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Resolving the *Diplodia* complex on apple and other *Rosaceae* hosts

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Key words

apple
black-rot
Botryosphaeriaceae
canker
Diplodia
phylogeny

Abstract *Diplodia* species are known as pathogens on many woody hosts, including fruit trees, worldwide. In this study a collection of *Diplodia* isolates obtained mostly from apple and other *Rosaceae* hosts were identified based on morphological characters and DNA sequence data from ITS and EF1-α loci. The results show that the diversity of species associated with twig and branch cankers and fruit rot of apples is larger than previously recognised. Four species were identified, namely *D. seriata* and *D. malorum* (which is here reinstated for isolates with *D. mutila*-like conidia). *Diplodia intermedia* sp. nov. is closely related to *D. seriata*, and *D. bulgarica* sp. nov. is morphologically and phylogenetically distinct from all *Diplodia* species reported from apples.

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INTRODUCTION

Species of *Diplodia*, like other members of the family *Botryosphaeriaceae*, are known to be pathogens, endophytes and saprophytes on a wide range of mainly woody hosts (Crous et al. 2006, Slippers & Wingfield 2007). Some of the more important pathogenic species include *D. pinea*, which causes crown wilt, dieback, cankers, shoot and tip blight, and root disease on pines (Eldridge 1961); *D. mutila*, the cause of black rot and canker of apples and *D. seriata*, which causes frog-eye leaf spot, black rot and canker of apples (Stevens 1933, Laundon 1973, Brown & Britton 1986, Brown-Rytlewski & McManus 2000); and *D. corticola* the cause of canker and dieback of cork and other oaks (Alves et al. 2004). There have been conflicting reports on the pathogenicity of some of the species. Thus, Larignon et al. (2001), Epstein et al. (2008) and Savocchia et al. (2007) considered *D. seriata* to be a primary and virulent pathogen of grapevines while Phillips (1998, 2000), van Niekerk et al. (2004) and Laveau et al. (2009) found it to be saprophytic or weakly pathogenic on this host. Also, *D. seriata* is regarded as an important pathogen causing canker, leaf spot and fruit rot of apple in the USA (Stevens 1933, Brown & Britton 1986, Brown-Rytlewski & McManus 2000) but as a weak secondary pathogen on the same host in England and New Zealand (Laundon 1973). These differences may be due to variations in virulence between strains, or they may be a result of the incomplete knowledge of the taxonomy of the genus, which in turn hampers accurate species recognition and identification. It is also possible that in species with a broad host range, such as *D. seriata*, virulence of any given isolate may vary according to the host that is being attacked.

Diplodia is a large genus with more than 1 000 species currently recognised. A search of MycoBank (March 2012; www.mycobank.org) revealed 1 244 names while a search of Species Fungorum (March 2012; www.speciesfungorum.org) lists 1 242 names. The genus was introduced by Montagne (1834) with *D. mutila* as the type species. As explained by Phillips et al. (2005) the concept of *Diplodia* has changed over the years and has been regarded as including species with dark brown, 1-septate conidia. However, the genus is typified by *D. mutila*, which has hyaline, aseptate conidia that can become brown and septate with age. Phillips et al. (2005) provided an emended description of the genus. Briefly, *Diplodia* is circumscribed by having uni- or multilocular conidiomata lined with conidiogenous cells that form hyaline, aseptate, thick-walled conidia at their tips (Phillips et al. 2005). The conidiogenous cells proliferate internally giving rise to periclinal thickenings, or proliferate percurrently to form two or three annellations. Typically the conidia remain hyaline for a long time before they become brown and 1-septate, but in some species, such as *D. pinea*, *D. scrobiculata* and *D. seriata*, the conidia become coloured before discharge from the pycnidia and mostly remain aseptate (Phillips et al. 2005). No paraphyses are found in the conidiomata of *Diplodia* species.

Despite the relatively simple generic definition, species are less easily defined. This is largely because there are few distinguishing morphological features. For many years species in *Diplodia* were defined on the basis of host association, which resulted in a proliferation of species names. According to Slippers et al. (2004) host is not of primary importance in species differentiation in the *Botryosphaeriaceae* and thus many of the names in *Diplodia* are likely to be synonyms.

Since 2003 several new species have been described in *Diplodia* and these species were recognized mainly from DNA sequence data and minor differences in conidial morphology. For example, *D. scrobiculata* was differentiated from *D. pinea* on the basis of consistent grouping of isolates in multiple gene genealogies inferred from six protein coding genes and six microsatellite loci (de Wet et al. 2003).

Amongst the species with hyaline conidia, recently described new species include *D. rosulata* with characteristic rosulate colonies (Gure et al. 2005), and *D. corticola* with large conidia (Alves et al. 2004). *Diplodia africana* (Damm et al. 2007),

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Table 1 Isolates of *Diplodia* species considered in this study.

