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; 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 multiocular 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),
**Table 1** Isolates of Diplodia species considered in this study.

| Species                  | Accession number | Host                  | Location                                    | Collector       | GenBank Numbers |
|--------------------------|------------------|-----------------------|---------------------------------------------|-----------------|-----------------|
| "Batryosphaeria" tsugae  | CBS 418.64       | Tsuga heterophylla    | British Columbia, Lake Cowichan, Canada     | A. Funk         | DQ458888        |
| Diplodia corticola       | CBS 112547       | Quercus ilex          | La Rozuela, Córdoba                        | M.E. Sánchez    | AY259110        |
|                         | CBS 112549       | Quercus suber         | Requeixo, Aveiro, Portugal                 | A. Alves        | AY259100        |
| Diplodia africana        | CBS 120835       | Prunus persica        | Paarl, Western Cape, South Africa          | U. Damm         | EF445343        |
|                         | CBS 12104        | Prunus persica        | Paarl, Western Cape, South Africa          | U. Damm         | EF445344        |
| Diplodia bulgarica       | CBS 124135       | Malus sylvestris      | Plovdiv, Bulgaria                          | S. Bobev        | GQ923852        |
|                         | CBS 124254       | Malus sylvestris      | Plovdiv, Bulgaria                          | S. Bobev        | GQ923835        |
|                         | CBS 124136       | Malus sylvestris      | Plovdiv, Bulgaria                          | S. Bobev        | GQ923854        |
|                         | IRAN 1530C       | Malus domestica       | Gahvareh village, Kermanshah, Iran         | J. Abdollahzadeh| JX152582        |
|                         | IRAN 1532C       | Malus domestica       | Gahvareh village, Kermanshah, Iran         | J. Abdollahzadeh| JX152578        |
|                         | IRAN 1548C       | Malus domestica       | Gahvareh village, Kermanshah, Iran         | J. Abdollahzadeh| JX152584        |
| Diplodia cupressi        | CBS 168.87       | Cupressus sempervirens| Bet Dagan, Israel                           | Z. Solei        | DQ458893        |
|                         | CBS 261.85       | Cupressus sempervirens| Bet Dagan, Israel                           | Z. Solei        | DQ458894        |
| Diplodia intermedia      | CA 147           | Malus domestica (fruit rot) | Aveiro, Portugal                         | A. Alves        | GG238257        |
|                         | CBS 112556       | Malus sylvestris (canker) | Monte da Caparica, Setúbal, Portugal    | A.J.L. Phillips | AY259086        |
|                         | CBS 124134       | Cydonia sp. (fruit rot) | Torres Vedras, Portugal                   | S. Santos       | HM036528        |
|                         | CBS 124462       | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923858        |
|                         | IRAN 1595C       | Unknown woody plant   | Rezvanshahr, Rasht, Gilan, Iran            | J. Abdollahzadeh| JX152585        |
| Diplodia malorum         | CAP 330          | Pyracantha coccinea   | Plovdiv, Bulgaria                          | S. Bobev        | GQ923881        |
|                         | CAP 265          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923859        |
|                         | CAP 266          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923860        |
|                         | CAP 267          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923861        |
|                         | CAP 268          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923862        |
|                         | CAP 269          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923863        |
|                         | CAP 270          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923864        |
|                         | CBS 124130       | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923865        |
|                         | CBS 124253       | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923866        |
|                         | CAP 271          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923867        |
|                         | CAP 272          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923868        |
|                         | CAP 273          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923869        |
|                         | CAP 274          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923870        |
|                         | CAP 275          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923871        |
|                         | CAP 276          | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923872        |
|                         | CBS 112554       | Malus sylvestris      | Monte da Caparica, Setúbal, Portugal        | A.J.L. Phillips | GQ923895        |
| Diplodia mutila          | CBS 112553       | Vitis vinifera        | Montermor-o-Novo, Portugal                 | A.J.L. Phillips | AY259093        |
|                         | CBS 230.30       | Phoenix dactylifera   | California, USA                            | L.L. Huillier   | DQ458886        |
| Diplodia olivarum        | CAP 301          | Ceratonia siliqua     | Sicily, Italy                               | A. Soldi        | GG238273        |
|                         | CAP 275          | Olea europaea         | Cutifoliaro, Lecce, Puglia, Italy          | S. Frisullo     | EU392265        |
|                         | CAP 224          | Olea europaea         | Salice Salentino, Lecce, Puglia, Italy     | S. Frisullo     | EU392296        |
|                         | CAP 225          | Olea europaea         | Campi Salentino, Lecce, Puglia, Italy      | S. Frisullo     | EU392297        |
|                         | CBS 121887       | Olea europaea         | Italy, Puglia, Lecce, Bosco Belvedere, Scorrano | S. Frisullo     | EU392298        |
|                         | CAP 257          | Olea europaea         | Montesano Salentino, Lecce, Puglia, Italy  | S. Frisullo     | EU392299        |
|                         | CAP 168          | Olea europaea         | Scanzano, Matera, Basilicata, Italy        | S. Frisullo     | EU392261        |
|                         | CAP 169          | Olea europaea         | Scanzano, Lecce, Puglia, Italy             | S. Frisullo     | EU392262        |
|                         | CAP 339          | Pinus sp.             | Belgium                                     | S. Bobev        | GG236875        |
|                         | CBS 393.84       | Pinus nigra           | Putten, Netherlands                        | H.A. van der Aa | DG458895        |
|                         | CBS 109727       | Pinus radiata         | Stellenbosch, South Africa                 | W.J. Swart      | DG458897        |
|                         | CBS 109725       | Pinus patula          | Habinsaran, Indonesia                      | M.J. Wingfield  | DG458896        |
|                         | CBS 109943       | Pinus patula          | Indonesia                                  | M.J. Wingfield  | DG458898        |
| Diplodia rosulata        | CBS 116472       | Prunus africana       | Gambo, Ethiopia                            | A. Gure         | EU430266        |
|                         | CBS 119470       | Prunus africana       | Gambo, Ethiopia                            | A. Gure         | EU430267        |
Diplodia scrobiculata (CMW 189) and Diplodia seriata (CBS 112555) 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 EF-1-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 Junipers phoenicea in Sardinia (Lin-aldeddu 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 Junipers (Solé 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**

Isolates 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 (Swoford 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+I+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 taxon 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 10th 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) of trees in each analysis. ML analyses were performed on a starting tree automatically from the same tree space were sampled during each analysis. ML analyses were performed on a starting tree automatically generated by the software. Nearest-Neighbor-Interchange (NNI) was used as the heuristic method for tree inference and 1 000 bootstrap replicates were performed. Bootstrap 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 epithet 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.

**Conidiotoma** 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 periclinical 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 one-septate 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
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 Diplocycis 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. 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.
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, (X ± S.D. of 700 conidia = 28.1 ± 2.4 × 14.4 ± 1.4 µm, L/W = 1.9 ± 0.24).

Specimens examined. GERMANY, Rhineland, on Malus, 1870, J. 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 N° 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 (DAVPF 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, Phaeobathyx and Spencermartinsia (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 1886, 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 (BP199153). 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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