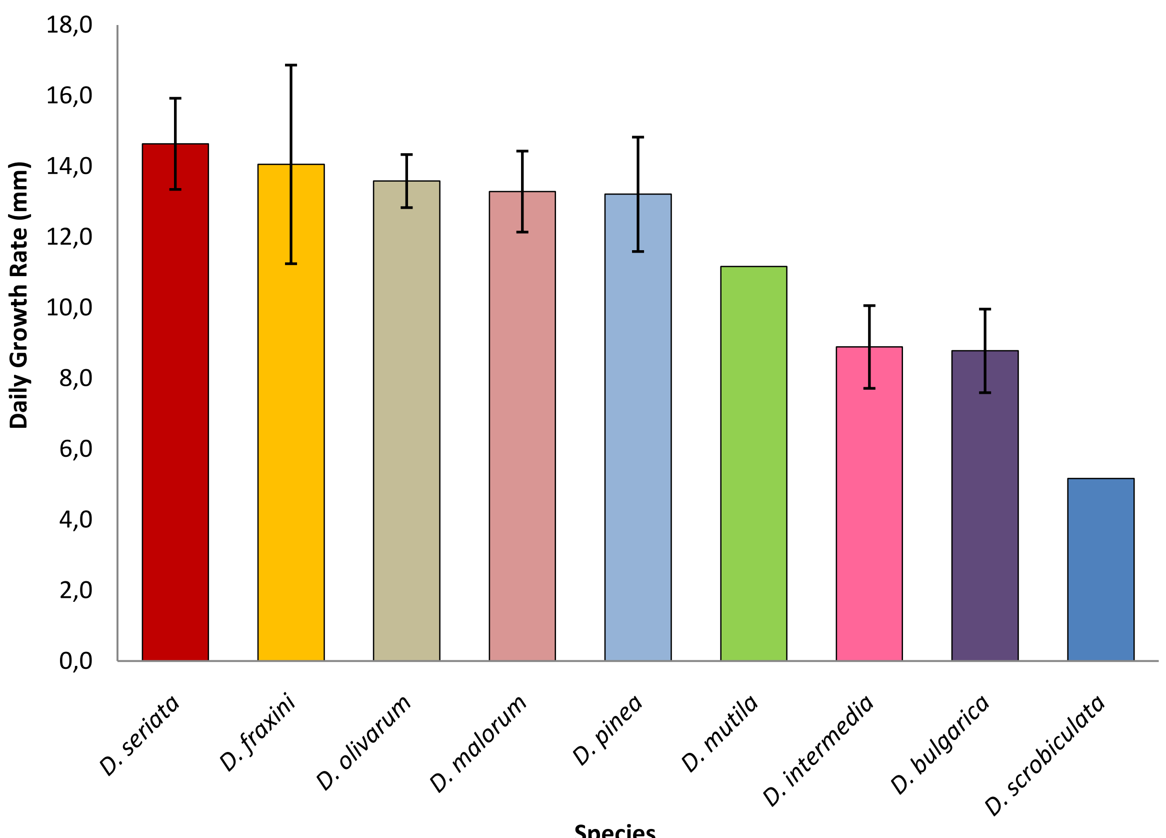

Figure 3.10. Daily mycelial growth rates (mm/day) at 25°C for the groups of species considered in this study. Error bars represent standard deviation.

When grouping the results of the mycelial growth regarding the different groups of species, it appears that the daily growth rates differed between the species of *Diplodia* at the temperature of 25°C. Isolates of *D. seriata* show the highest growth rate (14.6 mm/day) followed by isolates belonging to *D. fraxini* (14.1 mm/day) and *D. olivarum* (13.6 mm/day). Isolates of *D. malorum* and *D. pinea* have very similar growth rates (13.3 and 13.2 mm/day respectively) while *D. mutila* mycelium grows 11.2 mm/day. In contrast, isolates of *D. intermedia* (8.9 mm/day), *D. bulgarica* (8.8 mm/day) and *D. scrobiculata* (5.2 mm/day) are the species with slowest daily growth rates. However, mycelial growth of isolate CAP163, here representing *D. scrobiculata,* was atypically slow and it is believed that possibly this isolate could have been contaminated and, therefore, nothing will be concluded about this results for *D. scrobiculata.* It is significant to notice that both *D. mutila* and *D. scrobiculata* are based in one representative only.

Another approach taken into account in the mycelial growth rate analysis was the construction of growth curves and respective trendlines. Generally, all *Diplodia* species presented an almost linear relationship between mycelial growth (y) and time (x) (Appendix V). The linear regression equations are also shown as well as the correlation coefficient values ($R^2 \approx 1$), which indicate an excellent linear reliability. Based on the fact that each inoculum had the same dimensions in every measurement, time zero (0 hours) was considered for all the isolates as a mean for better graphic reading and comparison between the species.

As seen in Appendix V, *D. fraxini* and *D. pinea* show a slower growth in the first 24 hours. In this way and to maintain a better correlation between species. only the last 48h of growth were considered for these species.

In Table 3.2 it is clear that *D. fraxini* (0.59) and D*. seriata* (0.54) are the species that have faster growth, as shown by the higher slope values (m), whereas *D. bulgarica* (0.37), *D. intermedia* (0.37) and *D. scrobiculata* (0.23) exhibited slower growths. However, as stated above, the data for *D. scrobiculata* may not be reliable.

Table 3.2. Slope parameter (m) from the linear regression equations ($y = mx + b$) of the different species of *Diplodia* studied and respective correlation coefficient values (R^2).

Species	**Slope (m)**	**R^2**
D. fraxini	0.59	0.98
D. seriata	0.54	0.99
D. olivarum	0.50	0.99
D. malorum	0.49	0.99
D. mutila	0.46	0.99
D. pinea	0.43	0.97
D. intermedia	0.37	0.99
D. bulgarica	0.37	0.99
D. scrobiculata	0.23	0.99

Thus, based on the different linear regression equations, the growth of a species through time can be easily estimated.

3.3. Host association

In Figure 3.11 is represented the distribution of the species present in the collection and the number of isolates with respective hosts from which they were obtained.

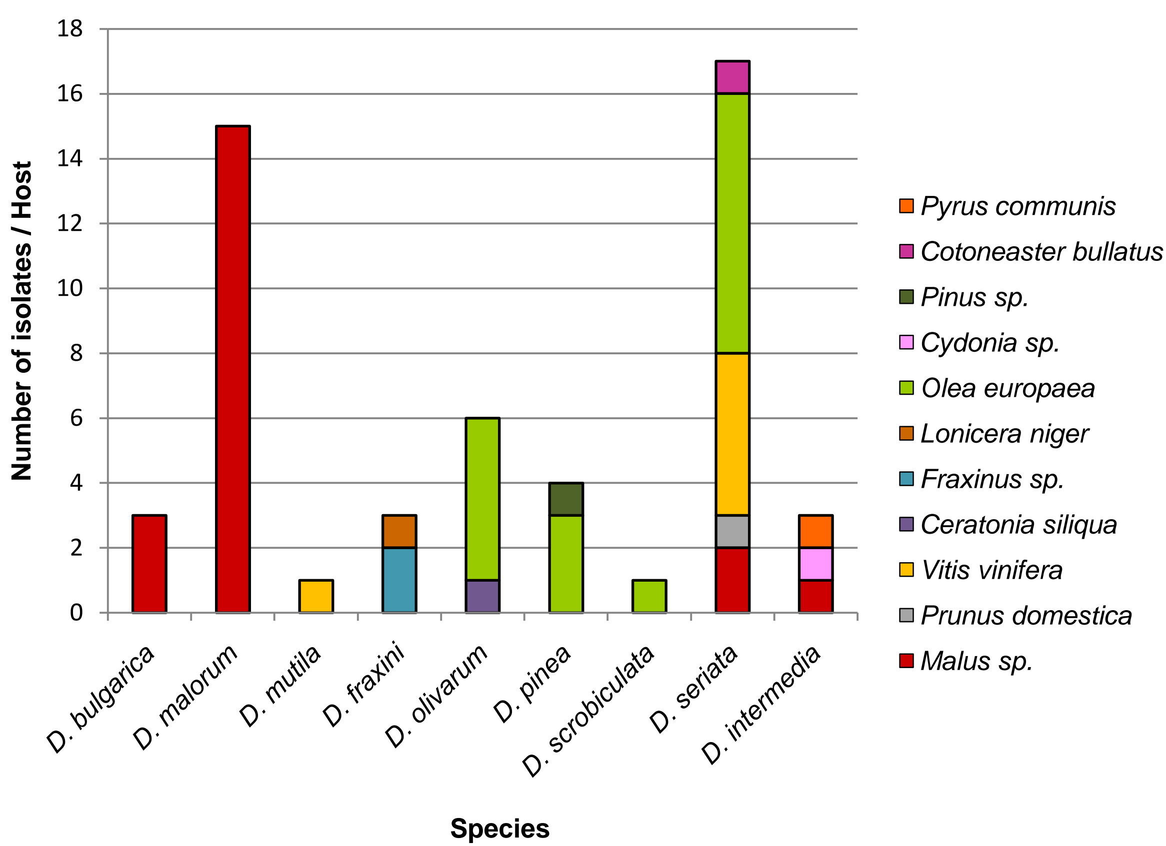

Figure 3.11. Variety of hosts from which the studied species and respective isolates were collected.

The small size of this particular collection and of the isolates representing each species are some of the features that must be taken into account while interpretating these data. Results presented here are generalistic and based exclusively on this sample collection. Thus no firm conclusions can be drawn in a specific matter and more strains from these and other hosts will have to be studied.

As depicted above and based on this specific collection of isolates some of the species in the collection are apparently generalistic and have a wide range of hosts like *Diplodia seriata* and *Diplodia intermedia*, while others as *Diplodia olivarum*, *Diplodia malorum* and *Diplodia bulgarica* seem to be restricted to one host only, respectively, *Olea europaea* and *Malus sp.*. In the case of *Diplodia pinea*, some of the isolates in the collection come from *Olea europaea* even though it is recognized as a specific inhabitant of *Pinus* sp..

All isolates within the group of *D. malorum* were obtained from *Malus* sp. trees, including *M. sylvestris* and *M. domestica* from Portugal, thus demonstrating host-specificity. Similarly, *D. bulgarica* was found only on *M. sylvestris* collected in Bulgaria.

All the isolates belonging to *D. intermedia* were from three different *Rosaceae* hosts, including *Malus* sp., *Cydonia* sp. and *Pyrus* sp.. These host species reside in the *Rosales* and *Rosaceae* and they are represented by one common sub-family, *Maloideae*. Moreover, they are all originally from Portugal.

The collection of isolates representing *D. seriata* showed a diverse range of hosts that includes angiosperms such as *Malus* sp., *Prunus domestica*, *Vitis vinifera*, *Olea europaea* and *Cotoneaster bullatus* collected in different countries such as Portugal, Italy and Bulgaria. Isolates within *D. olivarum* were from *Olea europeae*, similar to the work by Lazzizera *et al.* (2008), with the exception of one isolate which was collected from *Ceratonia siliqua*.

The *D. pinea* group contained one isolate from Bulgaria, from *Pinus* sp. a conifer host typical of this species (De Wet. 2008). and also three isolates from *Olea europaea* in Italy.

Diplodia fraxini is represented by three isolates, two of which were from *Fraxinus* sp. and the other from *Lonicera niger*.

Both *D. mutila* and *D. scrobiculata* were studied based on one isolate each. Thus, the hosts were *Vitis vinifera* and *Olea europaea*, respectively.

Since the isolates which represent *Diplodia bulgarica* and *Diplodia intermedia* were morphologically and phylogenetically distinct from the other species herein studied, they are described as new species in the present work.

3.4. Taxonomy

Diplodia mutila Fries in Montagne, *Ann. Sci. nat.*, sér. 2. 1: 302. 1834.(Fig. 3.12).

≡ *Sphaeria mutila* Fries, *Syst. Mycol.* 2: 424−425. 1823.

Teleomorph: "*Botryosphaeria*" *stevensii* Shoemaker, *Can. J. Bot.* 42: 1299. 1964.

= *Physalospora mutila* N. E Stevens, *Mycologia* 28: 333. 1936, as *Physalospora mutila* (Fries) N. E. Stevens comb. nov.

Synonyms of *D. mutila* are given by Stevens (1933).

The following description is based on CAP 062 = CBS 112553.

Conidiomata solitary or aggregated in clusters of up to five or more, immersed, partially erumpent when mature, dark brown to black, more or less globose, up to 600 μm diam, wall composed of three layers, an outer of dark brown, thick-walled *textura angularis*, a middle layer of dark brown thin-walled cells, an inner layer of thin-walled hyaline cells. *Ostiole* central, circular, papillate. *Conidiophores* absent. *Conidiogenous cells* 11.0−15.0 × 4.0−5.0 μm, holoblastic, discrete, cylindrical, hyaline, smooth, indeterminate, proliferating at the same level giving rise to periclinal thickenings, or proliferating percurrently to form one or two indistinct annellations. *Conidia* hyaline, aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded and rarely becoming pale brown and one-septate with age, (21.1−)24.7–25.7(–28.3) x (10.9−)13.3–13.8(−15.8) μm. $\bar{x}$ ± S.D. of 50 conidia = 25.2 ± 1.9 x 13.5 ± 0.9 μm. L/W ratio = 1.9 ± 0.2.

Specimens examined: **Portugal:** Alentejo, Montemor-o-Novo, *Vitis vinifera,* 1996, *A.J.L. Phillips* (CAP062) (epitype will be designated; culture ex-epitype CBS 112553 = CAP062).

Note - An epitype and respective culture ex-epitype CBS 112553 (CAP062) will be designated for *D. mutila* and representatives have already been sent to the CBS culture collection and CBS herbarium. This isolate (CAP062) conforms in all ways to the type material of *D. mutila* as described by Alves *et al.* (2004). Furthermore, it has been adopted unofficially as representative of the species as, for example, in the work of Damm *et al.* (2007).