Species	Accession number[1]	Host	Location	Collector	GenBank Numbers [2]	
					ITS	EF1-α
"Botryosphaeria" tsugae	CBS 418.64[3]	*Tsuga heterophylla*	British Columbia, Lake Cowichan, Canada	A. Funk	*DQ458888*	*DQ458873*
Diplodia corticola	**CBS 112547**	*Quercus ilex*	La Rozuela, Córdoba	M.E. Sánchez	*AY259110*	*DQ458872*
	CBS 112549	*Quercus suber*	Requeixo, Aveiro, Portugal	A. Alves	*AY259100*	*AY573227*
Diplodia africana	**CBS 120835**	*Prunus persica*	Paarl, Western Cape, South Africa	U. Damm	*EF445343*	*EF445382*
	CBS 121104	*Prunus persica*	Paarl, Western Cape, South Africa	U. Damm	*EF445344*	*EF445383*
Diplodia bulgarica	**CBS 124135**	*Malus sylvestris*	Plovdiv, Bulgaria	S. Bobev	GQ923852	GQ923820
	CBS 124254	*Malus sylvestris*	Plovdiv, Bulgaria	S. Bobev	GQ923853	GQ923821
	CBS 124136	*Malus sylvestris*	Plovdiv, Bulgaria	S. Bobev	GQ923854	GQ923822
	IRAN1530C	*Malus domestica*	Gahvareh village, Kermanshah, Iran	J. Abdollahzadeh	JX152582	JX152578
	IRAN1532C	*Malus domestica*	Gahvareh village, Kermanshah, Iran	J. Abdollahzadeh	JX152583	JX152579
	IRAN1548C	*Malus domestica*	Gahvareh village, Kermanshah, Iran	J. Abdollahzadeh	JX152584	JX152580
Diplodia cupressi	CBS 168.87	*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	*DQ458893*	*DQ458878*
	CBS 261.85	*Cupressus sempervirens*	Bet Dagan, Israel	Z. Solel	*DQ458894*	*DQ458879*
Diplodia intermedia	CAA 147	*Malus domestica* (fruit rot)	Aveiro, Portugal	A. Alves	GQ923857	GQ923825
	CBS 112556	*Malus sylvestris* (canker)	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	*AY259096*	GQ923851
	CBS 124134	*Cydonia* sp. (fruit rot)	Torres Vedras, Portugal	S. Santos	HM036528	GQ923851
	CBS 124462	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923858	GQ923826
	IRAN1559C	Unknown woody plant	Rezvanshahr, Rasht, Gilan, Iran	J. Abdollahzadeh	JX152585	JX152581
Diplodia sp.	CAP 330	*Pyracantha coccinea*	Plovdiv, Bulgaria	S. Bobev	GQ923881	GQ923849
Diplodia malorum	CAP 265	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923859	GQ923827
	CAP 266	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923860	GQ923828
	CAP 267	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923861	GQ923829
	CAP 268	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923862	GQ923830
	CAP 269	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923863	GQ923831
	CAP 270	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923864	GQ923832
	CBS 124130	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923865	GQ923833
	CAP 272	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923866	GQ923834
	CBS 124253	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923867	GQ923835
	CAP 275	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923868	GQ923836
	CAP 277	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923869	GQ923837
	CAP 278	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923870	GQ923838
	CAP 340	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923871	GQ923839
	CAP 341	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	GQ923872	GQ923840
	CBS 112554	*Malus sylvestris*	Monte da Caparica, Setúbal, Portugal	A.J.L. Phillips	*AY259095*	*DQ458870*
Diplodia mutila	CBS 112553	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259093*	*AY573219*
	CBS 230.30	*Phoenix dactylifera*	California, USA	L.L. Huillier	*DQ458886*	*DQ458869*
Diplodia olivarum	CAP 301	*Ceratonia siliqua*	Sicily, Italy	A. Sidoti	GQ923873	GQ923841
	CAP 222	*Olea europaea*	Cutrofiano, Lecce, Puglia, Italy	S. Frisullo	*EU392295*	*EU392272*
	CAP 224	*Olea europaea*	Salice Salentino, Lecce, Puglia, Italy	S. Frisullo	*EU392296*	*EU392273*
	CAP 225	*Olea europaea*	Campi Salentino, Lecce, Puglia, Italy	S. Frisullo	*EU392297*	*EU392274*
	CBS 121887	*Olea europaea*	Italy, Puglia, Lecce, Bosco Belvedere, Scorrano	S. Frisullo	*EU392302*	*EU392279*
	CAP 257	*Olea europaea*	Montesano Salentino, Lecce, Puglia, Italy	S. Frisullo	GQ923874	GQ923482
Diplodia pinea	CAP 166	*Olea europaea*	Scanzano, Matera, Basilicata, Italy	S. Frisullo	*EU392284*	*EU392261*
	CAP 168	*Olea europaea*	Scorano, Lecce, Puglia, Italy	S. Frisullo	*EU392285*	*EU392262*
	CAP 169	*Olea europaea*	Cutrofiano, Lecce, Puglia, Italy	S. Frisullo	*EU392286*	*EU392263*
	CAP 339	*Pinus* sp.	Belgium	S. Bobev	GQ923875	GQ923843
	CBS 393.84	*Pinus nigra*	Putten, Netherlands	H.A. van der Aa	*DQ458895*	*DQ458880*
	CBS 109727	*Pinus radiata*	Stellenbosch, South Africa	W.J. Swart	*DQ458897*	*DQ458882*
	CBS 109725	***Pinus patula***	Habinsaran, Indonesia	M.J. Wingfield	*DQ458896*	*DQ458881*
	CBS 109943	*Pinus patula*	Indonesia	M.J. Wingfield	*DQ458898*	*DQ458883*
Diplodia rosulata	**CBS 116472**	*Prunus africana*	Gambo, Ethiopia	A. Gure	*EU430266*	*EU430268*
	CBS 116470	*Prunus africana*	Gambo, Ethiopia	A. Gure	*EU430265*	*EU430267*

Diplodia scrobiculata	CMW 189	*Pinus banksiana*	USA	M.A. Palmer	*AY253292*	*AY624253*
	CBS 109944	*Pinus greggii*	Mexico	M.J. Wingfield	*DQ458899*	*DQ458884*
	CBS 113423	*Pinus greggii*	Mexico	M.J. Wingfield	*DQ458900*	*DQ458885*
	CAP 163	*Olea europaea*	Supersano, Lecce, Puglia, Italy	S. Frisullo	*EU392283*	*EU392260*
Diplodia seriata	**CBS 112555**	*Vitis vinifera*	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259093*	*AY573219*
	CBS 119049	*Vitis vinifera*	Italy	L. Mugnai	*DQ458889*	*DQ458874*
	CAP 154	*Vitis vinifera*	France	P. Larignon	*EU392303*	*EU392280*
	CAP 171	*Olea europaea*	Ruffano, Lecce, Puglia, Italy	S. Frisullo	*EU392305*	*EU392282*
	CAP 276	*Malus sylvestris*	Monte da Caparica, Portugal	A.J.L. Phillips	GQ923876	GQ923844
	CBS 124137	*Prunus domestica*	Plovdiv, Bulgaria	S. Bobev	GQ923877	GQ923845
	CBS 124138	*Malus sylvestris*	Plovdiv, Bulgaria	S. Bobev	GQ923878	GQ923846
	CAP 337	*Cotoneaster bullatus*	Plovdiv, Bulgaria	S. Bobev	GQ923879	GQ923847
	CBS 124139	*Vitis vinifera*	Plovdiv, Bulgaria	S. Bobev	GQ923880	GQ923848
	CBS 121885	*Olea europaea*	Casarano, Lecce, Puglia, Italy	S. Frisullo	*EU392289*	*EU392266*
	CAP 217	*Olea europaea*	Ruffano, Lecce, Puglia, Italy	S. Frisullo	*EU392293*	*EU392270*
	CAP 220	*Olea europaea*	Felline, Lecce, Puglia, Italy	S. Frisullo	*EU392294*	*EU392271*
Diplodia pseudoseriata	**UY107**	*Myrcianthes cisplatensis*	Uruguay	C. Perez	*EU080914*	*EU863178*
	CBS124907	*Hexachlamis edulis*	Uruguay	C. Perez	*EU080922*	*EU863179*
	CBS 124906	*Blepharocalyx salicifolius*	Uruguay	C. Perez	*EU080927*	*EU863181*
	UY1263	*Myrciaria tenella*	Uruguay	C. Perez	*EU080933*	*EU863182*
Diplodia alatafructa	CBS 124931	*Pterocarpus angolensis*	Sudwala Caves area, South Africa	J. Mehl & J. Roux	*FJ888460*	*FJ888444*
	CBS 124932	*Pterocarpus angolensis*	Sudwala Caves area, South Africa	J. Mehl & J. Roux	*FJ888461*	*FJ888445*
	CBS 124933	*Pterocarpus angolensis*	Buffelskloof Nature Reserve, South Africa	J. Mehl & J. Roux	*FJ888478*	*FJ888446*
Lasiodiplodia theobromae	**CBS164.96**	Fruit along coral reef coast	New Guinea	A. Aptroot	*AY640255*	*AY640258*
	CBS124.13	Unknown	USA	J.J. Taubenhaus	*DQ458890*	*DQ458875*