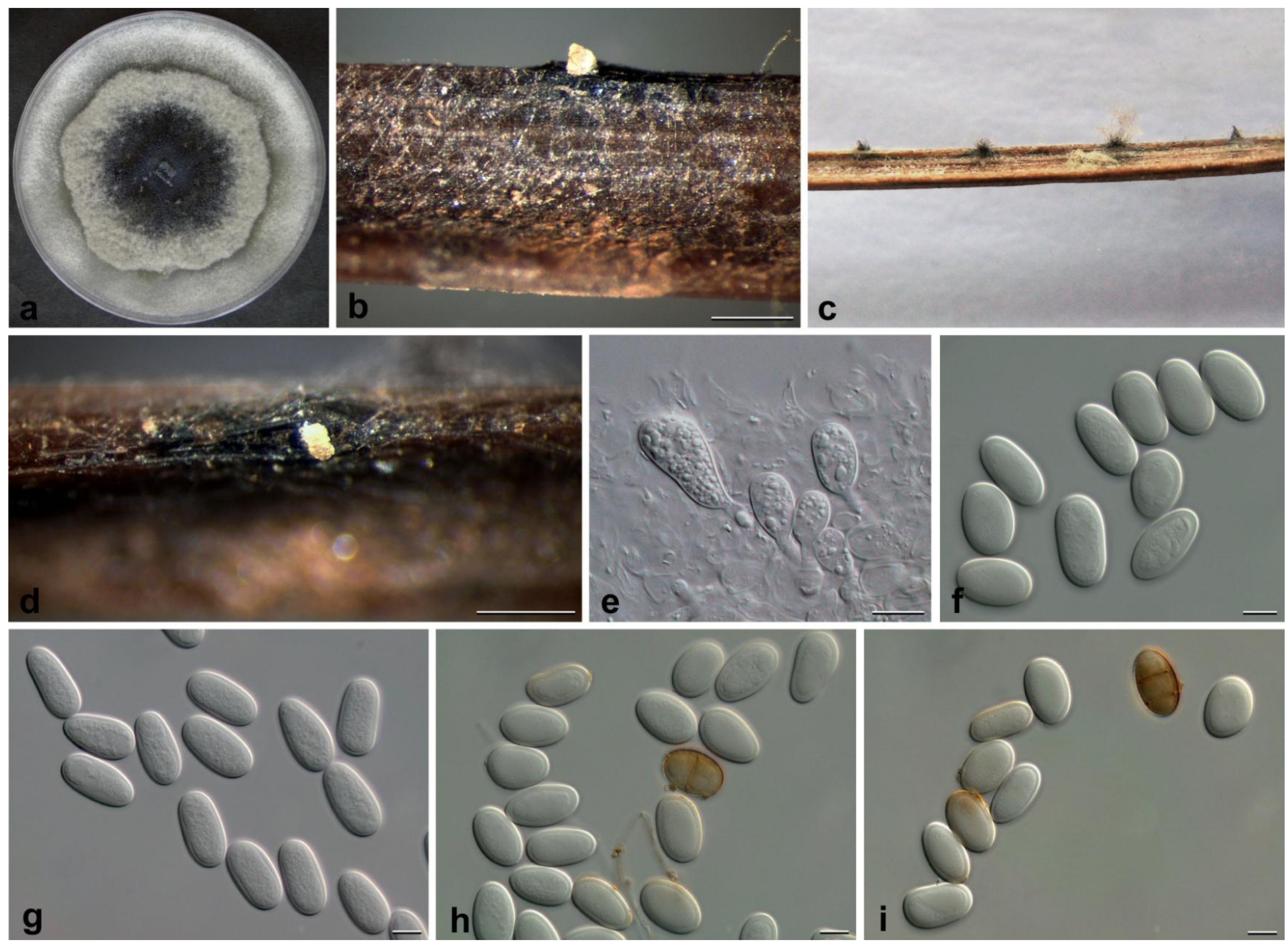

Figure 3.12. *Diplodia mutila*. **a)** Colony characteristics on potato dextrose agar. **b – d)** Conidiomata formed on pine needles in culture; with emerging conidia **(b, d)**. **e)** Conidiogenous cells. **f – i)** Conidia; hyaline, aseptate **(f, g);** hyaline, aseptate, with one- and two-septate brown conidia **(h, i)**. Scale bars: b, d = 0.5 mm; e – i =10 μm.

Diplodia olivarum A.J.L. Phillips, Frisullo & Lazzizera, *Fungal Diversity* 31: 67. 2008 (Fig. 3.13)

Conidiomata pycnidial, solitary, globose to ovoid, dark brown to black, wall composed of dark brown, thick-walled textura angularis, becoming thin-walled and hyaline towards the inner region, semi-immersed to erumpent, unilocular, with a short neck. *Ostiole* circular, central. *Conidiophores* hyaline, cylindrical, *Conidiogenous cells* hyaline, cylindrical, holoblastic forming a single conidium at the tip. proliferating internally to form periclinal thickenings or proliferating percurrently giving rise to 2–3 annellations. *Conidia* hyaline, aseptate, smooth, thick-walled, oblong to oval, widest in the middle, apex broadly rounded, base rounded or truncate, rarely becoming pale brown, internally verruculose, one-septate after discharge from the pycnidia, (20.8–)25.6–26.2(–31.1) x (8.3–)12.1–12.5(–16.9) µm. $\bar{x}$ ± S.D. of 250 conidia = 25.9 ± 2.2 x 12.3 ± 1.8 µm. L/W ratio = 2.1 ± 0.3.

Specimens examined: **Italy:** Puglia. Lecce: Cutrofiano. *Olea europaea.* 2004. *S. Frisullo* (CAP222). Salice salentino. *Olea europaea.* 2004. *S. Frisullo* (CAP224). Campi Salentino. *Olea europaea.* 2004. *S. Frisullo* (CAP225). Puglia: Brindisi. San Pietro Vernotico.

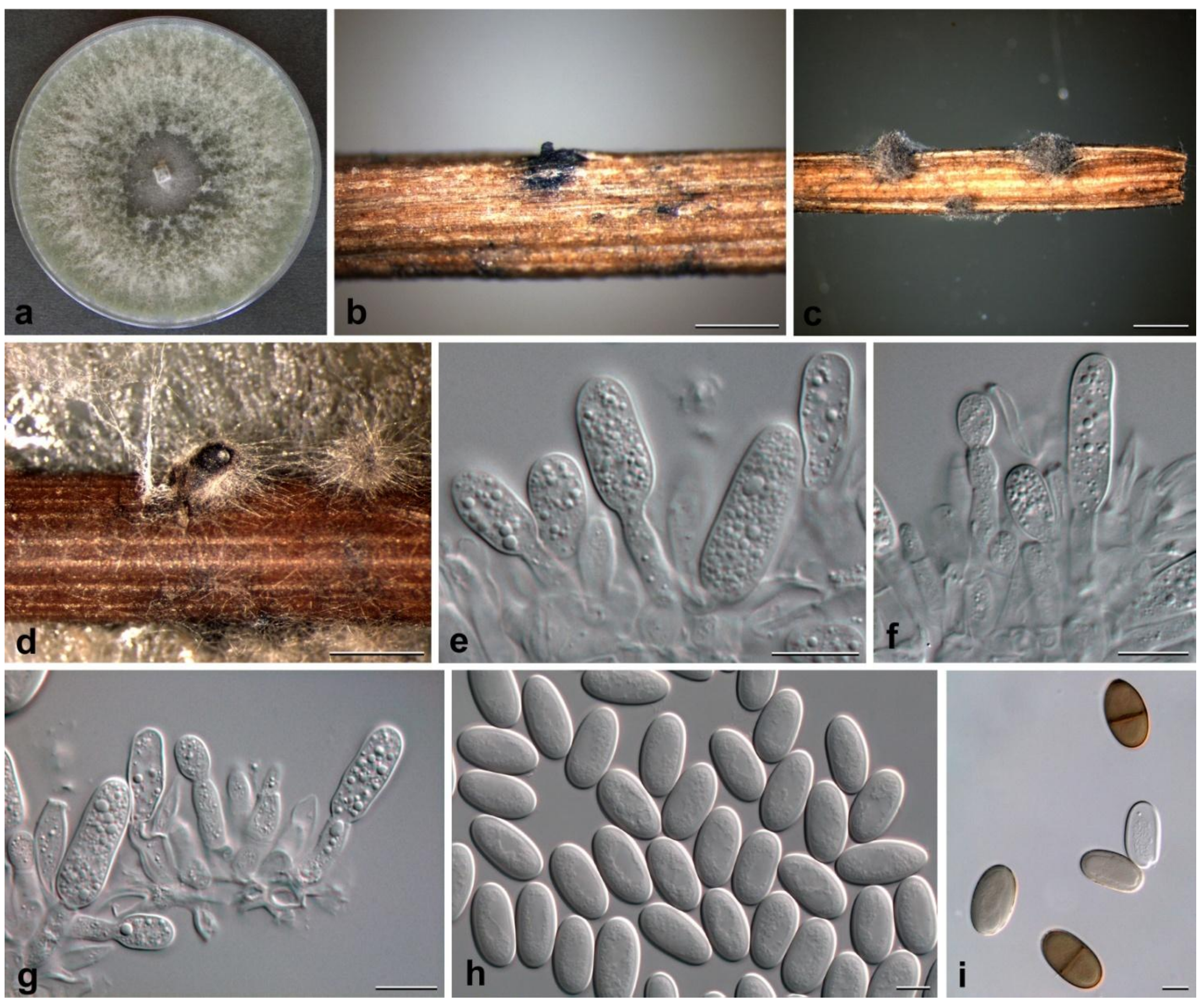

Figure 3.13. *Diplodia olivarum*. **a)** Colony characteristics on potato dextrose agar. **b – d)** Conidiomata formed on pine needles in culture; with emerging conidia **(d)**; **e – g)** Conidiogenous cells with developing conidia. **h, i)** Conidia; hyaline and aseptate conidia **(h)**; pale brown aseptate and one-septate conidia **(i)**. Scale bars: b = 0.5 mm; c, d = 1 mm; e – i =10 µm.

Diplodia malorum Fuckel. *Symb. Mycol.*: 395. 1870. (Fig. 3.14)

Conidiomata pycnidial, immersed, erumpent, dark brown to black, aggregated, internally white, ostiolate, ostiole circular, central, short papilla, *Conidiophores* absent. *Conidiogenous cells* cylindrical, thin-walled, hyaline, holoblastic, indeterminate, proliferating at the same level to produce periclinal thickenings, or proliferating percurrently giving rise to 2-3 or more indistinct annelations. *Conidia* oblong with broadly rounded ends, smooth-walled, thick-walled, hyaline, eguttulate aseptate, sometimes becoming pale brown with age and then dark brown and one-septate, three-septate rarely, (21.3–)27.8–28.3(–36.1) x (11.1–)14.3 –14.5(–19.2) µm. $\bar{x}$ ± S.D. of 700 conidia = 28.1 ± 2.4 x 14.4 ± 1.4 µm. L/W ratio = 2.0 ± 0.2.

Specimens examined: **Portugal:** Setúbal, Monte da Caparica, *Malus sylvestris*, 2002, *A.J.L. Phillips* (CBS112554 = CAP129). Monte da Caparica, *Malus sylvestris*, 2006, *A.J.L. Phillips* (CAP265, CAP266, CAP267, CAP268, CAP269, CAP270, CAP271, CAP272, CAP274, CAP275, CAP277, CAP278). Monte da Caparica, *Malus sylvestris*, 2007, *A.J.L. Phillips* (CAP340. CAP341). **Germany:** Hieme, on rotting fruit of *Malus* sp., *Fuckel*, 1870?, Fuckel Fungi Rhenani 1706, in M and K(M) 104760 (isotypes).

Note: After examination of the isotypes of *D. malorum* in Kew and Munich. it was apparent that the specimens observed here. obtained from Portugal. corresponded in all ways with the morphology of the isotypes. A dried specimen of isolate CAP274 will be designated as epitype and resulting culture as ex-epitype. Representatives have already been sent to the culture collection and herbarium of CBS.

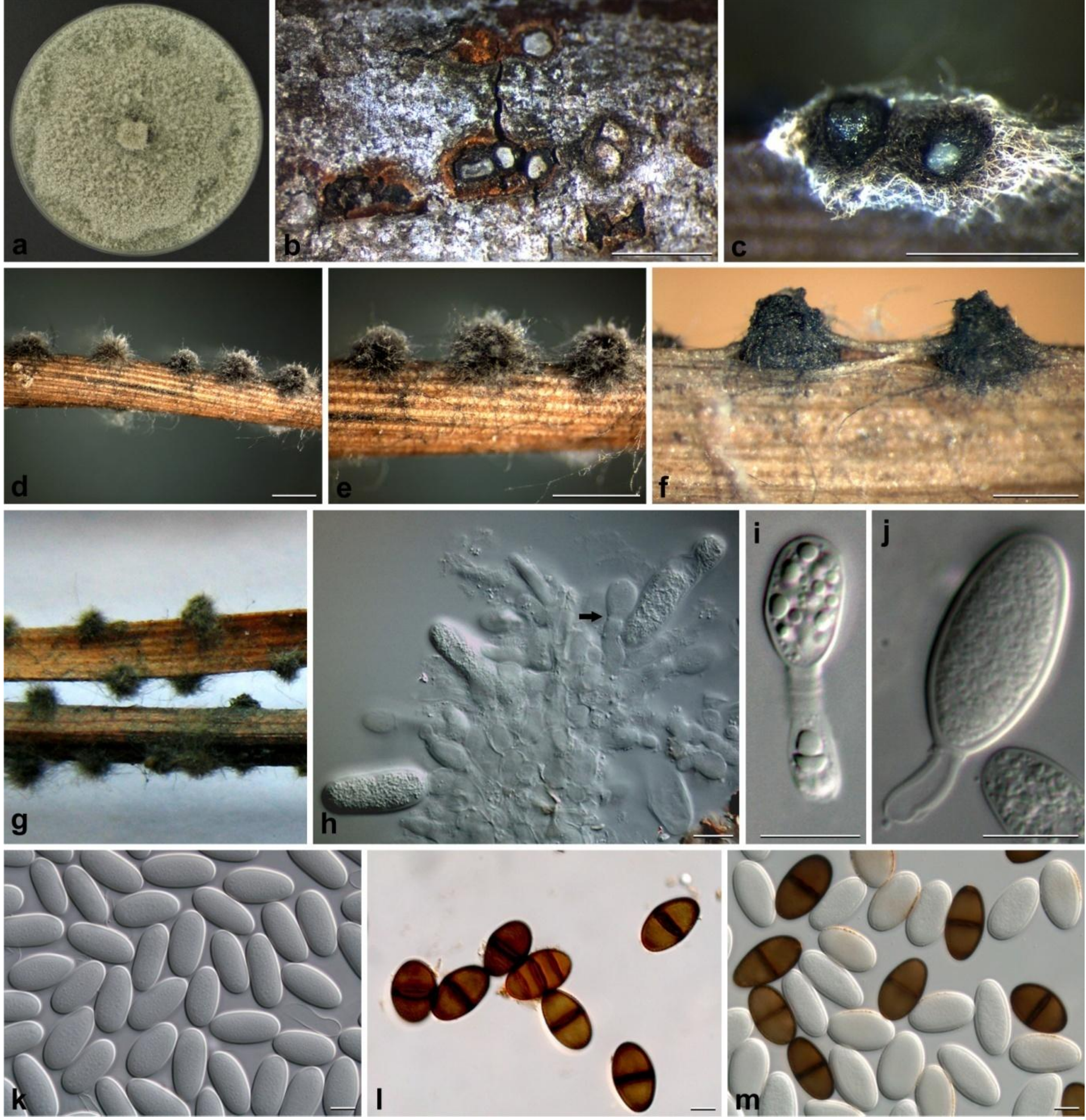

Figure 3.14. *Diplodia malorum.* **a)** Colony characteristics on potato dextrose agar. **b)** Conidiomata cut through horizontally showing a mass of conidia **c – g)** Conidiomata formed on pine needles in culture; with conidia oozing from a pycnidium **(d)**; **h – j)** Conidiogenous cells with developing conidia; conidiogenous cell that proliferated internally forming periclinical thickenings in a first stage, then made a single percurrent proliferation forming an annelation and lastly reverted to internal proliferation (black arrow) **(h)**; with several annelations **(i);** with one annelide **(j)**. **k – m)** Conidia; hyaline, aseptate conidia **(k);** brown, one or three-septate **(l);** hyaline aseptate and brown one-septate **(m).** Scale bars: b – g = 0.5 mm; h – m = 10 μm.