[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 Pretoria, South Africa.
[2] Sequence numbers in italics were retrieved from GenBank. All other sequences were obtained in this study.
[3] Ex-type strains in **bold** face.

D. olivarum (Lazzizera et al. 2008) and *D. cupressi* (Alves et al. 2006) were differentiated from *D. mutila* on the basis of their unique conidial morphology. All five species formed distinct clades in phylogenies based on ITS and EF1-α sequence data.

Although Slippers et al. (2004) suggested that host association may not be a suitable character for species differentiation in the *Botryosphaeriaceae*, many species in *Diplodia* do show some host preference. For example, *D. pinea* and *D. scrobiculata* occur only on conifers with rare reports on angiosperm hosts such as *Prunus* and *Olea* (Damm et al. 2007, Lazzizera et al. 2008). *Diplodia rosulata* has been found only on *Prunus* spp. (Gure et al. 2005), while *D. africana* initially found only on *Prunus* spp. (Damm et al. 2007) has meanwhile been reported as causing dieback on *Juniperus phoenicea* in Sardinia (Linaldeddu et al. 2011). *Diplodia olivarum* was considered to be restricted to *Olea* spp. (Lazzizera et al. 2008), and *D. cupressi* has been found only on *Cupressus* and *Juniperus* (Solel et al. 1987, Alves et al. 2006). *Diplodia corticola* has been found mainly on *Quercus* spp., although one isolate studied by Alves et al. (2004) was from *Tsuga* and another was from *Cercis*. More recently *D. corticola* has been reported from grapevines (Úrbez-Torres et al. 2010).

A collection of *Diplodia* isolates was obtained from stem cankers and fruit rots of mainly apple trees (*Malus*) and other *Rosaceae* hosts (*Cydonia*, *Pyracantha*, *Cotoneaster*) in Portugal, Bulgaria and Iran. The aim of this study was to determine the identity of the species. For this, the isolates were characterised in terms of morphology and their phylogenetic relationships to known species of *Diplodia*.

MATERIALS AND METHODS

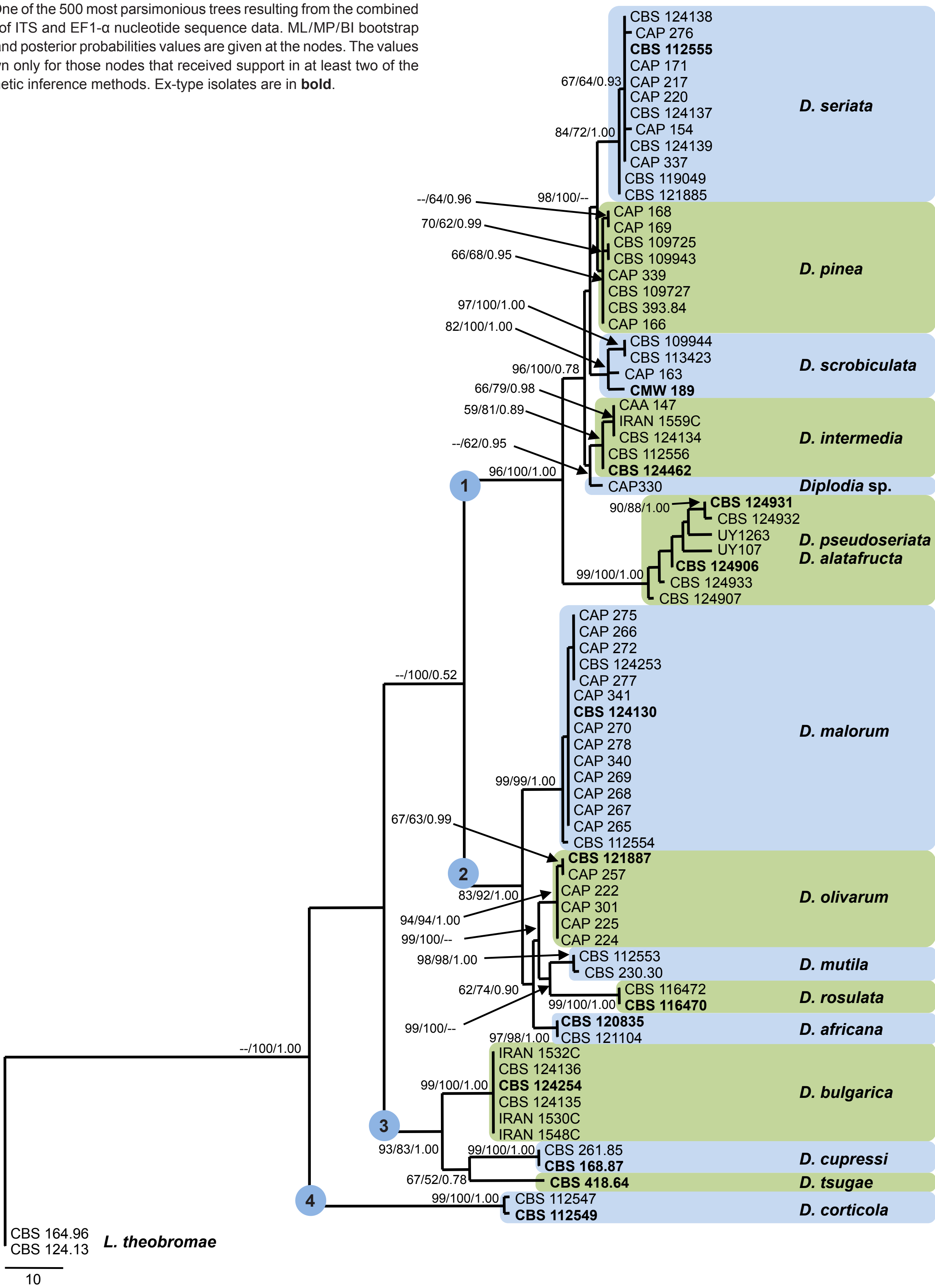
Isolates

Isolations were made by spreading ascospores or conidia on the surface of Difco (Becton, Dickinson & Co, Sparks, USA) potato-dextrose agar (PDA) and incubating overnight at 25 °C. Single germinating spores were transferred to fresh plates of PDA. Isolates were cultured on half-strength PDA (1/2 PDA) or on water agar supplemented with autoclaved pine needles (Smith et al. 1996) on the agar surface. Cultures were kept on the laboratory bench at about 20–25 °C where they received diffused daylight. Growth rates were determined on PDA plates incubated in the dark at 25 °C. Representative isolates and specimens were deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and nomenclatural data in MycoBank (Crous et al. 2004).

DNA isolation and amplification

DNA was isolated from fungal mycelium by the method of Möller et al. (1992). Procedures and protocols for DNA sequencing were as described by Alves et al. (2004). PCR reactions were carried out with *Taq* polymerase, nucleotides and buffers supplied by MBI Fermentas (Vilnius, Lithuania) and PCR reaction mixtures were prepared according to Alves et al. (2004), with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. All primers were synthesised by MWG Biotech AG (Elbersberg, Germany). The ITS region was amplified using the primers ITS1 and ITS4 (White et al. 1990) as described by Alves et al. (2004). The primers EF1-728F and EF1-986R (Carbone & Kohn 1999) were used to amplify part of the translation elongation factor 1-alpha (EF1-α) as described by Alves et al. (2006). The amplified PCR products were purified with the JETQUICK PCR Purification Spin Kit (GENOMED, Löhne, Germany). The PCR products were sequenced by STAB Vida Lda (Portugal).

Fig. 1 One of the 500 most parsimonious trees resulting from the combined analysis of ITS and EF1-α nucleotide sequence data. ML/MP/BI bootstrap support and posterior probabilities values are given at the nodes. The values are shown only for those nodes that received support in at least two of the phylogenetic inference methods. Ex-type isolates are in **bold**.



Phylogenetic analysis

Sequences were aligned with ClustalX v. 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 phylogenetic analyses using simple indel coding as implemented by GapCoder (Young & Healy 2003).

Phylogenetic analyses of sequence data were done using PAUP v. 4.0b10 (Swofford 2003) for Maximum-parsimony (MP) analyses, Mr Bayes v. 3.0b4 (Ronquist & Huelsenbeck 2003) for Bayesian Inference (BI) analyses and MEGA5 (Tamura et al. 2011) for Maximum-likelihood (ML) analyses. The general time-reversible model of evolution (Rodriguez et al. 1990), including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+Γ+G) was used for both ML and BI analyses. Trees were rooted to *L. theobromae* and visualized with TreeView (Page 1996).

Maximum-parsimony analyses were performed using the heuristic search option with 1 000 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. Maxtrees were set to 500, branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated from 1 000 bootstrap replications (Hillis & Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI).

Bayesian analyses employing a Markov Chain Monte Carlo method were performed. Four MCMC chains were run simultaneously, starting from random trees for 1 000 000 generations. Trees were sampled every 100th generation for a total of 10 000 trees. The first 1 000 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala & Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 9 000 trees. This analysis was repeated three times starting from different random trees to ensure trees from the same tree space were sampled during each analysis.

ML analyses were performed on a starting tree automatically generated by the software. Nearest-Neigbor-Interchange (NNI) was used as the heuristic method for tree inference and 1 000 bootstrap replicates were performed. Bootstrapping analysis was computed using ML with an estimated proportion of invariant sites and empirical base frequencies with the indicated outgroup sequences.

A comparison of highly supported clades (bootstrap support values ≥ 70 %) among trees generated from MP analyses of individual datasets was performed in order to detect conflict between individual phylogenies (Alves et al. 2008).

RESULTS

DNA phylogeny

Approximately 550 and 300 bases were determined for the ITS and EF1-α genes, respectively. New sequences were deposited in GenBank (Table 1) and the alignment in TreeBase (submission ID 12819). No major conflicts were detected between single gene phylogenies indicating that the genes could be combined. After alignment the combined ITS and EF1-α dataset consisted of 946 characters (including alignment gaps) for 74 ingroup taxa and 2 outgroup taxa. Of the 946 characters, 735 were constant and 18 were variable and parsimony-uninformative. Maximum parsimony analysis of the remaining 193 parsimony-informative characters resulted in 500 most parsimonious trees of 312 steps (CI = 0.753, RI = 0.955, HI = 0.247) and one is shown in Fig. 1. ML and BI analyses retrieved phylogenetic trees whose topologies were identical to the MP tree presented.

Four main clades were identified and in Fig. 1 they are labelled 1–4. Clade 1 is composed of species with brown, aseptate conidia that occasionally develop one or two septa. Six species can be resolved in this clade, although bootstrap support (MP and ML) for some of them was generally low. One of these species is described here as new. Clade 2 consists of five species with hyaline, aseptate conidia that later become brown and one septate. Clade 3 includes three species, one of which is here described as new, that have conidia that are hyaline or pale brown and become one-septate. Clade 4 is composed only of *D. corticola* whose conidia are similar to those of species in clade 2.