Diplodia bulgarica A.J.L. Phillips, J. Lopes & S.G. Bobev **sp. nov.** (Fig. 3.15).

This species will be formally described in a separate publication and the description here should not be cited.

Etym: in reference to the country were it was first found, Bulgaria.

Conidiomata pycnidial, produced on pine needles on WA after 7–21 days, solitary, immersed, partially erumpent when mature, dark brown to black, globose to ovoid, up to 600 µm diam and 700 µm high, wall composed of an outer of dark brown, thick-walled *textura angularis*, a middle layer of dark brown thin-walled cells, an inner layer of thin-walled hyaline cells, sometimes producing several small conidiomata all over the surrounding media. *Ostiole* central, circular, papillate. *Conidiophores* absent. *Conidiogenous cells* 9.0−18.0 × 2.0−5.0 µm, hyaline, cylindrical, holoblastic, forming a single conidium at the tip, discrete, smooth, indeterminate, proliferating internally giving rise to periclinal thickenings, or proliferating percurrently to form 1−5 annellations. *Conidia* aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded, initially hyaline but becoming pale brown soon after discharge from pycnidia and occasionally one-septate, (22.4−)25.0−25.7(−28.2) × (14.5−)16.6−17.0(−18.4) µm. $\bar{x}$ ± S.D. of 50 conidia = 25.4 ± 1.2 × 16.8 ± 0.7 µm. L/W ratio= 1.5 ± 0.1.

Specimens examined: **Bulgaria**: Plovdiv, *Malus sylvestris*, 2005, *S.G. Bobev* (CAP331, CAP332 and CAP333) (epitype will be designated; culture ex-epitype CAP333).

Figure 3.15. *Diplodia bulgarica*. **a)** Colony characteristics on potato dextrose agar. **b – f)** Conidiomata formed on pine needles in culture; with emerging conidia **(c, d)**; **g, h)** Conidiogenous cell with annelations in two different focal planes. **i, j)** Conidiogenous cells with developing conidia. **k, j)** Conidia; brown, aseptate conidia and one hyaline aseptate conidium **(k)**; brown, aseptate conidia and one dark, one-septate conidium **(l)**. Scale bars: b – f = 0.5 mm; g, h = 5 µm; i – l =10 µm.

Diplodia fraxini Fries, *Sum. veg. Scand.* 2: 417 (1849). (Fig. 3.16)

Conidiomata solitary, immersed in the host, dark brown to black, globose to ovoid, up to 560 µm diam and 400 µm high, wall composed of three layers, an outer of dark brown, thick-walled *textura angularis*, a middle layer of dark brown thin-walled cells, an inner layer of thin-walled hyaline cells. *Ostiole* central, circular, papillate. *Conidiogenous cells* 11.0−25.0 × 3.0−9.0 µm, holoblastic, discrete, cylindrical, hyaline, smooth, indeterminate, proliferating at the same level giving rise to periclinal thickenings, or proliferating percurrently to form one or two indistinct annellations. *Conidia* hyaline, aseptate, smooth, thick-walled, ovoid, straight, both ends broadly rounded, rarely becoming pale brown and septate when aged, (23.8−)27.4−27.9(−32.8) x (15.5−)18.1−18.5(−22.1) µm. $\bar{x}$ ± S.D. of 150 = 27.7 ± 1.8 × 18.2 ± 1.2 µm. L/W ratio = 1.5 ± 0.1.

Specimens examined: **Italy**: Sicily, *Fraxinus ornus*, 2006, *A. Sidoti* (CAP302). **Spain**: Cataluña, *Fraxinus excelsior*, (no date), *J. Luque* (JL375). **Spain**: Cataluña, *Lonicera niger*, (no date), *J. Luque* (JL453).

Note: The name *D. fraxini* was considered to be suitable for these isolates since two of them were from *Fraxinus* sp.. The type specimen could not be located during the course of this study and thus the identification could not be confirmed. Three isolates have been sent to the culture collection of CBS. This species is distinctive in the relatively wide conidia and L/W ratio of 1.5.

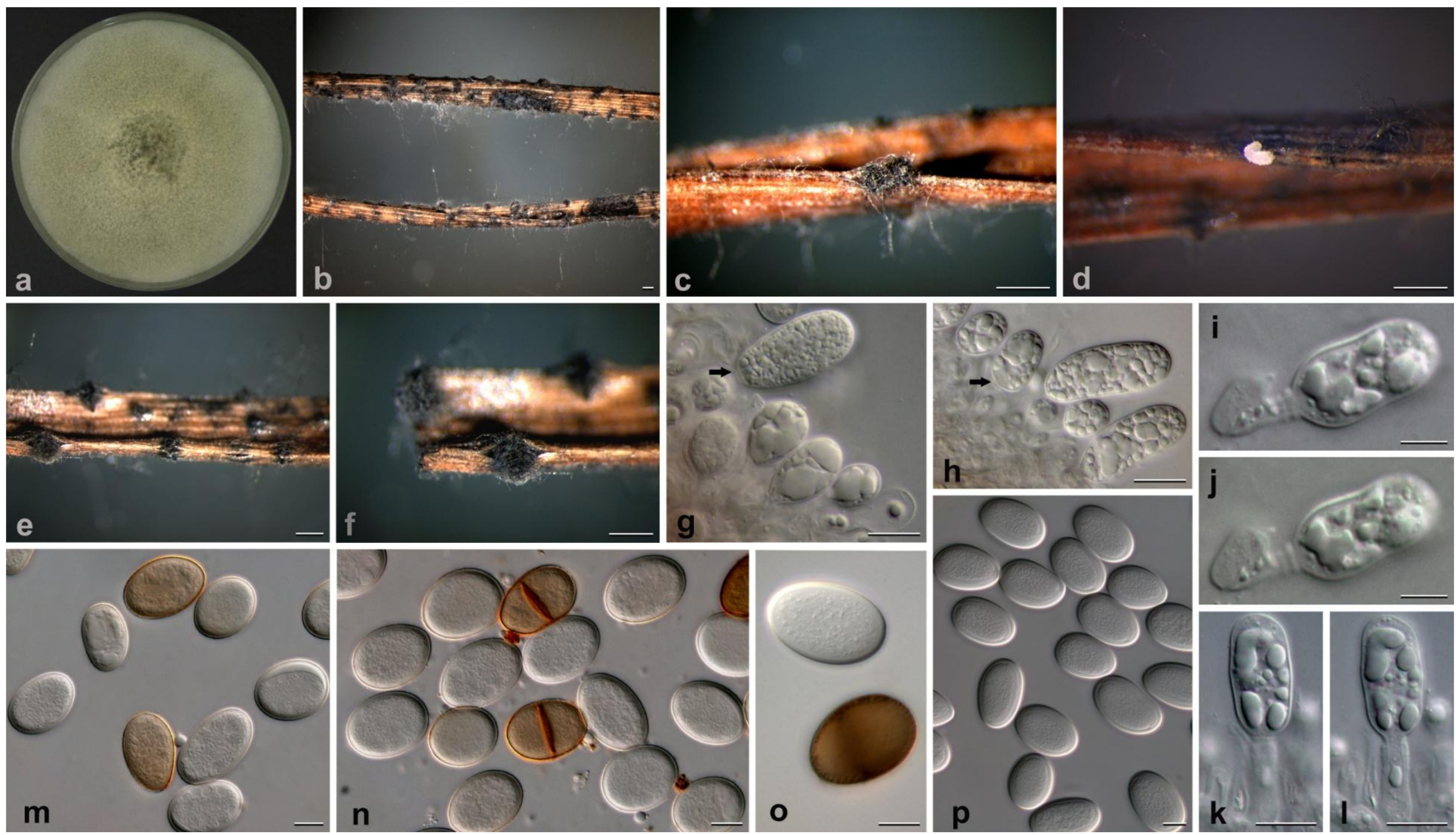

Figure 3.16. *Diplodia fraxini.* **a)** Colony characteristics on potato dextrose agar. **b – f)** Conidiomata formed on pine needles in culture; with conidia oozing from a picnidium **(f)**; **g – l)** Conidiogenous cells with developing conidia and with annelations (black arrow) **(g, h)**; at two different levels of focus, percurrently proliferating to form one or two annelides **(i – l)**. **m – p)** Conidia; brown and hyaline, aseptate conidia **(m)**; hyaline aseptate conidia and brown one-septate conidia **(n, o)**; hyaline aseptate conidia (**p)**. Scale bars: b – f = 0.5 mm; g – p = 10 μm.

Diplodia seriata De Not., *Mirom. Ital. Dec.* IV No 6. 1842. (Fig. 3.17)

= *Diplodia profusa* De Not., *Microm. Ital. Dec.* IV. no 8. 1842.
= *Diplodia pseudodiplodia* Fuckel, *Jb. Nassau. Ver. Naturk.* 23-24: 393. 1870.

Conidiomata pycnidial, separate or aggregated and confluent, immersed in the host, partially emergent at maturity, dark brown to black, ostiolate, nonpapillate, thick-walled, outer layers composed of dark brown *textura angularis*, inner layers of thin-walled hyaline *textura angularis*. *Conidiogenous cells* hyaline, thin-walled, smooth, cylindrical, swollen at the base, discrete, producing a single conidium at the tip, indeterminate, proliferating internally giving rise to periclinal thickenings or proliferating percurrently forming 2–3 annelations. *Conidia* aseptate, ovoid, widest in the middle, with obtuse apex and truncate or rounded base, initially hyaline but soon becoming dark brown and rarely one-septate before being released from the pycnidia, wall moderately thick, with a smooth outer surface and roughened on the inner suface, (17.7–)25.5–26.3(–33.4) × (8.1–)11.1–11.5(–16) µm. $\bar{x}$ ± S.D. of 250 conidia = 25.9 ± 3.1 × 11.3 ± 1.5 µm. L/W ratio= 2.3 ± 0.3.

Specimens examined: **Portugal:** Alentejo, Montemor-o-Novo, *Vitis vinifera,* 1996, *A.J.L. Phillips* (epitype CBS-H 19809; culture ex-epitype CBS 112555 = CAP063). Setúbal, Monte da Caparica, *Malus* sp., 2006, *A.J.L. Phillips* (CAP276). **Italy**: Puglia, Brindisi, *Olea europaea*, 2004, *S. Frisullo* (CAP255). **Bulgaria**: Plovdiv, *Prunus domestica*, 2004, *S. Bobev* (CAP328). Plovdiv, *Malus sylvestris*, 2006, *S. Bobev* (CAP335). *Vitis vinifera*, 2006, *S. Bobev* (CAP338).

Figure 3.17. *Diplodia seriata*. **a)** Colony characteristics on potato dextrose agar. **b)** Conidiomata cut trhough horizontally showing a mass of conidia, on the host. **c, d)** Conidiomata formed on pine needles in culture. **e – g)** Conidiogenous cells. **h – k)** Conidia; brown aseptate and two one-septate conidia **(h)**; brown aseptate conidia **(i – k)**. Scale bars: b **–** d = 0.5 mm; e – k =10 µm.

Diplodia pinea (Desm.) J. Kickx f., *Flora Crypt. Flandres* 1: 397. 1867. (Fig. 3.18)

= *Sphaeria pinea* Desm. 1842.

= *Sphaeropsis sapinea* (Fr. : Fr.) Dyko & Sutton in Sutton, *The Coelomycetes*, 120. 1980.

= *Sphaeria sapinea* Fr., *Systema mycologicum* 2: 491. 1823.

Conidiomata pycnidial, immersed, separate or aggregated, globose, dark brown, unilocular, thick-walled, wall composed of thin-walled textura angularis. Ostiole central, circular, papillate. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* formed from the cells lining the inner wall of the pycnidium, long lageniform, hyaline, holoblastic, discrete, determinate or indeterminate proliferating percurrently giving rise to annelations or internally to form periclinal thickenings. *Conidia* brown, oblong to clavate, straight, thick-walled, roughened on the inner surface of the wall, apex obtuse, base truncate, aseptate but sometimes becoming 1 or 2-septate, (30.0–)40.1–41.4(–54.6) × (10.6–)14.4–14.9(–21.0) µm. $\bar{x}$ ± S.D. of 200 conidia = 40.7 ± 4.6 × 14.7 ± 1.7 µm. L/W = 2.8 ± 0.5.

Specimens examined: **Italy:** Basilicata, Matera, Scanzano, *Olea europaea,* 2001, *S. Frisullo* (CAP166). Puglia, Lecce, Scorano, *Olea europaea,* 2001, *S. Frisullo* (CAP168). Cutofiano, *Olea europaea,* 2001, *S. Frisullo* (CAP169).

Notes - Three morphotypes (A, B and C) have been described for the species *Diplodia pinea* (Wang *et al.*, 1985; Palmer, Stewart & Wingfield, 1987). The A morphotype has fluffy mycelium and smooth conidia. In the B morphotype the mycelium is appressed and the conidial walls are pitted. In the C morphotype, the mycelium is fluffy, conidia are smooth but considerably larger than in the A morphotype (de Wet *et al.*, 2000).

Although an I morphotype, intermediate between the A and the B morphotypes, has been described (Hausner *et al.*, 1999), subsequent studies by Burgess, Wingfield & Wingfield (2001) showed that this fungus represents *Diplodia seriata*. Through a study of multiple gene genealogies and microsatellite markers, de Wet *et al.* (2003) showed that the B morphotypes are distantly related to *D. pinea* and represent a discrete taxon, which they described as *Diplodia scrobiculata*.

Figure 3.18. *Diplodia pinea*. **a)** Colony characteristics on potato dextrose agar. **b, c)** Conidiomata formed on pine needles in culture; brown conidia emerging from pycnidia **(c)** **d – g)** Conidiogenous cells; with annelations **(e, f)**. **h – k)** Brown aseptate conidia; two levels of focus show roughening on the inner face of the wall **(j, k)**. Scale bars: b, c = 0.5 mm; d – k =10 µm.

Diplodia scrobiculata J. de Wet, B. Slippers & M. J. Wingfield, *Mycol. Res*. 107 (5): 557-566. 2003 (Fig. 3.19)

Conidiomata pycnidial, covered in mycelium, dark, immersed in pine needles or in the agar, (100–) 150 (–250) µm diameter, single, papillate ostiole. *Conidiogenous cells* discrete, dark, smooth, 10 mm in diameter, holoblastic with limited percurrent proliferation seen as small number of annellations. *Conidia* clavate to truncate, brown, straight, thick-walled, apex obtuse, base truncate, aseptate, (28.9–)32.0–33.0(–37.4) × (10.7–)13.0–13.5(–15.1) µm. $\bar{x}$ ± S.D. of 50 conidia = 32.5 ± 1.7 × 13.2 ± 0.9 µm. L/W ratio = 2.5 ± 0.2.

Specimen examined: **Italy:** Puglia, Lecce, Supersano, *Olea europaea,* 2000, *S. Frisullo* (CAP163).