Taxonomy

A group of isolates from *Malus* in clade 2 lay within a distinct sub-clade separate from all other species and was supported by high bootstrap value and posterior probability. Morphologically these isolates correspond in all ways with the isotype of *D. malorum*. Therefore this name is re-instated for the species that is found on *Malus*, and an epitype is designated here. A group of isolates from *Malus* and one from *Cydonia*, morphologically similar to *D. pinea*, lay within a distinct sub-clade in clade 1 and were considered to represent a distinct species, which is described here as *Diplodia intermedia* sp. nov. A further species in clade 3 sister to *D. cupressi* and '*B.*' *tsugae* does not correspond to any known species and is described here as *D. bulgarica* sp. nov.

Diplodia bulgarica A.J.L. Phillips, J. Lopes & S.G. Bobev, *sp. nov*. — MycoBank MB519632; Fig. 2

Etymology. Named after Bulgaria where this species was first found.

Conidiomata pycnidial, produced on pine needles on WA after 7–21 d, solitary, immersed, partially erumpent when mature, dark brown to black, globose to ovoid, up to 600 μm diam and 700 μm high, mostly unilocular; wall composed of an outer layer 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* 9–18 × 2–5 μm, hyaline, smooth, thin-walled, cylindrical, slightly swollen at the base, holoblastic, forming a single conidium at the tip, discrete, indeterminate, proliferating internally giving rise to periclinal thickenings, or proliferating percurrently to form 1–5 annellations. *Conidia* aseptate, externally smooth, internally verruculose, thick-walled, oblong to ovoid, straight, both ends broadly rounded, (22.5–)24–27(–28) × (14.5–)15.5–18(–18.5) μm, 95 % confidence limits = 25–25.7 × 16.6–17 μm (mean ± S.D. of 50 conidia = 25.4 ± 1.2 × 16.8 ± 0.7 μm, L/W ratio = 1.5 ± 0.1), initially hyaline, soon becoming pale brown, later darkening and becoming 1-septate.

Specimens examined. Bulgaria, Plovdiv, *Malus sylvestris*, 2005, *S.G. Bobev* (CBS H-20189 holotype, culture ex-type CBS 124254). Additional isolates are given in Table 1.

Notes — This species is morphologically distinct from other *Diplodia* species reported from apples. Conidia are shorter and wider than both *D. intermedia* and *D. malorum*. Furthermore, the conidia are distinctive in that they become pale brown soon after they are formed. Phylogenetically this species is closely related to *D. cupressi* and '*B.*' *tsugae*.

Diplodia intermedia A.J.L. Phillips, J. Lopes & A. Alves, *sp. nov.* — MycoBank MB519633; Fig. 3

Etymology. Named after its morphology and phylogenetic position that are intermediate between *D. pinea* and *D. seriata*.

Ascomata unilocular, solitary or clustered, immersed, partially erumpent when mature, globose, up to 400 μm diam, dark brown to black, thick-walled, wall composed of outer layers of thick-walled, dark brown *textura angularis*, inner layers of thin-walled, hyaline *textura angularis*. *Ostiole* central, circular, non-papillate, periphysate. *Pseudoparaphyses* hyaline, branched, septate, constricted at the septum, 2–3 μm wide. *Asci* clavate, stipitate, bitunicate, 85–160 × 22–28 μm, containing eight