Note - Phylogenetically, isolate CAP163 is very closely related to *D. scrobiculata*. Furthermore, this isolate has some morphological differences that separate it from typical isolates of *D. scrobiculata*, namely smaller conidia. This may either reflect the deggree of variability within this species or that isolate CAP163 may represent a recently evolved lineage of Diplodia that could be regarded as a distinct species.

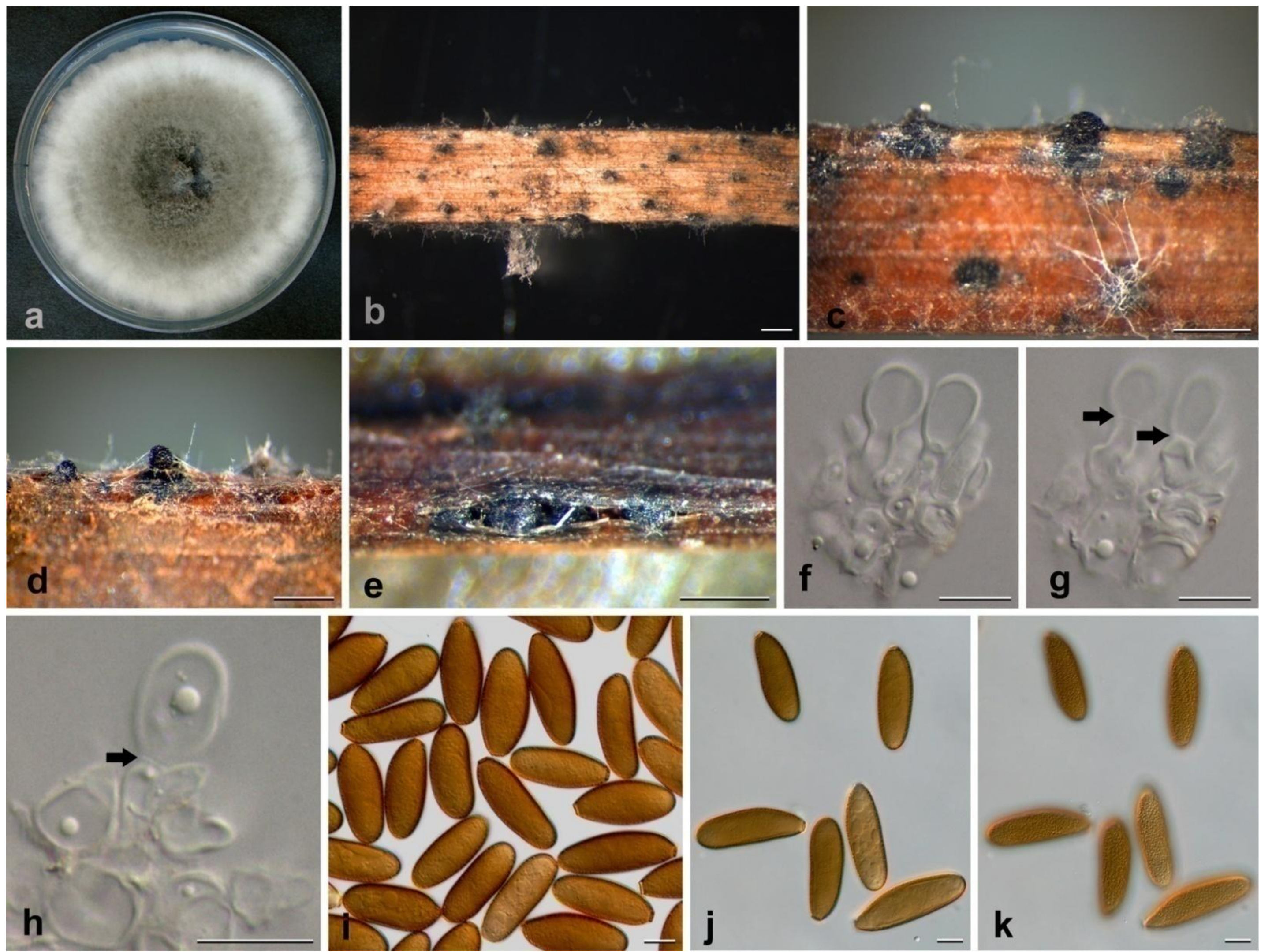

Figure 3.19. *Diplodia scrobiculata.* **a)** Colony characteristics on potato dextrose agar. **b – e)** Conidiomata formed on pine needles in culture; with conidia oozing from a pycnidium (**d**); **f – h)** Conidiogenous cells with developing conidia at two different levels of focus (**f, g**) showing annelations (black arrows). **i – k)** Brown, aseptate conidia; at two different levels of focus showing roughening of conidia walls (**j, k**). Scale bars: b – e = 0.5 mm; f – l = 10 µm.

Diplodia intermedia A.J.L. Phillips, J. Lopes & A. Alves **sp. nov.** (Fig. 3.20).

This species will be formally described in a separate publication and the description here should not be cited.

Etym: named for its phylogenetic position intermediate between *D. pinea* and *D. scrobiculata*.

Conidiomata pycnidial, solitary or clustered, immersed in the host, partially emergent at maturity, up to 380 µm diam and 360 µm high, dark brown to black, ostiolate, nonpapillate, thick-walled, outer and inner layers composed of dark brown and thin-walled hyaline *textura angularis*, respectively. *Conidiogenous cells* 11.0–22.0 × 4.0–5.0 µm, hyaline, thin-walled, smooth, cylindrical, swollen at the base, discrete, producing a single conidium at the tip, indeterminate. *Conidia* aseptate, ovoid to oblong, widest in the middle, with obtuse apex and truncate or rounded base. initially hyaline but soon becoming dark brown while in the pycnidia; wall moderately thick, with a smooth outer surface and roughened on the inner suface, (24.6–)30.3–30.9(–36.9) × (10.1–)13.0–13.6(–17.7) µm. $\bar{x}$ ± S.D. of 150 conidia = 30.6 ± 1.9 × 13.3 ± 1.8 µm. L/W = 2.3 ± 0.3. *Microconidia* abundant, aseptate, hyaline, smooth, ovoid to oblong with rounded ends, (5.4–)6.4–8.1(–9.5) × (3.8–)4.4–5.5(–6.8). Microconidiogenous cells not seen.

Specimens examined: **Portugal**: Setúbal, Monte da Caparica, *Pyrus communis*, 2002, *A.J.L. Phillips* (CAP130=CBS112556). Monte da Caparica, *Malus* sp., 2006, *A.J.L. Phillips* (CAP273). Torres Vedras, *Cydonia* sp., 2003, *S. Santos* (CAP150).

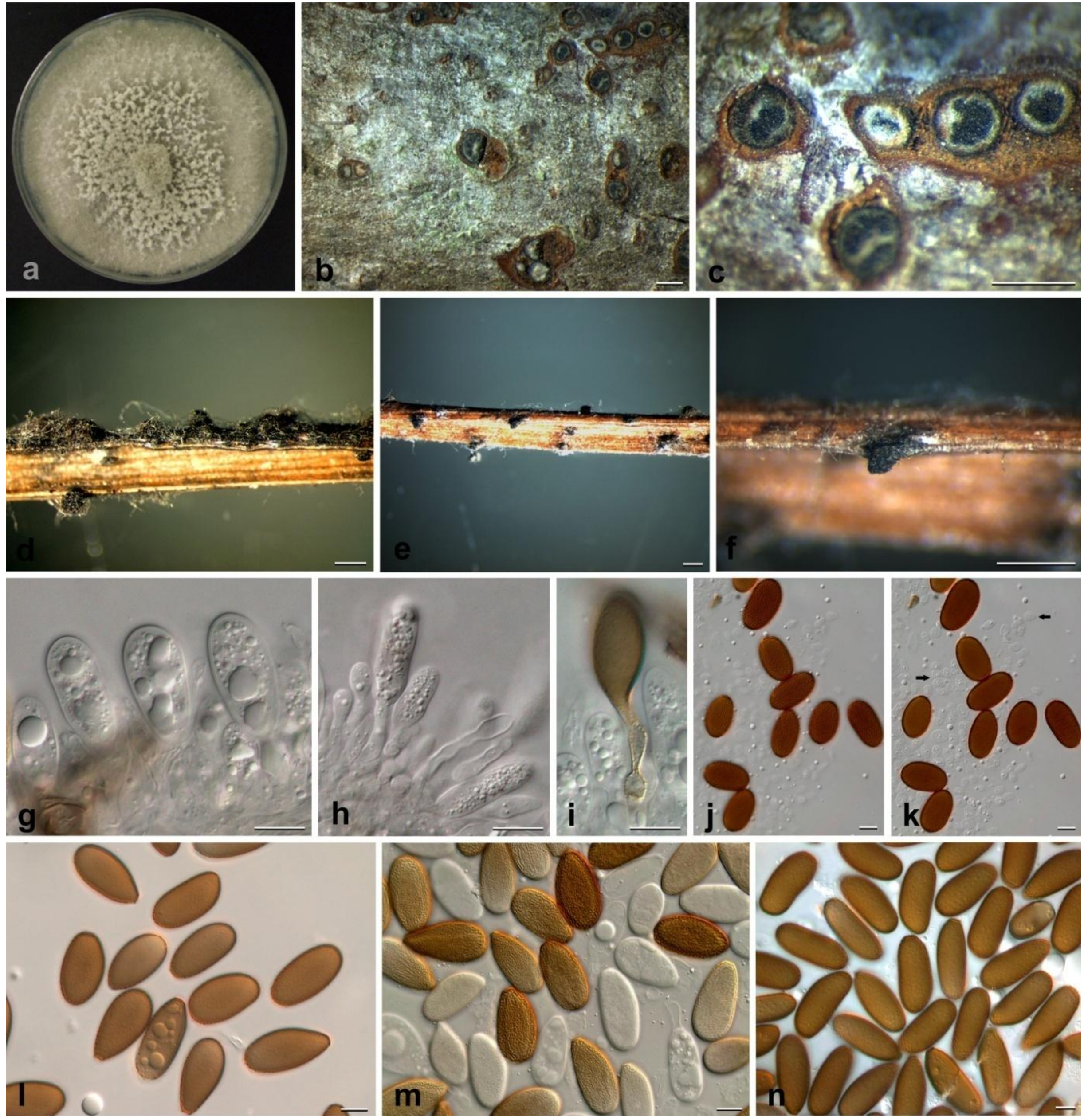

Figure 3.20. *Diplodia intermedia.* **a)** Colony characteristics on potato dextrose agar. **b, c)** Conidiomata cut through horizontally showing a dark mass of conidia, on the host. **d – f)** Conidiomata formed on pine needles in culture; with dark mass of conidia oozing from a pycnidium **(f)**. **g – i)** Conidiogenous cells with developing conidia. **j – n)** Brown, aseptate conidia; and some hyaline aseptate conidia **(m)**; at two different levels of focus showing roughening of conidia walls and several microconidia (black arrows) **(j, k)**. Scale bars: b – e = 0.5 mm; f – k = 10 μm.

Chapter 4

Discussion

Taxonomy of fungi is traditionally based on comparative morphology and on development of sexual and asexual reproduction structures. However, conventional methods for detection and identification are laborious and time-consuming (Wu *et al.*, 2003) and non- or low-sporulating fungi are more difficult to identify by means of conventional taxonomy.

In this work, the taxonomy and phylogenetic position of species of *Diplodia* were studied. A number of *Diplodia* species were recognized from a combination of morphological and molecular characters. Based on morphological and phylogenetic analyses two species were described as new (*D. bulgarica* sp. nov. and *D. intermedia* sp. nov.) while two existing species names that have been rarely used were recognised as distinct and valid (*D. malorum* and *D. fraxini*). The known species that could be distinguished here included both morphological forms (A and C morphotypes) of *D. pinea* (Swart and Wingfield, 1991), *D. scrobiculata*, which was previously known as the B morphotype of *D. pinea* (De Wet *et al.*, 2003), *D. mutila* (Alves *et al.*, 2004), *D. seriata* (Phillips *et al.*, 2007) and *D. olivarum* (Lazzizera *et al.*, 2008). Many of these species have been confused in the past due to their similarity to one another (e.g. Burgess *et al.*, 2001; Zhou and Stanosz, 2001). However, by using multiple gene genealogies, as has been shown in previous studies (De Wet *et al.*, 2000, 2003; Alves *et al.*, 2006), these species could be differentiated. The main aim of this work was to resolve the complex of species that are morphologically similar to *Diplodia mutila*. The approach was to integrate morphology with phylogenetic analyses.

The species studied in the present work could be divided into two major morphological groups, namely, 1) the mostly hyaline-spored species, i.e., species in which the conidia remain hyaline for a long time after discharge from pycnidia and 2) the dark-spored species with conidia that become brown while within the pycnidia. As depicted in Figure 3.3, the group of the hyaline-spored species are represented by clades I, II and IV, however the latter, corresponding to *D. corticola*, will not be discussed further here, while clade II represents the group of dark-spored species.

Clades I and II (Figure 3.3) are represented by the *Diplodia mutila* complex of species, i.e., species that are morphologically similar to *D. mutila*, the type species of the genus *Diplodia*, characterized by thick-walled conidia that remain hyaline for a long time (Alves *et al.*, 2004, Phillips *et al.*, 2005). The species representing this group besides *D. mutila*, are *D. olivarum*, *D. fraxini*, *D. africana*, *D. rosulata*, *D. malorum*, *D. bulgarica*, *D. cupressi* and *"Botryosphaeria" tsugae* A. Funk. However, only some of these species were studied morphologically in this work, namely *D. mutila*, *D. olivarum*, *D. fraxini*, *D. malorum* and *D. bulgarica.* This complex of species was well resolved in the present work and good phylogeneies were obtained with ITS and EF1-α datasets. Moreover, the individual clades within this group corresponded to phenotypic characters such as morphology and host

specificity. These species are morphologically similar, however some features may differentiate them.

Within Clade I, *D. malorum* and *D. fraxini* are recognized as valid names. In this regard, three isolates clustered together forming a group considered to be *Diplodia fraxini*. Although isolate CAP302 formed a separate internal branch, it differed from the other two isolates in only two deletions in the ITS region and two nucleotide substitutions in the EF-α sequence. These differences were not considered to be of any major significance and therefore these three isolates were considered to be the same species, but distinct from other species in the hyaline-spored group. The name *D. fraxini* was considered to be suitable for this clade because two of the isolates were from *Fraxinus*, the host with which this species was originally associated. However, the type specimen could not be located during the course of this study and thus its identification could not be confirmed. A group of isolates collected from *Malus* sp. in Portugal formed a well supported clade clearly separated from *D. mutila* and related species. It is considered here that both morphological and cultural features that characterize this species correlate with *Diplodia malorum*, introduced by Fuckel in 1870. Morphological features of the isolates studied here also correlated in all ways with features of isotypes of *D. malorum*, and thus it was considered that this name is applicable. The name *Diplodia malorum* has not been used for many years and no ex-type cultures are extant. In this way, it is reinstated in the present work, and an epitype will be designated, together with ex-epitype cultures.