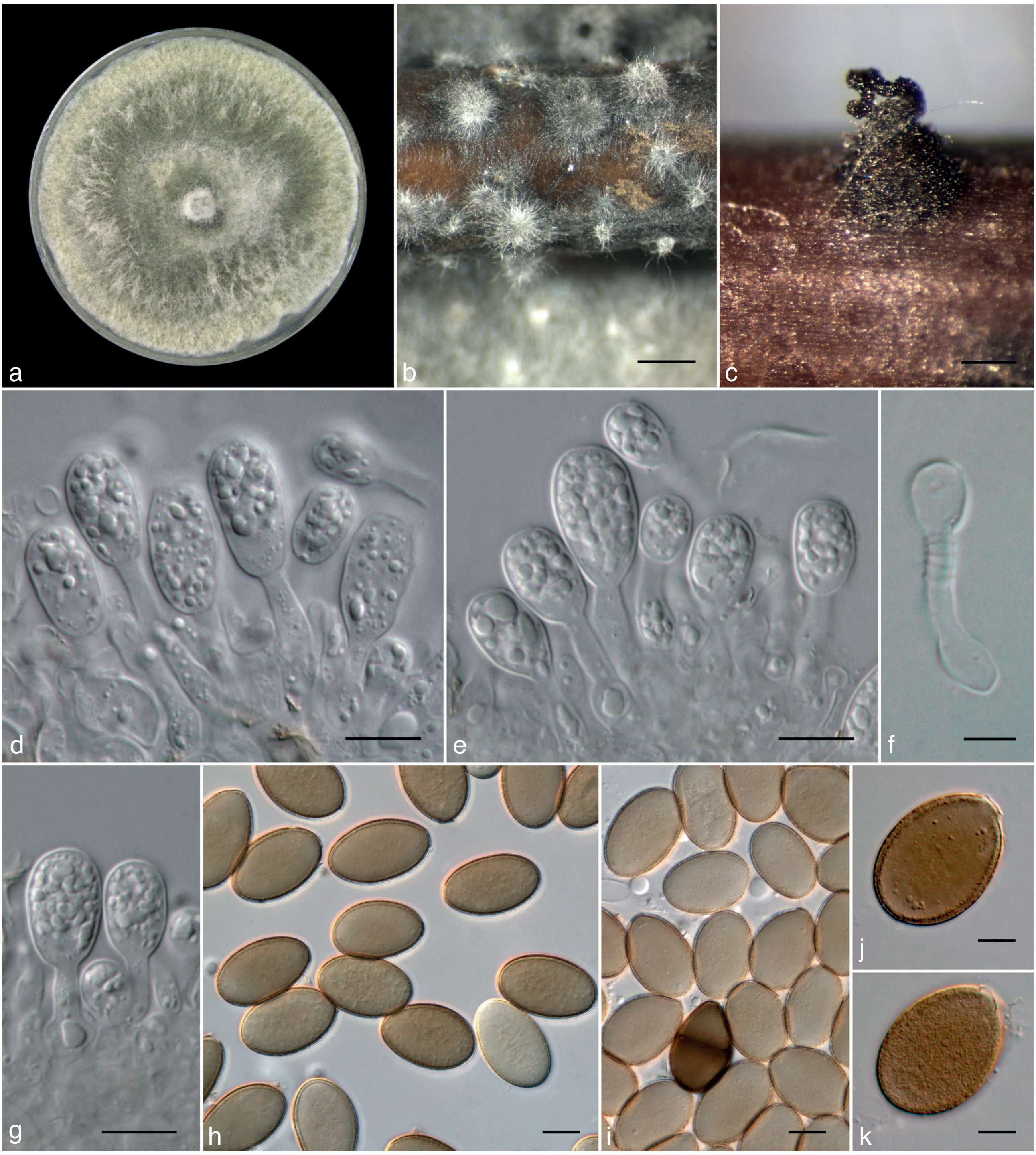


Fig. 2 *Diplodia bulgarica*. a. Culture growing on PDA; b. pycnidia developing on pine needles in culture; c. pycnidium on pine needle exuding conidia; d–g. conidiogenous cells with developing conidia; h. brown, aseptate conidia; i. brown aseptate conidia and a 2-celled conidium; j, k. conidium in two levels of focus showing finely verruculose inner surface of the conidium wall. — Scale bars: b = 500 µm; c = 200 µm; d–i = 10 µm; j, k = 5 µm.

ascospores biseriate in the ascus. *Ascospores* 32–37(–40) × 6–8 µm, fusiform, widest in the upper third, hyaline, thin-walled, smooth, aseptate. *Conidiomata* pycnidial, solitary or clustered, immersed in the host, partially erumpent at maturity, 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* 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, apex obtuse, base truncate or rounded, initially hyaline, becoming dark brown before release from the pycnidia, wall moderately thick, externally smooth, roughened on the inner surface, (24.6–)29–33.5(–36.9) × (10–)11–16(–17.5) µm, with 95 % confidence limits = 30.2–31.1 × 13–13.6 µm (mean ± S.D. of 150 conidia = 30.6 ± 1.9 × 13.3 ± 1.8 µm, L/W = 2.3 ± 0.3). *Microconidiogenous* cells not seen. *Microconidia* hyaline, aseptate, smooth, oblong, ends rounded, 5.5–9.5 × 4–6.5 µm.

Specimens examined. Portugal, Setúbal, Monte da Caparica, dead twigs of *Malus sylvestris*, Mar. 2006, *A.J.L. Phillips* (CBS H-20190 holotype, culture ex-type CBS 124462). Additional isolates listed in Table 1.

Notes — Phylogenetically this species is very closely related to *D. pinea*. However, on account of its smaller conidia, apparent preference for *Rosaceae* hosts, and the distinct clade it forms in the phylogenetic trees we consider it to represent a separate species.

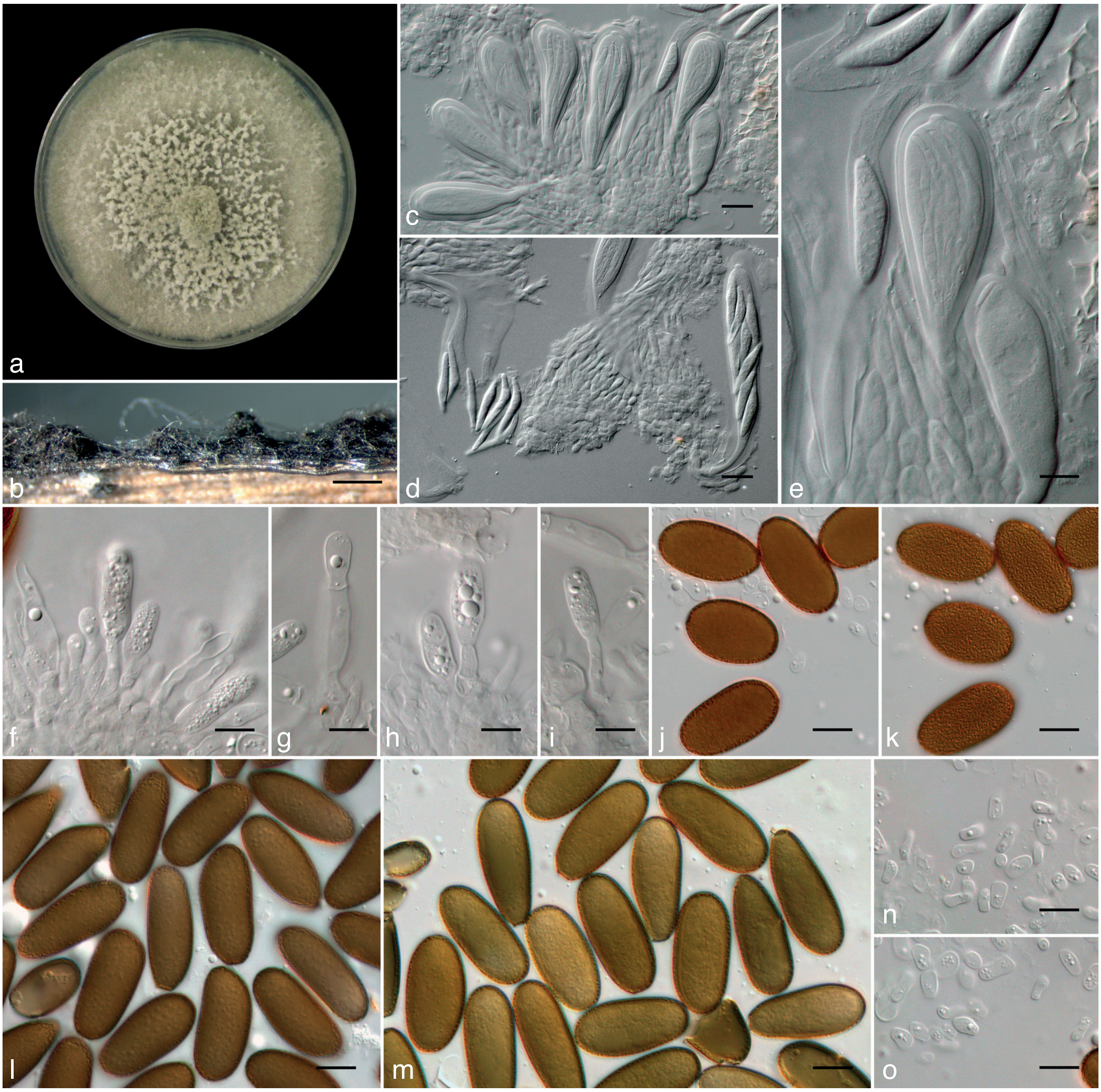


Fig. 3 *Diplodia intermedia*. a. Culture growing on PDA; b. pycnidia developing on pine needle; c. asci; d, e. ascus, ascospores and pseudoparaphyses; f–i. conidiogenous cells; j, k. conidia in two levels of focus to show finely verruculose inner surface of the conidium wall; l, m. conidia; n, o. microconidia. — Scale bars: b = 500 µm; c, d = 20 µm; e–m = 10 µm.