Clade II is composed of *D. cupressi*, *"B". tsugae* and a group of three isolates collected from apples trees in Bulgaria. These isolates were phylogenetically and morphologically distinct from the other two species in this clade (*D. cupressi* and *"B". tsugae*) and were thus considered to represent a new species recognized as *Diplodia bulgarica* sp. nov.

Although *D. bulgarica*, *D. fraxini* and *D. malorum* resemble *D. mutila*, they can be distinguished on the size of their conidia and the timing at which the conida darken. Thus, mature conidia of *D. malorum* are larger than those of *D. mutila*. While the range of conidial dimensions in *D. malorum* is 28–28.3 × 14.3–14.5 µm, those of *D. mutila* lie in the range of 24.7–25.7 × 13.3–13.8 µm. Furthermore, conidia of *D. fraxini* are also larger than conidia of *D. mutila* with dimensions ranging 27.4−27.9 × 18.1−18.5 µm, however, this species is easily distinguished from all the other species within this group on account of its wider conidia, with average widths exceeding 18 µm. Also, *D. fraxini* conidia are subglobose with an average length/width ratio of 1.5 that resembles *D. bulgarica* but is distictive from all the other species with higher ratios. Although the average ratio is similar to that of *D. bulgarica*, minimum value of *D. fraxini* is the smallest, 1.25. Regarding *D. bulgarica*, the length of its conidia overlap with those of *D. mutila*, 25−25.7 µm, but it has wider conidia up to 17 µm. Moreover, conidia

of *D. bulgarica* become pale brown soon after discharge from pycnidia and rarely brown and one-septate, and likewise, conidia of *D. malorum* become brown and one-septate frequently and in an early stage of maturity, while conidia of *D. mutila* and *D. fraxini* remain hyaline and aseptate for a long time before becoming coloured and seldomly septate. Furthermore, *D. bulgarica* formed a further clade (Clade II) together with *D. cupressi* and *"B". tsugae* and though they are all species morphologically similar to *D. mutila*, *D. bulgarica* can be distinguished by the early development of pigmentation of its conidia, whereas in *D. cupressi* and *"B". tsugae* they remain hyaline for a long time (Alves *et al.*, 2006; Funk, 1964). In fact, only hyaline conidia have been reported in *"B". tsugae.* (Funk, 1964). Moreover, mature conidia of *D. bulgarica* rarely form septa, while those of *D. cupressi* septate frequently (Alves *et al.*, 2006).

In general matters, although the ranges of dimensions of conidia of the hyaline-spored group of species overlap considerably, mean conidial lengths of *D. malorum* are greater than *D. fraxini*, followed by *D. olivarum*, *D. bulgarica* and *D. mutila*, although the values of the latter three are extremely close. In terms of width, conidia of *D. fraxini* are the widest, followed by *D. bulgarica*, *D. malorum*, *D. mutila* and lastly, *D. olivarum*, which has the narrowest conidia.

Clade III (Figure 3.3.) is represented by a group of dark-spored species, including the *D. pinea* complex of *D. pinea*, *D. intermedia* and *D. scrobiculata* together with *D. seriata*. The *Diplodia pinea* - *Diplodia seriata* complexes were also resolved into the known species. However, it was apparent that in this clade the species are very closely related while others are evolving or have emerged very recently and are thus difficult to separate with ITS and EF1-α alone. Some isolates clustered separately although they had few nucleotide differences from other species. For this reason it was thought necessary to include a third gene, β-tubulin, in the phylogenetic analysis.

Various attempts were made to try to create a phylogenetic tree using ITS, EF1-α and β- tubulin genes with the entire dataset studied in this work in order to improve resolution, especially in the *D. pinea* and *D. seriata* clades. However, the resulting trees showed no improvement and in some clades resolution was even worse . Thus, the best resolution for the larger dataset was in fact achieved with only the ITS region and EF1-α gene and for that reason all the conclusions regarding the whole dataset are based on the two-genes tree (Figure 3.3).

A smaller dataset, including only the dark-spored species, was used for the phylogenetic analyses using the three genes, leading to a slightly better resolution within this group of species (Figure 3.4). However, it seems that the β- tubulin gene is too conserved to give good resolution of the more recently evolved species as seems to be the case in *D. pinea* complex. In contrast, the EF1-α gene is very variable and contains much phylogenetic

information (Cho *et al.,* 1995). Thus, from the phylogenies, it seems that *D. pinea* may also be a complex of species that needs further studies to be resolved, possibly including further isolates and DNA sequences of further genes (e.g. de Wet *et al.*, 2003).

Three isolates (CAP130, CAP150 and CAP273) clustered within a group supported by high bootstrap. Thus, based on morphological features as well as DNA phylogeny, the isolates from this clade are considered to represent a new species, namely *Diplodia intermedia*. Morphologically, conidia of this species resemble those of *D. seriata*, however they are larger with dimensions ranging 30.3–30.9 × 13–13.6 µm, while conidial dimensions of *D. seriata* are 25.5–26.3 × 11.1–11.5 µm. Thus, conidia of *D. intermedia* are smaller than those of *D. pinea* with average lengths exceeding 40 µm and widths over 14 µm and conidia of *D. scrobiculata* with mean dimensions of 39.5 × 14 µm (de Wet *et al.*, 2003). Furthermore, this species can be easily differentiated on account of the microconidia, which are absent in the other two species. Phylogenetically, this species falls between *D. pinea* and *D. scrobiculata*, thus proving to be related to the *D. pinea* complex of species (Figures 3.3 and 3.4).

In the phylogenies, one isolate (CAP163) clustered close to the isolates from *D. scrobiculata* previously studied by de Wet *et al.* (2003) and from which it differed by one substitution in ITS, four in EF1-α and two in β-tubulin. These differences were not considered to be significant and probably represent normal variation within *D. scrobiculata.* Nevertheless, it differed morphologically on the dimensions of its conidia. In the work of de Wet *et al.* (2003), conidial dimensions of *D. scrobiculata* are (37.5–)39.5(–41.5) × (13–)14(–15.5) µm, whereas those of CAP163 are smaller ranging (28.9–)32–33(–37.4) × (10.7–)13–13.5(–15.1) µm. This may suggest either variability within the species or that this isolate is in fact a distinct species. However, on the basis of one isolate only it is not possible to achieve further conclusions.

Isolate CAP330 diverged from the other isolates morphologically and phylogenetically. However conidia from this isolate are similar to those of *D. seriata*, ranging (17.7–)25.5–26.3(–33.4), they tend to be shorter (21.6)23.7–24.5(–27) and therefore, shorter than all the other dark-spored *Diplodia* species studied here. Furthermore, conidiomata of this isolate are formed in clusters of 1–5 showing distinct morphologies of amorphous shapes, sometimes with long ostiolar necks, which is probably the most distinctive morphological character between this isolate and the others studied in this work. Based on the ITS, EF1-α and β-tubulin dataset, this isolate proved to be phylogenetically related to isolates from *D. intermedia* species (CAP130, CAP 150 and CAP273) with only one difference in the ITS sequence, 3–4 in EF1-α and 0–2 in β-tubulin sequence. Moreover, it is also related to isolates from *D. pinea* A diverging in two nucleotides in the ITS, three in EF1-α and none in the β-tubulin. However, this isolate did not cluster with any of these species and,

in the tree, stands apart from any of the species studied here. The phylogenetic position of this isolate remains uncertain. Furthermore, since only one isolate was available, no species name was applied to this isolate. However, it appears to be a distinct species and it is hoped that further isolates will be found and when they are studied the status of this species will be established.

On the whole, morphological features within the *Diplodia mutila* complex of species could differentiate some species, for example, *D. malorum* which has the longest conidia, and *D. fraxini* that comprises the widest ones. *D. bulgarica* shows an early development of pigmentation of its conidia that is rarely found in other species of this group. In this way, despite the great morphological similarity between species belonging to this complex, some differences are apparent especially in the conidial dimensions. Among the dark-spored species, *D. seriata* has the shortest conidia while *D. pinea* has the longest and wider ones. These morphological characters and others have been used to distinguish closely related species within "*Botryosphaeria*" anamorphs, such as *B. dothidea* (Slippers *et al.*, 2004a), *Neofusicoccum australis* (Slippers *et al.*, 2004b), *Dothiorella sarmentorum and Dothiorella iberica* (Phillips *et al.*, 2005). In this way, Alves *et al.* (2006) distinguished *D. cupressi* from *D. mutila* by its larger and frequently one-setate conidia and from *D. pinea* by the stage at which the conidia become pigmentated and by the size conidia, larger in *D. pinea*. Moreover, *D. olivarum* was differentiated from *D. mutila* on account of its smaller conidia (Lazzizera *et al.*, 2008). Several *Lasiodiplodia* species were also distinguished on the basis of their conidial morphology (Pavlic *et al.,* 2004; Burgess *et al.*, 2006; Alves *et al.*, 2008).

Diplodia corticola lies in a clade separated from all other species (Clade IV), however it has typical hyaline-spored *Diplodia* characteristics. This species is characterized by very large conidia and its teleomorphic state is very common in nature (Alves *et al.*, 2004), which is in contrast to other *Diplodia* species.

Regarding colony characteristics, these were similar in most *Diplodia* species. However, *D. rosulata* is differentiated by its specific petaloid-shaped colony morphology (Gure *et al.*, 2005).

The study of the morphological and cultural characteristics of the isolates was useful for the preliminary identification of the species, the most discriminative characters were color, dimensions and septation of the conidia. Some of the species could be recognized based on phenotypic characters. However, only with the phylogenetic analysis could most of them be identified and discriminated. Phylogenetic analyses of combined ITS, EF1-α nucleotide sequence data clearly separated all the species in *Diplodia*. Thus, in other studies some *Diplodia* species have been differentiated with basis on DNA sequence data of the ITS region as in the case of *D. corticola* (Alves *et al.*, 2004), combined ITS and EF1-α as with the species *D. africana* (Damm *et al.*, 2007) and *D. olivarum* (Lazzizera *et al.*, 2008) and

combined ITS, EF1-α and β-tubulin as in *D. cupressi* (Alves *et al.*, 2006). Moreover, multiple gene genealogies constructed from six protein coding gene regions and six microsatellite-rich loci were needed to distinguish *D. scrobiculata* (de Wet *et al.*, 2003).

In terms of host association of the *Botryosphaeriaceae*, certain well characterized species have in recent years been confirmed to infect a wide range of hosts using molecular data, e.g. *B. dothidea*, *D. seriata*, *N. parvum*, *N. australe*, *L. theobromae* (Burgess *et al.*, 2006; Pavlic *et al.* 2004; Slippers *et al.* 2004a, b, 2007; Phillips *et al.*, 2007). In contrast, other species are clearly specialized on certain host genera or specific plant families in a defined area, e.g. *D. pinea* and *D. scrobiculata* on *Pinus* and occasionally other conifers (Burgess *et al.* 2004), *N. eucalyptorum* and *N. eucalypticola* on *Eucalyptu*s (Smith *et al.* 2001; Slippers *et al.* 2004c), *D. cupressi* on *Cupressus* and *Juniper* (Alves *et al.*, 2006) and *D. corticola*, that has been found only on *Quercus* (Alves *et al.*, 2004). Thus, there is some evidence of host specialization among the species of *Diplodia* studied.

Four isolates (CAP166, CAP168 and CAP169 from *O. europaea* and CAP339 from *Pinus* sp.) grouped closely with *Diplodia pinea*. This species has been differentiated based on a variety of morphological types, that, in the past, have been referred to as the A, B, C and I morphotypes (Wang *et al.*, 1985; Palmer *et al.*, 1987; Smith and Stanosz, 1995; Hausner *et al.*, 1999; De Wet *et al.*, 2000, 2002). However, multiple gene genealogies have shown that *D. pinea* is represented by only two morphotypes (A and C). The B morphotype of *D. pinea* elevated to species status as *D. scrobiculata* (De Wet *et al.*, 2003) and the the I morphotype was shown to be *D. seriata* (Burgess *et al.*, 2001). *D. pinea* is a well known pathogen of *Pinus* spp. worldwide (Punithalingam and Waterston, 1970) and it has also been reported on several other hosts (Punithalingam and Waterston, 1970; Sutton, 1980; De Wet, 2008). The fungus was also reported on *Eucalyptus* spp. in Uruguay (Bettucci *et al.*, 1999; 2004), although unconfirmed and recently it was found on *Prunus* spp. in South Africa (Damm *et al.*, 2007) and on *O. europaea* in Italy (Lazzizera *et al.*, 2008). Three of the isolates of *D. pinea* used in this study, CAP166, CAP168 and CAP169 were from *O. europaea* from Italy and have been studied previously by Lazzizera *et al.* (2008). This author referred that those isolates were derived from olive orchards surrounded by pine trees and that it was likely that the high inoculum pressure resulted in a few infections of the olives by *D. pinea*. In this way and similarly to what was stated by Lazzizera *et al.* (2008) there is a possibility that *D. pinea* is an opportunist on *Olea* and should not be regarded as a major host. In a similar way, Damm *et al.* (2007) noted that *D. pinea* was isolated from pycnidia on the bark of pruning debris of *Prunus* and was not associated with necrosis within the host tissue. In contrast, isolate CAP339 from Bulgaria was originated from *Pinus* sp., conforming with other authors that *D. pinea* is a primary pathogen of this host.

Isolate CAP163 representing *D. scrobiculata*, which has only been reported on conifer hosts before (De Wet *et al.*, 2003, 2008), was collected from *O. europaea*. However, as with *D. pinea*, it is probable that the high levels of inoculum from the surrounding pine trees resulted in this infection and it is unlikely that *D. scrobiculata* is a primary pathogen of olives. On the other hand, it is possible that isolate CAP163 may represent a distinct species, which has recently evolved, but more isolates are needed to support this hypothesis.

Twelve of the isolates studied here were *D. seriata* which is a plurivorous species known to occur on a wide range of hosts (Punithalingam and Waller, 1973) and has a worldwide distribution. In recent years *D. seriata* has been recognized as a pathogen of *Vitis vinifera* in Australia (Castillo-Pando *et al.*, 2001) and South Africa (van Niekerk *et al.*, 2004). However, Phillips (1998, 2002) regarded this species as a weak pathogen or saprophyte on *Vitis*. In this study, isolates from this host were collected in Portugal, Italy and Bulgaria. More recently, this species has been reported from olive drupes (*Olea europaea*) in Spain (Moral *et al.*, 2008) and Italy (Lazzizera *et al.*, 2008), thus suggesting a possible role as a a pathogen on this host. In the present work, *D. seriata* was the species that was represented in a wider range of hosts than any other species.