Diplodia malorum Fuckel, Jahrb. Nassauischen Vereins Naturk. 23–24: 395. 1870. — MycoBank MB246351; Fig. 4

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 indistinct annelations. *Conidia* oblong with broadly rounded ends, smooth-walled, thick walled, hyaline, eguttulate, aseptate, becoming dark brown and 1-septate soon after release from the pycnidium, (24–)26–32(–36) × (12–)13–17.5(–18.5) µm, 95 % confidence intervals = 28–28.3 × 14.3–14.5 µm, ($\bar{x}$ ± S.D. of 700 conidia = 28.1 ± 2.4 × 14.43 ± 1.4 µm, L/W = 1.9 ± 0.24).

Specimens examined. Germany, Rhineland, on *Malus*, 1870, *J. Fuckel*, Fuckel, Fungi rhenani N° 1706 in K and M (isotypes). – Portugal, Setúbal, Monte da Caparica, *Malus sylvestris*, Feb. 2006, *A.J.L. Phillips* (CBS H-201888 epitype designated herein, culture ex-epitype CBS 124130). Additional isolates given in Table 1.

Notes — Since the time that it was introduced by Fuckel (1870) the name *D. malorum* has been used infrequently, while the name *D. mutila* was applied to the apple pathogen. However, as shown here, *D. malorum* is morphologically and phylogenetically distinct from *D. mutila*. The conidia are larger than those of *D. mutila* and they frequently become brown and 1-septate soon after discharge from the pycnidia. The characteristics of the isolates studied here correspond with the isotypes (Fungi rhenani Nº 1706 in K and M).

Diplodia tsugae (A. Funk) A.J.L. Phillips & A. Alves, *comb. nov.* — MycoBank MB801409

Basionym. *Botryosphaeria tsugae* A. Funk, Canad. J. Bot. 42: 770. 1964.

Specimens examined. Canada, British Columbia, near Bella Coola (Snootli Creek), on branches of *Tsuga heterophylla*, 11 Sept. 1963, *A. Funk* (DAVFP 15485 holotype, DAOM 96030 isotype, CBS H-6790 isotype, culture ex-isotype CBS 418.64).

Notes — When Funk (1964) introduced this species he did not apply a name to the anamorph, which he referred to as a *Macrophoma* species. When Crous et al. (2006) re-defined *Botryosphaeria* they removed this species to *Diplodia* but did not formally make a new combination. Since morphologically and phylogenetically this species is clearly a *Diplodia* species we recombine it in *Diplodia*.

DISCUSSION

In this work a collection of *Diplodia* isolates mainly from apples was studied in terms of morphology and phylogenetic position based on nucleotide sequence data from ITS and EF1-α loci. Two species are described as new, while one existing name that has rarely been used is recognised as valid and distinct. All species studied in this work lay within four distinct clades in a combined ITS plus EF1-α phylogeny. Each clade was characterised by distinct morphological features of the anamorphs. Isolates from apples and other hosts in *Rosaceae* were distributed between three of these clades.

The distinct morphologies associated with each clade deserves some comment. Clade 1 includes species with brown, aseptate conidia that occasionally develop one or two septa. Another distinctive feature of species in this clade is that their conidia become coloured at an early stage of development even before dehiscence from the conidiogenous cells. Within