Diplodia mutila has been regarded to as a generalist species able to infect a wide range of angiosperms (Jacobs and Rehner, 1998; Zhou and Stanosz, 2001; Alves *et al.*, 2004). However, it was only recently that the concept of *D. mutila* and the group of species close to it was clarified. It has been attested that *D. mutila* is a complex of morphologically similar species. Possibly, some of the species found in many hosts have been misidentified as *D. mutila*. Thus, in the past, morphological differences and mostly conidial morphologies have been regarded as natural variations whithin the species (e.g. Luque and Girbal, 1989). Only recently, Zhou and Stanosz (2001a, b) suggested that the name "*B". stevensii* (= *D. mutila*) might have been applied to more than one species. In this way, Alves *et al.* (2004) studied several "*Botryosphaeria"* isolates obtained from oaks that had been reported as "*B". stevensii* since 1989 (Luque and Girbal, 1989) and proved with basis on morphological characters and molecular data that the species found on *Quercus* was in fact *D. corticola*. Possibly, a similar situation occurred to *D. malorum* on *Malus* and most of the isolates found in that host were identified as *D. mutila,* whereas in fact they might have been *D. malorum*. However, in the present work, only one isolate of *D. mutila* was studied, and only two isolates were included in the phylogeny. To fully resolve the pathology and phylogeny of *D. mutila*, a larger collection of isolates from different hosts and geographical regions will have to be studied.

Apparently, *D. malorum* and *D. bulgarica* are specific to *Malus* sp. trees, however all the isolates studied here were collected from the same trees in Portugal and Bulgaria, respectively. Only very recently, in ongoing studies, has *D. bulgarica* been found in *Malus* in

Iran, thus raising the possibility of specificity to that host. Still, neither of these species have been found on other hosts.

The isolates representing *D. olivarum* were mostly from *O. europaea* as in the work by Lazzizera *et al.*, 2008. However, one isolate was from *Ceratonia siliqua*, which could suggest that this species is not host specific and can infect other hosts than olive. Nevertheless, being the only isolate of *D. olivarum* reported so far in a different host than *Olea*, may indicate that due to the proximity between the two hosts, this species was an opportunist on *Ceratonia siliqua* which probably should not be regarded as a primary host.

Diplodia fraxini was described by Fries (1849) and reported on *Fraxinus* sp. The isolates studied here and regarded as this species appear to have some kind of host specificity to *Fraxinus* and the fact that one of the isolates was from *Lonicera* sp. could be explained by proximity of both hosts. Nevertheless, with two isolates only representing *Fraxinus* host, nothing can be inferred for now and in the future more isolates need to be collected and studied not only from *Fraxinus* but also other hosts.

The three isolates studied here referring to the species *D. intermedia* appear to show association to hosts belonging to the *Rosaceae*, particularly the sub-family *Maloideae*. Thus, all isolates were collected in different areas of Portugal. This is also evident in the case of another isolate (CAA147) which belongs to this group and is being studied in other ongoing investigations, however, not taken into account in this work. So far, this species has not been found in any other country than in Portugal and only on *Maloideae*.

In general, it is apparent that the species in Clades I and II (*D. mutila* complex) are, to a certain extent, host specific and occur mostly on one particular host, as in the cases of *D. olivarum* (*Olea* sp.), *D. fraxini* (mostly *Fraxinus* sp.), *D. africana* (*Prunus persica*), *D. rosulata* (*Prunus africana*), *D. malorum* (*Malus* sp.), *D. bulgarica* (*Malus* sp.), *D. cupressi* (*Cupressus* sp. and *Juniperus* sp.) and *"B." tsugae* (*Tsuga* sp.). However, with the clarification of the concept of *D. mutila*, host specificity of this species remains to be determined.

The host specialization that seems to be apparent in some of the species studied here, could have important implications in management of the diseases, especially in terms of reducing the use (or misuse) of fungicides and other hazardous chemicals in treatments on certain crops. For example, if an olive orchard is surronded by an apple orchard which is infected with *Diplodia malorum*, it is most likely that this species will not infect the olive trees because of its host specialization to apple trees only. Therefore no fungicides for prevention will be needed. Moreover, if host specialization exists in *Diplodia* species, it suggests that breeding for tolerance may be possible.

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Appendix I

Table I. Dimensions of the hyaline and dark conidia of *Diplodia* species from reproductive structures obtained directly from the host.

	HAP 265			HAP 266			HAP 267			HAP 268			HAP 269		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	37	37	37	50	50	50	50	50	50	50	50	50
Sum	1249,6	618,58	101,819	850,63	416,55	75,97588	1266,98	700,3	90,69407	1397,35	730,66	96,18836	1275,48	577,62	111,0714
Mean	24,99	12,37	2,04	22,99	11,26	2,05	25,34	14,01	1,81	27,95	14,61	1,92	25,51	11,55	2,22
S.D.	1,63	1,05	0,23	1,73	1,05	0,19	2,08	0,97	0,16	1,74	0,94	0,21	1,09	0,89	0,20
Max	28,8	14,37	2,676622	27,32	14,09	2,533797	28,47	16,47	2,196486	31,79	16,65	2,318481	27,43	13,56	2,720374
Min	20,9	9,71	1,546852	18,97	9,53	1,71786	20,76	11,79	1,568913	23,98	12,56	1,500626	22,68	9,62	1,823344
Conf limit	0,452625	0,290101	0,06416	0,555985	0,338951	0,061721	0,577424	0,269588	0,043608	0,483037	0,261451	0,057406	0,302021	0,245358	0,05545
Low limit	24,54	12,08	1,97	22,43	10,92	1,99	24,76	13,74	1,77	27,46	14,35	1,87	25,21	11,31	2,17
Up limit	25,44	12,66	2,10	23,55	11,60	2,12	25,92	14,28	1,86	28,43	14,87	1,98	25,81	11,80	2,28

	HAP 270			HAP 271			HAP 272			HAP 273			HAP 274		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1215,28	596,35	102,2026	1212,47	642,42	94,82989	1254,93	665,8	95,0801	1239,53	599,92	103,5809	1279,94	702,36	91,60704
Mean	24,31	11,93	2,04	24,25	12,85	1,90	25,10	13,32	1,90	24,79	12,00	2,07	25,60	14,05	1,83
S.D.	1,38	0,90	0,13	2,09	0,70	0,23	2,57	1,62	0,22	1,48	0,64	0,16	1,83	1,03	0,19
Max	26,96	14,06	2,277674	29,56	14,57	2,488189	32,57	17,49	2,434281	28,52	13,85	2,477845	31,06	17,53	2,309756
Min	19,9	9,54	1,696948	18,42	11,34	1,424594	19,16	10,58	1,324567	21,39	10,52	1,7739	22,26	11,59	1,5
Conf limit	0,383468	0,24873	0,034659	0,579124	0,193967	0,063361	0,713559	0,450259	0,062239	0,411089	0,176028	0,045079	0,506589	0,284487	0,052543
Low limit	23,92	11,68	2,01	23,67	12,65	1,83	24,39	12,87	1,84	24,38	11,82	2,03	25,09	13,76	1,78
Up limit	24,69	12,18	2,08	24,83	13,04	1,96	25,81	13,77	1,96	25,20	12,17	2,12	26,11	14,33	1,88

	HAP 275			HAP 276			HAP 277			HAP 278		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1208,64	618,92	98,19544	1025,75	549,99	94,56258	1358,98	699,73	97,19987	1337,34	652,53	102,8664
Mean	24,17	12,38	1,96	20,52	11,00	1,89	27,18	13,99	1,94	26,75	13,05	2,06
S.D.	1,79	0,88	0,21	1,85	1,36	0,24	1,34	0,57	0,10	1,23	0,88	0,15
Max	27,53	14,63	2,446211	22,42	19,88	2,197211	29,86	15,13	2,264977	29,62	15,27	2,381313
Min	20,72	10,66	1,446343	10,48	9,77	0,527163	23,85	12,93	1,729181	24,23	11,47	1,748247
Conf limit	0,496603	0,245002	0,058249	0,511689	0,376633	0,066973	0,371225	0,157803	0,02833	0,341268	0,242657	0,042102
Low limit	23,68	12,13	1,91	20,00	10,62	1,82	26,81	13,84	1,92	26,41	12,81	2,02
Up limit	24,67	12,62	2,02	21,03	11,38	1,96	27,55	14,15	1,97	27,09	13,29	2,10

Appendix II

Table II. Dimensions of the hyaline conidia of *Diplodia* species from reproductive structures sporulated in culture.

	CAP 062			CAP 222			CAP 224			CAP 225			CAP 235		
	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1186,87	701	84,98428	1240,61	566,1	109,8193	1362,01	521,66	132,0277	1183,06	589,73	100,9476	1266,84	614,65	103,1991
Mean	23,74	14,02	1,70	24,81	11,32	2,20	27,24	10,43	2,64	23,66	11,79	2,02	25,34	12,29	2,06
S.D.	1,37	0,85	0,15	1,01	0,64	0,12	1,54	1,18	0,31	1,13	1,06	0,17	1,51	0,60	0,13
Max	26,48	15,59	2,042846	27,63	12,25	2,509862	30,4	13,55	3,440145	26,14	13,68	2,410582	32,27	14,2	2,487512
Min	20,61	11,93	1,391132	22,32	9,47	1,972339	22,93	8,27	2,111015	21,55	9,45	1,719193	22,84	10,81	1,723774
Conf limit	0,379978	0,23428	0,04122	0,281025	0,176276	0,033153	0,425961	0,328055	0,085449	0,31427	0,292698	0,04776	0,419044	0,16559	0,035788
Low limit	23,36	13,79	1,66	24,53	11,15	2,16	26,81	10,11	2,56	23,35	11,50	1,97	24,92	12,13	2,03
Up limit	24,12	14,25	1,74	25,09	11,50	2,23	27,67	10,76	2,73	23,98	12,09	2,07	25,76	12,46	2,10

	CAP 265			CAP 266			CAP 267			CAP 268			CAP 269		
	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50,00
Sum	1428,61	800,69	89,71487	1458,81	734,73	100,091	1385,63	676,81	102,6307	1530,18	821,27	93,16144	1352,96	746,5	90,90353
Mean	28,57	16,01	1,79	29,18	14,69	2,00	27,71	13,54	2,05	30,60	16,43	1,86	27,06	14,93	1,82
S.D.	1,33	1,28	0,15	2,02	1,40	0,23	1,79	0,81	0,16	2,96	1,12	0,13	1,23	0,87	0,13
Max	31,32	18,39	2,272	33,95	18,18	2,547417	33,43	16,87	2,380204	36,08	19,1	2,200271	29,41	16,95	2,127259
Min	25,96	13,45	1,525952	24,07	11,81	1,606236	24,66	12,39	1,691761	26,1	14,71	1,574955	24,67	13,16	1,527174
Conf limit	0,368766	0,355262	0,042915	0,560086	0,387346	0,062775	0,494896	0,224407	0,043423	0,819095	0,310769	0,035428	0,340768	0,240821	0,035615
Low limit	28,20	15,66	1,75	28,62	14,31	1,94	27,22	13,31	2,01	29,78	16,11	1,83	26,72	14,69	1,78
Up limit	28,94	16,37	1,84	29,74	15,08	2,06	28,21	13,76	2,10	31,42	16,74	1,90	27,40	15,17	1,85

	CAP 270			CAP 271			CAP 272			CAP 273			CAP 274		
	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W
Count	50	50	50	50	50	50	50	50	50	31	31	31	50	50	50
Sum	1408,96	690,44	102,336	1419,26	693,36	102,7544	1380,38	655,92	105,8197	878,66	440,47	62,52299	1527,44	636,44	120,4416
Mean	28,18	13,81	2,05	28,39	13,87	2,06	27,61	13,12	2,12	28,34	14,21	2,02	30,55	12,73	2,41
S.D.	1,14	0,86	0,13	1,24	0,84	0,16	1,49	1,02	0,20	2,46	1,82	0,23	1,72	0,78	0,20
Max	30,57	15,93	2,338944	30,98	15,33	2,510675	30,76	16,22	2,661844	33,25	16,58	2,416589	34,48	15,42	2,91709
Min	25,59	12,31	1,705545	26,09	11,71	1,762032	23,88	11,06	1,657213	21,69	10,73	1,593681	27,62	11,39	1,838521
Conf limit	0,317041	0,239741	0,035046	0,342812	0,232507	0,045713	0,412698	0,282292	0,054095	0,867122	0,641207	0,081078	0,476558	0,215057	0,055508
Low limit	27,86	13,57	2,01	28,04	13,63	2,01	27,19	12,84	2,06	27,48	13,57	1,94	30,07	12,51	2,35
Up limit	28,50	14,05	2,08	28,73	14,10	2,10	28,02	13,40	2,17	29,21	14,85	2,10	31,03	12,94	2,46

	CAP 275			CAP 277			CAP 278			CAP 301			CAP 302		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1230,89	700,27	88,07113	1413,24	675,98	104,8409	1533,94	784,46	98,38801	1259,41	678,22	93,50895	1298,91	875,53	74,38324
Mean	24,62	14,01	1,76	28,26	13,52	2,10	30,68	15,69	1,97	25,19	13,56	1,87	25,98	17,51	1,49
S.D.	1,11	0,78	0,10	1,29	0,73	0,15	1,90	1,18	0,21	1,43	1,16	0,19	1,17	0,99	0,10
Max	27,89	16,12	1,949884	30,48	15,07	2,446355	34,49	19,24	2,51682	27,96	16,89	2,409836	28,26	20,2	1,719819
Min	21,3	12,23	1,515509	24,91	12,14	1,722683	26,15	13,08	1,482722	20,79	10,76	1,378922	23,81	15,49	1,253818
Conf limit	0,306811	0,215412	0,027589	0,357811	0,202921	0,041416	0,526294	0,32767	0,056921	0,395865	0,321899	0,052861	0,324198	0,273517	0,027626
Low limit	24,31	13,79	1,73	27,91	13,32	2,06	30,15	15,36	1,91	24,79	13,24	1,82	25,65	17,24	1,46
Up limit	24,92	14,22	1,79	28,62	13,72	2,14	31,21	16,02	2,02	25,58	13,89	1,92	26,30	17,78	1,52

	CAP 340			CAP 341			JL 375			JL 453		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1287,93	733,14	88,18926	1341,73	718,08	93,75156	1428,07	911,23	78,54415	1420,81	952,68	74,80353
Mean	25,76	14,66	1,76	26,83	14,36	1,88	28,56	18,22	1,57	28,42	19,05	1,50
S.D.	1,52	0,98	0,15	1,26	0,77	0,15	1,42	1,05	0,10	1,51	1,18	0,11
Max	29,45	16,77	2,313433	29,6	15,96	2,365564	31,13	20,47	1,747253	32,76	22,09	1,790834
Min	22,74	12,56	1,497558	24,6	11,79	1,648496	25,47	16,38	1,350477	25,69	16,8	1,299683
Conf limit	0,421112	0,271206	0,041986	0,348003	0,213396	0,042154	0,392449	0,289669	0,027335	0,417288	0,326504	0,030909
Low limit	25,34	14,39	1,72	26,49	14,15	1,83	28,17	17,93	1,54	28,00	18,73	1,47
Up limit	26,18	14,93	1,81	27,18	14,57	1,92	28,95	18,51	1,60	28,83	19,38	1,53

Table III. Dimensions of the dark conidia of *Diplodia* species from reproductive structures sporulated in culture.

	CAP 063			CAP 130			CAP 150			CAP 163			CAP 166		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1063,17	494,64	108,0259	1493,05	612,7	122,1259	1566,8	648,29	121,3273	1625,24	661,01	123,5571	2122,4	708,95	150,7999
Mean	21,26	9,89	2,16	29,86	12,25	2,44	31,34	12,97	2,43	32,50	13,22	2,47	42,45	14,18	3,02
S.D.	1,95	0,63	0,26	1,67	0,60	0,18	2,15	1,01	0,20	1,73	0,90	0,23	3,80	1,32	0,37
Max	28,58	11,26	2,826355	33,49	13,31	2,85901	36,89	15,33	2,909014	37,39	15,11	3,213084	49,37	17,29	4,287063
Min	17,73	8,12	1,5746	26,43	10,71	2,13463	24,55	10,89	1,973907	28,91	10,7	2,100282	35,51	10,59	2,336381
Conf limit	0,540776	0,175043	0,071641	0,463388	0,166865	0,049881	0,595177	0,279095	0,055963	0,478956	0,2482	0,064074	1,052852	0,364835	0,102206
Low limit	20,72	9,72	2,09	29,40	12,09	2,39	30,74	12,69	2,37	32,03	12,97	2,41	41,40	13,81	2,91
Up limit	21,80	10,07	2,23	30,32	12,42	2,49	31,93	13,24	2,48	32,98	13,47	2,54	43,50	14,54	3,12

	CAP 168			CAP 169			CAP 255			CAP 265			CAP 269		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1902,15	684,43	140,3444	2202,95	747,43	150,0628	1266,84	614,65	103,1991	1383,81	856,19	81,32328	1356,37	740,19	91,87601
Mean	38,04	13,69	2,81	44,06	14,95	3,00	25,34	12,29	2,06	27,68	17,12	1,63	27,13	14,80	1,84
S.D.	3,79	1,51	0,38	4,32	1,96	0,52	1,51	0,60	0,13	2,14	1,28	0,19	1,70	0,87	0,14
Max	49,23	17,91	3,872881	54,58	20,99	4,441603	32,27	14,2	2,487512	32,09	19,59	2,069751	31,56	16,33	2,09701
Min	31,93	11,21	2,04905	35,45	11,3	2,100524	22,84	10,81	1,723774	23,15	13,73	1,309852	23,99	13,01	1,539234
Conf limit	1,05051	0,417779	0,105476	1,198574	0,542586	0,144823	0,419043	0,165589	0,035788	0,592288	0,354728	0,052181	0,47114	0,241228	0,039135
Low limit	36,99	13,27	2,70	42,86	14,41	2,86	24,92	12,13	2,03	27,08	16,77	1,57	26,66	14,56	1,80
Up limit	39,09	14,11	2,91	45,26	15,49	3,15	25,76	12,46	2,10	28,27	17,48	1,68	27,60	15,05	1,88

	CAP 271			CAP 273			CAP 276			CAP 328			CAP 330		
	Length	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**	**Length**	**Width**	**L/W**
Count	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1434,46	704,55	102,4316	1533,14	733,16	106,9051	1368,81	585,84	117,2657	1351,46	507,15	133,6985	1204,39	601,92	100,3152
Mean	28,69	14,09	2,05	30,66	14,66	2,14	27,38	11,72	2,35	27,03	10,14	2,67	24,09	12,04	2,01
S.D.	1,31	0,98	0,20	1,65	2,23	0,34	2,00	0,75	0,22	1,24	0,62	0,20	1,38	0,64	0,15
Max	31,19	15,83	2,575581	34,06	17,69	3,134731	31,21	13,74	2,940613	29,53	12,14	3,121535	27,02	13,57	2,370175
Min	25,45	12,04	1,701203	27,82	10,02	1,637841	22,87	10,17	1,877778	24,56	9,07	2,248952	21,61	10,3	1,592483
Conf limit	0,362273	0,272272	0,056794	0,45617	0,61679	0,093528	0,555624	0,208764	0,062154	0,343752	0,172051	0,054791	0,381489	0,177824	0,042756
Low limit	28,33	13,82	1,99	30,21	14,05	2,04	26,82	11,51	2,28	26,69	9,97	2,62	23,71	11,86	1,96
Up limit	29,05	14,36	2,11	31,12	15,28	2,23	27,93	11,93	2,41	27,37	10,32	2,73	24,47	12,22	2,05

	CAP 333			CAP 335			CAP 338			CAP 339		
	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W	Length	Width	L/W
Count	50	50	50	50	50	50	50	50	50	50	50	50
Sum	1269,21	838,72	75,76271	1399,36	668,73	104,9521	1287,99	568,26	113,6892	1915,16	794,24	120,9701
Mean	25,38	16,77	1,52	27,99	13,37	2,10	25,76	11,37	2,27	38,30	15,88	2,42
S.D.	1,23	0,69	0,09	1,87	0,84	0,17	2,23	0,84	0,21	3,36	1,05	0,25
Max	28,18	18,41	1,724379	32,39	16	2,52442	33,4	14,32	2,830653	46,65	18,16	3,077677
Min	22,44	14,53	1,363305	24,79	11,59	1,62875	22,49	9,81	1,90872	29,99	13,86	1,870867
Conf limit	0,342258	0,191268	0,024321	0,518052	0,232577	0,047334	0,617511	0,233381	0,05774	0,931268	0,289911	0,068646
Low limit	25,04	16,58	1,49	27,47	13,14	2,05	25,14	11,13	2,22	37,37	15,59	2,35
Up limit	25,73	16,97	1,54	28,51	13,61	2,15	26,38	11,60	2,33	39,23	16,17	2,49

Appendix III

Table IV. Comparison of the mean conidial dimensions of some isolates obtained from the host and from culture.

	CAP 265			**CAP 266**			**CAP 267**		
	Width	Length	W/L	Width	Length	W/L	Width	Length	W/L
Host	24,99	12,37	2,04	22,99	11,26	2,05	25,34	14,00	1,81
Culture	28,57	16,01	1,79	29,18	14,69	2,00	27,71	13,54	2,05

	CAP 268			**CAP 269**			**CAP 270**		
	Width	Length	W/L	Width	Length	W/L	Width	Length	W/L
Host	27,95	14,61	1,92	25,51	11,55	2,22	24,31	11,93	2,04
Culture	30,60	16,43	1,86	27,06	14,93	1,82	28,18	13,81	2,05

	CAP 271			**CAP 272**			**CAP 273**		
	Width	Length	W/L	Width	Length	W/L	Width	Length	W/L
Host	24,25	12,85	1,90	25,10	13,32	1,90	24,79	11,99	2,07
Culture	28,39	13,87	2,06	27,61	13,12	2,12	28,34	14,21	2,02

	CAP 274			**CAP 275**			**CAP 276**		
	Width	Length	W/L	Width	Length	W/L	Width	Length	W/L
Host	25,59	14,05	1,83	24,07	12,38	1,96	20,52	11,00	1,89
Culture	30,55	12,73	2,41	24,62	14,01	1,76	27,38	11,72	2,35

	CAP 277			**CAP 278**		
	Width	Length	W/L	Width	Length	W/L
Host	27,18	13,99	1,94	26,75	13,05	2,06
Culture	28,26	13,52	2,10	30,68	15,69	1,97

Appendix IV

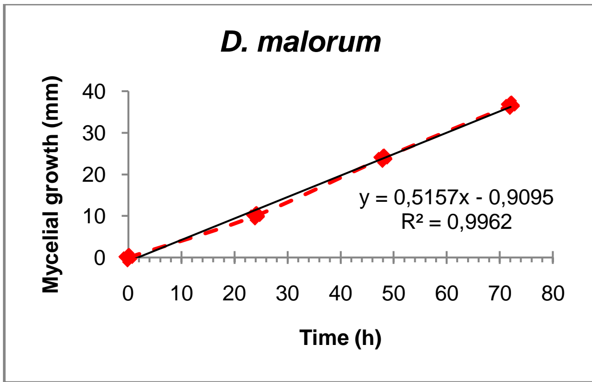
Table V. Mycelial growth rates for the isolates studied.

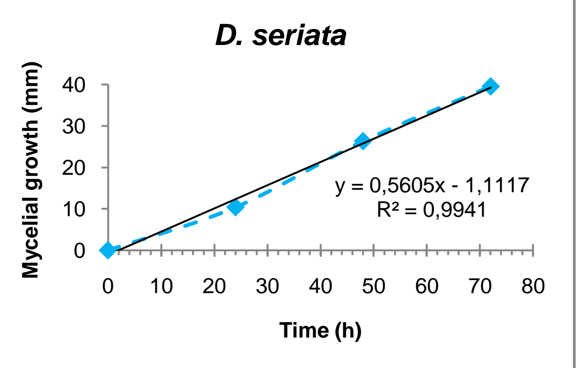
ISOLATES	MYCELIAL GROWTH (cm)												Growth rate in the last 48h (cm)	Diary growth rate (cm)
	24 h				48 h				72 h					
	1	2	3	**M**	1	2	3	**M**	1	2	3	**M**		
CAP 062	0,8	1,3	1,1	**1,07**	2,0	2,3	2,4	**2,23**	3,2	3,2	3,5	**3,30**	2,23	1,12
CAP 129	1,0	0,7	0,9	**0,87**	2,6	2,4	2,5	**2,50**	3,2	4,0	4,0	**3,73**	2,87	1,43
CAP 167	0,8	1,0	1,0	**0,93**	1,9	1,9	2,0	**1,93**	2,5	2,6	2,7	**2,60**	1,67	0,83
CAP 207	1,2	1,9	1,8	**1,63**	3,0	3,6	3,6	**3,40**	4,3	4,4	4,4	**4,37**	2,73	1,37
CAP 208	1,2	1,7	1,8	**1,57**	3,0	3,5	3,6	**3,37**	4,3	4,4	4,4	**4,37**	2,80	1,40
CAP 221	0,6	1,3	1,3	**1,07**	1,6	2,8	2,7	**2,37**	2,7	3,9	3,9	**3,50**	2,43	1,22
CAP 222	0,9	1,4	1,3	**1,20**	2,3	2,8	2,9	**2,67**	3,7	4,1	4,0	**3,93**	2,73	1,37
CAP 224	1,0	1,5	1,4	**1,30**	2,4	3,0	3,0	**2,80**	3,4	4,1	3,9	**3,80**	2,50	1,25
CAP 225	0,7	1,1	1,3	**1,03**	2,2	2,4	2,7	**2,43**	3,6	3,8	4,3	**3,90**	2,87	1,43
CAP 228	1,1	1,2	1,3	**1,20**	2,2	2,4	2,7	**2,43**	3,6	3,8	4,3	**3,90**	2,70	1,35
CAP 229	1,2	1,3	0,4	**0,97**	2,8	2,8	2,5	**2,70**	4,0	4,0	3,9	**3,97**	3,00	1,50
CAP 230	1,0	1,1	1,0	**1,03**	3,0	3,1	3,2	**3,10**	4,3	4,3	4,4	**4,33**	3,30	1,65
JL 375	0,7	0,0	0,6	**0,43**	2,2	1,5	1,9	**1,87**	3,8	3,2	3,5	**3,50**	3,07	1,53
JL 453	0,8	0,0	0,0	**0,27**	2,5	0,0	1,0	**1,17**	4,3	3,4	2,7	**3,47**	3,20	1,60
CAP 063	1,0	1,0	0,9	**0,97**	2,7	2,7	2,9	**2,77**	3,7	4,1	4,2	**4,00**	3,03	1,52
CAP 148	0,5	0,6	0,6	**0,57**	2,2	2,3	2,3	**2,27**	3,9	4,0	3,9	**3,93**	3,37	1,68
CAP 150	1,1	1,2	1,1	**1,13**	2,2	2,3	2,3	**2,27**	3,1	3,0	2,7	**2,93**	1,80	0,90
CAP 154	1,3	1,2	1,2	**1,23**	2,8	2,8	2,9	**2,83**	4,1	4,0	4,1	**4,07**	2,83	1,42
CAP 160	1,2	1,1	1,2	**1,17**	2,9	2,7	2,8	**2,80**	4,3	4,0	4,0	**4,10**	2,93	1,47
CAP 163	0,6	0,6	0,6	**0,60**	1,3	1,1	0,9	**1,10**	1,8	1,7	1,4	**1,63**	1,03	0,52
CAP 166	1,0	1,0	1,0	**1,00**	2,3	2,3	2,4	**2,33**	3,5	3,6	3,5	**3,53**	2,53	1,27
CAP 168	0,8	0,4	0,5	**0,57**	2,3	2,0	2,0	**2,10**	3,7	3,3	3,5	**3,50**	2,93	1,47
CAP 169	0,7	0,5	0,7	**0,63**	2,3	2,0	2,0	**2,10**	3,7	3,3	3,5	**3,50**	2,87	1,43
CAP 171	0,8	0,7	0,8	**0,77**	2,6	2,5	2,6	**2,57**	4,0	4,0	4,2	**4,07**	3,30	1,65