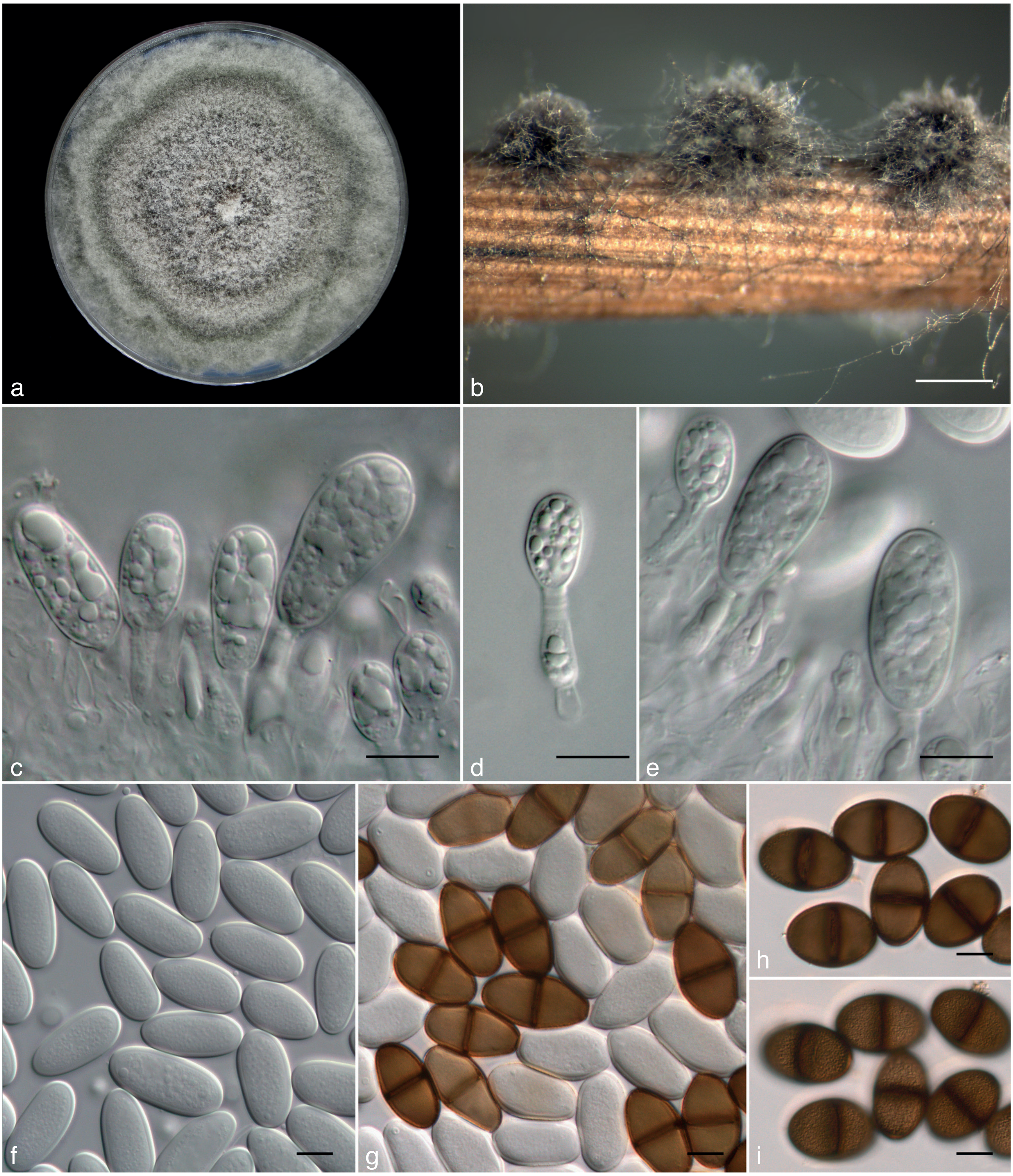


Fig. 4 *Diplodia malorum*. a. Culture growing on PDA; b. pycnidia formed on pine needles; c–e. conidiogenous cells; f. hyaline conidia; g; hyaline and 1-septate brown conidia; h, i. brown conidia at different levels of focus to show the finely verruculose inner surface of the wall. — Scale bars: b = 500 μm; c–i = 10 μm.

the *Botryosphaeriaceae* this feature is seen only in *Dothiorella*, *Phaeobotryon* and *Spencermartinisia* (Phillips et al. 2008). The main character used to separate species within this clade is conidial dimensions. In practice this can be difficult to apply on account of the variability within a species and the overlap of dimensions between the species. Nevertheless, they can all be distinguished phylogenetically, although for some species, such as *D. pinea* and *D. intermedia*, support for the branches is low.

Diplodia pseudoseriata was recently described from native *Myrtaceae* trees in Uruguay (Pérez et al. 2010) while *D. alatafructa* was described from *Pterocarpus angolensis* in South Africa (Mehl et al. 2011). In the phylogeny constructed in the present work isolates of both of these species clustered in a single clade suggesting that they represent a single phylogenetic species. Judging from the original descriptions these two species also appear to be morphologically indistinguishable. Therefore it seems that they should be regarded as synonyms and given that *D. pseudoseriata* was the first name to be published it takes priority over *D. alatafructa*. This possibility will be addressed in a future publication currently in preparation.

Both *D. seriata* (as *Botryosphaeria obtusa*) and *D. mutila* (as *Botryosphaeria stevensii*) have been implicated in fruit rot (black rot) and cankers of apples (Stevens 1933, Laundon 1973, Brown & Britton 1986, Brown-Rytlewski & McManus 2000). Stevens (1933) found that in the United States *D. seriata* was the most common cause of black rot while *D. mutila* was confined mainly to the west coast. In the present study the *Diplodia* species isolated from *Malus* and other *Rosaceae* were *D. bulgarica*, *D. intermedia*, *D. malorum* and *D. seriata*. This study has shown that *D. seriata* s.l. is a complex of species, two of which are associated with fruit rot and canker of apples and other *Rosaceae*, namely *D. intermedia* and *D. seriata*. This was based on five isolates of *D. intermedia* and only two of *D. seriata*, from three widely separate regions in Portugal and one sample from Bulgaria. Both species are easily confused on the basis of morphology only and therefore it is virtually impossible to know if previous reports regarding the black rot pathogen of apple were referring to *D. intermedia*, *D. seriata* or both. Given the data presented it may be premature to suggest that the cause of black rot of apples is *D. intermedia* and not *D. seriata*, but it does indicate that a more complete phylogenetic and pathogenicity study of the black rot fungus should be done on collections from a wider geographical range.

Although *D. mutila* has also been implicated as the cause of apple black rot and canker, the evidence presented here suggests that the name *D. malorum* is more correctly applied to isolates from *Malus* species. Slippers et al. (2007) initially regarded *D. malorum* to be a more appropriate name for the anamorph of '*B.*' *obtusa* but they rejected this possibility after studying the type specimen in G (Fungi rhenani 1706) and that was later confirmed by Phillips et al. (2007). The current study clearly supports this view and shows that *D. malorum* is morphologically and phylogenetically distinct from *D. seriata*, which is the anamorph of '*B*'. *obtusa*. It is likely that given the morphological similarity between *D. malorum* and *D. mutila* both species have been confused in the past. Again, the data presented here relates only to one geographical region in Portugal but the type specimen of *D. malorum* is on a rotten apple collected from Germany. On the other hand, the occurrence of *D. mutila* on apples and its association with cankers cannot be ruled out, since it has been reported in previous studies and the ITS sequences (GenBank accessions AF243406 and AF243407, Zhou & Stanosz 2001) from those isolates are 100 % identical to the ITS sequence of typical *D. mutila* (Alves et al. 2004). The lack of an ex-type culture, or other cultures linked to the holotype of *D. mutila* hampers this kind of study, but Alves et al. (2004) provided a detailed description of this species based on an isolate from grapevines in Portugal (CBS 112553), an isotype of *D. mutila* (K99664) and the lectotype of *Physalospora mutila* (BPI99153). They showed that CBS 112553 correlated closely with the morphology of *D. mutila*. This culture has subsequently been cited as typical of *D. mutila* and has been referred to as a standard isolate for this species (Alves et al. 2006, Damm et al. 2007, Lazzizera et al. 2008). In the future more studies should be done in order to confirm the occurrence and pathogenicity of *D. mutila* towards apples.

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