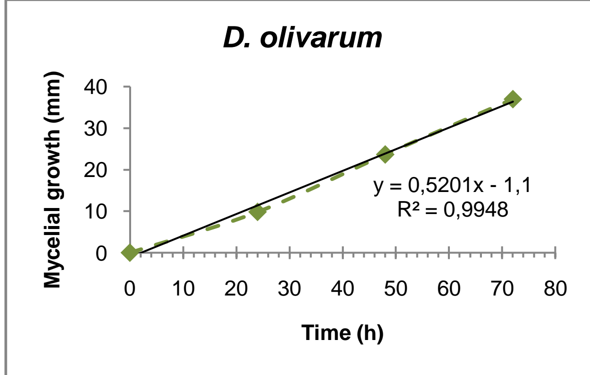
ISOLATES	MYCELIAL GROWTH (cm)												Growth rate in the last 48h (cm)	Diary growth rate (cm)
	24 h				48 h				72 h					
	1	2	3	**M**	1	2	3	**M**	1	2	3	**M**		
CAP 172	1,3	1,4	1,4	**1,37**	2,9	3,0	2,9	**2,93**	4,1	4,2	4,0	**4,10**	2,73	1,37
CAP 255	1,4	1,4	1,4	**1,40**	3,1	3,1	3,1	**3,10**	4,4	4,5	4,4	**4,43**	3,03	1,52
JL 562	0,7	0,8	0,9	**0,80**	1,5	1,7	1,9	**1,70**	2,5	2,8	2,5	**2,60**	1,80	0,90
JL 593	1,0	0,8	0,8	**0,87**	1,9	1,7	1,8	**1,80**	2,8	2,7	2,6	**2,70**	1,83	0,92
JL 515	1,0	1,1	1,1	**1,07**	3,0	3,2	3,0	**3,07**	4,5	4,6	4,3	**4,47**	3,40	1,70
JL 574	1,1	0,9	1,1	**1,03**	3,0	2,7	3,2	**2,97**	4,5	4,4	4,5	**4,47**	3,43	1,72
CAP 170	1,2	1,3	1,2	**1,23**	3,0	3,1	3,0	**3,03**	4,4	4,4	4,4	**4,40**	3,17	1,58
CAP 265	0,9	1,0	0,9	**0,93**	2,5	2,7	2,4	**2,53**	3,8	3,8	3,5	**3,70**	2,77	1,38
CAP 266	1,0	1,1	1,1	**1,07**	2,7	2,6	2,5	**2,60**	3,9	3,6	3,4	**3,63**	2,57	1,28
CAP 267	1,0	1,1	0,9	**1,00**	2,4	2,2	2,2	**2,27**	3,8	4,0	3,8	**3,87**	2,87	1,43
CAP 268	1,1	0,9	1,1	**1,03**	2,7	2,0	2,7	**2,47**	4,0	3,3	4,1	**3,80**	2,77	1,38
CAP 269	0,8	0,8	0,8	**0,80**	2,0	2,0	2,0	**2,00**	3,4	3,5	3,5	**3,47**	2,67	1,33
CAP 270	0,8	0,8	0,8	**0,80**	1,8	1,9	2,0	**1,90**	3,2	3,6	3,3	**3,37**	2,57	1,28
CAP 271	1,0	1,0	1,1	**1,03**	2,5	2,8	2,8	**2,70**	4,0	4,1	4,2	**4,10**	3,07	1,53
CAP 272	0,9	0,9	1,0	**0,93**	2,1	2,1	2,2	**2,13**	3,6	3,1	3,5	**3,40**	2,47	1,23
CAP 273	1,0	0,9	0,8	**0,90**	2,0	2,1	1,9	**2,00**	2,9	2,9	2,9	**2,90**	2,00	1,00
CAP 274	1,1	1,2	1,1	**1,13**	2,6	2,5	2,4	**2,50**	3,8	3,9	4,0	**3,90**	2,77	1,38
CAP 275	1,1	1,1	1,1	**1,10**	2,6	2,5	2,6	**2,57**	3,9	3,6	3,9	**3,80**	2,70	1,35
CAP 276	1,5	1,4	1,5	**1,47**	3,4	3,4	3,5	**3,43**	4,5	4,5	4,3	**4,43**	2,97	1,48
CAP 277	1,0	1,1	1,0	**1,03**	2,4	2,7	2,5	**2,53**	3,5	4,1	3,4	**3,67**	2,63	1,32
CAP 278	0,8	0,7	0,8	**0,77**	1,1	1,2	1,2	**1,17**	1,6	1,5	1,6	**1,57**	0,80	0,40
CAP 279	1,0	1,1	0,9	**1,00**	2,8	3,0	2,7	**2,83**	4,3	4,3	3,8	**4,13**	3,13	1,57
CAP 301	0,8	0,8	0,7	**0,77**	2,1	2	2	**2,03**	3,4	3,4	3,4	**3,40**	2,63	1,32
CAP 302	0,7	0,6	0,6	**0,63**	1,7	1,6	1,5	**1,60**	2,9	2,7	2,8	**2,80**	2,17	1,08
CAP 326	0,3	0,4	0,4	**0,37**	1,0	1,2	1,0	**1,07**	2,0	2,1	1,8	**1,97**	1,60	0,80

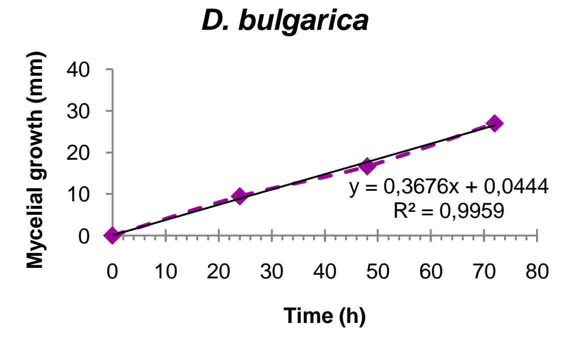
ISOLATES	MYCELIAL GROWTH (cm)												Growth rate in the last 48h (cm)	Diary growth rate (cm)
	24 h				48 h				72 h					
	1	2	3	M	1	2	3	M	1	2	3	M		
CAP 327	0,6	0,5	0,7	**0,60**	1,4	1,2	1,3	**1,30**	1,8	1,7	2,0	**1,83**	1,23	0,62
CAP 328	0,5	0,7	0,7	**0,63**	2,0	2,0	2,0	**2,00**	3,3	3,3	3,3	**3,30**	2,67	1,33
CAP 329	0,4	0,3	0,4	**0,37**	0,8	0,9	1,2	**0,97**	1,4	1,6	1,8	**1,60**	1,23	0,62
CAP 330	0,5	0,5	0,5	**0,50**	1,3	1,1	1,3	**1,23**	2,2	1,9	2,0	**2,03**	1,53	0,77
CAP 331	1	0,8	0,8	**0,87**	1,6	1,4	1,5	**1,50**	2,8	2,9	2,8	**2,83**	1,97	0,98
CAP 332	1	1	1	**1,00**	1,8	1,9	1,8	**1,83**	2,4	2,6	2,5	**2,50**	1,50	0,75
CAP 333	1	0,9	1	**0,97**	1,6	1,8	1,6	**1,67**	2,7	2,8	2,8	**2,77**	1,80	0,90
CAP 334	0,4	0,4	0,3	**0,37**	0,9	0,9	1,5	**1,10**	1,6	1,7	3,0	**2,10**	1,73	0,87
CAP 335	0,6	0,7	0,6	**0,63**	2,0	2,0	1,9	**1,97**	3,4	3,4	3,2	**3,33**	2,70	1,35
CAP 336	0,5	0,5	0,6	**0,53**	2,7	2,6	2,8	**2,70**	4,5	4,5	4,4	**4,47**	3,93	1,97
CAP 337	0,4	0,4	0,3	**0,37**	1,5	1,6	1,2	**1,43**	2,8	2,9	2,6	**2,77**	2,40	1,20
CAP 338	0,5	0,5	0,6	**0,53**	1,6	0,2	2,0	**1,26**	3,2	3,3	3,4	**3,30**	2,77	1,38
CAP 339	0,4	0,4	0,4	**0,40**	1,3	1,2	1,3	**1,27**	2,6	2,7	2,6	**2,63**	2,23	1,12
CAP 340	1,9	1,0	1,1	**1,33**	2,7	2,3	2,4	**2,47**	3,6	3,4	3,6	**3,53**	2,20	1,10
CAP 341	1,1	1,1	0,9	**1,03**	2,4	2,4	2,1	**2,30**	3,4	3,2	3,4	**3,33**	2,30	1,15
CAP 342	0,9	1,1	1,0	**1,00**	2,1	2,5	2,2	**2,27**	3,9	4,2	3,7	**3,93**	2,93	1,47
CAP 343	0,6	0,8	0,8	**0,73**	1,2	1,3	1,2	**1,23**	2,2	2,2	2,0	**2,13**	1,40	0,70
CAP 344	0,8	0,6	0,7	**0,70**	0,9	0,9	0,8	**0,87**	2,0	1,7	1,8	**1,83**	1,13	0,57
CAP 345	1,0	0,9	0,9	**0,93**	2,2	2,2	2,2	**2,20**	3,2	3,8	3,8	**3,60**	2,67	1,33
CAP 346	1,0	1,0	0,9	**0,97**	2,3	2,3	2,2	**2,27**	4,1	4,1	4,0	**4,07**	3,10	1,55
CAP 347	0,8	0,7	0,7	**0,73**	1,9	1,8	1,9	**1,87**	3,7	3,5	3,5	**3,57**	2,83	1,42
CAP 348	0,5	0,7	0,5	**0,57**	1,1	1,2	1,2	**1,17**	1,8	1,9	1,8	**1,83**	1,27	0,63
CAP 235	0,9	0,8	0,8	**0,83**	2,3	2,1	2,3	**2,23**	3,8	3,6	3,8	**3,73**	2,90	1,45
CAP257	0,9	0,6	0,8	**0,77**	2,4	1,7	2	**2,03**	3,6	3,2	3,5	**3,43**	2,67	1,33

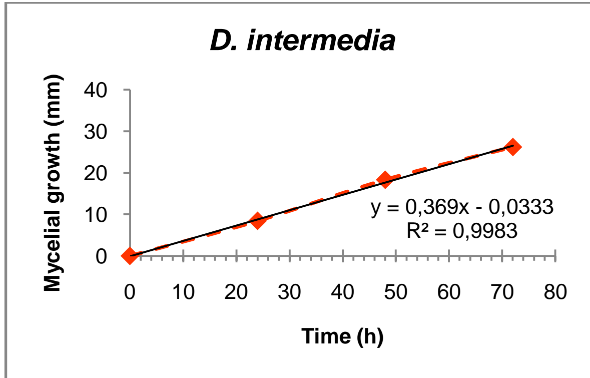
Appendix V

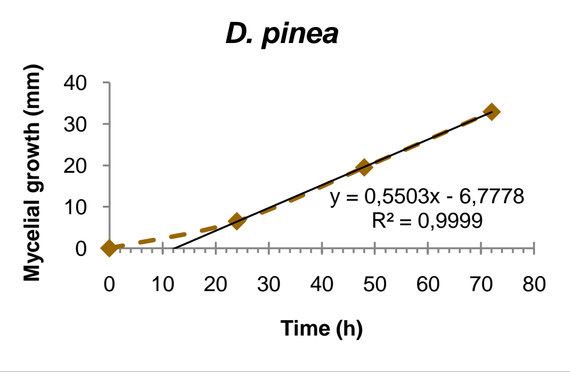


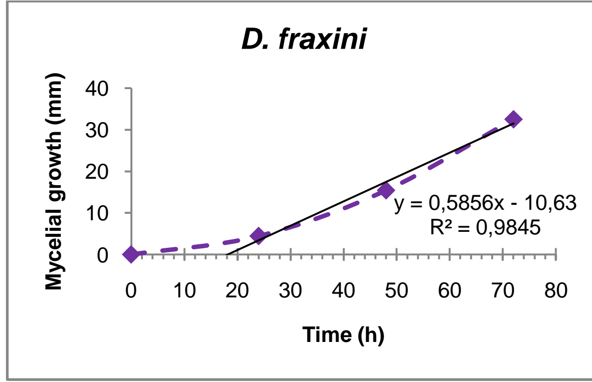


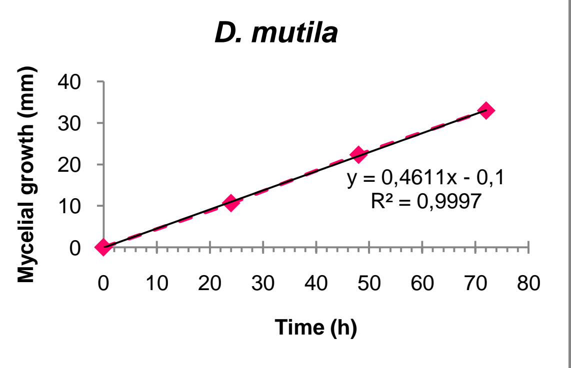












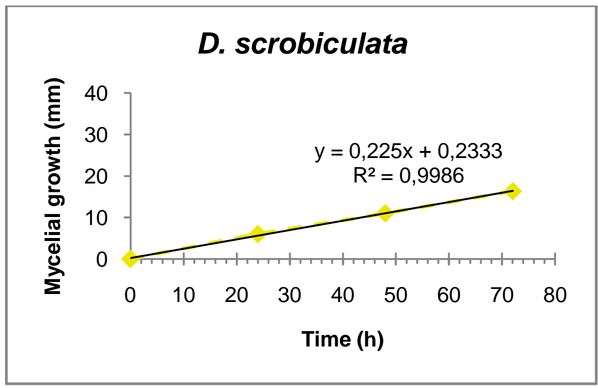


Figure I - Mycelial growth curves and respective trendlines of the species of *Diplodia* sp.

Appendix VI

Table VI. Morphological characteristics of the *Diplodia* species considered in the work.

Species	Morphology of conidia		Dimensions of conidia (μm)			
	Image	Description	Count	Length × Width	Ratio Length / Width	Mean ± Standard Deviation
D. mutila		hyaline, aseptate, smooth, thick–walled, oblong to ovoid, straight, both ends broadly rounded, rarely becoming pale brown and septate when aged.	50	(21.1–) 24.7 – 25.7 (–28.3) × (10.9–) 13.3 – 13.8 (–15.8)	1.9 ± 0.2	25.2 ± 1.9 x 13.5 ± 0.9
D. fraxini		hyaline, aseptate, smooth, thick–walled, ovoid, straight, both ends broadly rounded, rarely becoming pale brown and septate when aged.	150	(23.8–) 27.4 – 27.9 (–32.8) x (15.5–) 18.1 – 18.5 (–22.1)	1.5 ± 0.1	27.7 ± 1.8 x 18.3 ± 1.2
D. malorum		oblong with broadly rounded ends, smooth–walled, thick walled, hyaline, eguttulate aseptate. A few dark brown, one-septate conidia seen, but not within the pycnidium.	700	(21.3–)28.0 – 28.3(–36.1) x (11.1–)14.3 – 14.5 (–19.2)	2.0 ± 0.2	28.1 ± 2.4 x 14.4 ± 1.4

D. olivarum		hyaline, aseptate, smooth, thick–walled, oblong to oval, widest in the middle, apex broadly rounded, base rounded or truncate, rarely becoming pale brown, internally verruculose, one septate after discharge from the pycnidia	250	(20.8–) 25.6 – 26.2 (–31.1) x (8.3–) 12.1 – 12.5 (–16.9)	2.1 ± 0.3	25.9 ± 2.2 x 12.3 ± 1.8
D. bulgarica		aseptate, smooth, thick-walled, oblong to ovoid, straight, both ends broadly rounded, initially hyaline but becoming pale brown soon after discharge from pycnidia and occasionally one- septate.	50	(22.4–) 25.0 – 25.7 (–28.2) x (14.5–) 16.6 – 17.0 (–18.4)	1.5 ± 0.1	25.4 ± 1.2 x 16.8 ± 0.7
D. seriata		aseptate, ovoid, widest in the middle, with obtuse apex and truncate or rounded base. Initially hyaline but become dark brown before being released from the pycnidia. Wall moderately thick, with smooth outer surface and roughened inner suface.	250	(17.7–) 25.5 – 26.3 (–33.4) x (8.1–) 11.1 – 11.5 (–16.0)	2.3 ± 0.3	25.9 ± 3.1 x 11.3 ± 1.5

D. pinea		oblong to clavate, straight, aseptate, thick–walled, ornamented on the inner surface of the wall, apex obtuse, base truncate. wall dark brown, internally roughened, sometimes becoming 1–septate.	150	(30.0–) 40.1 – 41.4 (–54.6) x (10.6–) 14.4 – 14.9 (–21.0)	2.8 ± 0.5	40.7 ± 4.6 x 14.7 ± 1.7
D. intermedia		aseptate, ovoid to oblong, widest in the middle, with obtuse apex and truncate or rounded base. initially hyaline but soon becoming dark brown while in the pycnidia; wall moderately thick, with a smooth outer surface and roughened on the inner suface.	250	(24.6–) 30.3 – 30.9 (–36.9) x (10.0–) 13.0 – 13.6 (–17.7)	2.3 ± 0.3	30.6 ± 1.9 x 13.3 ± 1.8
D. scrobiculata		clavate to truncate, dark brown, straight, thick-walled, apex obtuse, base truncate, aseptate.	50	(28.9) 32.0 – 33.0 (–37.4) x (10.7–) 13.0 – 13.5 (–15.1)	2.5 ± 0.2	32.5 ± 1.7 x 13.2 ± 0.9